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Adaptation in rural water, sanitation, and hygiene programs: A qualitative study in Nepal

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ABSTRACT

Adaptations are modifications made to programming to improve effectiveness or contextual fit, and are important for program improvement. However, adaptations can be detrimental if they do not preserve an intervention's underlying theory of change. We present a case study of 45 adaptations made to rural WaSH programming in Nepal, identified through qualitative interviews with implementers conducted in June through August 2019. For each adaptation, we characterized its target outcomes and implementers' motivations for making the adaptation, and we assessed the adaptation's intended and unintended effects on program quality. Participants described adaptations to both interventions (e.g., changes to hygiene promotion messages) and implementation strategies (e.g., sanctions to enforce toilet construction, such as denying work permits to households without a toilet). Adoption was the most common target outcome, specifically increasing toilet construction. Other target outcomes included feasibility of program delivery, acceptability of messages or WaSH products, reach of program activities in the community, and sustainability. Implementers were commonly motivated by intense pressure to meet national open defecation free targets. Most adaptations achieved their target outcomes. However, sanctions adaptations had substantial unintended negative effects. Implementers reported that sanctions were unpopular with communities and had poor sustainability. In contrast, non-sanctions adaptations that targeted outcomes of feasibility, acceptability, and sustainability had few unintended negative consequences. Our findings suggest that adaptations to promote rapid adoption of toilet construction do not consistently achieve sustained behavior change. Furthermore, adaptations to improve feasibility of program delivery or cost and acceptability of WaSH products can indirectly improve adoption even when it is not an explicit target outcome.

1. Introduction

Realizing the health and development benefits of water, sanitation, and hygiene (WaSH) programs requires both evidence-based interventions and their effective implementation. While WaSH is a well-documented determinant of health (Clasen et al., 2015; Freeman et al., 2014, 2017), effective implementation at scale remains a challenge. Many national programs have struggled to realize health benefits (Cameron et al., 2013; Clasen et al., 2014; Patil et al., 2014; Sinharoy et al., 2017), with challenges such as poor implementation and adaptation to the local context implicated for poor performance (Boisson et al., 2014; Cameron et al., 2019; Chambers, 2009; Mbuya et al., 2015; Parvez et al., 2021; Routray et al., 2017).

Adaptations are modifications made to programming to improve effectiveness and contextual fit (Aarons et al., 2012; Kirk et al., 2020), which are often needed as interventions are brought to scale. For

Abbreviations: WaSH, water, sanitation, and hygiene; CLTS, community-led total sanitation; ODF, open defecation free; MADI, model for adaptation design and impact; CFIR, consolidated framework for implementation research; NGO, non-governmental organization; SSH4A, Sustainable Sanitation and Hygiene for All program; FCHV, female community health volunteer.

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example, budget constraints may necessitate adaptations to ensure that implementation at scale remains affordable. Interventions may also be implemented in different contexts than where they were originally designed, requiring adaptations to ensure that programs are contextually appropriate and accepted by target populations (Aarons et al., 2017; Milat et al., 2015).

Both interventions and implementation strategies can be adapted. Interventions are the practices, products, policies, and procedures designed to improve health and wellbeing (Brown et al., 2017). Implementation strategies are the methods or techniques used to improve the delivery, adoption, and sustainability of those interventions (Powell et al., 2015). For example, community-led total sanitation (CLTS) is an intervention that uses negative emotions of shame and disgust to “trigger” grassroots action within communities to become open defecation free (ODF). CLTS has been widely applied and adapted as the foundation of rural sanitation programming in over fifty countries (Galvin, 2015; Sigler et al., 2015; Venkataramanan et al., 2018). Adaptations to CLTS interventions include adding dramas and songs that target positive local motivations for latrine construction or omitting activities that are perceived as offensive or inappropriate for local norms. Adaptations to CLTS implementation strategies include providing training to grassroots community leaders or external incentives for achieving ODF status, such as cash prizes (Chambers, 2009; Deak, 2008; Venkataramanan, 2016; Zuin et al., 2019).

Adaptations are an important aspect of program improvement that are typically needed throughout program design and implementation (Moore et al., 2013). Yet systematic documentation of adaptation processes in WaSH delivery is limited. In some cases, studies have found that documentation of adaptation in WaSH may be intentionally suppressed, as deviation from program protocols can be negatively perceived and discouraged (Venkataramanan, 2016). There is some evidence of systematic adaptations of WaSH interventions in preparation for pilot testing and scale-up (Nordhauser and Rosenfeld, 2020). However, in global health and development, adaptations are typically made after the intervention design and piloting stage, in response to emergent needs and challenges encountered during implementation and sustainment (Moore et al., 2013).

Furthermore, adaptations can have detrimental effects, particularly if they do not carefully preserve an intervention’s underlying theory of change (Kirk et al., 2020; Perez Jolles et al., 2019). For example, while adaptations to CLTS are common, they may not always be systematically planned or supported by evidence. Studies have documented implementers who omit activities that elicit strong disgust and cause participant discomfort, even though feelings of disgust are central to CLTS’s theory of change (Venkataramanan, 2016). These adaptations may reduce intervention effectiveness, but their full range of effects on program performance have not been comprehensively documented.

A better understanding of adaptations and their effects would support improvements to WaSH service delivery. Various models and frameworks exist in the field of implementation science to describe different types of adaptations and understand their effects on program performance. These models also propose possible mediators and moderators of adaptations’ effects, such as how and why adaptations were made (Kirk et al., 2020; Moore et al., 2013; Wiltsey Stirman et al., 2019). Adaptation models and frameworks can help stakeholders more comprehensively evaluate adaptations’ potential positive and negative effects and promote adaptations that improve contextual fit while preserving a program’s theory of change.

We apply constructs from several adaptation models and frameworks to study adaptations in rural WaSH (Kirk et al., 2020; Moore et al., 2013; Stirman et al., 2013; Wiltsey Stirman et al., 2019). The purpose of this study is to systematically document what adaptations were made to rural WaSH programs using adaptation models and frameworks and to assess how these adaptations affected program outcomes. We use a case study approach of adaptations made to WaSH programming in Nepal, using data collected through qualitative interviewers with program implementers. Specific objectives were to characterize the adaptations that implementers made to rural WaSH programs, describe implementers’ processes and motivations for making adaptations, and assess adaptations’ intended and unintended effects on program quality outcomes.

This study provides an example of how adaptation models and frameworks can be applied to better understand adaptations’ effects on WaSH program performance. We document a range of positive and negative adaptation effects and provide guidance to inform selection of effective adaptations and mitigate unintended negative consequences. We describe implementers’ processes and motivations for making adaptations, and we make recommendations to better support and incentivize adaptations that improve program performance.

2. Methods

2.1. Conceptual frameworks

This study was informed by the model for adaptation design and impact (MADI) (Kirk et al., 2020). Briefly, the MADI combines frameworks and constructs that describe adaptations, how those adaptations are made, and their impacts. The MADI proposes that adaptations have both intended and unintended outcomes, and these outcomes are moderated by the process and rationale for making the adaptation (Kirk et al., 2020). The purpose of utilizing this framework was to ensure comprehensive documentation of adaptations through use of well-defined constructs and to improve our ability to analyze relationships between adaptation characteristics, process and motivations, and outcomes.

Our conceptual model for this study used constructs from the MADI to characterize adaptations made by rural WaSH implementers, processes for making these adaptations, and their intended and unintended outcomes. We added to the MADI implementers’ motivations for making adaptations as an additional potential moderator. The conceptual model for this study is depicted in Fig. 1. Specific constructs are described below.

2.1.1. Adaptation characteristics

We characterized adaptations based on three dimensions: *what* was adapted, the *target outcomes* of the adaptation, and *who* made the adaptation. We categorized *what* was adapted as either the intervention or implementation strategies (Brown et al., 2017; Powell et al., 2015). *Target outcomes* are the outcomes or impacts that participants intended to improve by making the adaption. We classified target outcomes as either implementation outcomes or health and wellbeing impacts (Proctor et al., 2011). Implementation outcomes are the intermediate outcomes necessary for realizing health and wellbeing impacts. We specified eight implementation outcomes defined by Proctor et al. (2011): acceptability, adoption, appropriateness, cost, feasibility, fidelity, reach, and sustainability. We assessed *who* made the adaptation using the organizational affiliation and implementation role of the participant describing it. Table 1 provides definitions of all adaptation characteristics constructs.

We define the implementation outcome of adoption as “intention, decision, or action to uptake a WaSH behavior or technology” (Table 1). To assess this construct, we differentiated between three measures of adoption. At the household level, we considered construction of WaSH infrastructure (e.g., toilets, handwashing stations). Following construction, we considered individual household members’ use of constructed infrastructure. Household construction is a necessary condition for use, but construction does not guarantee use. Thus, we treated the two as separate measures. As a community-wide measure of sanitation adoption, we also considered eligibility or declaration of ODF status. In Nepal, villages become eligible for ODF declaration when all households have constructed a toilet (regardless of use), and ODF is declared once the government officially certifies 100% household toilet construction.

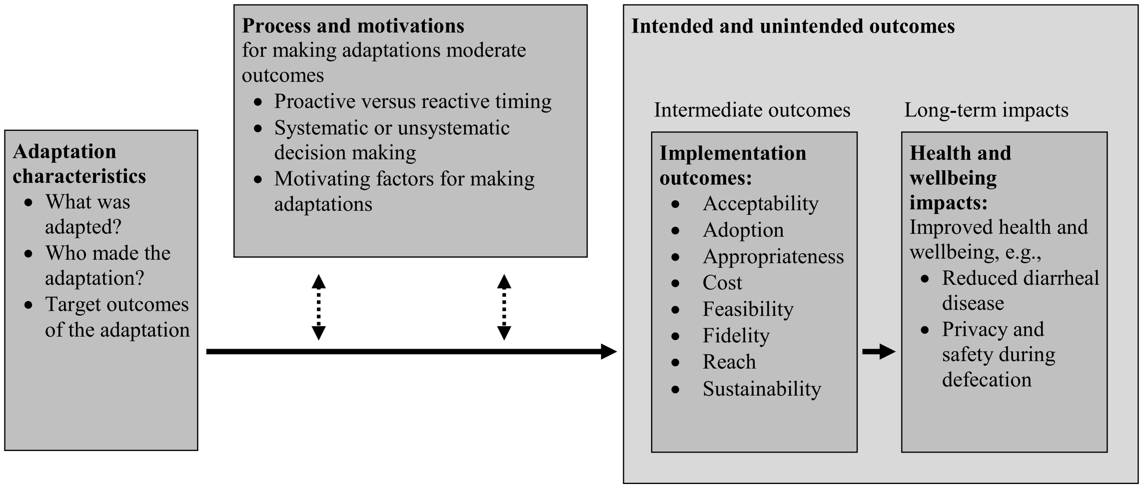

Fig. 1. Conceptual model depicting relationships between adaptation characteristics, process, and motivations for making adaptations, and intended and unintended outcomes. The model combines constructs from frameworks describing adaptation characteristics (Kirk et al., 2020; Moore et al., 2013; Stirman et al., 2013; Wiltsey Stirman et al., 2019) and implementation outcomes (Proctor et al., 2011). Adapted from the model for adaptation design and impact (Kirk et al., 2020).

Table 1
Constructs to describe adaptation characteristics of what was adapted, the target outcomes, and by whom.

Adaptation characteristics	Constructs and definitions
What was adapted?	*Intervention*: changes to the practices, products, policies, and procedures designed to improve health and wellbeing of beneficiaries *Implementation strategies*: changes to the methods or techniques used to improve the delivery, adoption, and sustainability of interventions
Target outcomes of the adaptation	*Implementation outcomes:* intermediate effects of adaptations to interventions or implementation strategies, which are precursors to achieving health and wellbeing impacts *Acceptability*: Perception that a given WaSH program or its component parts is agreeable and satisfactory *Adoption*: Intention, decision, or action to uptake a WaSH behavior or technology *Appropriateness*: Perceived fit, relevance, or compatibility of the WaSH program with context, or perceived fit of the program to address a specific issue or problem *Cost*: Cost of interventions and/or implementation efforts *Feasibility*: The extent to which the WaSH program can be successfully delivered or used within a given context *Fidelity*: *The* extent to which the WaSH program is delivered as originally developed and specified in program plans and protocols *Reach*: Reach of the WaSH program among the target population *Sustainability*: The extent to which WaSH behaviors and technologies are maintained and institutionalized within the target population *Health and wellbeing impacts*: Changes in determinants of health (e.g., water quality, environmental cleanliness) or health status (e.g., enteric disease) of recipient households or communities
Who made the adaptation?	Organizational affiliation (government, development or civil society organizations, private sector) and job description of participant describing the adaptation

2.1.2. Process and motivations

Process and motivations for making adaptations are depicted as potential moderators of adaptations' effects in our conceptual model (Fig. 1).

We used two constructs to describe the implementers' processes for making adaptations: timing (proactive versus reactive) and whether the process was systematic (Kirk et al., 2020; Moore et al., 2013; Wiltsey Stirman et al., 2019) (Table 2). We considered adaptations to be systematic if implementers applied any of the following to evaluate potential outcomes or fitness for purpose: consulting with implementation stakeholders, consulting with communities, and formative research or evaluations (Kirk et al., 2020). Table 2 provides definitions of all adaptation process constructs.

We classified motivations for making adaptations using constructs described by the consolidated framework for implementation science research (CFIR) (Damschroder et al., 2009). Briefly, the CFIR proposes 39 constructs that can motivate implementers to make adaptations. Constructs are characteristics of the intervention itself, implementing individuals, inner setting (organizations directly implementing the adaptation), outer setting (external organizations and entities that may support, regulate, or receive the adaptation but do not directly implement, such as government agencies establishing WaSH policies), and implementation process (e.g., planning, reflecting, and evaluating). Supplementary Information file 1 gives a full listing of motivation constructs as applied in this study.

Table 2
Constructs to characterize processes for developing and implementing adaptations.

Adaptation process	Constructs and definitions
Timing	*Proactive*: adaptations that are made in response to anticipated needs before the need arises *Reactive*: adaptations that are made in response to unanticipated needs after the need arises
Systematic decision making	*Systematic:* adaptations that are informed by deliberate and methodical efforts to evaluate potential outcomes and fitness for purpose *Consulting with implementing stakeholders*: Soliciting feedback from implementing stakeholders about an adaptation *Consulting with communities*: Soliciting feedback from communities about an adaptation, or discussions with communities to understand local needs and preferences to inform adaptation design *Formative research or evaluation*: Testing an adaptation or its components, or applying learnings from evaluations of similar interventions or implementation strategies *Unsystematic:* adaptations that are made without systematic efforts to evaluate potential outcomes or fitness for purpose

2.1.3. Intended and unintended outcomes

Intended and unintended consequences are depicted in our conceptual model as resulting from adaptation characteristics and moderators related to processes and motivations (Fig. 1). Assessing both intended and unintended outcomes accounts for the possibility that adaptations may have complex, unanticipated effects beyond their original target outcomes. We define intended outcomes as target outcomes that were achieved, while unintended outcomes were not targeted for change but were nonetheless affected by the adaptation. We classify intended and unintended outcomes following the definitions of implementation outcomes and health and wellbeing impacts described in Table 1.

2.2. Study design

We assessed adaptations described by rural WaSH implementers in Nepal primarily at the district- and sub-district levels. Data for this study were collected between June and August of 2019 via qualitative interviews with implementers from government, non-governmental organizations (NGOs), multilateral organizations, civil society organizations, and the private sector.

We conducted this research in rural areas of Nepal delivering the Sustainable Sanitation and Hygiene for All (SSH4A) program (SNV, 2018). Briefly, SSH4A is a program implemented by the SNV Netherlands Development Organization to improve delivery of WaSH through building capacity of local governments to lead and steer WaSH service delivery and by providing technical assistance for implementation efforts by non-governmental partners in local NGOs, civil society, and the private sector. For this study, we recruited participants from local governments and non-governmental implementing partners in SSH4A program areas. SSH4A began in Nepal in 2008, and implementation had been ongoing in all study areas for multiple years.

2.3. Study setting

WaSH policy in Nepal is guided by the government's National Sanitation and Hygiene Master Plan (hereafter the "Master Plan") (Department of Water Supply and Sewerage, 2011). The Master Plan outlines responsibilities for different levels of government and defines "guiding principles" for implementation—such as using a no subsidy approach. Details of specific activities or program plans are determined at the subnational level, though CLTS-style triggering is a key component of sanitation and hygiene promotion in many areas. Implementation is decentralized and, at the time of this study, was primarily coordinated at the district and municipal levels.

The Master Plan established national WaSH targets, including achieving country-wide ODF status by 2017. National ODF targets were not met by 2017 and, at the time of this research, remained a focus of WaSH activities in non-ODF areas. Nation-wide ODF was declared shortly after the conclusion of field activities for this research, on 30th September 2019. The Master Plan also outlines targets for post-ODF declaration called "Total Sanitation." Total Sanitation indicators comprise five key behaviors related to toilet use, handwashing at critical times, safe drinking water, food hygiene, and solid waste management. Targets to achieve Total Sanitation are set for 2030 (Department of Water Supply and Sewerage, 2011). Access to at least basic water is estimated at 91%, and over 99% of households have a handwashing facility (WHO/UNICEF, 2020).

Relevant stakeholders involved in WaSH implementation in Nepal are diverse and include international and local NGOs and multilateral organizations (hereafter "development organizations"); civil society organizations, including women's, mothers', and journalist groups; government; and the private sector. For the purpose of this study, we considered government implementers to be individuals in elected offices (e.g., mayors, ward chairpersons) and paid government jobs (e.g., technicians and engineers). We considered small business owners and other implementers working in for-profit entities as private sector. We considered implementers employed by NGOs and multilateral organizations as development organization employees. We considered all other participants (e.g., women's and journalist group members) as civil society.

Men outnumber women in the Nepali WaSH sector. Elected officials, government employees, and development organization employees are predominantly men. Women predominantly fill unpaid implementation roles within women's and mother's groups and as female community health volunteers (Anderson et al., 2021).

2.4. Study population and recruitment

We recruited participants from four rural districts: Siraha and Mahottari districts in the Terai of the Central Region and Salyan and Surkhet districts in the hills of the Mid-Western Region. Districts were selected to capture ethnically, culturally, and politically diverse perspectives and to reflect a range of geographic challenges encountered in the flat low-land areas of the Terai versus more mountainous and remote terrain in the hills. At the time of this research, Salyan and Surkhet had been declared ODF; Siraha and Mahottari had not.

We worked with local program coordinators to identify stakeholders who were highly involved in implementation, had worked multiple years in WaSH, and/or were influential in making adaptations. Local program coordinators were NGO staff who had worked in the district for at least five years. They provided a list of 15–25 relevant individuals per district. From these lists, we purposively sampled 10–14 participants per district for diverse representation from local-level implementers to regional-level supervisors across government and development organizations, and private sector and civil society at the village levels. Our sample included only program implementers. We did not include program recipients.

2.5. Data collection

We collected data through semi-structured qualitative interviews. During interviews, we asked participants to describe a "new solution" (i.e., adaptation) they had made to improve WaSH programming. To characterize the nature of adaptations, we asked participants to identify how the solution was different from what was done previously, the purpose of the solution, and its target outcomes. To assess the factors influencing implementers' motivations for adaptation, we asked participants to describe the factors that motivated them to adopt and sustain their new solutions over time. To assess intended and unintended outcomes, we asked participants to describe whether the adaptation was successful and how they defined and measured success, with additional probes about adoption, sustainability, and acceptability. These outcome measures reflect participants' perceptions of adaptation success, and we did not triangulate these findings with quantitative outcomes measures or interviews with program beneficiaries.

We developed an initial interview guide in English, translated it into Nepali, and pre-tested in a district bordering Siraha. Pre-testing assessed question appropriateness, ordering, and effectiveness at eliciting information relevant to the study objectives. We also iteratively revised the interview guide throughout data collection based on emergent themes.

A team of one interviewer and one notetaker (A.K.G, D.M.A) conducted interviews in private offices or meeting rooms at participants' workplaces or at the home or a nearby community center for participants without a formal office. We conducted interviews in Nepali, Hindi, Maithili, or English, following the preference of the participant. Interviews lasted approximately one hour. When participants gave permission to audio-record ($n = 45$, 85%), we transcribed recordings directly into English for analysis.

2.6. Analysis

We assessed the characteristics of adaptations, implementers' processes and motivations for making them, and adaptations' intended and unintended outcomes. We coded each interview to identify adaptations made by each participant. For each adaptation, we coded adaptation characteristics as what was adapted, by whom, and target outcomes, using the constructs in Table 1. We coded for constructs related to the process (proactive versus reactive timing, systematic decision making) and motivations (CFIR constructs) for making adaptations. We coded for intended and unintended outcomes using the implementation outcomes and health and wellbeing impacts in Table 1.

We used NVivo qualitative data analysis software (Melbourne, Australia) for coding. We conducted an initial round of coding with a subset of eight transcripts, reflecting on emergent themes and revising codes, as necessary. A single author (D.M.A.) conducted the initial round of coding, and two other members of the author team (S.A.B, M.C.F) reviewed the results and suggested revisions to the codebook. The same author (D.M.A) that conducted initial coding then proceeded to code the full dataset and synthesize the data as described below.

Following coding, we synthesized coded text from each interview relating to the same adaptation into a matrix in Microsoft Excel (Redmond, Washington), with rows for each adaptation and columns describing the corresponding characteristics, processes, motivating factors, and outcomes. Using this matrix, we developed inductive codes to aggregate similar adaptations under archetypes of either intervention or implementation strategy adaptations. For example, we aggregated adaptations to engage women in program implementation and adaptations to engage municipal government officials in planning under the implementation strategy archetype "engaging new stakeholders."

Our results present the characteristics, processes and motivations, and intended and unintended outcomes of these archetypes. The full disaggregated matrix of all individual adaptations can be found in Supplementary Information file 2.

Table 3
Intervention adaptation archetypes and their corresponding target outcomes, process and motivations, intended and unintended outcomes. All adaptations were reactive except two examples under hardware safety and/or functionality adaptations. Target outcomes are the outcomes and impacts that participants intended to achieve by making the adaptation. Intended outcomes are participants' perceived effects on target outcomes. Unintended outcomes are participants' perceived effects on non-target outcomes: (+) indicates positive unintended effects; (−) indicates negative unintended effects. (±) indicates mixed effects.

Intervention adaptations	Examples	Target outcomes	Process	Motivations	Intended outcomes	Unintended outcomes
Hardware safety and/ or functionality Modification of existing hardware systems or addition of new hardware systems to ensure that WaSH services are safe and/or adequate to meet demand	Adding chlorination to the municipal water supply Purchase of a generator to ensure water treatment plant operability during electrical outages	Feasibility – ensure technical specifications are met for hardware systems to provide adequate service	Formative research & evaluation – surveys, site assessments, and other safety data used to inform adaptation development	Patient needs & resources – existing systems used within communities do not provide safe and/or adequate service Relative advantage – adaptations are the only viable option given other technical or geologic limitations (e.g., water or electricity availability)	Improvements in target outcomes for feasibility by meeting technical specifications for operation	(−) Cost – new hardware systems to overcome technical challenges are often higher cost (±) Acceptability – low initial willingness to pay, but increases over time as households observe improved service reliability
Hardware user preferences Modification of existing hardware systems or addition of new hardware systems to ensure that WaSH services are compatible with user preferences	Upgrading from poured cement slabs to prefabricated latrine pans installed with decorative tile floors	Acceptability – align product designs with user preferences, typically to make more sales Cost – ensure that products match consumers' willingness to pay Other – to increase profit and personal livelihood	Consulting with communities – small business owners update hardware designs to better meet consumers' stated preferences	Patient needs and resources – consumer demands for new and different products Increased sales, profit, and personal livelihood for implementers	Improvements in target outcomes for acceptability of designs to consumers, cost of products, and profit and personal livelihoods	(+) Adoption – designs that meet consumer demand increase purchases and toilet coverage within the community
Triggering messages Modification of behavior change triggering messages delivered to households and communities	Adding religious content to messaging to encourage households to be pure and holy through WaSH Switching from in-person delivery of menstrual hygiene messages to video-based format	Adoption – increase toilet coverage and achieve ODF targets; promote WaSH behaviors Appropriateness – improve relevance of messages to local conditions, cultural norms, and/or behavioral drivers Health and wellbeing impacts - reduce WaSH-related diseases or improve health and wellbeing generally	Mixture of systematic and unsystematic approaches. Local-level implementers relied on lay knowledge of communities (unsystematic). More senior development organization staff applied a mix of consulting implementing stakeholders, communities, and formative research and evaluations (systematic).	External policies and incentives – targets set under the national Master Plan; sense of urgency created when targets are not met Patient needs and resources – need to address locally relevant drivers and barriers to behavior change Cosmopolitanism (i.e., networks with other organizations) – meetings with technical advisers and other implementing partners discuss challenges and offer solutions Reflecting and evaluating – previous messages observed to be ineffective and/or irrelevant to local context	Improvements in target outcomes for adoption of WaSH infrastructure and behaviors, appropriateness of messages to local context, and community health and wellbeing	(+) Reach – adaptations to use women's and mothers' groups as implementation personnel expand program coverage area beyond what NGOs can achieve with limited staff (+) Acceptability - improving relevance of messages to local context typically also improves likeability of messages

2.7. Ethics

This study was ruled as non-human subjects research by the Institutional Review Boards of the University of North Carolina-Chapel Hill (IRB # 19–0945) and Emory University. Local approval for study activities was obtained from the Nepali Ministry of Water Supply. Participants were informed of the study purpose and provided written consent before enrollment.

3. Results

The purpose of this study was to comprehensively document what adaptations are made to rural WaSH programs, and assess their effects on program outcomes. We identified 45 adaptations described by 48 study participants. Not all study participants described an adaptation to WaSH programming. We grouped these 45 adaptations into ten different adaptation archetypes.

For each archetype, we present characteristics of who made the adaptation and its target outcomes. We describe processes for making adaptations based on its timing and whether the process was systematic, following constructs defined in Table 2. We assess motivations for making adaptations using constructs of the CFIR. Finally, we assess unintended and intended outcomes, based on whether target outcomes were achieved and any other unintentional effects on implementation outcomes or health and wellbeing. Tables 3 and 4 summarize these characteristics, processes and motivations, and target outcomes for each adaptation archetype. A narrative summary of key trends is provided below.

3.1. Sample characteristics

Our sample comprised implementers at the municipal, district, and regional levels (Table 5). Participants were predominantly development organization employees (n = 17) and elected government officials (n = 11). Most participants were men (n = 35, 73%), as is consistent with broader sector-wide trends in Nepal of underrepresentation of women in WaSH. The average age of participants was 41 years, and approximately half of participants (58%) had held their current implementation role for less than four years.

3.2. Characteristics of adaptations

To document what adaptations implementers made to rural WaSH programming, we characterized adaptations based on what was adapted, by whom, and target outcomes. We summarize what was adapted and target outcomes for intervention and implementation strategy adaptation archetypes in Tables 3 and 4, respectively. Fig. 2 maps the participant demographics of who made different adaptation archetypes.

3.2.1. What was adapted?

We identified three archetypes of intervention adaptations: hardware safety and/or functionality, hardware user preferences, and triggering message adaptations. We identified seven archetypes of implementation strategy adaptations: monitoring tools, engaging new stakeholders, financing mechanisms, forming new governance structures, sanctions for behavioral enforcement, rewards for behavior incentives, and supply chain strengthening. The most common implementation strategy adaptations were engaging new stakeholders (n = 10) and sanctions for behavioral enforcement (n = 7). The most common intervention adaptations were hardware user preferences (n = 6) and triggering messages (n = 8).

3.2.2. Who made the adaptation?

Intervention adaptations for hardware safety/functionality were made by technical government employees (e.g., municipal utility committee members and WaSH technicians), and intervention adaptations for hardware user preferences were made predominantly by private sector participants. Only two adaptations for hardware user preferences were reported by development organization employees. One entailed encouraging households to use low-cost local materials (e.g., mud, bamboo) for toilet superstructures rather than expensive materials like concrete or brick, and the other entailed moving the location of water sources to be closer to toilets to facilitate handwashing and anal cleansing behavior preferences. Municipal-level NGO implementers primarily reported intervention adaptations to triggering messages.

Implementation strategy adaptations to engage new stakeholders occurred at all levels from village to district and were reported by government, development organizations, and civil society participants. Adaptations for form governance structures and develop supply chains were made by senior development organization employees at the regional level. Sanctions adaptations were primarily implemented by elected government officials. The three non-government participants who reported sanctions adaptations had either participated in or observed committees patrolling to enforce sanctions but were not directly involved in their design. Sanctions were designed in government committees, and elected government officials made the final decision to apply sanctions.

3.2.3. Target outcomes

For both intervention and implementation strategy adaptations, most target outcomes were implementation outcomes, of which adoption was the most common. For adaptations made prior to ODF declaration, participants typically reported household toilet construction and/or achieving ODF declaration as the adoption target. After ODF declaration, adoption targets focused on toilet use or other WaSH behaviors (e.g., handwashing). Adoption was the only target outcome for the implementation strategy adaptation of sanctions to enforce behavior.

Intervention adaptations for hardware safety/functionality and user preferences and implementation strategy adaptations for supply chain strengthening and forming governance structures were the only adaptations where participants did not report adoption as a target outcome. Feasibility was a target outcome for adaptations for hardware safety/functionality, forming new governance structures, and supply chain strengthening. These adaptations were perceived as necessary technical fixes to ensure that intervention hardware was available and suitable for local geographic conditions. For example, one hardware functionality adaptation comprised incorporating solar-powered pumping to municipal water systems where electrical grids were absent or unreliable, but energy was needed to lift water up to municipalities in the hills. Cost was an important target outcome for adaptations to financing mechanisms to ensure that WaSH hardware was available at prices consumers were willing and able to pay.

Appropriateness was cited as a target outcome only for intervention adaptations to triggering messages, where message content or format was adjusted to locally relevant behavioral drivers. Acceptability was a target outcome for intervention adaptations related to hardware user preferences but was otherwise not described as a target outcome. Acceptability was particularly important for participants in the private sector, who sold products and needed to adapt to consumer preferences to ensure product sales and the success of their businesses. Sustainability and reach were cited as target outcomes for implementation strategy adaptations of engaging new stakeholders in order to create a sense of ownership among local governments and communities to sustain WaSH activities or to reach a larger target population.

Health and wellbeing impacts were less common target outcomes. Participants cited improvements in health and wellbeing as a target outcome for adaptations to triggering message content and context, monitoring tools, engaging new stakeholders, financing mechanisms, and rewards for behavior incentives. In all cases, improvements in health were not the sole target outcome, and other implementation outcomes were also indicated as targets.

Table 4
Implementation strategy adaptation archetypes and their corresponding target outcomes, motivations, intended and unintended outcomes. Target outcomes are the outcomes and impacts that participants intended to achieve by making the adaptation. Intended outcomes are participants' perceived effects on target outcomes. Unintended outcomes are participants' perceived effects on non-target outcomes: (+) indicates positive unintended effects; (−) indicates negative unintended effects. (±) indicates mixed effects.

Implementation strategy adaptations	Examples	Target outcomes	Process	Motivations	Intended outcomes	Unintended outcomes
Monitoring tools Development of systems to track progress towards WaSH goals, typically by assigning identity cards or other visual markers to households	Green, yellow, or red stickers placed on households to mark permanent (cement or durable material), temporary (mud or bamboo), or no toilet ownership	Adoption – increase toilet coverage Feasibility – identify lagging program areas to prioritize activities Health and wellbeing impacts - reduce WaSH-related diseases or improve health and wellbeing generally	Consulting with implementing stakeholders – development and decision to use was made in municipal and district WaSH coordination committees	Tension for change - strong movement among implementers that WaSH is important and current conditions are unacceptable Leadership engagement – commitment at all levels of the organization to improve governance and support implementation Complexity – adaptation is easy for implementing partners to understand	Improvements in target outcomes for adoption of toilets	(+) Sustainability – engaging local government leaders in monitoring stickers promotes sustained government leadership (−) Acceptability – households feel shamed when assign monitoring stickers that visibly indicate their "unclean" status to others
Engaging new stakeholders Inclusion of additional stakeholders in planning, implementation and/ or monitoring; new stakeholder groups comprised children, women, and/or political or social leaders.	Formation of journalists' WaSH coordination committee to report on progress towards WaSH targets and hold stakeholders accountable "Political triggering" to convince local politicians of the benefits of sanitation and secure public commitments to improve WaSH	Adoption – increase toilet coverage and achieve ODF targets, promote toilet use Sustainability – increase sense of ownership among community members to sustain WaSH improvements Reach – spread program messages and activities to a wider audience Health and wellbeing impacts - reduce water-related diseases or improve health and wellbeing generally	Mixture of systematic and unsystematic approaches. Local-level implementers relied on lay knowledge of communities (unsystematic). More senior government and development organization staff applied a mix of consulting implementing stakeholders, communities, and formative research and evaluations (systematic).	Tension for change – strong movement among implementers that WaSH is important, current conditions are unacceptable; commitments made to achieve WaSH targets External policies and incentives – targets set under the national Master Plan; sense of urgency created when targets are not met Cosmopolitanism – meetings with technical advisers and other implementing partners to discuss challenges and offer solutions Reflecting and evaluating – observations and monitoring data indicate effectiveness	Improvements in target outcomes for adoption and sustainability of WaSH behaviors, reach of program activities to community households	(+) Feasibility – program implementation becomes easier as WaSH develops into a social movement
Financing mechanisms Reducing tariffs or providing in-kind subsidies or pairing WaSH programs with loan or income generation opportunities to reduce financial barriers	Providing government land by roadsides on which landless households may construct toilets Providing loans for toilets through a partnership between municipal government and a local lending cooperative	Cost – improve affordability of WaSH hardware Adoption – increase toilet coverage and achieve ODF targets Health and wellbeing impacts – improve wellbeing, particularly in relation to economic livelihoods	Consulting with implementing stakeholders – Subsidies and tariff changes required consensus among stakeholders	Patient needs and resources – perceived mismatch between household ability or willingness to pay and prices of WaSH goods and services	Improvements in target outcomes for adoption (toilets constructed and ODF targets met) and cost (willingness/ability to pay)	(±) Sustainability – money from income generation and loan programs revolves within the community to support ongoing WaSH maintenance and investment; toilets built with in-kind subsidies are perceived as temporary
Forming governance structures Formation of governing bodies to coordinate and supervise activities of WaSH stakeholders	Formation of district WaSH coordination committee to supervise and facilitate program delivery, e.g., ensuring that stakeholders adhere to no-subsidy policies, monitoring ODF status	Feasibility – improve ability of stakeholders to successfully deliver programs	Consulting with implementing stakeholders – development and decision to use was made in district WaSH coordination committees	Tension for change - strong movement among implementers that WaSH is important, current conditions are unacceptable; commitments made to achieve WaSH targets Leadership engagement – commitment at all levels of the	Improvements in target outcomes for feasibility of implementation and coordinating efforts of stakeholders	(+) Adoption – improved accountability and monitoring through new governance structures speeds progress towards ODF targets (+) Acceptability – formal regulation of program activities and

(continued on next page)

Table 4 (*continued*)

Implementation strategy adaptations	Examples	Target outcomes	Process	Motivations	Intended outcomes	Unintended outcomes
				organization to improve governance and support implementation External policies and incentives – targets to achieve ODF by 2017 set under the national Master Plan Peer pressure – perception that the district is in "last place" relative to other districts for achieving ODF		shared goals reduces tension among partners formerly using contrasting approaches (+) Reach – governance structures that engage local government increase the program's coverage area
Sanctions for behavior enforcement Introducing negative reinforcement for undesirable behaviors, typically applied at the individual or household levels	"Sanitation card" is given to households upon constructing a toilet, and this card is needed to receive government services (e.g., work permits, citizenship documents) Arrest and fines issued to open defecators	Adoption – increase toilet coverage and achieve ODF targets, promote toilet use	Mixture of unsystematic and limited consulting with implementing stakeholders When stakeholders were consulted, decisions were often made among government stakeholders only against the advice of development organizations.	Tension for change – strong movement among implementers that WaSH is important, current conditions are unacceptable; commitments made to achieve WaSH targets External policies and incentives – targets set under the national Master Plan; sense of urgency created when targets are not met Peer pressure – perception that the municipality or district is backwards, inferior, or otherwise lagging on meeting WaSH targets relative to other areas Patient needs and resources – perception of non-toilet owning households as lazy or defiant; belief that households could easily build toilets but choose not to Relative advantage – perception that all other options have been tried and sanctions necessary to achieve behavior change	Some improvements in target outcomes for adoption as households constructed toilets for sanctions policies incentivizing toilet ownership/ construction, fewer changes in toilet use	(−) Acceptability – strong objection to sanctions among community members, confrontation between implementers and communities (e.g., yelling, cursing) (−) Sustainability – sanctions do not address underlying behavioral drivers and households revert to open defecation once no longer sanctioned
Rewards for behavior incentives Introducing positive reinforcement mechanisms for performing desirable behaviors or meeting WaSH targets	Cash prizes are given to municipalities that achieve ODF status	Adoption – improve WaSH conditions in the community Health and wellbeing impacts – reduce WaSH-related disease and improve health	Consulting with implementing stakeholders – development and decision to use was made in district WaSH coordination committees with support from development organizations	Tension for change – strong movement among implementers that WaSH is important, current conditions are unacceptable; commitments made to achieve WaSH targets External policies and incentives – targets set under the national Master Plan; sense of urgency created when targets are not met Cosmopolitanism – meetings with technical advisers and other implementing partners to discuss challenges and offer solutions	Improvements in target outcomes for adoption and rapid achievement of ODF	None described
Supply chain strengthening Strengthening systems for producing WaSH hardware at the local level	Providing training and wages to local youth to manufacture concrete rings for latrine pits	Feasibility – to ensure that products are available to meet demand for hardware	Consulting with implementing stakeholders – development and decision to use was made in district WaSH	Culture and values – strengthening supply chains aligns with organizations' mission to promote social and economic welfare	Improvements in target outcomes for feasibility of supply chains to meet local demand	(+) Cost – locally produced goods are more affordable (+) Adoption – local producers encourage toilet purchase among

(continued on next page)

Table 4 (*continued*)

Implementation strategy adaptations	Examples	Target outcomes	Process	Motivations	Intended outcomes	Unintended outcomes
			coordination committees	through jobs Patient needs and resources – local supply chains insufficient to meet demand		community members to increase product sales

Table 5
Participant demographics.

Participant characteristics	Number of participants
Location	
Mahottari	12
Salyan	10
Siraha	14
Surkhet	12
Gender	
Men	35
Women	13
Organizational affiliation	
Elected officials	*11*
Municipal or sub-municipal level	10
District level	1
Government employees	*6*
Municipal or sub-municipal level WaSH specialists	2
District level WaSH specialists	2
School teachers and headmasters	2
Development organizations	*17*
Municipal-level implementers	5
Program coordinators and supervisors	5
Senior staff[a]	7
Other	*14*
Small business owners and masons	5
Women's and mothers' group members	3
Journalists	2
Disabled persons organization representative	1
FCHV[b]	1
Financial cooperative manager	1
Social activist	1
Time in current position[c]	
0–1 year	7
2–3 years	21
4–5 years	9
>5 years	11

[a] Senior development organization staff include non-governmental organization regional supervisors, directors, and chairpersons, and technical advisers from multilateral organizations.
[b] FCHV (female community health volunteer). Denotes participants for whom FCHV was their only implementation role. The sample included two other participants who held elected or paid employment as their primary job but also served as FCHVs.
[c] Not applicable to participants without formal job titles (e.g., social activists).

3.3. Process and motivations for making adaptations

In our conceptual model (Fig. 1), we propose that implementers' processes and motivations for making adaptations may moderate adaptations' effects on intended and unintended outcomes. We assessed implementers' processes for making adaptations using constructs of timing and whether implementers applied any methods for systematic decision making, as described in previous sections. We assessed motivations for making adaptations in five domains: characteristics of the intervention, implementing individuals, inner setting, outer setting and implementation process. Processes and motivations for intervention and implementation strategy adaptation archetypes are summarized in Tables 3 and 4, respectively.

3.3.1. Adaptation process

Of all adaptations described by participants, most were reactive (n = 43, 96%). Only two were proactive. These two proactive adaptations were made when developing a proposal to the Asian Development Bank to fund a new municipal water treatment plant. During proposal development, the municipality and water users' committee recognized electrical blackouts as a likely barrier to plant operation and adapted the proposal to incorporate budget line items for a generator. Budget line items for a chlorination system were also added in anticipation of high fecal contamination levels.

Intervention adaptations for hardware safety/functionality were most commonly made using a systematic process of formative research, such as application of safety data, engineering surveys, or other site assessments. Intervention adaptations for hardware user acceptability typically used a systematic process of consulting communities. These adaptations were most often made by small business owners based on consumers' stated preferences in order to sell more products and increase profits.

For implementation strategy adaptations, the most common process used was systematic consultation with at least some other implementation stakeholders. Adaptations for monitoring tools, engaging new stakeholders, financing, forming governance structures, rewards for behavioral incentives, and supply chain development all used some consultation with other implementing stakeholders. Typically, these adaptations were developed in municipal or district WaSH coordination subcommittees comprising government and development organization stakeholders. Sanctions adaptations were typically developed by government implementers in consultation with other government stakeholders, without seeking—or in some cases explicitly disregarding—input from communities or development organizations who opposed sanctions. Government participants who reported disregarding opposition to sanctions often did so because they believed they knew what would be best for communities and that opposition was due to laziness or greediness of households not wanting to construct toilets without subsidies.

Adaptations developed using unsystematic processes were most often adaptations to triggering messages or engaging new stakeholders. Where we did not identify any systematic decision-making strategies, often participants reported that adaptations developed organically from their "own thinking" or experiences working, without formal consultation with others or research and evaluation. Participants describing unsystematic processes were often local NGO municipal-level implementers who lived in communities where they worked and had lay knowledge of local WaSH conditions and norms.

3.3.2. Motivations for adaptations

For all adaptation archetypes, constructs in the outer setting were the strongest motivators. For intervention adaptations to hardware for safety/functionality and user preferences, local community needs and preferences were a common motivator. Consumer demands for new products were a key motivator for hardware adaptations targeting user preferences, as was a desire to increase profit and personal income among small business owners. For adaptations for hardware safety/functionality, considerations of the local geography (e.g., drying of gravity-fed water sources) or other technical requirements (e.g., need for electricity but poor reliability of municipal grid) were key motivations.

For implementation strategy adaptions where adoption was a key target outcome (engaging new stakeholders, monitoring tools, financing

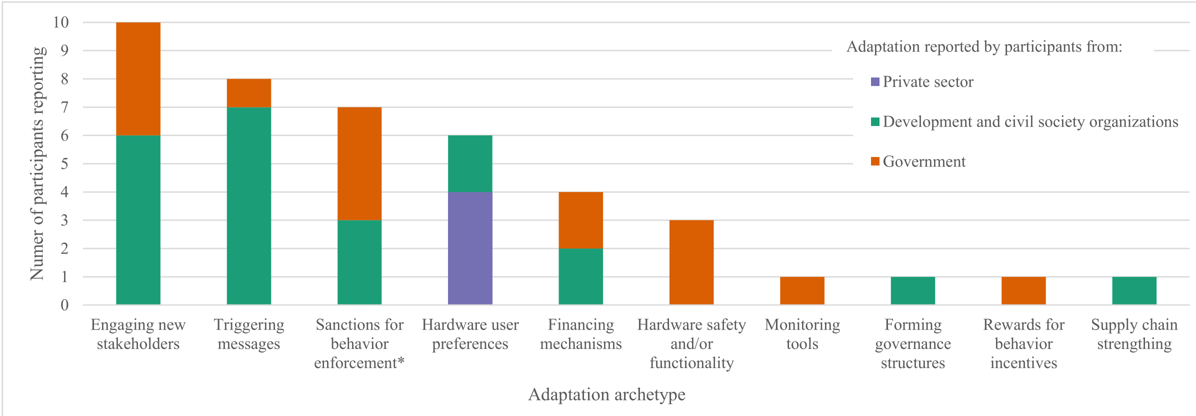

Fig. 2. Distribution of intervention and implementation strategy adaptation archetypes based on the demographic characteristics of participants describing the adaptation. *Non-government participants who reported sanctions for behavior enforcement participated in or observed committees patrolling to enforce sanctions, but this adaptation was designed in government committees and the final decision to use sanctions was made by elected government officials.

mechanisms, sanctions and rewards for enforcing behavior), we found that external policies set by the federal government to achieve ODF by 2017 were an important motivator. There was strong tension for change; implementers felt that the current situation of open defecation was unacceptable and that toilets were urgently needed to achieve ODF. In many cases, public commitments had been made to achieve ODF, and implementers were motivated to uphold these commitments. For some, peer pressure to compete with neighboring districts or municipalities was also a motivator, with feelings of shame for being "behind" or "in last place" on progress to achieve ODF.

There were several key differences in motivations we identified for sanctions versus other implementation strategy archetypes targeting adoption. Sanctions were additionally motivated by the perception that non-toilet owing households were defiant or lazy. Participants reporting sanctions believed that households could construct toilets but chose otherwise, and they believed that sanctions were the only way to force behavior change after all other approaches (e.g., triggering messages, engaging new stakeholders) had failed. In contrast, other adaptations targeting adoption were more commonly motivated by evaluating and reflecting on prior data and experiences suggesting non-sanctions adaptations would be effective and meeting with technical advisers and other stakeholders to discuss challenges and propose solutions.

3.4. Intended and unintended outcomes

We assessed both intended and unintended outcomes based on our conceptual model that adaptations would affect target outcomes (intended outcomes) and on have other positive and negative effects on other non-target outcomes (unintended outcomes). Intended and unintended outcomes for intervention and implementation strategy adaptations are presented in Tables 3 and 4 The original target outcomes for each adaptation archetype are also included in these tables.

Participants perceived that adaptations typically had the intended effects on their target outcomes. No participant reported that their adaptation had failed to meet its target outcomes, though five participants could not describe any effects on target outcomes, in part because the adaptations were newly implemented, and insufficient time had elapsed to meaningfully observe any effects.

Most adaptations had unintended effects on at least one non-target outcome. Intervention adaptations for hardware safety/functionality were reported to increase costs. This in turn decreased acceptability among households who were unwilling to pay increased prices or tariffs for goods, though household acceptance increased over time as adaptations increased service functionality and reliability.

Intervention adaptations to hardware for user preferences targeted acceptability and cost of products, to make more sales and increase personal profit and livelihood among small business owners. Higher acceptability and lower cost of products subsequently increased adoption of WaSH hardware and behaviors in the community, though participants were not specifically targeting behavior change among community members. Similarly, adaptations to triggering messages to increase adoption of WaSH behaviors and appropriateness of messages to local WaSH conditions also had unintended effects on acceptability, as households found messages delivered by local people about local conditions to be more likeable.

Implementation strategy adaptations to form new governance structures and strengthen supply chains were intended to improve feasibility but also had positive impacts on adoption and reach, as better coordinated efforts among partners created more effective and efficient program delivery, and low-cost WaSH products were widely marketed by manufacturers to increase sales. Similarly, implementation strategy adaptations to engage new stakeholders primarily targeted adoption but also increased feasibility of program delivery by helping to build a widespread social movement and support for WaSH.

Introducing sanctions to enforce behavior were the only adaptation to have exclusively negative unintended outcomes, although participants perceived sanctions to be effective in meeting short-term adoption targets to construct toilets and/or become eligible for ODF declaration. However, participants that used sanctions adaptations also described receiving "curses" or "abuse" from households and perceived that sanctions had low acceptability among the community. Furthermore, participants reported that sanctions had poor sustainability. While households constructed toilets to avoid sanctions, toilet use was not sustained after sanctions were avoided, and participants reported that some households returned to open defecation despite toilet construction. Adaptations to monitoring tools that placed visible markers on households not meeting WaSH indicators also negatively impacted acceptability, as participants reported that households felt shamed for being marked as "unclean."

4. Discussion

The purpose of this study was to document what adaptations are made to rural WaSH programs, and to assess how these adaptations affect program outcomes. Theory and empirical evidence in adaptation literature shows that while adaptations are designed to improve effectiveness or contextual fit, they can have negative impacts on program performance when they are poorly aligned with a program's theory of change (Kirk et al., 2020; Perez Jolles et al., 2019; Venkataramanan, 2016). As such, we conducted a case study of adaptations to WaSH programming in Nepal to assess the range of positive and negative outcomes adaptations that may have, as well as the motivations and processes that influence those outcomes.

We hypothesized that implementers' processes and motivations for making adaptations would influence effects on intended and unintended outcomes. This hypothesis was grounded in adaptation models and empirical evidence suggesting that adaptations that are systematically designed and weigh potential tradeoffs between outcomes (e.g., rapid adoption versus sustainability) are more likely to have positive effects on program performance (Kirk et al., 2020; Moore et al., 2013; Wiltsey Stirman et al., 2019).

Our findings support this hypothesis. We found numerous examples of adaptations introducing sanctions that were motived by intense pressure to meet ODF targets, which were made without systematic processes. These sanctions, when made under intense pressure to meet ODF targets and without systematic processes to weigh potential trade-offs, did increase toilet construction but not long-term behaviors of toilet use. Additionally, participants reported that these sanctions had negative effects on other outcomes such as acceptability and sustainability, and in extreme cases negative impacts on health and wellbeing (e.g., sanctions to disenroll children from school or deny work permits to households). In contrast, other adaptations that targeted a wider range of implementation outcomes and were systematically developed were perceived to have more lasting positive impacts.

As such, we caution implementers to carefully consider the implications of adapting programs to prioritize a single outcome such as rapid adoption at the cost of other outcomes such as acceptability, appropriateness, and sustainability, or indeed long-term health and wellbeing of participants. This study suggests that adaptations to promote rapid adoption can have trade-offs that diminish acceptability and long-term sustainability. Furthermore, adaptations to improve feasibility of program delivery or affordability and acceptability of WaSH products can indirectly improve adoption, even when it is not an explicit target outcome. Systematically consulting with stakeholders and evidence and weighing trade-offs between outcomes is important for developing effective adaptations and mitigating negative consequences.

We applied adaptation models and frameworks retrospectively to examine adaptations that had already been implemented (Kirk et al., 2020; Moore et al., 2013; Wiltsey Stirman et al., 2019). However, these same models can be applied prospectively to help implementers systematically design adaptations and evaluate the range of possible intended and unintended outcomes. Application of these or similar structured tools (e.g. Miller et al., 2020) to more comprehensively evaluate adaptations before they are implemented at scale may help to avoid widespread negative unintended consequences.

4.1. Adaptation characteristics

We characterized adaptations as either intervention or implementation strategy adaptations. Intervention adaptations were more commonly reported by private sector participants, while implementation strategy adaptations were only reported by government, development, and civil society organizations. This is likely reflective of the roles filled by each stakeholder group. Implementation was coordinated in WaSH committees led by government with participation from development and civil society organizations. Participants in these committees more commonly reported adaptations to implementation strategies. Private sector participants were rarely engaged in decision making surrounding implementation done by these committees and perceived their role in the sale of WaSH hardware as separate from other program activities. However, the success of private sector participants in adapting hardware to meet user preferences suggests that inclusion of private sector representatives in WaSH coordination committees may be a potential strategy to increase program acceptability.

Adoption was the most common target outcome for all adaptations, specifically to increase toilet construction and/or use. In many cases, participants specified the rate of adoption was important and that adaptations were made to achieve ODF as quickly as possible. For adaptations where adoption was the primary reported target outcome, national government targets to achieve ODF by 2017—a target which was not met at the time of this study—were a strong motivator, and participants shared a sense of urgency and importance in achieving ODF targets. Country-wide ODF was declared in September 2019, one month following data collection, which may have heightened the sense of urgency for ODF declaration when interviews were conducted.

4.2. Processes and motivations

There are several possible root causes of the urgency and importance placed on adoption and achieving ODF targets. High importance of meeting ODF targets may be driven in part by results-based financing (RBF) of program activities and evaluation strategies emphasizing sanitation coverage. Under RBF, implementers are required to pre-finance program activities and are reimbursed by program funders only if they meet certain pre-specified performance targets (Anderson et al., 2018; Witter et al., 2012). In Siraha and Mahottari districts, a major funding source was an RBF under which the number of people with new access to improved sanitation facilities and the number of people using improved sanitation facilities were performance targets (Ticani et al., 2020). No participants specifically described financial incentives under RBF as a motivator, but organizations' desire to receive full payment may contribute to pressure on local-level staff even if they are not fully aware of program financing structures. RBF may also influence how external policies and incentives are defined by national offices and how program targets are communicated to field teams, even when field teams themselves do not directly bear the financial risk. Overall, the importance placed on sanitation coverage and ODF in program evaluation, regardless of financing mechanism, likely contributes to prioritization of adoption over other outcomes.

Performance metrics used to evaluate government stakeholders may also drive motivations and competition for rapid ODF attainment. The number of municipalities declared ODF is a criterion in performance evaluations of Nepali civil servants (Government of Nepal National Planning Comissions, 2013; Secretariat of National Sanitation and Hygiene Coordination Committee, 2020). Government stakeholders who used sanctions were often motivated by the perception that all other feasible alternatives had been tried and sanctions were a necessary last resort to meet ODF targets.

Perception of sanctions as a last resort may be exacerbated by loss of expertise in some government offices due to government restructuring. In 2015, Nepal adopted a new federalist constitution (Goverment of Nepal, 2015), which empowered local governments to regulate WaSH but also led to loss of institutional knowledge as officials and experts were reassigned to new areas (Chaudhary, 2019; Thapa et al., 2019; World Bank, 2018). Newly elected government stakeholders with limited WaSH experience and technical advisory support may be less aware of alternative behavior change strategies and therefore more likely to apply sanctions. Nepal's constitution and national WaSH strategy gives broad authority to municipal governments to regulate WaSH, and implementation is highly decentralized. One senior development organization official indicated that the formal policy of international NGOs and multilateral organizations headquartered in Kathmandu was to not endorse sanctions. However, convincing municipal governments not to apply sanctions was difficult, given their expanded autonomy under the constitution.

While many participants reported strong pressure to meet ODF targets, not all enacted sanctions in response. Participants who received support for systematic decision making (e.g., consulting with development organizations and government technical experts, conducting formative research) made non-sanctions adaptations in response to challenges with low adoption (e.g., engaging new stakeholders or changes to triggering messages content).

Previous studies have suggested that nonsystematic adaptations can have negative unintended consequences (Moore et al., 2013). Previously observed examples of detrimental adaptations in WaSH include when implementers omit activities that are time-consuming to improve convenience (Venkataramanan, 2016). However, we found that hardware adaptations made by small business owners were motivated by a desire to increase business profitability. These adaptations had positive impacts on acceptability and cost of WaSH hardware, and by extension unintended positive impacts on adoption of WaSH products and behaviors. Similarly, adaptations made by village-level implementers to improve appropriateness of triggering messages were often made based on their intuition and lay knowledge of communities. Under the adaptation framework we applied in this study, these adaptations were considered unsystematic, though they successfully improved program outcomes. This suggests that the construct of systematic adaptation may need revision in a WaSH context. Adaptions motivated by non-evidence-based reasons (e.g., increased business profit) may still have positive outcomes, provided they target relevant outcomes in a program's theory of change. In WaSH, implementers recruited from local communities often have deep lay knowledge of the local context that makes them effective implementers (Anderson et al., 2021). While this lay knowledge does not conform to conventional research paradigms, our study suggests it is an important contribution to systematic decision making for adaptation.

4.3. Intended and unintended consequences

Participants perceived that their adaptations had the desired effects on target outcomes but also perceived that most adaptations had unintended impacts on at least one other outcome. Many unintended impacts were positive. For example, outcomes of acceptability and adoption were closely related. Participants reported that adaptations that successfully improved acceptability also increased adoption, and vice versa.

However, we also documented negative unintended outcomes. In particular, participants stated that sanctions adaptions to promote rapid ODF declaration had mixed impacts on adoption. According to participants, some households constructed toilets to avoid sanctions but continued open defecation. Sanctions to enforce WaSH behavior have been previously documented in multiple countries, particularly under CLTS approaches where communities are encouraged to create their own grassroots action plans (Bartram et al., 2012; Ficek and Novotný, 2018). Previously documented sanctions range from social embarrassment, such as sending children with whistles to disturb open defecators, to more extreme cases, such as throwing rocks or opting not to prosecute cases of sexual assault and rape perpetrated against open defecators (Devine, 2009; Kar and Chambers, 2008; Mahbub, 2008; Pattanayak et al., 2009). Participants in our study reported primarily top-down sanctions in which government officials would restrict access to services (e.g., work permits, citizenship papers) for non-toilet owning households. Participants described little to no community consultation in developing these sanctions. We found some examples of adaptations to engage community groups to patrol open defecation sites, but these groups were accompanied by top-down measures by police to enforce arrests and fines for open defecations.

Evidence on the effectiveness of government-imposed sanctions without grassroots community support is minimal. The original handbook on CLTS suggests that community-imposed sanctions for open defecators can be a sign of progress towards sustainable behavior change (Kar and Chambers, 2008). However, government-imposed sanctions without community support vitiate the theory of change of CLTS, where a social movement for sanitation is triggered from within the community as community members realize the value of sanitation and are motivated to end their open defecation behaviors (Venkataramanan et al., 2018). Our evidence suggests that top-down sanctions adaptations without community support may not be effective in achieving meaningful behavior change. We did not find any examples of grassroots sanctions where development was led by community members, likely because our sample did not include community members without a formal implementation role. Further study of informal sanctions developed within communities may yield different results and would help determine in what contexts sanctions adaptations lead to long-term, sustained behavior change.

Extreme cases of sanctions also raise questions about harmful impacts on health and wellbeing. Denial of work permits and other services has potential to negatively impact household economic wellbeing. By extension, these sanctions may also affect other outcomes like food security or school enrollment, and indeed ability to invest in safe, sustainable WaSH infrastructure, which can be prohibitively expensive for poor households (Hutton, 2012). One municipal official reported disenrolling children of non-toilet owning households from schools, though this was ultimately only enforced for a few days until being dropped out of concern for child rights and wellbeing. These examples raise concerns that intense pressure to meet WaSH coverage targets can drive implementers to make adaptations that lose sight of overall long-term health and wellbeing goals. Intense punishments like disenrolling school children may help meet WaSH targets but have potentially severe consequences for overall wellbeing. Implementers should weight the benefits of meeting WaSH targets through punishments against the potential impacts of those punishments, and should not impose sanctions that violate human rights or harm overall wellbeing regardless of their impacts on WaSH.

4.4. Next steps for improving adaptation in rural WaSH

We propose several strategies for improving adaptations and mitigating negative unintended consequences. Soliciting and meaningfully incorporating feedback from community members throughout the design and implementation of adaptations may mitigate unintended negative outcomes and avoid undue harm to poor and vulnerable households. We found that participants making adaptations with low acceptability reported hearing complaints or other negative feedback from communities, but rarely took meaningful action to address concerns. Participatory design with community members is a well-recognized practice for developing appropriate, feasible, and acceptable interventions, both in WaSH and other sectors (Budge et al., 2021; Cole et al., 2013; Foulds et al., 2021), which applies to adaptations as well (Kirk et al., 2020).

Similarly, building capacity and support for local-level implementers to engage in systematic adaptation and promoting collaboration between diverse stakeholders may mitigate negative unintended consequences. Theories on adaptation propose that systematic consideration of evidence and stakeholder consultation can improve outcomes (Escoffery et al., 2018, 2019; Kirk et al., 2020). Our findings support this theory: we found that adaptations with minimal negative unintended outcomes were encouraged by and developed with diverse stakeholders. Village-level implementers had deep knowledge of the local context but less commonly engaged in formal data collection or evaluation. Technical experts and advisors engaged in more formal data collection and formative research but were less intimately familiar with the local context. Encouraging collaboration between diverse stakeholders can ensure that the necessary skillsets are present to develop successful adaptations (Chen et al., 2012). Building capacity of local-level implementers for data collection and evaluation on adaptation effectiveness may also empower local-level implementers to develop effective adaptations without external support.

Finally, efforts to identify and incentivize target outcomes that are better aligned with programs' theories of change may yield more successful adaptations. Toilet construction is an appealing target and performance indicator that it is easy to define and objectively measure. However, this and other studies have shown it is a poor indicator of adoption, as households may construct but not use toilets (Garn et al., 2017). Performance targets that focus exclusively on toilet construction may incentivize implementers to make trade-offs between rapid ODF attainment and long-term sustainability.

Funding recipients and implementers at the local level understandably feel pressured to meet performance targets, particularly when payment or job security is conditional on meeting those targets. Funders and national governments have a responsibility to set realistic goals and appropriate performance indicators that are aligned with a program's theory of change. While measuring sustainable behavior change over years is not realistic in many cases, this research suggests that other implementation outcomes such as acceptability or appropriateness may be more feasible short-term measures that are necessary to achieve long-term health and wellbeing goals. Some indicators for evaluating program performance beyond adoption have been proposed (e.g., inclusion of diverse stakeholders and vulnerable groups in demand creation activities as an indicator of reach and appropriateness (SNV, 2019)) but not yet validated. Further research to develop robust WaSH-specific measures for implementation outcomes would help in evaluating future adaptations.

5. Limitations

To assess intended and unintended outcomes, we asked participants to self-report their perceptions. We did not triangulate these findings with community members, nor did we measure quantitative outcomes such as toilet coverage. Recall and reporting bias are therefore relevant concerns. Participants may be less likely to recognize unintended consequences if effects are subtle or not intentionally monitored. Our consent process emphasized that study findings were for research purposes only and would be anonymized. However, participants may still have been unwilling to report negative unintended consequences.

Our dataset was limited to adaptations made after WaSH program implementation had already been ongoing for multiple years. Adaptations made during program design and early implementation stages may likely have different characteristics, motivations, and processes. Similarly, adaptations made in earlier stages of program design and implementation may have different outcomes, as learning and experience gained over time may have allowed for more informed decision making.

Most adaptations that participants described related to sanitation; we found few related to water and hygiene. This is likely reflective government priority to meet national ODF targets, which were set for 2017 and not met at the time of this research. Total Sanitation targets, which include water and hygiene behaviors, were set for 2030 and lacked the same urgency as sanitation targets. We expect that adaptations related other WaSH behaviors may differ from adaptations we found in this study.

6. Conclusions

Implementers at all levels, from regional to municipal, and all stakeholder roles reported making adaptations with the intent of improving rural WaSH programs. In Nepal, toilet construction was the most common target outcome for adaptations. Pressure to meet ODF targets set by the national government was a strong motivator for making these adaptations. While participants reported that adaptations typically achieved their target outcomes, they often also reported unintended trade-offs. Notably, participants described that sanctions adaptations to enforce toilet construction had hindered acceptability and sustainability among the community.

Adaptations that prioritize a single outcome such as adoption may make unintended tradeoffs with other outcomes like appropriateness or acceptability and ultimately hinder long-term progress towards improving health and wellbeing. This concern is particularly relevant when external factors, such as policies and financing structures, incentivize a single outcome like toilet construction that is necessary but not sufficient to achieve program goals and may be only weakly associated with a program's theory of change.

When adaptations were informed by local knowledge of context and community preferences and developed through systematic processes of stakeholder consultation and data collection, they often had more positive impacts on program performance. Adaptation models and frameworks can support implementers to systematically describe adaptations and weigh potential tradeoffs between a wider range of outcomes to better improve program performance.

Author contributions are as follows

Darcy Anderson: conceptualization, investigation, formal analysis, writing – original draft, writing – review and editing. **Ankush Kumar Gupta:** investigation, writing – review and editing. **Sarah Birken:** methodology; writing – review and editing. **Zoe Sakas:** formal analysis, writing – review and editing. **Matthew Freeman:** conceptualization, writing – review and editing, supervision, funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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Addressing the preprint dilemma

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At the height of a media frenzy, a 2017 research preprint reported a "horrifyingly large" increase of fetal deaths due to lead exposure from the 2014-15 Flint, Michigan Water Crisis (Grossman and Slusky, 2017). *The Washington Post, The Economist, PBS NOVA* and *Science* ran major stories that sometimes used this exact same phrasing (Ingraham, 2017; Roy and Edwards, 2021). The reported 58% increase in fetal death rate was not statistically significant and also did not appear in the final 2019 peer-reviewed paper (Grossman and Slusky, 2017, 2019).

While several follow-up analyses, including our own epidemiological study, later found no evidence of anomalous fetal death rates during the crisis, the original preprint claim still dominates Google search results and has been cited as a fact in recent stories from *The Atlantic, Axios*, and *Detroit Free Press*, and a new *BBC* documentary (Roy and Edwards, 2021). The assertion of a "horrifyingly large" spike in fetal death rates became part of the lived experience of Flint residents creating real trauma that cannot be undone (Schaefer, 2017).

There is little guidance as to the circumstances under which scientists should consider releasing preliminary data. The COVID-19 global pandemic has ushered in a "deluge" of over 30,000 preprints in 2020 alone, which are responsible for 21% of all documented COVID-19 article retractions (n = 40/192) as of November 23, 2021 (Else, 2020; Retraction Watch, 2021). A recent commentary rightly argued that the proliferation of preprints has led to "outsourcing peer-review to practicing physicians and journalists" and lowering of publication standards in the midst of a crisis (London and Kimmelman, 2020).

As whistleblowers on the Flint Water Crisis who felt compelled to release revelatory scientific results to the media and the public while bypassing peer-review, we offer four principles and a checklist that researchers could consider before releasing preprints in real or perceived public health emergencies.

1. You must be right and act with high integrity

Corollary: One must be extremely cautious about releasing preprint results, because you are fully responsible for all failures and oversights and errors can cause real harm.

When we publicly alleged in August–September 2015 that Flint had dangerous water lead levels and was breaking Federal law, we drew upon data from a citizen science campaign that collected hundreds of samples across the city and decades of our professional experience interpreting such data. Our reputations and scientific credibility were briefly attacked by government agencies, before the crisis was eventually acknowledged and a Federal emergency declared vindicating our scientific position (Roy and Edwards, 2019). However, if we had been proven wrong, any damage to our reputations would have been deserved in our opinion.

By the same token, even if it is impossible to be 100% certain that you are right, you should do everything possible to ensure your preprint data and findings are accurate, thorough and fairly presented. A commitment to research integrity, which is the "active adherence to the ethical principles and professional standards essential for the responsible practice of research" (Korenman, 2006) is sacred and non-negotiable. A scientist who wrongly cries "Wolf!" undermines the credibility of the scientific enterprise as well as themselves.

2. The issue in question must be urgent, posing an ongoing or imminent endangerment to the public or exposing potentially illegal activity

Releasing our datasets, results and conclusions online in Flint eventually exposed government failure to implement corrosion control

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treatment and the potential imminent endangerment of a population who was repeatedly being assured their water was safe (Roy and Edwards, 2019). We openly discussed our concern about possible deaths due to *Legionella* bacteria exposure, which was also eventually proven valid (Schwake et al., 2016). Our team had to counter professional criticisms from colleagues that we had "crossed an imaginary line" in exposing the crisis, by citing the First Canon of Civil Engineering that an engineer "shall hold paramount the health, safety, and welfare of the public," and our "productive disobedience" in doing so was later recognized (Edwards et al., 2016; Sedlak, 2016; Zuckerman and Ito, 2017).

In contrast, the Flint fetal deaths preprint and accompanying press release and YouTube video came two years after residents had been protected from water lead exposure and a declared Federal emergency that mobilized the U.S. National Guard and the Federal Emergency Management Agency (Grossman and Slusky, 2017; Roy and Edwards, 2021; University of Kansas, 2017). While the economist authors may have been following their field's norm of releasing findings as preprints beforehand, emphasizing statistically insignificant results in the midst of a media frenzy, potentially contributed to widespread publicity and resident distress. Even if the finding was eventually proven to be correct (which it was not), there was never an imminent endangerment and no harm could have arisen from awaiting peer-review.

3. Embrace and enforce professional accountability

Perverse incentives in academia exacerbate pressures to obtain funding and publicity at the expense of quality science (Edwards and Roy, 2017). The Chinese phrase for "crisis" (wēijī) may not mean "danger" plus "opportunity" as is often claimed, but it does so for academics, who face asymmetric incentives and disincentives (Lenardic et al., 2021 PREPRINT). Specifically, it appears that those taking risks and being proven correct can reap the rewards, but if they are later proven wrong there are few consequences because the error was "just a preprint."

Scientists uploading rushed and erroneous preprints to the public domain should face greater consequences for reckless behavior. Preprint servers should enforce stringent screening procedures and adequately warn readers of preprint limitations (Flanagin et al., 2020). The scientific community may eventually develop and enforce tangible "disincentives" in the form of retractions and academic sanctions, but this process would require a long time because it can sometimes take years before it is understood that preprint results[1] are wrong.

Proposals by U.S. State Medical Boards to consider disciplining and suspending licenses of doctors who spread verifiable misinformation about COVID-19 and vaccines, is an example that scientific societies and professional licensing boards should consider when devising preprint policies for their members (Alba and Frenkel, 2021).

4. Choose your words with extreme care to prevent unnecessary panic or hyperbolic media headlines

Media outlets may bear some responsibility for propagating hype and spreading panic by inaccurately characterizing scientific claims. A recent peer-reviewed study found 43% of analyzed COVID-19 stories in the media did not clarify when results of studies were preprints or preliminary in nature – *The New York Times* ranked in the bottom 20% of 15 analyzed media outlets for its failure to put results in context (Fleerackers et al., 2021). The headlines and university press releases can also persist indefinitely online and are not always corrected (Ingraham, 2017; University of Kansas, 2017). A recent survey of researchers probing the benefits and challenges of preprints found that 79% were appropriately worried about "premature media coverage" (Flanagin et al., 2020).

Moreover, since reporters are vulnerable to falsely equivocating preliminary findings to facts, it is wise to adopt open science practices and take control of your preprint, beyond measures taken by preprint servers (Besançon et al., 2021; Flanagin et al., 2020; Ravinetto et al., 2021). In our case, we provided the media with summaries of key findings and in-depth analyses by maintaining a website that provided all data to anyone who asked for it in an open science format.

A checklist to consider

In a world where instant publication and viral dissemination of preliminary results can occur at the click of a button, preprint dilemmas are a serious ongoing concern (Fox, 2015; Sheldon, 2018). Before we decided to publish our Flint data online in 2015, we asked ourselves a series of questions (Table 1) that were answered affirmatively at the time and now even with benefit of hindsight.

We found another important good exemplar that checked all boxes in our list. Early preprints by aerosol scientists, warning about airborne transmission of SARS-CoV-2 via microdroplets, contradicted the conventional wisdom of public health authorities (Allen and Marr, 2020; Brosseau, 2020; Morawska and Cao, 2020). In one case, attempts for timely publication through peer review were unsuccessful *(L. Marr, Personal Communication, Aug 25, 2021)*, and we believe the scientists were vindicated in using the preprint option to correct dangerous misconceptions (Allen and Marr, 2020).

We note that there are also other personal considerations in deciding whether to go the preprint route. Sharing valuable data via preprints can allow other researchers to gain insights without completely compromising research priority and future citations, even though some academics do take "non-peer review" data and insights without appropriate citation for use in their peer-reviewed journal articles. And there can also be negative consequences of speaking up on one's career and mental health: several non-academic whistleblowers within government agencies were wrongfully terminated for exposing data about high waterborne lead in the 2001-04 Washington D.C. lead in drinking water crisis years before it became public knowledge (Roy and Edwards, 2019).

Successfully resolving preprint dilemmas will always require

Table 1
Should you release your preliminary data or preprint during a real or perceived emergency? A Checklist.

No.	Question
1	Are your findings accurate and meet high standards for quality and confidence?
2	Do you personally have access to the raw data[a]?
3	Are you working in your area of expertise?
4	Is there an ongoing crisis, illegal activity, or imminent and substantial endangerment to the public that will cause harm by waiting for peer review?
5	Are the scientific or public health authorities wrong or severely deficient in aspects of their current assessment or guidance?
6	Are you willing to publicly and immediately acknowledge any error(s) if your results are found to be inaccurate and accept adverse consequences?
7	Have you composed a brief summary for non-experts that acknowledges your findings are preliminary and are subject to limitations?
8	Are you willing to risk losing academic priority in peer-reviewed citations on behalf of the greater good?

[a] The Lancet retracted a high-profile peer-reviewed study on hydroxychloroquine/chloroquine for treatment of COVID-19, because the authors were unable to access and verify primary data sources (Mehra et al., 2020). Preprints are also vulnerable to pushing findings based on faked data, as appears to be the case in the recently retracted preprint on ivermectin treatment for COVID-19 (Davey, 2021).

[1] While citation practices are evolving to flag preprints (e.g., the suggestion to use a "PREPRINT" descriptor for citations in-text [Crotty, 2018] that we have adopted in this article for a preprint reference [Lenardic et al., 2021 PREPRINT]), their indiscriminate use in peer-reviewed articles can skew the permanent scientific record.

scientists to assess each individual situation, use a high degree of technical competence and integrity to consider risks and rewards, and releasing only accurate, methodologically sound, carefully worded and ethically defensible preprints for the right reasons.

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Author contributions

S.R. and M.A.E. wrote the Commentary.

Declaration of competing interest

M.A.E. and S.R. worked with Flint residents to expose the Flint Water Crisis, and their data, testimony and emails have been subpoenaed in several lawsuits. They are not party to any of these lawsuits. M.A.E. has been subpoenaed as a fact witness in many of the lawsuits, but he has refused all financial compensation for time spent on those activities.

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Alterations of fecal antibiotic resistome in COVID-19 patients after empirical antibiotic exposure

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ABSTRACT

As the COVID-19 pandemic spread globally, the consumption of antibiotics increased. However, no studies exist evaluating the effect of antibiotics use on the antibiotic resistance of intestinal flora in COVID-19 patients during the pandemic. To explore this issue, we collected 15 metagenomic data of fecal samples from healthy controls (HCs) with no use history of antibiotics, 23 metagenomic data of fecal samples from COVID-19 patients who received empirical antibiotics [COVID-19 (abx+)], 18 metagenomic data of fecal samples from antibiotics-naïve COVID-19 patients [COVID-19 (abx-)], and six metagenomic data of fecal samples from patients with community-acquired pneumonia [PC (abx+)] from the Sequence Read Archive database. A total of 513 antibiotic-resistant gene (ARG) subtypes of 18 ARG types were found. Antibiotic treatment resulted in a significant increase in the abundance of ARGs in intestinal flora of COVID-19 patients and markedly altered the composition of ARG profiles. Grouped comparisons of pairs of Bray-Curtis dissimilarity values demonstrated that the dissimilarity of the HC versus the COVID-19 (abx+) group was significantly higher than the dissimilarity of the HC versus the COVID-19 (abx-) group. The *mexF*, *mexD*, *OXA_209*, major facilitator superfamily transporter, and *EmrB_QacA* family major facilitator transporter genes were the discriminative ARG subtypes for the COVID-19 (abx+) group. IS621, qacEdelta, transposase, and *ISCR* were significantly increased in COVID-19 (abx+) group; they greatly contributed toward explaining variation in the relative abundance of ARG types. Overall, our data provide important insights into the effect of antibiotics use on the antibiotic resistance of COVID-19 patients during the COVID-19 epidemic.

1. Introduction

Coronavirus disease 2019 (COVID-19, or SARS-CoV-2) has rapidly spread worldwide (Auerswald et al., 2021), and it is exacting substantial medical and economic tolls upon humanity. This disease has been declared as a "Public Health Emergency of International Concern" by the World Health Organization (WHO) and is being treated with various antivirals, antibiotics, and antifungals (Miranda et al., 2020). For example, many COVID-19 patients presenting with mild disease without pneumonia or moderate disease with pneumonia are treated with antibiotics (WHO, 2020). Severely ill COVID-19 patients are treated with a variety of antibiotics to take control of the condition (Fehr and Perlman,

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2015). A recent review showed that 72% (1450/2010) of hospitalized COVID-19 patients received antibiotics (Rawson et al., 2020). Current evidence indicates low rates of bacterial/fungal co-infection in patients with COVID-19, yet high prescription rates of broad-spectrum antibiotic agents have been reported (Rawson et al., 2020). In the short term, antibiotics exert a variety of roles during the pandemic, but the long-term use of antibiotics and their misuse will contribute to the development of antibiotic resistance (Miranda et al., 2020). This should be expected a marked increase in antibiotic resistance during this pandemic.

Human intestinal flora are considered an important reservoir of antibiotic-resistant genes (ARGs), which have posed immense threats to public health over the last two decades (Feng et al., 2018; Laxminarayan et al., 2013). The bacteria carrying out antibiotic resistance can persist in human intestines for several years, even after short periods of antibiotic intake (Clemente et al., 2015; Hu et al., 2013). Moreover, it is generally recognized that horizontal gene transfer (HGT) is an important evolutionary force for the spread of the ARGs among bacteria (Forsberg et al., 2014; Goossens et al., 2005). In the COVID-19 pandemic, it is unclear whether the use of empirical antibiotics will have a net-positive or net-negative impact on the emergence and development of antibiotic resistance of intestinal flora. Knowing the impact of the use of empirical antibiotics on the antibiotic resistance of intestinal flora is essential for designing antibiotic stewardship programs during the COVID-19 pandemic.

In the work described herein, we performed a metagenomic analysis on 62 fecal samples obtained from the U.S. National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database. To the best of our knowledge, this is the first study to characterize the alterations of fecal resistome in COVID-19 patients after empirical antibiotic exposure. This study aimed to report the impact of antibiotic use on antibiotic resistance during this pandemic to characterize the broad implications of the pandemic for health and social care systems. This issue must be approached with caution so that the long-term challenges of antibiotic resistance are addressed while preserving access to effective drugs.

2. Materials and methods

2.1. Data collection and information

To explore this issue, we selected and downloaded a total of 62 fecal metagenomic datasets from NCBI BioProject No. PRJNA624223 (Zuo et al., 2020), including 15 metagenomic data of fecal samples from healthy individuals with no history of antibiotic intake in the past 3 months, 23 metagenomic data of fecal samples from COVID-19 patients who received empirical antibiotics and without discontinued use of antibiotics during stool collection, 18 metagenomic data of fecal samples from antibiotics-naïve COVID-19 patients during stool collection, and six metagenomic data of fecal samples from community-acquired pneumonia patients who received empirical antibiotics. See Supplementary Table 1 for detailed accession number information relating to specimens. The basic information of subjects is provided in Supplementary Table 1. FastQ files for all SRA accessions were obtained from the NCBI SRA repository using prefetch and fastq-dump from the SRA toolkit (https://github.com/ncbi/sra-tools).

2.2. Bioinformatics analysis

High-quality microbial sequencing data were generated by removing low-quality and short reads and human host sequences using the "metaWRAP read_qc" module (Uritskiy et al., 2018). Metagenomic sequencing yielded 33.1 million microbial reads per sample on average, with data analysis performed using MetaPhlAn3 software, a bioinformatics pipeline developed for taxonomic classification from metagenomic sequence data (Beghini et al., 2021). ARG-like reads were determined in all samples using ARGs-OAP v2.0 (Yin et al., 2018). The default parameters of ARGs-OAP v2.0 were used, i.e., a sequence identity of 80%, an E-value cutoff of 10^{-7}, and an alignment length of more than 25 amino acids. The Structured Antibiotic Resistance Genes (SARG) v2.0 database, provided by the ARGs-OAP v2.0 software developers, was used to search ARGs. The SARG database was created by concatenating the Antibiotic Resistance Genes Database (ARDB; http://ardb.cbcb.umd.edu/) (Liu and Pop, 2009) and the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/) (Jia et al., 2017), which contained 24 ARG types (e.g., tetracycline-resistant gene) and 1244 ARG subtypes (e.g., *tetA* and *tetB*).

ARG-like and 16S rRNA gene sequences were prescreened using customized Perl scripts. We calculated and normalized ARG abundance (copies of ARG per 16S rRNA) using the following equation (Li et al., 2015):

$$Abundance = \sum_{i}^{n} \frac{N_{i(ARG-like\ \ sequence)} \times L_{\text{reads}} / L_{i(ARGs\ \ reference\ \ sequence)}}{N_{16S\ \ sequence} \times L_{\text{reads}} / L_{16S\ \ sequence}} \times N_{16S\ \ copy\ \ number},$$

where $N_{i(\text{ARG-like sequence})}$ is the number of the ARG-like sequences aligned to one specific ARG reference sequence; $L_{i(\text{ARGs reference sequence})}$ is the length of the corresponding specific ARG reference sequence; $N_{16S\ \text{sequence}}$ is the number of the 16S rRNA gene sequence identified from the metagenomic reads; $L_{16S\ \text{sequence}}$ is the mean length of 16S rRNA genes sequence (1432 bp) in the Greengenes reference database (http://greengenes.secondgenome.com/downloads/database/13_5) (DeSantis et al., 2006); n is the number of the mapped ARG reference sequence belonging to the ARG type or subtype; L_{reads} is the sequence length of the metagenomic reads (150 nt).

MGE-like reads were identified and annotated in all samples by an aligned custom MGE database (https://github.com/KatariinaParnanen/MobileGeneticElementDatabase) (Pärnänen et al., 2018). Relatively strict criteria for MGE-like reads annotation with a sequence identity of 80%, an E-value cutoff of 10^{-7}, and an alignment length of more than 25 amino acids were used to reduce the number of false positives. We calculated and normalized MGE abundance (copies of ARG per 16S rRNA) using the following equation:

$$Abundance = \sum_{i}^{n} \frac{N_{i(MGE-like\ \ sequence)} \times L_{\text{reads}} / L_{i(MGEs\ \ reference\ \ sequence)}}{N_{16S\ \ sequence} \times L_{\text{reads}} / L_{16S\ \ sequence}} \times N_{16S\ \ copy\ \ number},$$

where $N_{i(\text{MGE-like sequence})}$ is the number of the MGE-like sequence aligned to one specific MGE reference sequence; $L_{i(\text{MGEs reference sequence})}$ is the length of the corresponding specific MGE reference sequence; $N_{16S\ \text{sequence}}$ is the number of the 16S rRNA gene sequence identified from the metagenomic reads; $L_{16S\ \text{sequence}}$ is the mean length of 16S rRNA genes sequence (1432 bp) in the Greengenes reference database (http://greengenes.secondgenome.com/downloads/database/13_5) (DeSantis et al., 2006); n is the number of the mapped MGE reference sequence belonging to the MGE type or subtype; L_{reads} is the sequence length of the metagenomic reads (150 nt).

2.3. Statistical analysis

Statistical analysis and graphical plotting were performed using R software. Alpha and beta diversity, principal coordinates analysis (PcoA), Procrustes analysis, permutational multivariate analysis of variance (PERMANOVA), and partial redundancy analysis (pRDA) were computed using the Vegan R package. We used the Kruskal-Wallis rank-sum test to compare the four groups and applied the Wilcoxon rank-sum test to assess the statistical significance between the two groups. LEfSe was performed to compare the characteristics of different resistance genes from the HC group, COVID-19 (abx-) group, COVID-19 (abx+) group, and PC (abx+). Spearman's rank correlation between the microbiota and ARG was calculated with Hmisc in R. Network visualization was conducted using the Gephi platform (https://gephi.org/). The correlations of the relative abundance of ARGs and MGEs were evaluated by correlation and best random forest model.

3. Result

3.1. Antibiotic resistome differences among HC group, COVID-19 (abx-) group, COVID -19 (abx+) group, and PC (abx+) group

In total, we detected 18 ARG types and 513 ARG subtypes in the fecal microbiota of the study samples. Tetracycline, multidrug, beta-lactam, macrolide-lincosamide-streptogramin (MLS), aminoglycoside, and bacitracin resistance genes were the main classes of ARGs with high relative abundance [Fig. 1(a)]. The total relative abundance of ARGs did not show any significant difference between the HC group and COVID-19 (abx-) group. Notably, the relative abundances of total ARGs in the COVID-19 (abx+) group and PC (abx+) group were significantly higher than those in the HC group [Fig. 1(b)]. The average relative abundances of ARGs in the COVID-19 (abx+) group and PC (abx+) group were 4.77 and 5.08 times higher than the HC group, respectively. No significant differences were identified in the number of ARGs between any two of the four groups [Fig. 1(c)].

Beta diversity was analyzed using PCoA by Bray-Curtis, showing that no significant difference only between the COVID-19 (abx+) and PC (abx+) groups was observed [Fig. 2(a)]. Note that empirical antibiotic exposure modifies not only the abundance of ARGs, but also their composition in the gut microbiome. We compared the within-group variation of the ARG composition based on Bray-Curtis distance and found that the dissimilarity among the subjects in the COVID-19 (abx+) group was statistically higher than those of other groups [Fig. 2(b)]. The magnitudes of the Bray-Curtis distance of four groups fall in the order COVID-19 (abx+)>PC (abx+)>COVID (abx-)>HC. Further grouped comparisons of pairs of Bray-Curtis dissimilarity values demonstrated that the similarity of the HC versus the COVID-19 (abx+) group was significantly lower than the similarity of the HC versus the COVID-19 (abx-) group [Fig. 2(c)]. In addition, the dissimilarity of the HC versus the COVID-19 (abx+) group was significantly higher than the dissimilarity of the HC versus the PC (abx+) group, which revealed that antibiotic treatment has a greater impact on COVID-19 patients compared to CAP patients.

Next, we compared differential enrichment of ARGs (linear discriminant analysis). The discriminative ARGs were differentially colored for the different groups. The *OXA_209*, major facilitator superfamily transporter, *EmrB_QacA* family major facilitator transporter genes, *mexF*, and *mexD* were the representative ARG subtypes for the COVID-19 (abx+) group. The mean relative abundance of major facilitator superfamily transporter, *EmrB_QacA* family major facilitator

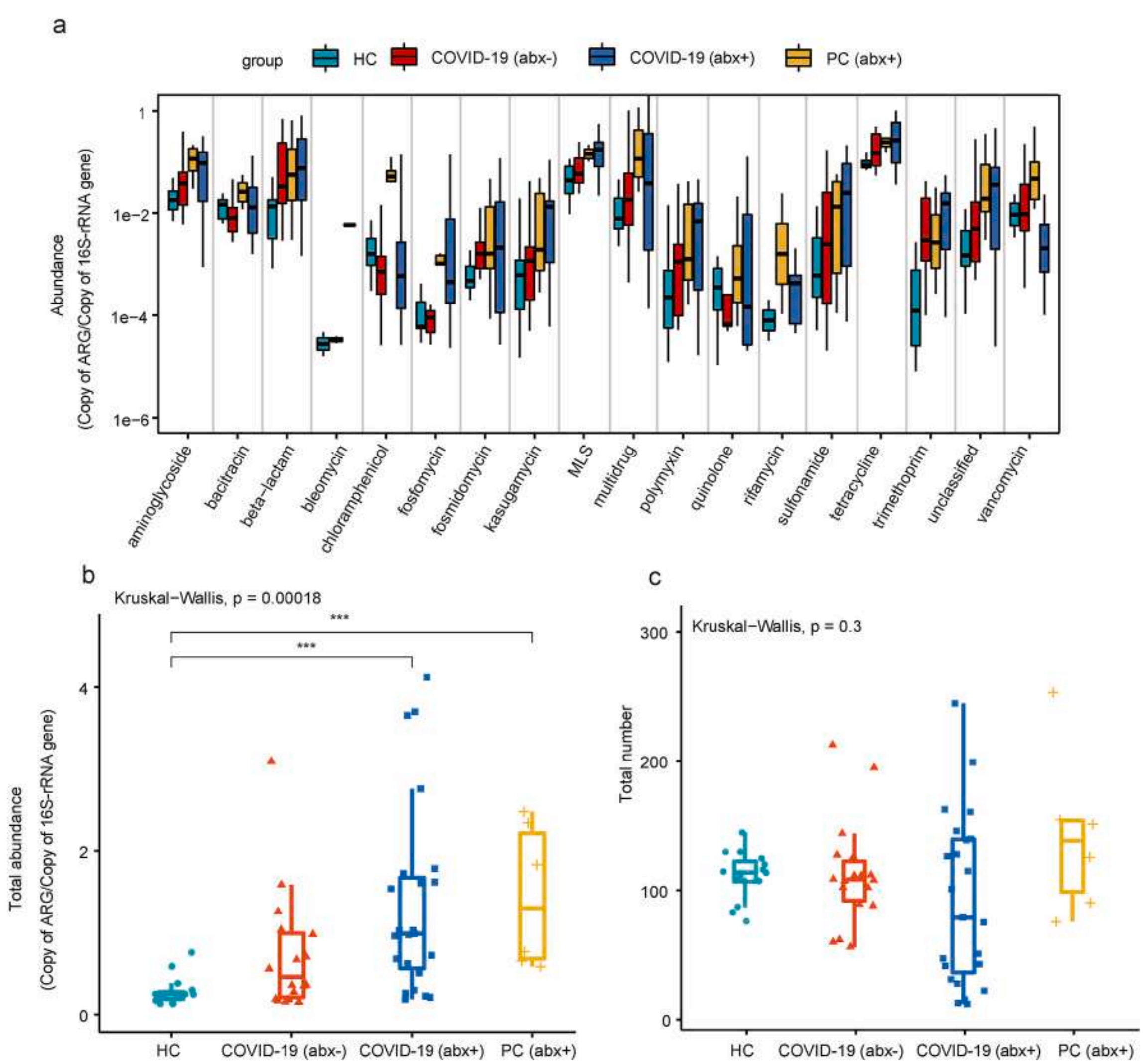

Fig. 1. (a) Comparison of antibiotic resistance gene (ARG) abundance at type level. Boxplots of (b) total relative abundance and (c) number of ARGs in four groups included in present study.

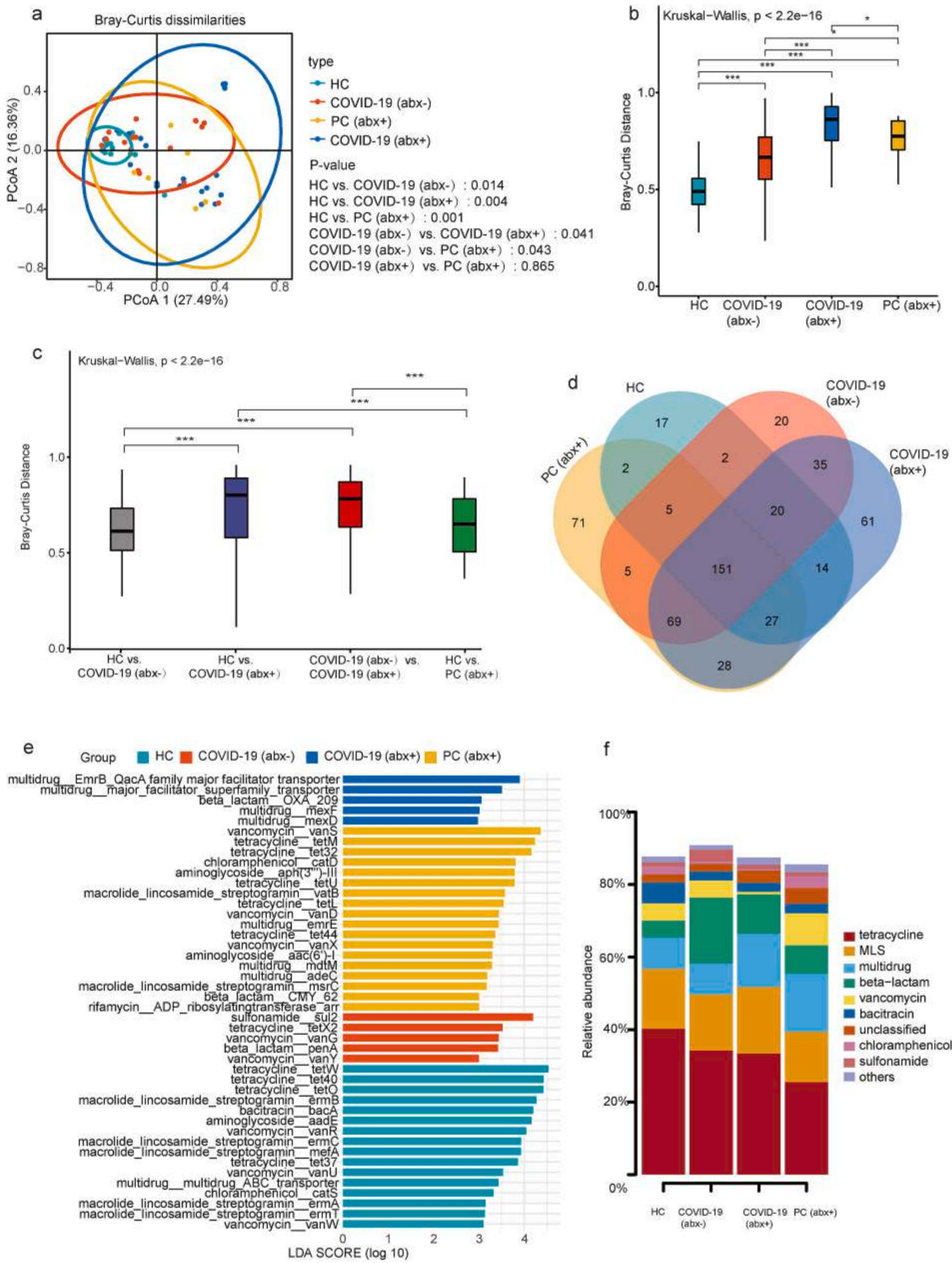

Fig. 2. (a) Beta diversity was analyzed using principal coordinates analysis (PCoA) by Bray Curtis, showing segregation of the HC group, COVID-19 (abx-) group, COVID-19 (abx+) group, and PC (abx+) group. Beta-diversity boxplots based on Bray-Curtis distances of metagenomics samples from the (b) same group (intra-beta diversity) and (c) between groups (inter-beta diversity). (d) Linear discriminant analysis effect size (LEfSe) histogram used to compare the HC group, COVID-19 (abx-) group, COVID-19 (abx+) group, and PC (abx+) group. (e) Venn diagram showing shared and unique ARG subtypes amongst four groups. (f) Shared ARGs and abundance comparisons in the HC group, COVID-19 (abx-) group, COVID-19 (abx+) group, and PC (abx+) group. In the stacked bar plot, the percentage (%) of a specific ARG in one of the four groups is equal to the ratio of its corresponding abundance to the sum of the abundances of the ARG in the four groups.

transporter genes, *mexF*, and *mexD* increased by more than 625.79-, 179.32-, 116.89-, and 341.18-fold, respectively, in the COVID-19 (abx+) group than in the HC group. The emergence and dissemination of multi-drug resistant (MDR) bacteria pose a grave public health problem (Nagarajan et al., 2018). In addition, *sul2*, *vanG*, *tetX2*, *penA*, and *VanY* genes displayed discriminativity in COVID-19 (abx-) subjects. We found that a greater proportion of *vanS*, *tetM*, *tet32*, *catD*, *aph(3''')-III*, *tetU*, *vatB*, *tetL*, *vanD*, *emrE*, *tet44*, *vanX*, *aac(6')-I*, *mdtM*, *adeC*, *msrC*, *CMY_62*, and *ADP_ribosylatingtransferase_arr* were detected in the PC (abx+) group [Fig. 2(e)].

3.2. Shared ARGs among HC group, COVID-19 (abx-) group, COVID-19 (abx+) group, and PC (abx+) group

Fig. 2(d) is a Venn diagram showing shared and unique ARG subtypes amongst all groups. A total of 151 ARGs were common to the four groups despite the total abundance levels. The total number of ARGs in the HC group, COVID-19 (abx-) group, COVID-19 (abx+) group and PC (abx+) was 238, 307, 405, and 358, respectively. The total number of ARGs in the COVID-19 (abx+) group was higher than in the HC group, COVID-19 (abx-) group, and PC (abx+). We found 17 unique ARG subtypes in the HC group and identified 20, 61, and 71 unique ARG subtypes in the COVID-19 (abx-), COVID-19 (abx+), and PC (abx+) groups, respectively. More unique ARGs were identified in the COVID-19 (abx+) and PC (abx+) groups [Fig. 2(d)]. Notably, compared with the HC group, the COVID-19 (abx+) group had 193 unique ARGs, including 156 beta-lactam resistance genes, 16 MDR genes, four aminoglycoside resistance genes, five MLS resistance genes, three trimethoprim resistance genes, two tetracycline resistance genes, two chloramphenicol resistance genes, one bleomycin resistance gene, one vancomycin resistance gene, one rifamycin resistance gene, one polymyxin resistance gene, and one unclassified resistance gene. A stacked column plot further showed detailed information on the types of shared ARGs and their abundance comparison among the HC, COVID-19 (abx-), COVID-19 (abx+), and PC (abx+) groups. Among these shared ARGs, MDR genes were more abundant in the COVID-19 (abx+) and PC (abx+) groups [Fig. 2(f)].

3.3. Correlation network of cooccurring ARG subtypes and microbial taxa

Procrustes tests depicted a correlation of 0.804 between ARGs and microbial communities (Procrustes sum of squares, 0.415; $P = 0.001$; number of permutations, 999) [Fig. 3(a)]. This result indicates that the ARG profile overall was significantly correlated with microbial communities. The microbial community composition shapes ARG distribution in the gut microbiota. Network analysis showed the detailed co-occurrence patterns between the specific ARG subtypes and microbial taxa. It was indicated that the potential hosts of ARGs could be tracked using non-random co-occurrence patterns in network analysis (Li et al., 2015; Pehrsson et al., 2016; Su et al., 2015). The correlation network consisting of 150 edges and 145 nodes was constructed based on the significant positive correlations (q-value <0.01, $r > 0.6$) between ARG subtypes and microbial taxa that occurred in at least 20% of samples [Fig. 3(b)]. The detailed cooccurrence patterns between microbial taxa and ARG subtypes are presented in Supplementary Table 2.

We speculated that 48 bacterial species were the possible hosts of 97 ARG subtypes conferring resistance to 12 kinds of antibiotics based on the co-occurrence results. Among them, *E. coli* was predicted to harbor 54 ARGs, 25 of which were MDR genes (*TolC*, *acrA*, *acrB*, *acrF*, *emrA*, *emrB*, *emrD*, *emrK*, *mdfA*, and *mdtA* et al.), with the balance being one kasugamycin resistance gene (kasugamycin resistance protein *ksgA*), one aminoglycosides resistance gene (*aadA*), one sulfonamide resistance gene (*sul2*), two fosmidomycin resistance genes (*rosB* and *rosA*), two tetracycline resistance genes (*tet34* and *tetA*), one polymyxin resistance gene (*arnA*), two MLS resistance genes (*macA* and *macB*), 12 beta-lactam resistance genes (*OXA-9*, *TEM-1*, *TEM-118*, *TEM-177*, *TEM-178*, *TEM-187*, *TEM-205*, *TEM-6*, *TEM-75*, *TEM-91*, class C beta-lactamase, and metallo-beta-lactamase), and several unclassified resistance genes. Some species (*Akkermansia muciniphila*, *Alistipes indistinctus*, *Clostridium leptum*, *Eggerthella lenta*, *Gordonibacter pamelaeae*, *Ruthenibacterium lactatiformans,* and *Streptococcus oralis*) were also predicted to harbor four or more ARG subtypes. Other bacterial species were speculated to carry three or one ARG subtypes. For instance, *K. pneumoniae* carried one ARG subtypes to fosfomycin (*fosA*), *Prevotella copri* carried one ARG subtype to tetracycline (*tet37*), and *Erysipelatoclostridium ramosum* carried three ARG subtypes to vancomycin (*vanH* and *vanD*) and MLS (*ermT*).

3.4. MGEs in fecal microbiome among HC group, COVID-19 (abx-) group, COVID -19 (abx+) group, and PC (abx+) group

According to metagenomic analysis, we detected a total of 19 MGE types among all samples. Transposase had the highest relative abundance, followed by the plasmid. Here, we show the top 15 abundant MGEs and their differences among the four groups. Notably, the COVID-19 (abx+) group had a significantly higher abundance of integrase, *IS621*, *ISCR*, plasmid, *qacEdelta*, and transposase than the HC group. The PC (abx+) group had a significantly higher abundance of *Tn916*, *IS621*, plasmid, and transposase compared to the HC group (Fig. 4). We evaluated the relative role of MGEs and phylum-level microbial composition to explain the relative abundance of ARGs based on partial redundancy analysis (pRDA). The MGEs and microbial community showed contribution to the ARGs of 17% and 18%, respectively, and their joint effect was 21%. Our pRDA analyses showed that the explanatory proportion of MGEs and microbial community for the total ARG composition was close to equal [Fig. 5(a)].

The normalized abundance of MGEs was significantly positively correlated with the normalized abundance of ARGs detected [Fig. 5(b)]. Subsequently, we revealed the correlation between ARG types and MGEs in the fecal microbiome. Transposase, *qacEdelta*, *IS621*, *IS200*, and *ISCR* contributed more significantly toward explaining variation in the relative abundance of ARG types. Transposase was significantly related to aminoglycoside, beta-lactam, sulfonamide, and trimethoprim resistance genes. *QacEdelta* was significantly associated with aminoglycoside, bacitracin, beta-lactam, fosmidomycin, kasugamycin, multidrug, sulfonamide, and unclassified resistance genes. *IS621* was significantly correlated with fosmidomycin, kasugamycin, multidrug, polymyxin, trimethoprim, and unclassified resistance genes. *IS200* was significantly related to beta-lactam, fosmidomycin, kasugamycin, multidrug, polymyxin, trimethoprim, and unclassified resistance genes. *ISCR* was significantly correlated with beta-lactam, fosmidomycin, kasugamycin, rifamycin, sulfonamide, and trimethoprim resistance genes [Fig. 5(c)].

4. Discussion

Up to 70% of the patients with COVID-19 receive antibiotic treatment either in the outpatient or inpatient setting (Langford et al., 2021). There are two main reasons that patients with COVID-19 potentially receive antibiotic therapy. First, COVID-19 symptoms may be similar to bacterial pneumonia. Second, patients with COVID-19 may acquire a secondary bacterial infection that requires antibiotic treatment (Knight et al., 2021). There are potential threats during the COVID-19 pandemic that could drive antibiotic resistance. Since the beginning of the COVID-19 pandemic, the dynamics of antibiotic resistance have remained uncertain. Meanwhile, the impact of empirical antibiotic exposure on fecal resistome in COVID-19 patients remains largely unknown.

Consistent with past research, tetracycline resistance genes were found to be dominant in gut flora (Hu et al., 2021; Pal et al., 2016; Feng et al., 2018). Interestingly, the total ARG abundances in the COVID (abx+) group were the highest when compared with the HC and COVID (abx-) groups. This finding confirmed the severe emergence of ARGs in the gut microbiota of COVID-19 patients after empirical antibiotic

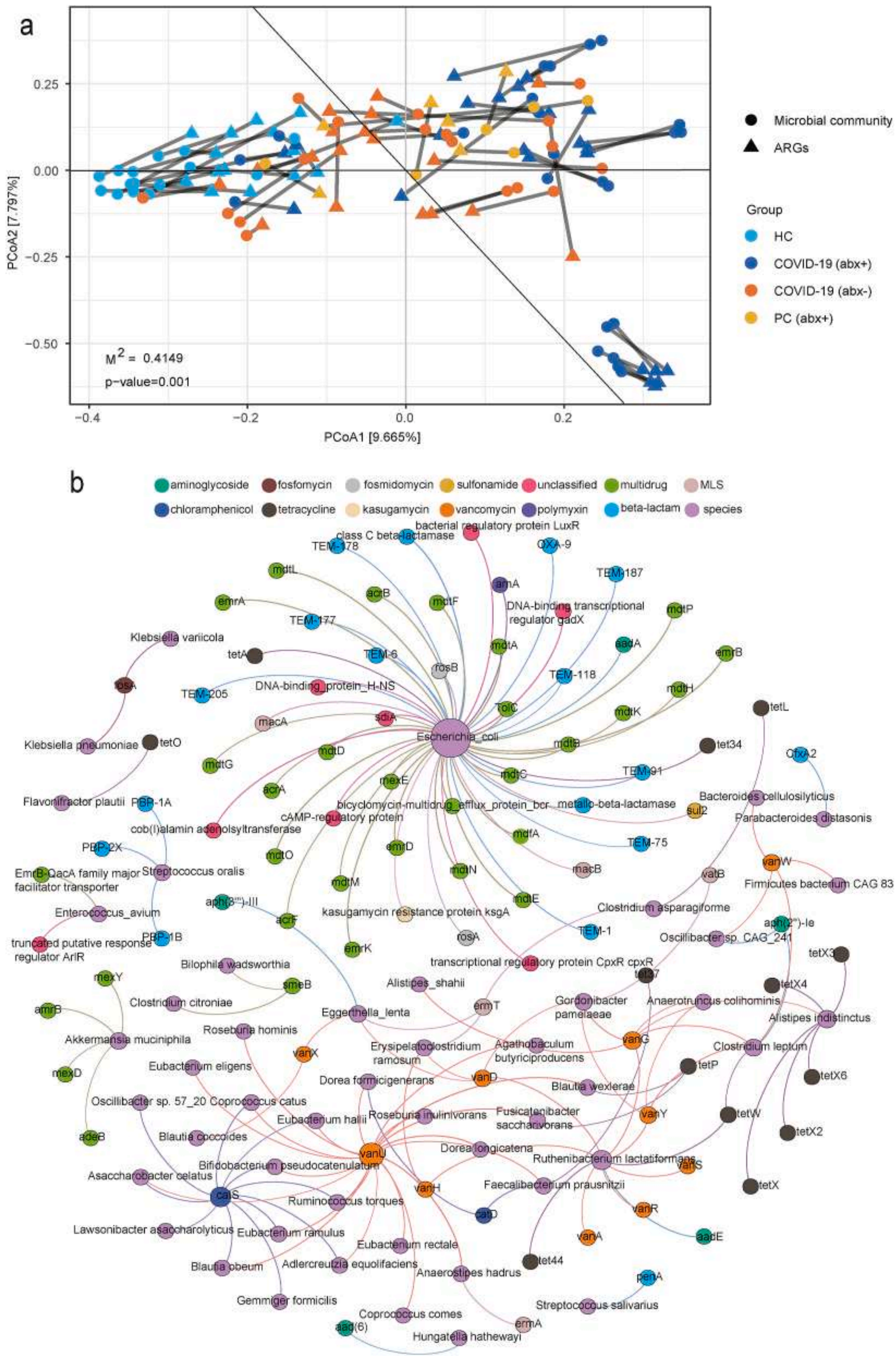

Fig. 3. (a) Procrustes analysis of correlations between ARGs and the microbial community. Statistical significance was verified by comparing the similarity of squared distances between matched samples in Procrustes analyses. P-value <0.05 indicated a significant correlation between antibiotic resistance and microbial community composition. (b) Network analysis showing co-occurrence pattern between microbial taxa and ARG subtypes based on Spearman's correlation analysis. An edge represents a strong (Spearman's r > 0.6) and significant (P < 0.01) correlation. The size of each node is proportional to the number of connections. The purple nodes represent microbial species, while the other nodes are colored according to ARG types. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

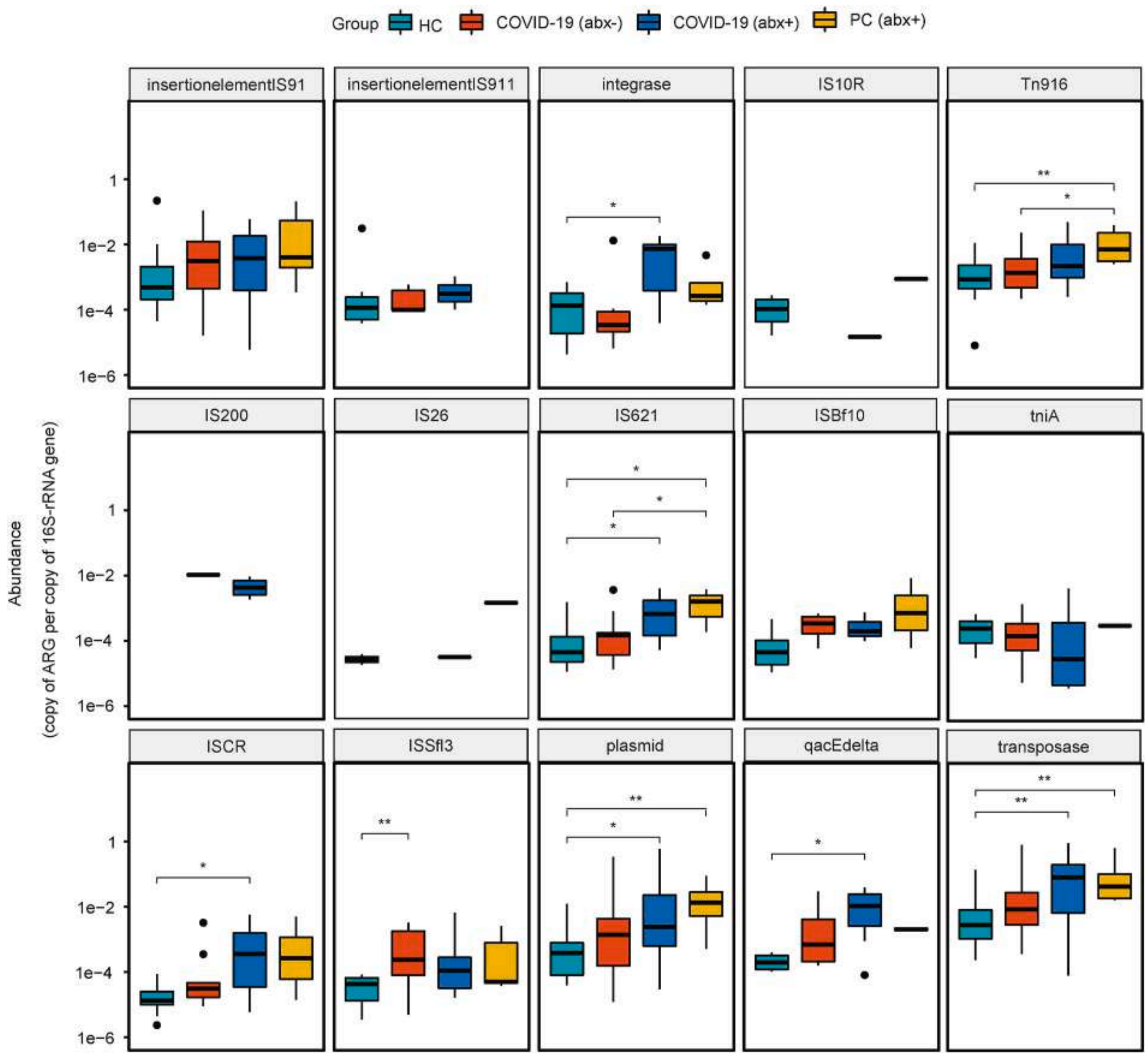

Fig. 4. Boxplots showing relative abundances and differences of MGEs among the HC group, COVID-19 (abx-) group, COVID-19 (abx+) group, and PC (abx+) group.

exposure. In addition to gut microbiota, a similar result was recently reported that ARGs dramatically increased in the oropharyngeal microbiome of COVID-19 patients (Ma et al., 2021). In addition, we found that the total ARG abundances in the PC (abx+) group were also significantly higher than those in the HC group. Furthermore, the results of a recent study indicated that the total relative abundance of detected ARGs was significantly higher in individuals with diseases (non--COVID-19) who received antibiotic therapy for 1 month than that in healthy individuals (Duan et al., 2020). Collectively, these results highlight the need for the rational use of antibiotics in clinical settings.

Although antibiotic resistance is a common phenomenon, antibiotic consumption is a leading cause of the emergence of antibiotic resistance as well as of the acquisition, development, and spread of the resistome (Forslund et al., 2013; Wright, 2007). As with most infections, the initial antibiotic choice in COVID-19 patients is often empirical (Huang et al., 2020). For patients, broad-spectrum empirical antibiotic therapy increases not only antibiotic drug resistance but also the risk of adverse side effects (Llor and Bjerrum, 2014). It is worth stressing that antibiotic-resistant infections pose a major and growing threat to global health, and these infections kill an estimated 700,000 people worldwide annually (O'Neill, 2016). If appropriate measures are not taken, this number is expected to rise to 10 million per year by 2050 (Roope et al., 2019). Our results emphasize the necessity for decreasing unnecessary antibiotic use in the clinical management of COVID-19.

Antibiotic use favors the selection of resistant microbes. Increasing antibiotic resistance in intestinal flora of COVID-19 (abx+) patients can be explained by bystander selection (Tedijanto et al., 2018). Although antibiotic treatment is focused on controlling the pathogenic bacteria that cause co-infection in the lungs of COVID-19 patients, currently available antibiotics usually have antibacterial activity against many bacterial species and disseminate extensively throughout the body (Sullivan et al., 2001). Thus, the bacteria that constitute the human microbiome are subject to the selection pressure exerted by antibiotic consumption (Gustafsson et al., 2003; Lindgren et al., 2009; Nyberg et al., 2007). Hence, we speculate that antibiotic resistance in the microbiota of other body sites may also increase in addition to the gut. This is worthy of further research in the future.

COVID-19 has resulted in an exponential increase in global biocide use, possibly inducing further antibiotic selection pressure and contributing to the selection and development of highly resistant microorganisms (Getahun et al., 2020; Ruiz, 2021). Compared with the HC group, a vast majority of unique ARGs in the COVID-19 (abx+) group are against beta-lactam antibiotics. A recent study by Peng et al. also found that beta-lactam resistance genes have increased in abundance during

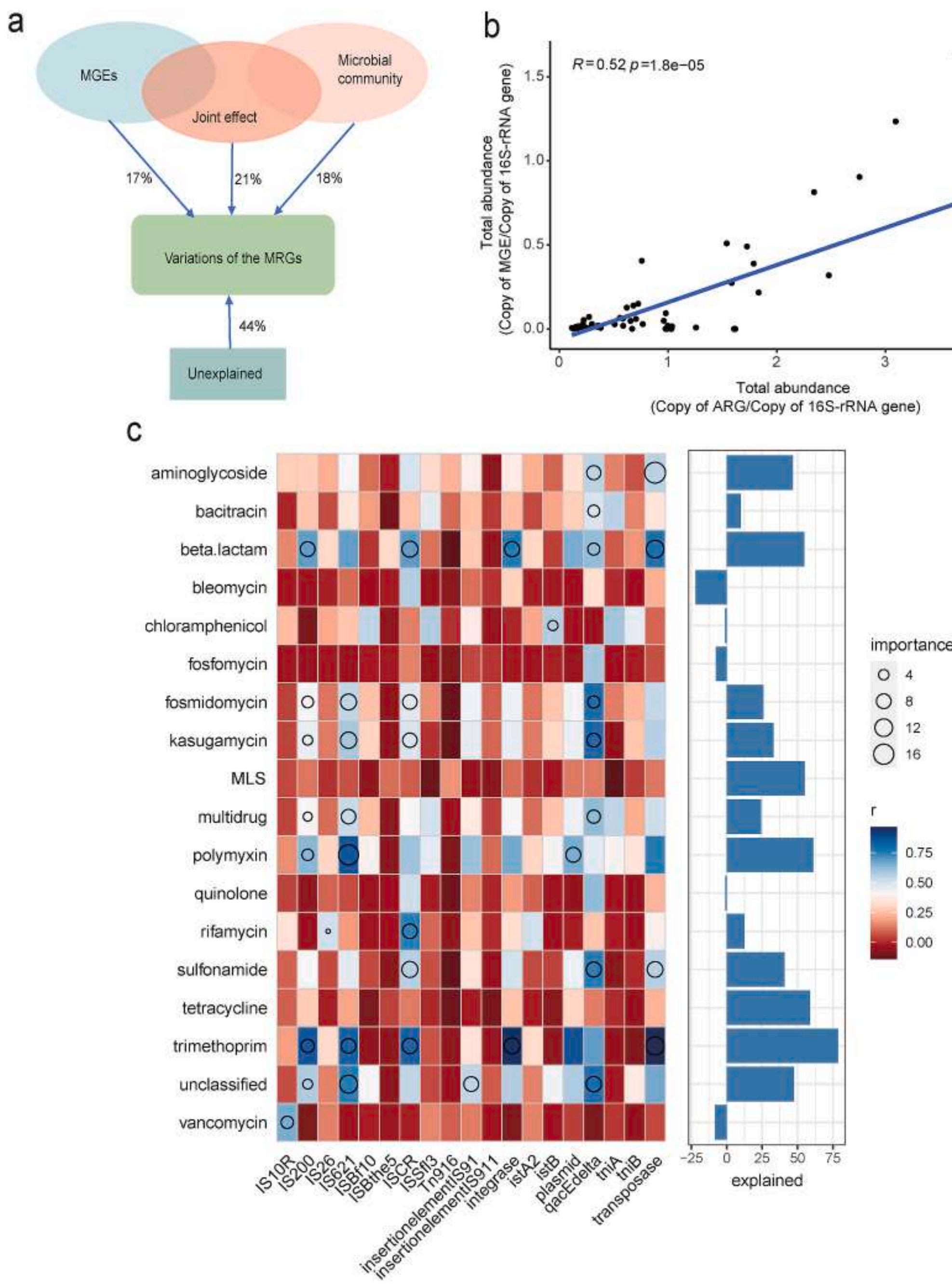

Fig. 5. (a) Total abundance of MGEs significantly correlated with the total abundance of detected ARGs based on Pearson's correlation. (b) pRDA differentiating the effect of microbial communities (at the phylum level) and MGEs on the profile of ARGs. (c) Associations of the relative abundance of ARGs and MGEs evaluated by correlation and best random-forest model. Circle size represents the variable importance (that is, proportion of explained variability estimated with out-of-bag cross-validation). Colors represent Spearman correlations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the COVID-19 pandemic (Peng et al., 2021). Four MDR genes (*mexD*, *mexF*, major facilitator superfamily transporter, and *EmrB_QacA* family major facilitator transporter) were significantly more abundant among COVID-19 (abx+) patients. MDR transporters, or efflux pumps, play a prominent role in acquired clinical antibiotic resistance (Higgins, 2007; Webber and Piddock, 2003). The emergence and dissemination of MDR bacteria can impair the clinical utility of major antibiotic agents, which could pose serious consequences (Kumar et al., 2013). The worldwide advent of MDR bacteria represents a serious public health issue. Previous research based on metagenomic analysis revealed ARG exchange events between environmental bacteria and clinical pathogens (Forsberg et al., 2012). This implies that receiving antibiotic administration not only increases the COVID-19 patient's own MDR genes of intestinal flora, but may also pollute the surrounding environment. In the future, with the wide spread of MDR bacteria, more clinically available antibiotics may prove to be ineffective.

Within-individual heterogeneity of ARG composition in the COVID-19 (abx+) group was found to be significantly higher compared with the HC, COVID-19 (abx-) groups, and PC (abx+). Meanwhile, the heterogeneity of ARG composition between the HC and COVID-19 (abx+) groups was significantly higher than between the HC and COVID-19 (abx-) groups. Procrustes analysis demonstrated that the microbial community composition shapes ARG distribution in the gut microbiota. We surmise that this heterogeneity found in ARG composition may also exist in intestinal flora. This serious disorder may have a negative impact on subsequent treatment, which is not conducive to the recovery of COVID-19 patients. Compared with antibiotics-naïve COVID-19 patients, the ARG composition of COVID-19 patients receiving antibiotic administration could require more time to return to the level of a healthy population. Whether this effect persists in the long term must be confirmed in investigations with a long-term follow-up.

Infections due to antibiotic-resistant bacteria are a major public health threat. The co-occurrence pattern network implied that 48 species might be potential hosts of 97 ARG subtypes. Among them, *E. coli* harbored maximum numbers of ARGs, 60% of which were MDR genes. A survey of antimicrobial resistances of fecal *Enterobacteriacea* in healthy people, short-term hospital patients, and long-term hospital patients also found that *E. coli* were the most highly representative carriers of resistance in all three groups (Osterblad et al., 2000). These results are similar to those of Feng et al. (2018), but *E. coli* increased the resistance to aminoglycoside, sulfonamide, and kasugamycin in our results. We posit that this could imply the emergence of *E. coli* with stronger antibiotics resistance. Considering that *E. coli* can co-infect COVID-19 patients (Zhu et al., 2020), the prevalence of ARGs is of special concern.

According to previous studies, *Streptococcus oralis* (Byers et al., 2000), *Klebsiella pneumoniae* (Martin and Bachman, 2018), *Klebsiella quasipneumoniae* (Brisse et al., 2014), *Klebsiella variicola* (Maatallah et al., 2014), *Streptococcus salivarius* (Olson et al., 2019), *Enterococcus avium* (Lee et al., 2004), *Prevotella copri* (Posteraro et al., 2019), *Alistipes indistinctus* (Parker et al., 2020), *Erysipelatoclostridium ramosum* (Zakham et al., 2019), *Eggerthella lenta* (Bo et al., 2020), and *Anaerotruncus colihominis* (Lau et al., 2006) can be pathogens or potential pathogens that can cause diseases in humans. The fact that these ARG-carrying pathogens are able to resist antibiotics taken by humans represents a high risk to human health.

HGT is a common event in the microbial ecosystem of the human intestinal tract (McInnes et al., 2020). A concerning result is that, compared to the HC group, the abundances of *IS621*, *qacEdelta*, transposase, and *ISCR* were significantly increased in the COVID-19 (abx+) group. These four MGEs also contributed significantly toward explaining variations in the relative abundance of ARG types. For COVID-19 patients, the elevated abundance of these MGEs may increase the opportunity for horizontal gene transfer of ARGs in gut microbiota (Abe et al., 2020; Lu et al., 2018; Larsson et al., 2018). What is more worrying is that HGT in this niche has the potential to mediate ARG transfer from commensal organisms into potential pathogens (Broaders et al., 2013). Consequently, it is possible for *E. coli*, *K. pneumoniae*, *enterococci*, and other opportunistic pathogens inhabiting the human gut to acquire resistance determinants from other members of gut flora (McInnes et al., 2020). ARGs that are carried on mobile genetic elements in pathogens could give rise to an immediate threat to the successful treatment of clinical bacterial infections.

5. Conclusions

The results of this study indicate that the use of empirical antibiotics has a significantly negative impact on the antibiotic resistance of intestinal flora. To reduce the potential long-lasting effects on antibiotic resistance and promote access to effective antibiotics, the development of treatment guidelines to limit unnecessary antibiotic exposure and of measures to maintain conventional surveillance of antibiotic resistance must be at the forefront of research in the post-COVID-19 era.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Antibiotic-resistant *Escherichia coli* isolated from dairy cows and their surrounding environment on a livestock farm practicing prudent antimicrobial use

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A B S T R A C T

On a livestock farm where antimicrobial administration and its history had been managed for prudent use of antimicrobials, we surveyed antibiotic-resistant *Escherichia coli* strains isolated from cow feces and the surrounding environment (i.e., rat and crow feces, and water samples from a drainage pit and wastewater processing tank) every month for 1 year. Two strains (1.7%) in cow feces were resistant to tetracycline, whereas all other strains were susceptible to all other antimicrobials. Among 136 strains isolated from cows and wild animals, only one ampicillin-resistant strain was identified. The antibiotic resistance rate in the drainage from the barn was 8.3% (10/120), and all strains showed susceptibility for 8 months of the year. Tetracycline resistance was common in all resistant strains isolated from animal feces and water samples; all tetracycline-resistant strains carried *tet*A. These results strongly support the proper use and management of antibiotics on farms to minimize the outbreak and spread of antibiotic-resistant bacteria.

1. Introduction

Antibiotics are indispensable for the life support and health management of humans and animals and have been widely used for the treatment of infectious bacterial diseases. However, antibiotic-resistant bacteria can arise by administration of antibiotics to humans and animals, and they are excreted and discharged into the environment as hazardous microbes (Sawant et al., 2007; Jia et al., 2017; Menz et al., 2019; Tullo et al., 2019; Dafale et al., 2020). Currently, there are worldwide concerns regarding the outbreak and spread of infectious diseases caused by antibiotic-resistant bacteria. Indeed, the annual death toll worldwide from antibiotic-resistant bacteria is reported to be 700,000, but this number could exceed 10 million by 2050 (O'Neil, 2014). The World Health Organization and Centers for Disease Control and Prevention (CDC) have selected antibiotic-resistant bacteria that pose a threat to the world and published research data that warn of the seriousness of the problem (CDC, 2019; Willyard, 2017). The spread of antibiotic-resistant bacteria is also serious in Japan. According to a report by the National Center for Global Health and Medicine, 8000 deaths occur annually due to methicillin-resistant *Staphylococcus aureus* and fluoroquinolone-resistant *Escherichia coli* (Tsuzuki et al., 2020).

Antibiotics are used on livestock farms to treat animal diseases and to effectively utilize the nutritional components in feed. In fact, more antibiotics are used on farms than are used in humans. The annual amount of antibiotics used in Japan is 581.3 ton/year for humans and 915.5 ton/year for livestock animals including feed additives (AMR Clinical Reference Center, 2018). The most important meat-producing countries, such as China, the USA, and Brazil, all use large amounts of antibiotics during meat production, while Japan and countries in Europe also use antibiotics on a large scale (Center for Disease Dynamics, Economics and

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Policy, 2015). Consequently, livestock farms are recognized as an important source of antibiotic-resistant bacteria, which may even be transmitted to humans via meat (Asai, 2008). In the Netherlands, methicillin-resistant *S. aureus* (MRSA) derived from pigs has also infected livestock industry personnel (van Loo et al., 2007). In addition, transmission of antibiotic-resistant bacteria from livestock-derived compost to fields (Sengeløv et al., 2003; Zhang et al., 2020) and vegetables (Marti et al., 2013) has been confirmed. Furthermore, studies have shown that antibiotic-resistant bacteria can spread from livestock wastewater to natural rivers (Wei et al., 2011) and may be transported into the natural environment via small animals (Furness et al., 2017; Zanardi et al., 2020; Nishimura et al., 2021).

Managing the amount and careful administration of antibacterial substances used will suppress the spread of antibiotic-resistant bacteria (Fujimoto et al., 2021; Nicola et al., 2021). However, maintaining the productivity of livestock farms is often difficult while properly using antibiotics. Thus, to promote the proper use of antibiotics in livestock animals, it is necessary to accumulate and share data on the actual conditions and antibiotic resistance rates from practical cases.

In this study, we focused on barn-reared dairy cows at the Sumiyoshi Livestock Science Station (known as Sumiyoshi Farm) attached to University of Miyazaki, Japan, where the administration of antibiotics and their history for all individuals has been recorded and managed. Antibiotic-resistant *E. coli* (AR-*E. coli*) isolated from cows and the surrounding environment was surveyed every month for 1 year. The strains of *E. coli* were isolated not only from the feces of dairy cows but also the feces of wild rats and wild crows living around the barn on the livestock farm. In addition, water samples were collected from the drainage pit and wastewater processing tank. The actual state of AR-*E. coli* on this livestock farm was then examined based on the resistance rate of strains collected from samples, the antibiotic resistance profile, and antibiotic administration history.

2. Materials and methods

2.1. Survey outline

The reasons for focusing on dairy cows were as follows: (1) dairy cows are extremely important industrial animals; (2) their rearing conditions are maintained and managed; (3) they are directly administered antibiotics for the treatment of mammitis and the dose is strictly controlled; (4) the rearing period of dairy cows is longer than that of beef cattle and/or swine; and (5) since the cows excrete a large amount of feces in the barn and surrounding area during rearing, they continuously affect the environment surrounding the farm. In addition, we assumed that rats and crows were vectors for antibiotic-resistant bacteria. The black rat (*Rattus rattus*) nests in the dairy barn, eats livestock feed and spilled feed mixed with cow feces, and drinks water from the water dispenser. The large Japanese field mouse (*Apodemus speciosus*), which lives in copse areas outside the barn, was also investigated as a contrast to the black rat. Carrion crows (*Corvus corone*) have been observed flying into the barn and pecking at the cows' feed and feces. Drainage is mixed with dairy cow manure, waste milk, and washing water from the barn. In addition, *E. coli* was targeted because it exists in the intestinal tract of warm-blooded animals and has the characteristic of easily acquiring antibiotic resistance depending on the antibiotics used in the host (Looft et al., 2012); *E. coli* can also adapt and survive in the natural environment (Ishii et al., 2006); several types of *E. coli* strains can cause diverse intestinal and extraintestinal diseases in healthy humans by means of individually acquired virulence factors, including Shiga toxins (Kaper et al., 2004). *Escherichia coli* are one of the most important bacteria because of the fear that antibiotic-resistant strains could spread from livestock farms.

2.2. Sampling

The Sumiyoshi Farm is the first facility in Japan to obtain GAP certification (certified in July 2014), which is an international initiative, in the field of livestock. As of March 2020, the number of farms with Global GAP certification for livestock farms in Japan is three management bodies (the total number of management bodies in Japan 63,790) (Ministry of Agriculture, Forestry and Fisheries, Japan, 2020; 2021). The farm manages to ensure various components of sustainability, including food safety and environmental conservation. In total, 12 surveys were conducted, once per month, from July 10, 2018 to June 25, 2019 for 1 year at the dairy barn in the Sumiyoshi Farm and in the surrounding area. At this farm, the dairy cow feed is self-mixed and does not contain antibacterial substances as feed additives. In addition, the administration of antibiotics for treatment is performed under the direction of a farm management veterinarian and the administration history (administration date and dose) is recorded. The farm has been prudently using antimicrobials for >10 years. Images of the dairy barn and each sampling point are shown in Fig. 1. The number of dairy cows reared during the survey period was 32–37, with an average of 34 per month.

The feces of dairy cows, black rats, and crows were collected in the barn and surrounding area. Ten fresh fecal samples excreted from each individual dairy cow were randomly selected and collected in a sterilized 50-mL polyethylene tube with a sterile spatula. All cow feces samples could be collected in 12 surveys, for a total of 120 samples. Black rats were captured by setting an adhesive mouse sheet (Sankyo-Shodoku Co., Tokyo, Japan) in the barn. As a result of conducting a survey 12 times, we captured three individual black rats in October 2018, two in December 2018, one in January 2019, and two in February 2019 (eight individuals in total). In the other survey months, we could not capture black rats. Rat feces were collected from the anus of each captured individual with a sterile cotton swab and placed in a sterile 15-mL polyethylene tube together with the sterile swab. During sampling, we observed crows flying to the trees near the dairy barn where their feces fell to the ground. Therefore, a survey of crows' feces was additionally conducted during the period from November 2018 to June 2019. Crows' feces could be collected each time in eight monthly surveys from November 2018 to June 2019. Fresh feces confirmed to be excreted from crows flying to the barn area were collected with a sterile cotton swab and placed into a 15-mL polyethylene tube. Finally, field mice were captured by setting up a live trap (Sherman Trap; H.B. Sherman Traps Inc., FL, USA) at a copse 300–500 m away from the Sumiyoshi Farm dairy barn. We conducted a survey to capture field mice in October 2018 and captured five individuals. The feces excreted from individual field mice were collected with tweezers and placed into 15-mL polyethylene tubes.

The drainage from the barn was collected from the drainage pit (i.e., the pit water) using a dipper and placed into a sterilized 1-L polyethylene bottle. The drainage and washing wastewater generated from the entire rearing facility on the farm, including the pit water, are transported to a wastewater treatment facility where the water is processed in an aerobic batch-type tank. The wastewater treatment system sequentially stores wastewater in a terete reaction tank (capacity 45.5 m^3, diameter 13 m, height 3.5 m). The wastewater is treated under aerobic and anaerobic conditions by turning the aeration on and off. The mixed liquor suspended solids are not controlled in the reaction tank. When the reaction tank is full, the treated water is subsequently sprayed onto the fields. The stored wastewater in the tank (known as tank water) was collected in a 1-L polyethylene bottle. During the survey period, due to a breakdown of the treatment facility, it was not possible to sample the tank water during the period from October 2018 to January 2019.

All samples were placed in a cool box without a refrigerant after collection and taken back to the laboratory. *E. coli* isolation was conducted within 3 h after the survey.

Fig. 1. Images of the survey area and sampling points at the Sumiyoshi Livestock Science Station.

2.3. Collection of *E. coli* strains

Escherichia coli were isolated from each sample using the membrane filter method. For cow and field mouse feces, approximately 0.1 g of fecal sample was dispensed into a sterilized 15- mL tube using a sterile cotton swab, and then 10 mL of sterile physiological saline water was added to prepare a suspension. The physiological saline water was adjusted to 0.90% sodium chloride in ion-exchange distilled water and then sterilized. Similarly, 10 mL of saline water was added to the sample tubes containing cotton swabs with the feces of black rats or crows without weighing the feces. These suspensions were then serially diluted from 10- to 10^3-fold. The diluent was filtered through a membrane filter (diameter: 47 mm; pore size: 0.45 μm; Advantec, Tokyo, Japan) and the filters were placed on CHROMagar ECC agar plates (CHROMagar, Paris, France) for incubation at 37 °C for 24 h. After incubation, blue colonies putatively identified as *E. coli* were picked from the ECC agar plates and purified by repeated single-colony isolation on the same medium. The isolates were incubated on brain heart infusion agar plates (1.5% agar; Becton, Dickinson and Company, NJ, USA) at 37 °C for 18 h and then species were identified. By this series of isolations, 10 strains of *E. coli* were isolated and collected from each fecal sample. If less than 10 positive colonies were found, all positive strains were isolated. The pit water and tank water were serially diluted from 10- to 10^4-fold. Similarly, the diluents were filtered through membrane filters and placed on CHROMagar ECC agar plates. In the same manner as the fecal samples, 10 strains of *E. coli* were isolated from each water sample.

2.4. Identification of *E. coli*

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis was used for species identification (Suzuki et al., 2018). An aliquot (1.0 μL) of colony suspension was spotted directly onto a 384-well stainless-steel target plate (MTP 384; Bruker Daltonics, Billerica, MA, USA). Following air-drying for 10 min, a template was overlaid with 1.0 μL of the matrix solution. All samples were analyzed using an Autoflex III TOF/TOF (Bruker Daltonics, Billerica, MA, USA) operated in the linear positive mode within a mass range of 2000–20,000 Da based on the manufacturer's instructions. For database construction and validation, measurements were performed in the auto-execute mode using Flex Control 3.4 software (Bruker Daltonics). The software settings were as follows: linear positive: 3–20 kDa; detector gain: 2691 V; laser shots: 40–200; laser power: 30%. A Bruker bacterial test standard (part no. 8255343, Bruker Daltonics) was used for instrument calibration. Recorded mass spectra were analyzed with the MALDI Biotyper Compass (Bruker Daltonics) under standard settings. The MALDI Biotyper output is a log score value from 0.000 to 3.000; the *E. coli* identification score was >2.000.

2.5. Determination of minimum inhibitory concentration

An antibiotic susceptibility test was performed on one strain from each fecal sample identified. Ten isolated strains were randomly numbered. Then, *E. coli* isolates were identified using MALDI-TOF-MS. Among the identified *E. coli* isolates, the isolate with the lowest number of colonies was selected for the antibiotic susceptibility test. In addition, 10 identified *E. coli* strains isolated from pit and tank water were tested. The minimum inhibitory concentration (MIC) of each antimicrobial agent was determined via the microliquid dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). The *E. coli* isolates were cultured at 37 °C for 18 h in Mueller–Hinton broth (Becton Dickinson, Sparks, MD, USA) and then diluted to a final concentration corresponding to the 0.5 McFarland turbidity standard with fresh Mueller–Hinton broth. Inocula were then applied to the microplate surface containing graded concentrations of each antimicrobial agent in a microplate well (Eiken Chemical Co., Tokyo, Japan). The plates were incubated at 37 °C for 18 h before MICs were determined. MIC breakpoints for resistance (susceptibility: S, intermediate resistance: I, resistant: R) were based on the CLSI criteria.

The antimicrobials used in the current study (all from Wako Pure Chemical Industries, Ltd., Osaka, Japan, unless otherwise stated) included ampicillin (ABPC; breakpoints concentrations; S ≤ 8, I = 16, R ≥ 32 μg/mL) as a representative penicillin; cefazolin (CEZ; S ≤ 2, I = 4, R ≥ 8 μg/mL) and cefotaxime (CTX; S ≤ 1, I = 2, R ≥ 4 μg/mL) as representative cephem antimicrobials; imipenem (IMP; S ≤ 1, I = 2, R ≥ 4 μg/mL) as a representative carbapenem; gentamicin (GM; S ≤ 4, I = 8, R ≥ 16 μg/mL) and kanamycin (KM; S ≤ 16, I = 32, R ≥ 64 μg/mL) as representative aminoglycosides; tetracycline (TC; S ≤ 4, I = 8, R ≥ 16 μg/mL) as a representative tetracycline; nalidixic acid (NA; S ≤ 16, R ≥ 32 μg/mL) and ciprofloxacin (CPFX; S ≤ 1, I = 2, R ≥ 4 μg/mL) as representative fluoroquinolones; sulfamethoxazole/trimethoprim (SMX/TMP; S ≤ 2/38, R ≥ 4/76 μg/mL) as a compound; and chloramphenicol (CP; S ≤ 8, I = 16, R ≥ 32 μg/mL; Sigma-Aldrich) as a representative phenicol. The reference *E. coli* strain ATCC25922 was used for quality control.

2.6. Detection of the tetracycline resistance gene tet by PCR analysis

For the strains that were resistant to tetracycline, the types of tetracycline resistance gene, *tet*, were detected by PCR analysis. The target types of *tet* gene were *tetA*, *tetA* (P), *tetB*, *tetB* (P), *tetD*, *tetH*, *tetL*, *tetM*, *tetT*, and *tet37* (Aminov et al., 2001; Jin et al., 2002; Call et al., 2003; Diaz-Torres et al., 2003). The sequence information of each *tet* was referred to in the comprehensive antibiotic resistance database (CARD, Alcock et al., 2020). The primers and probes specific for each *tet* gene were designed using Primer3 web tool (Untergasser et al., 2012; Table S1) and purchased from Integrated DNA Technologies (IDT). DNA was extracted using the InstaGene matrix (Bio-Rad, Laboratories Inc., USA) according to the manufacturer's recommendations. The reaction was conducted with 20-μL volume containing 10 μL of SsoAdvanced Universal Probe Supermix (Bio-rad Laboratories Inc., USA), 2-μL of primer probe mix (primer: 5 μM; probe: 2.5 μM), 3 μL of nuclease-free water, and 5 μL of template DNA. A thermal cycler (CFX-96 Touch, Bio-Rad Laboratories Inc., USA) was used for the PCR reaction. The reaction conditions for PCR were 95 °C (30 s), and reactions at 95 °C (10 s) and 60 °C (30 s) for 40 cycles. The specificities of the *tet* assays were compared using a standard DNA (Table S2) that was designed based on sequence information in CARD (Alcock et al., 2020) and were purchased from IDT. The endpoint fluorescence of the sample and standard DNA at each thermal cycle was measured. When a fluorescence signal from sample confirmed until 40 cycles, the sample DNA was considered positive. Nuclease-free water was used as a negative control. Reactions for the DNA template and control DNA were run in two replicates to detect *tet*.

2.7. Statistical analysis

To examine the statistical differences in the proportions of antibiotic-resistant strains in the different sampling environments, we used Fisher's exact test following Holm's multiple comparison test; we used fisher. multcomp in theRVAideMemoire package under R ver. 3.6.3. In this process, only the Cow, Pit, and Tank environments were compared owing to the smaller sample size for the other environments.

3. Results and discussion

3.1. Antibiotic resistance rate and resistance profiling

An antibiotic susceptibility test was conducted on 341 strains of *E. coli* (120, 8, 5, 8, 120, and 80 from cows, black rats, Japanese field mice, crows, pit water, and tank water, respectively) isolated and identified from each sample (Table S3). Fig. 2 shows the resistance rate to the antibiotic agent(s) in the strains from each type of sample. Fisher's exact test results showed no significant differences in the resistance rate among the Cow, Pit, and Tank samples (p = 0.102–785). In addition, the ratio of susceptible strains and strains resistant to 1–4 antibiotics is shown in Fig. S1. Tetracycline resistance was common to all resistant

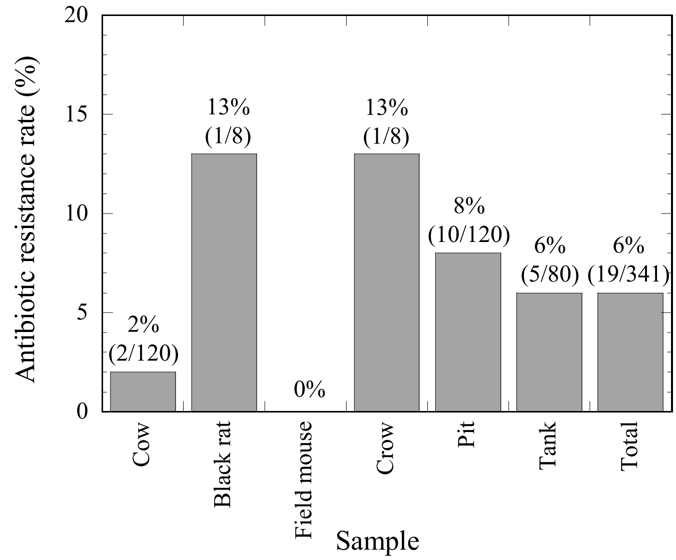

Fig. 2. Resistance (%) to one or more antibiotics in the *Escherichia coli* strains isolated from each sample.

strains sampled from animal feces and water, except for one strain in the tank water. The antibiotic resistance rate of *E. coli* from cows, which play a leading role as source from which the strains spread, was 1.7% (2 resistant strains of 120 strains). From the March 2019 samples, only two strains were resistant to tetracycline while the other strains were susceptible to all antibiotics. According to national drug resistance statistical data from Japan in 2018 (AMR Clinical Reference Center, 2018), the resistance rate of tetracycline in *E. coli* from healthy cattle in livestock farms is 26.5% on average, which is 13-fold higher than the resistance rate detected in this study. In addition, referring to data from the AMR Clinical Reference Center (2018), the resistance rates of specific antibacterial drugs were as follows: ABPC: 11.6%; CEZ: 0.5%; CTX: 0.0%; GM: 0.0%; KM: 0.0%; CPFX: 0.5%; NA: 2.1%; CP: 4.8%; and SMX/TMP 5.3%. Data from the AMR Clinical Reference Center (2018) were based on the test results of the isolated *E. coli* strains cultured in a regular medium (without antibacterial agents) (Kijima-Tanaka et al., 2003), which was similar to the data analyzed in this study. Consistent with this survey result and AMR report, CTX-, GM-, and KM-resistant *E. coli* were not detected, and it is considered that farms in Japan are not the source of these antibacterial-resistant bacteria. Notably, these resistance rates largely differ from the results obtained in this study, which showed susceptibility to each antibiotic. Indeed, the antibiotic resistance rates of dairy cows on the studied farm were extremely low in comparison to the rates in other cattle in Japan and overseas. (DeFrancesco et al., 2004; Sawant et al., 2007; Cheney et al., 2015). High resistance rates of 33.3%–93% have been reported for TC-resistant *E. coli* from dairy cows in many farms in Asia, the UK, and the USA (DeFrancesco et al., 2004; Sawant et al., 2007; Cheney et al., 2015, Hennessey et al., 2020; Sobur et al., 2019), and farms would be one of the sources of TC-resistant *E. coli*. In addition, resistant strains have not been detected in Japanese farms; CTX-, GM-, KM-resistant strains have been detected as follows: CTX, 3.1% (Cheney et al., 2015); GM, 0.3%–12.76% (DeFrancesco et al., 2004; Cheney et al., 2015, Sobur et al., 2019); and KM, 42.55% (Sobur et al., 2019). It is inferred that there are differences in the use and management of antibacterial agents on farms between Japan and overseas countries in Asia, the UK, and the US.

The antibiotic resistance rate of *E. coli* from black rats captured in the barn was 12.5% (1 of 8 samples); the resistant strain showed resistance to ampicillin and tetracycline. The resistance rate of *E. coli* from crows was also 12.5% (1 of 8 samples), with the only resistant strain showing resistance to tetracycline. The antibiotic resistance rate of *E. coli* from wild animals living around the barn was lower than that in livestock in domestic farms in Japan based on a large-scale study that compared antimicrobial-resistant bacteria from different regions in Japan (Yoshizawa et al., 2020). Our data confirm that the acquisition and transmission of AR-*E. coli* to wild animals around the barn from the feces of dairy cows had not occurred to a great extent at Sumiyoshi Farm. In addition, antibiotic-resistant strains of *E. coli* were not detected in Japanese field mice caught in the copse area away from the barn.

The antibiotic resistance rate of samples from the pit water was 8.3% (10 of 120 samples). Among 12-month samples, resistant strains were observed in 4 months. Manure and waste milk were mixed in the pit water, and the pollutants had extremely high solid content. Nevertheless, antibiotic-resistant strains were rarely detected throughout the year. Of the survey months, the detection of resistant strains was concentrated in June, July, and August (Table S3). During this period, four strains of multidrug-resistant (i.e., resistant to four antibiotics) *E. coli* were detected from pit water. Two resistance patterns were observed for the four antibiotics: ABPC– TC– NA–CPFX (three strains) and ABPC–GM–KM–TC (one strain). From June (mean daily maximum temperature: 27.1 °C) to the early part of July in the subtropical rainy season, conditions were hot and humid, and cefazolin was frequently administered to treat mammitis (Table 1). In the midsummer from July (30.8 °C) to August (31.6 °C), doses of benzylpenicillin alone and kanamycin–benzylpenicillin were increased for the treatment of mammitis. ABPC-resistant strains were detected in the pit water from June to August. One KM-resistant strain was detected in the pit water in August, when the dose of kanamycin was the highest (Table S4). The increased antibiotic doses used for the treatment of mammitis likely gave rise to the resistant strains detected in the pit water; however, these resistant strains were not retained in this water, with the resistance rate shown to be extremely low in September. The resistance rate of the tank water was 6.3% (5 of 80 samples), similar to the resistance rate of the pit water. In April, one strain resistant to three antibiotics was detected with a resistance pattern of ABPC–TC–CP. However, AR-*E. coli* was rarely detected in the tank water during the survey period, which was in agreement with the data obtained from the pit water. In Miyazaki City, in which Sumiyoshi Farm is located, the antibiotic resistance rates of *E. coli* in sewage and the urban river water were previously reported as 59.5% and 28.5%, respectively (Ogura et al., 2020). Therefore, the antibiotic resistance rates of the pit water and treated tank water on the studied farm were much lower than the rates found in water bodies in the urban city.

3.2. Classification of tet tetracycline resistance genes

The types of *tet* gene, i.e., tetracycline resistance genes, were analyzed in all tetracycline-resistant strains (18 strains) in this study. Fig. 3 shows a comparison of the presence or absence of *tet* detected with the antibiotic resistance profile of strains. The *tet*A gene, which is the oldest known gene for encoding the tetracycline efflux protein, was detected in all tetracycline-resistant strains isolated from cow and crow feces and from pit and tank water. Additionally, *tet*B was detected in 17 strains excluding 1 strain from pit water. Conversely, *tet*M, which encodes a ribosomal protection protein detected in the feces of many domestic animals in Japan (Kobayashi et al., 2007), was found only in one strain from pit water. These results are consistent with a previous report that among the known tetracycline-resistant determinants, tetracycline efflux genes, especially *tet*A and *tet*B, are prevalent, but ribosomal protection genes, including *tet*M, are rarely detected in tetracycline-resistant *E. coli* strains (Chopra and Roberts, 2001), it has been detected in *E. coli* strains isolated from diverse human and animal sources (Bryan et al., 2004). Since the use of tetracycline was discontinued before 2013 on the entire farm including the barn, the low resistance rate of tetracycline and the possession of *tet*A and *tet*B genes likely indicate the positive implications for the environment around the farm. Tetracycline-resistant *E. coli* carrying *tet*A was below the detection limit in 1 week in the environment, and a correlation has been reported between the number of copies of tetracycline resistance gene in farm compost and the amount of tetracycline remaining (Yoshizawa et al., 2020). Thus, the tetracycline-resistant *E. coli* in this study was derived from outside the farm and may have been brought in by wild animals, but the details are unknown. It has been indicated that the feces of wild and migratory birds may be a potential factor in the spread of antibiotic-resistant *E. coli* in dairy farms (Fahim et al., 2019).

3.3. Milk production and antimicrobial doses

On Sumiyoshi Farm, the quality of milk (including residual antibiotics) produced by dairy cows administered antibiotics is strictly managed in accordance with “Food Sanitation Act” and “Japanese veterinary public health legislation: ministerial ordinance concerning compositional standards, etc. for milk and milk products, Ministry of Health and Welfare.” Thus, milk with a guaranteed quality is continuously produced every day from the farm (monthly production: 6762–13,371 kg; average = 9648 kg). Given that the tetracycline resistance rate of *E. coli* from the feces of cows was ≤2% and no other resistant *E. coli* isolate was found in cows during our 1-year study, we conclude that AR-*E. coli* are under control in cows reared on the farm. The monthly number of rearing cows, amount of antibacterial drugs used, and drug administration per number of cows are shown in Table 1. Indeed, if dairy cows are reared using the levels of antibiotics shown Table 1, it seems to be possible to control the expression of AR-*E. coli* on a farm. The number of treatment days using antibiotics per dairy cow per

Table 1
The head of cows per month, the amount of antibiotics used, and the amount of antibiotics used per head.

Antibiotics															
Sampling		Cow		Cephalonium		Cefazolin		Benzylpenicillin		Kanamycin and benzylpenicillin				Sulfamonomethoxyn	
			Administration	Udder		Udder		Intramuscular		Udder				Intramuscular	
Year	Month	heads		(mg, titer)	(mg/ head)	(mg, titer)	(mg/ head)	(mg, titer)	(mg/ head)	(mg, titer)	(mg/ head)	(mg, titer)	(mg/ head)	(mg, titer)	(mg/ head)
2018	July	32		0	0	12,000	375	3600	113	10,800	338	6480	203	0	0
	August	32		0	0	0	0	18,000	563	14,400	450	8640	270	0	0
	September	33		4000	121	0	0	1200	36	0	0	0	0	6000	182
	October	33		3000	91	4000	121	0	0	3600	109	2160	65	0	0
	November	34		1000	29	0	0	12,600	371	0	0	0	0	0	0
	December	37		1000	27	0	0	0	0	0	0	0	0	2000	54
2019	January	34		0	0	4000	118	0	0	4800	141	2880	85	2000	59
	February	34		1000	29	0	0	1800	53	0	0	0	0	0	0
	March	33		0	0	0	0	0	0	0	0	0	0	4000	121
	April	33		0	0	3000	91	1650	50	0	0	0	0	4000	121
	May	34		0	0	0	0	0	0	0	0	0	0	0	0
	June	33		1000	0	3000	91	0	0	0	0	0	0	0	0

Strains code	Antibiotics											tet gene									
	ABPC	CEZ	CTX	GM	KM	TC	NA	IPM	CPFX	CP	SMX/TMP	*tet* A	*tet* A(P)	*tet* B	*tet* B(P)	*tet* D	*tet* H	*tet* L	*tet* M	*tet* T	*tet* 37
Cow 3_1	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Cow 3_6	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Black rat 6	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Crow 2	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 4_6	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 6_3	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	R≥32	S≤1	R≥4	S≤8	S≤1	+	+	+							
Pit 6_4	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	R≥32	S≤1	R≥4	S≤8	S≤1	+							+		
Pit 7_1	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 7_2	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 7_3	R≥32	I=4	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 8_3	R≥32	S≤2	S≤1	R≥16	R≥16	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+				+			
Pit 8_4	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	R≥32	S≤1	R≥4	S≤8	S≤1	+		+							
Pit 8_6	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 8_7	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Tank 4_4	R≥32	I=4	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	R≥32	S≤1	+		+							
Tank 5_3	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+				+			
Tank 6_2	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Tank 9_1	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							

(μg/mL)

S : Susceptibility
I : Intermediate resistance
R : Resistance

Fig. 3. Detection of *tet* genes conveying antibiotic resistance in tetracycline-resistant *Escherichia coli* strains. Abbreviations: ABPC, ampicillin; CEZ, cefazolin; CTX, cefotaxime; GM, gentamicin; KM, kanamycin; TC, tetracycline; NA, nalidixic acid; IPM, imipenem; CPFX, ciprofloxacin; CP, chloramphenicol; SMX/TMP, sulfamethoxazole/trimethoprim.

year raised in Japan is estimated to be 15.5 days/year (Abe et al., 2021). In contrast, the number of treatment days for dairy cows on this farm was 2.4 days/year. Accordingly, we infer that this farm properly implements antibiotics administration compared with general domestic farms in Japan.

3.4. Conclusions

In a survey lasting 1 year with data collected monthly, we confirmed that the antibiotic resistance rate of *E. coli* in the animal feces and wastewater sampled from Sumiyoshi Farm, on which antibiotic use is strictly monitored and controlled, was maintained at extremely low levels compared with the levels of antibiotic resistance typically reported on domestic farms in Japan. Moreover, the problematic ESBL-producing and fluoroquinolone-resistant *E. coli* were not detected, despite 341 strains being analyzed. Antibiotic resistance may be kept low on Sumiyoshi Farm because antibiotics are used appropriately by the veterinarian supervisor, the administration history is recorded every day for all livestock individuals, and the rearing environment is strictly managed. In a previous study, Walk et al. (2007) analyzed *E. coli* strains from 30 conventional and 30 organic dairies and concluded that it takes a conventional farm approximately 8 years to acquire the lower resistance profile of an organic farm. The low rate of antibiotic resistance noted in this case study of the Sumiyoshi Farm, which has acquired GLOBAL G.A.P. and has been continuing to improve antibiotic use-related practices for >10 years, is consistent with the predictions from the previous study (Walk et al., 2007). From the results of our survey, we conclude that the outbreak and spread of antibiotic-resistant bacteria are markedly reduced in farms that practice the prudent use and management of antibiotics.

Credit authorship contribution statement

Yoshihiro Suzuki: Conceptualization, Methodology, Investigation, Writing – Original Draft, Writing — Review & Editing, Supervision. Hayate Hiroki: Investigation. Hui Xie: Investigation, Visualization. Masateru Nishiyama: Investigation, Validation. Shinsuke H. Sakamoto: Investigation, Validation. Ryoko Uemura: Validation — Review & Editing. Kei Nukazawa: Writing — Review & Editing. Yoshitoshi Ogura: Validation. Toru Watanabe: Investigation, Validation. Ikuo Kobayashi: Investigation — Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113930.

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A review of legionnaires' disease and public water systems – Scientific considerations, uncertainties and recommendations

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ABSTRACT

Legionella is an opportunistic premise plumbing pathogen and causative agent of a severe pneumonia called Legionnaires' Disease (LD). Cases of LD have been on the rise in the U.S. and globally. Although *Legionella* was first identified 45 years ago, it remains an 'emerging pathogen." *Legionella* is part of the normal ecology of a public water system and is frequently detected in regulatory-compliant drinking water. Drinking water utilities, regulators and public health alike are increasingly required to have a productive understanding of the evolving issues and complex discussions of the contribution of the public water utility to *Legionella* exposure and LD risk. This review provides a brief overview of scientific considerations important for understanding this complex topic, a review of findings from investigations of public water and LD, including data gaps, and recommendations for professionals interested in investigating public water utilities. Although the current literature is inconclusive in identifying a public water utility as a sole source of an LD outbreak, the evidence is clear that minimizing growth of *Legionella* in public water utilities through proper maintenance and sustained disinfectant residuals, throughout all sections of the water utility, will lead to a less conducive environment for growth of the bacteria in the system and the buildings they serve.

1. Introduction

Legionella is an opportunistic premise plumbing pathogen and causative agent of legionellosis. Legionellosis is a disease grouping which includes Legionnaires' Disease (LD), a severe pneumonia often requiring treatment in a hospital, Pontiac fever, a generally milder illness, and additionally extrapulmonary infections. An estimated 8–18,000 people are hospitalized annually with LD in the United States, and yet only about 10% of cases are clinically diagnosed (Adams et al., 2013; Cassell et al., 2019). From 2007 to 2018, the incidence of LD has more than tripled (NAS, 2019). *Legionella* has been identified as the leading cause of waterborne outbreaks in the US (Benedict et al., 2017), and, nationally, health insurers paid $434 million dollars annually for LD alone (Collier et al., 2012).

The bacteria are commonly found in the freshwater environment and reproduce in high numbers inside free-living amoeba in warm (25–45 °C) stagnant water (Fliermans et al., 1981). The primary human exposure route to *Legionella* is the inhalation of aerosolized water containing the microorganism, typically from showers, whirlpool spas and outdoor cooling equipment, humidifiers, misters and respiratory therapy devices. LD cannot be transmitted person-to-person or by swallowing contaminated water, however aspiration is also an important mode of disease transmission. Older adults, smokers, individuals with immunocompromised conditions and comorbidities are at higher risk of LD (Silk et al., 2013). Yet, a large majority of cases (as many as 96%) are sporadic with no identified source (Orkis et al., 2018a).

Legionella pneumophila, especially serogroup (SG1), is the most common etiologic agent of LD in the U.S., accounting for approximately 85–90% of reported clinical cases (Fields et al., 2002; Yu et al., 2002) though that differs in other continents. Since a urinary antigen test specific to *L. pneumophila* SG1 is the primary means of diagnosing LD in the U.S., and although infections by other serogroups may be captured by this test, generally infections with other species and serogroups are being missed (Mercante and Winchell, 2015; Muyldermans et al., 2020).

Over 40 years after *Legionella* was first discovered, the Water Research Foundation identified *Legionella* as an 'emerging pathogen' (Jang et al., 2014). Indeed, when outbreak investigations are covered by news and media, it is often suggested that the public water system may be the source of the outbreak and water utilities may continue to become the subject of litigation as building owners more frequently test the

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incoming water during public health outbreak investigations (AWWA, 2017). A recent LD outbreak in Flint, Michigan in 2014–2015, occurring after water utility-wide deficiencies, has brought attention to the role of public water systems in LD outbreaks (Rhoads et al., 2017). There are additional instances when public water systems have been implicated in LD outbreak investigations (Cohn et al., 2015; Rhoads et al., 2020). Public health professionals, drinking water regulators and water utilities alike are increasingly required to have a productive understanding of the evolving issues and complex discussions of the contribution of the public water utility to *Legionella* exposure and LD risk. To this end, this review provides a brief overview of scientific considerations important for understanding this complex topic, a review of findings from investigations of public water and LD, including data gaps, and recommendations for professionals interested in investigating public water utilities and risk of LD.

2. *Legionella* growth in public water

Legionella is part of the normal ecology of a public water system and is frequently detected in regulatory-compliant drinking water (Hsu et al., 1984; USEPA, 2016). Currently there are no national drinking water regulations for *Legionella* in the U.S. The United States Environmental Protection Agency (USEPA) considers *Legionella* to be controlled if and when water systems treat their water for the removal/inactivation of Giardia and viruses. The USEPA states that *Legionella* can enter a facility from the source water, and the "environmental conditions and processing of the water once it enters a building can lead to the growth of *Legionella*, which could result in increased risks of infection" (Hsu et al., 1984; USEPA, 2016). This paradigm is also proposed by American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE), an industrial professional society which promulgates standards and guidelines reflecting best practices and current science for the building industry (ASHRAE, 2020).

Legionella is commonly detected as part of the complex water utility pipe biofilms (Waak et al., 2018). *Legionella* contained inside amoebae, in the environmentally hardy cyst, frequently escape the treatment plant unimpaired and enter the distribution system and ultimately indoor plumbing, where *Legionella* and their protozoan hosts are incorporated within biofilms (Nisar et al., 2020). The protozoan hosts facilitate the replication and viability of *L. pneumophila* (Nisar et al., 2020). Numerous reports describe the existence of *L. pneumophila* harbored within protozoans from thermally-, chemically-, and UV radiation-treated potable water supplies and storage reservoirs (Kim et al., 2002).

Additional mechanisms of colonization may involve insufficient cleaning of water mains during construction or repair (Colbourne and Trew, 1986) and leaking mains affected by transient negative pressure spikes that pull in soil/sewage line microorganisms. Other mechanisms of colonization of premise plumbing distinct from public water system "seeding" of building premise plumbing could include improper or insufficient backflow prevention from cross-connections between potable and non-potable water and introduction from elevated building water storage tanks.

Areas of low flow frequently are sites of increased sedimentation and reduced disinfection residual as well as a source of additional nutrients for *Legionella* growth. Corroded mains can provide greatly expanded surface area for biofilm formation (LeChevallier et al., 1993; NAS, 2019). Tuberculation also increases the availability of iron, which is a nutrient for *Legionella.* Extended water age in a distribution network is known to be associated with a loss of chlorine residual due to reaction with the main and any corrosion products, biofilm lining the main, sediments in the main and soluble organic material in the water.

Under conditions conducive for growth, *Legionella* that may be present in low numbers within the biofilm can begin to proliferate, whether in the biofilm of water utility-owned distribution pipes or within building premise plumbing. Although even low levels of *Legionella* may be associated with cases of LD (Demirjian et al., 2015), the active growth and explosion of high numbers of *Legionella* are most anticipated to cause disease despite a lack of known infectious dose (CDC, 2018). Proliferation of the organism remains the largest health concern because it indicates the presence of viable and pathogenic bacteria (Colbourne and Dennis, 1985). Factors known to influence *Legionella* growth within buildings include temperature, disinfection, hydraulic conditions, presence of nutrients, pipe materials and presence of distal devices; factors which may be relevant to public water utilities (NAS, 2019).

2.1. *Disinfection level*

There are requirements for free chlorine residual levels in surface water systems (Pressman, 2020). However, these regulations are intended to control gastrointestinal pathogens, and not specifically *Legionella* or their associated biofilm protozoan hosts. These regulations require a detectable residual (0.05 mg/L) at 95% of sampling locations throughout the utility. Evidence of the influence of disinfection level and type on *Legionella* occurrence and LD risk is strong. Localized low chlorine residuals (<0.2 mg/L) could be an indicator of insufficient bacterial control. LeChevallier (2019b) suggests that utilities should maintain a chlorine disinfectant residual of at least 0.1 mg/L in all parts of the distribution system, while much higher levels are recommended for health care building systems. The World Health Organization (WHO) estimated that healthcare facilities need 0.3–0.5 mg/L to keep *Legionella* proliferation under control and as much as 50 mg/L is needed to kill *Legionella* embedded within biofilm (WHO, 2007). Notably, some protozoans are more resistant to chlorine when infected by *Legionella* (Boamah et al., 2017).

2.2. *Disinfection type*

Disinfectants used to create a residual in the distribution system are chlorine, chlorine dioxide and chloramines. A microbiological survey of a water system before and after the disinfection type was switched from chlorine to chloramine found that while *Legionellae* was widely distributed in source water and in the distribution system, and was the dominant biofilm bacteria in some samples, it was not detectable in the distribution system in the months after the switch to chloramine disinfection (Pryor et al., 2004). Chloramine disinfection has been demonstrated to reduce *Legionella* detections in multiple water surveys (Donohue et al., 2014; Flannery et al., 2006; Moore et al., 2006; Pryor et al., 2004) and to reduce hospital-associated LD outbreaks (Heffelfinger et al., 2003; Kool et al., 1999). The greater disinfection efficacy of chloramine is thought to occur because chloramines are selectively reactive, allowing deeper penetration into biofilms (Xue et al., 2014), and because chloramines can kill *Legionella* inside amoebae (Dupuy et al., 2011). However more research is needed to understand the exact mechanism for improved effectiveness (NAS, 2019).

2.3. *Water source*

Some research suggests systems using a surface water supply are more likely to be associated with LD (Wullings and van der Kooij, 2006), which may be expected because surface waters typically have the amoebae that supports *Legionella* growth. However, *Legionella* is known to naturally exist in groundwater, and higher levels of *Legionella* have been found in homes served by private groundwater wells as compared to those served by public water (Mapili et al., 2020). An ecological analysis of census tract LD incidence rates in NJ did not confirm an increased association among census tracts served by public water utilities with a surface water source compared to census tracts served by groundwater (Gleason et al., 2017).

2.4. *Other factors*

A growing body of research suggests that warm, wet, humid weather is associated with increased incidence of LD, which is likely an indicator that these meteorological conditions increase the proliferation of *Legionella* in the water environment (Fisman et al., 2005; Gleason et al., 2016; Passer et al., 2020).

3. *Legionella* occurrence in public water utilities

Despite the acceptance of that fact that *Legionella* is detected in regulatory compliant drinking water, there remains a lack of consensus regarding the prevalence of *Legionella* in distributed water, and, further, whether there would be an acceptable detection concentration. A limited amount of research provides *Legionella* sample results from public water distribution systems and even less real-world evidence of factors which may increase *Legionella* detection in a water utility distribution system (Donohue et al., 2019).

A detailed picture of *L. pneumophila* in a municipal water system was obtained by States et al. (1987). Raw water, water at different stages in the treatment plant, and finished water in several reservoirs were tested by a culture method. There was no detection in raw water, sporadic detection at different points in the treatment process, but persistent detection in the three reservoirs (one covered, two open). Chlorine residuals were always >0.2 mg/L. Other culture-based studies have found *Legionella* throughout water utility distribution systems (Stout et al., 1992; Tison and Seidler, 1983). *Legionella* concentrations, as measured by the quantitative polymerase chain reaction method (qPCR), were found to decline with each step of the treatment process (Lin et al., 2014). *L. pneumophila* was detected by qPCR in 25% of the source water samples collected from 25 drinking water treatment plants in the U.S. but in only 4% of treated water samples (King et al., 2016).

Analysis of sediment samples from municipal drinking water storage tanks in 18 community water systems across ten U.S. states using qPCR found potential opportunistic pathogens dominated with the highest detection of occurrence being *Mycobacterium* spp., followed by *Legionella* spp. with a 66.7% detection frequency (Lu et al., 2015). Diverse *Legionella* spp. including *L. pneumophila*, *L. pneumophila* SG1 and *L. anisa* were identified, each of which might cause legionellosis. All sampled tanks had detectable residual chlorine, 39% were from surface water-based systems, and temperatures ranged from 2 to 29 °C. However, unfortunately, there was no reported analysis of occurrence by these factors. A small-scale field study of 35 residential water meter biofilms found *L. pneumophila*, through molecular techniques, in 14% of samples, and occurrence was in only one area, indicating that environmental differences in the water distribution system may impact *Legionella* occurrence (Schwake et al., 2015).

A national occurrence study (Donohue et al., 2019) of cold-water samples (n = 108) taken during 2009–2014 at building and residential cold-water taps, using two sensitive primer/prober qPCR sets, found that a quarter of the taps showed presence of low levels of *L. pneumophila* SG1 in at least one of the sampling events. Large buildings and residences exhibited a similar number of detections of *L. pneumophila* SG1 (21–24%), and there was no difference between newer and older residences. Considerable lack of persistence (sites testing positive more than once) was observed. Buildings showed more persistence of *L. pneumophila* SG1 than residences and persistent detection at a building location tended to correlate with the detected concentration of *L. pneumophila*. A one-year sampling campaign throughout the Paris drinking water system found that the presence of *L. pneumophila* as quantified by qPCR fluctuated over space and time (Perrin et al., 2019). Lechevallier (2019a; 2019b) found *L. pneumophila* in a small number of tap samples from ten community water systems tested with Legiolert (a culture-based method with detection based on a *Legionella*-specific enzyme) during warm weather months (July–October 2018). Of the 573 tap samples, only 14 were positive, and were associated with low residual chlorine and higher water temperature. Almost all source water and treatment plant effluent samples had no detectable *Legionella*, which may be due in part to the high concentration of disinfectant leaving the plant.

Despite a lack of known infectious dose, alert and action levels for *L. pneumophila* have been established in a few European countries, based on WHO guidance (Hamilton et al., 2019; Van Kenhove et al., 2019). The levels range from 100 to 100,000 colony-forming units in culture per liter (CFU/L), based in part on different intended purposes, such as protecting at-risk populations and triggering different responses.

4. LD outbreak and public water system investigations

4.1. Flint, MI investigation

The largest investigation of the role of public water as a cause of an LD outbreak occurred after the City of Flint (in Genesee County, Michigan) switched the source of their drinking water from water purchased from the Detroit Water and Sewerage Department to the Flint River in 2014. The new source water was treated to bring the finished water to a free chlorine level that was more than double the level that it had been with the original source water but lacked sufficient corrosion control (Rhoads et al., 2017). The lack of corrosion control led to widespread iron corrosion in the mains and leaching of lead from leaded plumbing. There were areas of the distribution system that barely maintained a chlorine residual, water had a higher temperature in the summer months than with the old water source, and there were increased main breaks which may have increased microbial contamination (Rhoads et al., 2017). Flint switched its source water back to purchased drinking water in October 2015 and implemented enhanced corrosion control. During the same time frame, outbreaks of LD occurred during the summers of 2014 and 2015, receding in 2016. Given the timing of the community outbreaks of LD, it was hypothesized that they were related to the water utility failures. The National Academy of Sciences (2020) report on management of *Legionella* presents a timeline of events. It is instructive to examine the available epidemiologic investigation findings, environmental sampling results and genomic comparisons, and the often-disparate conclusions of investigators regarding the role of the utility - which are reviewed in the sections that follow.

4.2. Flint, MI – epidemiologic investigation data

The final case numbers provided by the Michigan Department of Health and Human Services (MDHHS) are 90 cases and 12 deaths (MDHHS, 2018a, b). During 2014–2015, 61 of the LD cases (68%) lived in a residence not serviced by the City of Flint water system during their incubation period, while 56% of cases, or as many as 65% when restricted to cases with complete exposure history, were patients at McLaren Hospital, making the hospital the most frequently visited location among all of the case patients. The MDHHS report concluded that McLaren Hospital was a common source that explains the majority of cases and that the 2015 outbreak ended before the switch back to Detroit water.

Alternatively, other investigators provide analysis indicating the Flint Water was associated with the LD outbreaks. An analysis of LD incidence as a function of free chlorine residual and estimated water age in the Flint Water system mains found a 7.2-fold increase of census tract incidence of LD after the water was switched to the Flint River, and the risk subsided after the switch back (Zahran et al., 2018). When average weekly chlorine levels were <0.5 mg/L, the likelihood of a census tract presenting with LD increased 2.9 times (95% CI 1.4, 6.3) and increased 3.9 times (95% CI 1.7, 8.7) when the chlorine levels were <0.2 mg/L. Notably, McLaren Hospital was located in the high water-age, low--chlorine part of the distribution system. However, other possible sources of exposure including poor building water management at McLaren Hospital were not adequately addressed by the authors. Additional criticisms included the inclusion of only 25 out of 42 cases exposed at McLaren Hospital, inadequate resolution of case residence issues (MDHHS, 2018b), poor exposure assessment for City of Flint water, how cases were classified as commuters, certain model assumptions, misclassification of case illness onset, and that some LD cases classified as receiving City of Flint water only did so two months after the switch back of water source (Smith et al., 2019).

An independent retrospective investigation of the outbreak (based on 86 cases) including epidemiologic, environmental sampling and genomic data found evidence for three sources of the outbreak: 1) McLaren Hospital, 2) residences receiving City of Flint water, and 3) cooling towers and other outdoor aerosol exposures (Smith et al., 2019). Smith et al. (2019), concluded that there was strong evidence for a hospital-associated outbreak in 2014 and 2015, as well as some evidence that in 2014 a proportion of cases were associated with residences served by City of Flint water and select cooling towers in Flint. Importantly, Smith et al. noted the lack of timely sampling of all potential sources. The most frequently reported exposure was to McLaren Hospital (49%). Investigators also found that individuals receiving City of Flint water were at increased risk of developing LD than other Genesee County residents in 2014 but not 2015. Notably, after excluding cases with exposure to McLaren Hospital, the relative incidence rate of LD in Genesee County was still higher than expected. Unfortunately, only a minority of the hospital-associated cases had whole genome molecular subtyping, and, among the eight cases in 2015 that were tested, only three were of a *Legionella* type that matched the *Legionella* typing in the hospital plumbing, sampled a year later in 2016 (Garner et al., 2019; Smith et al., 2019).

4.3. Flint, MI – environmental sampling data

Both hot- and cold-water culture detections of *L. pneumophila* SG1 in McLaren Hospital were found in 75% of the sampled patients' rooms, and, though the hospital hyperchlorinated their water system on three separate occasions, *L. pneumophila* SG1 was found in more than 95% of patients' areas that were sampled (Smith et al., 2019). McLaren Hospital had higher *Legionella* proliferation than any other building sampled (Schwake et al., 2016). A review of McLaren Hospital records of *Legionella* occurrence in their plumbing system and remedial actions shows that high concentrations of *L. pneumophila* SG1 (>10,000 CFU/L) had been detected in the hospital plumbing system in 2014 and 2015 at several different locations (Smith et al., 2019). McLaren conducted a final remedial event, which included a two-phase water system remediation and water use restrictions. Hospital-associated cases decreased within days, though within a couple of months the Flint system had also switched back to its original water source.

Legionella spp. but not *L. pneumophila* were detected in cold-water taps in single-story buildings in an August 2015 sampling (Schwake et al., 2016). Smaller sampling studies at the same time found DNA markers (qPCR) of *Legionella* spp., but no *L. pneumophila* (Flint Water Study, 2019a, b, c). Additional sampling in March 2016 of homes and small business in Flint were all culture negative. Alternatively, Garner et al. (2019) found a more widespread pattern of *L. pneumophila* detection in sampling during June and August 2016, including in cold-water taps in residences. Garner et al. (2019) suggested that detection of *L. pneumophila* in cold-water taps indicates that its presence was not due to a faulty temperature setting on a hot water heater, but rather represents the community water system as a reservoir for the organism.

4.4. Flint, MI – genomic linkage data

Whole-genome sequencing of clinical *Legionella pneumophila* isolates collected during the second of the two outbreaks in Flint was compared with water isolates collected the following year from Flint tap water, after the switch back to Detroit water (Garner et al., 2019). A genetically diverse range of *L. pneumophila* was found across clinical and water isolates, and investigators hypothesize that the LD outbreak could have originated from a variety of different exposure sources. A second genomic comparison of clinical samples and environmental isolates from McLaren's plumbing system and from one cooling tower found environmental isolates from McLaren Hospital in 2016 and 2017 were ST1 and together with three ST1 clinical isolates, formed a genetic cluster. Unfortunately, it is hard to make a strong conclusion based on such a small genetic cluster in environmental samples taken 1–2 years later.

4.5. Flint, MI - summary of findings

The lines of epidemiologic data into sources of the 2014 and 2015 LD outbreaks in Flint, MI are disparate. Despite compelling associations with chlorine residuals and water main breaks with LD case rates, study limitations suggest these findings should be interpreted carefully. Environmental sampling in the community was mixed, may not have included buildings in the high water-age, low chlorine area, and in some cases took place a year after the second outbreak. Although genomic evidence is rarely available in any given investigation of LD, the availability of genomic data in this investigation remained limited and inconclusive. Together, the epidemiologic evidence of shared exposure to McLaren Hospital among most cases and environmental sampling data in McLaren Hospital indicating *Legionella* proliferation, suggest the hospital as the source of the outbreak. However, because the hospital was located in a high water-age zone of the system, it is further possible these issues were compounded by deficiencies in the public water utility.

4.6. Other LD outbreak investigations

Although limited, some additional LD outbreak investigations have explored distributed public water as a source of exposure with varying findings. Following an LD outbreaks (22 cases in 2000 and eight cases in 2006) in the city of Rennes, France (population approximately 200,000), *Legionella* isolates were collected from across the city's entire water distribution system and cooling towers from 2000 to 2009 (Sobral et al., 2011). A few clones were found to colonize the entire water supply system in the city but were not related to the two outbreaks.

An investigation into a multi-year outbreak in Italy that included sampling of 48 points of the unchlorinated municipal water system gave only one positive result in a public drinking water fountain (Scaturro et al., 2015). Investigators also performed residential home sampling including from case-patient homes and a selection of control homes. In patient homes ($n = 22$), 52% had culture detection of *Legionella,* while 14 out of 16 control homes were negative (12.5% positivity). Along with cooling tower maintenance and disinfection measures, a 0.2 mg/L chlorine disinfection was applied to the municipal water system. Although cases reduced slightly, numbers remained elevated in this area for five years when case numbers returned to background occurrence levels, which coincided with cooling towers going offline due to the shutdown of several factories. The investigators concluded that this investigation remains a reminder of how difficult it can be to identify a source of exposure and the need for clinical isolates for comparison.

In New Jersey, a multiyear sporadic series of LD cases led to an environmental investigation into a section of a community water system (Cohn et al., 2015). The community outbreak included up to eleven SG1 urine antigen-confirmed cases over the course of five years. The case investigation determined that the five-year rate of LD in an area of the community water system near a water storage tank was eight times greater than in the rest of the service area and almost 20 times higher than in the rest of the state. More cases continued to occur in the area after the five-year period. An environmental investigation identified conditions conducive for *Legionella* growth in that area, particularly low chlorine residuals (<0.1 mg/L) during warm weather months over several years, stagnant water in the storage tank, and no flushing program for the distribution system. Two regulatory samples had no chlorine residual. As part of the investigation, *Legionella pneumophila* SG1 was detected by culture at 50% of the sample sites during maintenance flushing of the water mains in the area. The findings from the investigation were not sufficient to conclude there was a direct association with the outbreak and the community water system due to the lack of combined clinical and environmental sequence-based typing and the fact that the sampling of flushed water from the mains occurred several years afterward.

In Quincy, Illinois, 58 cases of LD occurred during a 2015 outbreak (Rhoads et al., 2020). Although deficiencies in the Veterans' Home were associated with many of the cases, an additional four community-acquired cases were not associated with the Veterans' Home, and investigators expanded their investigation to evaluate possible deficiencies with the water utility. About three to six months before the outbreak, the water utility switched their primary disinfectant and corrosion control, which resulted in a decrease in the chlorine residual throughout the system, though no regulatory violations occurred. Although no conclusions are drawn on whether the community water system was responsible for the outbreak, the authors recommend additional water quality monitoring, distribution system management and clinical monitoring whenever major changes in water treatment or changes in distribution system operation occur.

5. Data gaps and limitations

The lack of clinical and timely environmental *Legionella* isolates remains a major limitation in establishing links between the disease clusters and the drinking water utility (Rhoads et al., 2020; Scaturro et al., 2015). This seems to mirror the state of the science in general and explain why so many outbreaks do not have a clear exposure pathway. Since the introduction of a urinary antigen test (UAT) which provides rapid results for the *L. pneumophila* serogroup 1 antigen, respiratory specimens often are not collected because ordering the UAT is faster and easier. Typically, the request for collecting clinical isolates requires the recognition of a defined outbreak, which would also trigger the collection of environmental isolates. The timeframe for realizing there is a problem, and the subsequent investigation of a water utility is typically lengthy, and this affects all aspects of the investigation. Proliferation in the water mains may occur weeks or more prior to the mobilization/-sloughing of the biofilm. This lag time may also be accompanied by the lack of persistence of specific *Legionella* colonies. As noted above, Donohue et al. (2019) observed that only a small proportion of specific subtypes of *Legionella* persisted between one sample event and the next. Rhoads et al. (2017) noted that in the Flint, MI investigation, appropriate sampling was not done during the most relevant time frame.

Importantly, there is no established guideline for investigating a public water utility during an LD outbreak or cluster. Furthermore, in the U.S., investigators can use a variety of culture and non-culture methods for laboratory detection of *Legionella* which can lead to differing results and conclusions and impact comparably of investigation findings. Internationally, culture-based methods are standard and have the added benefit of identifying *non-pneumophila Legionella* that may cause infection; however, these methods may miss more than 90 percent of active infectious cells present (i.e., active but not able to be grown in culture, though possibly able to seed growth in the body) (USEPA, 2017), and reliance on culture-based methods may exaggerate treatment efficacy (Ashbolt, 2015). Molecular methods provide an alternative to these limitations. For instance, qPCR is a culture-independent approach for pathogen enumeration, the advantages of which include a low detection limit, high specificity, and high throughput which provides rapid results (as much as 10–12 days faster than culture) however, this method cannot differentiate between living and dead organisms (Wang et al., 2012). Although growing in utility and availability, the use of non-culture based molecular methods for detection of *Legionella* in potable water samples are not standardized and vary meaningfully across and within commercial, academic and environmental laboratories (Mercante and Winchell, 2015).

The burden of evidence to conclusively confirm the water utility was the source of an LD outbreak may not be achievable. Even when utility deficiencies that create conditions conducive for the growth of *Legionella* are identified, individuals must be exposed to aerosols. Since aerosol generating devices such as showers and cooling towers are privately owned and operated, finding a link between a potable water isolate and clinical isolate would not conclusively confirm that the water utility was the source of the exposure. Instead, such a conclusion may require cumulative evidence of linkages between potable water isolates from independent buildings and residences serviced by the water utility. The lack of clinical and timely environmental isolates for comparisons, no established guidance for investigating a public water utility, and the delayed timing of investigations further compound the burden of proof.

6. Recommendations

Public health investigators need to quickly recognize an outbreak of LD and rapidly respond with both clinical and environmental inquiries. The Centers for Disease Control and Prevention (CDC) recommend that disease investigators consider contacting the local water authority to determine issues or changes that could have contributed to *Legionella* growth (e.g., modifications to potable water disinfection, water main breaks, major construction activity, water service interruptions) (CDC, 2020). Outbreak investigations need to access and consider detailed disinfectant residual data, because the system will only have been cited if disinfectant was non-detectable. In addition, chlorine residual test sites in the distribution system are often not sufficiently close to the case residences to reliably estimate the chlorine residual in smaller, usually slower flowing, mains with older water close to those residences. Regulatory sample sites are typically buildings with easy access, such as schools, police and fire stations and commercial buildings which are usually close to larger mains and larger roadways. Water regulators may be able to request additional water quality monitoring in identified high-risk areas of public water utility distribution systems.

Along with considering disinfection residual data, regular flushing of mains, especially in low-flow areas, a need that is frequently ignored because of insufficient water system staffing, funding, or lack of realization of the value, should be examined. As suggested by CDC, information about utility maintenance events, water main breaks or fire suppression events should be collected when investigating clusters of disease (CDC, 2020). If the data from a public water system suggest the presence of conditions conducive to *Legionella* growth, cold-water tap sampling in the area should be performed for *Legionella* monitoring. Widespread building vacancies, as occurred in many business areas following COVID-19 shutdowns or following natural disasters as occurred following Hurricane Sandy, may also impact water use and increase *Legionella* growth in buildings.

Only a small fraction of reported LD cases is observed as part of an outbreak event. In the absence of a reported common source such as the common plumbing in a building or a sudden unexplained increase in reported cases, disease clusters may go undetected due to unusual geographic cluster patterns or high baseline of disease (Edens et al., 2019; Orkis et al., 2018b). To enhance surveillance detection of clusters, some jurisdictions are implementing prospective legionellosis cluster detection systems (Greene et al., 2016). Surveillance methods that can more effectively and efficiently identify geographic clusters in time would help investigators search out broader common exposures such as local or system-wide public water utility deficiencies more rapidly. A clinical response should also be planned. Without clinical isolates to compare to environmental samples by whole genome sequencing, there is no gold standard available for exposure assessment. Healthcare professionals should be encouraged to collect lower respiratory specimens from patients being evaluated for suspected LD.

Active monitoring for *Legionella* in all public water systems, in the absence of disease, may not be warranted due to potential for false negatives leading to a false sense of security, false positives which could lead to financial burdens (Whiley, 2016), and uncertainty in regard to the interpretation of positive findings. For example, LeChevallier

(2019a) noted that a public water utility was advised that a "do not drink" order would be issued if *L. pneumophila* was detected and subsequently decided to not participate in a *Legionella* monitoring project. However, others highlight scenarios whereby water control measures meet recommendations, but widespread *Legionella* colonization exists, which would only be detected through monitoring (Collins and Walker, 2017). *Legionella* monitoring as part of a public health investigation would be informative for establishing effective reduction of *Legionella* growth in buildings following water utility interventions such as increasing chlorine residuals, through enhanced flushing programs, engineering improvements (e.g., cleaning and lining projects), water storage tank cleaning, and storage tank draw down protocols to refresh the water.

In summary, public health investigators need to continue to develop and refine tools for detecting clusters in space and time and subsequently explore possible underlying sources of exposure such as undiagnosed public water utility deficiencies; to continue to promote the collection of clinical specimens, and to conduct investigations within a faster timeframe. Proactive examination of water quality data and close cooperation between public health authorities, environmental protection authorities and water utilities can be invaluable for protecting the public from *Legionella*, as well as preventing future problems. Although current literature is not conclusive in identifying a public water utility as a sole source of an LD outbreak, the evidence is clear that minimizing growth of *Legionella* in public water utilities through proper maintenance and sustained disinfectant residuals throughout all sections of the water utility will lead to a less conducive environment for growth in the system and the buildings the system serves.

Declaration of competing interest

None.

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A review of studies on blood lead concentrations of traditional Mexican potters

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ABSTRACT

Background: Traditional Mexican potters and their families have been occupationally exposed to lead for centuries; however, studies on blood lead levels (BLL) and their adverse health impact on this population are scarce. There is no safe BLL, even at 1 μg/dL there are associated health effects.
Objective: To systematize and characterize Mexican potters' historic lead exposure through their BLL and associated health outcomes.
Methods: Using PRISMA guidelines, we conducted a systematic review through January 2021 of published studies on BLL and associated health outcomes in Mexican potters.
Results: Fifteen studies containing data from 1980 to 2013 met the inclusion criteria and were published between 1980 and 2018. Study populations ranged from n = 5 to n = 457, and included adult potters (mean BLL 37.9 ± 16.2 μg/dL) and/or their children (mean BLL 22.5 ± 10.5 μg/dL). Studies reported on general lead poisoning symptoms, neurotoxic and nephrotoxic outcomes as well as correlated biomarkers.
Conclusions: Our results confirm high occupational and para-occupational lead exposure. Despite governmental and non-governmental initiatives to promote lead-free glazes, lead continues to be used by traditional potters and their families.

1. Background

Pottery is one of the oldest human expressions for artistic and utilitarian objects in cultures worldwide. Pottery can be made by firing clay at temperatures above 600 °C, and techniques such as burnishing and glazing have been used to obtain impermeable and shiny finishes (Tite et al., 1998). The use of lead-based glazes was introduced in pottery around the Roman era, and in Mexico, since the 16th century. These glazes have the advantage of being reliable and melting at low (<1,000 °C), uneven temperatures. Traditional kilns in Mexican pottery workshops reach uneven, low temperatures, since they are handcrafted with adobe bricks (usually by the artisan) and use wood (often collected from surrounding forests) as fuel (Spielholtz and Kaplan, 1980). Pottery will frequently need a first firing before being decorated and a second after the last glazing for the final vitrification process. This process can take months of work and potters frequently rely on the sale of their products as their main source of income, hence risking their production to a different, less predictable technology is a risk they cannot undertake.

Potters who use lead-based glazes and their families are exposed to high occupational and environmental lead levels (Hibbert et al., 1999). There is no safe level of lead exposure, and it can have detrimental prenatal health effects and throughout life; additionally, potter families frequently live in rural and poverty conditions, making them more susceptible to toxicity due to malnourishment (Talpur et al., 2018) and to a less favorable environment for mitigating the effects (Marshall et al., 2020). In many cases, the work and living areas are interconnected resulting in the cooking stove being adjacent to the kiln, and the glazing space. Frequently, it is women who are in charge of the glazing process, simultaneously with other family responsibilities like cooking and child care (Tamayo-Ortiz and Navia-Antezana, 2018).

In Mexico, the current guidelines indicate blood lead level (BLL) limits of 10 μg/dL for occupationally exposed women (SSA, 2011), and 5 μg/dL for children (SSA, 2000). Most traditional potters are not in the formal sector of the economy, making health and social benefits (e.g., maternity leave or pension plan) provision more complicated, and keeping track of their health through medical records, practically

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impossible. In 1993, the Mexican Health Secretariat issued the Official Mexican Standard in Environmental Health to regulate the use of lead compounds that has since then been updated (SSA, 2013). There is also a specific regulation for lead in pottery (SSA, 2016), and since 1994 there is a program to help traditional potters transition to lead-free glazes. However, lack of surveillance and enforcement, and a strong embedded cultural tradition have resulted in the persistent production of low-temperature, lead-glazed pottery.

There are no longitudinal studies or systematic reviews of blood lead levels (BLL) or their health effects that could shed light on the impact of the different governmental and non-governmental initiatives since the 1990's to reduce or eliminate lead exposure in traditional potters' communities. Therefore, we aimed to characterize and systematize Mexican potters' lead exposure by reviewing all published studies to date reporting their BLL and associated health outcomes.

2. Methods

We conducted a systematic review using the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (Tricco et al., 2018). For studies with available data, we compared BLL of potters to those of non-potter groups, we explored the association with specific pottery activities and with health effects, and we identified the communities where the studies were conducted.

2.1. Research question

A PECO (participants, exposure, comparison, outcome) question was developed to address "What are the blood lead levels of Mexican potters and their families resulting from occupational and para-occupational exposure?", how do these levels vary according to age and sex, the type of activity (ies) they carry out, and over time?" (Table S1).

2.2. Literature search strategy and selection criteria

Based on the research question, a search in the MEDLINE (PubMed), Scopus, and LILACS electronic databases was performed until January 2021, using the following combination of key terms in English or Spanish: "lead/plomo", "Pb", "lead oxide/óxidos plomo", "blood/sangre", "Mexico", "occupational/ocupacional", "workers", "ceramics/cerámica", "potters/alfareros", and "occupational exposure" for PubMed-Scopus and LILACS, respectively. The search key terms were combined with their corresponding Medicine's Medical Subject Headings (MeSH) term when the database search was launched in PubMed. Boolean operators ("OR"; "AND") were applied to link search terms to the research question:

2.3. Database: ovid MEDLINE (R) 1966 to February week 2, 2021

Search: (("lead"[MeSH Terms] OR "lead"[All Fields] OR "Pb" [All Fields] OR "lead oxide" [All Fields]) AND ("Blood*"[All Fields]) AND ("Mexico"[MeSH Terms] OR "Mexico"[All Fields]) AND ("occupational groups"[MeSH Terms] OR ("occupational"[All Fields] AND "groups"[All Fields]) OR "occupational groups"[All Fields] OR "worker"[All Fields] OR "workers"[All Fields] OR "workers"[All Fields] OR "occupational exposure"[All Fields])) Sort by: Publication Date.

The scope of the computerized literature search was expanded according to the reference lists of retrieved articles by manual search.

Eligible studies met the following inclusion criteria: (1) epidemiological studies (cross-sectional, case-control, case-crossover, case reports, cohort, and quasi-experimental or experimental studies) with original data, (2) reporting the exposure measurement by blood lead levels (BLLs) in the study population and comparison group, and (3) published in English or Spanish. We excluded studies that used exposure biomarkers other than BLL. Special attention was given to include studies of BLL specifically in potters, since many studies are available on BLL and other occupations, and to exclude studies on the use of lead-glazed pottery without occupational exposure.

2.4. Data extraction and analysis

Following an initial phase of removing duplicates and completely irrelevant records, two independent reviewers (J.E. and P.F.) screened records for potentially eligible titles and abstracts and subsequently reviewed full texts to determine inclusion in the review. Disagreements were resolved with a third reviewer (M.T-O.) via consensus.

Three reviewers (J.E., P.F., and M.T-O.) independently extracted the following study-level data from records by using a common data collection spread-sheet: authors, publication year, research design, study location, the period of study, population characteristics, inclusion and exclusion criteria, sample size, biological matrix analyzed, determination laboratory technique, BLL, and other relevant results. The BLL data reported was extracted, focusing the systematic review on summary statistics of central tendency and spread, mainly, geometric or arithmetic mean or median and standard deviation or interquartile range. Where these measures were not reported but the raw data was, the measures were calculated directly by the authors. We used t-tests for independent groups to determine the magnitude and statistical significance of the differences in BLL between potters and non-potters. We illustrate the geographic spread of the studies using a map of Mexico locating each referenced study. Lastly, as a reference for the expected health effects of lead exposure, we crossed the BLLs of each study with the toxicological profile for lead of the Agency for Toxic Substances and Disease Registry (ATSDR). Statistical significance was assigned at a p-value <0.05. Stata version 14.1 (StataCorp, 2015) was used for all summary statistics.

2.5. Quality assessment

The quality of primary studies included in this review was assessed using a 13-item specific scale to assess study quality based on the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) (Vandenbroucke et al., 2007) principles and was developed by consensus among the three authors (Table S2). Each item was scored as 0 = no description, 1 = limited description, 2 = good description, for the introduction, methods, results, and discussion. The items covered domains such as study design, recruitment and description of participants, and global quality. The score was expressed in arbitrary units (a.u.) ranging from 0 to 27 (high quality). P.F. and MT-O independently evaluated quality, and disagreements were resolved after discussion.

3. Results

We identified a total of 142 articles and kept 32 records for full-text review following the removal of duplicates (n = 110) and irrelevant records. We excluded seven full-text records for not having BLL data, not being sufficiently specific on the population of interest, or because they were only describing previously reported data. Also, eight records were excluded due to the unavailability of the full text. Exclusions resulted in 15 studies eligible to assess the exposure of interest, with a total sample of 1836 subjects (Fig. 1) (Chantiri-Pérez et al., 2003; Estrada-Sánchez et al., 2017; Fernandez et al., 1997; Flores-Ramírez et al., 2012; Hernández-Serrato et al., 2003; Jones et al., 2013; Molina-Ballesteros et al., 1980, 1981, 1982, 1983; Ortiz-Ortiz et al., 2017; Saavedra Juárez et al., 2010; Sánchez Alarcón et al., 2012; Tamayo-Ortiz and Navia-Antezana, 2018; Torres-Ortiz et al., 2006). During the period between 1984 and 1996 there were no publications according to our search criteria.

3.1. Quality assessment of included studies

The mean (SD) of the quality assessment of the 15 selected studies

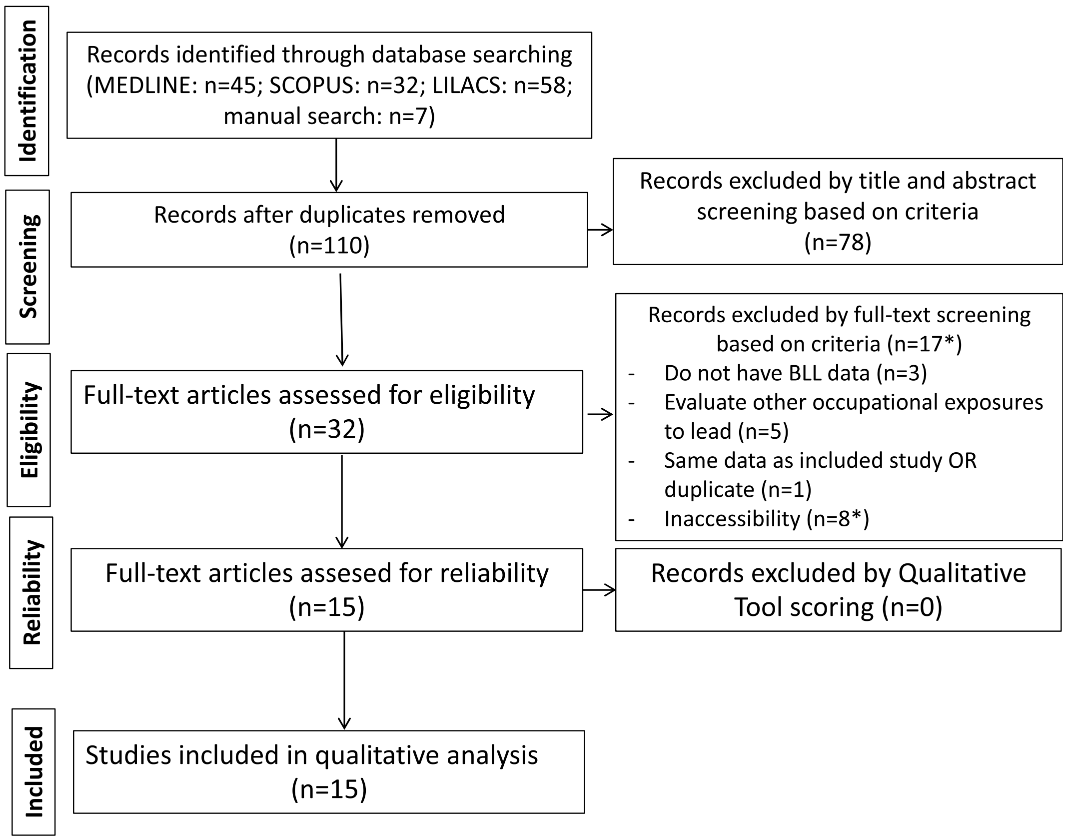

Fig. 1. PRISMA Flow Diagram for Systematic Review of BLL in pottery-making groups.

was 23 (1.8) a.u. (range 19–25) for a satisfactory level. The extent of the scale was 0–27, with 12 questions with a score between 0 and 2, and one question with a score between 0 and 3 (Table A3). All papers included a complete abstract, an introduction that described the study's scientific context and purpose, objectives and/or hypothesis, and precision of results obtained (items 1, 2a, 2b, 4, 11, and 13), and only one paper did not describe the statistical methods (item 8).

All studies were conducted between 1980 and 2013, the majority (53%) from 2002 to 2010. It is essential to highlight that between the years 1984 and 1996, and after 2018, no studies of BLL or health effects in potters were published. Considering the type of epidemiological design, fourteen studies had a cross-sectional design and one, a case-crossover design.

All studies were conducted in Mexico, mainly in: Tlaxcala, Jalisco, and Michoacán ($n = 3$ in each state), and Veracruz ($n = 2$). One study included an analysis of pottery workshops of seven Mexican states (Colima, Michoacán, Oaxaca, Puebla, Tlaxcala, México and Jalisco). Twelve studies were performed in urban areas, and the remaining, in rural areas ($n = 3$). Population sample sizes ranged from 5 to 456 individuals, with sample sizes >100 in five studies (Table 1). Participants were almost evenly distributed between male and female (51.3%), and most were older than 16 years of age (57.6%). The population groups evaluated in the studies were either only adults (51.3%), only children (35.3%), or both (13.4%). Occupational exposure was evaluated in 461 potters and compared to 321 non-potter adults as a low-risk group, a population without occupational exposure to lead (general population) in the same study location. Para-occupational exposure was evaluated in 377 children. In three studies, researchers reported that out of 694 studied households, 49.7% had a kiln inside the home.

3.2. Blood lead levels (BLLs)

We identified fourteen studies that reported lead levels in venous blood. One study estimated the blood lead level through soil concentration by the Integrated Exposure Uptake Biokinetic (IEUBK) model. In seven studies BLLs were determined through atomic absorption spectrometry (AAS) or graphite furnace atomic absorption spectrometry (GFAAS).

The mean BLL in potters was almost double that of the BLL in the non-potter group (37.9 ± 16.2 vs. 22.5 ± 10.5 μg/dL, $p < 0.001$). There were significant differences in the mean BLL between and within the study groups in sex and age ($p < 0.001$). Males and those occupationally exposed had the highest BLLs of all participants (Table 2). The difference in BLL by age varied less among potters (36.1 ± 16 μg/dL in <19 years old vs. 35.5 ± 19.7 μg/dL in ≥19 years old) than among the non-potter group, where those ≥19 years of age had higher BLL than younger subjects (18.8 ± 9.7 μg/dL in <19 years old vs. 20.8 ± 13 μg/dL in ≥19 years old). Non-significant differences in the BLL were observed between potters in urban and rural areas. The mean BLL by study site showed the highest levels in Metepec-Mexico (65.1 ± 2.3 μg/dL), followed by Tonala-Jalisco (52.8 ± 12.2 μg/dL), and Rancho Nuevo-Veracruz (49.1 ± 4.3 μg/dL). Subgroup analysis by workforce exposure showed that potters had higher BLL than para-occupationally exposed workers (38.3 ± 15.8 μg/dL vs. 32.8 ± 13.6 μg/dL, respectively). Analysis by type of activity performed in the pottery workshop was included in four studies and showed that firing (50.7 ± 12.9 μg/dL), glazing (45.8 ± 4.4 μg/dL), and sieving (43.9 μg/dL), were associated with the highest BLLs. BLLs were significantly higher among individuals from workshops/homes with a kiln ($p < 0.001$).

Four studies reported mean soil and food lead concentrations. In Trinidad Tenexyecac (Tlaxcala), soil samples had the highest levels (62–5,187 mg/kg), and these were above the National Guidelines for residential soils (400 mg/kg, NOM-147-SEMARNAT/SSA1-2004). Similar findings were reported in Chapantongo (Hidalgo), where the mean lead levels of surface soil samples were 1,600 mg/kg(Jones et al., 2013). Regarding food Pb concentrations, 87.5% of the 48 food samples

Table 1
Studies describing BLLs in potters and non-potters' families in different localities in Mexico published from 1980 to 2013.

Study no. Author, publication year	Study location (State and location)	Study year	Population characteristics (n)	Analytical matrix/method	Blood Lead Level (BLL) μg/dL: (Mean ± SD)	Results and other health outcomes
1. Molina-Ballesteros et al., 1980.	Jalisco: Tonala (potters exposed to lead) and Rosario (potters non-exposed to lead).	1980	n = 301 Adults and children (n = 167 exposed potters, 134 non-exposed potters).	Lead in peripheral venous blood. Atomic absorption spectrometry (AAS).	**Men** **Group I age 0–9 years** Exposed (n = 6): 82.7 ± 30.8 Non-exposed (n = 18): 18.8 ± 7.5; **Group II > 9 years:** Exposed (n = 68): 57.0 ± 16.3 Non-exposed (n = 35): 21.1 ± 7.2 **Women** **Group I age 0–9 years** Exposed (n = 3): 79.7 ± 3.2 Non-exposed (n = 14): 20.5 ± 6.6; **Group II >9 years** Exposed (n = 81): 51.6 ± 19.8 Non-exposed (n = 67): 21.6 ± 8.2	Exposed groups showed the highest BLL. The difference in the BLLs between the two populations was highly significant.
2. Molina-Ballesteros et al. (1981)	Mexico:Metepec and Mexico City	NA	n = 71 Potters n = 45, Farmers n = 12, inhabitants of Mexico City n = 14.	Lead in peripheral venous blood- AAS. Urinary zinc protoporphyrin IX (Z-PPF). Urinary delta-aminolaevulinic acid (ALA-U). Hemoglobin and Hematocrit.	Potters: 65.1 ± 2.3 Farmers: 38.6 ± 3.3 Inhabitants 28.1 ± 1.3	Individual interviews complaints of asthenia, and articular pains only in potters who glazed. No differences found between groups in hemoglobin and hematocrit measurements. A significant difference found between BLL, Z-PPF, and ALA-U levels between potters and other groups. The correlation coefficient between BLL and ALA-U was 0.71.
3. Molina-Ballesteros et al., 1982	Jalisco: Tonala	1980	n = 233 Children (5–15 years old) Potter families n = 153 Non-potter families n = 80	Lead in peripheral venous blood-AAS. Urinary Z-PPF and ALA-U. Hemoglobin - Hematocrit	Potters 39.5 ± 19.6 Non-Potters 24.8 ± 7.7 **Male** Potters: 40.6 ± 20.9 Non-potters: 26.2 ± 7.5 **Female** Potters: 38.2 ± 18.1 Non-potters: 24 ± 7.7	Higher levels of ALA-U and Z-PPF in the exposed group compared to the unexposed group. 43% of boys and 41% of the girls had BLL up to 40 μg/dl in the exposed group. Non-significant differences by sex.
4. Molina-Ballesteros et al. (1983)	Jalisco: Tonala	NA	n = 63 Children (10 years old) Potter families n = 33 Non-potter families n = 30	Lead in peripheral venous blood-AAS	Potter families: 63.39 ± 15.8 Non-potter families: 26.27 ± 7.9	Total IQ ($p < 0.01$), verbal IQ ($p < 0.01$), and performance IQ ($p < 0.025$) scores for potters were lower than for the non-potters. On average, a significant disparity between chronological and mental age. Potters had an inverse correlation between BLL and the total, verbal, and performance IQ ($p < 0.05$). A higher percentage of moderate deficiencies in potters compared to non-potters (33.9 vs. 7.4%). Four children from potter families were diagnosed with probable brain injury.
5. Olaiz Fernandez et al., 1997	Michoacan: Tzintzuntzan Tzintzunzita, and Colonia Lazaro Cardenas	NA	n = 457 Potters and their families n = 181 (4–16 years old); n = 276 (17–74 years old)	Lead in capillary blood-Anodic stripping voltammetry (ASV). For samples with concentrations >25 μg/dL confirmed by peripheral venous blood- AAS.	Men (n = 146): 23.9 Women (n = 310): 22.9	Non-differences by sex in BLLs. BLL was higher among individuals from households with an open kiln (n = 147) (31.7 vs. 19.2 μg/dL, $p < 0.01$). Lack of a water pipe in the home (i.e., insufficient drainage), dust floor, use of lead-glazed pottery for cooking, and an open kiln in the household were associated with BLL >15 μg/dl.
6. Hernández-Serrato et al. (2003)	Oaxaca: Santa María de Atzompa	NA	n = 413 Adult Potters and non-potters (>15 years old)	Lead in peripheral venous blood. Graphite furnace atomic	Potters (n = 217): 48.24 ± 12.97 Non-potters (n = 196): 39.07 ± 2.64 Women (n = 257): 42.18 ± 13.34 Men (n = 156): 46.69 ± 14.16	Hyperuricemia was associated with BLL above 40 μg/dL (OR = 1.74, 95% CI: 1.12–2.61). Serum creatinine was associated with sex, age, and BLL.

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Table 1 (*continued*)

Study no. Author, publication year	Study location (State and location)	Study year	Population characteristics (n)	Analytical matrix/method	Blood Lead Level (BLL) μg/dL: (Mean ± SD)	Results and other health outcomes
				absorption spectrometry (GFASS)	Potters wives (n = 80): 37.2 ± 12.9 Farmers (n = 30): 43.15 ± 13.05 Students (n = 25): 38.67 ± 11.87 Merchants (n = 11): 33.18 ± 10.08 Employees (n = 50) 41.0 8 ± 14.02 Molding pottery pieces (n = 172): 47.3 ± 12.4 Glazing (n = 83): 48.6 ± 12.4 Firing (n = 114): 50.7 ± 12.8 Having a kiln (n = 189): 49.2 ± 12.9	
7. Chantiri-Pérez et al., 2003	Veracruz: Chavarrillo	2000	n = 20 Potter families Women (n = 6) Children (n = 14):1–18 years old	Lead in capillary blood- ASV	Overall: 34.57 ± 15.72 Children: 38.06 Women: 24.1 Breastfeeding women (n = 2) 36.4 and their children: 37.0 Kilns in the household (n = 9): 43.78 ± 15.9 Non-kilns in the household (n = 11): 27.02 ± 10.7 Molding clay (n = 11): 27.07	80% of the study population prepare food in lead-glazed ceramics, and 30% store food in them.
8. Torres-Ortiz et al. (2006)	Veracruz: Rancho Nuevo	NA	n = 28 Potters n = 14 Non-potters n = 14	Lead in capillary blood- ASV	Potters: 52.5 (27.3–78.8). Non-potters: 27.0.	Potter women's BLL was 34.9% higher than the men's. The concentration of potter's enzyme δ-ALA-D activity is reduced up to 77%, indicating chronic lead poisoning.
9. Sánchez Alarcón et al., 2012	Tlaxcala: San Pablo del Monte	2007	n = 50 Adults Potters n = 40 Non-potters n = 10	Lead in capillary blood- ASV	Potters: 28.9 ± 13 Non-potters: 8.3 ± 1.8 Men: 31.1 ± 13 Women: 18.6 ± 6.3 **By workforce** Sieving (n = 3):, 43.9 Glazing (n = 10): 36.8 Decoration (n = 9): 32.8 Administrator (n = 3): 24.0 Throwing on the wheel (n = 5): 22.6 Sculpting clay(n = 4): 22.0 Assistant (n = 4): 17.7 μg/dL	14.3% of the soil samples exceeded the USEPA criteria lead hazard in soil (400 mg/kg) for residential use. Lead concentration ranged: 3.4–525 mg/kg
10. Saavedra Juárez et al., 2010	Michoacan:Capula	2002	n = 150 Children (8–15 years old) from Potter families n = 50 with BLL	No data	n = 9: BLL >25 mg/dL; n = 28: BLL 10–25 mg/dL; n = 14: BLL< 10 mg/dL.	BLL≥ 25 mg/dL is associated with lower weight and height than BLLs <25 mg/dL ($p < 0.01$). n = 24 (16%) and n = 19 (12.6%) had lower attained weights and heights for age, respectively. Symptoms possibly associated with chronic lead poisoning include headache (67%), abdominal pain (59%), nausea (57%), and nervousness (50%).
11. Flores-Ramírez et al., 2012	Tlaxcala: Trinidad Tenexyecac,	2008–2009	n = 72 Children (4–9 years old) living close to lead-contaminated sites & residence time >2 years	Lead in peripheral venous blood- GFASS	Median 19.2 μg/dL, IQR: 14.8–25.0 μg/dL	Lead concentrations in soil (median 1,126 mg/kg, IQR: 800-1,510 mg/kg) exceeded the National Guidelines for agricultural, residential and commercial land use (<400 mg/kg; NOM-147-SEMARNAT/SSA1-2004). Trinidad soil samples had the highest levels, above the National Guidelines. High and significant correlation between BLL and soil lead concentration ($r = 0,8244$; $p < 0.001$). The 25th percentile of Trinidad BLLs exceeded the National Guidelines of Health (10 μg/dL; NOM-199-SSA1-2000).

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Table 1 (*continued*)

Study no. Author, publication year	Study location (State and location)	Study year	Population characteristics (n)	Analytical matrix/method	Blood Lead Level (BLL) μg/dL: (Mean ± SD)	Results and other health outcomes
12. Jones et al. (2013)	Hidalgo: Chapantongo//Case-crossover	NA	n = 5 Children (9–18 years old) Potter families n = 71 soil samples	Lead in capillary blood- ASV X-Ray fluorescence (XRF)	Baseline: 19.3 ± 6.37 μg/dL Follow up 3 months post intervention: 8.88 ± 1.23 μg/dL Follow up 12 meses post intervention: 8.26 ± 2.07 μg/dL	Mean Lead in surface soil measurements (n = 71): 1,600 mg/kg (range 30 ± 5–28,635 ± 514 mg/kg). Mean Lead in surface soil measurements within the workshop (n = 34): 2,652 mg/kg Mean Lead in surface soil measurements post remedial activities in the workshop: 322 mg/kg (range 12 ± 4 - 1,538 ± 34 mg/kg). The mean soil lead level in the workshop exceeds the EPA standard by approximately 6.6 times. The median BLL for the group in the follow-up had decreased on average by 54% (from 19.3 to 8.8 μg/dL). The group, on average, experienced a decline of 57% in their BLLs over the initial period and 12 months after. The U.S. EPA Integrated Exposure Uptake Biokinetic (IEUBK) model predicted a 9.2 μg/dL BLL for children.
13. Estrada-Sánchez et al. (2017)	Colima, Estado de México, Jalisco, Michoacán, Oaxaca, Puebla and Tlaxcala	2009–2010	N = 19 Children from Potter families Soil samples	XRF- Soil samples and Integrated Exposure Uptake Biokinetic (IEUBK) to estimate BLL	26.4 ± 2.8 μg/dL (range 23.2–29.8)	n = 149 environmental measurements in pottery workshops had a lead geometric mean soil concentration: 1,098.4 ppm (CI95% 898-1,344). 50% of the readings exceeded 5,000 ppm. Estimated BLLs were five times higher than the US Centers for Disease Control (CDC) guidelines (5 μg/dL). Applying different mathematical estimation models (Lanphear and Schwartz), n = 11 children would probably decrease between 7.13 and 8.84 IQ points due to lead exposure.
14. Ortiz-Ortiz,2017	Tlaxcala: San Pablo del Monte	2009–2010	n = 44 Male potters (25–50 years old): n = 26 General population (22–36 years old): n = 9 (3–14 years old): n = 38 Food samples n = 48	Lead in capillary blood- ASV and venous blood-GFAAS Lead analysis in food samples (rice, beans samples, and mole samples) cooked in glazed ceramic- GFAAS	Potters: 32.2 μg/dL, IQR = 16.1 Children: 5.4 μg/dL; IQR = 5.9 Adults: 8.3 μg/dL; IQR = 2.7	Correlation between ASV and GFAAS (r = 0.952; p < 0.001). 87.5% of the food samples exceed the maximum level of Lead food of the Health National guidelines (0.5 μg/g) (NOM-247-SSA1-2008), and 100% exceed the maximum level of Lead (0.2 μg/g) in the Codex General Standard for Contaminants and Toxins in Food and Feed (WHO and FAO, 2015).
15. Tamayo-Ortiz and Navia-Antezana, 2018	Michoacan: Santa Fe de la Laguna	2002	n = 9 Female potters (15–40 years old)	Lead in venous blood Inductively coupled plasma mass spectrometry (ICP-MS) XRF- lead in bone	14.8 ± 8.9	Cancellous bone had a mean concentration of 84.8 ± 68 μg/g; cortical bone a mean concentration of 93.2 ± 81.2 μg/g. BLL and cancellous bone of r = 0.88; BLL and cortical bone r = 0.84 Cancellous and cortical bone r = 0.98.

AAS: Atomic absorption spectrometry; ALA-U: delta-aminolevulinic acid in urine; ASV: anodic stripping voltammetry; BLL: Blood Lead Levels; GFASS: Graphite furnace atomic absorption spectrometry; ICP-MS: inductively coupled plasma mass spectrometry; IQ: intelligence quotient; IEUBK: Integrated Exposure Uptake Biokinetic; LCS: Lead Care II System; NA: Not available; NOM: Official Mexican Standard of the Ministry of Health; Z-PPF: Zinc Protoporphyrin in blood; XRF: X-ray fluorescence. r: correlation coefficient.

Table 2
Characteristics of studies describing blood lead levels in studies of Mexican Potters published from 1980 to 2013.

Subgroups within studies	Potters		Non-potters	
	N (%)	BLL Mean ± SD	N (%)	BLL Mean ± SD
Total	998 (100)	37.9 ± 16.2	838 (100)	22.5 ± 10.5
Sex				
Male	353 (35.4)	42.9 ± 15.7	225 (26.8)	21.5 ± 9.1
Female	342 (34.3)	32.8 ± 17.3	289 (34.5)	23.5 ± 10.6
Unspecified data	303 (30.4)	30.3 ± 12.9	324 (38.7)	19.5 ± 1.6
Age				
<19 years	377 (37.8)	36.1 ± 16	207 (24.7)	18.8 ± 9.7
≥19 years	461 (46.2)	35.5 ± 19.7	321 (38.3)	20.8 ± 13
Unspecified data	160 (16)	42.1 ± 14.7	310 (37)	23.1 ± 5.5
Study area				
Urban	903 (90.4)	37.8 ± 16.6	813 (97)	20.9 ± 11.4
Rural	95 (9.6)	38.5 ± 17.3	25 (3)	27 ± N.A
Locality of study - State				
Tonala and El Rosario - Jalisco	353 (35.4)	52.8 ± 12.2	244 (29.1)	22.7 ± 2.3
Tzintzuntzan, Tzintzunzita, Capula, Santa Fe de la Laguna and Colonia Lazaro Cardenas - Michoacan	206 (20.6)	31.7 ± N.A	310 (37)	19.2 ± N.A.
Chavarillo and Rancho Nuevo - Veracruz	23 (2.3)	49.1 ± 4.3	25 (3)	27 ± N.A.
San Pablo del Monte and Trinidad Tenexyecac -Tlaxcala	138 (13.8)	24.6 ± 3.6	38 (4.5)	7.2 ± 0.6
Chapantongo-Hidalgo	5 (0.5)	19.3 ± 6.4		8.3 ± 2.1
Metepec - Mexico	45 (4.5)	65.1 ± 2.3	25 (3)	32.7 ± 5.3
Santa María de Atzompa - Oaxaca	217 (21.7)	48.2 ± 13	196 (23.4)	39.1 ± 2.6
Seven States: Colima, Michoacán, Oaxaca, Puebla, Tlaxcala, México, Jalisco	11 (1.1)	26.4 ± 2.7		
Workforce exposure (n = 15 studies)				
Number studies	9/15	11/15		
Occupational	377 (37.8)	38.3 ± 15.8		
Para occupational	461 (46.2)	32.8 ± 13.6		
Workforce exposure by type of activity (n = 4 studies*)				
Firing	114 (11.4)	50.7 ± 12.9		
Glazing and decoration	147 (14.7)	45.8 ± 4.4		
Sieving process	3 (0.3)	43.9 ± N.A.		
Sculpting clay	187 (19)	32.1 ± 13.4		
Administrator	3 (0.3)	24 ± N.A.		
Pottery wheel user	5 (0.5)	22.6 ± N.A.		
Assistant	4 (0.4)	17.7 ± N.A.		
Kiln in workshop/house (n = 3 †)				
Number Households reported	694 (100)	31.4 ± 14.4		
Yes	345 (50.2)	41.6 ± 8.6		
No	349 (49.7)	21.2 ± 6.1		

BLL: blood lead level; N.A. No available; S.D.; Standard deviation. *Studies describe data workforce exposure by type of activity: Molina-Ballesteros,1981; Chantiri, 2000; Hernandez-Serrato,2003; Sanchez-Alarcon,2007. † Studies that included data of the use of kiln in the pottery workshop: Olaiz-Fernandez,1997; Chantiri, 2000; Hernandez-Serrato,2003.

exceed the national and international food safety standards("Codex-alimentarius," n.d.) in San Pablo del Monte (Tlaxcala) (Ortiz-Ortiz et al., 2017), which is an additional source of metal exposure. Additionally, Chantiri-Pérez et al., 2003 report that a significant percentage of potter families prepare food in glazed ceramics and store food inside them (Chantiri-Pérez et al., 2003).

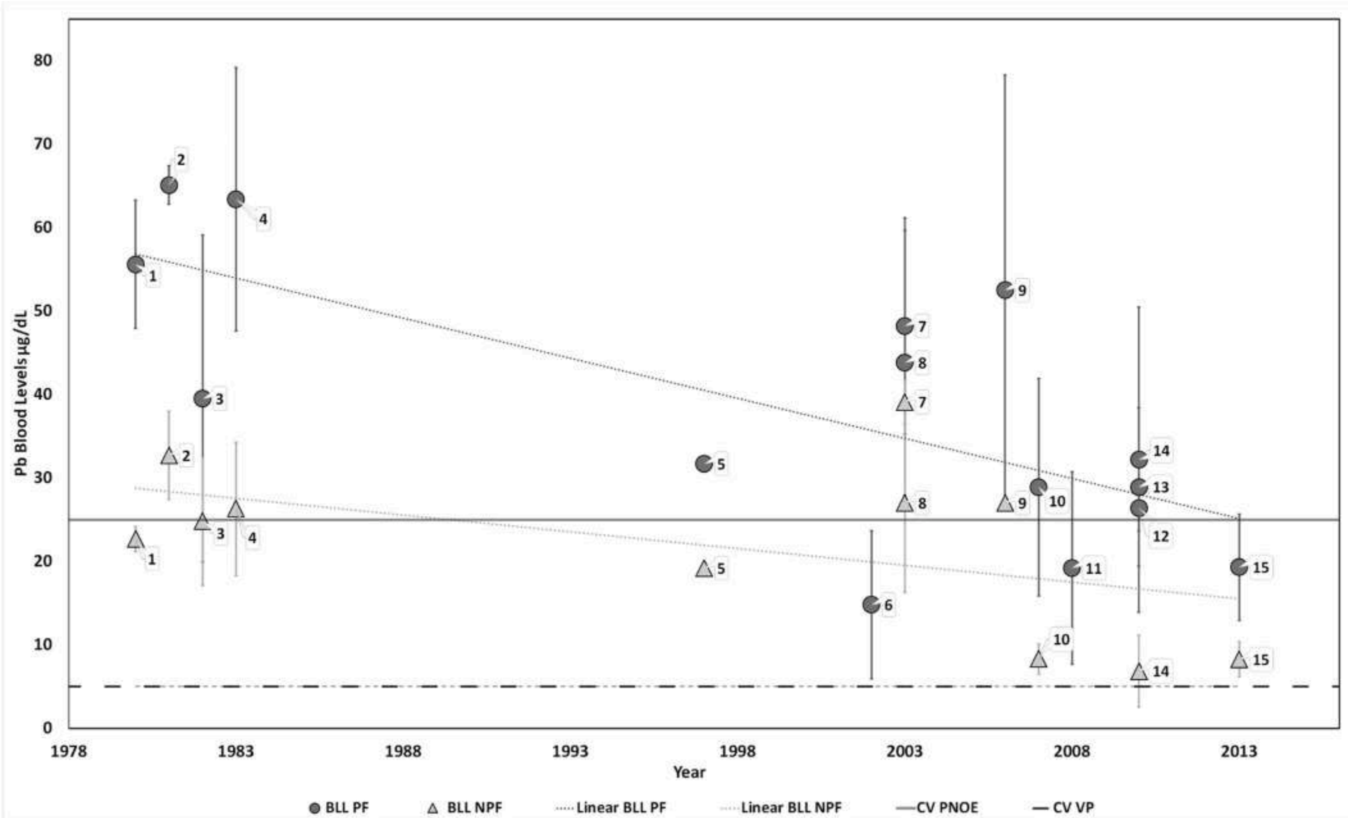
Fig. 2 shows the time course of BLL in the studies analyzed. Average BLL in potters decreased from the highest level of 65.1 ± 2.3 μg/dL in 1980 to 19.3 ± 6.4 μg/dL in 2013. Non-potters' BLLs also decreased from 28.1 ± 1.3 μg/dL in 1981 to 5.4 μg/dL IQR = 5.9 μg/dL in 2009. Potters' BLLs were consistently higher than those of non-potters throughout time. However, the potter s' BLLs temporal trend has a more significant decrease in comparison with non-potters' (0.9 vs. 0.4 μg/dL per year) throughout the period analyzed. Supplemental Figs. 1 and 2 show BLLs for children and adults, respectively. Children and adults exhibited mean BLLs >60 μg/dL in the earlier studies, and although both showed a decline over the years, mean BLL of para-occupationally exposed children and potters could still be > 10 μg/dL.

The different study locations and the Mexican states most representative of their traditional pottery production can be seen in Fig. S3.

3.3. Health effects

Of the total number of studies included in this review, nine evaluated health effects and/or other biomarkers of lead exposure in the potter population. The health effects reported were: osteoarticular pain (Molina-Ballesteros et al., 1981) and kidney function alterations (Hernández-Serrato et al., 2003) in adults, and intellectual deficits, delayed growth, and chronic lead poisoning symptoms in potters' children(Estrada-Sánchez et al., 2017; Molina-Ballesteros et al., 1983; Saavedra Juárez et al., 2010). Biomarkers of lead exposure measured were: hemoglobin and hematocrit(Molina-Ballesteros et al., 1981), delta-aminolevulinic acid in urine (ALA-U) and in blood (ALA-B) (Torres-Ortiz et al., 2006), zinc protoporphyrin (Z-PPF) in urine(Molina-Ballesteros et al., 1981), and bone lead levels(Tamayo-Ortiz and Navia-Antezana, 2018). A significant difference in these biomarkers levels was reported in the potter population in comparison to other groups. Among the effects on kidney function, Hernandez-Serrato et al. reported a higher prevalence (46.2%) of hyperuricemia (blood acid uric >7.0 mg/dL) in the individuals with BLLs above ≥40 μg/dL(Hernández-Serrato et al., 2003).

Table 3 shows the expected health effects in adults and children chronically exposed to lead (using the ATSDR toxicological profile for lead as a reference), for the BLLs found in each study. None of the studies found the average or median BLLs to be below 10 μg/dL; n = 7 studies found BLL in the range of 10–30 μg/dL, with 18 associated health effects ranging from lung disease (asthma) to effects on sperm characteristics (Estrada-Sánchez et al., 2017; Fernandez et al., 1997; Flores-Ramírez et al., 2012; Jones et al., 2013; Saavedra Juárez et al., 2010; Sánchez Alarcón et al., 2012; Tamayo-Ortiz and Navia-Antezana, 2018). The remaining eight studies(Chantiri-Pérez et al., 2003; Hernández-Serrato et al., 2003; Molina-Ballesteros et al., 1980, 1981, 1983; Ortiz-Ortiz



BLL: Blood Lead Levels (mean±SD); PF: pottery-making families; NPF: Non-pottery-making families; CV PNOE: Criteria value for blood lead level for the non-occupationally exposed population (25 µg/dL) according to the official Mexican standard of the Ministry of Health (NOM-199-SSA1-2000 SSA); CV VP: Criteria value for blood lead level in the vulnerable population (childhood, pregnant and breastfeeding women) (5 µg/dL) – Modification NOM-199-SSA1-2000 SSA. *Report interquartile range. Studies included: 1. Molina-Ballesteros et al, 1980; 2. Molina-Ballesteros et al.,1981; 3. Molina-Ballesteros et al, 1982†; 4. Molina-Ballesteros et al, 1983; 5. Olaiz-Fernandez et al.,1997; 6. Tamayo-Ortiz et al.,2018†; 7. Hernandez-Serrato et al.,2003; 8. Chantiri et al.,2003†; 9. Torres-Ortiz et al., 2006; 10. Sanchez-Alarcon et al,2007; 11. Flores-Ramirez et al.,2008†; 12. Estrada-Sanchez et al.,2010†; 13. Saavedra et al.,2010†; 14. Ortiz-Ortiz et al.,2017 and 15. Jones et al.,2013. †These studies were carried out in years other than their publication.

Fig. 2. Comparison of blood lead levels from pottery-making families vs. non-pottery-making families, Mexico 1980–2013.

et al., 2017; Torres-Ortiz et al., 2006) reported BLL above 30 µg/dL for potters and their families, increasing the risk of developing multiple deleterious health effects that affect most organs and systems (i.e. skeletal, muscular, lymphatic, respiratory, digestive, nervous, endocrine, cardiovascular, urinary, and reproductive systems) (Table 3).

4. Discussion

Our study is the first systematic review of BLL and health outcomes in Mexican potters. Only 15 studies with well-documented potters' lead exposure were published during the past 41 years in Mexico. The results suggest that the BLLs of Mexico's traditional potters have been historically high and differ according to the specific activities they carry out. BLLs reported can be associated to detrimental health effects (Table 3) and were well above the reference value for occupationally exposed workers, according to the Mexican health normativity, 30 µg/dL for male workers and 10 µg/dL for women, NOM-047-SSA1-2011(SSA, n.d.). Children's BLLs reported also surpass the Mexican reference level of 5 µg/dL NOM-199-SSA1-2000("NORMA Oficial Mexicana NOM-199-SSA1-2000," n.d.).

Only five studies reported BLLs associated health outcomes(Estrada-Sánchez et al., 2017; Hernández-Serrato et al., 2003; Molina-Ballesteros et al., 1981, 1983; Saavedra Juárez et al., 2010), and no follow-up studies were found. Lead is amongst the most studied toxicants, with long-term health effects across the lifespan-from gestation (as lead will readily cross the placental barrier(Saylor et al., 2021; Schnaas et al., 2006)) to older age, affecting all organ systems(Can et al., 2008; Harari et al., 2018; Lanphear et al., 2018; Shefa and Héroux, 2017). Although BLLs in potters seem to have decreased over the years, there are several considerations, 1) most of the recent studies had small study populations (n < 100) and some with a wide age range, 2) studies might have underestimated the potters' highest BLL since traditional potters have production cycles (i.e. not constantly glazing or firing), and none of them accounted for the timing of exposure with respect to the BLL measurement; the half-life of lead in blood is approximately 30 days compared to decades in bone, reflecting chronic exposure("Toxicological Profiles | ATSDR," 2021). One study included an assessment of lead in bone, with 3 participants measuring over 100 µg/g of lead in cortical bone(Tamayo-Ortiz and Navia-Antezana, 2018). These results are considerably higher than previously reported measures for occupationally exposed workers in Europe and the US, ranging from 13.0 µg/g of lead in cortical bone in Swedish primary lead smelter workers, to 54.8 µg/g of lead in cortical bone in UK precious metal smelter workers (Popovic et al., 2005), and 43.3 µg/g of lead in cortical bone in Mexican workers in a printing workshop(Aguilar-Madrid et al., 1999). Therefore, although BLLs could be low at the time of the studies, chronic occupational exposure could result in high bone lead levels, representing an endogenous source of lead exposure, especially during pregnancy and menopause("Toxicological Profiles | ATSDR," 2021). Another possible explanation of the decrease observed in BLLs may be the success of several endeavors by governmental and non-governmental institutions to introduce lead-free glazes. For example, in 1994 Mexico's National Fund for the Development of Arts and Crafts (FONART) launched its first lead-free pottery program. However, the studies reviewed did not include information on whether the potters participated or not in such programs. Furthermore, currently only a small number of potters can

Table 3
Mexican potters studies classification in accordance to the Blood lead levels and documented health effects in adults and children exposed to lead (ATSDR, 2020).

Mexican potters studies - maximum blood lead levels reported: mean (range)	Blood lead levels (μg/dL)	Documented health effects in adults and children chronically exposed to lead* (ATSDR, Toxicological Profile of Lead, 2020) *not an exhaustive list
	<10	Decreased lung function
		Increased bronchial responsiveness
		Lung disease (asthma and obstructive lung disease) in children
		Atherosclerosis
		Heart disease and cardiac function
		Mortality due to cardiovascular disease
		Decreased hemoglobin and platelet count
		Abdominal discomfort or colic
		Anemia, altered heme synthesis in children
		Decreased glomerular filtration rate
		Altered levels of thyroid hormones
		Intellectual deficits
		Altered mood and behaviors
		Altered neuromotor and neurosensory function
		Effects on sperm
		Effects on reproductive hormones
Olaiz Fernandez et al., 1997	>10-30	Lung disease (asthma)
Sánchez Alarcón et al., 2012		Increased blood pressure and hypertension
Saavedra Juárez et al., 2010		Atherosclerosis
Flores-Ramírez et al., 2012		Heart disease and cardiac function
Jones et al. (2013)		Mortality due to cardiovascular disease
Estrada-Sánchez et al. (2017)		Decreased platelet count
Tamayo-Ortiz and Navia-Antezana, 2018		Anemia and decreased blood hemoglobin
		Altered heme synthesis
		Osteoporosis
		Decreased bone mineral density (adults)
		Increased levels in liver function tests
		Decreased glomerular filtration rate
		Enzymuria or Proteinuria
		Altered levels of thyroid hormones
		Intellectual deficits
		Altered mood and behaviors
		Altered neuromotor and neurosensory function
		Effects on sperm
Molina-Ballesteros et al., 1982	>30-50	Decreased lung function
Hernández-Serrato et al. (2003)		Increased blood pressure and hypertension
Chantiri-Pérez et al., 2003		Atherosclerosis
Ortiz-Ortiz,2017		Heart disease
		Mortality due to cardiovascular disease
		Abdominal discomfort, colic, or pain
		Gastrointestinal symptoms
		Anemia and decreased blood hemoglobin
		Altered heme synthesis
		Muscle soreness/weakness
		Decreased bone mineral density
		Decreased glomerular filtration rate
		Enzymuria or Proteinuria
		Decreased serum vitamin D levels
		Intellectual deficits
		Effects on sperm
		Effects on reproductive hormones
		Decreased fertility
Molina-Ballesteros et al. (1980)	>50	Lung disease (asthma)
Molina-Ballesteros et al. (1981)		Increased blood pressure and hypertension
Molina-Ballesteros et al. (1983)		Atherosclerosis
Torres-Ortiz et al. (2006)		Mortality due to cardiovascular disease
		Abdominal colic or constipation
		Anemia and decreased blood hemoglobin
		Altered heme synthesis
		Decreased bone mineral density
		Decreased glomerular filtration rate
		Enzymuria or Proteinuria
		Kidney impaired tubular transport or histopathological changes
		Altered levels of thyroid hormones
		Decreased serum vitamin D levels
		Intellectual deficits
		Altered mood and behaviors
		Altered neuromotor and neurosensory function
		Peripheral neuropathy
		Effects on sperm
		Effects on reproductive hormones
		Decreased fertility

apply these lead-free glazes since their kilns are not technologically adequate (uneven and lower temperatures).

While other countries successfully eliminated lead poisoning among potters centuries ago(Meiklejohn, 1963), this is a contemporary and prevalent problem that is not exclusive to Mexico. Studies carried out in potters' communities from Brazil, Barbados, and Tunisia, also showed high lead exposure among artisans and their families, exceeding environmental and occupational safety levels. In a rural potter community in Barbados, Koplan et al. described elevated mean BLL in potters (49 μg/dL) their family members (35 μg/dL), as well as high concentrations of Pb in soil (up to 320,000 μg Pb/g) (Koplan et al., 1977). In Tunisia, potters' BLL reached up to 54 μg/dL, with a mean of 22 μg/dL(Chaouali et al., 2018). In Brazil, two studies reported higher BLL in potters (median 7.9 vs. 1.5 μg/dL), and children (median 2.8 vs. 0.1 μg/dL) from areas in proximity to the pottery workshop in contrast with the control group(Bah et al., 2020). Furthermore, the geometric mean of Pb dust deposition rates in pottery workshops was approximately 18 times higher than the average observed in the reference group (geometric mean (SD) 1,463 (±290,000) vs. 82 (46) μg/m^2/30 days) (de J Bandeira et al., 2021). In 17 potter households in Mexico, Hibbert et al. found personal air Pb concentrations of up to 454 μg/m^3 for potters firing and glazing, soil Pb concentrations ranging between 0.39 and 19.8 mg/g, and dust Pb loading on different surfaces (eg. hands, clothes and household items) ranges from 172 to 33,060 μg/ft^2 (Hibbert et al., 1999). Studies have shown that children of these communities have higher BLL values than children living near mining waste sites, metallurgical areas, or an informal lead battery recycling workshop(Ansari et al., 2020; Flores-Ramírez et al., 2012; Soto-Ríos et al., 2017).

We are aware that other countries besides Barbados, Tunisia and Brazil, such as Ecuador, China, Peru and some other European countries (Counter et al., 2000, 2015; Flores et al., 2016; Fralick et al., 2016; Sheets, 1999) might still have artisans producing lead-glazed pottery, nonetheless, studies reporting BLLs of potters are scarce. For example, studies from Ecuador reported high BLL in children associated with the use of lead-glazed ceramics(Counter et al., 2015). In Mexico, results of the 2018 National Health and Nutrition Survey showed that 17.4% of 1–4-year-old children had BLL >5 μg/dL with an OR of 3.27 (95% CI 2.34, 4.58) associated to the use of traditional pottery(Tellez-Rojo et al., 2020).

Our review had several limitations, for example, no studies of BLL or health effects in potters were published between the years 1984 and 1996. However, we consider this a finding of our study, since this lack of information does not reflect the continued use of lead glazes and sales of traditional pottery during these years. Another limitation was the small number of potter's communities included in the studies, although they are well-known for their pottery production, they are far from being representative of the universe of existing potter communities throughout Mexico and across the study period. As can be seen in the map of Mexico (Supplementary Fig. 3), these are only a few in the extension of the Mexican territory and of the states that are well known for traditional pottery production, which are shown shaded in the map.

Given the scarce research in occupational and paraoccupational exposure to lead in Mexican potters and their families, this review was also limited by the subsequent few available published articles, and even fewer of these studies that also evaluated associated health effects, and none that followed-up the subjects in time. The latter issue has not allowed the evaluation of trends in the aforementioned populations' exposures or ruling out their potentially biased (underestimation or overestimation of ranges and averages due to ill-timing) one-time, short-term, measurements. Since there are also not many groups of researchers dealing with this problem, we were faced with information gaps in space (geographic scope) and time (years when no research was done).

Our results confirm high occupational and para-occupational lead exposure in Mexican traditional potters and their families in the last four decades despite the governmental and non-governmental initiatives to promote lead-free glazes. W e conclude that as long as the use of lead-based glazes continues, there is a need to focus more on exposure prevention and interventions-rather than conducting observational studies. We suggest a holistic strategy in traditional pottery communities should have five components: 1. Environmental: to replace the source of exposure by optimized lead-free glazes, plus new regulations on the presence of lead in the environment; 2. Health: to monitor the BLL in traditional potter's children/communities and train first-line family caregivers in a timely diagnosis of acute and chronic lead poisoning; 3. Scientific research: to conduct longitudinal intervention studies, that will allow for a clear evaluation of intervention strategies 4. Educational amelioration: for children with neurobehavioral and cognitive problems, as well as improvement of their nutritional status, and 5. Raising awareness: sensitized potters that transition to lead-free glazes will not only eliminate their exposure but can act as key actors in diminishing the general population's exposure as well. Today an estimated 10,000 potter families continue to use lead glazes in workshops located mainly in rural areas and indigenous communities("FONART- Manual pruebas," n.d.). Lead-glazed low-temperature pottery is readily available for consumers across Mexico, highlighting the need for continuity to this important occupational and public health problem.

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Appendix A. Supplementary data

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Assessing exposures to per- and polyfluoroalkyl substances in two populations of Great Lakes Basin fish consumers in Western New York State

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ABSTRACT

Background: Fish and other seafood are an important dietary source of per- and polyfluoroalkyl substances (PFAS) exposure in many areas of the world, and PFAS were found to be pervasive in fish from the Great Lakes area. Few studies, however, have examined the associations between Great Lakes Basin fish consumption and PFAS exposure. Many licensed anglers and Burmese refugees and immigrants residing in western New York State consume fish caught from the Great Lakes and surrounding waters, raising their risk of exposure to environmental contaminants including PFAS. The aims of this study were to: 1) present the PFAS exposure profile of the licensed anglers and Burmese refugees and 2) examine the associations between serum PFAS levels and local fish consumption.

Methods: Licensed anglers (n = 397) and Burmese participants (n = 199) provided blood samples and completed a detailed questionnaire in 2013. We measured 12 PFAS in serum. Multiple linear regression was used to assess associations between serum PFAS concentrations and self-reported consumption of fish from Great Lakes waters.

Results: Licensed anglers and Burmese participants reported consuming a median of 16 (IQR: 6–36) and 88 (IQR: 44–132) meals of locally caught fish in the year before sample collection, respectively (data for Burmese group restricted to 10 months of the year). Five PFAS were detected in almost all study participants (PFOS, PFOA, PFHxS, PFNA and PFDA; 97.5–100%). PFOS had the highest median serum concentration in licensed anglers (11.6 ng/mL) and the Burmese (35.6 ng/mL), approximately two and six times that of the U.S. general population, respectively. Serum levels of other PFAS in both groups were generally low and comparable to those in the general U.S. population. Among licensed anglers, Great Lakes Basin fish meals over the past year were positively associated with serum PFOS ($P < 0.0001$), PFDA ($P < 0.0001$), PFHxS ($P = 0.01$), and PFNA ($P = 0.02$) and the number of years consuming locally caught fish was positively associated with serum PFOS ($P = 0.01$) and PFDA ($P = 0.01$) levels. In the Burmese group, consuming Great Lakes Basin fish more than three times a week in the past summer was positively associated with serum PFOS ($P = 0.004$) and PFDA ($P = 0.02$) among the Burmese of non-Karen ethnicity, but not among those of Karen ethnicity, suggesting potential ethnic differences in PFAS exposure.

Conclusions: Great Lakes Basin fish consumption was associated with an increase in blood concentrations of some PFAS, and especially of PFOS, among licensed anglers and Burmese refugees and immigrants in western New York State. In the Burmese population, there may be other important PFAS exposure routes related to residential history and ethnicity. Continued outreach efforts to increase fish advisory awareness and reduce exposure to contaminants are needed among these populations.

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1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic chemicals that have been used in industrial and commercial applications worldwide since the 1950s. PFAS are used for coatings and products that resist water, stains, grease, and oil and can migrate into the environment during production and use. Some long-chain PFAS are highly persistent in the environment and bioaccumulate in organisms (Agency for Toxic Substances and Disease Registry [ATSDR], 2021). The two most studied PFAS, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS), were voluntarily phased out of manufacturing in the U.S. starting in 2000–2002. However, these legacy compounds persist in the environment and are still manufactured and used globally (ATSDR, 2021; United States Environment Protection Agency, 2015). The major non-occupational sources of PFAS exposure in humans are contaminated drinking water and seafood rather than inhalation or dermal contact; however, the relative contribution of different sources varies widely by PFAS, populations, and demographic groups (Centers for Disease Control and Prevention [CDC], 2017; Sunderland et al., 2019; Wu and Kannan, 2019). The European Food Safety Authority estimated that fish and other seafood was the dominant contributor to the chronic dietary exposure to PFOS in adults (up to 86% of exposure) (European Food Safety Authority, 2018). Once absorbed, PFAS with longer carbon chains such as PFOS and PFOA are persistent in the human body, with elimination half-lives ranging from several years to three decades (ATSDR, 2021).

Concentrations of PFAS in fish vary depending on factors including the bioaccumulation potential of a particular PFAS, fish species, environmental media (water, sediment), and proximity to industrial sources and densely populated areas (Domingo and Nadal, 2017; Jian et al., 2017). In the Great Lakes region, some PFAS, especially PFOS, have been shown to bioaccumulate in fish and other aquatic species used as human food sources (Klecka et al., 2010; Sinclair et al., 2006; Wu and Kannan, 2019). A recent U.S. EPA study measured concentrations of 13 PFAS in a sample of common fish species from 157 randomly selected nearshore Great Lake sites. Six PFAS were detected in over 65% of fish tissue composites, with PFOS being detected in all composites and representing the predominant PFAS in the fish samples (Stahl et al., 2014).

Human biomonitoring studies in North America, Europe, and Asia have found positive associations between fish and shellfish consumption and levels of PFAS in human blood (Christensen et al., 2017; Haug et al., 2010; Lee et al., 2017; Lindh et al., 2012; Liu et al., 2017; Yamaguchi et al., 2013). For instance, analyses of data from the U.S. National Health and Nutrition Examination Survey (NHANES) found that even though the level of fish consumption was low in the general U.S. population, fish consumption in the past 30 days was associated with elevated serum levels of PFOS, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), and perfluoroundecanoic acid (PFUnDA) (Christensen et al., 2017). A study of the general adult population in South Korea showed that eating raw or cooked fish was associated with higher serum PFDA, PFNA, and PFOS (Lee et al., 2017). Studies of anglers in Germany, France and the U.S., with various levels of freshwater fish consumption, also found associations between fish intake and higher serum PFAS concentrations, especially PFOS (Christensen et al., 2016; Denys et al., 2014; Holzer et al., 2011). In a sample of Wisconsin male anglers, consumption of locally caught fish, including Great Lakes fish, was associated with higher levels of PFOS, PFOA, PFDA, PFNA, PFUnDA, and perfluoroheptane sulfonic acid (PFHpS), but not with perfluorohexane sulfonic acid (PFHxS) (Christensen et al., 2016). In contrast, the authors found that consumption of commercially purchased fish (store or restaurant) was only associated with higher PFHxS levels. Some biomonitoring studies have assessed PFAS levels in indigenous populations who practice subsistence fishing and reported varying levels of serum PFAS, which could be due to environmental levels of PFAS and diverse dietary patterns (Dallaire et al., 2009; Lindh et al., 2012). These data suggest that populations with higher consumption of fish/seafood, including anglers, people living in coastal areas, and especially those practicing subsistence fishing, may be at higher risk of exposure to PFAS and associated health effects (Fair et al., 2019; Kannan, 2011).

Current epidemiological evidence suggests associations between PFAS exposure, especially PFOA and PFOS, and various human health outcomes (ATSDR, 2021; Rappazzo et al., 2017; Sunderland et al., 2019). Potential health effects of PFAS exposure include increased total cholesterol and low-density lipoprotein cholesterol (Averina et al., 2021; Mora et al., 2018; Zeng et al., 2015), pregnancy-induced hypertension or pre-eclampsia (Savitz et al., 2012; Stein et al., 2009; Stone et al., 2021), increases in serum enzymes and decreases in serum bilirubin indicative of liver damage (Darrow et al., 2016; Gleason et al., 2015; Jain and Ducatman, 2019; Mora et al., 2018; Stratakis et al., 2020), increased risk of kidney or testicular cancer (Barry et al., 2013; Shearer et al., 2021; Steenland and Winquist, 2021), decreased antibody response to vaccines (Grandjean et al., 2012; Grandjean et al., 2017; Grandjean et al., 2017; Pilkerton et al., 2018), and small decreases in birth weight (Bach et al., 2015; Steenland et al., 2018; Verner et al., 2015). Evidence suggests that PFOA is possibly carcinogenic to humans (Barry et al., 2013; International Agency for Research on Cancer, 2016; Shearer et al., 2021), and the U.S. EPA has concluded that PFOA has suggestive evidence of carcinogenic potential (U.S. EPA, 2016). Studies of prenatal or childhood PFAS exposure and neurodevelopmental outcomes offer inconsistent evidence (Carrizosa et al., 2021; Harris et al., 2021; Lenters et al., 2019; Liew et al., 2018; Skogheim et al., 2020; Skogheim et al., 2021). While further studies are needed to assess associations between PFAS and human health, it is important to monitor levels of PFAS in human populations and identify sources of exposure.

From 2010 through 2015, the New York State (NYS) Department of Health (DOH) and the ATSDR collaborated on a biomonitoring project (Healthy Fishing Communities Project) to assess exposure to environmental contaminants, including PFAS, in two populations (Wattigney et al., 2019). The project focused on licensed anglers and Burmese refugees and immigrants who consumed fish from the four Great Lakes Areas of Concern (AOCs) and surrounding waters in western NYS. AOCs are areas in the Great Lakes watershed which have significant environmental degradation. Both populations consume more locally caught fish than the general population, resulting in increased risk of exposure to environmental contaminants. Refugees and immigrants from Burma residing in the City of Buffalo, New York, are known to supplement their diets by regularly eating fish caught from local waters (Zremski, 2016). Many of the Burmese have limited awareness of local fish advisories, which likely contributes to their increased risk of exposure to PFAS from subsistence fishing (Liu et al., 2018). In this study, we examined serum PFAS levels and their associations with local fish consumption among the two populations of interest.

2. Materials and methods

All study activities were approved by the federal Office of Management and Budget (Control Number 0923–0044) and NYS DOH Institutional Review Board.

2.1. Study populations and recruitment

2.1.1. Licensed anglers

The first population of interest was licensed anglers who resided near the four AOCs in western NYS, i.e., Buffalo River, Niagara River, Eighteenmile Creek, and Rochester Embankment, and who consumed locally caught fish. Using the NYS Department of Environmental Conservation (DEC) 2010–2011 database of fishing licenses, we created a sampling frame consisting of persons aged 18–69 years who purchased a seasonal or lifetime fishing license and lived in a ZIP code within a 10-mile buffer of the AOCs. Details of the recruitment process have been published elsewhere (Wattigney, 2019). Briefly, a random sample of licensed anglers (n = 13,369) were mailed a screening survey to determine eligibility, and 2126 recipients (16%) completed the survey either by mail, by phone, or online. The eligibility criteria included living at the listed address for at least one year and consumption of at least one meal of fish caught from the AOCs or surrounding waters within the past year. Among the 883 eligible respondents, 409 participated in the study from February through October 2013, and 397 provided a blood sample and completed a detailed questionnaire.

2.1.2. Burmese refugees and immigrants

The second study population comprised refugees and immigrants from Burma and their descendants who lived in the City of Buffalo and who ate locally caught fish. Much of the Burmese population in Buffalo was concentrated in three contiguous neighborhoods along the Niagara River AOC and within three to five miles of the Buffalo River AOC. Due to low income levels and cultural dietary norms (high in fish and fish products), many of the Burmese subsist on locally caught fish (Judelsohn et al., 2017). During the 2013 fishing season (July to October), we used respondent driven sampling (RDS) to recruit the Burmese people. The sample characteristics and details of the sampling method have been reported in a previous publication (Liu et al., 2018). Eligible participants were adult refugees and immigrants from Burma who had lived in the City of Buffalo at least one year and consumed at least 12 meals of fish caught in the AOCs or surrounding waters within the past year. The fish consumption eligibility criterion was set higher than for the licensed anglers, because formative research indicated high levels of local fish consumption among this refugee population (Wattigney et al., 2019). Individuals were eligible irrespective of how they obtained the fish (i.e., whether they caught the fish themselves or ate someone else's catch). Among 206 Burmese participants recruited via RDS, 199 provided blood samples and completed a detailed interview.

2.2. Data collection

Data collection procedures for all participants included obtaining informed consent, height and weight measurements, serum sample collection, and administration of a detailed questionnaire by a trained interviewer. Following standard collection procedures provided by the NYS DOH Wadsworth Center Laboratory, all blood samples were collected by a trained phlebotomist, and serum separation was performed by centrifugation about one hour after collection. More details about serum sample collection can be found elsewhere (Savadatti et al., 2019). Each participant was provided with $75 for their time and effort to participate in the study. All the procedures were conducted at a clinic that provided privacy for the specimen collection and interview.

The questionnaires consisted of items about sociodemographic characteristics, residential history, lifestyle, consumption of fish from the AOCs and surrounding waters, and awareness of the local fish advisories. Maps and charts were used to assist participants in identifying local waters and fish species. Because the Burmese consume local fish year-round, past-year consumption of fish from local waters was assessed by season. The questionnaires also asked about consumption of select store-bought fish (grouper, shark, swordfish, salmon, and tuna) among both groups of participants; consumption of three types of store-bought shellfish (shrimp, snails, and mussels) was also assessed among the Burmese participants. Interviews with Burmese participants were administered in the participant's native language (e.g., Karen, Burmese, or other) and the responses were recorded on paper forms in English.

2.3. Serum PFAS analysis

All serum samples were analyzed for 12 PFAS at the Wadsworth Center Laboratory. PFAS in serum samples were measured using ultra-performance liquid chromatography (UPLC) (Acquity I Class; Waters, Milford, MA, USA) coupled with electrospray triple quadrupole tandem mass spectrometry (ESI-MS/MS) (API 5500; AB SCIEX, Framingham, MA, USA), after ion-pairing extraction (Kannan et al., 2004). The target analytes in the serum were separated using an Acquity UPLC BEH C18 (1.7 mm, 50 × 2.1 mm) column from Waters (Milford, MA, USA). PFAS analytes were monitored by multiple reaction monitoring mode under negative ionization. Quantification of all analytes was based on the isotope-dilution method. Instrumental calibration was verified by matrix-matched calibration standards with a 13-point calibration curve within the range of 0.01–100 ng/mL. Laboratory measurements underwent extensive quality control and quality assurance review, including tolerance limits for operational parameters, measurement of quality control sample in each analytical run to detect unacceptable performance in accuracy or precision, and verification of traceable calibration materials. Internal quality control materials (Standard Reference Material, 1958; fortified human serum), two-three blank samples prepared with HPLC water and matrix matched calibration curves were included throughout each analytical run. Method performance was monitored through successful participation in four external quality assessment schemes in human serum that included investigated congeners in spiked serum, including CDC proficiency testing and quality control schedule. Limits of detection (LODs) ranged from 0.0400 to 0.400 ng/mL.

2.4. Statistical analysis

We performed descriptive analysis of the interview data to characterize the sample of licensed anglers and Burmese immigrants and refugees in terms of demographics and fish consumption. Among licensed angler participants, total consumption of locally caught fish for the past year was estimated by summing the reported times of eating all types of fish species. Since Burmese participants reported high consumption of local fish and consumed it throughout the year, their consumption of locally caught fish was assessed differently and by season. For this group, the total number of meals consumed in the past ten months (summer, fall, and winter) was calculated; the spring data were not used in the calculation due to a high portion of missing data. Serum PFAS data were analyzed to determine distribution among study participants. For each PFAS, we calculated the percentage of samples with detectable concentrations and select percentiles, including the median, 25th, 75th, and 90th percentile. Correlations between PFAS were assessed using Spearman's rank correlation.

We used multiple linear regression to examine associations between local fish consumption and PFAS serum levels. The PFAS selected to perform regression analysis for this study had a high detection rate (97.5% or higher) in both groups (PFOS, PFOA, PFHxS, PFNA, PFDA). Non-detect PFAS results were assigned a value equal to the LOD divided by the square root of 2 following NHANES guidelines (CDC, 2019). A natural log transformation was performed on PFAS concentrations and total past-year fish meals to address the normality assumption for regression models. Consumption of locally caught fish in summer was typically reported as a weekly frequency (e.g., 2 times per week) among the Burmese and was dichotomized (>3 or ≤ 3 times per week) by collapsing adjacent categories with similar geometric means of PFOS, the predominant PFAS detected. Exponentiated model coefficients were calculated and presented as percent increase when appropriate for better interpretation of the results.

Demographic and background variables were evaluated first for associations with PFAS levels and fish consumption variables using bivariate analyses, including t-tests and correlation tests. Variables examined included age, gender, body mass index (BMI), race/ethnicity, education, years of residence in study area, ever living in a refugee camp (for the Burmese group), cigarette smoking, use of chewing tobacco or snuff, store-bought fish/shellfish meals, consumption of fish paste among Burmese participants, and popular hobbies among licensed anglers (e.g., gardening, metal work, and woodworking). Potential covariates that were associated with PFAS levels at $P < 0.2$ in bivariate analyses were included in initial multiple regression models. Covariates included in the final models were associated with at least one PFAS at P

< 0.05 in the multiple regression analysis. For the licensed angler group, a final model was created including a measure of local fish consumption (total meals of locally caught fish over past year or the number of years consuming locally caught fish), continuous age, gender, race (white or other), education (below associate degree or other) and smoking (current smokers or other) for each PFAS. Final models for Burmese participants consisted of a measure of local fish consumption (total meals of locally caught fish in three seasons or meals of locally caught fish in summer), continuous age, gender, education (median-split years of schooling, ≤ 4 or >4 years), ever living in a refugee camp, and years living in Buffalo. Since there was evidence of ethnic differences in PFAS exposure, separate models for the Karen and non-Karen Burmese participants were also created. For each model, diagnostic plots were used to examine whether the assumptions of linearity, normality, and homoscedasticity were met. All statistical analyses were performed with SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

Selected characteristics for the 397 licensed angler participants are shown in Table 1. Median age was 54 years (interquartile range [IQR]: 44–63). Most licensed anglers were male (86%), non-Hispanic white (84%), and had some college or higher education (63%). The participants reported having lived in the area for a median of 47 years (IQR: 33–57) and having eaten AOC fish for a median of 20 years (IQR: 10–40). For the past year, licensed angler participants reported consuming a median of 16 meals of locally caught fish (IQR: 6–36) and a variety of fish species (range, 1 to 20). The most commonly reported fishing locations in each area (reported by 30% or more participants) and the most popular fish species consumed among licensed anglers are listed in Table 1. This group of participants was largely aware of local fish advisories (82%). A median of 22 meals (IQR: 11–45) of select store-bought fish was consumed in the past year among licensed anglers.

Table 2 presents descriptive data for the 199 Burmese participants. Almost all participants were born in Burma (97%, i.e., Myanmar), and a significant proportion (84%) had lived in a refugee camp prior to coming to the U.S. They had resided in Buffalo for a median of four years (range, 1–9 years). The median age of Burmese participants was 38 years (IQR: 30–46). About 61% of them were women and 51% were of Karen ethnicity. Most of the Burmese had no more than four years of education (53%) and less than half (38%) could read some English. Only 29% of the Burmese were employed, and many (84%) received public assistance through the Supplemental Nutrition Assistance Program. This group consumed locally caught fish, including frozen fish, from nearby AOC waters throughout the year. Local fish were most frequently consumed during summer (June to August), with a median of 39 fish meals (three

Table 1
Demographic characteristics and fish consumption of licensed angler study participants (n = 397)[a].

Demographics	Median (IQR)	Fish Consumption	Median (IQR)
Age	54 (44, 63)	Number of years eating AOC fish	20 (10, 40)
BMI	29 (26, 33)	Past year AOC fish meals	16 (6, 36)
Years residing in study area	47 (33, 57)		**Number (%)**
	Number (%)	Popular fishing locations, by area	
Gender		Niagara and Erie counties (n = 259)	
Female	56 (14%)	Lake Erie	236 (91%)
Male	341 (86%)	Upper Niagara River	151 (58%)
Race/ethnicity		Lake Ontario	151 (58%)
Hispanic	14 (4%)	Lower Niagara River	128 (49%)
Non-Hispanic white	332 (84%)	Eighteenmile Creek	79 (31%)
Non-Hispanic black	37 (9%)	Monroe county (n = 138)	
Non-Hispanic others	10 (3%)	Lake Ontario	116 (84%)
Education		Irondequoit Bay/Creek	93 (67%)
High school graduate or less	148 (37%)	Ponds of Greece	72 (52%)
Some college or Associate's degree	131 (33%)	Lake Ontario creeks	61 (44%)
Bachelor's degree or higher	118 (30%)	Braddock Bay	58 (42%)
Annual family income		Lower Genesee River	41 (30%)
Less than $25,000	32 (9%)	Local fish commonly consumed in the past 12 months	
$25,000 to less than $50,000	88 (24%)		
$50,000 to less than $75,000	85 (23%)	Yellow perch	270 (68%)
$75,000 or more	167 (45%)	Walleye	203 (51%)
Employed in the past 12 months	278 (70%)	Smallmouth bass	138 (35%)
Ever smoked at least 100 cigarettes	259 (65%)	Rainbow/steelhead trout	117 (29%)
Currently smoke cigarettes	86 (22%)	Largemouth bass	104 (26%)
Use chewing tobacco or snuff	13 (3%)	Awareness of local fish advisories	320 (82%)

[a] Percentiles and percentages were calculated based on non-missing responses. Twenty-five (6%) participants had missing data on annual family income; all other variables reported in this table had less than 2% missing data.

Table 2
Demographic characteristics and fish consumption of Burmese study participants (n = 199)[a].

Demographics	Median (IQR)	Fish Consumption	Median (IQR)
Age	38 (30, 46)	Past year AOC fish meals by season	
BMI	26 (23, 29)	Summer (June–August)	39 (26, 52)
Years residing in Buffalo, NY	4.0 (2.6, 5.0)	Fall (September–October)	18 (9, 27)
		Winter (November–March)	22 (0, 44)
	Number (%)	Total of 3 seasons, 10 months	88 (44, 132)
Gender			**Number (%)**
Female	121 (61%)	Popular fishing locations	
Male	78 (39%)	Upper Niagara River at Unity Island Park	167 (84%)
Ethnicity		Upper Niagara River at Broderick Park	113 (57%)
Karen	93 (51%)	Black Rock Canal at Unity Island Park	81 (41%)
Burman	36 (20%)	Ponds on Unity Island Park	64 (32%)
Karenni	25 (14%)	Local fish commonly consumed in the past 12 months	
Other (Chin, Kachin, Rakhine, other)	28 (15%)		
Lived in refugee camps	168 (84%)	Quillback	145 (73%)
Years of education		Common carp	134 (67%)
0 to 4	102 (53%)	Minnow	118 (59%)
5 to 16	91 (47%)	White perch	101 (51%)
Read some English	76 (38%)	White bass	99 (50%)
Currently employed	58 (29%)	Brown bullhead	96 (48%)
Receive food stamps	167 (84%)	Consume homemade fish paste	62 (31%)
Currently smoke cigarettes	42 (21%)	Consume store bought fish paste	156 (78%)
Use chewing tobacco or snuff	55 (28%)	Awareness of local fish advisories	79 (41%)

[a] Percentiles and percentages were calculated based on non-missing responses. About 9% of data on ethnicity were missing, and all other variables reported in this table had less than 3% missing data.

times a week) eaten in the previous summer. For ten months in the past year, they consumed a median of 88 meals of locally caught fish (IQR: 44–132). The primary Burmese ethnic group in our study was the Karen who reported consuming more local fish meals (median = 88 meals) than the other Burmese participants (median = 79 meals) (Fig. 1). Burmese participants reported eating a large variety of fish species and typically caught fish from the upper Niagara River around Unity Island. About 41% of the Burmese participants reported being aware of local fish advisories. Store-bought fish/shellfish were consumed a median of 104 times (IQR: 36–156) in the past year, and fish paste was also commonly consumed in this group. Additional descriptive data on characteristics of the study participants can be found in Savadatti et al. (2019).

Ten of the 12 measured PFAS were detected in our samples. Table 3a shows the distribution of PFAS concentrations for each group of participants. As a reference, median PFAS levels in the general U.S. population aged 20 years and older from the NHANES 2013–14 report are also included (CDC, 2019). Five PFAS were detected in almost all (>97%) participants and were selected for regression analyses to assess associations with local fish consumption (PFOS, PFOA, PFHxS, PFNA and PFDA). Among licensed angler participants, PFOS had the highest median concentration (11.6 ng/mL), followed by PFOA, PFHxS, PFNA, and PFDA (medians ranged from 0.31 to 2.7 ng/mL). PFOS was also the predominant PFAS among the Burmese group (median, 35.6 ng/mL), followed by PFDA, PFHxS, PFOA, and PFNA (medians ranged from 1.5 to 2.1 ng/mL). In the Burmese group, the Karen and Burman ethnic groups had the highest median serum PFOS (39.4 and 37.6 ng/mL, respectively); the median PFOS level among the Karenni group was much lower (21.2 ng/mL), but still several times higher than that of the general U.S. population (5.60 ng/mL). All other ethnic groups had small sample sizes (less than 12 participants) (Fig. 2).

PFAS pairs showed positive correlations ranging from weak to strong (Table 3b). PFOS and PFDA were the most strongly correlated in both study groups (Spearman correlation coefficient [Spearman r]: 0.76 in licensed anglers and 0.94 in the Burmese). The least correlated pairs in licensed anglers were PFHxS and PFDA (Spearman r: 0.23). Among the Burmese participants, PFHxS was not associated with PFOS, PFNA, or PFDA, and had a weak association with PFOA (Spearman r: 0.19).

Multiple linear regression results for licensed angler participants are presented in Table 4. After adjusting for age, gender, race, education, and smoking, past-year AOC fish meals were positively associated with PFOS ($P < 0.0001$), PFDA ($P < 0.0001$), PFHxS ($P = 0.01$), and PFNA ($P = 0.02$) levels. The model results show stronger associations of past-year AOC fish consumption with PFOS and PFDA, which correspond to about 7% increase in PFOS and PFDA levels with a 50% increase in past-year AOC fish meals, or about 12% increase in PFOS and PFDA levels with a 100% increase in past-year AOC fish meals. For this group, the number of years consuming AOC fish was also positively associated with PFOS ($P = 0.01$) and PFDA ($P = 0.01$), controlling for the same covariates. The adjusted R^2 values of the models in Table 4 ranged between 0.03 and 0.15. The models showing significant associations of past-year AOC fish consumption with PFOS and PFDA had adjusted R^2 values of 0.15 and 0.14, respectively.

In the licensed angler group, age was positively associated with levels of PFOS, PFNA and PFOA. Male sex was associated with increased PFOS, PFHxS and PFOA levels. White race and current smoking were both associated with decreased PFOS and PFDA. Additionally, there was a weak association between higher education and increased PFNA and PFHxS levels among this group (results not shown).

Tables 5a–5c shows the multiple regression results for Burmese participants, adjusted for age, gender, education, ever living in a refugee camp, and years lived in Buffalo. For the overall Burmese group, we did not find significant associations of past-year or summer AOC fish consumption with the PFAS assessed (Table 5a). In stratified analyses, however, we found associations between summer AOC fish consumption and some PFAS by ethnicity, although levels of reported local fish consumption were similar among ethnic groups. Specifically, summer AOC fish meals among Burmese participants of Karen ethnicity were not associated with any PFAS (Table 5b), but among individuals of non-Karen ethnicity, PFOS ($P = 0.004$) and PFDA ($P = 0.02$) levels were significantly higher among those consuming AOC fish more than three times a week in the past summer (Table 5c). Among non-Karen participants, the results suggest about 76% increase in PFOS and 56% increase in PFDA for those consuming AOC fish more than three times a week compared to others consuming it less frequently, in summer. The adjusted R^2 values of the Burmese models in Tables 5a–5c ranged between 0.03 and 0.25. The non-Karen models showing significant associations of summer AOC fish consumption with PFOS and PFDA had adjusted R^2 values of 0.15 and 0.11, respectively.

In the Burmese group, age was associated with increased PFOA and

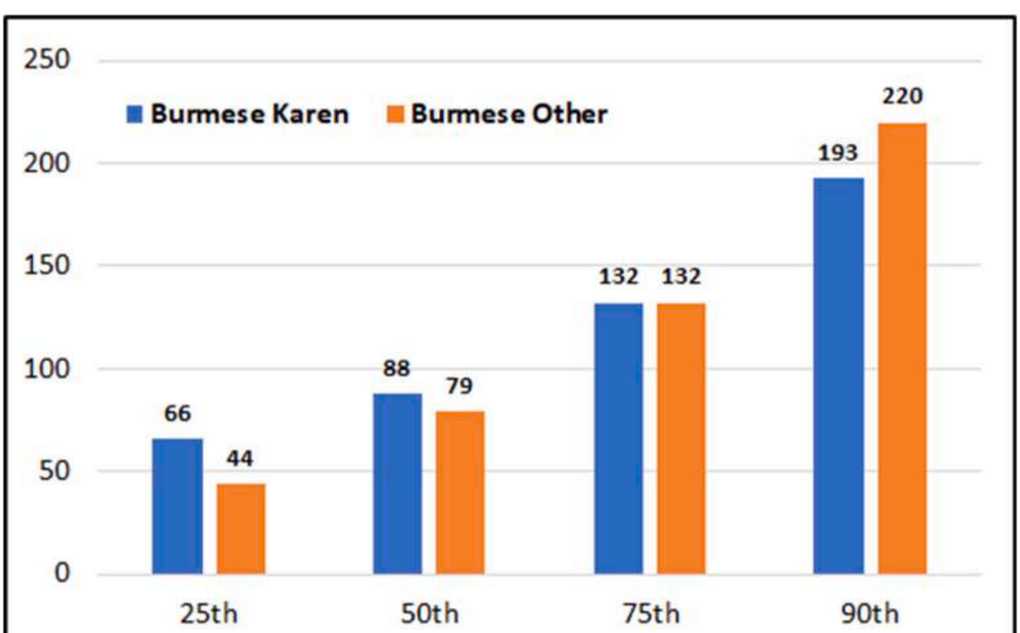

Fig. 1. Percentiles of locally caught fish meals in the past year (10 months) among the Burmese Karen and other Burmese participants.

Table 3a
Distribution of serum PFAS concentrations in study participants and in the general U.S. population (ng/mL).

	Licensed Anglers (n = 397)		Burmese (n = 199)		NHANES [b]
PFAS	% > LOD [a]	Median (25th, 75th, 90th percentile)	% > LOD [a]	Median (25th, 75th, 90th percentile)	Median (95% CI)
Perfluorooctane sulfonic acid (PFOS)	100	11.6 (8.0, 18.5, 35.4)	100	35.6 (20.2, 51.9, 94.3)	5.60 (5.10–6.00)
Perfluorooctanoic acid (PFOA)	99.8	2.7 (1.9, 3.4, 4.4)	100	1.6 (1.1, 2.0, 2.6)	2.07 (1.90–2.27)
Perfluorohexane sulfonic acid (PFHxS)	99.8	2.4 (1.7, 3.4, 5.1)	100	1.9 (1.3, 2.9, 8.0)	1.40 (1.30–1.60)
Perfluorononanoic acid (PFNA)	99.8	0.99 (0.72, 1.4, 1.9)	100	1.5 (1.0, 2.3, 3.2)	.700 (.600–.800)
Perfluorodecanoic acid (PFDA)	97.5	0.31 (0.20, 0.49, 0.97)	100	2.1 (1.3, 3.0, 5.5)	.200 (.200–.200)
Perfluoroundecanoic acid (PFUnDA)	72.5	0.15 (<LOD, 0.28, 0.48)	98.5	1.0 (0.63, 1.4, 2.7)	< LOD
Perfluorododecanoic acid (PFDoA)	29.0	< LOD (<LOD, 0.04, 0.08)	97.0	0.16 (0.10, 0.26, 0.45)	< LOD
N-methyl-perfluorooctane sulfonamido acetic acid (MeFOSAA)	86.2	0.15 (0.07, 0.29, 0.53)	93.5	0.18 (0.10, 0.33, 0.51)	< LOD
Perfluoroheptanoic acid (PFHpA)	89.7	0.14 (0.09, 0.21, 0.31)	65.3	0.12 (0.03, 0.22, 0.35)	< LOD
Perfluorohexanoic acid (PFHxA)	72.8	0.11 (<LOD, 0.18, 0.23)	26.1	< LOD (<LOD, 0.05, 0.10)	–
Perfluorodecane sulfonate (PFDS)	0	–	0	–	–
Perfluorooctane sulfonamide (PFOSA)	0	–	0	–	< LOD

[a] Laboratory method limit of detection (LOD): 0.04 ng/mL for PFNA, PFHpA, N-MeFOSAA, PFHxA, PFDoA and PFOSA; 0.10 ng/mL for PFOA, PFHxS, PFDA and PFUnDA; 0.20 ng/mL for PFOS; 0.40 ng/mL for PFDS.
[b] Median levels of PFAS in the general U.S. population aged 20 years or older, from the National Health and Nutrition Examination Survey (NHANES) 2013–14, are provided as a reference. The median estimate for PFOSA is not available for NHANES 2013–14 and is from NHANES 2011–12. The NHANES 2013-14 LOD was 0.1 ng/mL for all the PFAS analytes. PFHxA and PFDS were not included in NHANES 2013–14.

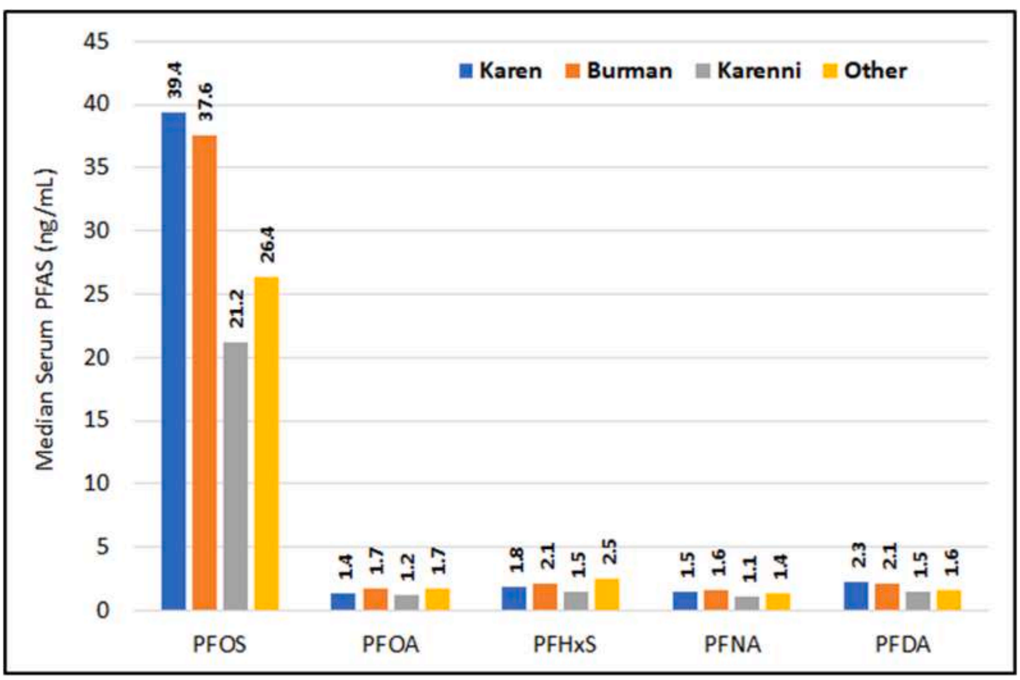

Fig. 2. Median serum concentrations of primary PFAS for Burmese participants by ethnic group.

PFNA levels but associated with decreased PFHxS levels. Male sex was associated with increased PFOS, PFOA and PFNA levels, but associated with decreased PFHxS levels. The number of years lived in Buffalo was associated with increased PFOS and PFNA levels and showed a borderline association with increased PFDA levels. More years of schooling was associated with lower levels of PFOS and PFDA. Ever living in a refugee camp was associated with lower PFOA levels (results not shown).

4. Discussion

This study presents valuable human biomonitoring data for PFAS in two different populations, licensed sport anglers and Burmese refugees and immigrants, who consumed fish caught from New York's Great Lakes basin. Licensed anglers were predominantly older white males with relatively high income and education levels, who had resided in western NYS for many years and were largely aware of local fish advisories. The Burmese participants were younger and with low income, education, and English proficiency, who had lived in Buffalo, NYS for several years and had relatively lower awareness of local fish advisories. The Burmese group consumed a large amount of locally caught fish, showing a much higher level of consumption than the licensed anglers. PFOS was the predominant compound among the 12 measured PFAS in

Table 3b
Spearman correlation coefficients between serum PFAS concentrations in study participants [a].

	PFOS	PFOA	PFHxS	PFNA	PFDA
Licensed Anglers (n = 397)					
	PFOS	PFOA	PFHxS	PFNA	PFDA
PFOS	–				
PFOA	**0.412**	-			
PFHxS	**0.373**	**0.366**	-		
PFNA	**0.583**	**0.496**	**0.325**	-	
PFDA	**0.760**	**0.300**	**0.226**	**0.663**	-
Burmese (n=199)					
	PFOS	PFOA	PFHxS	PFNA	PFDA
PFOS	–				
PFOA	**0.298**	-			
PFHxS	0.009	**0.188**	-		
PFNA	**0.750**	**0.589**	0.114	–	
PFDA	**0.945**	**0.271**	0.007	**0.724**	–

[a] Bold typeface indicates $P < 0.05$.

Table 4
Associations between fish consumption and serum PFAS levels in licensed angler study participants.

PFAS[b]	Model 1[a]: Past year AOC fish meals[b] (n = 382)[c]			Model 2[a]: Number of years eating AOC fish (n = 385)[c]		
	β	(95% CI)	P-value	β	(95% CI)	P-value
PFOS	**0.16**	**(0.10, 0.22)**	**<0.0001**	**0.0062**	**(0.0013, 0.011)**	**0.01**
PFOA	−0.011	(-0.049, 0.027)	0.6	0.00077	(-0.0026, 0.0042)	0.7
PFHxS	**0.061**	**(0.013, 0.11)**	**0.01**	0.0028	(-0.0014, 0.0069)	0.2
PFNA	**0.053**	**(0.0075, 0.098)**	**0.02**	0.0035	(-0.00033, 0.0073)	0.07
PFDA	**0.17**	**(0.12, 0.23)**	**<0.0001**	**0.0060**	**(0.0012, 0.011)**	**0.01**

[a] Adjusting for age, sex, race, education, and current smoking. Bold typeface indicates $P < 0.05$.
[b] Natural log-transformed.
[c] Sample size after excluding observations with missing values.

Table 5a
Associations between fish consumption and serum PFAS levels in all Burmese study participants (n = 193) [a].

PFAS [c]	Model 1 [b]: Past year (10 months) AOC fish meals [c]			Model 2 [b]: Summer AOC fish meals (>3 vs. ≤3 times/week)		
	β	(95% CI)	P-value	β	(95% CI)	P-value
PFOS	0.047	(-0.092, 0.19)	0.5	0.13	(-0.096, 0.36)	0.3
PFOA	−0.033	(-0.12, 0.054)	0.5	0.061	(-0.082, 0.20)	0.4
PFHxS	0.050	(-0.11, 0.21)	0.5	0.030	(-0.23, 0.29)	0.8
PFNA	−0.032	(-0.14, 0.074)	0.6	−0.019	(-0.19, 0.16)	0.8
PFDA	0.023	(-0.11, 0.16)	0.7	0.13	(-0.092, 0.35)	0.3

[a] Sample size after excluding observations with missing values.
[b] Adjusting for age, sex, education, lived in refugee camps, and years lived in Buffalo. Bold typeface indicates $P < 0.05$.
[c] Natural log-transformed.

both participant groups. Compared to the NHANES adult population (median for 2013–2014, 5.60 ng/mL), the licensed angler group had a higher median serum PFOS level (11.6 ng/mL), and the Burmese group had a substantially higher median serum PFOS level (35.6 ng/mL). Serum levels of other PFAS in both groups were generally low and comparable to those in the general U.S. population. The strong

Table 5b
Associations between fish consumption and serum PFAS levels in Burmese Karen participants (n = 92) [a].

PFAS [c]	Model 1 [b]: Past year (10 months) AOC fish meals [c]			Model 2 [b]: Summer AOC fish meals (>3 vs. ≤3 times/week)		
	β	(95% CI)	P-value	β	(95% CI)	P-value
PFOS	−0.18	(-0.40, 0.040)	0.1	−0.16	(-0.47, 0.15)	0.3
PFOA	−0.064	(-0.22, 0.089)	0.4	0.13	(-0.089, 0.34)	0.2
PFHxS	0.15	(-0.14, 0.43)	0.3	0.088	(-0.32, 0.49)	0.7
PFNA	−0.17	(-0.33, −0.0063)	0.04 [d]	−0.11	(-0.35, 0.12)	0.3
PFDA	−0.16	(-0.38, 0.050)	0.1	−0.13	(-0.44, 0.17)	0.4

[a] Sample size after excluding observations with missing values.
[b] Adjusting for age, sex, education, lived in refugee camps, and years lived in Buffalo.
[c] Natural log-transformed.
[d] The parameter estimates suggested a weak negative association.

Table 5c
Associations between fish consumption and serum PFAS levels in Burmese non-Karen participants (n = 87) [a].

PFAS [c]	Model 1 [b]: Past year (10 months) AOC fish meals [c]			Model 2 [b]: Summer AOC fish meals (>3 vs. ≤3 times/week)		
	β	(95% CI)	P-value	β	(95% CI)	P-value
PFOS	0.19	(-0.021, 0.40)	0.08	**0.57**	**(0.19, 0.94)**	**0.004**
PFOA	−0.010	(-0.13, 0.11)	0.9	0.038	(-0.19, 0.27)	0.7
PFHxS	−0.051	(-0.25, 0.15)	0.6	−0.054	(-0.42, 0.31)	0.8
PFNA	0.066	(-0.093, 0.23)	0.4	0.17	(-0.13, 0.46)	0.3
PFDA	0.12	(-0.087, 0.32)	0.3	**0.45**	**(0.079, 0.81)**	**0.02**

[a] Sample size after excluding observations with missing values.
[b] Adjusting for age, sex, education, lived in refugee camps, and years lived in Buffalo. Bold typeface indicates $P < 0.05$.
[c] Natural log-transformed.

correlations between serum PFOS and PFDA levels in both study groups were consistent with previous results of fish sample analyses. Among all analyzed PFAS, PFOS and PFDA had the highest percentages of detection (100% and 92%, respectively) in Great Lakes fish tissue samples (Stahl et al., 2014).

Besides measuring the body burden of PFAS, this study also provides evidence on the association of local fish consumption with serum PFAS levels in these populations. Among licensed anglers, higher consumption of locally caught fish over the past year was positively associated with higher serum PFOS, PFDA, PFHxS, and PFNA. In this group, the number of years consuming locally caught fish, which is a crude measure of long-term consumption, also showed positive associations with PFOS and PFDA. In the Burmese group, we found positive associations of local fish consumption in the past summer with levels of PFOS and PFDA among the Burmese of non-Karen ethnicity.

Our findings of associations between local fish intake and serum concentrations of some PFAS, especially PFOS, are consistent with previous studies of anglers and other populations with various levels of fish consumption (Christensen et al., 2016, 2017; Dallaire et al., 2009; Denys et al., 2014; Haug et al., 2010; Holzer et al., 2011; Lindh et al., 2012). A 2012–2013 study of male anglers 50 years and older from Wisconsin, U. S., who also consumed fish from Great Lakes waters, is comparable to our licensed angler group (Christensen et al., 2016). The Wisconsin anglers had a similar level of local fish consumption and higher median serum PFOS (19.0 ng/mL) than that of our licensed anglers, which could be partially due to older ages in that group (men 50 years and older). Consistent with our findings, consumption of locally caught fish in the Wisconsin anglers was associated with higher levels of PFOS, PFNA and PFDA. In contrast to our findings, this study did not find a similar association for PFHxS but found a positive association between local fish consumption and PFOA levels. Differences in these results may be due to different concentrations of PFHxS and PFOA in consumed local fish, a smaller sample size in the Wisconsin angler study (n = 154), and different methods used in recruitment (online survey, flyers, and other methods) and statistical analysis (ordinal logistic regression for quartiles of PFAS outcomes). Our results in the licensed angler group are also consistent with recent findings of serum levels of PFOS, PFNA, and PFDA being related to fish consumption in the past 30 days in the general U.S. population (Christensen et al., 2017).

In our Burmese group, the high median level of serum PFOS and the positive association with summer/recent local fish consumption among the non-Karen were in agreement with those found in studies of indigenous groups with high levels of local fish/seafood consumption in other countries (Dallaire et al., 2009; Hansen et al., 2016; Lindh et al., 2012). The findings of positive associations between serum PFOS and PFDA and local fish consumption in the non-Karen Burmese participants also agree with our results for the same two compounds in the licensed angler group. This is one of the first studies, to our knowledge, that reports biomonitoring data on serum PFAS of a subsistence fishing population in the Great Lakes region. However, several studies have found associations between consumption of Great Lakes fish and levels of polychlorinated biphenyls (PCBs), mercury, and dichlorodiphenyldichloroethylene (DDE) in Native Americans (Fitzgerald et al., 2001, 2004, 2007; Gerstenberger et al., 1997, 2000), and PCBs in people of Hmong descent in Wisconsin, U.S., who practice subsistence fishing (Schantz et al., 2010).

Since our Burmese participants had only lived in Buffalo or the U.S. for a few years (median = 4) and because PFOS has a long half-life, their elevated serum PFOS levels may in part reflect their dietary or environmental exposures while living in Burma and/or in refugee camps, which were mostly located in Thailand and some in Malaysia. Their PFOS levels were likely to be elevated relative to the U.S. general population or local anglers when they arrived in the U.S. A study assessing the diet of Burmese refugees living in a large camp in Thailand showed that fermented fish was the main source of animal protein and refugees also supplemented their diet by purchasing fresh fish and fish products (Banjong, 2003). Recent studies have shown widespread PFAS contamination of the water system in Thailand. PFOS was detected in rivers, groundwater, drinking water, and at the highest concentrations in industrial wastewater, which is a major source of PFOS contamination (Boontanon et al., 2013; Hongkachok et al., 2018). In addition, an analysis of freshwater fish samples from various Asian countries, including Thailand and Malaysia, reported that PFOS was commonly present in the fish samples and was the predominant PFAS (Murakami et al., 2011).

Our finding of ethnic differences in PFAS exposure was unexpected. Possible explanations include exposure to different environments and foods in refugee camps, which may have contributed to varying body burdens of PFAS prior to arriving in the U.S., and some existing dietary differences between the ethnic groups including a higher consumption of fish paste among the Karen Burmese. It is also possible that differences in the primary sources (waterbodies) and species of locally caught fish consumed by the ethnic groups also may have contributed to varying serum PFAS levels and their associations with local fish consumption.

Exposure to environmental contaminants in the general U.S. population has long been associated with ethnicity and health disparities (Belova et al., 2013; Payne-Sturges and Gee, 2006; Xue et al., 2014). This has been the case for contaminants associated with fish and shellfish intake, as illustrated by analyses of NHANES data that have consistently shown the highest concentrations of blood methyl mercury among Asian populations (CDC, 2019; Hightower et al., 2006; McKelvey et al., 2007). Our results for the Burmese group provide insight into levels of serum PFAS in an immigrant population whose cultural heritage and low income likely contribute to their reliance on local fish for subsistence and higher risk of exposure to environmental pollutants, as opposed to sport fishing populations or the general U.S. population.

Results of this study also suggest the importance of continuing to monitor PFAS concentrations in fish sampled from Great Lakes waterbodies and develop consumption advisories based on up-to-date fish testing data. The NYS DEC has been conducting statewide fish sampling to assess PFAS contamination in local fish. Their 2010–2018 data show that PFAS are pervasive in Great Lakes fish, with PFOS being the greatest contributor to total PFAS (NYSDEC, 2019). While PFAS were not specifically identified in NYS fish consumption advisories at the time of outreach being conducted for this project, exposures were partially mitigated by the general fish consumption advisories (eat up to four, half pound meals per month) and some existing specific advisories for other contaminants. Subsequently, NYS has issued PFAS advisories for some NYS waters as warranted by fish testing results (NYS DOH, 2017). Prior research suggested that knowledge of local fish advisories can impact fish consumption behaviors in anglers, including populations who practice subsistence fishing, thus helping reduce the risks of exposure to environmental contaminants in fish (Lauber et al., 2018; Oken et al., 2012; Silver et al., 2007). Since the 1980s, NYS DOH has developed brochures and materials to inform anglers and families of healthy choices related to fish they catch. DOH's fish consumption advice is available in multiple languages, online and in print with more than 40 separate titles, and can be browsed by NYS region, waterbody, and fish species. DOH has also conducted some targeted outreach to refugee and native American populations, by learning about their cultural practices and learning styles, to provide linguistically and culturally appropriate materials and messages to these populations.

Our study has limitations that should be considered in interpreting the findings and for planning future research. First, our results for licensed anglers may be affected by non-response bias (Savadatti et al., 2019). The response rate of licensed anglers to participate in the eligibility screening (16%) was somewhat lower than that in a large survey of licensed anglers in the Great Lakes region (24%), which could be due to different eligibility criteria and the additional requirement to provide biological samples in our study (Bruce Lauber et al., 2017). Although previous studies on licensed anglers found that non-respondents likely fished less and were less aware of local fish advisories, there are mixed findings on whether non-respondents ate less locally caught fish than respondents (Bruce Lauber et al., 2017). Second, our group sizes were small in the ethnicity-stratified analysis for the Burmese group, which could have limited the statistical power of the multivariable regression analyses in detecting associations. Third, our measure of PFAS exposure via local fish consumption was only based on the number of fish meals reported, since more detailed data on fish intake (e.g., serving size) and the PFAS concentrations in local fish species were not available. Improving the quantification of exposure to PFAS through fish intake and identifying other possible exposure routes (e.g., contaminated drinking water, indoor air and dust, consumer products) may improve the models. This study collected limited data on the consumption of store-bought fish, and future research could benefit from collecting consumption information on all sources of fish and shellfish (Christensen et al., 2016). Last, for the Burmese participants, data or reference levels on PFAS exposure prior to immigration to the U.S. were not available, which limited interpretation of the findings. Future studies should monitor changes in serum PFAS in anglers and other populations at higher risk of exposure to verify that they reflect the decreasing trends of serum PFAS in the general U.S. population. In addition, more efforts are needed to assess the health effects associated with long-term exposure to PFAS, together with other potential contaminants such as methyl mercury, through eating locally caught fish.

Overall, results from our two diverse participant groups suggest that consumption of fish caught from the Great Lakes Basin is associated with higher body burdens of some PFAS, and especially of PFOS. Populations consuming a relatively high amount of locally caught fish due to cultural or economic reasons, such as the Burmese refugee and immigrant population targeted in this study, are at greater risk of exposure to those PFAS. It is important to monitor PFAS concentrations in fish sampled from the Great Lakes region and other waterbodies that might have been contaminated with PFAS. NYS has general statewide advice to eat up to four meals per month of sportfish and has issued specific PFAS advisories for some NYS waters when warranted. Presentation of the fish consumption advice has been informed by both qualitative and quantitative data about the fishing and eating behaviors of anglers and families. NYS DOH will continue to use results from biomonitoring and interviews with these populations to create culturally appropriate materials and target outreach to populations at risk.

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Agency for Toxic Substances and Disease Registry.

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Assessing short-term impacts of $PM_{2.5}$ constituents on cardiorespiratory hospitalizations: Multi-city evidence from China

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A R T I C L E I N F O

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A B S T R A C T

Apart from concentrations of particulate mass, $PM_{2.5}$-associated effects on health may largely depend on its chemical components. However, little is known regarding the underlying effects of specific $PM_{2.5}$ constituents. The study included nearly 1 million hospital admissions from five Chinese cities during 2015–2017. Based on the modified Community Multiscale Air Quality model, our study simulated daily concentrations of $PM_{2.5}$ and five main components. We used a time-stratified case-crossover design with conditional logistic regression models to estimate short-term effects of $PM_{2.5}$ constituents on cause-specific hospital admissions. Per interquartile range increase in exposure to $PM_{2.5}$, elemental carbon, organic carbon, nitrate, sulfate and ammonium at lag 04-day was related to an excess risk (ER%) for non-accidental admissions of 1.6% [95% confidence interval: 1.1–2.0], 1.9% [1.3–2.4], 1.0% [0.5–1.6], 1.2% [0.4–2.0], 1.2% [0.9–1.5] and 1.4% [0.9–1.9], respectively. Great heterogeneities of constituents-admission associations existed in diverse causes and constituents. This study provided multi-center high-quality evidence that hospital admissions, particularly those for ischemic heart disease (ER% ranging from 2.3 to 5.4% at lag 04-day) and pneumonia (1.9–5.1% at lag 4-day), could be triggered by short-term exposures to ambient $PM_{2.5}$ constituents. Relatively stronger constituents-admission associations were found among females for respiratory causes and the elderly for cardiovascular causes.

1. Introduction

Hospital admission is an endpoint of special concern in epidemiological studies to measure broad health impacts of air pollution on the general population (Liu et al., 2018a; Villeneuve et al., 2006). A large body of time-series and case-crossover studies have revealed the association of short-term exposure to ambient $PM_{2.5}$ (particulate matter with aerodynamic diameter ≤2.5 μm) with cause-specific hospital admissions, particularly caused by cardiopulmonary diseases (Chang et al., 2015; Groves et al., 2020; Tian et al., 2019). However, the magnitudes of $PM_{2.5}$-associated effect estimates largely varied by season and studies (Dominici et al., 2006; Li et al., 2020), which could be partially explained by substantial differences of $PM_{2.5}$ constituents across study periods and locations (Achilleos et al., 2016; Hu et al., 2017c). Identifying the most harmful particulate constituents to human health is of great necessity and urgency, so as to make efficient public health intervention actions and comprehensively reduce $PM_{2.5}$-related risks (Achilleos et al., 2017; Bell et al., 2011).

$PM_{2.5}$ is a mixture of various chemical substances originating from primary emissions and secondary transformations. The major $PM_{2.5}$

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components generally include water-soluble inorganic ions, such as sulfate (SO_4^{2-}), nitrate (NO_3^-) and ammonium (NH_4^+), followed by elemental carbon (EC) and organic carbon (OC) (Hu et al., 2017c; Liang et al., 2016). Existing epidemiologic evidence, concentrated in developed regions such as North America and Western Europe (Samoli et al., 2016; Yang et al., 2019), has associated aforementioned $PM_{2.5}$ constituents with hospitalization for acute exacerbations of diseases, while constituents-morbidity relationships largely varied across regions and causes (Atkinson et al., 2015; Ito et al., 2011). For instance, a national research covering 119 US urban communities reported that EC, OC, NO_3^- and NH_4^+ were associated with cardiovascular hospitalizations, while only OC was related to respiratory hospital admissions (Peng et al., 2009). Another multi-city study in Southern Europe linked EC and SO_4^{2-}, rather than OC and NO_3^- with increased risks of cardiovascular and respiratory admissions (Basagana et al., 2015). Due to a wide lack of ground-based measurements (Hu et al., 2017c), the differential associations of $PM_{2.5}$ constituents with morbidity for cardiopulmonary subcategories remain largely unknown, particularly in western pacific countries such as China.

To enhance scientific understanding of $PM_{2.5}$ constituents-related health impacts among Chinese population, we conceived a large-scale case-crossover study to examine short-term associations between major $PM_{2.5}$ constituents and hospital admissions in five Chinese cities during January 1, 2015 to December 31, 2017. Further, we sought to identify whether constituents-associated effects differed by cause, sex and age.

2. Methods

2.1. Data collection

2.1.1. Hospital admission

During January 1, 2015 to December 31, 2017, our data of 7 hospitals were derived from the authoritative official Health Expense Accounting System of Hubei Province, China (Wang et al., 2020b; Yao et al., 2020). The process of selecting hospitals simply was divided into three stages (Fig. S1). First, the government selected 5 cities (i.e., Wuhan-13 districts or counties, Yichang-13, Huanggang-10, Shiyan-8, Jingmen-5) with different geographic locations and economic development levels from 17 prefecture-level cities in Hubei province (Fig. S2). Second, due to Wuhan and Yichang with large population, two districts or counties were allocated to them according to gross domestic product, and other cities randomly included one district or county (Wang et al., 2020b). Third, a best tertiary hospital for each region was chosen as the sample institution considering the level of medical and health resource allocation, the degree of informatization, etc (Yao et al., 2020).

The daily hospital admission data in 7 tertiary hospitals were drawn from the Hospital Information System (HIS) (Wang et al., 2020b). All tertiary hospitals in China were requested to implement the three-level quality control management to guarantee the completeness, consistency and accuracy of medical records (Wang, 2013). First, the doctor in charge records medical information of inpatients in a nationally standardized format and submits archives to the HIS. Second, the diseases' diagnosis is coded using the 10th revision of the International Classification of Diseases (ICD-10) by certified professional medical coders. A full-time quality control physician checking group is formed in the medical record department with a 100% checking rate. Third, an expert group will randomly check medical records every week and reconfirmed ICD code. For each inpatient, we extracted its hospital admission date, causes for admission, and demographic information such as sex and age. Based on ICD-10 codes, we defined the main outcomes as hospital admissions for non-accidental (A00–R99), cardiovascular (I00–99) and respiratory diseases (J00–99). The subsets of cardiovascular diseases included hypertension (I10–15), ischemic heart disease (IHD, I20–25) and stroke (I60–69) that was further divided into ischemic stroke (I63) and hemorrhagic stroke (I61–62). Upper respiratory tract infection (URTI, J00–J06), pneumonia (J18) and chronic obstructive pulmonary disease (COPD, J40–44 and J47) were included in respiratory diseases.

2.1.2. Environmental exposures

Daily concentrations of ambient $PM_{2.5}$ mass and its major constituents (i.e., EC, OC, SO_4^{2-}, NO_3^- and NH_4^+) and Ozone (O_3) at the horizontal resolution of 36 × 36 km during 2015–2017 were estimated by the modified Community Multiscale Air Quality (CMAQ) model (v5.0.1). The modified CAMQ model was generally used to estimate the concentrations of air pollutants, and would update with time and study sites. The data collection, methodology details, and specific model performances were described in professional atmospheric studies, which were conducted by our team based on consistent assumptions (Hu et al., 2014, 2016, 2017b, 2017c; Wang et al., 2014).

In brief, to drive the air quality simulations, four datasets of environmental exposures inputted in the modified CMAQ model were: 1) the meteorological parameters, generated using the Weather Research and Forecasting (WRF), v3.6.1; 2) the anthropogenic emissions, based on the Multi-resolution Emission Inventory for China (MEIC), v1.0, (http://www.meicmodel.org); 3) the biogenic emissions, generated by the Model for Emissions of Gases and Aerosols from Nature (MEGAN), v2.1; 4) the open biomass burning emissions, based on the Fire Inventory from the National Center for Atmospheric Research (FINN). By calculating mean fractional bias and error, we found that the ensemble predictions of $PM_{2.5}$ mass agreed favorably with hourly observations at 422 sites in 60 large cities from the China National Environmental Monitoring Center (http://www.cnemc.cn/sssj/) (Hu et al., 2017b). The modified CMAQ model with more detailed gas-phase photochemical mechanism and updated secondary organic aerosol yields can be applied to simulate surface concentrations and determine precursor contributions to secondary organic aerosol, and thus predict concentrations of secondary inorganic and organic components in $PM_{2.5}$ (Stewart et al., 2017; Ying et al., 2014, 2015). Overall, the model performance of $PM_{2.5}$ compositions met the criteria of mean fractional bias and error recommended by the U.S. Environmental Protection Agency (Hu et al., 2017b, 2017c). The exposure data were developed for mainland China and has been widely used in epidemiological research on health effects (Hu et al., 2017a; Lee et al., 2019; Wang et al., 2019). For instance, it not only has been applied in a nationwide study to investigate the associations between $PM_{2.5}$ constituents and cause-specific mortality across China (Yang et al., 2020), but also has been applied to estimate premature mortality attributable to particulate matter in China (Hu et al., 2017a).

We overlaid the natural cities' polygons with the gridded spatial data of $PM_{2.5}$ mass and its constituents at the horizontal resolution of 36 × 36 km, and calculated mean values of covered grids to extract estimates of average concentrations in each city. During the study period (1096 days), there is around 1.2% missing data for $PM_{2.5}$ constituents (13 days). Over the same period, daily meteorological covariates for each city, including average temperature (∘C), relative humidity (%), sunshine duration (hour), wind speed (m/s), and atmospheric pressure (hPa), were derived from the China Meteorological Network (http://data.cma.cn).

2.2. Design and analysis

2.2.1. Study design

In our study, we employed a time-stratified case-crossover (TSCC) design to investigate short-term associations of hospital admissions with exposure to $PM_{2.5}$ and its constituents. Each patient's admission date was designed as a "case" day, while his/her "control" days (3–4 days) were selected using time strata defined by the same day of the week (DOW) within the same year and month (Ding et al., 2017; Szyszkowicz et al., 2012). For example, a patient was admitted to hospital on 15 August 2017 ("case" day), August 1st, 8th, 22nd, and 29th in 2017 should be served as corresponding "control" days. This self-matching design could well control most individual confounders including sex, age, race, body mass index, smoking, drinking, metabolic and other time-invariant behavior factors, as well as zip code level variables, such as socioeconomic status, area level measure of ethnicity, and population density (Carracedo-Martínez et al., 2010; Wei et al., 2019). The time-stratified layout of a month could eliminate the potential confounding effects caused by seasonality, long-term time trend and DOW (Lumley and Levy, 2000). By comparing the exposure of fine particulates components on the case day with those on the control days, we could assess the risk of constituents-associated hospitalization for research subjects.

2.2.2. Statistical analysis

Spearman correlation coefficient (r_s) was applied to measure correlations between fine particulates, $PM_{2.5}$ constituents and meteorological conditions. Conditional logistic regression (CLR) models were adopted to separately evaluate short-term effects of ambient exposures to major $PM_{2.5}$ chemical components on hospital admissions for cause-specific diseases (i.e., non-accidental, cardiovascular and respiratory diseases, stroke, IHD, hypertension, pneumonia, COPD and URTI). $PM_{2.5}$ mass or individual constituent was included as a linear term in the CLR model (Chang et al., 2015; Michikawa et al., 2021). To account for the delayed or cumulative effects of particulate pollutants, we prespecified various lag patterns, including single-day lags (lag 0- to lag 4-day) and moving-average lags (lag 01- to lag 04-day) (Wang et al., 2020a; Yang et al., 2020). For instance, lag 0-day refers to exposures on the hospital admission day, and lag 02-day was 3-day moving average exposures of the admission day and 2 days prior to admission. We controlled for the nonlinear confounding effects of meteorological factors by using a natural cubic spline (NCS) function, with 3 degrees of freedom (df) for both average temperature at lag 02-day and relative humidity on current day (Chen et al., 2017; Zhang et al., 2020b).

Using maximum likelihood estimation method, we separately estimated odds ratios (OR) with 95% confidence intervals (CI) for hospital admissions associated with per interquartile range (IQR) increase in exposures to $PM_{2.5}$ and its components (Samoli et al., 2016; Sun et al., 2019). Estimated associations in our study were presented as changes in excess risks (ER%) calculated by $[(\mathrm{OR}-1) \times 100\%]$. Linearity for exposure-response curves between specific $PM_{2.5}$ constituents and risks of hospitalization were checked using likelihood ratio tests, by comparing models incorporating linear and NCS smoothing terms for the pollutant exposures. Additionally, to verify the robustness of our results derived from one-stage pooled analysis, we also applied a two-stage method as a secondary analysis. City-specific constitute-hospitalization association were estimated in the first stage, and the random effects meta-analyses were used to pool the city-specific estimates.

To identify potential vulnerable populations, we further conducted subgroup analyses stratified by sex (male, female), and age group (0–44, 45–64 and 65+ years). Two-sample z-test was implemented to examine whether between-subgroup differences in the estimated effects were substantial, based on the point estimate (PE) and standard error (SE) (Tian et al., 2019; Yang et al., 2020). PE is the logarithm transformed values of OR, i.e., β coefficient derived from CLR models for each stratum. For instance, effect differences between sex could be tested using the formula as follows:

$$z = \frac{\mathrm{PE}_{\mathrm{male}} - \mathrm{PE}_{\mathrm{female}}}{\sqrt{\mathrm{SE}(\mathrm{E}_{\mathrm{male}})^2 - \mathrm{SE}(\mathrm{E}_{\mathrm{female}})^2}}$$

Several sensitivity analyses were conducted to check the robustness of our main results by varying regression modelling choices. First, in line with previous studies (Peng et al., 2009; Sarnat et al., 2015), we additionally adjusted for total $PM_{2.5}$ mass when investigating constituents-hospitalization associations. Second, our study performed two-pollutant analyses by simultaneously incorporating O_3 and one $PM_{2.5}$ constituent into the CLR model. Third, NCS dfs for temperature and relative humidity were changed from 2 to 6 df. Additionally, we included NCS terms of other meteorological covariates (i.e., atmospheric pressure, wind speed, and sunshine duration) for modelling adjustments.

All analyses were conducted in R software (version 4.0.5, R Foundation for Statistical Computing, Vienna, Austria). We used the "ggcorrplot" package for Spearman's correlation analysis, the "splines" package for NCS smoothing, the "survival" package for CLR modeling, and the "meta" package to pool the city-specific estimates. For all tests, two-sided effects of $p < 0.05$ were considered statistically significant.

3. Results

Table 1 summarizes the characteristics of admission cases in cities of interest from January 1, 2015 to December 31, 2017. A total of 978,571 cases (male 51.8%) were included for non-accidental causes, wherein cardiovascular and respiratory diseases accounted for 15.5% (151,913 cases) and 12.9% (126,485 cases), respectively. Stroke, IHD and hypertension shared three quarters of total cardiovascular diseases, and over half of admissions for respiratory diseases were due to COPD, pneumonia and URTI. Mean (standard deviation) age at hospital admission was 48.4 (22.9) years, and 26.5% were aged over 65 years.

Table 2 describes the distribution characteristics of $PM_{2.5}$ mass and specific constituents and meteorological factors during 2015–2017. Daily mean exposures to $PM_{2.5}$ mass, EC, OC, NO_3^-, SO_4^{2-} and NH_4^+ on the day of non-accidental admissions were 55.3, 2.4, 5.4, 12.6, 13.3, and 8.3 μg/m^3, respectively. The selected 5 species represented 4.3–24.0% of the total $PM_{2.5}$ levels, wherein SO_4^{2-} was the most abundant constituent. Over the study period, the IQRs of daily mean $PM_{2.5}$, EC, OC, NO_3^-, SO_4^{2-} and NH_4^+ across five cities were 44.6, 1.9, 5.3, 15.3, 11.4 and 8.0 μg/m^3, respectively (Table S1). $PM_{2.5}$ and its main components were highly positively correlated, with Spearman correlation coefficients ranging from 0.74 to 0.98 (Fig. 1). City-specific summary characteristics in daily hospital admission for cause-specific diseases and meteorological factors are detailed in Supplementary Tables S1 and S2.

Fig. 2 estimates excess risks of hospital admission for total non-accidental, cardiovascular and respiratory diseases at various lag days associated with per IQR rise in exposure to $PM_{2.5}$ and its constituents. Total admissions for non-accidental causes showed significant associations with all selected fine particulate constituents, with greatest risks occurring at lag 04-day. Per IQR increase in exposure to $PM_{2.5}$ (44.6 μg/

Table 1
Summary statistics of study population for hospital admission in five Chinese cities, 2015–2017.

Characteristic	Value	Percent
Total non-accidental (ICD-10 code: A00–R99)	978,571	100.0
Case days	978,571	/
Control days	3,325,016	/
Sex		
Male	506,507	51.8
Female	472,064	48.2
Age, years	48.4 ± 22.9	/
0–44	379,692	38.8
45–64	339,894	34.7
≥65	258,985	26.5
Cardiovascular diseases (ICD-10 code: I00–I99)	151,913	15.5
Hypertension (I10–I15)	25,906	2.6
IHD (I20–I25)	42,157	4.3
Stroke (I60–I69)	47,353	4.8
Respiratory diseases (ICD-10 code: J00–J99)	126,485	12.9
URTI (J00–J06)	20,010	2.0
Pneumonia (J18)	25,016	2.5
COPD (J40–J44, J47)	25,632	2.6

Note: Values are frequency, or mean ± SD; Abbreviations: ICD-10, the International Classification of Diseases–Tenth Revision; IHD, ischemic heart disease; URTI, upper respiratory tract infection; COPD, chronic obstructive pulmonary disease; SD, standard deviation.

Table 2
Descriptive distributions of $PM_{2.5}$ mass and constituents, and meteorological conditions on case days and control days in five Chinese cities, 2015–2017.

Variables	Mean	SD	% of $PM_{2.5}$ mass	Percentile		
				25th	50th	75th
On case days (n = 978,571)						
Pollutant, μg/m^3						
$PM_{2.5}$	55.3	58.4	100.0	21.0	35.6	64.8
EC	2.4	3.1	4.3	0.8	1.4	2.6
OC	5.4	7.4	9.8	1.3	2.6	6.3
NO_3^-	12.6	12.2	22.7	3.2	8.2	17.9
SO_4^{2-}	13.3	20.7	24.0	2.9	5.2	14.1
NH_4^+	8.3	10.1	15.0	2.1	4.5	9.9
Meteorological factors						
Temperature, °C	17.0	8.5	/	9.6	17.7	24.4
Relative humidity, %	76.4	13.7	/	68.0	78.0	87.0
On control days (n = 3,325,016)						
Pollutant, μg/m^3						
$PM_{2.5}$	55.0	58.3	100.0	21.0	35.6	64.3
EC	2.4	3.0	4.3	0.8	1.4	2.6
OC	5.4	7.3	9.8	1.3	2.6	6.3
NO_3^-	12.4	13.1	22.6	3.1	8.1	17.8
SO_4^{2-}	13.2	20.8	24.1	2.9	5.2	13.9
NH_4^+	8.2	10.1	14.9	2.1	4.4	9.8
Meteorological factors						
Temperature, °C	17.1	8.6	/	9.6	18.0	24.5
Relative humidity, %	76.5	13.7	/	68.0	77.0	87.0

Abbreviations: SD, standard deviation; IQR, interquartile range; $PM_{2.5}$, fine particulate matter; EC, elemental carbon; OC, organic carbon; NO_3^-, nitrate; SO_4^{2-}, sulfate; NH_4^+, ammonium.

m^3), EC (1.9 μg/m^3), OC (5.3 μg/m^3), NO_3^- (15.3 μg/m^3), SO_4^{2-} (11.4 μg/m^3) and NH_4^+ (8.0 μg/m^3) was related to an ER% for non-accidental admissions of 1.6% [95% CI: 1.1–2.0], 1.9% [1.3–2.4], 1.0% [0.5–1.6], 1.2% [0.4–2.0], 1.2% [0.9–1.5] and 1.4% [0.9–1.9] at lag 04-day, respectively (Table 3). The smoothed exposure-response curves exhibit approximately linear trends between $PM_{2.5}$ constituents and non-accidental admissions at lag 04-day (Fig. S3). Evidently increased risks were also found in admissions for cardiovascular and respiratory diseases with different lag patterns. As checked for sub-causes in Figs. S4 and S5, most of the significant effects on cardiovascular hospitalizations (i.e., stroke, IHD, and hypertension) were observed at lag 4- and 04-day, and substantial associations with respiratory admissions (i.e., COPD, pneumonia, and URTI) of $PM_{2.5}$ components were concentrated on lag 1- and lag 4-day.

Fig. 3 illustrates excess risks of cause-specific hospital admissions associated with per IQR increase in $PM_{2.5}$ mass and its constituents. Great heterogeneities of constituents-admission associations existed in causes and $PM_{2.5}$ constituents. Significantly elevated risks were associated with exposures to $PM_{2.5}$ components, with an exception of OC for total cardiovascular causes, as well as NO_3^- for total respiratory causes. The excess risks across $PM_{2.5}$ constituents exhibited a good consistency in sub-causes including IHD (ER% ranging from 2.3 to 5.4% at lag 04-day) and pneumonia (1.9–5.1% at lag 4-day), wherein the estimated effect for NO_3^- exposure was stronger than those for other constituents. Stroke admission was associated with EC (ER% = 2.1% [95% CI: 0.0–4.3] at lag 04-day) and sulfate exposure (0.9% [0.2–1.7] at lag 3-day). The effect estimates of $PM_{2.5}$ constituents were more potent in hospital admission from ischemic stroke (Fig. S7) than hemorrhagic stroke (Fig. S8), while associations with various $PM_{2.5}$ constituents were not of significance. Increased risks of COPD admission were linked with EC (1.9% [0.1, 3.9] at lag 4-day), OC (2.7% [0.6, 4.9] at lag 4-day) and NH_4^+ (2.1% [0.3, 3.9] at lag 4-day) (Tables S3 and S4). No evident associations were observed between hypertension and URTI hospitalizations and exposures to ambient $PM_{2.5}$ constituents.

Table 3 lists subgroup-specific associations between hospital admissions and exposures to $PM_{2.5}$ and constituents, stratified by sex and age. Results revealed to-some-extent heterogenicity in $PM_{2.5}$ constituents-associated risks across causes and subgroups, despite the between-subgroup differences were not always statistically significant. Generally, females were at greater risks in admission for non-accidental

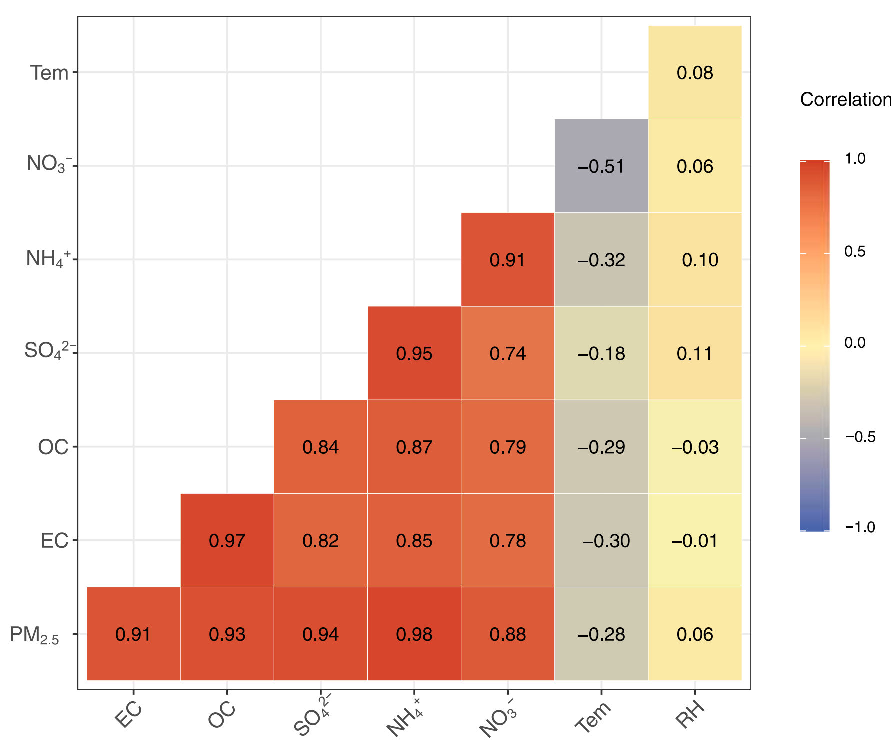

Fig. 1. Spearman's correlation coefficients between $PM_{2.5}$ mass, its constituents and weather variables. Abbreviations: $PM_{2.5}$, fine particulate matter; EC, elemental carbon; OC, organic carbon; NO_3^-, nitrate; SO_4^{2-}, sulfate; NH_4^+, ammonium; Tem, temperature; RH, relative humidity.

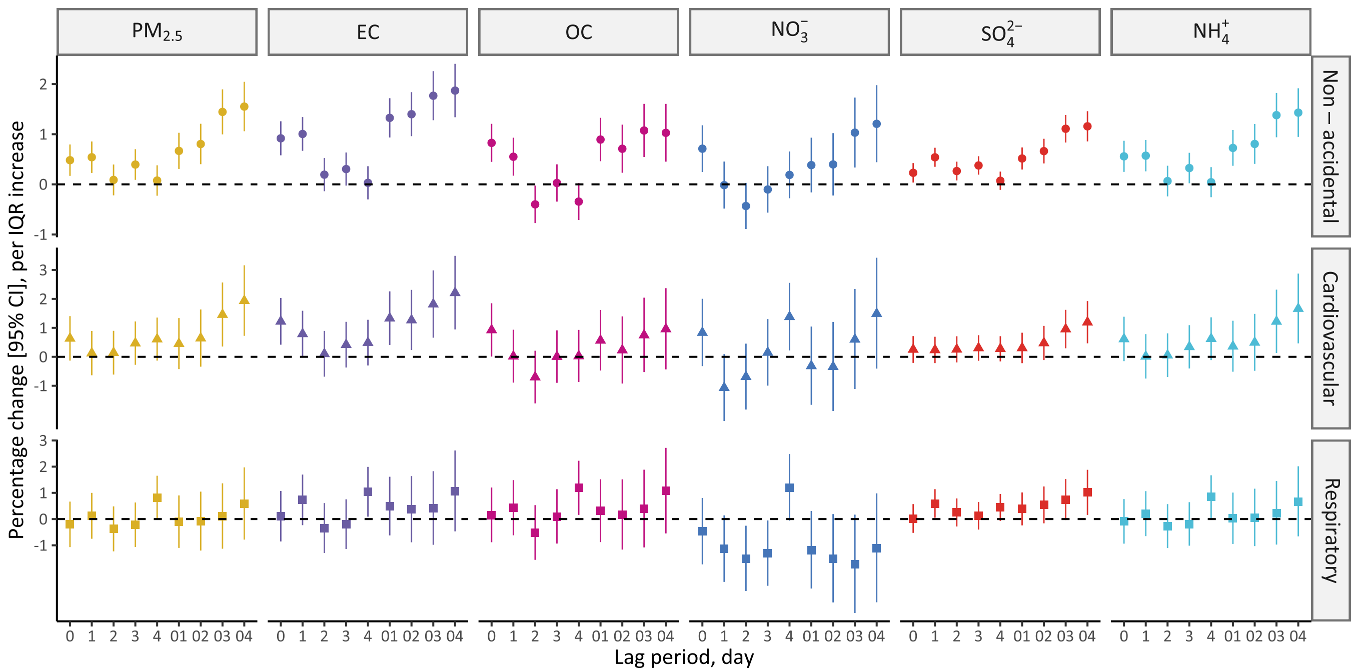

Fig. 2. Percentage changes [with 95% CIs] in daily hospital admissions for non-accidental, cardiovascular and respiratory diseases per IQR increase in $PM_{2.5}$ mass and its constituents on different lag days in 5 Chinese cities, 2015–2017 [a]. Note: [a] The IQRs of $PM_{2.5}$, EC, OC, NO_3^-, SO_4^{2-} and NH_4^+ were 44.6, 1.9, 5.3, 15.3, 11.4 and 8.0 μg/m^3, respectively; Abbreviations: CI, confidence interval; IQR, interquartile range; $PM_{2.5}$, fine particulate matter; EC, elemental carbon; OC, organic carbon; NO_3^-, nitrate; SO_4^{2-}, sulfate; NH_4^+, ammonium.

causes, and elevated risks of respiratory admission were only seen in female. Significant sex differences were identified in SO_4^{2-} (p for interaction = 0.023) for non-accidental causes, as well as $PM_{2.5}$ (0.039), OC (0.030) and NO_3^- (0.040) for respiratory causes. In terms of COPD and pneumonia admission, greater effect estimates were found in females than males (Table S4). More evident constituents-admission associations were found among the elderly, and groups aged over 65 years were susceptible to increased constituent exposure for non-accidental causes (ER% ranging from 1.5 to 2.7% at lag 04-day) and cardiovascular causes (1.3–3.3% at lag 04-day). Similarly, clear age patterns were observed in IHD, hypertension and COPD hospitalizations, suggesting higher vulnerability to particulates pollution among the older patients (Table S3). However, significant effects of components on pneumonia hospitalization existed groups aged below 44 years (Table S4).

Our two-stage results for $PM_{2.5}$ and its constituents were presented in supplementary materials (Fig. S6). Overall, risk estimates derived from the two methods are highly comparable. For instance, per IQR increase in exposure to $PM_{2.5}$ was related to an odds ratio for non-accidental admissions of 1.016 [95% CI: 1.011, 1.020] in our pooled analysis and 1.016 [1.010, 1.023] in the two-stage analytic protocol. After additionally adjusting for total $PM_{2.5}$ mass, the estimated excess risks for EC and SO_4^{2-} remained positive, while the impacts of NH_4^+, $NO_3^-$and OC attenuated probably due to the collinearity between chemical components and $PM_{2.5}$. Associations of constituents with hospital admissions did not substantially change by altering dfs (2–6) for temperature and relative humidity, further adjusting for O_3, and including NCS terms of atmospheric pressure, wind speed and sunshine duration. Overall, sensitivity analyses confirmed the robustness of our main findings (Table S5).

4. Discussion

Based on near one million samples of hospital admission, this research first systematically evaluated the short-term impacts of major $PM_{2.5}$ constituents on cause-specific hospitalizations in China. Our case-crossover study provided clear evidence on elevated risks of hospital admissions associated with exposures to major $PM_{2.5}$ constituents, i.e., EC, OC, NO_3^-, SO_4^{2-} and NH_4^+. Estimated excess risks varied largely by $PM_{2.5}$ constituents and causes of hospital admission, as well as sex and age groups. These findings in our study may contribute to better understandings of $PM_{2.5}$-induced health impacts.

Because particulates may affect lung function (Mordukhovich et al., 2015; Santilli et al., 2015) and likely hinder the immune system from clearing bacteria, viruses and other pathogens in the lung (Ostro et al., 2009). Despite the main causes of URTI and pneumonia being biologic in nature, it is quite possible that ambient $PM_{2.5}$ exposures could affect the exacerbation of diseases to the point of hospitalization. It is not yet clear how the constituents of $PM_{2.5}$ influence various cardiopulmonary diseases. The underlying biological mechanisms for BC and OC associated cardiopulmonary risks were probably related to mediation in DNA methylation (Niranjan and Thakur, 2017), promotion of oxidative stress (Brook et al., 2010; Mordukhovich et al., 2015), vasoconstriction, systemic inflammation and elevation of blood pressure (Hvidtfeldt et al., 2019; Magalhaes et al., 2018). NO_3^- could affect endocrine homeostasis by converting into nitric oxide, modifying the essential proteins, as well as directly interfering with the migration of chloride ions and iodide (Poulsen et al., 2018). SO_4^{2-} and NH_4^+ might induce systematic inflammation and coagulation (Lin et al., 2017b; Zhang et al., 2020a). More toxicological studies will be needed to study the mechanism of different ingredients on various diseases.

Existing international investigations provided population-based evidence for associations of hospital admission with EC and OC exposures (Ito et al., 2011; Kim et al., 2012). Our multi-city study in China showed that raised risks of cardiorespiratory hospitalization triggered by short-term exposures to these carbonaceous constituents with an exception of OC for total cardiovascular causes, which was consistent with a case-crossover study in five South-European cities (Basagana et al., 2015). Using billing claims information for US Medicare enrollees in 119 counties, Peng and collogues linked exposures to EC and OC during 2000–2006 with elevated risk of hospitalization for total

Table 3
Percentage changes [with 95% CIs] in risks of hospital admission for non-accidental, cardiovascular and respiratory diseases associated with per IQR change in $PM_{2.5}$ mass and its constituents[a], stratified by sex and age.

	$PM_{2.5}$	EC	OC	NO_3^-	SO_4^{2-}	NH_4^+
Non-accidental	**1.6 [1.1, 2.0]**	**1.9 [1.3, 2.4]**	**1.0 [0.5, 1.6]**	**1.2 [0.4, 2.0]**	**1.2 [0.9, 1.5]**	**1.4 [0.9, 1.9]**
Sex						
Male †	**1.1 [0.4, 1.8]**	**1.4 [0.6, 2.1]**	0.5 [−0.3, 1.3]	1.0 [−0.1, 2.1]	**0.8 [0.4, 1.2]**	**1.1 [0.4, 1.7]**
Female	**2.0 [1.3, 2.7]**	**2.4 [1.6, 3.2]**	**1.5 [0.7, 2.4]**	**1.4 [0.3, 2.5]**	**1.5 [1.1, 1.9]** *	**1.8 [1.1, 2.5]**
Age, years						
0–44 †	0.8 [0.0, 1.7]	0.9 [0.0, 1.9]	0.2 [−0.8, 1.2]	0.1 [−1.2, 1.4]	**0.8 [0.3, 1.3]**	**0.9 [0.1, 1.7]**
45–64	**1.6 [0.8, 2.5]**	**1.8 [0.9, 2.7]**	**1.1 [0.1, 2.0]**	**1.4 [0.1, 2.8]**	**1.1 [0.6, 1.6]**	**1.4 [0.6, 2.2]**
≥65	**2.3 [1.3, 3.2]** *	**2.7 [1.8, 3.7]** *	**1.9 [0.8, 2.9]** *	**1.9 [0.5, 3.4]**	**1.5 [1.0, 2.1]**	**2.0 [1.1, 2.9]**
Cardiovascular	**1.9 [0.7, 3.2]**	**2.2 [0.9, 3.5]**	**1.0 [-0.4, 2.4]**	**1.4 [0.2, 2.6]**	**1.2 [0.5, 1.9]**	**1.7 [0.5, 2.9]**
Sex						
Male †	**2.0 [0.2, 3.8]**	**2.2 [0.3, 4.2]**	0.8 [−1.3, 2.9]	1.1 [−0.6, 2.9]	**1.1 [0.0, 2.2]**	**1.9 [0.1, 3.7]**
Female	**1.9 [0.3, 3.6]**	**2.2 [0.5, 3.9]**	1.1 [−0.8, 3.0]	**1.6 [0.0, 3.2]**	**1.3 [0.3, 2.3]**	1.5 [−0.1, 3.2]
Age, years						
0–44 †	−1.1 [−5.1, 3.2]	−1.6 [−6.0, 3.1]	−2.9 [−7.5, 2.0]	−2.9 [−6.6, 0.9]	0.8 [−1.8, 3.5]	0.0 [−4.1, 4.1]
45–64	1.2 [−0.8, 3.2]	1.0 [−1.1, 3.2]	−0.1 [−2.4, 2.3]	0.8 [−1.1, 2.8]	0.4 [−0.8, 1.7]	1.0 [−0.9, 3.1]
≥65	**2.9 [1.2, 4.6]**	**3.3 [1.6, 5.0]**	**2.1 [0.2, 4.0]**	**2.4 [0.8, 4.1]** *	**1.7 [0.7, 2.7]**	**2.3 [0.6, 4.0]**
Respiratory	0.8 [0.0, 1.7]	**1.0 [0.1, 2.0]**	**1.2 [0.2, 2.2]**	1.2 [−0.1, 2.5]	**1.0 [0.2, 1.9]**	**0.8 [0.0, 1.7]**
Sex						
Male †	−0.2 [−1.5, 1.1]	−0.1 [−1.6, 1.4]	−0.2 [−1.7, 1.4]	−0.3 [−2.2, 1.6]	0.3 [−1.0, 1.7]	−0.1 [−1.3, 1.2]
Female	**1.6 [0.5, 2.7]** *	**1.8 [0.6, 3.1]**	**2.2 [0.8, 3.5]** *	**2.4 [0.7, 4.1]** *	**1.5 [0.4, 2.6]**	**1.5 [0.5, 2.6]**
Age, years						
0–44 †	0.6 [−0.6, 1.8]	0.4 [−1.0, 1.9]	0.6 [−0.9, 2.0]	1.3 [−0.4, 3.0]	1.0 [−0.3, 2.2]	0.7 [−0.4, 1.8]
45–64	0.0 [−1.9, 2.0]	0.2 [−1.9, 2.4]	−0.1 [−2.4, 2.3]	−0.4 [−3.4, 2.7]	1.6 [−0.4, 3.6]	−0.1 [−2.0, 1.9]
≥65	**1.6 [0.1, 3.2]**	**2.1 [0.5, 3.7]**	**2.8 [0.9, 4.7]**	1.9 [−0.5, 4.4]	0.5 [−1.0, 2.0]	**1.7 [0.2, 3.3]**

Notes.
[a]The IQRs of $PM_{2.5}$, EC, OC, NO_3^-, SO_4^{2-} and NH_4^+ were 44.6, 1.9, 5.3, 15.3, 11.4 and 8.0 μg/m^3, respectively; Bold font face indicates a statistically significant percentage change ($p < 0.05$).
† The subgroup was selected as the reference for z-test;
*$p < 0.05$, p-value for difference between the effects in two subgroups.
Abbreviations: CI, confidence interval; IQR, interquartile range; $PM_{2.5}$, fine particulate matter; EC, elemental carbon; OC, organic carbon; NO_3^-, nitrate; SO_4^{2-}, sulfate; NH_4^+, ammonium.

cardiovascular diseases (Peng et al., 2009), but Levy et al. failed to capture the adverse effects of OC through 2000–2008 (Levy et al., 2012). The heterogeneity in OC effects on cardiovascular health could be supported by a number of components-focused studies using death as endpoints (Achilleos et al., 2017; Dai et al., 2014). One underlying explanation could be the composition differences in the primary and secondary constituents across periods and regions, resulting in toxicity discrepancies of mixture OC (Liang et al., 2016). We also observed the effects of EC and OC on hospital admission for major cardiorespiratory subcategories, which echoed with previous epidemiological findings (Michikawa et al., 2021; Zhang et al., 2020b).

Heterogeneity exists in associations between water-soluble inorganic ions in ambient $PM_{2.5}$ (i.e., SO_4^{2-}, NO_3^- and NH_4^+) and premature mortality and excess morbidity (Heo et al., 2014; Li et al., 2015). Several studies in developed countries indicated significant effects of secondary inorganic aerosols on human health, suggesting SO_4^{2-}-associated increased risks of respiratory hospitalization in Spain and Italy (Basagana et al., 2015), NH_4^+ for cardiovascular mortality in Seoul, Korea (Son et al., 2012) and NO_3^- for nonaccidental death among the elderly in Atlanta, USA (Klemm et al., 2011). Besides, the California Teachers Study Cohort reported positive associations of NO_3^- and SO_4^{2-} with cardiopulmonary disease mortality, especially for IHD (Ostro et al., 2010, 2015) in accordance with our results. However, in the Denver Aerosol Sources and Health study, sulfate and nitrate showed no statistically significant associations with hospitalization for cardiovascular and respiratory causes (Kim et al., 2012). Two time-series studies among a privately insured population in Greater Houston, USA, assessed the relationship between levels of aforementioned inorganic ions and total emergency department visits (Liu et al., 2016a) and all-cause emergency hospital admission (Liu et al., 2016b), respectively. They only found an IQR increase in SO_4^{2-} (1.6 μg/m^3) was associated with an elevated risk of 1.22% [95% CI: 0.23–2.23%] in total emergency department visits. Our case-crossover study in China indicated per IQR increase in exposure to NO_3^- (15.3 μg/m^3), SO_4^{2-} (11.4 μg/m^3) and NH_4^+ (8.0 μg/m^3) was related to an excess risk for non-accidental admissions of 1.2% [0.4–2.0%], 1.2% [0.9–1.5%] and 1.4% [0.9–1.9%] at lag 04-day, respectively. These discrepancies in findings between studies were not well documented due to sparse toxicological evidence, but might be explained by levels of $PM_{2.5}$ constituents and sociodemographic characteristics in the study location, as well as health outcomes and study design.

Previous epidemiology studies suggested long lag effects of ambient $PM_{2.5}$ for total respiratory admissions (Kim et al., 2012; Lall et al., 2011; Peng et al., 2009). Ostro et al. found stronger associations between EC and respiratory hospitalization among children at lag 3-day than at lag 0-day (Ostro et al., 2009). Our findings showed the maximum effect estimates at lag 4-day. One potential mechanism may be that respiratory disease exacerbations take long time resulting from pulmonary oxidative stress/inflammation (Kim et al., 2012; Ostro et al., 2009). In contrast, more immediate estimated effects at lag 0-day were observed for total cardiovascular hospitalizations, which might be attributed to immediately cardiac responses following autonomic nervous system activation (Lall et al., 2011; Metzger et al., 2004). Besides immediate effects, our study suggested stronger cumulative lag effects at lag 04-day on CVD hospitalizations. It is plausible to some extent since exposure to ambient air pollution can affect blood pressure for days (Byrd et al., 2016; Giorgini et al., 2016). There is no evident lag pattern for cardiorespiratory subcategories. Future epidemiology and physiological mechanism studies are needed to explore the delayed effects and clarify the causes of the lag.

The magnitude of evidence linking mass of $PM_{2.5}$ mixture to excess morbidity for specific diseases has grown substantially in past decades, including hospital admission for IHD (Ostro et al., 2015), stroke (Crichton et al., 2016), pneumonia (Lv et al., 2017) and COPD (Dominici et al., 2006). Apart from concentrations of particulate mass, $PM_{2.5}$-associated effects on health may largely depend on its chemical

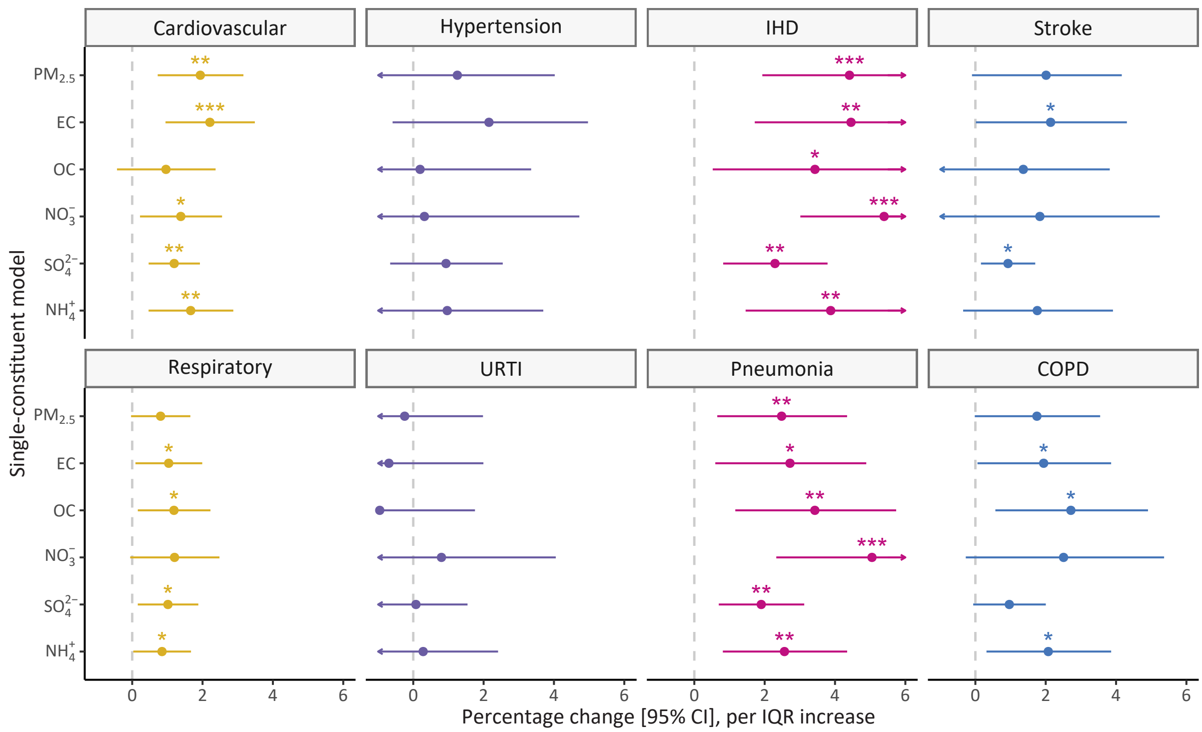

Fig. 3. Percentage changes [with 95% CIs] in cause-specific cardiovascular and respiratory hospital admission per IQR change in $PM_{2.5}$ mass and constituents [a]. Note: [a] The IQRs of $PM_{2.5}$, EC, OC, NO_3^-, SO_4^{2-} and NH_4^+ were 44.6, 1.9, 5.3, 15.3, 11.4 and 8.0 μg/m^3, respectively; Abbreviations: CI, confidence interval; IQR, interquartile range; $PM_{2.5}$, fine particulate matter; EC, elemental carbon; OC, organic carbon; NO_3^-, nitrate; SO_4^{2-}, sulfate; NH_4^+, ammonium.

ingredients (Achilleos et al., 2017). To date, limited epidemiological studies evaluated associations of $PM_{2.5}$ components with cause-specific hospitalization, especially in regions with heavy particulate pollution (Yang et al., 2019). This multi-city study in China observed consistently increased risks associated with aforementioned five PM $_{2.5}$ constituents for IHD and pneumonia admission, addressing the crucial research gap to some extent. In the five-county Denver metropolitan area, Kim et al. found EC and OC rather than NO_3^- and SO_4^{2-} were related to IHD hospitalization, and these four components had no significant effects on pneumonia admission (Kim et al., 2012). Our investigation further identified stroke admission was associated with EC and SO_4^{2-} exposure, and increased risks of COPD admission were linked with EC, OC and NH_4^+. However, two single-city analyses in Houston reported no significant impacts on stroke and COPD from carbonaceous constituents and water-soluble inorganic ions (Liu et al., 2016a). The underlying reasons for obviously stronger effects of constituents in our study probably were the levels of $PM_{2.5}$ were much higher in five Chinese cities (40.0–83.4 μg/m^3) than in Denver (8.0 μg/m^3) and Houston (12.0 μg/m^3). Additionally, sporadic evidence associated exposures to major $PM_{2.5}$ components with other specific admission, such as asthma (Mohr et al., 2008), congestive heart failure (Metzger et al., 2004) and myocardial infarction (Zanobetti and Schwartz, 2006). Our findings can provide valuable evidence linking selected $PM_{2.5}$ components with morbidity for further control of particulate matter pollution in China.

Sex differences in associations of particulate pollution with hospital admission have been of wide interest in air pollution epidemiology (Samoli et al., 2016; Wang et al., 2020a), while the effect modification is not well consistent across previous studies (Bell et al., 2015; Di et al., 2017). Our in-depth analyses for major $PM_{2.5}$ constituents identified that women were more susceptible to EC, NO_3^- and NH_4^+ exposures for respiratory admissions, but showed no differences for cardiovascular causes. Including 28 million cases (≥18 years) from the urban employee basic medical insurance database spanning 184 major Chinese cities, Tian and colleagues observed slightly higher risk of total $PM_{2.5}$ in males for cardiovascular admission (Tian et al., 2019). Based on about 12.6 million Medicare beneficiaries (≥65 years) residing in 213 US counties, point estimates were significantly higher for women than men for cardiorespiratory hospitalization (Bell et al., 2015). Sex differences may relate to psychosocial determinants, behavioral, and structural of health, such as occupation, stress, socio-economic levels, and family structure, as well as life style (e.g., diet, exercise, smoking, and drinking) (Denton et al., 2004). In addition, activity patterns at the study site may cause different exposures levels between men and women (Frazier et al., 2009; Martin et al., 2014). For instance, men spent less time outside than women in Los Angeles, but not Baltimore (Frazier et al., 2009).

Existing evidence supporting effect modification by age varied across health endpoints. For particulate-related cardiovascular admission, the estimated risks were consistently higher in old individuals than those in younger groups (Brook et al., 2010). In our subgroup analyses, obviously stronger constituents-admission associations also were found among the elderly aged over 65 years for several cardiovascular causes, in line with previous studies (Lin et al., 2018; Pinault et al., 2016). Higher risk among the elderly could be related to degradation of biological function and immune defense systems, as well as prevalence of pre-existing diseases (Lin et al., 2017a; Yang et al., 2018). In terms of respiratory subcategories, the estimates were generally higher in individuals aged 0–44 than in those aged over 65 for pneumonia, while COPD showed the reversal trend. The different pathogenesis of diseases and complex biological mechanisms for particulates components may partially explain the heterogeneity in cause-specific subpopulations (Brook et al., 2010; Zhang et al., 2020b). For instance, children are of special concern in respiratory health, because of their biologic characteristics (e.g., immune system, immature lungs and higher breathing rates) and behavior (e.g., more time spent outdoors) (Miller et al., 2002; Schwartz, 2004). From the perspective of $PM_{2.5}$ components, our findings may partially explain the inconsistency of cardiorespiratory diseases in the age-specific vulnerability to fine particulate matters.

Some limitations should be acknowledged in this study. First, we used $PM_{2.5}$ constituents and weather data from population level rather than individual exposures (Sun et al., 2019; Yang et al., 2020), which may introduce somewhat inevitable exposure misclassification. But this non-differential bias usually tends to cause an underestimate of the health risk from air pollution (Zanobetti et al., 2009). Second, there may be a lag when using the date of hospitalization as a proxy for the date of onset. Nevertheless, hospital admission has been broadly as a proxy to assess the short-term impact of air pollution on morbidity (Atkinson et al., 2015). Third, patients may be admitted to the hospital again within a month. The probability that the date of re-admission within one month is the comparison date is narrow (Tian et al., 2017). Fourth, we failed to conduct comparative assessments for metal constituents in $PM_{2.5}$ mass due to data unavailability for our study locations, though there are already some monitoring data of metal constituents in several megacities in China (Liu et al., 2018b; Xin et al., 2015). Additionally, individual behavioral and socioeconomic factors (e.g., smoking status and education) were not considered in our analyses, but these time-invariant cofounders were assumed to be well controlled by the case-crossover design (Lumley and Levy, 2000). Finally, we did not distinguish the source of ambient particulate pollution and call for more epidemiology research on it in the future.

5. Conclusion

In summary, our study revealed that short-term exposures to major $PM_{2.5}$ components contributes substantially to an increased risk of hospital admission in China. Great heterogeneities of constituents-admission associations existed in diverse causes and $PM_{2.5}$ constituents. Along with focusing on particulate components sources, more air pollution research targeting vulnerable populations are called for in the future, so as to refine air quality control strategies and provide significant public health benefits.

Author contributions

Yuanyuan Zhang: Methodology, Software, Writing - original draft; Linjiong Liu: Software, Writing - review & editing; Liansheng Zhang: Supervision, Writing - review & editing; Chuanhua Yu: Data curation; Xuyan Wang: Data curation; Zhihao Shi: Data curation; Jianlin Hu: Data curation, Writing - review & editing; Yunquan Zhang: Conceptualization, Formal analysis, Methodology, Software, Visualization, Data curation, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113912.

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Assessment of the 20L SODIS bucket household water treatment technology under field conditions in rural Malawi

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ABSTRACT

Two billion people worldwide consume unsafe drinking water. The problem is particularly pronounced in Sub-Saharan Africa, where more than a quarter of the population relies on unimproved surface water sources. Based on the principles of solar water disinfection (SODIS), a new household water treatment technology, the SODIS bucket, was developed to improve the microbial quality of water from these sources based on controlled tests in a laboratory setting. This study set out to evaluate the efficacy of the technology in a field setting, in rural communities in the Chikwawa District in southern Malawi.

SODIS experiments were carried out in two different vessels (1-L PET bottles and 20-L polypropylene SODIS buckets), over three months using unprotected water sources normally used by community members. Vessels were exposed to direct sunlight for 8 h per day in a village setting and were sampled at regular intervals to determine total coliforms, *E. coli*, turbidity, UV transmittance and UV dose.

In these experiments, the SODIS bucket reached inactivation targets for *E. coli* (<1 CFU/100 mL) in two of seven experiments and for total coliforms in one of seven for total coliforms (<50 CFU/100 mL), despite having greater UV doses than were seen in the evaluation carried out under controlled conditions during the bucket's development. PET bottles reached inactivation targets for both *E. coli* and total coliforms in five of seven experiments. There was no single factor that could be identified as preventing adequate inactivation, but the role of organic matter, inconsistent nature of the water source, and vessel size, when coupled with organic matter, were identified as contributing factors. This study highlights the need for further prototyping to provide a suitable pre-treatment step for unprotected water sources, and the importance of field testing with real-life parameters to ensure new technologies are context appropriate.

1. Introduction

Access to safe drinking water is essential to human health. Unsafe drinking water is estimated to cause 485,000 diarrhoeal deaths annually through the transmission of infectious diseases, of which up to 90% are children (WHO, 2019, 2007). Even when diarrhoea does not result in death it has long-lasting effects; delaying growth and development by reducing the intake of calories and nutrients. This puts at risk the 144 million people who are dependent on unimproved surface water sources such as rivers, lakes, ponds, and canals globally (UNICEF and WHO, 2019). In Sub-Saharan Africa, this problem is particularly pronounced, where 26% of the population is reliant on unimproved water sources (UNICEF and WHO, 2019), and inadequate Water Sanitation and Hygiene (WASH) has been attributed to 60% of all diarrhoeal deaths (Prüss-Ustün et al., 2019). Even improved water sources such as public taps, tube wells, protected dug wells, and rainwater systems are not always safe; two billion people use a drinking water source contaminated with faeces, or consume drinking water which has been subject to post collection contamination as a result of household storage (WHO, 2019; Wright et al., 2004). Therefore, a large proportion of the population does not have access to water which is safely managed as described in the United Nations Sustainable Development Goals (UN

Abbreviations: (HWTS), Household water treatment and safe storage; (LMICs), Low and Middle Income Countries; (PSA), Plataforma Solar de Almeria.

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SDG) framework; improved sources located on premises, available when needed, and free from faecal and priority chemical contamination (UNICEF and WHO, 2019).

In the absence of a piped water supply on premises, people in both urban and rural locations may use household water treatment and safe storage (HWTS) to improve and maintain water quality and thereby reduce the risk of waterborne diseases (Sobsey, 2002). To realise the full health benefits of improved water quality from HWTS, the technology must not only be effective, but users must treat their water continuously and consistently (Brown and Clasen, 2012; Enger et al., 2013). The effectiveness of HWTS is limited by user uptake and adherence to correct, consistent and sustained use, with one study finding that when HWTS use reduced from 100% to 90% of the volume of water consumed, the predicted health benefits, measured as disability affected life years, reduced by up to 96% (Brown and Clasen, 2012).

There are several factors that can affect uptake and continued use of HWTS in a community. Ojomo et al. (2015) identified 47 enablers and barriers to sustained and scaled HWTS use. These factors can be individual (the target households and their communities), or organisational (implementing organisations and governments) or both. Individual factors include user demand for a HWTS, preferences for certain types of technology, ease of incorporations into normal routine, and time taken to use the technology. Challenges shared by individuals and organisations include product supply of the HWTS systems as well as spare parts, and the provision of user guidance for households on the technical activities related to HWTS practices. Organisations must overcome difficulties collaborating with existing community programs, resource availability, and standardisation and certification of technologies (Ojomo et al., 2015).

To address some of the issues around standardisation and certification of technologies, the World Health Organisation (WHO) has developed a performance specification for HWTS systems based on health-based microbiological performance targets to choose an adequate system (WHO, 2011). The specification takes a tiered approach to performance targets defined across categories of highly protective systems, protective systems and interim systems (WHO, 2011). To date the WHO program has evaluated 30 proprietary HWTS products fitting into the categories of membrane ultrafiltration, ceramic filtration, flocculation-disinfection, flocculation bio-filtration, UV disinfection, chemical disinfection, and solar disinfection products (WHO, 2016, 2019a,2019b).

Solar disinfection of water dates to 2000 B.C.E. in ancient India. In modern times it has been studied as a treatment technology at least since the seminal work carried out by Acra and colleagues in the 1980s (Acra et al., 1980, 1984; Sobsey, 2002). Since then, researchers have further developed this technology into a viable HWTS technology in the form of the SODIS bottle system (McGuigan et al., 2012). The system consists of a 1- to 2-L plastic Polyethylene Terephthalate (PET) bottle left in the sun to absorb UV light. The primary mechanism of disinfection is UV inactivation, but there is also a synergistic effect provided by combining mild heat water temperatures with UV exposure (McGuigan et al., 2012). Field trials of the SODIS bottle have proven that, when used correctly, it can reduce rates of infant diarrhoea by up to 45% (Conroy et al., 1996; McGuigan et al., 2012). McGuigan et al. (2012) review of solar water disinfection technologies concluded that the SODIS PET bottle system is a proven technology.

In addition to PET bottles, research has been carried out using glass jars which are capable of transmitting 90% of UV-A radiation (Acra et al., 1980). However, glass bottles are heavy and pose a safety risk if they are broken (McGuigan et al., 2012). Plastic bags made of both PET (Walker et al., 2004) and low-density polyethylene (LDPE) (Dunlop et al., 2011) have both been trialled and can be more efficient than PET bottles as their shape can maximise the surface area of the water while minimising the depth of water for light to penetrate (McGuigan et al., 2012).

One shortcoming of the SODIS bottle system is the limited capacity of each bottle. The WHO advises that individuals need a minimum of 20 L of water per person per day for drinking and cooking (Reed and Reed, 2013). This makes use of small capacity bottles tedious and labour intensive. There have been previous attempts to develop larger volume vessels such as a 25-L borosilicate glass vessel in Kenya (Nalwanga et al., 2013) and 19-L polycarbonate water dispenser containers in Spain, Bahrain, and India (Keogh et al., 2015). Although both technologies were effective, neither has been implemented in the field at scale.

In rural Malawi, 13% of the population is dependent on unimproved water sources, with the remainder receiving a basic level of service from an improved source which is subject to post collection contamination (WHO, 2019). As such, there remains a significant need for effective HWTS in this setting, which must be cognisant of the enablers and barriers to uptake (Ojomo et al., 2015). Although the use of HWTS is supported through Malawi national water and environmental health policies, in practice this has tended to focus on safe water storage, and use of point of use chlorination during outbreaks of cholera to date (Rowe, 2012). As such, to examine the opportunity for a more sustained and affordable HWTS, a transdisciplinary method was used to develop a new, locally sourced, 20-L transparent polypropylene (PP) buckets, also known as the SODIS bucket (Morse et al., 2020). This design took into account not only the need for a greater volume of water but was also already familiar to users due to widespread use of plastic buckets, was low cost, required minimal maintenance, and minimized the impact on household chore time (Morse et al., 2020).

Controlled microbiological evaluation against the performance of already-proven 1.5-L PET bottles at Plataforma Solar de Almeria (PSA) in Spain showed the 20-L SODIS buckets were highly effective for solar disinfection of bacterially contaminated water and demonstrated similar inactivation kinetics to 1.5-L PET bottles therefore making them a good large volume alternative (Polo-Lopez et al., 2019). The objective of this study was to test efficacy of the SODIS bucket against standard SODIS PET bottles under field conditions and determine whether it should be further developed as a HWTS.

2. Materials and methods

2.1. Study area

Chikwawa District, in the southwest of Malawi, was selected by the WATERSPOUTT project (www.waterspoutt.eu) as the location to develop and field test the 20-L SODIS bucket system. Located in the Shire Valley, groundwater is highly saline in areas, making it unsuitable for domestic consumption (Monjerezi and Ngongondo, 2012). As a result, although groundwater boreholes are installed in several communities, many households still prefer to use unprotected surface water sources for drinking water. These sources have high levels of bacterial and faecal contamination making them unfit for human consumption (Pritchard et al., 2008). The water quality is also impacted by Malawi's tropical climate, with a hot and rainy season from mid-November to April and a cool and dry season from mid-May to mid-August. (Ministry of Natural Resources, Energy and Environment, 2020); consequently, the Shire Valley is extremely flood-prone at this time of year (DoDmA, 2015). This flooding disrupts the quantity and quality of water, and water access in the area (GFDRR, 2011).

Development and initial testing of the SODIS bucket began with a transdisciplinary study carried out by Morse et al. (2020). This study identified households from 17 villages (total population: 3290) within the district to participate in development and testing of the SODIS bucket prototype. Of the 46 water sources used in the villages, 27 were unimproved, characterised as: canal/irrigation channel ($n = 6$), river/dam/lake/pond/stream ($n = 5$) or unprotected dug well ($n = 16$). The 19 improved sources were characterised as: borehole/deep well ($n = 15$), protected dug well ($n = 1$), private/public tap ($n = 3$). With this in mind, the field study to test the efficacy of the SODIS bucket was nested within these participating populations.

2.2. SODIS vessels

SODIS experiments were carried out in two different vessels: 1-L PET bottles and 20-L PP SODIS buckets constructed locally in Blantyre, Malawi, to the same specification as the SODIS buckets used by Polo--Lopez et al. (2019). The key differences between the PET bottles and PP SODIS buckets were the container material properties and the vessel dimensions. PET transmits a greater total amount of UV radiation than PP, but this radiation is restricted to the UV-A and visible light wavelengths, as it is effectively opaque to UV-B radiation (Polo-Lopez et al., 2019). PP transmits less total radiation but includes transmission of more lethal UV-B radiation (Polo-Lopez et al., 2019). The other factor to consider, the vessel size, affects the optical path length through water in the vessel. Vessel dimensions are shown in Fig. 1. The vessel size becomes relevant when you consider that the light attenuating effect of natural waters increases exponentially with water depth (Kirk, 2010).

Fig. 2. Experiment set up A locally made table, UV photometer is shown on the ground.

2.3. Experimental methods

The experiments were carried out in Malawi between October 28, 2019 and January 25, 2020 and consisted of two phases. In phase one, 16 of the 46 unprotected water sources were examined to determine the water matrix characteristics and the levels of microbial contamination of each source. Characteristics examined were, temperature, UV_{254} transmittance (filtered and unfiltered), turbidity, and *E. coli* concentration. From this assessment, four sites were selected for use in the remainder of the study. The criteria for selection were having a turbidity below 30 NTU, and ease of access to the site.

In phase two, the SODIS buckets and PET bottles were assessed concurrently. Water for the experiment was taken directly from sources in a single 20-L bucket that was distributed evenly across four 20-L SODIS bucket until each was full (80-L total). One of the buckets was then used to fill 15 x 1-L PET bottles. A schematic of the different number and type of vessels in each experiment is shown in Fig. 1.

The bottles and buckets were arranged on a table, made by a member of the village, to mimic the conditions used by the households in the study group (Fig. 2). The table was set up in a location approved by the village chief, and the vessels were exposed to natural UV radiation. The experiments began between 7am and 8am and ran for 8 h.

Although the objective of the research was to assess the effectiveness of solar water disinfection using SODIS buckets, carrying out experiments in PET bottles provided a benchmark for its performance to be assessed against. The effectiveness of SODIS in PET bottles has been well documented in both laboratory and field conditions (Joyce et al., 1996). Comparing the effectiveness of a solar water disinfection technology to that of SODIS in PET bottles is a common practice that has been used in several contemporary studies (Castro-Alfarez et al., 2017; Keogh et al., 2015; Lawrie et al., 2015) including Polo-Lopez et al. (2019) that studied the effectiveness of SODIS buckets under controlled conditions.

A dark control SODIS bucket and three dark control PET bottles were also included in the experiment to determine if inactivation of bacteria was occurring independently from UV exposure. These controls used the same water as the SODIS experiments and were stored in a shaded area and covered with a cloth bag. This inhibited UV exposure and helped to maintain an ambient temperature equivalent to water stored inside a home in the same location.

2.4. Sampling methods

After homogenization of source water, three samples were taken from a bucket at $t = 0$ h to determine the initial total coliform and *E. coli* concentrations. Samples were taken from PET bottles at 2-h time steps but only at 4, 6 and 8 h for SODIS buckets. At $t = 8$ h samples were taken

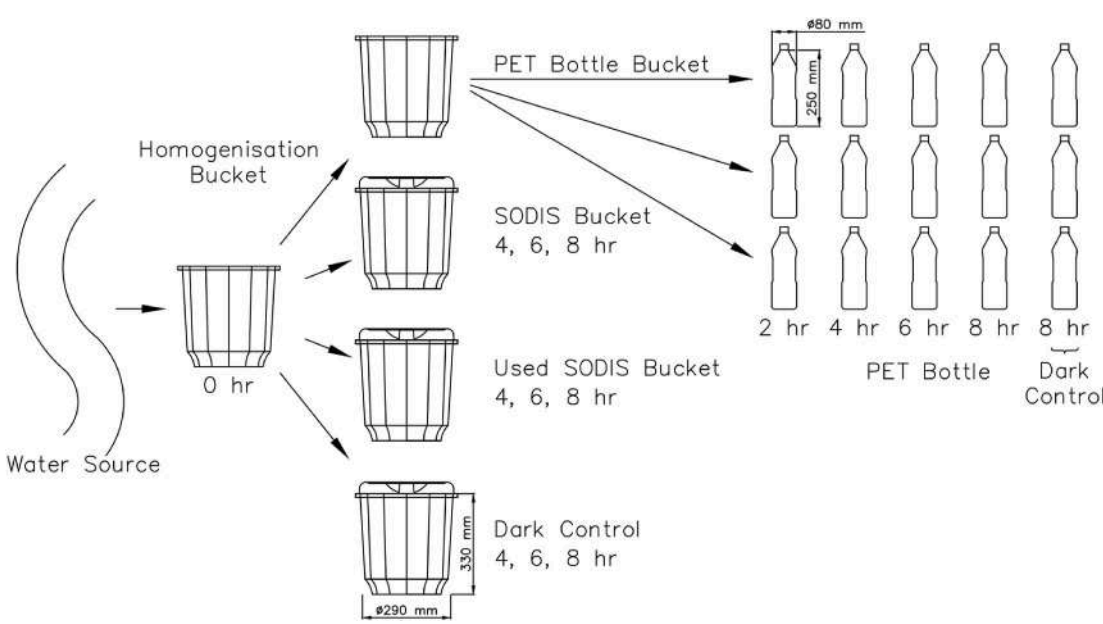


Fig. 1. Method Setup Schematic of homogenization process, experimental set up and vessel sizes.

from the three dark control bottles and the dark control SODIS bucket. For both PET bottles and SODIS buckets, samples were collected in triplicate with samples from PET bottles collected from three different vessels and for the SODIS buckets, the three samples were collected from the same vessel. The details of the sampling schedule are shown in a table in the supplementary material.

Before sampling, particles in the water were resuspended by either shaking for PET bottles or stirring with a sterilised instrument for SODIS buckets. Samples were put on ice in a cooler and were refrigerated within 12 h. The testing of physical and chemical parameters of the water matrix were carried out on site at the time of sampling.

2.5. Inactivation targets

In Malawi, the maximum permissible level of faecal coliforms in drinking water is 50 CFU/100 mL, for *E. coli* this limit is < 1 CFU/100 mL (MBS, 2005) which is also the WHO guideline value for microbial water quality (WHO, 2011). To determine if the SODIS bucket system could achieve this level of inactivation, WATERSPOUTT had set treatment targets to below detection limits in 100 mL for *E. coli*. The duration of solar exposure recommended for SODIS to be effective was set as 6 h based on previous testing (McGuigan et al., 2012; Meierhofer and Wegelin, 2002; Polo-Lopez et al., 2019).

2.6. Analytical methods

E. coli and Total Coliform concentration was determined as most probable number (MPN) by enzyme substrate coliform test using the IDEXX Colilert™ 24-h system. Where coliform concentrations exceeded 2400 MPN samples were diluted between 10^{-1} and 10^{-3} to enable quantification. Tests were carried out within 24 h of field sampling.

Physical and chemical characteristics were measured in the field using handheld instruments. Turbidity was measured using a Wag-WT3020 turbidity meter (Wagtech). UV_{254} transmittance is a surrogate measurement for natural organic matter (NOM) (Cho et al., 2006; Crittenden et al., 2012) and was measured using a P254C UV Photometer (Trojan Technologies). UV transmittance was measured in unfiltered samples and also samples that had been filtered through 45-μm filter paper to differentiate between dissolved and undissolved NOM.

Solar water disinfection is dependent on both total UV dose as well as intensity (Ubomba-Jaswa et al., 2008). To normalize results for the different weather conditions encountered during each experiment, solar radiation was measured in the 290–390 nm band every 30 min using a handheld PCE UV-34 radiation detector (PCE group). The sensor was set up to be aligned to the path of the sun. Using the memory function, the maximum and minimum irradiance was recorded every 30 min. The mid-point between these two readings was considered the average irradiance intensity for that time step. At every reading, the sensor was realigned to face toward the sun again. The UV dose was calculated using equation (1), integral UV intensity over time.

$$UV\ Dose = \int_{t_1}^{t_2} UVIntensity dt \tag{1}$$

2.7. UV transmittance of used SODIS buckets samples

The optical properties of the plastic materials were evaluated by recording their transmission spectra with a UV–Vis–NIR spectrophotometer (Varian Cary 500, Palo Alto, California, USA). Three independent and replicated measurements of the plastic samples were considered.

2.8. Data analysis

Results from coliform testing were initially recorded as MPN/100 mL and then converted to log units for analysis. As the different water sources had varying *E. coli* concentrations, the results are shown in log units of *E. coli* concentration remaining rather than log removal as would be conventional in a controlled test where initial *E. coli* concentrations were the same. When determining the mean coliform count, statistically outlying results were removed. In statistical analysis there is value in including outliers and as a rule they should not be removed from a data set. The decision to remove outliers in this research was based on the small number (3) of samples to be tested for coliforms and the disproportionate effect one outlying result would have on the average of a sample. Outliers were determined using Tukeys 1.5 IQR rule (Tukey, 1977), equation (2) and equation (3). Data that remained after cleaning was then averaged and used for further analysis.

$$Upper\ Limit > Q_3 + 1.5 \times (Q_3 - Q_1) \tag{2}$$

$$Lower\ Limit < Q_1 - 1.5 \times (Q_3 - Q_1) \tag{3}$$

The students t-test was used to determine if the difference between two sets of measurement was statistically significant. This was calculated using the *t*-test function in Microsoft Excel. All tests were type two (unpaired, samples of equal variance). Where there was an expectation of the direction of change, such as an increase in temperature, a one-tailed test was used. Where there was no expectation of the direction in which a value would change, a two-tailed test was used. The level of significance was set at a = 0.05.

2.9. Ethical approval

Ethical approval for this study was obtained from the National Health Sciences Research Committee (approval number 1823) in Malawi.

3. Results

3.1. Source water quality characteristics

In phase one of this research 16 of the 46 water sources used by households in the study group were evaluated for their water quality characteristics. The results (Table 1) showed the water sources to have a high turbidity with 9 of 16 water sources having a turbidity of over 30 NTU. This is the recommended maximum turbidity for SODIS treatment (Meierhofer and Wegelin, 2002). UV_{254} transmittance varied from 92%–20% in unfiltered samples and between 94% and 46% in filtered samples. *E. coli* concentrations varied between 4 and 6,488 MPN/100 mL with a median of 588 MPN/100 mL indicating that the water sources used by the study group were contaminated with faecal coliform bacteria and required treatment before consumption. Four sources were selected for use in the subsequent phase of the study (highlighted in grey in Table 1). The criteria for selection are discussed in the experimental methods section.

During the phase two SODIS experiments it was found that the composition of the water matrix at the selected sources had changed since the assessment in phase one. This change was attributed to the inconsistent nature of open water sources, and the onset of the first rains of the wet season carrying sediment from runoff into surface waters. Chikwawa experiences extreme seasonal variation, with the dry season (May–October) having an average rainfall of 5 mm, and the rainy season (November–April) having an average of 13 mm with a peak average of 226 mm in January (Weatherspark, 2021).

At two locations, KUT-A and MAF-D, turbidity increased to over 300 NTU and 500 NTU, respectively. In all cases, there was a reduction in UV_{254} transmittance, to between −3% and −26% of the original measurement, indicating an increase in NOM in the water. Given the initial criteria for source selection included turbidity of less than 30 NTU, source KUT-A was abandoned before the first experiment. The change in water quality at source MAF-D occurred after the first experiment had

Table 1
Microbial and physical source water quality data for phase one.

Water source code	Temperature [C]	Turbidity [NTU]	UV_{254} transmittance unfiltered [%]	UV_{254} transmittance filtered [%]	*E. coli* [MPN/100 mL]
BIA-A	35	153	29%	83%	6,488
DZI-A	29	2	92%	92%	4
DZI-B	29	28	58%	74%	435
DZI-C	29	70	50%	81%	2,282
KUT-A	28	23	72%	81%	1,553
MAF-A	33	40	54%	80%	1,300
MAF-B	33	93	20%	46%	2,600
MAF-C	29	23	75%	90%	205
MAT-A	30	39	67%	83%	91
MUO-A	31	21	69%	85%	6
NAM-A	31	32	66%	92%	135
NYA-A	33	33	55%	68%	727
NYM-A	30	29	54%	62%	4,611
NZA-A	30	46	66%	92%	325
SAL-A	30	29	65%	94%	187
SAL-B	31	58	61%	93%	816

already been carried out and was abandoned for the second experiment. Both sources were replaced with alternative open water sources (KUT-B and MAF-E) with lower turbidity within the same village (Table 2).

3.2. *E. coli* inactivation **in SODIS buckets and PET bottles**

E. coli removal in SODIS Buckets **and PET bottles** from all seven experiments is shown in Fig. 3. These experiments were carried out under different solar conditions and results have been normalised by plotting them against the cumulative UV dose over 8 h. The maximum and minimum 8-h cumulative UV doses were 1,367 and 742 kJ/m^2 respectively, while the average was 1,083 kJ/m^2. The maximum water temperature recorded was 50.6 °C in a SODIS Bucket and 50.3 °C in a PET bottle. PET bottles were on average 0.5 °C warmer than SODIS buckets when T = 0 recordings were omitted. In SODIS buckets only two of the experiments (02-MAF-D and 07-MUO-A) reached the inactivation target for *E. coli* of below detection. The lowest dosage required was approximately 450 kJ/m^2 in experiment 02-MAF-D. In PET bottles five of the seven experiments achieved the inactivation target in 6 h. Experiments 01-KUT-B and 03-NYM-A did not reach the target. Results from the dark controls showed a median change in *E. coli* MPN of 0.22 Log (SD 0.22) in PET Bottles and 0.25 (SD 0.25) in SODIS Buckets.

Total coliform removal in SODIS Buckets and PET bottles was measured in all seven experiments. Only one of the SODIS bucket experiments reached the inactivation target of 50 MPN for total coliforms at a UV dose of approximately 900 kJ/m^2 In PET bottles five of the seven experiments achieved the inactivation target at UV doses between 700 and 1350 kJ/m^2. Graphs showing the results are presented in supplementary material [S1].

A comparison of the inactivation of *E. coli* in SODIS buckets and PET bottles from experiments 06-NYM-A and 07-MUO-A are shown in Fig. 4. These examples are shown as they represent experiments where inactivation targets were met at both high and low total UV doses relative to the other experiments in the study. The SODIS bucket was less effective at inactivating *E. coli* in all seven experiments with an average difference in inactivation of 0.94 log ($p < 0.01$), at all comparable timesteps. For total coliforms, this difference was greater at 1.46 log ($p < 0.01$).

3.3. *Comparison of new and used SODIS buckets*

SODIS buckets that had previously been given to families within the study group for home water treatment were obtained and included in the phase two experiments and a comparison of inactivation in the used and new buckets was made in six of the seven experiments. For *E. coli* (Fig. 5), used buckets had an average reduction value of 0.01 log (p = 0.88) less than in new buckets at each timestep. For total coliforms, the average difference was greater at 0.09 log (p = 0.04) less at each timestep.

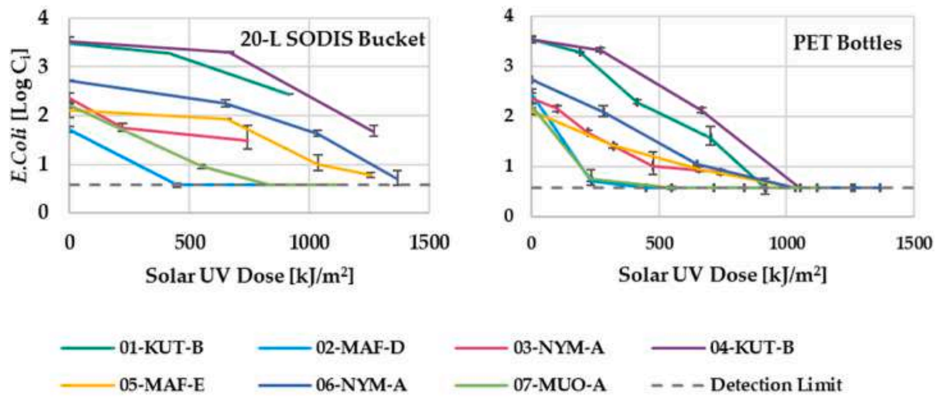
During the experiments it was observed that the used buckets and buckets used by villagers in the testing areas had an opaqueness to them caused by abrasion to the buckets surface. To establish if this opaqueness had an effect on the inactivation process samples of used buckets were tested for UV transmittance. The reduction in UV transmission by natural aging was measured in four used SODIS buckets (not those used in the SODIS bucket experiments). There is a noticeable difference in UV transmittance for the used buckets. The lowest for the 9-month-old samples, with values of 28.5% and 17.3% versus 33.3% and 33.8% for 6-month-old samples (Table 3). Samples from a bucket previously received were also kept in the dark as reference (sample 0 Months).

3.4. *Factors influencing microbial inactivation*

A comparison of *E. coli* inactivation in PET bottles for the two experiments carried out at water source KUT-B and NYM-A are shown in Fig. 6. These experiments were carried out using the same water source three weeks apart under different solar conditions. At water source KUT-B, (experiment 01 and 04) turbidity increased from 17 to 32 NTU between experiments but other water quality characteristics remained

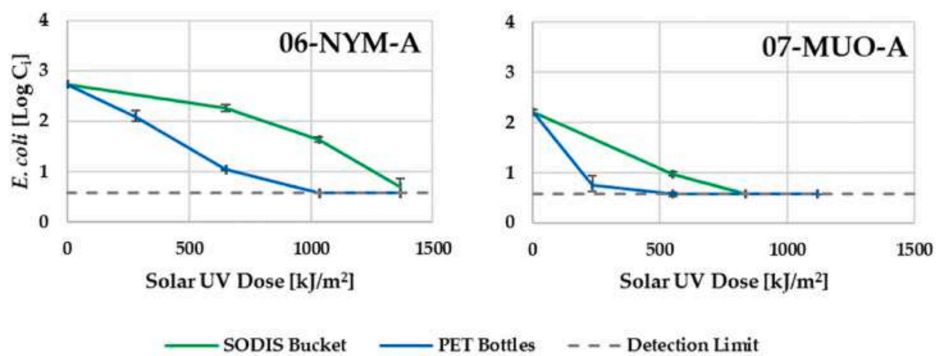
Table 2
Microbial and physical source water quality data for phase two.

Water source code	Temperature [C]	Turbidity [NTU]	UV_{254} transmittance unfiltered [%]	UV_{254} transmittance filtered [%]	*E. coli* [MPN/100 mL]
01-KUT-B	28	17	50%	58%	3,382
02-MAF-D	31	21	73%	87%	278
03-NYM-A	29	20	47%	58%	229
04-KUT-B	29	32	47%	63%	3,399
05-MAF-E	28	49	35%	65%	127
06-NYM-A	27	42	40%	57%	530
07-MUO-A	31	29	57%	77%	162



(a) (b)

Fig. 3. *E. coli* removal (a) SODIS buckets and (b) PET bottles in all seven experiments. Error bars indicate the standard error of the mean calculated from triplicate samples.



(a) (b)

Fig. 4. Comparison of *E. coli* inactivation in PET bottles and SODIS Buckets (a) 06-NYM-A and (b) 07-MUO-A. Error bars indicate the standard error of the mean calculated from triplicate samples.

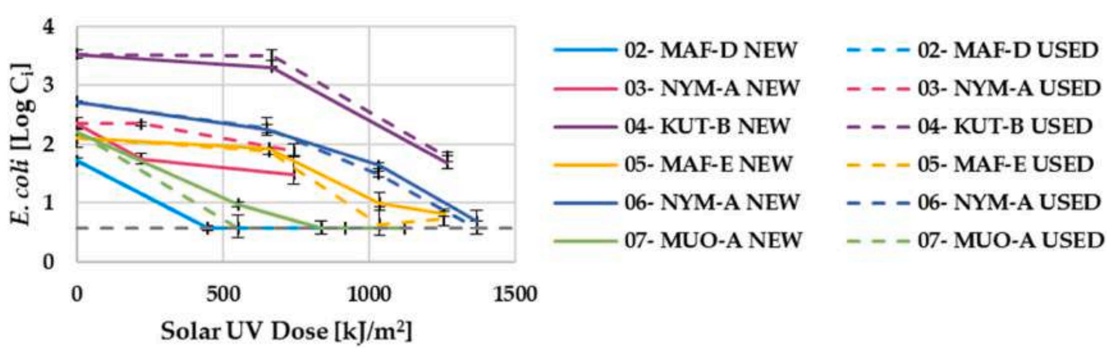


Fig. 5. Comparison of *E. coli* inactivation in new and used SODIS buckets New (solid lines) vs used (dashed lines) SODIS buckets in all experiments.

Table 3
Transmittance of samples from the buckets after solar exposure for 6 and 9 months.

	0 months	6 months	6 months	9 months	9 months
UV-A (%)	70.6	33.4	34.0	17.4	28.7
UV-B (%)	57.0	21.3	18.2	10.6	17.0
UV-C (%)	0.0	0.0	0.0	0.0	0.0

largely unchanged. UV intensity was higher in experiment 04 with a cumulative UV dose of 1,268 kJ/m^2 compared to 916 kJ/m^2 for experiment 01. At water source NYM-A, (experiment 03 and 06) turbidity increased from 20 to 42 NTU between experiments but as with KUT-B, other water quality characteristics remained largely unchanged. Here, the difference in UV intensity is more pronounced with experiment 06 receiving a cumulative UV dose of 1,367 kJ/m^2 compared to 742 kJ/m^2 for experiment 03. This difference in intensity is also evident in the maximum temperature of experiment 06 of 48 °C compared to 41 °C for experiment 03, while the difference was only 1 °C at KUT-B with 49 °C for experiment 01 and 50 °C for 04.

The relationship between cumulative *E. coli* inactivation and UV dose was compared and a strong correlation was found in both SODIS buckets, $R^2 = 0.83$ and PET bottles $R^2 = 0.88$.

The influence of turbidity in the source water, when inactivation is

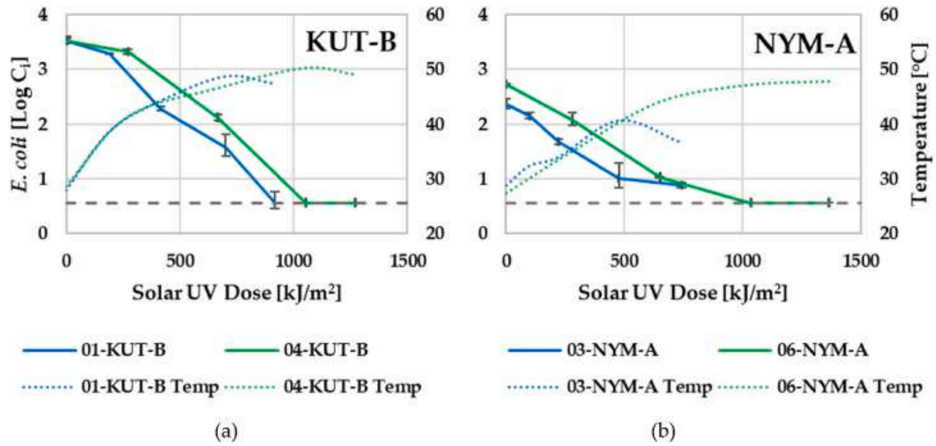


Fig. 6. Comparison of *E. coli* inactivation under different solar conditions PET bottles at water source (a) KUT-B and (b) NYM-A on two different days with different UV doses. Error bars indicate the standard error of the mean calculated from triplicate samples.

normalised to account for the different UV dose, has some correlation to *E. coli* inactivation in SODIS buckets, $R^2 = 0.63$, and almost no correlation in PET bottles $R^2 = 0.15$.

The amount of NOM (measured as UV_{254} transmittance) in the water is strongly correlated to inactivation of *E. coli* when inactivation is normalised to account for the UV dose both in SODIS buckets, $R^2 = 0.72$, and in PET Bottles, $R^2 = 0.80$.

4. Discussion

This study assessed the effectiveness of the SODIS bucket under field conditions to consistently meet the water quality standards laid down by the Malawi Bureau of Standards (MBS, 2005) and WHO (WHO, 2011).

We found that SODIS buckets were not able to consistently achieve the inactivation target that was set or replicate the results achieved under controlled conditions (Polo-Lopez et al., 2019). Although the experiments carried out in Malawi had much lower initial *E. coli* concentrations, median of 2.4 log compared to 6 log at PSA (Polo-Lopez et al., 2019)) we were still unable to reach complete inactivation even with much higher UV doses of 450–1350 kJ/m^2 compared to 250–300 kJ/m2 at PSA (Polo-Lopez et al., 2019).

Of the parameters measured in this study, the single biggest predictor of *E. coli* inactivation was total UV dose. However, it should also be kept in mind that inactivation occurs not only by UV oxidation of bacterial cells but also by the pasteurising effect caused by mild heat temperatures (McGuigan et al., 2012, 1998). Therefore, an increase in UV intensity, that increases water temperature, rather than an increase in duration of exposure is more beneficial to the SODIS process. The UV intensity for these experiments was typically higher at maximums of between 44 and 58 W/m^2 compared to a maximum of 28 W/m^{2}at PSA (Polo-Lopez et al., 2019). This would suggest that UV intensity was not the limiting factor in this instance. When we compare the results in PET bottles, the PSA evaluation achieved a 5-log reduction in *E. coli* with a UV dose of approximately 400 kJ/m^2. Again, it is difficult to directly compare the results in Malawi given the lower initial *E. coli* concentrations, but complete inactivation of *E. coli* required higher doses.

Previous studies have reported no observable difference in inactivation as a result of vessel volume ranging from 0.5 to 20-L systems, including controlled comparisons of the PP SODIS bucket with PET bottles (Gómez-Cuoso et al., 2012; Kehoe et al., 2001; Polo-Lopez et al., 2019). However, the results of this study showed a greater inactivation of *E. coli* occurring at the same UV doses in PET bottles compared to SODIS buckets. We attribute this finding to the attenuation of light as an exponential function of the photon path length through water. When high levels of NOM (measured as UV_{254} transmittance) are present, it follows that the SODIS bucket with a longer path length would be less effective than a smaller PET bottle with a shorter path length. These results would indicate that vessel volume does play a role in inactivation when NOM is present in high levels.

SODIS guidelines suggest a maximum turbidity of 30 NTU for successful water treatment (Meierhofer and Wegelin, 2002). Unlike many lab-based experiments, the water used in Malawi was taken from natural sources which vary in water quality characteristics not only from source to source but from day to day due to local weather events. In this study the initial turbidity was above this guideline in nearly half of the experiments, but although the effectiveness of the SODIS process tended to reduce with increased turbidity the correlation was not strong. Results from Kehoe, et, al. (2001) found that in high turbidity waters (>100 NTU) the UV dose required to achieve complete inactivation increased but was achievable with exposures of up to 8.5 h. They concluded that water above 300 NTU may need to be pre-treated by filtering or decanting to be treated effectively by SODIS. In light of these prior studies, and the results of phase one and two testing, which showed turbidity levels as high as 500 NTU in some water sources, shows that the issue of turbidity should not be set aside, but rather considered alongside other environmental factors such as concentration of NOM and solar conditions.

Published literature shows a large variability in experimental outcomes when different locations and water sources are used, and with a wide range of turbidity. Research carried out by Keogh et al. (2015) testing 19-L polycarbonate containers using low turbidity water at PSA, in Bahrain and India achieved a 4-log reduction of *E. coli* at UV doses of 250, 730 and 750 kJ/m^2, respectively. An additional test using high turbidity water (100 NTU) at PSA required 300 kJ/m^2 to achieve the same result. The water sources used in this study reflect the water sources available to the study participants, both in terms of the water quality and in the variability of its characteristics. These results show that although the SODIS bucket is effective in some situations, it is not a one size fits all HWTS solution. The effect of the variation in water quality can be mitigated by the use of pre-treatment devices such as the cloth filters developed by Morse et al. (2020). However, it should be noted that cloth filters are limited to removing particulate matter and will have limited effectiveness on dissolved or colloidal organic matter which in this study have been shown to have an effect in the larger SODIS buckets. Combining these two processes may increase the effectiveness of SODIS but may not be totally effective in all situations.

The effect of material degradation on the SODIS process was investigated by testing a used SODIS bucket alongside new SODIS buckets. Although the results showed that the new SODIS buckets had consistently lower *E. coli* counts at the same UV dose, this difference was of such a small magnitude that it appears to make no meaningful difference to overall treatment success. This conflicts with the measurement of light transmission through six-, and nine-month-old used SODIS buckets which found that UV light transmission through SODIS buckets reduced over time. It would be logical to expect this would lead to a reduction in UV transmittance and therefore inactivation effectiveness. The buckets being used by participants in the area had an opaqueness that had developed over time. Field researchers carrying out the experiments noted it was extremely easy to scratch the buckets if using anything abrasive to wipe the buckets clean between experiments. The samples that were tested for UV transmission were cleaned with sack cloth that could easily have abraded the surface. Despite there being little difference between the used and new buckets in this research, the effect of user cleaning methods should be investigated further as part of any future field trials.

A key objective of this study was to validate the relative efficacy of the 20L PP SODIS bucket in a real-life situation against that of both standard SODIS use, and results of the controlled testing of Polo-Lopez et al. (2019). The results clearly demonstrate that there was a high level of variation across water sources found within the field trial population, reflecting the diversity of water quality which changed not only by site and type, but also by day due to the influence of weather and use. This lack of consistency in water quality, compounded by the impact of user handling on the SODIS bucket demonstrates why it is so important to validate proposed water treatment systems through real-world field trial analysis. The value of assessing prototypes in their proposed settings cannot be underestimated to determine not only their scientific efficacy, but also unanticipated uses, practices and subsequent outcomes.

5. Limitations

Only five different water sources were used for experiments. This means that the data does not accurately reflect the full range and distribution of water quality conditions encountered by the trial group. Therefore, the results and conclusions that have been drawn are only applicable to water sources that fall within the range of conditions that were tested. This combined with the limited number of experiments means that the relationship between water quality characteristics and inactivation have limited statistical power. Although some of the correlations found in this study are strong, further experiments with a greater variance in individual characteristics are necessary to confirm these findings.

The methodology for this study was designed to assess the effectiveness of solar water disinfection. Therefore, the experimental methodology required that the effect of settlement in SODIS buckets be negated by resuspending settled particles before biological sampling. It is possible that SODIS could produce greater reductions in bacterial concentrations if the effect of particle settlement were considered.

Solar irradiances were measured directly, normal to the position of the sun. Ideally for the purpose of comparison with Polo Lopez et al. (2019), this study would have utilised a global pyranometer to also measure global irradiance. However, this equipment was not available, and it should be assumed that measurements of irradiance in this study are underestimates compared to the global irradiance.

6. Conclusions

The SODIS bucket did not consistently reach the bacterial inactivation targets, despite having greater UV doses than were seen in the initial controlled evaluation at PSA. No single factor could be identified as preventing adequate inactivation, but the role of organic matter and vessel size were contributing factors, whereas the role of turbidity did not have as great an impact as was expected. Future research should further examine the roles of organic matter and vessel size and should focus on the variety of conditions encountered in the real-world settings where SODIS is likely to be deployed. We would recommend that all proposed water treatment systems be tested in the environment in which they are intended to be used to ensure that they are fit for purpose.

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Data statement

Data for this study is available https://doi.org/10.5281/zenodo.5081924.

Author contributions

Steven Brockliss – Data curation, formal analysis, methodology, investigation, original draft.

Kondwani Luwe – investigation, review and editing.

Giuliana Ferrero – conceptualisation, funding acquisition, methodology, resources, supervision, validation, review and editing.

Tracy Morse - conceptualisation, funding acquisition, methodology, supervision, validation, resources, review and editing.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113913.

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Association between per- and polyfluoroalkyl substances and risk of gestational diabetes mellitus

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ABSTRACT

Background: Existing evidence suggests that perfluoroalkyl and polyfluoroalkyl substances (PFASs) exposure might contribute to the incidence of gestational diabetes mellitus (GDM). This study aimed to perform a meta-analysis to identify the association between PFAS and the risk of GDM.
Methods: We systematically searched PubMed, Ovid, Cochrane Library, and Web of Science databases for appropriate articles about the association between PFASs exposure and the risk of GDM before September 28, 2020. Odds ratios (OR) with 95% confidence intervals (CIs) were summarized by Stata 16.0 through fixed effect models according to heterogeneity. We also carried out subgroup analyses by geographic location, blood sampling time of subjects, method of chemical analysis, study design, sample size, and sampling year. In addition, a sensitivity analysis was conducted to explore the robustness of the results.
Results: A total of eight studies involving 5654 pregnant women were included in the meta-analysis. Perfluorooctanoic acid (PFOA) exposure was positively and significantly associated with the risk of GDM (OR = 1.27, 95% CI: 1.02–1.59). Exposure to other types of PFASs such as perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS), perfluorononanoic acid (PFNA) was not statistically significantly associated with the risk of GDM with the pooled effect estimates of 0.97 (95% CI: 0.86–1.09), 1.03 (95% CI: 0.86–1.24), and 0.80 (95% CI: 0.55–1.16) respectively.
Conclusion: We conducted a meta-analysis to investigate the association between PFASs exposure and GDM and found that PFOA concentration was significantly associated with a higher risk of GDM, which is of great significance for the prevention and control of GDM in public health. Further studies are needed in order to establish causality and clarify the potential mechanism.

1. Introduction

Endocrine-disrupting chemicals (EDCs) were defined as compounds, either natural or synthetic, which interfered with hormones in the body's endocrine system through environmental or inappropriate developmental exposures (Gore et al., 2015). Various EDCs are common in our daily lives, such as bisphenol A, polychlorinated biphenyls and phthalates, among which, due to global drinking water contamination, perfluoroalkyl and polyfluoroalkyl substances (PFASs) have received unprecedented attention (Burger et al., 2007; Caserta et al., 2011). PFASs are a type of ubiquitous anthropogenic contaminants (Eriksson et al., 2013; Kantiani et al., 2010; Lau et al., 2007), and the most frequently studied including perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS),

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perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), and perfluoroundecanoic acid (PFUnDA) (Birru et al., 2021). They have been used in industrial and commercial supplies such as lubricants, carpets, food packaging materials, waterproof fabrics, and foam extinguishing agents (Calafat et al., 2007; Schaider et al., 2017; Trier et al., 2011). Due to the widespread use and the resulting emissions of PFASs, a wide range of these substances have been detected in the environment, wild animals, plants, and humans worldwide (Houde et al., 2006). Humans are extensively exposed to PFASs, especially PFOA and PFOS, which was evident from the biological monitoring, particularly in blood (Kato et al., 2014; Stahl et al., 2011). Due to their carbon-fluorine bonds, PFASs are biologically persistent with long half-lives in the human body, and the mean serum half-lives of PFASs vary from 2.3 to 8.5 years (Olsen et al., 2007).

Apart from the universal presence and long half-lives of PFASs, animal studies also showed that PFASs exposure was associated with a wide range of adverse effects, including impaired glucose metabolism and insulin hypersensitivity (Yan et al., 2015), immune system disturbance (Lau et al., 2007; Seacat et al., 2002), alteration in serum lipid levels (Seacat et al., 2002), and the disruption of endocrine hormones (Biegel et al., 1995; Fuentes et al., 2006). Moreover, extensive epidemiological studies also reported an increased prevalence of obesity (Halldorsson et al., 2012), type 1 or 2 diabetes (Su et al., 2016; Sun et al., 2018), and gestational diabetes (Matilla-Santander et al., 2017; Shapiro et al., 2016) in higher PFASs exposure groups.

Gestational diabetes mellitus (GDM), which is one of the most common complications of pregnancy, has experienced an ascending incidence over the past decade (Melchior et al., 2017). GDM is defined as a temporary impaired glucose tolerance that occurs during pregnancy (Dabelea, 2007; Hartling et al., 2012). According to the latest estimates of International Diabetes Federation (IDF) in 2019, the global prevalence of GDM was 14%, equivalent to approximately 20 million births annually (Federation, 2019). A systematic review involving 79 064 Chinese participants from 25 studies showed that the total incidence of GDM in mainland China was 14.8% (Gao et al., 2019). GDM leads to adverse health consequences among women, such as type 2 diabetes and preeclampsia (Lowe et al., 2012; Sweeting et al., 2016), and their offspring are more susceptible to fetal macrosomia and obesity in childhood (Josefson et al., 2020; Kc et al., 2015). Several cohort studies demonstrated a positive correlation between PFOA exposure and the risk of GDM (Matilla-Santander et al., 2017; Preston et al., 2020; Wang et al., 2018a), whereas other studies reported the opposite results (Shapiro et al., 2016; Valvi et al., 2017). Beyond that, there was inconsistent evidence regarding the association between PFOS exposure and GDM as well: both a positive relationship (Liu et al., 2019; Matilla-Santander et al., 2017; Preston et al., 2020; Xu et al., 2020) and a negative relationship have been reported (Shapiro et al., 2016; Valvi et al., 2017; Wang et al., 2018b).

To date, there was increasing evidence that PFASs may be associated with GDM, but the association between PFASs and GDM has not been systematically reported. Thus, we conducted a meta-analysis to review the existing epidemiological studies in order to systematically and comprehensively evaluate the impact of PFASs exposure on the risk of GDM.

2. Methods

This meta-analysis was conducted on the basis of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement to explore the association between the dependent variable (PFAS) and the independent variable (GDM) (Moher et al., 2009).

2.1. Selection criteria

2.1.1. Inclusion criteria

1. Human-based epidemiological studies such as cohort studies, case-control studies and cross-sectional studies that explore the association between PFASs and gestational diabetes mellitus were included.
2. The exposure factors of the study were PFASs.
3. The outcomes of the study were gestational diabetes mellitus, including insufficient glucose tolerance, fasting plasma glucose, 1-h postprandial plasma glucose, and 2-h postprandial plasma glucose.

2.1.2. Exclusion criteria

1. Mechanism studies, animal studies, reviews, letters, conference reports and abstracts, comments, editorials, and announcements.
2. Studies unrelated to maternal diabetes mellitus and PFASs exposure.
3. Studies lacking available statistical, such as odds ratios (OR), hazard ratios (HR), and 95% confidence intervals (CI).
4. Research articles published in other languages apart from English.

2.2. Search strategy

We performed a systematic search in four databases: PubMed, Ovid, the Cochrane library, and Web of Science for the period between inception and September 28, 2020, by using a combination of the following search terms:

#1 PFAS OR PFAA OR PFOA OR PFOS OR PFHxS OR PFNA OR perfluorochemical OR perfluoroalkyl OR polyfluoroalkyl OR perfluorinated OR perfluorooctanoic OR perfluorooctane OR perfluorooctane sulfonate OR perfluorohexane sulfonate OR perfluorononanoate
#2 pregnant OR gestational OR pregnancy OR maternal OR reproductive OR prenatal OR (pregnant women)
#3 diabetes OR (diabetes mellitus) OR endocrine OR (blood glucose) OR (plasma glucose) OR (glucose tolerance) OR (glucose homeostasis) OR (fasting glucose) OR glycemic OR metabolic
#4 #1 AND #2 AND #3

The titles and abstracts of all primary articles were independently reviewed and screened by two researchers. Any discrepancy was resolved by discussion. In all studies, GDM was diagnosed according to corresponding standards including the International Association of Diabetes and Pregnancy Study Groups (IADPSG) and the Society of Obstetricians and Gynaecologists of Canada (SOGC), and we did not impose additional restrictions on this.

All the articles retrieved through the advanced search in the databases were imported to the Endnote X9 for reference management.

2.3. Data extraction

Two researchers independently extracted the characteristics from eligible studies: first author, year of publication, research design, sample size, sampling year, country, type of PFASs, exposure time, effect estimates (OR, HR, and 95% CIs), method of chemical analysis and adjusted variables.

2.4. Assessment of quality

Two researchers independently assessed the quality of the included articles using the Newcastle Ottawa Scale (NOS) (Wells et al., 2014). Differences in grading were discussed with a third researcher to reach a consensus. The scale was used to evaluate the quality of cohort studies and case-control studies, and contained eight items in three categories. Each item was given a maximum of one star in selection and exposure/outcome categories, and a maximum of two stars in comparability assessment (Stang, 2010). According to the NOS, we scored the studies into high quality with at least 7 stars, medium quality awarding 4–6 stars, and low quality if studies received less than 4 stars (Wen et al., 2015).

2.5. Statistical analysis

We conducted a meta-analysis of PFASs and GDM to compute separate forest plots. We only analyzed the four most studied PFASs, namely PFOA, PFOS, PFHxS and PFNA. Since each study had different criteria for selecting chemicals, we mainly focused on the detectable rate of PFASs. 1999–2008 National Health and Nutrition Examination Survey (NHANES) data found that four of twelve PFASs were detected in 95% of serum samples, which were PFOA, PFOS, PFHxS, and PFNA (Kato et al., 2011). Similarly, 2013–2014 NHANES data showed that the U.S. general population was generally exposed to four long-chain PFASs, which were also PFOA, PFOS, PFHxS, and PFNA (CDC, 2019). Additionally, PFASs that were detected in the majority of human blood samples were PFOA, PFOS, PFHxS, and PFNA in a birth cohort in China (Tian et al., 2018). The OR, HR and their corresponding 95% CIs were extracted from adjusted models in each study. The included studies used the concentration of PFASs as a categorical variable, divided into tertiles or quartiles to compare the risk of GDM at different exposure levels. We calculated the pooled estimates by comparing the highest categories with the lowest categories of PFASs concentration (Sun et al., 2020). All statistical analyses were performed using Stata version 16.0 (StataCorp, College Station, TX, USA). The appropriate effect sizes were included, namely OR and HR that can be combined in the meta-analysis (Khreis et al., 2017). The effect size (ES) was calculated by ES = ln (OR), and the standard error (SE) of effect size was calculated as SE = [ln (UC) – ln (LC)]/3.92 (UC and LC represent upper and lower confidence limits respectively). The %weight represented the size of information (i.e., sample size, number of events, and confidence interval) and was calculated as weight $= 1/(SE^2)$. The I^2 statistic was used to evaluate the heterogeneity between studies, and values of 25%, 50%, and 75% were considered to be low, moderate, and high heterogeneity, respectively (Higgins et al., 2003). The findings were statistically significant with $P < 0.05$. Random effect model was applied when the effect estimate was heterogeneous ($I^2 > 50\%$ or $P < 0.1$), otherwise performed the fixed effect models.

Stratified analyses were applied based on the geographic location, blood sampling time of subjects, method of chemical analysis, study design, sample size, and sampling year. The leave-one out sensitivity analysis was applied by omitting one study at a time to explore the robustness of all included studies. We also performed a meta-analysis after excluding studies that evaluated PFASs exposure after diagnosing GDM. Since fewer than ten studies were included, we did not assess publication bias (Sutton et al., 2000).

3. Results

3.1. Study selection

Electronic literature retrieval (September 28, 2020) and manual retrieval yielded 1745 articles for this meta-analysis after the removal of duplicate papers. Among which, 1728 were excluded after reviewing their titles and abstracts, the reason was listed as follows: 199 articles were reviews, 868 articles were irrelevant to the research subject, 137 articles were not research, 413 articles were animal studies and 111 articles were mechanism research. Of the remaining 17 full-text articles, 9 were excluded due to incomplete statistics, only abstract and irrelevant exposure or/and outcome (Fig. 1). A total of 8 studies were finally included.

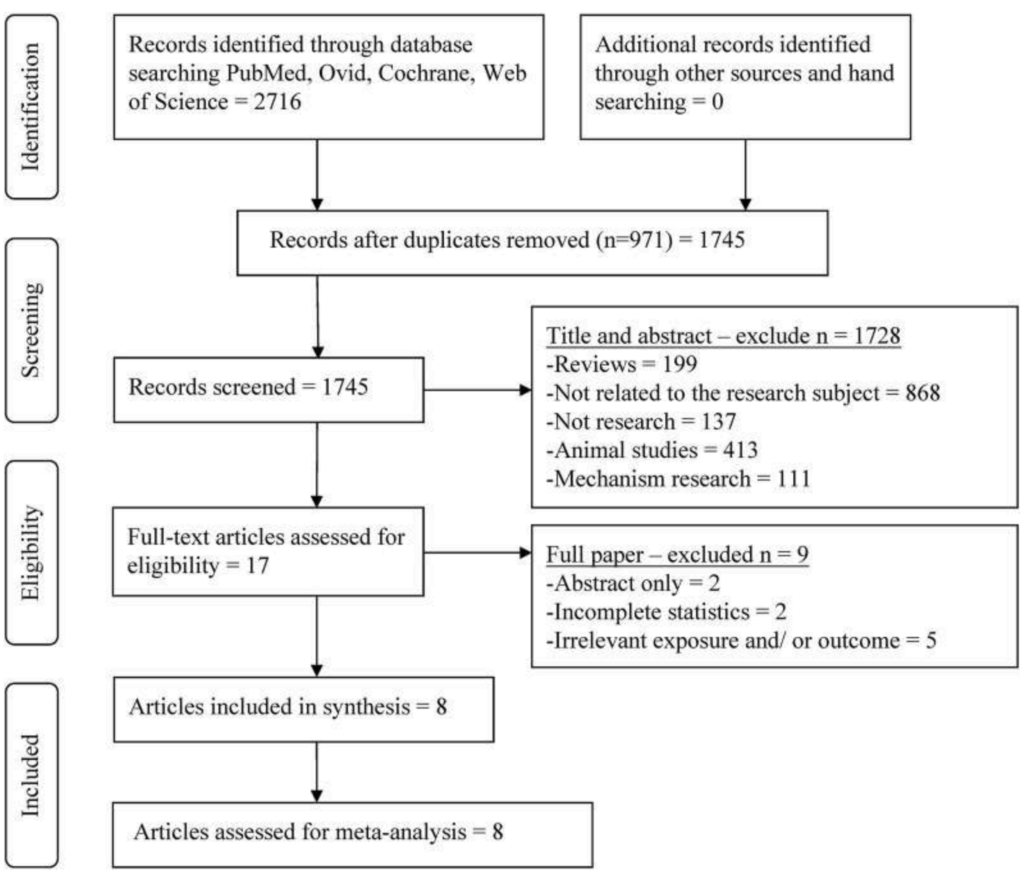

Fig. 1. PRISMA flow diagram of the retrieved eligible articles.

3.2. Study characteristics

Characteristics of all included studies were summarized in Table 1. There were five cohort studies and three case-control studies, and all of them were population-based. Four studies were conducted in China while the other four studies were conducted in Canada, Spanish, Denmark, and the USA respectively. All studies measured the concentrations of PFASs in blood samples from pregnant women, and the sample size ranged from 187 to 1369 with an overall total of 5654 subjects. All studies provided multiple sets of exposures, with different effect estimates and 95% CIs. Among them, five studies measured the concentration of four types of PFASs (PFOA, PFOS, PFHxS, and PFNA), two studies measured three types of PFASs (PFOA, PFOS, and PFHxS), and one study measured only two types of PFASs as PFOA and PFOS. Of the included studies, four studies determined the relationship between the PFASs concentration in the first trimester and the risk of GDM (Liu et al., 2019; Preston et al., 2020; Shapiro et al., 2016; Wang et al., 2018a), two studies measured the PFASs concentration in the second trimester (Matilla-Santander et al., 2017; Xu et al., 2020), and two in the third trimester (Valvi et al., 2017; Wang et al., 2018b). The outcome definition of all eight articles on GDM met our inclusion criteria. Among them, the assessment of GDM in four studies was consistent with the recommendation of IADPSG. Two studies defined GDM in accordance with guidelines from SOGC (Petrović, 2014), one study defined GDM according to National Diabetes Data Group (NDDG), and one study used the Carpenter/Coustan diagnostic criteria (Berggren et al., 2011) (Supplementary Table S2). Most studies adjusted for BMI ($n = 8$), age ($n = 6$) and parity ($n = 5$). However, fewer studies adjusted for confounding factors including education ($n = 4$), ethnicity ($n = 3$), tobacco exposure ($n = 3$) and income ($n = 2$). The exposure levels of four PFASs in the included studies were shown in Supplementary Table S1.

3.3. Quality assessment

Eight included eligible studies were evaluated for quality, of which five cohort studies scored 8 points with high quality, and the remaining three case-control studies were considered as high quality with score 7.

3.4. Systematic review

Among eight studies on the association between PFASs and GDM, none of them demonstrated any significant association between PFOA and GDM (Liu et al., 2019; Matilla-Santander et al., 2017; Preston et al., 2020; Shapiro et al., 2016; Valvi et al., 2017; Wang et al., 2018a, 2018b; Xu et al., 2020) and between PFOS and GDM (Liu et al., 2019; Matilla-Santander et al., 2017; Preston et al., 2020; Shapiro et al., 2016; Valvi et al., 2017; Wang et al., 2018a, 2018b; Xu et al., 2020). Whereas, seven of them did not demonstrate any significant association between PFHxS and GDM (Liu et al., 2019; Matilla-Santander et al., 2017; Preston et al., 2020; Shapiro et al., 2016; Valvi et al., 2017; Wang et al., 2018b; Xu et al., 2020), while five did not demonstrate any significant association between PFNA and GDM (Matilla-Santander et al., 2017; Preston et al., 2020; Valvi et al., 2017; Wang et al., 2018b; Xu et al., 2020). Four of the included studies originated from Asia, and the remaining articles reporting studies in West, including Canada, Denmark, America, and Spain.

3.5. Meta-analysis

Data extracted from eight studies were analyzed to compare the risk of GDM for pregnant women with the highest and lowest categories of PFASs concentration: eight studies for PFOA and PFOS exposure, seven studies for PFHxS exposure, and five studies for PFNA exposure.

3.5.1. Association between PFOA exposure and GDM

Five cohort studies and three case-control studies reported a statistically significant association between PFOA exposure and the risk of GDM. The pooled estimate was OR 1.27 (95% CI: 1.02–1.59). From the eight studies reporting PFOA exposure and GDM risk, six studies showed a positive association, two studies presented a negative association, but the estimate sizes did not reach statistical significance. Due to the absent heterogeneity of the studies ($I^2 = 0.0\%$, $P = 0.686$), a fixed effect model was used to show the correlation between PFOA exposure and GDM (Fig. 2).

3.5.2. Association between PFOS exposure and GDM

Five cohort studies and three case-control studies on the association between PFOS and GDM were included. The overall result provided evidence that PFOS exposure was not statistically significantly associated with the risk of GDM with a pooled OR of 0.97 (95% CI: 0.86–1.09). Considering the low heterogeneity of the included studies ($I^2 = 10.5\%$, $P = 0.349$), a fixed effect model was adopted to show the relationship between PFOS and GDM (Fig. 3).

3.5.3. Association between PFHxS exposure and GDM

Among four cohort studies and three case-control studies, the pooled estimate (OR) was 1.03, which was not statistically significant (95% CI: 0.86–1.24). Moreover, there was no significant heterogeneity across studies ($I^2 = 0.0\%$, $P = 0.973$), so a fixed effect model was used (Fig. 4).

3.5.4. Association between PFNA exposure and GDM

Three cohort studies and two case-control studies were included in the analysis to examine the association between PFNA exposure and the risk of GDM. No evidence has shown a significant association between PFNA exposure and the risk of GDM with a pooled effect size of OR = 0.80 (95% CI: 0.55–1.16) (Fig. 5). According to the result of an absent heterogeneity ($I^2 = 0.0\%$, $P = 0.844$), we performed an overall analysis via a fixed effect model.

3.6. Subgroup analysis

PFOA exposure was found to have a positive and statistically significant association with GDM. PFOS and PFNA exposure had a negative correlation with GDM, while PFHxS exposure was positively correlated with GDM, but none of the analyses for PFOS, PFHxS and PFNA yielded significant differences within the subgroups. In order to explore the association analyses more deeply, we further performed subgroup analyses according to study characteristics. The results of all stratified analyses were shown in Table 2. Subgroup analysis was conducted based on geographic location, blood sampling time of subjects, method of chemical analysis, study design, sample size, and sampling year.

When stratified by geographic location, the pooled estimate of PFOA and GDM was 1.38 (95% CI: 1.07–1.78, $I^2 = 0.0\%$, $P = 0.731$) for Asian countries, but 1.03 (95% CI: 0.68–1.57, $I^2 = 0.0\%$, $P = 0.542$) for Western countries. When stratified by trimester of PFOA measurement, the study was categorized as first, second and third trimesters. In the first trimester group, the pooled estimate was 1.50 (95% CI: 0.98–2.30); the second and third trimester results approximated the first trimester group (OR = 1.24, 95% CI: 0.75–2.04; OR = 1.19, 95% CI: 0.89–1.60). The results of the heterogeneity from subgroup analyses indicated that if the blood samples were detected in the third trimester, it would yield higher heterogeneity ($I^2 = 59.1\%$). When stratified using UPLC or HPLC to measure the concentration of PFOA, the pooled estimate for UPLC was 1.34 (95% CI: 1.05–1.73, $I^2 = 0.0\%$, $P = 0.749$), but HPLC showed no statistically significant association (OR = 1.06, 95% CI: 0.67–1.68, $I^2 = 3.2\%$, $P = 0.356$). When stratified by study design, the association of case-control studies was positive (OR = 1.35, 95% CI: 1.03–1.76, $I^2 = 0.0\%$, $P = 0.671$), and the result of cohort studies indicated no statistically significant association (OR = 1.13, 95% CI: 0.77–1.67, $I^2 = 0.0\%$, $P = 0.485$). A significant positive association between PFOA exposure and GDM was revealed in studies with sample sizes of less than 1000 (OR = 1.29, 95% CI: 1.01–1.64, $I^2 = 6.2\%$, $P = 0.371$), but not in studies

Table 1
Characteristics of all included studies in the meta-analysis.

Study	Research design	Sample size	Sampling year	Country	Exposure time	PFASs	Estimate size (95% CI)	Method of chemical analysis	Adjusted variables	NOS
Shapiro et al., 2016	Cohort	1146	2008–2011	Canada	In first trimester	PFOA	0.9 (0.3, 2.3)	UPLC-MS/MS	Maternal age, pre-pregnancy BMI, race and education	8
						PFOS	0.7 (0.3, 1.7)			
						PFHxS	1.2 (0.4, 3.5)			
Wang et al., 2018a	Cohort	385	2013.9–2014.12	China	In early term of pregnancy	PFOA	1.98 (0.70, 5.57)	UPLC-Q/TOF-MS	Pre-pregnant BMI, pregnant age, family history of diabetes, husband smoking status, family per capita income, baby sex, averaged intake of food and physical activity and energy intake	8
						PFOS	0.71 (0.29, 1.75)			
Matilla-Santander et al. (2017)	Cohort	1214	2003–2008	Spain	At approximately 13 gestational weeks	PFOA	1.25 (0.50, 3.13)	HPLC-MS/MS	Country of birth, pre-pregnancy body mass index, previous breastfeeding, parity, gestational week at blood extraction, physical activity and relative mediterranean diet score	8
						PFOS	2.07 (0.85, 5.01)			
						PFHxS	1.15 (0.42, 3.12)			
						PFNA	0.70 (0.28, 1.75)			
Valvi et al. (2017)	Cohort	604	1997–2000	Denmark	At 34 gestational weeks	PFOA	0.66 (0.30, 1.48)	HPLC-MS/MS	Maternal age at delivery, education, parity, pre-pregnancy BMI and smoking during pregnancy	8
						PFOS	0.56 (0.26, 1.19)			
						PFHxS	1.00 (0.48, 2.07)			
						PFNA	0.65 (0.31, 1.36)			
Preston et al. (2020)	Cohort	1369	1999–2002	America	At the first prenatal visit (median: 9.7 gestational weeks)	PFOA	1.4 (0.7, 2.9)	HPLC-MS/MS	Maternal age, pre-pregnancy BMI, prior history of GDM/parity, smoking, race/ethnicity and education	8
						PFOS	1.5 (0.7, 3.0)			
						PFHxS	1.0 (0.5, 2.2)			
						PFNA	1.0 (0.5, 2.0)			
Wang et al. (2018b)	Case-control	84/168	2013.1–2013.3	China	At 1–2 days before delivery	PFOA	1.31 (0.95, 1.80)	UPLC-MS/MS	BMI, gestational weight gain, ethnic groups, maternal education, parity, maternal drinking during pregnancy, and household income	7
						PFOS	0.96 (0.85, 1.09)			
						PFHxS	1.07 (0.86, 1.35)			
						PFNA	1.25 (0.37, 4.28)			
Xu et al. (2020)	Case-control	165/330	2017.7.1–2019.1.31	China	At 16–20 gestational weeks	PFOA	1.23 (0.71, 2.35)	UPLC-Q/TOF-MS	Maternal age, sampling time, parity and BMI	7
						PFOS	1.01 (0.63, 2.48)			
						PFHxS	0.79 (0.46, 1.31)			
						PFNA	0.70 (0.34, 1.67)			
Liu et al. (2019)		63/126	2013.8–2015.6	China		PFOA				7

(continued on next page)

Table 1 (*continued*)

Study	Research design	Sample size	Sampling year	Country	Exposure time	PFASs	Estimate size (95% CI)	Method of chemical analysis	Adjusted variables	NOS
	Case-control				At first trimester prenatal visit		1.88 (0.85, 4.15)	UPLC-MS/MS	Maternal age, BMI in early pregnancy, fetal sex, and serum triglyceride and total cholesterol	
						PFOS	1.39 (0.55, 3.49)			
							PFHxS			
1.13 (0.47, 2.74)										

The incidence of GDM in each cohort study was 3.8%, 9.1%, 4.4%, 8.1%, and 6.2%, respectively.

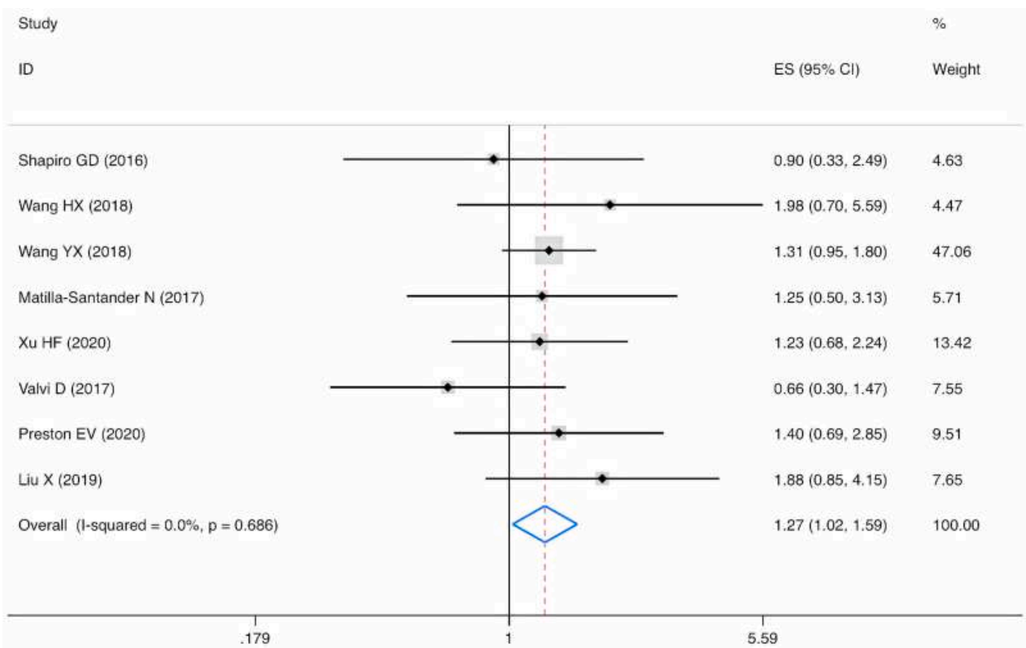

Fig. 2. Forest plot (fixed effect model) for ORs and their corresponding 95% CIs for the association between PFOA exposure and the risk of gestational diabetes mellitus.

more than 1000 (OR = 1.22, 95% CI: 0.75–2.00, $I^2 = 0.0\%$, $P = 0.783$). In addition, when stratified by sampling years, a significant positive association between PFOA exposure and GDM was observed in the studies after 2010 (OR = 1.34, 95% CI: 1.05–1.73, $I^2 = 0.0\%$, $P = 0.749$). In analyses investigating the effect of all six study characteristics on the risk of GDM, there was no evidence of significant heterogeneity.

3.7. Sensitivity analysis

Sensitivity analysis was utilized to determine the stability of the results by omitting single articles on by one to confirm if the results were not influenced by any article. The result of the sensitivity analysis showed the effect size of the association between PFOA exposure and GDM ranged from low 1.23 (95% CI: 0.98–1.55) by excluding the article by Liu (Liu et al., 2019), to high 1.34 (95% CI: 1.07–1.69) by excluding the article by Valvi (Valvi et al., 2017). The effect size of the association between PFOS exposure and GDM ranged from low 0.96 (95% CI: 0.85–1.07) by excluding the article by Matilla-Santander (Matilla-Santander et al., 2017), to high 1.02 (95% CI: 0.75–1.38) by excluding the article by Wang (Wang et al., 2018b). The effect size of the association between PFHxS exposure and GDM ranged from low 0.96 (95% CI: 0.70–1.31) by excluding the article by Wang (Wang et al., 2018b), to high 1.07 by excluding the article by Xu (Xu et al., 2020) (95% CI: 0.88–1.30). The effect size of the association between PFNA exposure and GDM ranged from low 0.73 (95% CI: 0.47–1.14) by excluding the article by Preston (Preston et al., 2020), to high 0.86 by excluding the article by Valvi (Valvi et al., 2017) (95% CI: 0.56–1.31). The analyses after removal of single articles were not significantly different with the analyses of all articles, which indicated that the removal of any article did not influence the overall statistical results. Furthermore, we analyzed six studies that evaluated PFASs exposure before diagnosing GDM, considering that the concentration of PFASs can change across pregnancy and the diagnosis of GDM may impact the concentration of PFASs. The meta-analysis result indicated that PFOA exposure might be positively associated with GDM (OR = 1.38, 95% CI: 1.00–1.91). The pooled OR and 95% CI showed no statistical significance of PFOS (OR = 1.14, 95% CI: 0.82–1.59), PFHxS (OR = 0.95, 95% CI: 0.68–1.34), and PFNA (OR = 0.82, 95% CI: 0.52–1.28) exposure in the fixed effect model (Figs. S1–S4). In summary, the results of this meta-analysis were robust and reliable.

4. Discussion

The association of PFASs exposure on glucose metabolism disorders remained uncertain since previous epidemiological studies have reported conflicting results. Eight studies were eligible to be included in the meta-analysis involving 578 cases of GDM from 5654 pregnant women. In this study, we performed analyses by combining studies with the same type of PFASs to summarize the association.

Our results indicated that only PFOA exposure was associated with a

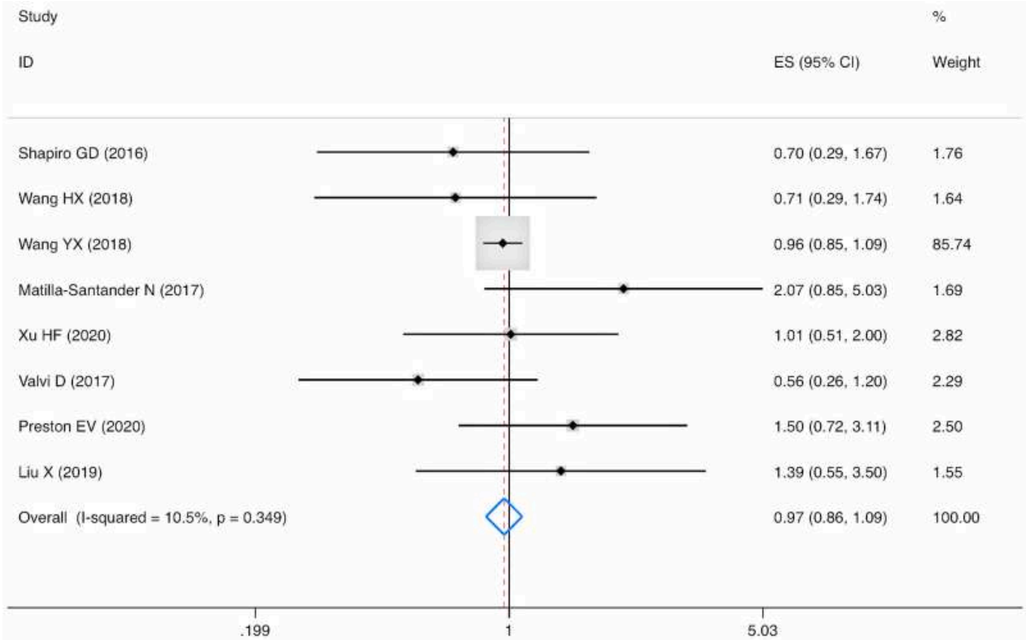

Fig. 3. Forest plot (fixed effect model) for ORs and their corresponding 95% CIs for the association between PFOS exposure and the risk of gestational diabetes mellitus.

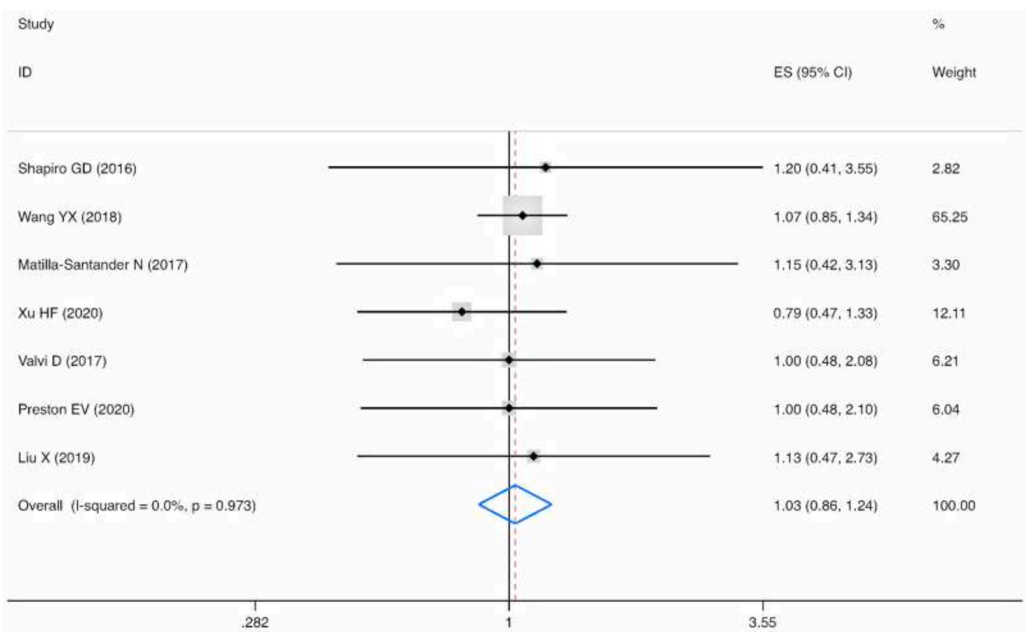

Fig. 4. Forest plot (fixed effect model) for ORs and their corresponding 95% CIs for the association between PFHxS exposure and the risk of gestational diabetes mellitus.

statistically significant increase in the risk of GDM. PFHxS was positively associated with the risk of GDM, but this relationship was not statistically significant. Exposure to the following PFASs may decrease the risk of GDM: 0.97 (95% CI: 0.86–1.09) in the PFOS group and 0.80 (95% CI: 0.55–1.16) in the PFNA group, but neither of these two associations was statistically significant.

In previous studies, there were six studies (Liu et al., 2019; Matilla-Santander et al., 2017; Preston et al., 2020; Wang et al., 2018a, 2018b; Xu et al., 2020) that showed a positive association between PFOA exposure and GDM, which were consistent with our result (OR = 1.27, 95% CI: 1.02–1.59). However, Shapiro et al. (2016) found that exposure to PFOA showed a negative correlation with GDM risk. This finding from regression analyses was based on small numbers of cases in each quartile, so the negative result could be due to type II error. The inconsistency may also be related to uncontrolled confounding factors, such as dietary intake and family history of diabetes. Contrary to our findings Valvi et al. (2017) did not find an association between PFOA exposure and GDM, which may be partly because the diagnosis of GDM based on medical records, as OGTT is only performed in women considered higher risk, possibly leading to an underestimation of GDM cases. In the studies of PFOS exposure, the results of Wang et al. (2018a) (OR = 0.71, 95% CI: 0.29–1.75), Shapiro et al. (2016) (OR = 0.70, 95% CI: 0.30–1.70), and Valvi et al. (2017) (OR = 0.56, 95% CI: 0.26–1.19) were in agreement with our study result (OR = 0.97, 95% CI:

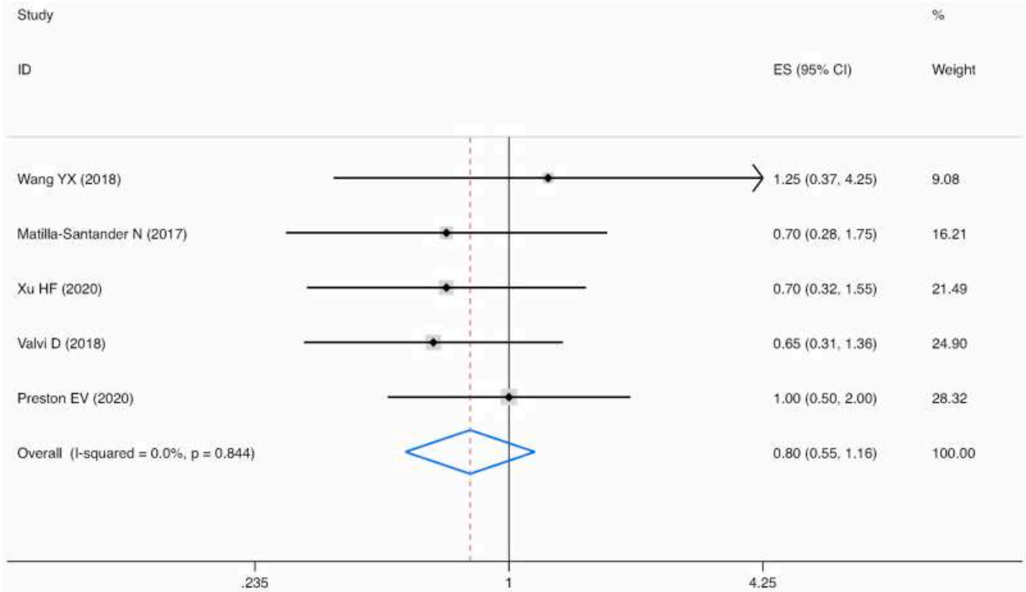

Fig. 5. Forest plot (fixed effect model) for ORs and their corresponding 95% CIs for the association between PFNA exposure and the risk of gestational diabetes mellitus.

Table 2
Subgroup analysis of PFASs (PFOA, PFOS, PFHxS, and PFNA) exposure and the risk of gestational diabetes mellitus.

	Geographic location		Exposure time			Method of chemical analysis		Design		Sample size		Sampling year	
	Asia	Western	First trimester	Second trimester	Third trimester	UPLC	HPLC	Cohort	Case-control	<1000	>1000	Before 2010	After 2010
PFOA													
Number	4	4	4	2	2	5	3	5	3	5	3	3	5
OR (95% CI)	1.38 (1.07, 1.78)	1.03 (0.68, 1.57)	1.50 (0.98, 2.30)	1.24 (0.75, 2.04)	1.19 (0.89, 1.60)	1.34 (1.05, 1.73)	1.06 (0.67, 1.68)	1.13 (0.77, 1.67)	1.35 (1.03, 1.76)	1.29 (1.01, 1.64)	1.22 (0.75, 2.00)	1.06 (0.67, 1.68)	1.34 (1.05, 1.73)
I^2 (%)	0.0	0.0	0.0	0.0	59.1	0.0	3.2	0.0	0.0	6.2	0.0	3.2	0.0
*P**	0.731	0.542	0.662	0.977	0.118	0.749	0.356	0.485	0.671	0.371	0.783	0.356	0.749
*P***	0.246		0.686			0.373		0.464		0.854		0.373	
PFOS													
Number	4	4	4	2	2	5	3	5	3	5	3	3	5
OR (95% CI)	0.96 (0.85, 1.09)	1.04 (0.69, 1.55)	1.05 (0.69, 1.59)	1.32 (0.77, 2.27)	0.95 (0.84, 1.07)	0.96 (0.85, 1.08)	1.15 (0.73, 1.81)	0.97 (0.67, 1.40)	0.97 (0.86, 1.09)	0.95 (0.84, 1.07)	1.31 (0.82, 2.10)	1.15 (0.73, 1.81)	0.96 (0.85, 1.08)
I^2 (%)	0.0	54.8	0.0	36.5	46.8	0.0	64.5	44.4	0.0	0.0	36.7	64.5	0.0
*P**	0.785	0.085	0.416	0.210	0.170	0.813	0.060	0.126	0.733	0.563	0.206	0.060	0.813
*P***	0.733		0.468			0.438		0.981		0.194		0.438	
PFHxS													
Number	3	4	3	2	2	4	3	4	3	4	3	3	4
OR (95% CI)	1.03 (0.84, 1.25)	1.05 (0.69, 1.61)	1.08 (0.65, 1.79)	0.86 (0.54, 1.36)	1.06 (0,86, 1.32)	1.03 (0.85, 1.26)	1.03 (0.65, 1.63)	1.05 (0.69, 1.61)	1.03 (0.84, 1.25)	1.02 (0.84, 1.24)	1.08 (0.64, 1.83)	1.03 (0.65, 1.63)	1.03 (0.85, 1.26)
I^2 (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*P**	0.566	0.989	0.957	0.515	0.862	0.749	0.971	0.989	0.566	0.767	0.955	0.971	0.749
*P***	0.908		0.693			0.997		0.908		0.842		0.997	
PFNA													
Number	2	3	1	2	2	2	3	3	2	3	2	3	2
OR (95% CI)	0.83 (0.43, 1.62)	0.79 (0.51, 1.23)	1.00 (0.50, 2.00)	0.70 (0.38, 1.28)	0.77 (0.41, 1.46)	0.83 (0.43, 1.62)	0.79 (0.51, 1.23)	0.79 (0.51, 1.23)	0.83 (0.43, 1.62)	0.74 (0.45, 1.22)	0.88 (0.51, 1.53)	0.79 (0.51, 1.23)	0.83 (0.43, 1.62)
I^2 (%)	0.0	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*P**	0.436	0.678	–	1.000	0.370	0.436	0.678	0.678	0.436	0.657	0.543	0.678	0.436
*P***	0.896		0.742			0.896		0.896		0.663		0.896	

*P** for heterogeneity within each subgroup. *P*** for heterogeneity between subgroups. CI, confidence interval.

0.86–1.09). In a Spanish INMA Birth Cohort study, the association found by Matilla-Santander (OR = 2.07, 95% CI: 0.85–5.01) was positive; however, the results did not appear significant since the estimates for GDM were based on fewer cases and were less precise (Matilla-Santander et al., 2017). In the studies of PFHxS exposure, the results of Shapiro et al. (2016) (OR = 1.20, 95% CI: 0.40–3.50) and Liu et al. (2019) (OR = 1.13, 95% CI: 0.47–2.74) were consistent with ours (OR = 1.03, 95% CI: 0.86–1.24). Xu et al. (2020) reported that PFHxS exposure was associated with a lower risk of GDM (OR = 0.79, 95% CI: 0.46–1.31), which was inconsistent with our finding. Xu believed that any GDM that occurred during the third trimester was not detected, so the correlation between serum PFHxS level and the prevalence of GDM may be underestimated. In the three studies of Matilla-Santander (Matilla-Santander et al., 2017) (OR = 0.70, 95% CI: 0.28–1.75), Valvi (Valvi et al., 2017) (OR = 0.65, 95% CI: 0.31–1.36), and Xu (Xu et al., 2020) (OR = 0.70, 95 %CI: 0.34–1.67), PFNA exposure and GDM risk were all negatively correlated. Nevertheless, Wang's findings (Wang et al., 2018b) (OR = 1.25, 95% CI: 0.37–4.28) were inconsistent with ours, which possibly because of the existence of unmeasured causative factors including sociodemographic factors and other environmental toxicants related to PFASs exposure. Considering that the reported findings appeared inconsistent, further laboratory studies are warranted. Based on subgroup analyses, we found that the Asian population with high PFOA exposure had a higher risk of GDM, whereas studies from the West did not find an association. Of note, four studies from Asia all defined GDM according to IADPSG. The cut-off values for OGTT of this guideline were lower than other diagnosis criteria, resulting in more positive cases.

The regulation mechanism of PFASs on glucose homeostasis was complex that involves multiple pathways. Previous studies have observed that high exposure to PFASs may decrease insulin sensitivity or cause an increase in blood glucose level. One possible mechanism is that exposure to PFASs might induce oxidative stress by increasing fatty acid oxidation (Guruge et al., 2006), so that peroxisome proliferator-activated receptor alpha (PPAR-α) is induced to express (Fang et al., 2012), and the activation of PPAR-α can further increase the liver glucose output by up-regulating the expression of G6PC gene (Fleisch et al., 2017; Im et al., 2011). There were other studies that indicated exposure to PFASs can cause hypothyroidism in pregnant women (Lee and Choi, 2017), which could promote the risk of hypoglycemia (Wang, 2013). PFASs may alter TH homeostasis during pregnancy by increasing TSH levels and reducing T3 and T4 levels, thereby disrupting downstream glucose metabolism (Birru et al., 2021). In our study, PFOA and PFHxS were positively correlated with GDM, while PFOS and PFNA were negatively correlated with GDM, indicating that the effect on glucose homeostasis may vary by different species of PFASs. These discrepancies might be in part attributed to their different chemical structures considering that PFASs are a broad class of organic chemicals with varying carbon backbone length, isomeric structure, and charged carboxylate or sulfonate functional groups like PFOA has a carboxyl and PFOS has a sulfonic acid group (Jensen and Leffers, 2008).

There were several strengths of this study. To begin with, the selection of studies was on the basis of a specific search strategy. Secondly, compared to individual studies with limited sample sizes, this analysis included a larger sample size of GDM cases. A large number of samples not only allow us to better explore the relationship between PFASs and the risk of GDM, but also facilitate us to perform detailed subgroup analyses on the data. Besides, there has been no systematic review of studies focusing on the relationship between multiple types of PFASs and GDM so far. No statistically significant correlation between PFOA exposure and GDM risk was detected in previous research, but a statistically significant positive association appeared after pooling the data in our study. In addition, eight included articles were all of high quality with 7 or 8 scores to ensure the reliability of the results in this meta-analysis.

Despite the strengths, there were several limitations in our study. We only included studies that used PFASs concentration as a categorical variable, that is, the highest quantile compared to the lowest quantile, and we excluded studies that used concentration as a continuous variable. This may probably be a risk of bias here, due to the different quantile standards for PFASs concentrations in various studies. Of the included studies, there were only two studies evaluating blood glucose levels. Due to the limited statistical data, more studies on PFASs and OGTT blood glucose data are necessary. In addition, not all included original studies measured potential confounding factors (such as age, parity, tobacco exposure, etc.) that might affect the results of the present meta-analysis. To exemplify, when tobacco exposure was unadjusted, PFOA exposure was statistically significant and positively associated with GDM, but when adjusted, the association was not statistically significant. Moreover, the participants of the included studies were recruited by hospitals and there may be selection bias, so even though they had high quality scores, the results should be interpreted with caution and more population-based studies are required in the future.

5. Conclusion

In this meta-analysis, we found that exposure to PFOA was associated with an increased risk of GDM. Further animal experiments and large-scale cohorts should endeavor to establish potential causality and clarify the role of PFASs exposure in the development of GDM in pregnant women. It is very necessary to monitor ambient PFASs exposure, so as to prevent and control GDM and elevate maternal and infant health protection.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113904.

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Association of ambient air pollution with risk of hemorrhagic stroke: A time-stratified case crossover analysis of the Singapore stroke registry

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ABSTRACT

Background: Haemorrhagic stroke (HS) is a major cause of mortality and disability. Previous studies reported inconsistent associations between ambient air pollutants and HS risk.
Objective: We evaluated the association between air pollutant exposure and the risk of HS in a cosmopolitan city in the tropics.
Methods: We performed a nationwide, population-based, time-stratified case-crossover analysis on all HS cases reported to the Singapore Stroke Registry from 2009 to 2018 (n = 12,636). We estimated the risk of HS across tertiles of air pollutant concentrations in conditional Poisson models, adjusting for meteorological confounders. We stratified our analysis by age, atrial fibrillation and smoking status, and investigated the lagged effects of each pollutant on the risk of HS up to 5 days.
Results: All 12,636 episodes of HS were included. The median (1st-to 3rd-quartile) daily pollutant levels from 22 remote stations deployed across the island were as follows: ($PM_{2.5}$ = 15.9 (12.7–20.5), PM_{10} = 27.3 (22.7–33.4), O_3 = 22.5 (17.3–29.8), NO_2 = 23.3 (18.8–28.4), SO_2 = 10.2 (5.6–14.4), CO = 0.5 (0.5–0.6). The median (1st-to 3rd-quartile) temperature (°C) was 27.9 (27.1–28.7), that of relative humidity (%) was 79.4 (75.6–83.2), and that of total rainfall (mm) was 0.0 (0.0–4.2). Higher levels of CO were significantly associated with an increased risk of HS (3rd tertile vs 1st tertile: Incidence Rate Ratio (IRR) = 1.06, 95% CI = 1.01–1.12). The increased risk of HS due to CO persisted for at least 5 days after exposure. Individuals under 65 years old and non-smokers had a higher risk of HS when exposed to CO. O_3 was associated with increased risk of HS up to 5 days (3rd tertile vs 1st tertile: $IRR_{day\ 1}$ = 1.07, 95% CI = 1.02–1.12; $IRR_{day\ 5}$ = 1.07, 95% CI = 1.02–1.13).
Conclusion: Short-term exposure to ambient CO levels was associated with an increased risk of HS. A reduction in CO emissions may reduce the burden of HS in the population.

1. Introduction

Haemorrhagic stroke (HS) is a major cause of mortality and disability across the globe. HS has an overall prevalence of 116.6 per 100,000 people worldwide and occurred most commonly in developed countries and in Asians (Feigin et al., 2015; van Asch et al., 2010). HS accounted

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Table 1
Characteristics of patients with haemorrhagic stroke in 2009–2018 (n = 12636 episodes).

Demographics	
Age, median (1st- to 3rd-quartile)	63 (53–75)
Male gender, n (%)	7000 (55.4)
Ethnicity, n (%)	
Chinese	10115 (80.1)
Malay	1820 (14.4)
Indian	512 (4.0)
Others	189 (1.5)
Current/ex-smoker, n (%)	3352 (29.9)
Co-morbidities	
Diabetes mellitus, n (%)	3292 (26.1)
Hypertension, n (%)	10327 (81.7)
Hyperlipidemia, n (%)	6996 (55.4)
Atrial fibrillation/flutter, n (%)	1223 (9.7)
History of ischemic heart disease, n (%)	1786 (32.9)

for approximately 10–15% of strokes worldwide, but was associated with higher rates of mortality, morbidity, and financial burden than ischemic stroke (Feigin et al., 2015; Poon et al., 2014; van Asch et al., 2010).

Air pollution refers to the presence of harmful airborne substances that arises from the complex interaction between natural and anthropogenic environmental conditions (Mayer, 1999). Air pollution is considered a serious public health problem and its important constituents comprises but is not limited to particulate matter of aerodynamic diameter of ≤ 2.5 μm ($PM_{2.5}$) and ≤ 10 μm (PM_{10}), ozone (O_3), nitrogen dioxide (NO_2), sulphur dioxide (SO_2) and carbon monoxide (CO) (Brunekreef and Holgate, 2002; Cheong et al., 2019). According to reports from the World Health Organization, low- and middle-income countries experience a disproportionately higher disease burden from air pollution, and its noxious effects are felt most in the Pacific and Southeast Asia region (Aik et al., 2020; Chan et al., 2020; Cheong et al., 2019; Foreman et al., 2018; Ho et al., 2018; Ho et al., 2018; Ho et al., 2019; Rajarethinam et al., 2020; World Health Organization, 2021). In Southeast Asia, agricultural slash-and-burn techniques leads to extensive land fires and haze which has an important influence on air quality (Sharma and Balasubramanian, 2018; Tacconi, 2016).

In the Global Burden of Disease Study 2015), which analysed data across 25 years, ambient air pollution was found to have contributed substantially to diseases such as ischaemic heart disease, cerebrovascular disease, chronic obstructive pulmonary disease and their resultant mortality and disability-adjusted life years loss (Cohen et al., 2017; Ljungman and Mittleman, 2014; Shah et al., 2015). Indeed, there is a growing body of evidence on the association between exposure to air pollution and HS (Chien et al., 2017; Nzwalo et al., 2019; Qian et al., 2019; Yorifuji et al., 2011; Zhang et al., 2018). Existing studies suggest that short term exposure to $PM_{2.5}$, PM_{10}, and ozone were significantly associated with the risk of HS (Yorifuji et al., 2011; Zhang et al., 2018), intracerebral haemorrhage (Chien et al., 2017; Nzwalo et al., 2019), and fatal intracerebral haemorrhage (Qian et al., 2019).

While these studies reported associations between ambient air pollutants and HS, these associations were inconsistent and higher-quality studies are urgently needed (Ljungman and Mittleman, 2014; Shah et al., 2015). Moreover, the majority of studies examining the short-term relationship between air quality and HS risk were undertaken in temperate settings (Ljungman and Mittleman, 2014; Shah et al., 2015). Few studies have explored the association between ambient air pollutants and HS in a tropical setting. Thus, we aimed to evaluate the association between individual ambient air pollutants and the risk of HS in a cosmopolitan city in the tropics.

Table 2
Descriptive summary of the daily number of haemorrhagic stroke cases, meteorological factors and air pollutants in 2009–2018 (n = 3652 days).

	Mean (SD)	Median (first- to third-quartile)	Minimum	Maximum
Number of HS	3.5 (1.9)	3 (2–5)	0	11
Meteorological factors				
Average temperature, °C	27.8 (1.1)	27.9 (27.1–28.7)	22.8	30.8
Relative humidity, %	79.5 (5.4)	79.4 (75.6–83.2)	59.2	96.9
Total rainfall, mm	5.5 (12.6)	0.0 (0.0–4.2)	0.0	216.2
Air pollutants				
$PM_{2.5}$, μg/m^3	18.4 (12.9)	15.9 (12.7–20.5)	5.1	274.4
Tertile 1	11.3 (1.7)	11.6 (10.3–12.6)	5.1	13.6
Tertile 2	15.9 (1.4)	15.9 (14.8–17.0)	13.6	18.6
Tertile 3	27.8 (18.7)	22.9 (20.5–27.4)	18.6	274.4
PM_{10}, μg/m^3	30.0 (15.8)	27.3 (22.7–33.4)	9.7	335.9
Tertile 1	20.5 (2.8)	21.0 (18.9–22.7)	9.7	24.3
Tertile 2	27.3 (1.8)	27.3 (25.7–28.9)	24.3	30.9
Tertile 3	42.0 (22.3)	36.4 (33.4–41.5)	30.9	335.9
O_3, μg/m^3	24.3 (10.1)	22.5 (17.3–29.8)	4.4	76.0
Tertile 1	14.5 (3.2)	15.2 (12.3–17.3)	4.4	18.8
Tertile 2	22.6 (2.3)	22.4 (20.6–24.6)	18.8	26.9
Tertile 3	35.7 (7.7)	33.5 (29.8–39.8)	26.9	76.0
NO_2, μg/m^3	23.9 (7.0)	23.3 (18.8–28.4)	6.8	53.4
Tertile 1	16.5 (2.7)	17.0 (14.7–18.8)	6.8	20.3
Tertile 2	23.3 (1.8)	23.3 (21.7–24.9)	20.3	26.4
Tertile 3	31.8 (4.7)	30.4 (28.4–33.9)	26.4	53.4
SO_2, μg/m^3	10.8 (6.1)	10.2 (5.6–14.4)	2.0	45.0
Tertile 1	4.6 (1.3)	4.5 (3.5–5.6)	2.0	7.0
Tertile 2	10.0 (1.6)	10.2 (8.6–11.4)	7.0	12.8
Tertile 3	17.8 (4.5)	16.5 (14.4–19.7)	12.8	45.0
CO, mg/m^3	0.54 (0.16)	0.51 (0.45–0.59)	0.24	3.35
Tertile 1	0.41 (0.05)	0.42 (0.38–0.45)	0.24	0.47
Tertile 2	0.52 (0.03)	0.52 (0.49–0.54)	0.47	0.57
Tertile 3	0.69 (0.20)	0.65 (0.60–0.72)	0.57	3.35

Abbreviations: carbon monoxide (CO); haemorrhagic stroke (HS); nitrogen dioxide (NO_2); ozone (O_3); particulate matter of aerodynamic diameter of ≤ 2.5 μm ($PM_{2.5}$); particulate matter of aerodynamic diameter of ≤ 10 μm (PM_{10}); sulphur dioxide (SO_2); standard deviation (SD).

2. Methods

2.1. Setting

This study was conducted in Singapore, a heavily-urbanised, densely populated island city-state in Southeast Asia, with a population of 5.7 million over a land area of 725.7 km^2 (population density of 7810 per km^2) (Department of Statistics Singapore, 2021). Singapore is situated 1.5° north of the equator, with a climate characterized by warmer

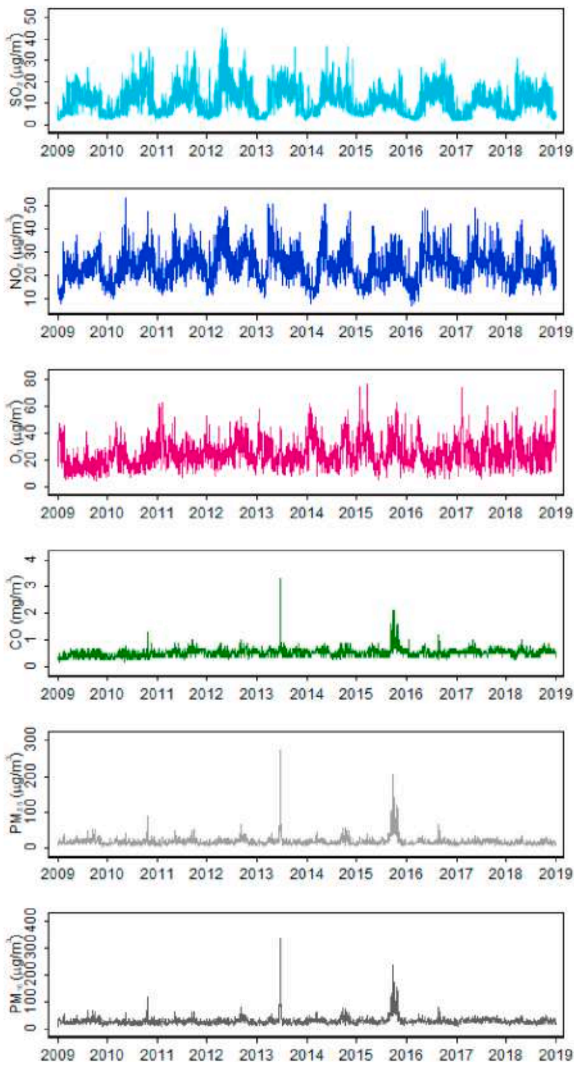

Fig. 1. Trend of daily air pollutant concentrations in Singapore from 2009–2018.
Fig. 1 showing the daily average concentration of individual air pollutants over the study period.
Abbreviations: carbon monoxide (CO); nitrogen dioxide (NO_2); ozone (O_3); particulate matter of aerodynamic diameter of $\leq$ 2.5 μm ($PM_{2.5}$); particulate matter of aerodynamic diameter of $\leq$ 10 μm (PM_{10}); sulphur dioxide (SO_2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ambient temperatures, high humidity levels and abundant rainfall, and experiences transboundary smoke haze from time to time (Meteorological Service Singapore, 2021). Seven restructured general hospitals, along with several private hospitals, deliver tertiary health care and manages more than 95% of HS patients (Venketasubramanian, 2020). Based on annual reports from the Singapore Stroke Registry, the annual age-standardized incidence of HS in Singapore between 2009 and 2018 remained stable, ranging between 30.2 and 34.0 per 100,000 population. The age-standardized mortality rate for HS decreased from 8.9 per 100,000 cases in 2010 to 5.9 per 100,000 cases in 2018 (National Registry of Diseases Office, 2020).

2.2. Air quality and meteorological exposures

The primary exposures evaluated in this study were the daily mean ambient concentrations of the following air pollutants: $PM_{2.5}$, PM_{10}, O_3, NO_2, SO_2 and CO. The National Environmental Agency (NEA), Singapore is the custodian of meteorological data in Singapore, and manages the long-term archive and quality control of national climate data (National Environment Agency). The NEA monitors the daily pollutant levels via 22 remote stations deployed across the island, and collects daily data for temperature, humidity and rainfall via 11 weather stations across Singapore. The air quality exposure and meteorological data from 2009 to 2018 used in this study were retrieved from the NEA, and has been described in detail previously (Ho et al., 2018).

2.3. Study population and outcome data

The Singapore Stroke Registry (SSR) receives stroke case notifications from all public healthcare institutions via hospital in-patient discharge summaries, medical claim listings, and the national death registry (Venketasubramanian et al., 2015). The International Classification of Diseases (ICD) diagnosis codes were used to identify stroke cases, comprising ICD-9 codes 430 to 437 (excluding 432.1 and 435) prior to 2012, and ICD-10 codes I60 to I68 (excluding I62.0, I62.1) from 2012 onward. The registry coordinators confirmed the diagnosis of HS by assessing patients' medical records before extracting detailed individual-level clinical data. All the cases collected in the SSR were diagnosed as HS by a medical practitioner, defined as the presence of neurological deficit lasting more than 24 h, supported by appropriate neuroimaging. All daily reported cases of HS in Singapore from Jan 1, 2009 to Dec 31, 2018 were obtained from the SSR. The date of presentation to the hospital was taken as the event date as this was the earliest available date that was objective. The registry data were subject to annual audits for accuracy and inter-rater reliability. Outlier and illogical data were flagged and reviewed for final consensus among the registry coordinators. The Centralised Institutional Review Board and the Domain Specific Review Board granted approval for this study with a waiver of patient consent (CIRB Ref: 2017/2380).

2.4. Statistical analyses

Baseline characteristics (including age, gender, ethnicity, smoking status, and co-morbidities of patients) of patients diagnosed with HS were summarized using median and 1st-to 3rd-quartile for continuous variables, and numbers and percentages for categorical variables. We also computed and reported the mean (standard deviation), median (1st-to 3rd-quartile), and the minimum and maximum number of HS cases diagnosed daily between 2009 and 2018. The daily meteorological factors (average temperature, relative humidity, and total rainfall) and air pollutant measures (ambient concentrations of $PM_{2.5}$, PM_{10}, O_3, NO_2, SO_2 and CO) were summarized and reported similarly as well. The effects of each pollutant up to a maximum of 5 days before the incidence of HS (lag day 0) were reported as lag day 1, 2, 3, 4, and 5 respectively. The daily average ambient concentrations of $PM_{2.5}$, PM_{10}, O_3, NO_2, SO_2 and CO from 2009 to 2018 were plotted to show the trend in the air pollutant concentrations over the study period.

A time-stratified case-crossover approach was used to examine the association between individual air pollutants and HS risk. For every day with at least one incidence of HS, the day was considered as a "case" and its "controls'' were derived using the same day-of-week in the same month and year. Conditional Poisson regression was used to compare the incidence rate ratio (IRR) of HS on the same day with exposure to the air pollutant across different concentrations by tertile (referencing the 1st tertile), accounting for over-dispersion and autocorrelation. All models included daily mean temperature, relative humidity and total rainfall as covariates, and the population-at-risk as an offset. We analysed each air pollutant in single-pollutant models to avoid multicollinearity among the pollutants (Ito et al., 2007; Tolbert et al., 2007).

We hypothesized that age, atrial fibrillation (previous or recent diagnosis) and smoking were potential modifiers of the relationship between ambient air pollutants and HS risk and stratified our analysis by these three sub-groups (Ho et al., 2018). We also investigated the

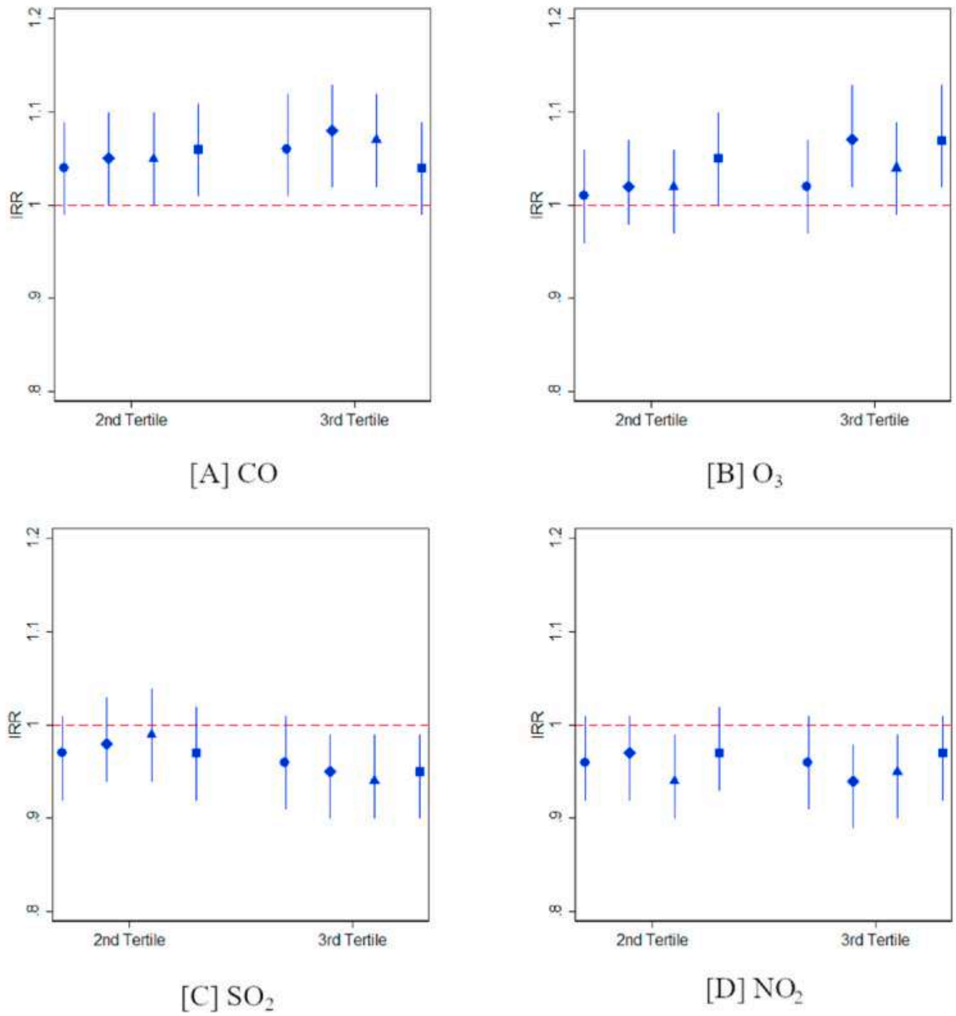

Fig. 2. Immediate and lagged associations between HS risk and air pollutant exposure. Fig. 2 showing the immediate and lagged associations between HS risk and [A] carbon monoxide (CO) exposure, [B] ozone (O_3) exposure, [C] sulphur dioxide (SO_2) exposure, and [D] nitrogen dioxide (NO_2) exposure. The point estimates are indicated by the solid circles (lag day 0), diamonds (lag day 1), triangles (lag day 3) and squares (lag day 5). The vertical lines depict the 95% confidence intervals of the point estimates while the horizontal dashed line depicts the null value. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

independent lagged effects of each pollutant on the risk of HS up to a maximum of 5 days before the incidence of HS (lag day 1, 2, 3, 4, and 5) (Ho et al., 2018). Two-tailed p values $< .05$ were considered statistically significant. Statistical analyses were performed using STATA SE 13 (StataCorp, College Station, Texas).

3. Results

3.1. Baseline characteristics of patients with HS between 2009 and 2018

We included all 12,636 episodes of HS reported to the SSR between 2009 and 2018. The median daily number of HS for the duration of the study was 3 (1st-to 3rd-quartile = 2–5). The median age was 63 year (1st-to 3rd-quartile = 53–75) and 55.4% were men. The majority were of Chinese (80.1%) ethnicity. Baseline characteristics of the study cohort are presented in Table 1.

3.2. Baseline weather and air quality measures between 2009 and 2018

A summary of the daily meteorological and air quality measures is described in Table 2. The median temperature was 27.9 °C (1st-to 3rd-quartile = 27.1–28.7) and the median relative humidity was 79.4% (1st-to 3rd-quartile = 75.6–83.2).

Fig. 1 shows the daily average concentration of individual air pollutants over the study period. NO_2 and SO_2 concentration levels broadly demonstrated similar trends, with at least one periodic within-year peak. $PM_{2.5}$, PM_{10} and CO concentrations appeared to have similar trends, with their highest concentrations recorded in June 2013 and October 2015, coinciding with periods of transboundary haze that Singapore experienced (Cheong et al., 2019). O_3 concentrations did not appear to exhibit a regular cyclical trend.

3.3. The associations between air pollutants and the risk of HS

Across the entire study cohort, after adjusting for the potential confounding effects of temperature, relative humidity and total rainfall, higher levels of CO were independently associated with an increased risk of HS. The 3rd tertile of CO exposure (3rd tertile vs 1st tertile: IRR = 1.06, 95% CI = 1.01–1.12) was significantly associated with a higher risk of HS on the same day compared to the 1st tertile of exposure (Fig. 2 [A]). No associations between other air pollutants and immediate HS risk were found (See Table S1 in Supplemental Digital Content).

3.4. Lagged effects of air pollutants on the risk of HS

HS risk persisted up to 5 days after CO exposure for the 2nd tertile (3rd tertile vs 2nd tertile: IRR = 1.06, 95% CI = 1.01–1.11) and up to three days for the 3rd tertile (Fig. 2[A]). O_3 was associated with increased risk of HS up to 5 days (Fig. 2[B]). SO_2 was associated with decreased risk of HS up to 5 days after exposure (Fig. 2[C]), and NO_2 was associated with decreased risk of HS up to 3 days after exposure (Fig. 2 [D]) (See Table S2 in Supplemental Digital Content).

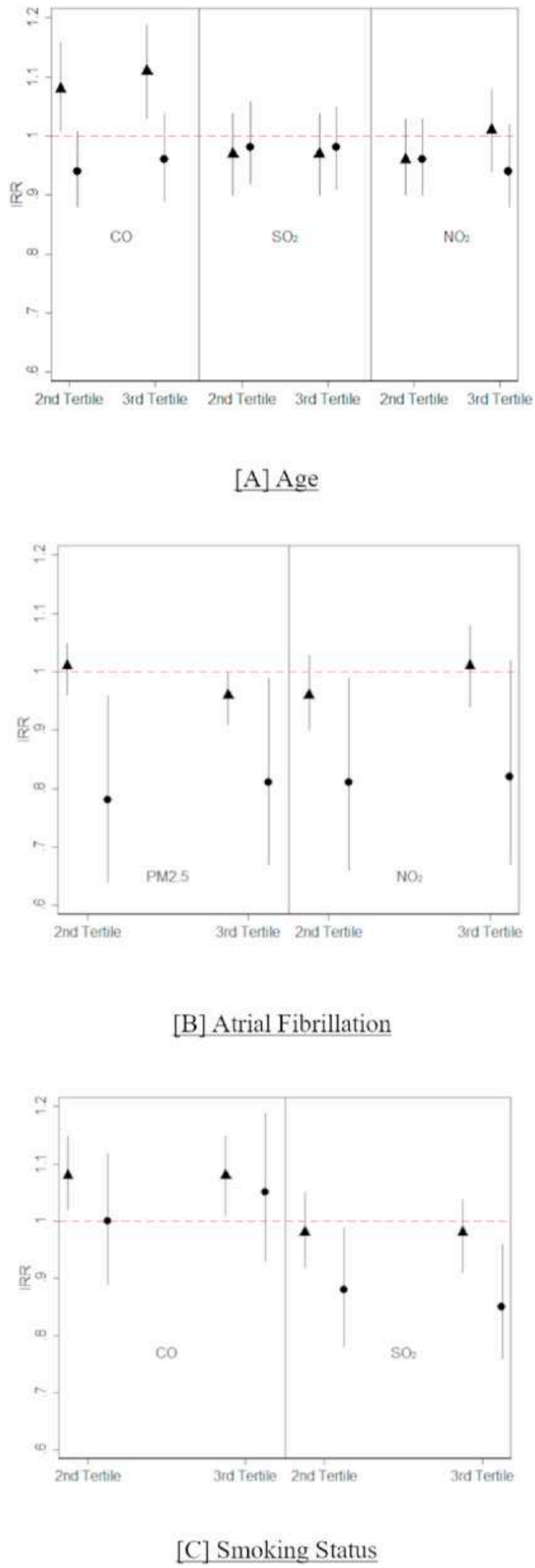

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Fig. 3. Associations between HS risk and air pollutant exposure by subgroups.
[A] Associations between HS risk and air pollutant exposure by age subgroups. The solid triangles refer to those <65 years while the solid circles refer to those ≥65 years of age. [B] Associations between HS risk and air pollutant exposure by atrial fibrillation subgroups. The solid triangles refer to those without atrial fibrillation while the solid circles refer to those with atrial fibrillation. [C] Associations between HS risk and air pollutant exposure by smoking subgroups. The solid triangles refer to those who were non-smokers while the solid circles refer to those who were ex- or present smokers. The vertical lines depict the 95% confidence intervals of the point estimates while the horizontal dashed line depicts the null value.
Abbreviations: carbon monoxide (CO); nitrogen dioxide (NO_2); particulate matter of aerodynamic diameter of ≤ 2.5 μm ($PM_{2.5}$); sulphur dioxide (SO_2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. *Effect modification by age, atrial fibrillation, and smoking status*

Patients who were <65 years of age had a higher risk of HS at both the 2nd and 3rd tertiles of CO exposure compared to those ≥65 years of age (Fig. 3[A]). Individuals with atrial fibrillation had a lower risk of HS at both the 2nd and 3rd tertiles of $PM_{2.5}$ exposure (Fig. 3[B]). compared to those without atrial fibrillation. Those with atrial fibrillation also had a lower risk of HS at the 2nd tertile of NO_2 exposure compared to those without atrial fibrillation. Non-smokers had a higher risk of HS when exposed to CO or SO_2 compared to current or ex-smokers (Fig. 3[C]).

4. Discussion

In this study, we investigated the short-term association between individual ambient air pollutants on the risk of HS in a cosmopolitan city in the tropics. To the best of our knowledge, this was the first study to show both a significant immediate and delayed influence of ambient CO exposure on HS risk, after adjusting for time-varying confounders and meteorological influences. Our results were consistent with the findings of a population-based study in China examining the immediate effects of exhaled CO on risk of stroke (Qiu et al., 2020).

Several animal studies have also suggested that exposure to CO or O_3 may lead to a higher risk of cerebrovascular diseases. Weekly episodic exposure to O_3 was associated with an increase in biomarkers of oxidative stress, microvascular thrombosis, and aortic vasoconstriction, suggesting possible mechanisms through which O_3 may be associated with stroke (Kodavanti et al., 2011). An experiment on 30 adult male rats also showed that exposure to CO has been associated with increased levels of heart-type fatty acid-binding protein (H-FABP), which has also been found to be elevated in patients with HS (Yardan et al., 2011). In addition, a population-based longitudinal study in South Korea found that there was a significant increase in risk of HS in those with CO poisoning compared to those without (Kim et al., 2020). The consistency of the results across these studies increases the plausibility of a true biological effect of CO exposure on HS risk. We hypothesize that exposure to CO may result in CO-induced catecholamine surges and dysregulation of cerebral autoregulation (Cha et al., 2016; Roderique et al., 2015). These effects may lead to changes in cerebral haemodynamics resulting in aneurysm rupture causing HS.

Our study also showed that the association between CO and risk of HS was higher in persons aged <65 compared to those aged 65 or older, and in non-smokers compared to smokers. Other studies have also reported similar observations of effect modification by age and non-smokers on air pollutant-driven stroke risk (Han et al., 2016; Ho et al., 2018; Nzwalo et al., 2019). One possible hypothesis was that individuals aged <65 were more likely to be physically active and thus experienced higher levels of ambient air pollutant exposure due to increased respiration during such activities compared to those aged 65 or older, increasing the risk for HS (Giles and Koehle, 2014). Unexpectedly, we found that non-smokers were at higher risk of CO-driven HS risk. Smokers may have been more likely to experience higher CO exposure than non-smokers because of tobacco use. Additional studies are required to understand if this is a biologically plausible association.

Air pollution is an important public health problem to which billions of people globally are involuntarily exposed to, with increasing evidence of detrimental effects on their cardiovascular health (Ljungman and Mittleman, 2014; Mannucci et al., 2019). Greater consideration for measures to reduce air pollutant emissions may potentially reduce the risk of HS. Increases in air pollutant concentrations should also prompt clinicians and individuals at higher risk to be on the lookout for early signs and symptoms of HS or to engage in preventive strategies. Tertiary healthcare facilities may also consider using anticipated periodic peaks in CO and O_3 levels for resource planning.

Our population-based study used well-defined outcome measures that reduced the potential for HS misclassification and were generalizable to the majority of the population who rely on the public healthcare system. The present study was set in Singapore which has a high population density and was exposed to fluctuations in air quality, allowing for a natural experiment to assess the relationship between pollutants and the risk of HS. However, as population-level exposures were analysed, our results are not generalizable to individuals. An individual's hereditary considerations, lifestyle habits, occupation exposure, and/or other factors that could directly or indirectly contribute to the risk of HS risk were also not accounted for. Our study was limited by unmeasured individual characteristics and potential exposures that may have confounded the HS risk estimates. Lastly, this study was unable to quantify periods of transboundary haze in the region, and further research investigating the effects of transboundary haze on the incidence of HS may be useful.

5. Conclusion

Short-term exposure to ambient CO and O_3 levels was associated with an increased risk of HS. Greater consideration for measures to reduce air pollutant and carbon monoxide emissions may be warranted to reduce the risk of HS, especially in persons at higher risk during periods of elevated pollutant exposure. Future studies to investigate this association using individual air pollutant exposures and characteristics are needed, as well as to investigate the pathophysiological mechanisms of carbon monoxide and ozone on the risk of HS.

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Availability of data and material

The authors do not own the data used in this study. Data requests for weather and air quality should be made to the NEA, Singapore (email: Contact_NEA@nea.gov.sg) and those for HS should be made to National Registry of Diseases Office (NRDO), Singapore (email: www.nrdo.gov.sg/enquiry). Access to the data is subject to approval from the respective agencies.

Declaration of competing interest

The authors declare no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113908.

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Association of short-term exposure to air pollution with recurrent ischemic cerebrovascular events in older adults

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A R T I C L E I N F O

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A B S T R A C T

The acute effects of ambient air pollution on recurrence of ischemic cerebrovascular events (ICEs) remains largely unknown. We therefore conducted a time-stratified case-crossover study of 43,896 patients who were 60 years or older and were admitted to hospital for recurrent ICEs including ischemic stroke and transient ischemic attack in Guangzhou, China during 2016–2019. Based on each patient's home address and pollutant data from its neighboring air quality monitoring stations, we used an inverse distance weighting method to assess exposures to particulate matter with an aerodynamic diameter ≤2.5 μm ($PM_{2.5}$), particulate matter with an aerodynamic diameter ≤10 μm (PM_{10}), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), carbon monoxide (CO) and ozone (O_3). Conditional logistic regression models were used to quantify exposure-response associations. During the study period, there were 43,896 case days and 149,131 control days. In single-pollutant models, each 10 μg/m^3 increase in exposure to PM_{10}, NO_2 and CO (mean exposure on date of admission and 1 day prior) was significantly associated with a 0.74% (95% confidence interval [CI]: 0.13–1.36%), 2.15% (1.38–2.93%) and 0.14% (0.07–0.21%) increase in odds of hospital admissions for recurrent ICEs, respectively, and no significant departures from linearity were detected. The association for NO_2 exposure remained consistent in 2-pollutant models, while the associations for PM_{10} and CO disappeared or changed materially with adjustment for other pollutants. Stronger association for NO_2 exposure was observed in cool season than that in warm season. We found that short-term exposure to ambient air pollutants, especially NO_2, was associated with increased risk of hospital admissions for recurrent ICEs in older adults.

1. Introduction

Air pollution continues to be a major environmental and public health issue globally. In 2016, over 90% of the world's population lived in places where air pollution levels exceeded the World Health Organization (WHO) limits (World Health Organization, 2018). Previous epidemiological studies have linked air pollution exposure to a variety of cardiovascular and cerebrovascular events, especially stroke (Gu et al.,

; CI, confidence interval; CO, carbon monoxide; *df*, degree of freedom; ICD-10, International Statistical Classification of Diseases and Related Health Problems 10th Revision; ICE, ischemic cerebrovascular event; IDW, inverse distance weighting; IQR, interquartile range; IS, ischemic stroke; MAE, mean absolute error; NO_2, nitrogen dioxide; O_3, ozone; $PM_{2.5}$, particulate matter with an aerodynamic diameter ≤2.5 μm; PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; R^2, coefficient of determination; SD, standardized deviation; SE, standard error; SO_2, sulfur dioxide; TIA, transient ischemic attack.

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2020; Nhung et al., 2020; Shah et al., 2015; Tian et al., 2018; Wang et al., 2014). A meta-analysis of 94 studies in 2015 concluded that exposure to particulate matter with an aerodynamic diameter ≤2.5 μm ($PM_{2.5}$), particulate matter with an aerodynamic diameter ≤10 μm (PM_{10}), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), carbon monoxide (CO) and ozone (O_3) had a marked association with hospital admissions for stroke or mortality from stroke (Shah et al., 2015). The acute adverse effects of air pollution exposure on stroke have drawn much concern worldwide.

It was reported that approximately 50% of patients who survived from an ischemic cerebrovascular event (ICE) including ischemic stroke (IS) and transient ischemic attack (TIA) were at underlying increased risk of recurrent stroke within a few days or weeks after first-ever stroke, with a pooled cumulative risk ranging from 3.1% at 30 days up to 39.2% at 10 years (Arsava et al., 2016; Mohan et al., 2011). Although there was extensive evidence on the association between short-term exposure to air pollution and ICEs, only a very limited number of studies have explored how air pollution contributes to the recurrence of ICEs and the findings are limited mainly due to their small sample size (ranging from 280 to 2,839 recurrent ICE cases) (Henrotin et al., 2010; Oudin et al., 2012; Suissa et al., 2013; Wellenius et al., 2012; Wing et al., 2017). Given recurrent ICEs pose a high disease burden globally (e.g., prolonged hospitalization, worsened functional outcome and increased mortality) and exposure to ambient air pollution is continuous and lifelong, it is of great significance to elucidate the acute effect of exposure to ambient air pollution on recurrent ICEs (Arsava et al., 2016).

In this study, we used a case-crossover design to explore the acute adverse effects of exposure to $PM_{2.5}$, PM_{10}, SO_2, NO_2, CO and O_3 on the recurrence of ICEs in Guangdong province, China and hypothesized that short-term exposure to certain criteria air pollutants was associated with increased risk of hospital admissions for recurrent ICEs in older adults.

2. Methods

2.1. Study setting

Guangzhou is the capital of Guangdong province in southern China. In 2019, its area was 7,434.4 km^2 and the resident population was 15.3 million. With the rapid development of society and economic activities, Guangzhou city has experienced a variety of air pollution issues in recent years (Sun and Zhou, 2017).

2.2. Study population

We obtained clinical data on hospital admissions for recurrent ICEs in Guangzhou during 2016–2019 from the Guangzhou Health Technology Identification and Human Resources Assessment Center. The data covered hospital admission information from all medical institutions in Guangzhou that provided inpatient care services. The number of institutions included in the data from 2016 to 2019 was 345, 346, 349 and 372, respectively. During the study period, we identified 54,805 patients who were 60 years or older, lived within Guangdong province and were admitted for recurrent ICEs in Guangzhou, China. By excluding patients without complete home address information or without air quality monitoring stations within a buffer of 25 km around their home address, we finally included 43,896 patients and collected their demographic data on sex, age, race, date of admission and source of hospital admission (including admission from emergency department and admission from outpatient department). This study was approved by the Ethical Committee of School of Public Health, Sun Yat-sen University with a waiver of informed consent.

2.3. Outcome

The study outcome was hospital admissions for recurrent ICEs, including IS (International Statistical Classification of Diseases and Related Health Problems 10th Revision [ICD-10] code: I63) and TIA (ICD-10 code: G45). A recurrent ICE was defined as the first recurrent IS or TIA after their first hospital admission for IS or TIA recorded in the hospital admission data from any medical institution in Guangzhou during 2016–2019.

2.4. Study design

We investigated the association between short-term ambient air pollution exposure and hospital admissions for recurrent ICEs using a time-stratified case-crossover study design, which has been widely used to assess acute human health effects of air pollution (Carracedo-Martínez et al., 2010; Di et al., 2017). In this design, each case serves as its own control by comparing referent exposures on the days before or after the case day. For each case, the case day was defined as the same day at admission, while the control days were defined as days with the same day of week in the same month and year as the chosen case day (Di et al., 2017). According to this approach, each case day was matched with 3 or 4 control days. This design can control for the effects of seasonality, day of week, long-term trends and individual-level time-invariant confounders.

2.5. Exposure assessment

The air pollution data including daily 24-h average concentrations of $PM_{2.5}$, PM_{10}, SO_2, NO_2, CO and daily maximum 8-h moving average concentrations of O_3 in Guangdong province with a 25 km buffer in 2016–2019 were obtained from the National Urban Air Quality Real-Time Publishing Platform in China. Among a total of 114 air quality monitoring stations, 8 were located outside the Guangdong province and were used to provide necessary air pollution exposure data for patients who lived close to the administrative boundary of Guangdong province (Fig. S1). We used an inverse distance weighting (IDW) method, which has been widely used as an effective spatial interpolation tool to predict the distribution of air pollutants using data from fixed monitoring stations (de Mesnard, 2013), to assess daily individual-level exposure to $PM_{2.5}$, PM_{10}, SO_2, NO_2, CO and O_3. For each case, we calculated the inverse distance weighted ($1/d^2$) average of concentrations at all monitoring stations within a buffer of 25 km around the patient's home address as the exposure on each of the case day and control days, where the d represents distance between the home address and each monitoring station (Hwang et al., 2011). To examine the acute effects of air pollution on hospital admissions for recurrent ICEs, we used single-day lag exposures (from lag 0 to lag 3 day) and moving average lag exposures (from lag 01 to lag 03 day). Consistent with most previous air pollution studies, we used lag 01 day exposure as the exposure metric in the main analysis (Di et al., 2017; Liu et al., 2019).

To validate the predictive accuracy of IDW method in air pollution exposure assessment, we performed a 10-fold cross-validation for each air pollutant. After randomly splitting data from the 114 monitoring stations into 10 subsets, we used the IDW method to predict exposures for each subset with data from the other 9 subsets. By repeating this process 10 times, all subsets were predicted. Finally, we used all predicted and measured air pollutant concentrations to calculate the coefficient of determination (R^2), mean absolute error (MAE) and bias between predicted and measured concentrations (Supplemental Methods).

2.6. Covariates

Using the gridded meteorological data (resolution: spatial, 0.0625° × 0.0625°; temporal, 1 day) from the National Meteorological Information Center in China, we retrieved daily 24-h average temperature (°C) and calculated relative humidity (%) at each patient's home address on each of the case and control days to control for potential confounding effects by meteorological conditions in Guangdong province during

2016–2019 (Supplemental Methods) (Bolton, 1980). Individual-level time-invariant covariates including sex, age, race and source of hospital admission were not considered as confounders because they remained constant in comparing case days and control days (Janes et al., 2005).

2.7. Statistical analysis

Spearman's correlation coefficients (*r*) were calculated to evaluate the correlation between exposure to air pollutants and meteorological conditions. We used conditional logistic regression models to estimate the association between exposure to air pollution and hospital admissions for recurrent ICEs, which included each air pollutant as a continuous variable in a separate model. Percent change in odds ([odds ratio − 1] × 100%) of hospital admissions for recurrent ICEs and its 95% confidence interval (CI) were estimated for each 10 μg/m^3 increase in exposure to each air pollutant. In all models, we adjusted for both daily 24-h average temperature and relative humidity using a natural cubic spline with 3 degrees of freedom (*df*). To explore potential nonlinear associations, we further included the exposure as a natural cubic spline ($df = 3$) in the model and plotted exposure-response curves. Likelihood ratio tests were used to test nonlinearity of the associations.

We conducted stratified analyses to examine if the association differed by sex (male, female), age (<75, ≥75 years), season (cold, warm), and source of hospital admission (emergency department, outpatient department). Warm season was defined as May to October (Wang et al., 2020), which was reported as the specific time window to examine the health effect of O_3 exposure, while the cool season was defined as November to December and January to April. We performed a 2-sample z-test using the stratification-specific point estimates (β = ln odds ratio) and their standard errors (SEs) to investigate possible effect modification by the stratification variable (Altman and Bland, 2003).

To evaluate the robustness of our results, we conducted several sensitivity analyses. First, we performed 2-pollutant models by further including each of the other pollutants in the same model, and compared the nested single- and 2-pollutant models by likelihood ratio tests. To avoid the influence of serious collinearity between air pollutants, we did not include exposure to $PM_{2.5}$ and PM_{10} in the same model. Second, we restricted the analyses to the patients living in Guangzhou only. Third, we adjusted for daily 24-h average temperature using a natural cubic spline with 6 *df* and relative humidity with 3 *df*. Finally, we restricted the analyses to recurrent IS. All data analyses were performed with R version 3.6.1. All statistical tests were 2-sided and a p value <0.05 was considered statistically significant.

3. Results

A total of 43,896 patients with 149,131 control days in 2016–2019 were included in the main analysis, with 96.5% of the patients (42,343 patients with 143,833 control days) living in Guangzhou (Fig. S1). Among the identified patients, 55.3% were aged ≥75 years, 51.4% were male, 99.7% were Han race, 50.4% were admitted in warm season and 74.7% were admitted from outpatient departments (Table 1).

The R^2 of IDW method performance for $PM_{2.5}$, PM_{10}, SO_2, NO_2, CO and O_3 was 0.87, 0.86, 0.45, 0.78, 0.42 and 0.86, respectively, indicating a high performance for exposure to $PM_{2.5}$, PM_{10}, NO_2 and O_3 but relatively low performance for exposure to SO_2 and CO. The MAE for $PM_{2.5}$, PM_{10}, SO_2, NO_2, CO and O_3 was 4.2 μg/m^3, 6.4 μg/m^3, 2.7 μg/m^3, 6.0 μg/m^3, 0.14 mg/m^3 and 11.4 μg/m^3, respectively (Table S1). Table 2 gives the distribution of exposure to air pollutants and meteorological conditions on the day of hospital admission. The mean exposure to $PM_{2.5}$, PM_{10}, SO_2, NO_2, CO and O_3 was 33.1 μg/m^3, 54.8 μg/m^3, 9.6 μg/m^3, 44.6 μg/m^3, 0.86 mg/m^3 and 110.3 μg/m^3, respectively. Strong correlation was observed between $PM_{2.5}$ and PM_{10} ($r = 0.95$). In addition, $PM_{2.5}$ and PM_{10} were both positively and moderately correlated with other gaseous pollutants (all $r \geq 0.50$). Similarly, a positive and moderate correlation between NO_2 and CO ($r = 0.60$) was observed. All pairwise correlation coefficients were statistically significant (all p <0.05; Table 3).

In single-pollutant models, short-term exposure to PM_{10} (lag 0, lag 01-lag 03 day), SO_2 (lag 0 day), NO_2 (lag 0-lag 3, lag 01–03 day) and CO (lag 0-lag 2, lag 01–03 day) were positively associated with odds of hospital admissions for recurrent ICEs (all p <0.05). Each 10 μg/m^3 increase in exposure (lag 01 day) to PM_{10}, NO_2 and CO was significantly associated with a 0.74% (95% CI: 0.13–1.36%), 2.15% (95% CI: 1.38–2.93%) and 0.14% (95% CI: 0.07–0.21%) increased odds of recurrent ICEs, respectively (all p <0.05; Fig. 1). The models including each air pollutant exposure as a natural cubic spline function did not show any departure from linearity for all these associations (all p for nonlinear trend >0.05; Fig. 2). In the stratified analysis, we observed significantly higher odds of hospital admissions for recurrent ICEs associated with NO_2 in cool season compared with that in warm season (p for effect modification <0.05; Table S2).

Table 1
Characteristics of the study population, 2016–2019.

Characteristic	Value
No. of recurrent ICE patients (case days)	43,896
No. of control days	149,131
Age (y), n (%)	
Mean (SD)	76.2 (8.7)
Median (IQR)	76.5 (14.0)
<75	19,639 (44.7)
≥75	24,257 (55.3)
Sex, n (%)	
Male	22,565 (51.4)
Female	21,331 (48.6)
Race, n (%)	
Han	43,757 (99.7)
Other	88 (0.2)
Unknown	51 (0.1)
Season at hospital admissions[a], n (%)	
Warm	22,126 (50.4)
Cool	21,770 (49.6)
Source of hospital admission, n (%)	
Emergency department	11,102 (25.3)
Outpatient department	32,794 (74.7)

Abbreviations: ICD-10, International Statistical Classification of Diseases and Related Health Problems 10th Revision; ICE, ischemic cerebrovascular event; IQR, interquartile range; and SD, standardized deviation.
[a] Warm season: from May to October; Cool season: from November to December and January to April.

Table 2
Distribution of air pollutants and meteorological conditions on the day of hospital admissions for ICEs.

	Mean (SD)	Percentiles				
		5th	25th	50th	75th	95th
Air pollutant						
$PM_{2.5}$, μg/m^3	33.1 (19.1)	11.8	19.6	28.7	42.3	67.8
PM_{10}, μg/m^3	54.8 (27.6)	22.4	35.1	48.0	68.7	108.8
SO_2, μg/m^3	9.6 (4.4)	4.4	6.4	8.8	11.7	17.8
NO_2, μg/m^3	44.6 (22.6)	17.4	31.6	42.4	57.0	88.8
CO, mg/m^3	0.86 (0.24)	0.54	0.70	0.82	0.97	1.29
O_3[a], μg/m^3	110.3 (50.9)	39.1	72.9	101.8	142.4	206.3
Meteorological condition						
Temperature, °C	23.8 (5.9)	12.9	19.7	24.7	28.6	31.3
Relative humidity, %	75.8 (13.6)	48.9	68.4	78.8	86.2	92.3

Abbreviations: CO, carbon monoxide; ICE, ischemic cerebrovascular event; NO_2, nitrogen dioxide; O_3, ozone; $PM_{2.5}$, particulate matter with an aerodynamic diameter ≤2.5 μm; PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; SO_2, sulfur dioxide; and SD, standardized deviation.
[a] Restricted to days in warm season (May to October) only.

Table 3
Spearman's correlation coefficients for air pollutants and meteorological conditions on the day of hospital admissions for ICEs.

Variable	PM_{10}	SO_2	NO_2	CO	O_3[a]	Temperature	Relative humidity
$PM_{2.5}$	0.95	0.58	0.68	0.56	0.60	−0.29	−0.43
PM_{10}	–	0.61	0.71	0.52	0.63	−0.22	−0.44
SO_2	–	–	0.46	0.32	0.33	−0.04	−0.47
NO_2	–	–	–	0.60	0.18	−0.27	−0.15
CO	–	–	–	–	0.14	−0.37	−0.08
O_3[a]	–	–	–	–	–	0.37	−0.54
Temperature	–	–	–	–	–	–	0.20

Abbreviations: CO, carbon monoxide; ICE, ischemic cerebrovascular event; NO_2, nitrogen dioxide; O_3, ozone; $PM_{2.5}$, particulate matter with an aerodynamic diameter ≤2.5 μm; PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; and SO_2, sulfur dioxide.
[a] Restricted to days in warm season (May to October) only.

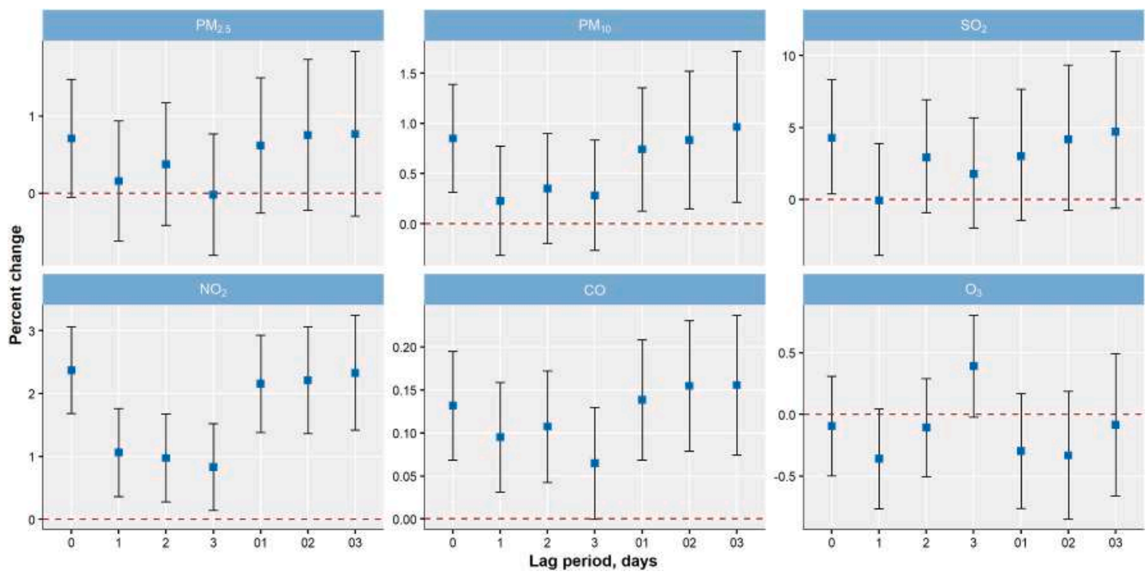

Fig. 1. Percent changes (95% CIs) in odds of hospital admissions for recurrent ICEs associated with each 10 μg/m^3 increase in exposure to ambient air pollutants *Abbreviations:* CI, confidence interval; CO, carbon monoxide; ICE, ischemic cerebrovascular event; NO_2, nitrogen dioxide; O_3, ozone; $PM_{2.5}$, particulate matter with an aerodynamic diameter ≤2.5 μm; PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; and SO_2, sulfur dioxide.

The results of 2-pollutant models showed that the association between NO_2 exposure and hospital admissions for recurrent ICEs remained significant and stable, while the associations for PM_{10} and CO became insignificant or turned negative when certain other pollutant (especially NO_2) was included in the same model (Table 4). In addition, the association between NO_2 exposure and hospital admissions for recurrent ICEs did not change materially (percent change: 3.67%, 95% CI: 2.44–4.91%; p for heterogeneity <0.001) with adjustment for $PM_{2.5}$, SO_2, CO and O_3 simultaneously in the same model. Restricting our analyses to patients who lived in Guangzhou yielded very similar results (Fig. S2). The results remained stable when we adjusted for temperature using a natural spline with 6 *df* (Fig. S3). Fig. S4 shows similar associations when we restricted the analyses to patients with recurrent IS.

4. Discussion

To date, this is the largest study exploring the acute effects of air pollution on recurrent ICEs. Using a case-crossover design, we quantitatively investigated the association of exposure to multiple criteria air pollutants with hospital admissions for recurrent ICEs in older adults in Guangzhou, China. We found consistent exposure-response association between NO_2 exposure and hospital admissions for recurrent ICEs, and the association was linear. Each 10 μg/m^3 increase in NO_2 exposure (lag 01 day) was significantly associated with a 2.15% increase in odds of recurrent ICEs, which was significantly stronger in cool season (3.16%) than that in warm season (0.98%).

The association between short-term ambient air pollution and hospital admissions for IS has been extensively studied (Chen et al., 2020; Liu et al., 2017a, 2017b; Tian et al., 2018), but only a limited number of studies specifically investigated recurrent ICEs or IS and the results remain inconsistent (Henrotin et al., 2010; Oudin et al., 2012; Suissa et al., 2013; Wellenius et al., 2012; Wing et al., 2017). In this study, we found significant and robust association of NO_2 exposure with both recurrent ICEs and recurrent IS; in contrast, the only study investigating NO_2 exposure and recurrent IS (n = 280) in Nice, France did not find any significant association for NO_2 (Suissa et al., 2013). Note that the sample size in the French study was much smaller (280 vs 43,896) and the mean NO_2 concentrations was lower (26.2 vs 44.6 μg/m^3) compared with that in our study. We did not observe any significant association for both $PM_{2.5}$ and O_3; however, significant associations were reported in two studies in France for O_3 exposure and one study in the US for $PM_{2.5}$ exposure (Henrotin et al., 2010; Suissa et al., 2013; Wellenius et al., 2012). No significant association was observed for PM_{10} in the Sweden study (Oudin et al., 2012), PM_{10} and SO_2 in the French study (Suissa et al., 2013) as well as $PM_{2.5}$ and O_3 in the Mexican study (Wing et al., 2017), which were consistent with our results. The inconsistence of results in studies on air pollution and recurrent ICEs might result from several reasons, including: variations of the level and sources of air pollution across countries and regions within a single country; small sample size in previous studies; strategies for exposure assessment; and different definition of the study outcome. Moreover, the complex collinearity between air pollutants with different concentration ranges

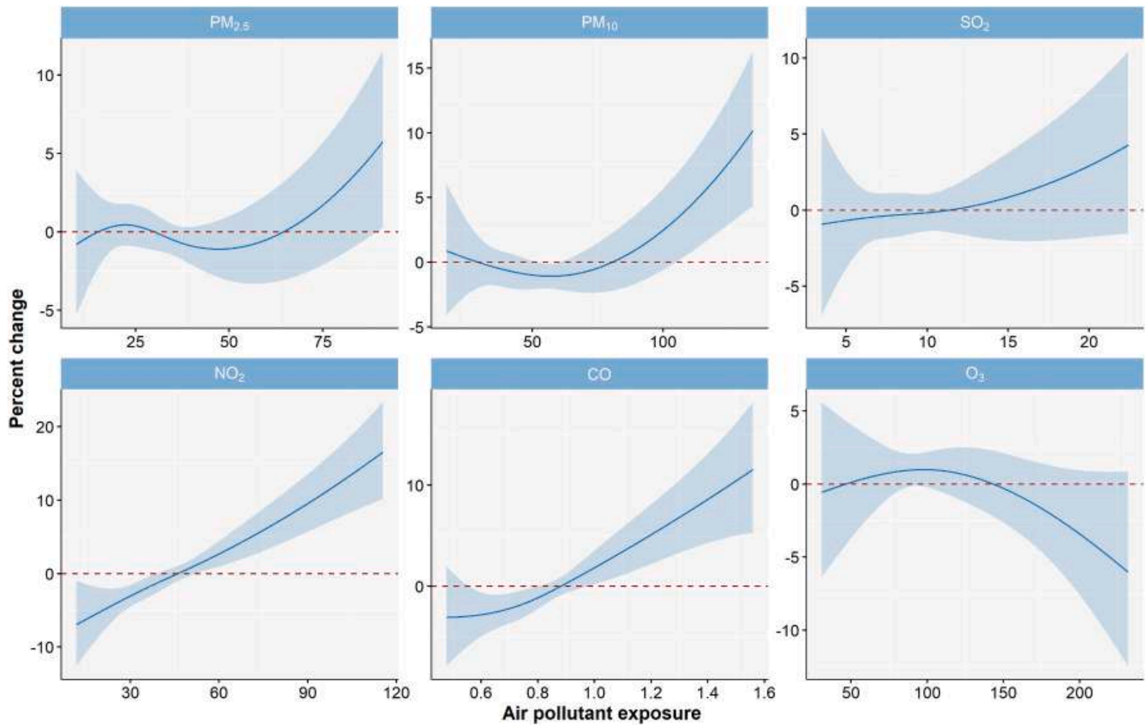

Fig. 2. Exposure-response curves between exposure to air pollutants (lag 01 day) and hospital admissions for recurrent ICEs. The blue solid lines and shaded regions represent percent changes in odds of hospital admissions for recurrent ICEs and their corresponding 95% CIs. Lag 01 day exposure refers to the mean of daily exposure on the day of admission and 1 day prior.

Abbreviations: CI, confidence interval; CO, carbon monoxide; ICE, ischemic cerebrovascular event; NO_2, nitrogen dioxide; O_3, ozone; $PM_{2.5}$, particulate matter with an aerodynamic diameter ≤2.5 μm; PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; and SO_2, sulfur dioxide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

might add uncertainty to the interpretation of the results and make it difficult to separate the effect of pollutants on recurrent ICEs in 2-pollutant models (Villeneuve et al., 2006).

In the past few decades, ambient NO_2 levels remained stable even slightly increased worldwide due to continuous increase of motorized vehicles (Chen et al., 2018). As one of the typical traffic-related air pollutants, NO_2 substantially contributed to ambient air pollution in most developed cities. In our study, we found a consistent association between NO_2 exposure and increased risk of hospital admissions for recurrent ICEs, indicating that exposure to traffic-related air pollution including NO_2 might trigger recurrent ICEs. Given that recurrent ICEs resulted in a significant disease burden for both individuals and the society, it is of great public health implications to take effective measures to control NO_2 emissions and reduce exposure to ambient NO_2 in areas experiencing significant NO_2 pollution, which may serve as an important initiative to reduce the recurrence of ICEs.

Although previous studies have linked exposure to air pollutants with ICE occurrence, the underlying pathophysiological mechanisms are still uncertain. Some studies have observed that inhaling air pollution including NO_2 may cause inflammatory response, increase plasma viscosity and induce vasoconstriction which may trigger atherosclerosis and thrombus formation (Brook et al., 2002; Peters et al., 1997; Schwartz, 2001; Villeneuve et al., 2006). Altered blood rheology, atherosclerosis and thrombus formation might induce obstruction in a stenotic artery, result in ischemia or impair the blood supply of brain tissue and finally increase the risk of cardiovascular and cerebrovascular events, which is generally supported by findings in animal studies (Sun et al., 2005; Suwa et al., 2002; Villeneuve et al., 2006; Zhu et al., 2012).

One unique strength of our study is that the sample size is the largest to date, which provided sufficient statistical power to identify potential association between short-term exposure to air pollution and recurrent ICEs. The hospital admission data was collected from all medical institutions with the capability to diagnose and treat stroke in Guangzhou, which covered a wide range of hospital admissions in Guangdong province during 2016–2019. Second, we used a time-stratified case-crossover design to estimate the associations, which allowed us to control the influence of seasonality, day of week, time trends and slowly varying individual-level confounding factors (e.g., age, sex and race). Besides, the case-crossover study design allowed us for the first time to use the IDW method to estimate more accurate individual-level exposure to ambient air pollutants compared with previous studies in which city- or regional-level exposure was used (Henrotin et al., 2010; Oudin et al., 2012; Suissa et al., 2013; Wellenius et al., 2012; Wing et al., 2017). Third, we investigated 6 major concerned air pollutants globally, and performed various sensitivity analyses to confirm the robustness of our results.

Our study also has some limitations. First, because hospital admission typically occurred after the onset of stroke symptoms, the date of stroke onset might be equal to or earlier than the date of admission, which might induce exposure misclassification. However, the exposure misclassification tended to be nondifferential. In addition, it was reported that 51.9% of patients were hospitalized within 6 h after the onset of stroke symptoms and those with a history of stroke had significantly less time-to-hospital than patients without stroke in China (Fang et al., 2011). Second, we calculated individual-level air pollutant exposure using the IDW method, but we were still unable to avoid the influence of individual variability in exposure due to personal activity and time spent indoors on personal air pollution exposure assessment in certain way. In addition, it should also be noted that the performance of the IDW method for both CO and SO_2 was relatively low, which might have contributed to their null or unstable associations with recurrent ICEs. The relatively lower R^2 of IDW method for SO_2 and CO may be attributable to their relatively greater spatial distribution variability. Previous studies have reported that more neighboring monitoring sites

Table 4
Percent changes (95% CIs) in odds of hospital admissions for recurrent ICEs associated with each 10 μg/m^3 increase in exposure (lag 01 day) to ambient air pollutants by single- and 2-pollutant models.

Air pollutant	Percent change (95% CI)	p for heterogeneity[a]
$PM_{2.5}$	0.62 (−0.25, 1.50)	
+ SO_2	0.51 (−0.58, 1.61)	0.67
+ NO_2	−2.39 (−3.61, −1.15)	<0.001
+ CO	−0.65 (−1.73, 0.43)	0.0015
+ O_3	1.39 (0.42, 2.37)	<0.001
PM_{10}	0.74 (0.13, 1.36)	
+ SO_2	0.88 (0.09, 1.68)	0.62
+ NO_2	−1.55 (−2.52, −0.58)	<0.001
+ CO	0.01 (−0.75, 0.78)	0.016
+ O_3	1.35 (0.67, 2.04)	<0.001
SO_2	3.00 (−1.46, 7.66)	
+ $PM_{2.5}$	2.60 (−2.91, 8.43)	0.024
+ PM_{10}	−0.43 (−5.91, 5.37)	0.0045
+ NO_2	−5.71 (−10.67, −0.47)	<0.001
+ CO	−0.34 (−4.98, 4.54)	0.0026
+ O_3	5.24 (0.48, 10.23)	0.0040
NO_2	2.15 (1.38, 2.93)	
+ $PM_{2.5}$	3.67 (2.54, 4.82)	<0.001
+ PM_{10}	3.50 (2.23, 4.79)	0.0028
+ SO_2	2.88 (1.93, 3.84)	0.042
+ CO	1.90 (0.96, 2.85)	0.71
+ O_3	2.74 (1.93, 3.56)	<0.001
CO	0.14 (0.07, 0.21)	
+ $PM_{2.5}$	0.17 (0.08, 0.25)	0.024
+ PM_{10}	0.13 (0.04, 0.22)	0.040
+ SO_2	0.14 (0.07, 0.22)	0.65
+ NO_2	0.04 (−0.05, 0.12)	<0.001
+ O_3	0.16 (0.08, 0.23)	0.0037
O_3[b]	−0.30 (−0.76, 0.17)	
+ $PM_{2.5}$	−0.89 (−1.50, −0.27)	0.0096
+ PM_{10}	−0.83 (−1.44, −0.22)	0.040
+ SO_2	−0.26 (−0.78, 0.26)	0.97
+ NO_2	−0.50 (−1.03, 0.03)	0.08
+ CO	−0.59 (−1.10, −0.07)	0.027

Abbreviations: CI, confidence interval; CO, carbon monoxide; ICE, ischemic cerebrovascular event; NO_2, nitrogen dioxide; O_3, ozone; $PM_{2.5}$, particulate matter with an aerodynamic diameter ≤2.5 μm; PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; and SO_2, sulfur dioxide.
[a] p values for heterogeneity were tested using likelihood ratio tests.
[b] Restricted to days in warm season (May to October) only.

(Wang et al., 2019) and more uniform spatial distribution of pollution (Qiao et al., 2018) may help improve the interpolation accuracy of IDW. Further studies with more accurate exposure assessment for these pollutants are warranted to confirm our findings. Third, the collinearity between the pollutants hindered us from distinguishing their respective effects on recurrent ICEs, and further studies are urged to identify the specific air pollutant which is directly responsible for increased risk of recurrent ICEs. Last, we only investigated adults 60 years or older in Guangzhou, China, though it was estimated that more than 60% strokes occurred among adults over 65 years (Feigin et al., 2014). Nonetheless, cautions should be made to generalize our results to other populations or age groups.

5. Conclusions

We found that short-term exposure to air pollutants, especially NO_2, was significantly associated with increased risk of hospital admissions for recurrent ICEs. Our findings provide further evidence on the adverse effects of air pollution (especially traffic-related air pollution) on cerebrovascular health. For ICE patients, it may be helpful to reduce exposure to ambient air pollution in preventing the recurrence of ICEs. The high disease burden of ICEs can be alleviated by applying effective measures to control air pollutant emissions and exposures. Further studies are warranted to confirm our findings in other regions or populations and to explore potential biological mechanisms.

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Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113925.

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Biomonitoring of exposure to Great Lakes contaminants among licensed anglers and Burmese refugees in Western New York: Toxic metals and persistent organic pollutants, 2010–2015

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ABSTRACT

Between 2010 and 2015, the New York State Department of Health (NYSDOH) conducted a biomonitoring program to gather exposure data on Great Lakes contaminants among licensed anglers and Burmese refugees living in western New York who ate locally caught fish. Four hundred and nine adult licensed anglers and 206 adult Burmese refugees participated in this program. Participants provided blood and urine samples and completed a detailed questionnaire. Herein, we present blood metal levels (cadmium, lead, and total mercury) and serum persistent organic pollutant concentrations [polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), dichlorodiphenyldichloroethylene (DDE), and *trans*-nonachlor]. Multiple linear regression was applied to investigate the associations between analyte concentrations and indicators of fish consumption (locally caught fish meals, store-bought fish meals, and consuming fish/shellfish in the past week). Licensed anglers consumed a median of 16 locally caught fish meals and 22 store-bought fish meals while Burmese refugees consumed a median of 106 locally caught fish meals and 104 store-bought fish/shellfish meals in the past year. Compared to the general U.S. adult population, licensed anglers had higher blood lead and mercury levels; and Burmese refuges had higher blood cadmium, lead, and mercury, and higher serum DDE levels. Eating more locally caught fish was associated with higher blood lead, blood mercury, and serum $\sum$PCBs concentrations among licensed anglers. Licensed anglers and Burmese refugees who reported fish/shellfish consumption in the past week had elevated blood mercury levels compared with those who reported no consumption. Among licensed anglers, eating more store-bought fish meals was also associated with higher blood mercury levels. As part of the program, NYSDOH staff provided fish advisory outreach and education to all participants on ways to reduce their exposures, make healthier choices of fish to eat, and waters to fish from. Overall, our findings on exposure levels and fish consumption provide information to support the development and implementation of exposure reduction public health actions.

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1. Introduction

The Great Lakes waterbodies contain 20 percent of the world's surface freshwater. They have supported one of the world's largest recreational and commercial freshwater resources for over a century. Historically, the Great Lakes Basin has been exposed to persistent contaminants as a result of heavy industry, manufacturing, and agriculture (Adriaens et al., 2002; NYSDEC, 2014; Wattigney et al., 2019). Many legacy contaminants including toxic metals such as cadmium, lead, mercury, and persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCs), continue to be found in and around the Great Lakes (Mitchell et al., 2019; Savadatti et al., 2019; Sherman et al., 2015).

The presence and persistence of legacy pollutants, including mercury, PCBs, and dichlorodiphenyltrichloroethane (DDT), have led to the routine surveillance of local fish and wildlife in the Great Lakes region (Bohr, 2014; Gewurtz et al., 2011; NOAA, 2017; US EPA, 2017). Multiple studies have reported contamination of Great Lakes Basin fish from PCBs, DDT, dichlorodiphenyldichloroethylene (DDE), toxaphene, chlordane, polybrominated diphenyl ethers (PBDEs), and per- and polyfluoroalkyl substances (PFAS) (Gandhi et al., 2017; McGoldrick and Murphy, 2016; Preddice et al., 2011). POPs and methylmercury bioaccumulate in greater concentrations in the fat and muscle tissue, respectively, in larger, older predatory fish (Turyk et al., 2012). Most importantly, Great Lakes Basin fish consumption is a concentrated dietary exposure to these pollutants in local populations.

Over four million adults in the Great Lakes region reported consuming locally caught fish in the previous year (Turyk et al., 2012). Native communities living around the Great Lakes Basin depend on locally caught fish for their diet (Turyk et al., 2012). Numerous studies have associated elevated body burdens of mercury and some POPs with Great Lakes Basin fish consumption (Cole et al., 2004; Fitzgerald et al., 1999). Lead, mercury, PCBs, and PBDEs have been associated with neurological deficits in cognitive function, such as IQ reduction and memory loss (Axelrad et al., 2007; Bellinger et al., 1991; Herbstman et al., 2010; Schantz et al., 2001; Stewart et al., 2008). Lead and mercury have also been associated with high blood pressure, heart disease, kidney disease, and reduced fertility (ATSDR, 1999, 2020). Most chlorinated organics are classified as probable human carcinogens (Steenland et al., 2004) and have been associated with various adverse health effects, such as reduced immune function, adverse reproductive outcomes, and altered thyroid and hormone regulation (Goncharov et al., 2008; Schell and Gallo, 2010). Conversely, fish provide an important source of protein with higher levels of nutrients such as omega-3 fatty acids, vitamins (D and B2), and minerals and lower levels of saturated fats compared to meats and poultry (Turyk et al., 2012). The American Heart Association (AHA) suggests eating fish rich in Omega-3 fatty acids at least twice a week, which can help reduce the risks of heart failure, coronary heart disease, cardiac arrest, and stroke (Rimm et al., 2018).

Between 2010 and 2015, the New York State Department of Health (NYSDOH) conducted an assessment of human exposure to Great Lakes contaminants by measuring body burdens among two populations in western New York (NY) at high-exposure risk, licensed anglers and Burmese refugees. The *Healthy Fishing Communities Program* was conducted in collaboration with the Agency for Toxic Substances and Disease Registry (Wattigney et al., 2019). In this paper we present whole blood concentrations of metals (cadmium, lead, and total mercury), and serum levels of summed PCBs ($\sum$PCBs), summed PBDEs ($\sum$PBDEs), DDE, and *trans*-nonachlor in the two populations. We also characterize Great Lakes Basin fish consumption by target groups and present associations between contaminant exposure (based on internal dose) and local fish consumption patterns.

2. Methods

2.1. Study populations and data collection

The Great Lakes Areas of Concern (AOCs) are defined as geographic areas "where significant impairment of beneficial uses has occurred as a result of human activities at the local level" (Environment Canada and United States Environmental Protection Agency [US EPA], 2012). The *Healthy Fishing Communities Program* focused on four AOCs in western NY: Buffalo River, Niagara River, Eighteenmile Creek, and the Rochester Embayment (Fig. 1). We targeted two populations who ate locally caught fish and had the potential for increased risk of exposure to persistent contaminants common to these four AOCs: (1) licensed anglers who lived in proximity to the focus AOCs, and (2) Burmese refugees, immigrants, and their descendants who lived in the City of Buffalo. Details on sample design, survey and biosample data collection, laboratory analysis, and other programmatic procedures have been described previously (Liu et al., 2018; Savadatti et al., 2019; Wattigney et al., 2019). Between February and October 2013, licensed anglers and Burmese refugees were recruited and enrolled in the program using random sampling and respondent-driven sampling (RDS), respectively. Briefly, a sampling frame of 94,077 licensed anglers aged 18–69 years living within a 10-mile buffer of four AOCs was extracted from the 2010–2011 NYS Department of Environmental Conservation fishing license database. Recruitment packages were mailed to 13,369 randomly selected licensed anglers. Of the 2126 (16%) respondents from whom surveys were received, 883 met the eligibility criteria for the biomonitoring study. In terms of RDS, the initial recruits were done by selecting five of participants from the Burmese community to serve as seeds. Each seed is given three referral coupons to recruit peers from their social network to participate in the project. A total of 311 coupons were distributed in 11 RDS events. 226 (73%) coupons were redeemed, of which 26 were ineligible. This resulted in 205 Burmese refugees (including 5 seeds) enrolled in the project.

Data collection procedures for all participants included obtaining informed consent, height and weight measurements, blood and urine sample collection, and interviews. Trained interviewers administered informed consent and a detailed questionnaire to eligible participants with the use of visual aids. For Burmese refugees, all study documents were presented in the participant's native language with the help of a trained interpreter. Questionnaire domains focused on demographics, residential history, lifestyle characteristics, and fish consumption history.

2.2. Fish consumption

Our primary exposure of interest was fish consumption, specifically the consumption of fish caught from the Great Lakes AOCs and surrounding waters. Questionnaire data were used to characterize fish consumption patterns among participants. Fish consumption variables examined were 1) the number of locally caught fish meals and store-bought fish meals in the past year, and 2) the number of years that participants had consumed locally caught fish and store-bought fish. Notice that years of locally caught fish consumption were not collected and both fish and shellfish were considered as a store-bought fish meal for Burmese refugees while only fish was considered as a store-bought fish meal for licensed anglers. We also considered if participants consumed fish/shellfish in the past week prior to being interviewed (Yes/No) for blood metals because of the shorter half-lives compared to serum POPs in human blood. Additionally, the annual number of fish paste meals consumed was collected for Burmese refugees. Data on locally caught fish meals in the past 12 months were collected by season for the Burmese refugees to account for their engagement in year-round fishing, while licensed anglers were asked about their number of fish meals in the past year, by species. However, the number of locally caught fish meals in the past year for Burmese refugees was estimated by

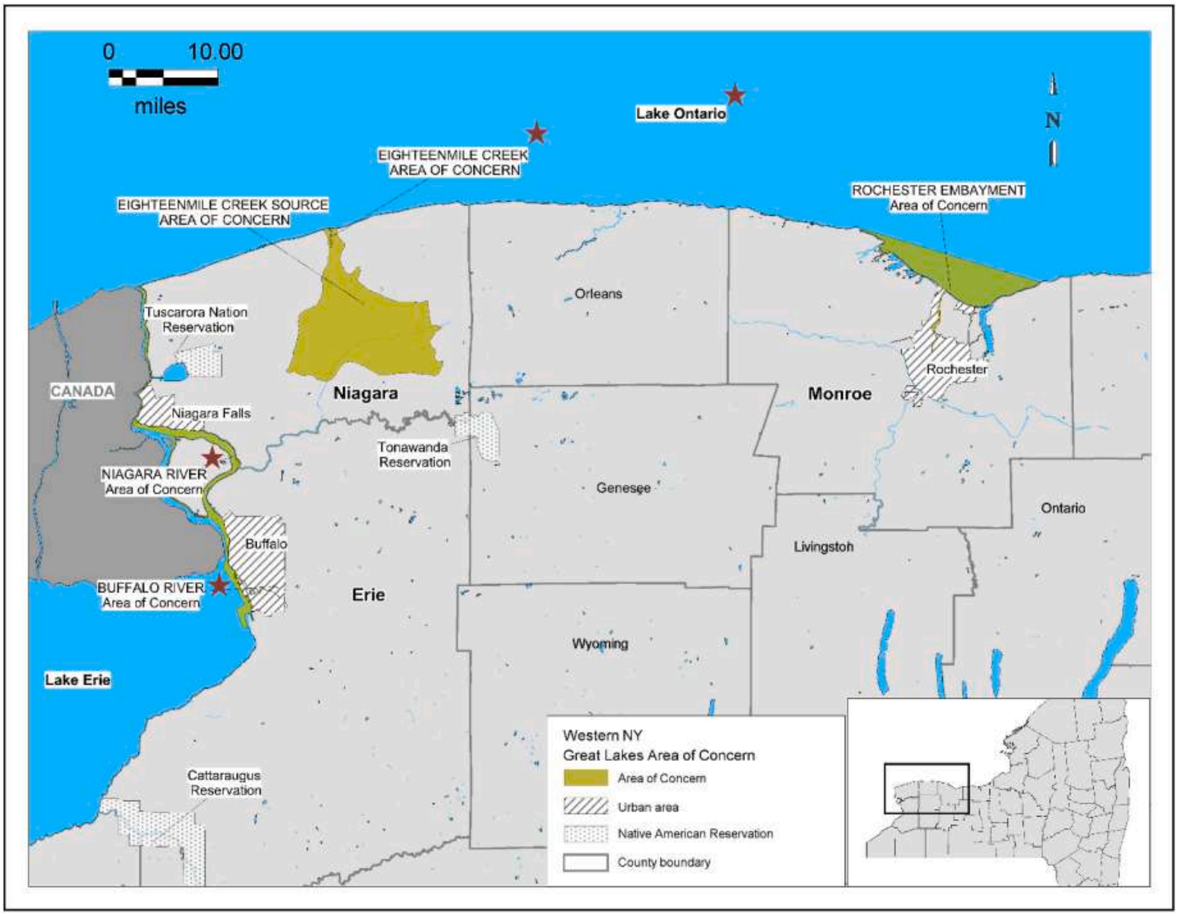

Fig. 1. Map of the Great Lakes and Areas of Concern (AOCs) under focus in the NYS Healthy Fishing Communities Program. Waterbodies included in the program are labeled with a star: four AOCs (Buffalo River, Niagara River, Eighteenmile Creek, the Rochester Embayment), plus Lake Erie and Lake Ontario.

three seasons (summer, fall, and winter) due to the presence of 40.3% of missing spring season data for this variable caused by a questionnaire error and was rescaled to an annual value. For licensed anglers, species-specific data were summed into a single variable to represent the number of locally caught fish meals in the past year.

2.3. Analytes

In this paper, we present blood metal concentrations (cadmium, lead, and total mercury) and serum concentrations of POPs, namely PCBs, PBDEs, and organochlorine pesticides (DDE and *trans*-nonachlor). Individual biosamples collected from participants were analyzed at the NYSDOH's Wadsworth Center, an approved clinical laboratory facility holding a permit under the Clinical Laboratory Improvement Amendments of 1988 (CLIA ′88). Metals were measured in whole blood samples using inductively coupled plasma mass spectrometry. PCBs, PBDEs, and organochlorine pesticides were analyzed in serum samples using gas chromatography coupled with high resolution mass spectrometry. Limits of detection (LOD) for each analyte, detection rates, and median measurements with interquartile ranges are presented in Supplementary Table S1. Quality assurance (QA) for the biomonitoring assays conducted at Wadsworth goes far beyond what is typically required under CLIA '88. For toxic metals in whole blood, the laboratory participates in six external QA schemes, and randomly repeats 2% of all samples. For serum POPs, the laboratory participates in three external QA schemes, and analyzes procedural blanks, matrix spikes, and standard reference materials with every batch of samples. Additional details on laboratory methods, including performance in the specific external QA schemes have been described elsewhere (Ma et al., 2013; Palmer et al., 2006; Savadatti et al., 2019; Shaw et al., 2013). POPs were adjusted for total serum lipids, unless specified (Phillips et al., 1989). $\sum$PCBs and $\sum$PBDEs were computed as a sum of 35 PCB congeners and a sum of five PBDE congeners (Fig. 2 footnote c and d). The proportions of measurements below the LOD were less than 40% for all analytes, except for some PCB congeners (PCB 52/73, 90/101/89, 105/127). Measurements below the LOD were imputed using LOD/ $\sqrt{2}$ per guidelines used for the National Health and Nutrition Examination Survey (NHANES) data (NCHS, 2010).

2.4. Statistical analysis

Descriptive analyses were conducted to present both demographic and fish consumption characteristics. Unadjusted bivariate associations of these characteristics with analyte levels were evaluated using Kruskal-Wallis tests for categorical variables and Spearman's correlation coefficients for continuous variables, respectively. The Kruskal-Wallis test and Spearman's correlation coefficients are rank-based non-parametric statistical methods used for non-normal distributed data. Medians and 95% confidence intervals (CIs) for analyte concentrations were calculated among licensed anglers and Burmese refugees, and comparisons were made to the medians from the NHANES data for adults aged 20 and over. Medians for blood metals were compared to those reported from NHANES 2013–2014, which corresponded to our sampling years (CDC, 2019a). Medians for POPs were compared to NHANES 2003–2004 since they were last measured in individual samples in NHANES 2003–2004 and measured in pooled samples from NHANES 2005–2006 onwards (CDC, 2019b). The NHANES medians of lipid-adjusted $\sum$PCBs and $\sum$PBDEs were calculated, adjusting for the

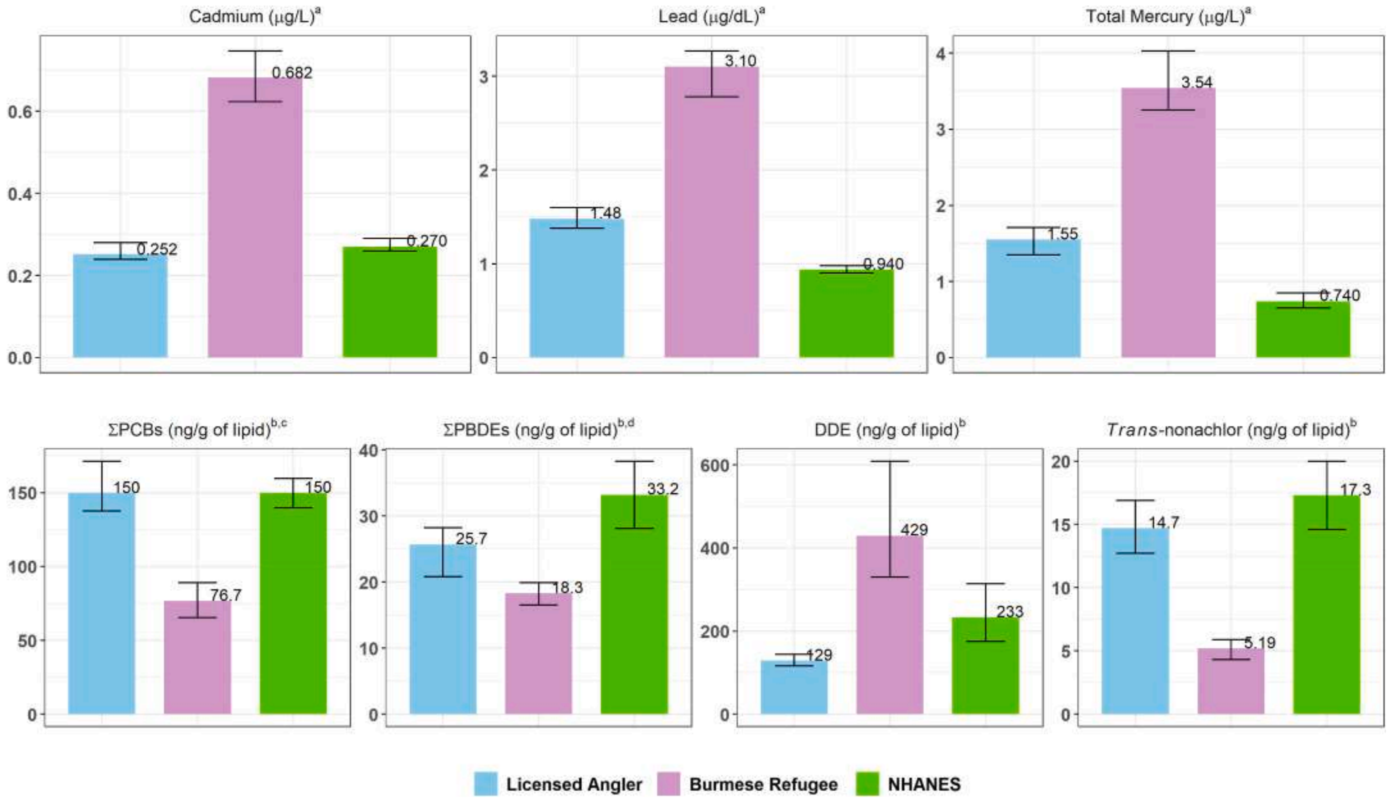

Fig. 2. Comparison of median (95% CI) concentrations in blood metals and serum POPs among licensed anglers, Burmese refugees, and NHANES U.S. adult population. Abbreviation: POPs, persistent organic pollutants; NHANES, National Health and Nutrition Examination Survey; CI, confidence interval. [a] NHANES 2013–2014 adults aged 20+ years. [b] NHANES 2003–2004 adults aged 20+ years. [c] For licensed anglers and Burmese refugees, ∑PCBs = sum of PCBs 31/28, 44, 49/43, 52/73, 66/80, 74/61, 87/117/125/116/111/115, 99, 90/101/89, 105/127, 110, 118/106, 128, 138/164/163, 146/161, 149/139, 151, 153, 156, 157, 167, 170/190, 172/192, 177, 178, 180, 183, 187/182, 189, 194, 195, 196/203, 199, 206, 209. For NHANES, ∑PCBs = sum of PCBs 28, 44, 49, 52, 66, 74, 87, 99, 101, 105, 110, 118, 128, 138/158, 146, 149, 151, 153, 156, 157, 167, 170, 172, 177, 178, 180, 183, 187, 189, 194, 195, 196/203, 199, 206, 209. [d] ∑PBDEs = sum of BDEs 28, 47, 99, 100, and 153 for licensed anglers, Burmese refugees and NHANES.

complex sampling design. NHANES samples with missing values for PCBs 118, 138/158, 153, 170, 180, and 187 were omitted and the rest of individual PCB and PBDE congeners with missing values were set to zero (Patterson et al., 2009).

To investigate analyte concentrations in relation to participants' fish consumption, we constructed linear regression models with a parsimonious set of covariates and multiple fish consumption measures after examining potential collinearity between all fish consumption variables. To derive a parsimonious set of covariates for each analyte, we used backward elimination regression of the natural log-transformed analyte concentrations and each covariate was kept based on the p-value (≤ 0.1). Important covariates including age, sex, and body mass index (BMI) were forced for inclusion in the backward elimination process (Gennings et al., 2012). Common covariates considered for inclusion in modeling for both licensed anglers and Burmese refugees were demographic (ethnicity/race, education, current unemployment) and lifestyle (smoking and tobacco/snuff use status). For the Burmese refugees, models also included residential history (length of residence [LOR] in the U.S. and living in refugee camps) and the use of Thanakar (a traditional cosmetic powder/paste). For licensed anglers, models included residential history (LOR at their current address and year house built) and family income level. Continuous fish consumption variables (fish meals and years of fish consumption) were natural log-transformed in the regression. Diagnostic plots for each model were used to examine whether the assumptions of linear regression were met. Several sensitivity analyses were conducted. To examine model uncertainty, we performed multiple linear regression with stepwise selection (entry and stay significance levels of 0.1 and 0.15) and with all covariates. For those covariates with potential nonlinear effects from bivariate analyses, regression models with quadratic terms and/or cubic splines were used to test for nonlinear relationships. To investigate the potential association between POP exposures and lipid levels, we modeled wet-weight serum POP concentrations with indirect adjustment for total serum lipids by incorporating total serum lipids (natural log-transformed) as a covariate in the multiple linear regression, instead of directly adjusting for total serum lipids by the method of Phillips et al. (1989). Additionally, wet-weight serum POP concentrations (unadjusted for total serum lipids) were also modeled in the multiple linear regression. For Burmese refugees, we used locally caught fish meals in the past year estimated based on the four seasons in the multiple regression, where missing locally caught fish meals spring season were imputed with the median. A result was considered to be statistically significant if the p-value was less than 0.05. All analyses were carried out using SAS version 9.4 (SAS Institute Inc., Cary, NC) and the R package *relaimpo* (Grömping, 2006).

3. Results

3.1. Descriptive analysis of study participants

Tables 1 and 2 present medians of blood metal and serum POP concentrations among licensed anglers and Burmese refugees, respectively, by categorical characteristics (e.g., sex, race/ethnicity, current unemployment status) and indicate whether analyte concentrations were significantly different by characteristics. Tables S2 and S3 present Spearman's correlation coefficients (Υ_s) between continuous covariates and analyte concentrations and indicate whether there were statistical dependencies between them. Additional descriptive results for both groups have been previously published (Savadatti et al., 2019).

Table 1
Categorical characteristics, fish consumption and medians of blood metals and serum POPs among licensed anglers.

Characteristic	n	Cadmium (μg/L)	Lead (μg/dL)	Total Mercury (μg/L)	∑PCBs (ng/g of lipid)	∑PBDEs (ng/g of lipid)	DDE (ng/g of lipid)	*Trans*-Nonachlor (ng/g of lipid)
Median (Interquartile ranges)								
Sex								
Male	353	0.245 (0.378)	**1.55 (1.22)**	**1.66 (2.38)**	149 (186)	25.7 (36.7)	129 (148)	15.0 (19.3)
Female	56	0.346 (0.367)	**1.18 (1.00)**	**1.13 (1.12)**	168 (146)	25.0 (42.2)	133 (229)	13.2 (23.8)
Race[a]								
White	352	**0.244 (0.354)**	**1.42 (1.09)**	1.64 (2.26)	**143 (170)**	**24.0 (33.4)**	**120 (145)**	13.3 (17.9)
Non-White	50	**0.407 (0.661)**	**1.87 (1.86)**	1.40 (1.99)	**194 (243)**	**34.7 (50.9)**	**194 (245)**	20.4 (27.6)
Education[b]								
< High School	25	**0.412 (0.616)**	**2.49 (1.42)**	**1.32 (2.57)**	177 (176)	28.2 (34.9)	174 (251)	19.0 (28.6)
High School	129	**0.291 (0.444)**	**1.56 (1.18)**	**1.42 (1.85)**	123 (179)	26.3 (43.7)	118 (153)	12.5 (15.5)
Some college (no diploma)	76	**0.293 (0.464)**	**1.58 (1.13)**	**1.09 (1.53)**	161 (207)	26.1 (36.6)	139 (144)	17.0 (19.3)
College (including associate degree)	179	**0.209 (0.206)**	**1.38 (0.922)**	**1.76 (2.39)**	153 (154)	24.3 (36.3)	130 (154)	14.7 (21.6)
Unemployment[a]								
Yes	120	**0.378 (0.570)**	1.51 (1.29)	1.40 (1.82)	**224 (169)**	25.0 (52.0)	**157 (204)**	**19.4 (21.9)**
No	288	**0.233 (0.299)**	1.45 (1.15)	1.67 (2.38)	**131 (144)**	26.0 (32.5)	**117 (139)**	**12.2 (17.3)**
Current smoker[a]								
Yes	88	**1.05 (0.899)**	**1.74 (1.73)**	**0.99 (1.55)**	**117 (129)**	24.3 (31.5)	**114 (121)**	11.2 (16.5)
No	318	**0.208 (0.179)**	**1.42 (1.15)**	**1.67 (2.27)**	**171 (189)**	25.7 (37.5)	**140 (175)**	15.1 (21.7)
Chewing tobacco/snuff use[a]								
Yes	15	0.259 (0.315)	1.49 (1.35)	1.04 (2.77)	94.6 (88.7)	28.3 (12.2)	141 (104)	11.9 (17.6)
No	391	0.251 (0.378)	1.49 (1.19)	1.57 (2.21)	152 (192)	25.2 (38.7)	129 (156)	14.8 (20.5)
Year house built[a]								
After 1977	101	**0.233 (0.210)**	**1.30 (0.988)**	1.46 (2.53)	148 (130)	25.0 (41.2)	147 (158)	14.6 (20.4)
1950 to 1977	165	**0.273 (0.494)**	**1.52 (1.29)**	1.42 (2.14)	165 (205)	26.1 (38.8)	137 (160)	15.0 (21.2)
Before 1950	128	**0.251 (0.372)**	**1.51 (1.16)**	1.63 (1.99)	137 (170)	24.0 (33.1)	111 (121)	13.2 (16.2)
Family income levels[a]								
< $35,000	73	**0.382 (0.595)**	**1.85 (1.42)**	**1.09 (1.77)**	171 (153)	29.0 (54.6)	151 (190)	17.7 (26.5)
$35,000-$75,000	139	**0.292 (0.362)**	**1.56 (1.26)**	**1.61 (2.00)**	174 (201)	20.9 (36.2)	126 (144)	14.6 (19.7)
≥ $75,000	172	**0.190 (0.177)**	**1.27 (0.969)**	**1.73 (2.57)**	136 (171)	25.1 (30.6)	128 (146)	13.1 (19.3)
Ate fish/shellfish in the past week[a]								
Yes	267	0.246 (0.332)	1.49 (1.29)	**1.76 (2.32)**	**177 (188)**	**26.6 (35.5)**	135 (171)	**16.0 (21.9)**
No	140	0.267 (0.650)	1.45 (1.05)	**1.00 (1.54)**	**121 (167)**	**20.0 (34.3)**	122 (119)	**11.0 (16.3)**
Locally caught fish meals in the past year[a]**, counts**								
≤16	210	0.248 (0.358)	**1.38 (0.928)**	**1.28 (0.87)**	**137 (162)**	24.5 (39.3)	120 (156)	13.2 (17.9)
>16	192	0.266 (0.409)	**1.66 (1.41)**	**1.71 (2.47)**	**172 (225)**	26.8 (33.7)	133 (143)	15.5 (21.9)
Store-bought fish meals in the past year[a]**, counts**								
None	26	0.347 (0.466)	1.76 (2.12)	**0.926 (1.22)**	167 (262)	25.8 (27.5)	141 (91.1)	14.3 (24.8)
≤22	181	0.258 (0.369)	1.52 (1.15)	**1.44 (1.90)**	149 (197)	23.5 (33.6)	120 (150)	15.0 (17.5)
>22	202	0.245 (0.372)	1.44 (1.18)	**1.67 (2.45)**	150 (155)	26.5 (41.7)	133 (164)	14.3 (21.2)
Years of locally caught fish consumption[a]								
≤23	204	0.248 (0.346)	**1.34 (1.04)**	1.44 (1.82)	**137 (157)**	27.0 (42.4)	119 (145)	**12.8 (17.6)**
>23	200	0.258 (0.444)	**1.59 (1.39)**	1.66 (2.40)	**181 (194)**	20.7 (33.6)	140 (162)	**17.2 (22.0)**
Years of store-bought fish consumption[a]								
≤40	205	**0.225 (0.393)**	**1.34 (1.18)**	1.55 (2.45)	**115 (150)**	26.5 (36.1)	**111 (123)**	**12.2 (16.6)**
>40	203	**0.291 (0.352)**	**1.61 (1.24)**	1.45 (2.15)	**196 (187)**	24.9 (34.6)	**158 (171)**	**17.7 (22.6)**

Note: Significant results (p-value < 0.05, Kruskal-Wallis Test) are bolded.
[a] Totals do not equal 409 because of missing data; a response of don't know or refusal is counted as missing.
[b] < High school: no high school diploma or GED; High school: high school diploma or GED; Some college: some college education without diploma; College: college degree including associate's degree.

Table 2
Categorical characteristics, fish consumption and medians of blood metals and serum POPs among Burmese refugees.

Characteristic	n	Cadmium (μg/L)	Lead (μg/dL)	Total Mercury (μg/L)	∑PCBs (ng/g of lipid)	∑PBDEs (ng/g of lipid)	DDE (ng/g of lipid)	*Trans*-Nonachlor (ng/g of lipid)
Median (Interquartile ranges)								
Sex								
Male	82	0.726 (0.741)	**3.72 (2.31)**	3.35 (2.70)	**95.2 (132)**	19.0 (16.6)	**902 (1618)**	6.50 (8.74)
Female	124	0.663 (0.522)	**2.71 (1.37)**	3.81 (2.74)	**68.3 (73.5)**	18.1 (20.0)	**293 (970)**	4.67 (5.28)
Ethnicity[a]								
Burman	38	0.633 (0.861)	3.04 (2.21)	**3.38 (2.71)**	**89.0 (121)**	**16.9 (20.1)**	531 (955)	5.60 (6.84)
Karenni	27	0.618 (0.456)	3.43 (1.85)	**2.10 (1.88)**	**41.3 (43.0)**	**28.9 (48.7)**	396 (1826)	3.60 (4.25)
Karen	96	0.712 (0.653)	2.99 (1.22)	**4.08 (3.13)**	**94.7 (117)**	**17.7 (17.2)**	305 (1038)	4.96 (6.83)
Other	28	0.695 (0.527)	2.68 (1.25)	**3.46 (3.49)**	**56.1 (67.2)**	**17.7 (18.2)**	721 (1599)	5.38 (4.57)
Unemployment[a]								
Yes	145	0.738 (0.745)	3.09 (1.90)	3.59 (2.65)	**72.0 (91.9)**	18.9 (19.6)	382 (1117)	4.74 (5.74)
No	59	0.592 (0.387)	3.13 (1.40)	3.26 (2.82)	**92.6 (124)**	16.1 (17.6)	569 (1659)	5.74 (6.77)
Current smoker[a]								
Yes	45	**1.31 (0.874)**	**3.79 (2.90)**	3.67 (2.46)	93.0 (115)	19.4 (16.7)	771 (1487)	**6.74 (9.06)**
No	156	**0.597 (0.386)**	**2.91 (1.46)**	3.43 (2.87)	69.9 (93.3)	17.3 (19.5)	397 (1098)	**4.61 (5.36)**
Chewing tobacco/snuff use[a]								
Yes	59	0.686 (0.639)	**3.41 (1.67)**	3.87 (3.29)	93.2 (94.1)	19.1 (24.2)	493 (987)	4.99 (4.69)
No	142	0.661 (0.654)	**2.82 (1.61)**	3.35 (2.70)	70.0 (95.8)	17.1 (18.5)	363 (1306)	5.35 (6.42)
Thanakar use[a]								
Yes	90	0.680 (0.511)	**2.72 (1.14)**	**4.06 (3.22)**	67.6 (105)	18.2 (19.1)	**317 (958)**	4.77 (5.75)
No	110	0.663 (0.678)	**3.30 (2.44)**	**3.25 (2.61)**	80.2 (88.4)	18.4 (18.7)	**524 (1487)**	5.53 (6.14)
Lived in any refugee camps[a]								
Yes	174	0.686 (0.703)	3.11 (1.81)	**3.60 (2.66)**	81.7 (111)	**18.9 (20.1)**	464 (1125)	**5.29 (6.01)**
No	28	0.606 (0.263)	2.65 (1.61)	**3.01 (2.06)**	56.3 (54.7)	**14.8 (8.93)**	393 (1258)	**3.52 (6.39)**
Ate fish/shellfish in the past week[a]								
Yes	179	0.682 (0.657)	3.09 (1.86)	**3.71 (2.71)**	77.5 (98.6)	18.2 (19.2)	398 (1176)	**5.41 (6.37)**
No	22	0.675 (0.834)	3.09 (1.47)	**2.81 (1.43)**	68.9 (72.2)	17.6 (28.1)	406 (1152)	**3.58 (4.05)**
Locally caught fish meals in the past year[a]**, counts**								
≤106	114	0.633 (0.622)	3.16 (1.54)	3.32 (2.71)	77.5 (95.8)	17.7 (18.9)	396 (1302)	5.29 (5.52)
>106	90	0.775 (0.715)	2.91 (1.96)	3.89 (3.25)	78.4 (105)	18.8 (19.6)	496 (1102)	5.03 (6.26)
Store-bought fish/shellfish meals in the past year[a]**, counts**								
None	17	0.630 (0.321)	2.97 (2.34)	4.31 (2.76)	94.7 (74.1)	20.9 (26.0)	641 (2105)	9.05 (9.64)
≤104	101	0.694 (0.663)	3.14 (1.38)	3.52 (2.74)	78.2 (101)	19.2 (19.4)	298 (1100)	5.05 (5.73)
>104	88	0.679 (0.715)	3.09 (2.06)	3.50 (2.81)	72.9 (104)	17.9 (18.7)	572 (1223)	4.75 (5.45)
Years of store-bought fish/shellfish consumption[a]								
≤28	98	**0.618 (0.509)**	3.14 (1.88)	3.34 (2.85)	77.8 (94.5)	18.1 (19.0)	396 (1070)	4.30 (5.17)
>28	97	**0.777 (0.646)**	3.08 (1.40)	3.70 (2.84)	76.9 (107)	18.7 (19.9)	426 (1189)	5.83 (6.91)
Annual fish paste meals[a]**, counts**								
≤156	92	0.714 (0.670)	2.83 (1.52)	3.49 (2.98)	65.3 (80.7)	18.7 (18.8)	452 (1095)	5.29 (5.89)
>156	51	0.687 (0.477)	3.14 (1.61)	3.34 (2.68)	75.9 (96.2)	15.1 (11.2)	373 (1100)	4.57 (5.37)

Note: Significant results (p-value < 0.05, Kruskal-Wallis Test) are bolded.
[a] Totals do not equal 206 because of missing data; a response of don't know or refusal is counted as missing.

A total of 409 licensed anglers, mostly non-Hispanic white males, participated in the program. Their average age was 52.3 years and their average BMI was 30.2 kg/m^2. A majority of licensed anglers had some college education and were currently employed with a family income of more than $35,000. The median LOR at their current address was 15.0 years. Approximately two-thirds of the licensed anglers reported eating fish/shellfish in the past week prior to being interviewed. Licensed anglers consumed a median of 16.0 locally caught fish meals and 22.0 store-bought fish meals in the past year. The median years of consuming locally caught fish and store-bought fish were 23.0 years and 40.0 years, respectively. Measurements of blood metals and serum POPs were valid in 400 and 396 licensed anglers, respectively. For the fish consumption variables, licensed anglers who reported fish/shellfish consumption in the past week had significantly higher concentrations of blood mercury, ∑PCBs, ∑PBDEs, and *trans*-nonachlor. Significant correlations were also found between locally caught fish meals in the past year and lead, mercury, and ∑PCBs; store-bought fish meals in the past year and mercury; years of locally caught fish consumption and blood lead and ∑PCBs, ∑PBDEs, and *trans*-nonachlor; and years of store-bought fish consumption and blood cadmium, lead, ∑PCBs, DDE, and *trans*-nonachlor.

Two hundred and six Burmese refugees participated, and they had an average age of 38.9 years and an average BMI of 26.2 kg/m^2. A majority were female and of Karen ethnicity, which is the second largest ethnic group in Burma (Hannah and Bangkok, 2009). The median of total years of education completed for the Burmese refugees was 4.00 years and most reported being currently unemployed. The median LOR in the U.S. for this group was 4.08 years. Only one-tenth of the Burmese refugees reported not eating fish/shellfish in the past week prior to being interviewed. Burmese refugees consumed a median of 106 locally caught fish meals and 104 store-bought fish/shellfish meals in the past year, respectively. The median length of store-bought fish/shellfish consumption was 28.0 years. The median consumption of fish paste in the past year was 156 fish paste meals. Measurements of blood metals and serum POPs were valid in 205 and 196 Burmese refugees, respectively. Burmese refugees who reported fish/shellfish consumption in the past week had elevated levels of mercury and *trans*-nonachlor. Additionally, there were significant correlations between consumption of locally caught fish meals in the past year and mercury, and years of consuming store-bought fish/shellfish and blood cadmium levels.

3.2. Biomonitoring results in comparison with NHANES

Fig. 2 shows the median concentrations and 95% CI for blood metal levels and serum POP concentrations among licensed anglers, Burmese refugees, and the general U.S. population aged over 20 years, using estimates from the NHANES data. Medians of blood metal levels were notably higher among Burmese refugees compared to NHANES.

Burmese refugees also had higher median concentrations of DDE than NHANES 2003–2004. Licensed anglers had moderately higher levels of blood lead and total mercury. Both groups had lower medians of serum concentrations for ∑PBDEs and *trans*-nonachlor compared to NHANES.

3.3. Correlations between analyte concentrations

Spearman's correlation coefficients between analytes for licensed anglers and Burmese refugees are shown in Table 3. Among licensed anglers, blood cadmium was positively correlated with blood lead but negatively correlated with blood mercury. Blood lead and mercury were significantly correlated with serum ∑PCBs, DDE, and *trans*-nonachlor. All serum POP concentrations were significantly correlated with each other; specifically, the relationships of ∑PCBs and *trans*-nonachlor, and DDE and *trans*-nonachlor ($\Upsilon_s = 0.456$ and 0.479, respectively) were substantially stronger compared to other relationships, which ranged from 0.128 to 0.388.

Among Burmese refugees, all blood metal levels were positively correlated with each other. Blood lead was also positively correlated with ∑PCBs and ∑PBDEs, while blood mercury was corelated with ∑PCBs ($\Upsilon_s = 0.259$), DDE ($\Upsilon_s = -0.181$), and *trans*-nonachlor ($\Upsilon_s = 0.302$). Among POPs, *trans*-nonachlor was positively correlated with ∑PCBs ($\Upsilon_s = 0.414$) and DDE ($\Upsilon_s = 0.183$).

3.4. Correlations between fish consumption variables

Table S4 shows Spearman's correlation coefficients between fish consumption variables among licensed anglers and Burmese refugees. Low correlations indicated that fish consumption variables could be independently included in multiple regressions. The number of fish meals and years of fish consumption were significantly correlated among licensed anglers ($\Upsilon_s = 0.417$). As expected, age was correlated with years of fish consumption among both groups (licensed anglers: $\Upsilon_s = 0.288$ for local fish years and $\Upsilon_s = 0.657$ for store fish years; Burmese refugees: $\Upsilon_s = 0.456$ for store fish/shellfish years). To reduce collinearity, only locally caught and store-bought fish meals, but not years of fish consumption, were included in multiple regressions for licensed anglers and Burmese refugees. However, to examine the predictive ability between years of locally caught fish consumption and locally caught fish meals in the past year, particularly for POPs among licensed anglers, another sensitive analysis was conducted by adding years of locally caught fish consumption into the final regression model.

3.5. Adjusted associations

Table 4 presents regression coefficients of fish consumption variables resulting from multiple regression modeling. Licensed anglers and Burmese refugees who reported consuming fish/shellfish in the past week had elevated blood mercury concentrations, compared to those who reported no consumption in the past week. Increased blood mercury levels among licensed anglers showed significant associations with increased locally caught fish meals and store-bought fish meals in the past year. Additionally, among the licensed anglers, higher numbers of locally caught fish meals were associated with higher blood lead levels and serum ∑PCBs; and higher numbers of store-bought fish meals were associated with higher levels of DDE. Multiple regression models of the two groups accounted for 20.1%–54.2% of the variability in blood metal concentrations and for 0.473%–23.0% of the variability in serum POP concentrations (Supplementary Tables S5 and S6). Among the total variances explained in the models, the contributions of fish consumption ranged from 0.33% to 31.6% in blood metal concentrations and 0.31%–12.97% in serum POP concentrations. The indicators of fish consumption with the highest contribution were "ate fish/shellfish in the past week" in the total mercury model for licensed anglers and "locally caught fish meals in the past year" in the *trans*-nonachlor model for Burmese refugees.

Additional regression model results including regression coefficients of the fish consumption variables, demographics, and other factors are shown in Supplementary Tables S5 and S6. Blood cadmium and lead were the highest among current smokers and males, respectively, for both the licensed anglers and Burmese refugees. Among licensed anglers, blood mercury was the highest among participants who consumed fish/shellfish in the past week; serum ∑PCBs was strongly positively associated with locally caught fish meals; *trans*-nonachlor was strongly positively associated with age; and ∑PBDEs and DDE were the highest among non-white licensed anglers. Among Burmese refugees, levels of blood mercury, ∑PCBs, and ∑PBDEs were most strongly associated with ethnicity; DDE was the highest among males; and *trans*-nonachlor was strongly positively associated with their LOR in the U.S.

All sensitivity analyses (regression with stepwise selection and all covariates) gave very similar results compared to that from the backward elimination, except that for licensed anglers, the effect of consuming locally caught fish meals was not significant on serum ∑PCBs in regression with stepwise selection (entry and stay significance levels of 0.15) and all covariates and the effect of consuming store-bought fish meals was not significant on serum DDE in regression with all covariates (Supplementary Tables S7 and S8). Particularly, the final list of covariates from the stepwise selection (entry and stay significance levels of 0.1) were the same with that from the backward elimination. Those covariates not selected by the backward elimination remained non-significant in regression with all covariates. In general, the adjusted R^2 was higher in regression with backward elimination than with

Table 3
Spearman's correlation coefficients between blood metals and serum POPs among licensed anglers and Burmese refugees.

Characteristic	Cadmium	Lead	Total Mercury	∑PCBs	∑PBDEs	DDE	*Trans*-Nonachlor
Licensed Anglers							
Cadmium, μg/L	–						
Lead, μg/dL	**0.292**	–					
Total Mercury, μg/L	**−0.128**	0.0859	–				
∑PCBs, ng/g of lipid	0.0217	**0.126**	**0.212**	–			
∑PBDEs, ng/g of lipid	−0.00703	0.0641	0.0878	**0.128**	–		
DDE, ng/g of lipid	0.0453	**0.228**	**0.171**	**0.388**	**0.296**	–	
***Trans*-Nonachlor, ng/g of lipid**	0.0581	**0.168**	**0.181**	**0.456**	**0.225**	**0.479**	–
Burmese Refugees							
Cadmium, μg/L	–						
Lead, μg/dL	**0.320**	–					
Total Mercury, μg/L	**0.143**	**0.172**	–				
∑PCBs, ng/g of lipid	−0.0208	**0.149**	**0.259**	–			
∑PBDEs, ng/g of lipid	0.0879	**0.222**	0.0656	0.0526	–		
DDE, ng/g of lipid	−0.0645	0.0221	**−0.181**	0.113	0.0745	–	
***Trans*-Nonachlor, ng/g of lipid**	0.0843	0.0428	**0.302**	**0.414**	0.134	**0.183**	–

Note: Significant results (p-value < 0.05) are bolded.

Table 4
Regression coefficients of multiple fish consumption variables from regressions of the natural log-transformed analyte concentrations.[a].

Cadmium[b]	Lead[c]	Total Mercury[d]	∑PCBs[e]	∑PBDEs[f]	DDE[g]	*Trans*-Nonachlor[h]
Licensed Anglers						
Ate fish/shellfish in the past week (reference: no)						
−0.308 (−0.169, 0.107)	−0.00334 (−0.116, 0.110)	**0.503 (0.311, 0.695)**	NA			
Ln(Locally caught fish meals in the past year)						
−0.0403 (−0.0948, 0.0142)	**0.0490 (0.00397, 0.0941)**	**0.134 (0.0594, 0.208)**	**0.102 (0.00383, 0.200)**	0.0134 (−0.0827, 0.110)	−0.0372 (−0.185, 0.111)	0.0272 (−0.116, 0.170)
Ln(Store-bought fish meals in the past year)						
0.0111 (−0.0433, 0.0654)	−0.0165 (−0.0620, 0.0290)	**0.0815 (0.00558, 0.157)**	0.0435 (−0.0539, 0.141)	0.0488 (−0.0486, 0.146)	**0.169 (0.0195, 0.319)**	0.0586 (−0.0853, 0.202)
Burmese Refugees						
Ate fish/shellfish in the past week (reference: no)						
0.127 (−0.118, 0.372)	0.157 (−0.0513, 0.366)	**0.328 (0.0506, 0.606)**	NA			
Ln(Locally caught fish meals in the past year)						
0.0120 (−0.0883, 0.112)	−0.00840 (−0.0942, 0.0773)	−0.0565 (−0.178, 0.0656)	−0.0838 (−0.272, 0.104)	−0.0193 (−0.171, 0.132)	0.138 (−0.190, 0.467)	0.166 (−0.0285, 0.360)
Ln(Store-bought fish/shellfish meals in the past year)						
0.0197 (−0.0289, 0.0682)	0.00172 (−0.0400, 0.0434)	−0.0171 (−0.0755, 0.0413)	−0.0282 (−0.124, 0.0680)	−0.0630 (−0.139, 0.0135)	0.0277 (−0.139, 0.195)	−0.0417 (−0.141, 0.0577)

Abbreviation: CI, confidence interval; Ln, natural log-transformation; NA, not applicable.
Note: Significant results (p-value < 0.05) are bolded.
[a] Fish variables included 1) locally caught fish meals in the past year and 2) store-bought fish or fish/shellfish meals in the past year; specifically, for modeling blood metals, 3) ate fish/shellfish in the past week (yes/no) was also included.
[b] The final covariates included in the model for cadmium were: sex, age, BMI, education, unemployment, family income levels, and current smoker for licensed anglers and sex, age, BMI, and current smoker for Burmese refugees.
[c] The final covariates included in the model for lead were: sex, age, BMI, race, unemployment, year house built, and current smoker for licensed anglers and sex, age, BMI, education, and current smoker for Burmese refugees.
[d] The final covariates included in the model for total mercury were: sex, age, BMI, education, unemployment, and current smoker for licensed anglers and sex, age, BMI, ethnicity, education, and lived in any refugee camps for Burmese refugees.
[e] The final covariates included in the model for ∑PCBs were: sex, age, BMI, and year house built for licensed anglers and sex, age, BMI, ethnicity, and LOR in the U.S. for Burmese refugees.
[f] The final covariates included in the model for ∑PBDEs were: sex, age, BMI, and race for licensed anglers and sex, age, BMI, ethnicity, and lived in any refugee camps for Burmese refugees.
[g] The final covariates included in the model for DDE were: sex, age, BMI, race, and LOR at the current address for licensed anglers and sex, age, BMI, and education for Burmese refugees.
[h] The final covariates included in the model for *trans*-nonachlor were: sex, age, and BMI for licensed anglers and sex, age, BMI, and LOR in the U.S. for Burmese refugees.

stepwise selection and all covariates. For those covariates with potential nonlinear effects, there was no strong evidence of nonlinear associations. Moreover, the use of direct/indirect lipid-adjusted and wet-weight serum POPs concentrations yielded very similar results, except that the association between consuming locally caught fish meals in the past year and wet-weight serum ∑PCBs was non-significant for licensed anglers. For Burmese refugees, wet-weight serum ∑PBDEs was positively associated with age and the association between BMI and wet-weight serum *trans*-nonachlor was non-significant (Supplementary Tables S9 and S10). Additionally, serum ∑PBDEs concentrations were negatively associated with years of locally caught fish consumption for licensed anglers (Supplementary Table S11).

4. Discussion

Licensed anglers and Burmese refugees in western NY were targeted in the *Healthy Fishing Communities Program* since they are at high risk of being exposed to persistent contaminants due to Great Lakes fish consumption. These two populations are very distinct. For example, licensed anglers are largely white men in their 50s and fishing for recreation while Burmese participants are mostly young adults (18–39 years) and fishing for subsistence. Our analyses quantified associations between fish consumption and persistent contaminants (cadmium, lead, total mercury, ∑PCBs, ∑PBDEs, DDE, and *trans*-nonachlor) and identified factors influencing these concentrations among both target groups.

Our results show that consuming fish/shellfish in the past week was associated with elevated blood mercury levels in both populations. Previous studies show that human dietary methylmercury intakes are absorbed into the blood stream and distributed to all organs and tissues within about four days (Carrier et al., 2001; WHO, 1990). Eating more locally caught fish meals in the past year was associated with higher blood levels of lead, mercury, and serum ∑PCBs, only among the licensed anglers. However, the association between locally caught fish meals and serum ∑PCBs levels among licensed anglers may be weak since it was almost non-significant in the regression with stepwise selection ($p = 0.08$), and its strength was also affected by total serum lipids (Aminov et al., 2013; Penell et al., 2014; Schisterman et al., 2009). For blood mercury and ∑PCBs, our findings among licensed anglers are not surprising since these contaminants have been found in predatory fish (lake trout and walleye) across the Great Lakes Basin (McGoldrick and Murphy, 2016). Although the Burmese refugees had higher fish consumption compared to licensed anglers, we did not find statistically significant effects of locally caught fish meals and store-bought fish/shellfish meals on concentrations of total blood mercury and PCBs among the Burmese. The different findings between licensed anglers and Burmese refugees could relate to the types of fish species consumed, the participants' distinct residential histories, and other factors. The most popular fish species (yellow perch, walleye, and smallmouth bass) that licensed anglers reported consuming are predatory and good bioaccumulators of mercury (Vermont Department of Environmental Conservation; Wiener et al., 2012), while the common fish species (quillback, common carp, and minnow) consumed by Burmese refugees are good bioaccumulators of lipophilic contaminants (Noyes and Stapleton, 2014; Pérez-Fuentetaja et al., 2010; West, 1995). For Burmese refugees, long-term locally caught fish consumption may contribute to mercury exposure, but the finding of a higher GM blood mercury level compared to licensed anglers may be caused by other factors, such as consuming wild waterfowl and using Thanakar. Over 60% of Burmese refugees had consumed wild waterfowl in the past year compared to 12.2% of licensed anglers. The use of Thanakar was one of the potential covariates in the backward elimination process but did not meet the criteria for inclusion in the final regression models. Furthermore, the Burmese participants were homogeneously high-end fish consumers, and the lack of lower end exposure could have influenced the non-statistically significant association with total blood mercury.

Having made the journey to the U.S. from a less developed and less industrialized nation, it is likely that the Burmese refugee participants were exposed to PCBs mainly after their arrival into the U.S., which may help explain a lower median of ∑PCBs in the Burmese group compared to the licensed angler group. This potential explanation takes into consideration the half-life of PCBs in serum which is ~10–15 years (Ritter et al., 2010), the median LOR of the Burmese refugees in the U.S. (4.08 years), a significant correlation between ∑PCBs and LOR in the U. S., and the lack of correlation between ∑PCBs and age in the Burmese group. These above-mentioned facts/findings may also explain the insignificant association between ∑PCBs and locally caught fish meals for the Burmese group. For lead, few studies have examined associations between blood lead levels and Great Lakes fish consumption although heavy metal contamination of Great Lakes waters has been reported (Forsythe et al., 2004, 2015). It is possible that licensed anglers may have been exposed to lead from fish tackle (we did not collect this data) or making their own lead fishing sinkers, jigs, or spinnerbait (Rattner et al., 2008; TACKLE, 2011; Watson and Avery, 2009) while Burmese refugees may catch fish using a traditional trap. These habits could be related to the amount of fish consumption and may explain the positive association between locally caught fish meals and higher lead levels.

Eating more store-bought fish meals was associated with higher blood mercury levels, only among licensed anglers. This is most likely because of canned tuna consumption which was the most popular store-bought fish among licensed anglers. According to previous studies, on average, 40% of canned tuna has mercury levels above the U.S. Environmental Protection Agency's safety levels (Burger and Gochfeld, 2004; Gerstenberger et al., 2010).

Although we observed a positive association between store-bought fish meals and DDE levels among licensed anglers: 1) their DDE median concentration was much lower than the NHANES median (128.7 ng/g of lipid vs. 233 ng/g of lipid); 2) there was no unadjusted correlation between DDE and store-bought fish meals; and 3) the explanatory power of this model was low (adjusted R^2: 0.0769). It is possible that this finding was due to chance. Despite insignificant associations between DDE and fish consumption among Burmese refugees, they had significantly higher DDE concentrations than licensed anglers and the general U.S. adult population. DDT continues to be used for vector control internationally (Peeters et al., 2015). It is likely that the Burmese refugees were exposed to DDT prior to their arrival to the U.S. (our results showed significant correlations between DDE, and age and years of education). The finding of a negative association between ∑PBDEs and years of locally caught fish consumption was likely due to chance because of low explanatory power. No associations between fish consumption and *trans*-nonachlor were observed; these results are consistent with previous reports (Christensen et al., 2016; Cole et al., 2002).

Consistent with the literature, blood cadmium was the highest among current smokers (Benedetti et al., 1994; Mannino et al., 2004; Tellez-Plaza et al., 2011). Our results show positive associations between being a current smoker and blood lead among licensed anglers; these results are consistent with previous reports (McKelvey et al., 2007). Additionally, we observed a negative association between being a current smoker and blood mercury among licensed anglers. It is possible that this association was confounded by participating in metal work as mercury levels among non-smokers increased upon exclusion of licensed anglers (both smokers and non-smokers) who reported no metal work in the past year prior to their interview.

Our study has several limitations. First, licensed anglers who participated in the program were more likely to be 60 years of age or older and more aware of local fish advisories and fished more, compared to non-participants. This results in potential selection bias. (Savadatti et al., 2019; Wattigney et al., 2019). In addition, the initial Burmese refugee 'seed' participants were selected in a nonrandom fashion, following the RDS methodology. However, RDS proved to be an effective methodology for recruitment, and the final sample represented the source population (Liu et al., 2018). Second, any recall issues with participants reporting historical fish consumption are likely to be random and non-differential. For Burmese refugees, interpreters were trained in all aspects of survey administration prior to program implementation, and interpreters were supervised by program staff. These procedures likely assisted with countering communication concerns that arose, minimizing some likelihood of information bias. Another limitation for the Burmese refugees is that an annual locally caught fish meal was estimated by three seasons. However, sensitivity analysis showed that our study results were not particularly affected by using different estimates of annual locally caught fish meals. The cross-sectional study design only allowed for a single timepoint measurement to examine associations, rather than establishing any cause-effect relationships. Also, for some contaminants such as ∑PBDEs, fish consumption is not the dominant exposure source. Important exposure routes (e.g. consumer products) are not addressed in our analysis due to lack of data on non-fish consumption sources of exposure. Thus, our analyses may not account for all potential confounders. There is also a possibility that few findings were due to chance because of multiple comparisons.

Since the 1980s, NYS has issued advice about fish to eat and fish to avoid in NYS waters as a result of contaminants detected in fish. Over the years, DOH has developed free brochures and materials to inform anglers and families about healthy choices related to fish they catch. Fish consumption advice focuses on who you are, where you fish, and what you catch. This advice is presented by NYS region so that anglers and families can more easily browse advice by waterbody and fish species. DOH's fish consumption advice is available in multiple languages, online and in print, with more than 40 separate titles, in addition to color coded maps, children's coloring books, magnets, and other promotional items to highlight the benefits of making healthy choices when eating fresh caught fish. DOH staff also support a robust outreach program targeted specifically to informing Hudson River anglers about PCB contamination in fish along a 200 mile stretch of the river.

NYS's presentation of fish consumption advice is informed by years of direct interaction with anglers and families, and both qualitative and quantitative data about their fishing and eating behaviors. For the most part, efforts have focused on the general NYS and susceptible populations (including women of childbearing age under 50, infants, and young children under 15). Although over the years, staff have done some targeted outreach to refugee and Native American populations to better target fish advisory materials and messages to these audiences by learning more about their cultural practices and learning styles. Some of these efforts are described in the research herein.

After sampling concluded, detailed fish advisory information was presented to the community including color coded maps of recommended waters to fish from, the best methods of fish preparation, fish consumption advice, and advice related to supermarket fish. The fish advisory information, specifically for supermarket fish consumption, closely follows the Food and Drug Administration (FDA) and Environmental Protection Agency guidelines and advice (EPA, 2019; FDA, 2020). In general, women of childbearing age and children are advised to limit the kinds of sportfish they eat and how often they eat them. Educational fact sheets including the NYSDOH fish advisory brochure for the region (NYSDOH, 2019) and the New York City Department of Health and Mental Hygiene's Eat Fish, Choose Wisely Protect Against Mercury brochure (NYCDOHMH, 2014) were made available. The risk/benefit trade-off in fish consumption is well documented in the literature and suggests that the benefits of eating fish, especially fish rich in Omega-3 fatty acids, exceeds the potential risk for human health (Mozaffarian and Rimm, 2006). The AHA advisory has also reaffirmed its recommendation to eat fish at least twice a week to prevent heart disease and stroke (Rimm et al., 2018). For our two target populations, fish consumption for licensed anglers did not meet the AHA recommendation while for cultural and economic reasons, fish consumption for Burmese refugees exceeded the FDA recommendation for susceptible populations. Thus, our *Healthy Fishing Communities Program* fish advisory outreach and education did not discourage eating fish but focused on informing people on ways to reduce exposure to contaminants by choosing better fish to eat (including store bought fish), better waters to fish from, and healthier ways to prepare fish (Savadatti et al., 2019). NYSDOH will continue to use results from biomonitoring and interviews with anglers and families to create culturally appropriate materials and target outreach to populations at risk.

In conclusion, our analyses support previous findings linking Great Lakes fish consumption to body burdens of mercury and $\sum$PCBs although mercury and $\sum$PCBs have shown declining trends in Great Lakes fish over time (McGoldrick and Murphy, 2016). In our analyses, store-bought fish consumption was associated with blood mercury concentrations among licensed anglers. For PBDEs, information on additional sources of exposure (e.g. house dust) is needed in order to capture the most important exposure routes (Gandhi et al., 2017; Johnson-Restrepo and Kannan, 2009). For a more thorough interpretation regarding analyte levels among the Burmese refugees, it would have been useful to have baseline data at the time of their arrival to the U.S. and/or additional information about sources of exposure prior to arrival.

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Agency for Toxic Substances and Disease Registry.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113918.

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Characterizing exposure to benzene, toluene, and naphthalene in firefighters wearing different types of new or laundered PPE

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ABSTRACT (291 WORDS)

The fire service has become more aware of the potential for adverse health outcomes due to occupational exposure to hazardous combustion byproducts. Because of these concerns, personal protective equipment (PPE) manufacturers have developed new protection concepts like particulate-blocking hoods to reduce firefighters' exposures. Additionally, fire departments have implemented exposure reduction interventions like routine laundering of PPE after fire responses. This study utilized a fireground exposure simulator (FES) with 24 firefighters performing firefighting activities on three consecutive days wearing one of three PPE ensembles (stratified by hood design and treatment of PPE): 1) new knit hood, new turnout jacket and new turnout pants 2) new particulate-blocking hood, new turnout jacket and new turnout pants or 3) laundered particulate-blocking hood, laundered turnout jacket and laundered turnout pants. As firefighters performed the firefighting activities, personal air sampling on the outside and inside the turnout jacket was conducted to quantify exposures to volatile organic compounds (VOCs) and naphthalene. Pre- and immediately post-fire exhaled breath samples were collected to characterize the absorption of VOCs. Benzene, toluene, and naphthalene were found to diffuse through and/or around the turnout jacket, as inside jacket benzene concentrations were often near levels reported outside the turnout jacket (9.7–11.7% median benzene reduction from outside the jacket to inside the jacket). The PPE ensemble did not appear to affect the level of contamination found inside the jacket for the compounds evaluated here. Benzene concentrations in exhaled breath increased significantly from pre to post-fire for all three groups (p-values < 0.05). The difference of pre-to post-fire benzene exhaled breath concentrations were positively associated with inside jacket and outside jacket benzene concentrations, even though self-contained breathing apparatus (SCBA) were worn during each response. This suggests the firefighters can absorb these compounds via the dermal route.

1. Introduction

Recent epidemiological studies have suggested firefighters have an increased risk for cancer. LeMasters et al. (2006) reported an increased risk for several types of cancer for firefighters (LeMasters et al., 2006). In 2010, the International Agency for Research on Cancer (IARC) classified firefighting to be possibly carcinogenic (Group 2B) (IARC 2010a). Studies conducted after the IARC meeting have further identified excess cancer risk for firefighters (Daniels et al., 2014; Glass et al., 2014; Lee et al., 2020; Tsai et al., 2015). More recent meta-analyses have provided additional support for firefighters' increased risks of specific types of cancer, including melanoma, testicular, bladder, prostate, and colorectal (Casjens et al., 2020; Jalilian et al., 2019; Soteriades et al., 2019). While there can be many causes for increased risk, Daniels et al. (2015) found a relationship between fire runs and leukemia and fire hours and lung cancer, suggesting firefighters' cancer risk is at least in part due to their occupational fireground exposures.

It has been well documented that structural fires produce compounds

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that include known (group 1), probable (group 2A) or possible (group 2B) carcinogens according to IARC, including benzene (group 1) and naphthalene (group 2B) (IARC 2010b, 2012). Firefighters' exposure to volatile organic compounds (VOCs) like benzene have been demonstrated through air samples taken during structure fires (Jankovic et al., 1991) and in the period immediately after fire suppression known as overhaul (Bolstad-Johnson et al., 2000). Several studies have also documented increased internal exposure to benzene by analyzing benzene in firefighters' breath or benzene metabolites in urine samples following firefighting (Caux et al., 2002; Fent et al. 2014, 2020; Wallace et al., 2019; Rosting and Olsen 2020). Firefighters can be exposed through the inhalation route, especially when self-contained breathing apparatus (SCBA) is not worn. However, in our most recent study, firefighters wore their SCBA throughout the fire exercise and benzene exhaled breath concentrations were still significantly increased post-fire (Fent et al., 2020), suggesting the dermal route of exposure is also important. Additionally, elevated skin temperature, which we know is common in firefighters, can also increase dermal absorption (Jones et al., 2003).

The protective hood is often considered one of the more vulnerable aspects of the firefighter personal protective equipment (PPE) ensemble from an exposure perspective (Avsec, 2019). As the fire service has become more aware of potential chemical exposures on the fireground, PPE manufacturers have developed new designs for protective hoods. The traditional protective hood is comprised of two layers of knit material, but newer designs have an added interstitial layer designed to block the penetration of particles (particulate-blocking hoods). In addition to the new types of PPE, fire departments have also become more consistent in laundering their PPE including turnout jackets, turnout pants, and hoods after fireground exposures. A recent study found various exposure reduction interventions, such as washing jackets and showering immediately post-fire, significantly reduced post-fire urinary levels of polycyclic aromatic hydrocarbon (PAH) metabolites (Burgess et al., 2020). However, repeatedly wearing and laundering turnout jackets may affect its protective properties both from physical and chemical hazards (Horn et al., 2021; Mayer et al., 2020).

A recent study found PAH contamination on PTFE filters under particulate-blocking hoods in the neck region of stationary mannequins that were placed in an exposure chamber called a fireground exposure simulator (FES) (Mayer et al., 2020). Another recent publication found PAH contamination on wipes taken from the neck region of firefighters wearing particulate-blocking and traditional knit hoods after conducting realistic simulated fireground operations in the same FES; although PAH levels were lower under the particulate-blocking hoods (Kesler et al., 2021). According to manufacturers, particulate-blocking hoods were designed to reduce the penetration of particles by 90% (Gore Fabrics, 2021; NFPA 2018b); however, these hoods were not designed to be vapor tight.

Only a few studies have examined the penetration of fireground contaminants to the interior of the structural firefighter turnout jacket. Mayer et al. (2020) found benzene concentrations inside the jacket were almost as high and sometimes higher than concentrations found outside the jacket. Kirk and Logan (2015) found inside turnout jacket PAH concentrations were 12 times lower compared to measurements outside the jacket, while Wingfors et al. (2018) found that total PAHs were 146 times lower when measured inside both turnout jacket and inside the base layer (i.e., clothing worn inside the turnout jacket) compared to outside the jacket.

Chemicals that deposit on thin skin areas like the neck are generally absorbed faster than in thicker skin areas like the plantar foot arch (VanRooij et al., 1993; Wester and Maibach 2000). While some less volatile compounds like the higher molecular weight PAHs bound to particulate matter can readily deposit onto skin as particulate and be absorbed transdermally, VOCs like benzene and lower molecular weight PAHs like naphthalene typically remain in vapor phase, and up to 1% of benzene vapor may be absorbed directly through skin (Franz 1984; Thrall et al., 2000). Additionally, these volatile compounds can condense and be absorbed through skin, especially if they are trapped against the skin rather than allowing for evaporation. Therefore, firefighters could absorb these volatile compounds transdermally if the compounds are able to permeate or penetrate the protective envelope of their full PPE ensemble.

The purposes of this study were threefold: 1) quantify the VOC (i.e., benzene, toluene, ethylbenzene, xylenes) and naphthalene concentrations inside and on the outside of turnout jackets worn by firefighters simulating fireground operations as part of training in the FES, stratified by treatment (new vs. laundered), 2) characterize the biological uptake of VOCs through breath samples taken following the fireground simulation for three PPE ensembles stratified by hood design and treatment of PPE (new knit hood, new turnout jacket and new turnout pants vs. new particulate-blocking hood, new turnout jacket and new turnout pants vs. laundered particulate-blocking hood, laundered turnout jacket and laundered turnout pants), and 3) explore the relationship between the VOC concentrations on the outside and inside turnout jacket and the VOC breath samples. This study was undertaken to increase our understanding of how different hood designs and laundering of turnout jackets, turnout pants, and hoods affects firefighters' exposures to VOCs.

2. Methods

2.1. Participants

Twenty-four firefighters were recruited from fire departments across 14 states in the United States of America (USA). The firefighters (23 male, 1 female; mean age: 39.3 years old) were required to have undergone a medical evaluation consistent with National Fire Protection Association (NFPA) Standard 1582 within 12 months prior to conducting the study (NFPA 2018a) and be fit tested for the SCBA used. Tobacco use was an exclusion criterion. Participants provided informed consent indicating they understood and voluntarily accepted the risks and benefits of participation in this study. This study was approved by the University of Illinois Institutional Review Board (IRB) (IRB approval # 17839).

2.2. Study protocol

The study protocol is described in detail elsewhere (Horn et al., 2020; Kesler et al., 2021). Briefly, the FES was developed from a steel intermodal shipping container, with the middle section serving as a combustion chamber generated by burning a commercially available sofa, and fire effluent funneled into two exposure chambers with 6 firefighters (3 in each chamber) undergoing training operations simultaneously (Horn et al., 2020; Mayer et al., 2020). Timing with the ignition of the sofa and ventilation of the exposure chambers was coordinated to create conditions that were similar to what is experienced during typical fireground operations. The scenarios were standardized to take 11 min (from ignition to firefighters exiting the FES). Four separate stations were set up for training and to simulate firefighting activities, including stair climbing (three steps up and down outside of the smoke chamber), crawling inside the chamber to simulate search as the chamber began to fill with smoke, hose advance inside the chamber (after which the sofa fire was suppressed by research staff members) and overhaul as the chamber doors were opened to allow smoke to passively vent to the environment (Table 1). All activities were conducted on 2-min work/rest cycles (e.g., 2-min stair climb, 2-min rest, 2-min search, etc.). After firefighting activities were completed, firefighters, while still on SCBA, were transported to an upwind processing tent where turnout jackets, turnout pants, and hoods were doffed.

2.3. Study design

Firefighters participated in groups of three while wearing one of

Table 1
Study protocol for simulated firefighting activities in the fireground exposure simulator (FES).

Time (Min)	Firefighter job assignment or task (n = 6 for each scenario)	Burn scenario
0:00	Stairs	Background – Exposure doors open
1:00		
2:00	Transition to FES & Rest	
3:00	Rest	Close Exposure Doors, Ignition
4:00	Search	
5:00		
6:00	Rest	
7:00		
8:00	Hose advance	
9:00		Suppression (~15 s)
10:00	Rest	Open front burner door
11:00		
12:00	Overhaul	Open exposure doors to vent
13:00		
14:00	Leave chamber for post-test	

three different PPE ensembles (all PPE were certified to the NFPA 1971 standard (NFPA 2018b)) including:

1. New Nomex® Knit Hood, New Turnout Jacket, and New Turnout Pants – Turnout gear (including jacket and pants) and hoods were new for the first trial and laundered between each wear, with a maximum of three launderings prior to completion of the study.
2. New Nomex Particulate-Blocking Hood, New Turnout Jacket, and New Turnout Pants– Turnout gear and hoods were new for the first trial and laundered between each wear, with a maximum of three launderings prior to completion of the study.
3. Laundered Nomex Particulate-Blocking Hood, Laundered Turnout Jacket, and Laundered Turnout Pants– Particulate-blocking hoods and turnout gear were exposed to smoke and laundered following NFPA 1851 guidelines (NFPA 2020) 40 times (protocols reported elsewhere (Horn et al., 2021) prior to human subject trials. Laundered particulate-blocking hoods were the same model and from the same manufacturer as the new particulate-blocking hoods.

Turnout jackets and pants were assigned to each firefighter based on chest and waist size. All protective hoods, turnout jackets, and turnout pants were laundered between each wear in a front-loaded extractor with warm water and detergent. Gear was subsequently transferred to a forced air cabinet at 105 °F to dry. For turnout jackets and pants, outer shell (Kevlar®/Nomex), moisture barrier (ePTFE film) and thermal liner (Kevlar/Lenzing FR® face cloth with Nomex batting) were selected because of their common use at the time of this study. Knit hoods were compliant two-layer Nomex material while particulate-blocking hoods had three layers, including an outer and inner layer of knit Nomex and a third interstitial layer (Horn et al., 2021).

This study was designed to evaluate the ingress of VOCs and naphthalene characterized by cleaning treatment and hood technology (1. new knit vs. 2. new particulate-blocking vs. 3. laundered particulate-blocking) worn by firefighters. Personal air sampling media was placed on the outside and inside of the turnout jacket prior to firefighters donning PPE. After firefighters completed the scenario and doffed their PPE, air sampling media was recovered by researchers. Firefighters then entered the data collection bay and provided post-fire exhaled breath samples to characterize the biological uptake of VOCs stratified by the three PPE ensemble and treatments. Pre-fire exhaled breath samples were provided prior to the scenario.

2.4. *Personal outside and inside jacket air sampling*

Personal air samplers (6 × 70-mm glass charcoal tubes and 13–8 X 75-mm glass OVS-XAD-7) were mounted on the outside of the turnout jacket at chest height to determine the magnitude of combustion byproducts (VOCs and PAHs, respectively) directly outside the firefighter's PPE ensemble. While VOCs were quantified for every fire response event (N = 72), PAHs were quantified for a subset of the population (N = 48). Pumps were calibrated using a low or medium flow Drycal Defender (MesaLabs, Lakewood, CO). All air samples had post-calibration flow rates that were within 5% of the pre-calibration flow rate. Pre-calibration flow rates were based on the target flow rates of 0.1 L/min for charcoal tubes and 1.0 L/min for OVS-XAD-7 tubes. One field blank was collected during each fire for each type of sampling media. After each trial, the samples were collected, capped, and stored in a freezer. The charcoal tubes were analyzed using NIOSH Method 1501 for BTEX (benzene, toluene, ethylbenzene, and xylenes) (NIOSH 2013). The OVS-XAD-7 tubes were analyzed using NIOSH Method 5506, and a subset of the samples were analyzed separately for particulate (captured on the filter) and vapor-phase PAHs (captured on the sorbent) using NIOSH Method 5506 (NIOSH 2013). Of the PAHs analyzed in this study, only naphthalene results are reported here because it is the most volatile PAH and has previously been the most abundant PAH found under hoods/jackets (Mayer et al., 2020). Other PAH results including total PAHs have been reported previously (Horn et al., 2020) and are available in Supplemental Materials (Table S1). The sampling time for outside personal air samples ranged from 6 to 11 min for OVS-XAD-7 samples and 11 min for charcoal tubes.

Passive personal air samplers (Tenax TA thermal desorption tubes) were clipped in the pocket inside the jacket of a subset of firefighters (N = 48) to determine the magnitude of VOCs and naphthalene inside the turnout jacket. One field blank was collected during each fire. The majority of field blanks resulted in non-detectable concentrations for all VOCs and PAHs, though negligible background levels of naphthalene were reported on some samples. After the fire was suppressed, the inside jacket passive air samples were still inside the enclosed jacket while the firefighters were transported from the FES to the air sampling process tent. Once the firefighters doffed their turnout jackets, the inside PPE samples were recovered, capped, and stored in a freezer in a manner consistent with the outside personal air samples. The tubes were thermally desorbed and analyzed following EPA TO-17 (EPA 1999). The sampling time for inside jacket personal air samples ranged from 13 to 19 min. Diffusion rates used in this study (1.3 ng/ppm*min for benzene, 1.67 ng/ppm*min for toluene) were reported in ISO Standard 16017–2. Diffusion rates used for naphthalene (2.14 ng/ppm*min) were reported in Lindahl et al. (2011). We multiplied the diffusion rate by the sample time, and then we divided the ng reported on the tube by this number. Results were then multiped by 1000 and reported as parts per billion (ppb). Naphthalene concentration was then converted to μg/m^3 to make results directly comparable to outside jacket samples.

2.5. *Exhaled breath sampling*

Exhaled breath samples were collected from firefighters before and immediately after each fire (n = 144 person events). Collections took place inside a laboratory building upwind of the FES and well after fire suppression was complete. Firefighters were instructed to take a deep breath in and then forcefully exhale their entire breath into the Bio-VOC™ sampler (Markes International, Inc., Cincinnati, OH), which serves to collect 129-mL of breath. This process was then repeated, resulting in 258-ml of breath for each sample collection. The collected air was pushed through Markes thermal desorption tubes (Carbograph 2TD/1TD dual bed tubes). The thermal desorption tubes were capped and stored at −20 °C until shipment to the U.S. Environmental Protection Agency (EPA) analytical laboratory. A field blank was collected during each sample collection period.

The method used to analyze the breath samples is described in detail elsewhere (Geer Wallace et al., 2017). Method detection limits (MDLs) ranged from 0.70 ng/tube for benzene to 1 ng/tube for toluene. The ng on tube was converted to ng/L by dividing by the total breath volume collected (0.258 L) and results are reported as parts per billion volume (ppbv).

2.6. Data analysis

Descriptive statistics were displayed as number of samples (N), number below limit of detection (N of non-detects), mean, median, and range for air concentrations by treatment and sampling location and for exhaled breath concentrations by PPE ensemble including hood design and treatment. Because we did not expect outside and inside turnout jacket results to be impacted by hood type, we presented these data stratified only by treatment (new vs. laundered), combining the results from the new knit and new particulate-blocking groupings. By contrast, hood type and treatment could influence the amount of benzene absorbed (i.e., exhaled breath concentrations), so this data has been stratified by the three different PPE groupings (1. new knit vs. 2. new particulate-blocking vs. 3. laundered particulate-blocking). LOD divided by square root of two was assigned to non-detectable concentrations due to moderate skewness (Hornung and Reed 1990).

Concentrations for air and exhaled breath samples were log transformed because corresponding distributions were skewed to the right. A paired *t*-test was utilized to examine whether the change in exhaled breath concentrations from pre to post-fire was significantly different from zero. Multiple comparisons were conducted to determine significant differences of concentrations from pre to post-fire among hood designs.

Univariable analyses were carried out using the exhaled breath concentration from pre to post-fire as the dependent variable. A mixed model with individual firefighter as a random effect was utilized to account for the statistical correlation among repeated measures from the same firefighter. Covariates treated as fixed effects included sampling location (outside and inside jacket samples) and treatment (new particulate-blocking and laundered particulate-blocking; no inside jacket samples were taken from the new knit grouping so they were omitted from this comparison). Due to the expected day-to-day variation between trials, the date of data collection was adjusted for in all models. Pearson correlation coefficients and corresponding statistical testing were also provided to measure and examine the linear correlations between the dependent variable and covariates. Statistical tests were two-sided at the 0.05 significance level. All analyses were performed in SAS version 9.4 (SAS Institute, Cary, NC).

3. Results

3.1. Personal VOC outside and inside jacket air concentrations

Table 2 outlines benzene and toluene outside and inside jacket personal air concentrations, stratified by treatment (new jacket vs. laundered). Benzene concentrations, both inside (new jacket median = 65,400 ppb; laundered jacket median = 62,200 ppb) and outside (new jacket median = 72,200 ppb; laundered jacket median = 75,900 ppb) the jacket were an order of magnitude higher than toluene concentrations inside (new jacket median = 1060 ppb; laundered jacket median = 1070 ppb) and outside jacket (new jacket median = 1890 ppb; laundered jacket median = 1790 ppb). There was a 9.7% median reduction in benzene concentrations measured inside the jacket compared to the outside concentrations for new jackets, while a 11.7% median reduction was observed for laundered jackets. However, the minimum benzene concentration found inside new jackets (727 ppb) was lower than the minimum concentration found inside laundered jackets (20,900 ppb). There was a 43.6% and 43.9% median reduction in toluene concentrations measured from inside the jacket compared to concentrations outside the jacket for new and laundered jackets, respectively. Overall, there were no significant differences in the % median reduction of benzene and toluene between the new and laundered groups. We analyzed for ethylbenzene and xylenes as well, but inside jacket samples had a detection rate below 50%, so these analytes were excluded from all analyses.

Table 3 summarizes naphthalene outside and inside jacket personal air concentrations after fireground exposure, which were analyzed for a subset of the study population. Naphthalene concentrations measured inside the jacket were much lower than concentrations reported outside the jacket (median reduction = 92.5% for new jacket, median reduction = 94.4% for laundered jacket). There were no significant differences between the % median reduction of naphthalene for the laundered and new jacket. The filter and sorbent of the OVS were measured separately for some (n = 17 new jacket, n = 7 laundered jacket) of the outside jacket samples. As expected, the vast majority (new jacket = 96.1%, laundered jacket = 98.7%) was captured on the sorbent, indicating naphthalene was primarily present in the chamber in vapor or gas phase.

3.2. VOC exhaled breath concentrations by PPE ensemble

Table 4 and Fig. 1 summarize the change in benzene exhaled breath concentrations (ppbv) from pre-to post-fire stratified by PPE ensemble (1. new knit vs. 2. new particulate-blocking vs. 3. laundered particulate-blocking). Firefighters in all three conditions had breath concentrations of benzene significantly increase (p-value < 0.05) from pre to post-fire. There were no significant differences in the amount of increase across the 3 conditions. The change in toluene concentrations from pre-to post-fire were also evaluated, and the new knit hood group saw increases that were significant (p-value = 0.050) (Fig. 2; Supplemental Materials, Table S2). To provide perspective, we compared the magnitudes of increasing benzene breath concentrations here to previous studies of firefighters (Supplemental Materials, Figs. S1 and S2). Overall, the change in benzene concentrations in breath here were similar to those measured in our previous study involving a controlled residential fire response. However, firefighters' breath concentrations of benzene in a study involving training fires with common fuel packages (e.g., pallet and straw and oriented strand board) did not increase as much as what we observed here.

Table 2
Benzene (ppb) and toluene (ppb) personal air concentrations collected from outside turnout jackets and inside turnout jackets during fire exposure stratified by treatment.

Analytes	Treatment	Sampling Location	N	N of Non-Detects	Mean	Median	Range	Median % Reduction
Benzene	*New jacket*	*Outside jacket*	48	0	83,800	72,200	18,100–169,000	9.7%
		Inside jacket	24	0	70,900	65,400	727–172,000	
	Laundered jacket	*Outside jacket*	24	0	83,200	75,900	17,700–251,000	11.7%
		Inside jacket	24	0	69,700	62,200	20,900–169,000	
Toluene	*New jacket*	*Outside jacket*	48	0	2260	1890	333–5320	43.6%
		Inside jacket	24	1	1220	1060	<LOD – 3960	
	Laundered jacket	*Outside jacket*	24	0	2270	1790	345–6990	43.9%
		Inside jacket	24	0	1170	1070	329 – 3350	

Table 3
Personal naphthalene (μg/m^3) air concentrations collected from outside turnout jackets and inside turnout jackets during fire exposure stratified by treatment.

Analytes	Treatment	Sampling Location	N	N of Non-Detects	Mean	Median	Range	Median % Reduction	% on Filter, % on Sorbent
Naphthalene	*New jacket*	*Outside jacket*	34	0	70,700	39,100	7640–344,000	92.5%	3.9%, 96.1%
		Inside jacket	24	8	1130	948	<LOD – 6250		N/A
	Laundered jacket	*Outside jacket*	14	0	93,800	43,000	8910–332,000	94.4%	1.3%, 98.7%
		Inside jacket	24	8	963	829	<LOD – 5020		N/A

Table 4
Change (post-pre) in benzene concentrations (ppbv) in exhaled breath samples collected from firefighters stratified by hood design and treatment.

Analytes	Hood Design/Treatment	N	Mean	Median	Range	p-value (Testing Difference of Post and Pre)	p-value (Comparisons of Hood Designs)	
Benzene	*New-Knit (K)*	24	17.0	15.6	0.94–42.6	**<0.001**	Reference	
	New-Blocking (B)	24	23.4	18.3	−0.85–105	**<0.001**	0.163	Reference
	Laundered-Blocking (L)	24	21.3	12.1	0.30–80.5	**<0.001**	0.344	0.645

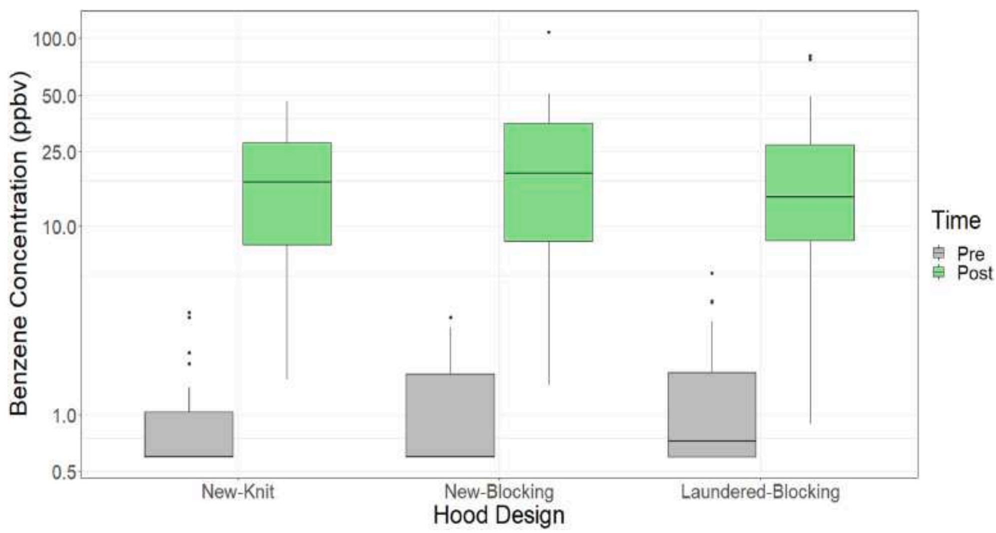

Fig. 1. Box-and-whisker plot of exhaled breath concentrations of benzene (ppbv) by PPE ensemble including hood design/treatment and sample collection time. The box represents the interquartile range (IQR), the horizontal line in each box represents the median, the upper whisker represents the upper fence 1.5 IQR above the 75th percentile, the lower whisker represents the lower fence 1.5 IQR below the 25th percentile, and the dots represent potential outliers.

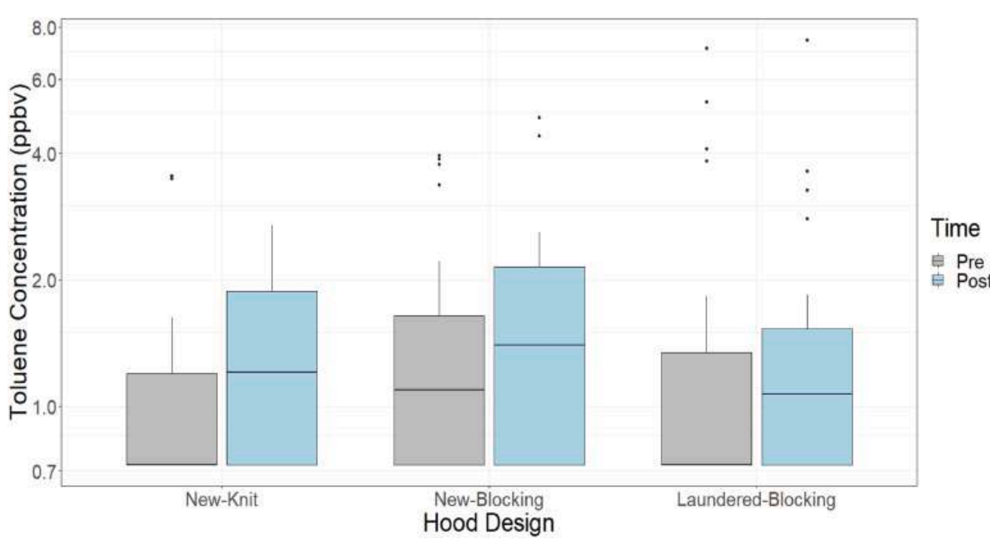

Fig. 2. Box-and-whisker plot of exhaled breath concentrations of toluene (ppbv) by PPE ensemble including hood design/treatment and sample collection time. The box represents the interquartile range (IQR), the horizontal line in each box represents the median, the upper whisker represents the upper fence 1.5 IQR above the 75th percentile, the lower whisker represents the lower fence 1.5 IQR below the 25th percentile, and the dots represent potential outliers.

3.3. Relationship between personal air and exhaled benzene concentrations

Table 5 summarizes the relationship between personal air and exhaled breath benzene concentrations for those wearing particulate-blocking hoods. Outside and inside jacket personal air concentrations of benzene (ppb) were both significantly associated (Pearson r = 0.620 and 0.593; p-values = 0.015 and 0.014, respectively) with the difference in firefighters pre-to post-fire exhaled breath concentrations of benzene (ppbv). However, the two correlation coefficients were not significantly different from each other (p-value = 0.723). Table S3 summarizes the relationship between personal air and exhaled breath toluene concentrations. No significant relationships were found between inside or outside jacket air sampling results and the difference in firefighters pre-to post-fire exhaled breath toluene concentrations.

When we stratified by treatment (Table 5), the association between the outside and inside jacket air concentrations of benzene and the difference in firefighters' pre-to post-fire exhaled breath concentrations of benzene was only statistically significant for the laundered group (p-values 0.006 and 0.049, respectively), but not the new group. For the laundered group, the outside personal air concentrations (Pearson r = 0.698) were slightly more correlated with exhaled breath concentrations compared to inside jacket benzene concentrations (Pearson r = 0.616), but the two correlation coefficients did not differ significantly from each other (p-value = 0.336). The change (post-pre) in toluene concentrations were only significantly related to the inside jacket personal air concentrations for the new group (Table S2).

4. Discussion

This study evaluated the protection of three different PPE ensembles characterized by cleaning treatment and hood design (1. new knit vs. 2. new particulate-blocking vs. 3. laundered particulate-blocking) that were worn by firefighters conducting training and simulating firefighting operations in a smoke-filled fireground exposure simulator. Specifically, this study characterized benzene, toluene, and naphthalene exposures for firefighters through personal outside and inside jacket air samples. Pre- and post-fire benzene and toluene exhaled breath concentrations were also quantified. Our results suggest that firefighters absorb combustion byproducts regardless of which of the three types of PPE ensembles that were included in this study.

4.1. Comparing personal VOC outside and inside jacket air concentrations by treatment

Personal outside jacket benzene concentrations (medians 72,200–75,900 ppb) in this study are higher than those reported in similar studies involving controlled training fires (median 37,900–40,300 ppb; 3000–31,700 ppb) (Fent et al. 2018, 2019a) and well above the NIOSH short term exposure limit (STEL) of 1000 ppb (NIOSH 2020). However, benzene concentrations from this study are an order of magnitude lower than those reported in our previous mannequin study that made use of the same fireground exposure simulator (medians 187,000–314,000 ppb) (Horn et al., 2020; Mayer et al., 2020) where the samplers were generally higher in the chamber and stationary. In the current study, firefighters were simulating fireground operations by crawling and staying lower in the structure which likely reduced the exposures (Horn et al., 2020). Overall, toluene concentrations outside the turnout jacket were well below the NIOSH STEL (150, 000 ppb; NIOSH, Pocket Guide). Outside jacket personal naphthalene concentrations (medians 39,000–43,000 μg/m^3), on the other hand, were well above the ACGIH excursion limit for coal-tar pitch volatiles (1000 μg/m^3; ACGIH 2018).

The laundered hoods, pants and jackets worn by firefighters in this study were previously placed on mannequins and repeatedly exposed and laundered 40 times. Ambient chamber and inside jacket benzene concentrations were characterized during four of the exposure trials (1st, 10th, 20th, and 40th), which revealed a trend where benzene ingress decreased slightly with each trial representing more laundered PPE (Mayer et al., 2020). We hypothesized that the softening of the turnout jacket textiles with repeated laundering might lead to a tighter fit on mannequins. However, in the current study, the two conditions (new vs 40-times laundered) did not significantly differ from each other in terms of protection, both showing relatively low level of protection from benzene (9.7–11.7% median reduction). Overall, laundering did not appear to impact the protection capability of turnout jackets for benzene. Due to the physical nature of firefighting that includes movements such as crawling, operating hand tools and handling hose lines, one could hypothesize that these physical actions, causing repeated compression and relaxation of air gaps in the PPE, could help draw air into the jacket and negate the positive impact of tighter fitting turnout jackets. Additional study is needed to further verify this hypothesis.

By contrast, we observed a higher reduction in toluene (43.6–43.9% median reduction) and naphthalene (92.5–94.4% median reduction) from outside to inside the turnout jacket than what was observed for benzene. These results are not entirely unexpected, as toluene and naphthalene have lower vapor pressures than benzene. As such, naphthalene and toluene are more likely to adsorb onto the fabric and other surfaces during the entrainment through or around the turnout jacket, resulting in lower concentrations inside the jacket. That combustion byproducts such as naphthalene and other higher molecular weight PAHs condense onto the turnout jacket and other station wear like hoods

Table 5
Correlation between inside and outside turnout jacket air samples (ppb) and the change in exhaled breath benzene concentrations (ppbv) stratified by treatment (excluding new knit hood grouping).

Outcome	Change in Pre- to Post-Fire Exhaled Breath Benzene Concentrations[a]			Pearson Correlation Coefficient	Testing the Difference of Correlation Coefficients Between Outside/Inside Jacket
Covariate	Estimate	SE	P-value		P-value
Outside Jacket Samples (B + L)	0.0002	0.0001	**0.015**	0.620	0.723
Inside Jacket Samples (B + L)	0.0002	0.0001	**0.014**	0.593	
Stratify by Treatment					
Outside New-Blocking (B)	0.0001	0.0002	0.645	0.547	0.801
inside New-Blocking (B)	0.0002	0.0001	0.116	0.578	
Outside Laundered-Blocking (L)	0.0003	0.0001	**0.006**	0.698	0.336
inside Laundered-Blocking (L)	0.0003	0.0001	**0.049**	0.616	

[a] The model was adjusted for date, a potential confounder.

and base layers that come in direct contact with skin is another justification for routinely laundering all of the various garment layers (e.g., base layer, station uniform, turnout jacket) worn during a fire response to reduce chronic exposure to these contaminants.

A subset of the outside jacket air samples were analyzed separately for gas and particulate phase of naphthalene, and the vast majority (>95%) of naphthalene was captured in the gas phase. This is to be expected in most occupational settings where naphthalene is produced but is especially likely under high heat conditions such as firefighting. Several studies have found that naphthalene is the most abundant PAH found in air samples taken from the fireground, and that it exists primarily in the gas phase (Fent et al., 2019a; Horn et al., 2020; Keir et al., 2020). It is important to note that the particulate-phase may be under-estimated using OVS samplers as the airflow across the filter will cause naphthalene to evaporate, but the impact of this is expected to be relatively minor over the short sampling periods in this study (<11 min). Relatively few studies have analyzed PAH concentrations inside turnout jackets, but Kirk and Logan (2015) found that naphthalene concentrations inside turnout jackets were an order of magnitude lower than outside the jacket. Overall, these findings suggest turnout jackets offer more protection against naphthalene and toluene compared to benzene.

Still, some of the naphthalene that penetrated through or around the turnout jacket could condense to the skin. In a previous study, we reported that naphthalene accounted for 75% of the total PAHs captured on PTFE filters under hoods placed on the neck region of mannequins (Mayer et al., 2020). Another recent study found naphthalene accounted for over 85% of total PAHs captured inside gear (Wingfors et al., 2018). It has been estimated that 10–30% of naphthalene applied to skin as a soil mixture can be absorbed dermally (Burnmaster and Maxwell. 1991). Previous studies have consistently measured increasing post-fire hydroxylated naphthalene urinary concentrations, even among firefighters who wore SCBA throughout the response (Fent et al., 2020). Our results indicate that naphthalene vapor ingress inside jackets and under hoods may be an important exposure pathway for firefighters and lead to dermal absorption of naphthalene. Further study is warranted.

4.2. *Impact of PPE ensemble on VOC exhaled breath concentrations*

When we compared the change in exhaled benzene concentrations in breath from pre to post-fire stratified by the PPE ensemble groupings (1. new knit vs. 2. new particulate-blocking vs. 3. laundered particulate-blocking), we found significantly higher post-fire versus pre-fire results for all 3 ensembles (p-value < 0.05). The pre-to post-fire increase in breath concentrations of benzene observed for all 3 ensembles was consistent with results from our previous simulated residential fireground study (Fent et al., 2020) and significantly higher than our recent training fire study (Fent et al., 2019b). The pre-to post-fire change in benzene exhaled breath concentrations from the current study (median increase for the three PPE ensembles = 15.6, 18.3, and 12.1 ppbv, respectively) was higher than the pre-to post-shift change in median exhaled breath concentrations measured in automotive mechanics (1.9 ppbv for smokers and non-smokers), a population known to have low level benzene exposures (Egeghy et al., 2002). Interestingly, median pre-shift breath concentrations of benzene for smokers (10.7 ppbv) from Egeghy et al. (2002) were lower than most of the post-shift concentrations (median = 17.2 ppbv) reported in the current study, suggesting firefighters' benzene exposures from firefighting may be higher than from smoking.

Toluene exhaled breath concentrations appeared to moderately increase from pre to post-fire, though only those wearing new knit hoods saw a difference that was statistically significant (p-value = 0.050). The exhaled breath fraction we collected represents the gas-exchange region of the lungs. Hence, timing of breath samples is critical as the compound of interest would have to be absorbed into the blood stream, but not yet fully metabolized, in order to measure it in breath. A recent study found increased toluene metabolites in urine samples taken from firefighters' post-fire (Rosting and Olsen 2020). It is possible that urinary analysis of toluene might better capture fire response exposures.

Ambient air concentrations of VOCs encountered after doffing jacket could also impact breath levels, but measures were taken to minimize this potential confounder (i.e., firefighters doffed PPE upwind of the fires and entered a climate-controlled laboratory for breath collection). In previous research, we found that firefighters had increased breath concentrations of benzene after firefighting even when they kept breathing air from SCBA until right before breath collection (Fent et al., 2020), providing strong evidence of the dermal route of absorption.

Interestingly, there were not significant differences in the pre-to post-fire change in exhaled breath benzene concentrations among the three ensembles. This is likely due to the volatile nature of benzene and the high level of ingress observed across the PPE ensembles. Also, the type of hood (particulate-blocking or knit) did not appear to influence the uptake of benzene, likely because the hoods and PPE interfaces were not vapor tight. As such, the neck region is one of the areas where we might expect to see the highest rate of benzene absorption.

4.3. *Evaluating relationship between personal air and exhaled benzene concentrations*

We also examined the relationship between benzene concentrations measured outside and inside jacket and exhaled breath concentrations of benzene for those wearing particulate-blocking hoods. Both inside and outside jacket benzene concentrations were significantly positively associated with the change in exhaled breath concentrations, and the correlation coefficients were similar. Additionally, none of the PPE ensembles provided much attenuation for benzene vapors. Overall, this indicates that benzene in the fire environment is a strong predictor of the post-fire levels measured in breath. Because is it assumed that the firefighters were well protected from the inhalation route (wearing SCBA throughout the exercise), we believe much of the benzene in breath likely came from the dermal route.

Some in the fire service have expressed concerns regarding repeated laundering of firefighter PPE, in particular particulate-blocking hoods, because it might damage the blocking layer and allow increased penetration (Kesler et al., 2021). There are also some concerns about the potential for cross-contamination during laundering, in particular for chemicals that have low water solubility like polybrominated diphenyl ethers (PBDEs) (Mayer et al., 2019). Though they were looking at PAHs rather than benzene, Kesler et al. (2021) found firefighters wearing laundered particulate-blocking hoods had significantly lower PAH neck skin contamination compared to those who wore new particulate-blocking hoods. The authors speculated that laundering may impact the surface area and surface coatings of the fibers in the hoods, which allowed the PAH contamination to embed deeper within the material of the laundered hoods compared to the new hoods, potentially transferring less PAH contamination to the skin. In the current study, we found that firefighters wearing the laundered turnout jacket, pants, and particulate-blocking hoods had a change in exhaled breath concentrations of benzene that were significantly associated with outside (p-value = 0.006) and inside (p-value = 0.049) jacket air concentrations of benzene, but this was not the case for those wearing new turnout jacket, turnout pants, and particulate-blocking hoods. While this could suggest that laundered PPE ensembles provided less protection than the new PPE ensembles, firefighters who wore new turnout jacket and pants with particulate-blocking hoods actually had a greater median increase (from pre to post firefighting) in breath concentrations of benzene than those who wore the same type of laundered jacket (Table 4). Hence, caution should be exercised in inferring that these findings reveal a meaningful change in chemical protection.

4.4. *Limitations*

This study has several important limitations to consider when interpreting these results. Sample sizes were relatively small, so statistical power was somewhat limited in the comparisons that were made. Only three different ensembles were included in this study, so caution should be exercised in extrapolating these results to all firefighter PPE ensembles. Naphthalene was not quantified in exhaled breath concentrations, so we were not able to explore an association between inside and outside jacket naphthalene concentrations and exhaled breath naphthalene concentrations. Note, however, that naphthalene would be difficult to measure in breath because of its lower vapor pressure compared to other VOCs. Because benzene was measured at relatively high levels outside and inside jacket, and was also detected with high frequency in breath, it represented the most complete data set for analysis. Variations in arm, torso and leg length were not considered in the sizing of the jacket for each firefighter, which could theoretically allow for more ingress of contaminants inside PPE. However, inclusion criteria for this study required participating firefighters to fit in the range of PPE sizes available for this study.

4.5. Future work

Future studies could further examine how the combination of repeated wear, exposure, and cleaning of PPE may impact the structural integrity over a longer period of time (e.g., 4–5 years) to reflect real life scenarios where turnout jackets and pants may be washed sparingly (e.g., 3–4 times a year). Studies could also explore the breakthrough mechanism (e.g., diffuse through or around) for the PPE ensemble for volatile chemicals such as benzene to identify ways to reduce firefighters' dermal exposure. Further quantification of biomarkers of other combustion products (e.g., PAHs) that firefighters might be exposed to during a fire response would be beneficial, particularly after exposure reduction measures have been put in place to evaluate their effectiveness.

5. Conclusions

Benzene, toluene, and naphthalene were found to diffuse through and/or around firefighting turnout jacket, and the attenuation of benzene was especially low (9.7–11.7% median reduction). Repeated laundering of the PPE ensemble including the turnout jacket, turnout pants, and particulate-blocking hood up to 40 times did not appear to reduce the protective properties of this PPE from any of the compounds. However, the firefighters' PPE ensemble as currently designed does not appear to provide sufficient protection against the most volatile compounds like benzene. Although the turnout jacket provided more attenuation against naphthalene and toluene than benzene, ingress still occurred. The change (post-pre) in exhaled breath benzene concentrations was significant for all three PPE ensembles evaluated (p-value < 0.05), suggesting the type of hood used in this study did not impact the level of protection. Air concentrations of benzene measured outside and inside turnout jacket were also significantly correlated with the pre-to post-fire change in exhaled breath concentrations of benzene in the firefighters (despite the use of SCBA). This suggests that ingress of certain volatile substances through or around the protective barriers of turnout jacket and protective hoods will contribute to the biological levels in firefighters via the dermal route, in addition to the potential for inhalation exposures after SCBA has been removed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113900.

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Co-development of a risk assessment tool for use in First Nations water supply systems: A key step to water safety plan implementation

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ABSTRACT

Despite several years of targeted interventions, First Nations drinking water systems in Canada remain under-resourced and require substantial improvements in both infrastructure and management to provide communities with safe drinking water. The purpose of this study was to co-develop a risk assessment process integral to the water safety planning methodology to determine if proactive risk assessment provides a beneficial management tool for First Nations water systems. We co-developed a risk assessment web-application with First Nations stakeholders to identify hazards and assess risk in six Atlantic region First Nations communities. Using this application, we were able to successfully identify high-risk hazards in each community, both risks specific to individual systems, and risks common at a regional level. Through semi-structured interviews we identified the following benefits of a risk assessment web application: increased communication, data ownership and centralized data management. However, challenges remain, including current fragmented governance realities, and liability concerns associated with adopting a new risk management strategy. Successful adoption of proactive risk management strategies in First Nations communities will depend on strong co-development of risk assessment tools, transparent communication between stakeholders and clearly defined data ownership and management practices.

1. Introduction

Indigenous communities in Canada experience more frequent and long-standing drinking water advisories, inadequate water supply and unacceptable water quality than non-Indigenous communities (Eggerston, 2008; Neeghan Burnside Ltd., 2011; Health Canada 2014; Murphy et al., 2015; Black and McBean, 2017a; Indigenous Services Canada, 2019). Despite decades of federal policy and program interventions, water system services remain disproportionally poor on reserves. The First Nations Water Management Strategy (2003–2008), the 2006 Expert Panel on Safe Drinking Water for First Nations, and the Plan of Action for First Nations Drinking Water (2006–2012) represent federal efforts aimed to secure safe drinking water for First Nations communities across Canada that have resulted in limited success (Morrison et al., 2015; Office of the Auditor General of Canada [OAG], 2021). Numerous studies have found that fragmented jurisdictional complexities (Swain et al., 2006; McCullough and Farahbakhsh, 2012), federal government-centric authority, limited community consultation (McCullough and Farahbakhsh, 2012; White et al., 2012), decreased community capacity (White et al., 2012), and infrastructure and resource gaps (Neegan Burnside Ltd, 2011; OAG, 2021) all present significant barriers to the provision of safe drinking water in First Nations communities.

Canadian and United Nations experts have stressed the importance of building water safety in First Nations communities from the bottom up through substantive community engagement and co-development (Swain et al., 2006; Anaya, 2013). The findings of the 2006 Expert Panel on Safe Drinking Water for First Nations identified the need to build capacity, make better use of existing resources, and find ways to work together with First Nations in a “formal, ongoing framework” (Swain et al., 2006, p. 59). The United Nations Human Right Council’s 2013 Report of the Special Rapporteur on the Rights of Indigenous Peoples included a summary of the situation of Indigenous peoples in Canada. The report concluded that “real partnership[s] with aboriginal peoples, through their own representative institutions, are vital to establishing long-term solutions.” Building First Nations operational

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capacity and competency through training and investment in human capital have been identified as key challenges that have been largely unaddressed or under-funded by federal programs (St. Germain and Sibbeston, 2007; Hrudey, 2013). Given the calls for community - government partnerships and increased support of operational capacity as necessary steps for safe drinking water in First Nations communities, an alternative approach to water systems management is needed. Beyond addressing the long-standing funding gaps that threaten drinking water safety (OAG, 2021), First Nations need a management framework that promotes co-development and knowledge mobilization between communities and the multiple federal agencies that are tasked with the provision of safe drinking water (Wilkes, 2011).

Water Safety Planning (WSP) is an international framework developed by the World Health Organization in 2004 that utilizes local system knowledge and risk management strategies to continuously improve drinking water quality (WHO, 2011). WSP is a cyclical and adaptive framework built on proactive hazard identification, risk mitigation, and operator knowledge, rather than traditional "end-of-pipe" water quality requirements (WHO, 2011; Hrudey, 2013). While there has been widespread implementation of WSP globally (WHO & IWA, 2017), there has been limited adoption within Canada at the provincial and regional levels, most notably in both Alberta and Ontario (AESRD, 2013; OMOE, 2013). Recently, there has been interest in using WSP in First Nations communities due in part to the inherent bottom-up nature of the management approach and the focus on operational competency and capacity (Hasan et al., 2011; Rondi et al., 2015). Work by Bradford et al. (2017) presents examples of indigenizing water governance in Canada and highlights the importance of strategies that promote "power with" policies instead over "power over" (Bradford et al., 2017). A study by Black and McBean (2017a) investigated how a WSP method could be adapted for Indigenous communities to advance the decolonization of water governance. The study found that bottom-up participatory methods possible through a WSP approach are paramount for Indigenous communities because they allow for re-engagement and re-empowerment around Indigenous decision-making. However, the authors noted that if WSP implementation is a part of a top-down or one-size-fits-all approach from the federal government, it will repeat the past mistakes of previous federal programs that fail to consider Indigenous realities. While some research exists on the applicability of WSPs in small and rural systems in Canada (Perrier et al., 2014; Lane et al., 2018; Kot et al., 2011), limited work has been done in collaboration with First Nations communities.

The purpose of this work was to engage in collaborative development with First Nations communities to create a risk assessment web-based application (RAWA) to facilitate the hazard identification and risk assessment processes central to a WSP management approach. The complete WSP cycle, as defined by the WHO WSP Handbook for small community water supplies, includes six steps and is intended for iterative incremental improvement of water treatment systems (WHO, 2012). The full cycle is presented in the supplemental information in Fig. S1. This research primarily addressed step 3 in the WSP cycle (hazard identification and risk assessment) through the development and refinement of risk assessment processes co-developed with First Nations stakeholders.

The objectives of this study were to: (1) co-develop a RAWA with First Nations water stakeholders in six communities to identify and prioritize hazards in the water treatment systems, (2) pilot the RAWA in the participating communities to evaluate water treatment system risks within and across communities and (3) determine the benefits and challenges of a risk-based approach to water management as a first step to implementing a community-driven WSP approach in First Nations communities.

1.1. Positionality statement

The authors of this study represent researchers from the Centre for Water and Resource Studies (CWRS) at Dalhousie University in Halifax, Nova Scotia. The CWRS, through its Director Dr. Graham Gagnon, has collaborated with the Atlantic Policy Congress of First Nations Chiefs Secretariat (APC) on numerous projects. This research was conducted with the support of the APC, which advocates a strong Indigenous voice supported by research and analysis, aimed at changing policies impacting First Nations. This study investigates the development and use of a RAWA in six First Nation communities, utilizing the CWRS and APC partnership to engage with community leaders and water system stakeholders. This project was conceived and conducted with the cooperation and consent of the First Nation community stakeholders involved. Stakeholders were consulted at multiple points during the research to ensure that data was accurately presented, communicated and ownership was clear in accordance with the First Nations Information Governance Centre principles of Ownership, Control, Access and Possession (OCAP) (First Nations Information Governance Centre, 2021). Dr. Lane visited the six communities and conducted the risk assessment development and research components of the study with community stakeholders between 2017 and 2019. Dr. Fuller provided insight to the results obtained from the WSP and aided in the preparation of the manuscript, providing critical revision of terminology and content. Mr. Dyment provided feedback during the initial development of the RAWA and provided a review of the thematic analysis and RAWA results in this paper prior to publication in his capacity as a First Nations drinking water system operator. Dr. Gagnon facilitated research activities with the APC to formulate the components of this study and guided the investigation of how an WSP framework could benefit First Nations communities. The authors acknowledge that we bring a post-positivist and pragmatist worldview to the evaluation of the WSP methodology in these water systems from our training as engineers. We have attempted to provide an analysis of how a WSP can address managing clean water in First Nation communities.

2. Material and method

The co-development of a First Nations informed web-based software application in six First Nations communities was achieved through ongoing community engagement, iterative software development cycles, and implementation and evaluation of the tool. The methodological approach is described in Fig. 1.

The four stages of the project included 1.) initial development of the RAWA, 2.) preliminary engagement session with First Nations communities and water stakeholders, 3.) co-development and implementation of the RAWA with participating systems, and 4.) a collaborative review process to receive feedback and refinement of the WSP tool.

2.1. Development of the RAWA

The RAWA developed for this study is a collection of risk assessment questionnaires organized with conditionally formatted question logic designed to estimate the likelihood and consequence of possible hazards in the drinking water treatment systems. The RAWA is designed to focus on the risk assessment component of the WSP process and CWRS researchers completed the initial steps of compiling hazards and defining system boundaries based on previous reports so that this research targeted the risk assessment process. Initial hazards and issues relevant to First Nations water systems were compiled by the authors prior to community visits, based on previous internal system assessment reports provided by the APC and hazard identification documentation from global case studies of WSP implementation (AESRD, 2013; New Zealand Ministry of Health, 2015). Questions and answers were drafted by the authors and risk score calculations were coded into the RAWA based on likelihood and consequence responses entered by users. Ideally, system experts would help draft potential hazards, but we created the initial hazards and related questions to avoid making the research process overly burdensome for participating operators and to facilitate the

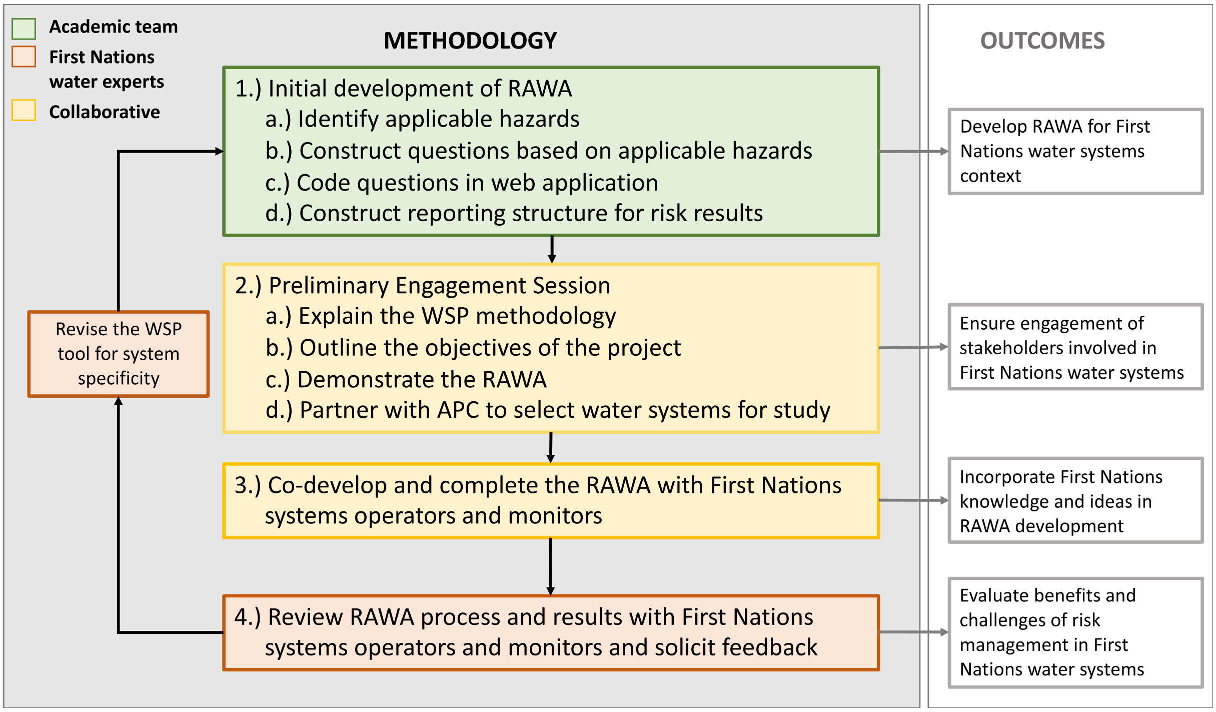

Fig. 1. The multistep process for developing and piloting the RAWA in participating communities Colours indicate which steps were led by the academic research team and First Nations water experts, and which steps were completed collaboratively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

necessary coding and database construction to build the RAWA. While later iteration did allow First Nations water experts to edit and alter hazard framing and question logic, it should be noted that this approach could have limited or influenced the hazard selection. The questions aimed to collect operator knowledge and system performance rather than directly attempting to quantify probabilities and impacts. This helped to prevent users from inadvertently answering questions in a biased way and placed emphasis on operator knowledge instead of the methodology used to quantify risk. A generic example of the question logic is shown in Fig. 2. Because question pathways were conditional on previous answers and because the question database includes hundreds of questions, it is not possible to present all questions included in these surveys here. A specific example of a conditionally formatted question from the chlorination survey, with associated question logic, is shown in Fig. S2.

Risk scores were calculated in the RAWA following a standard 5 × 5

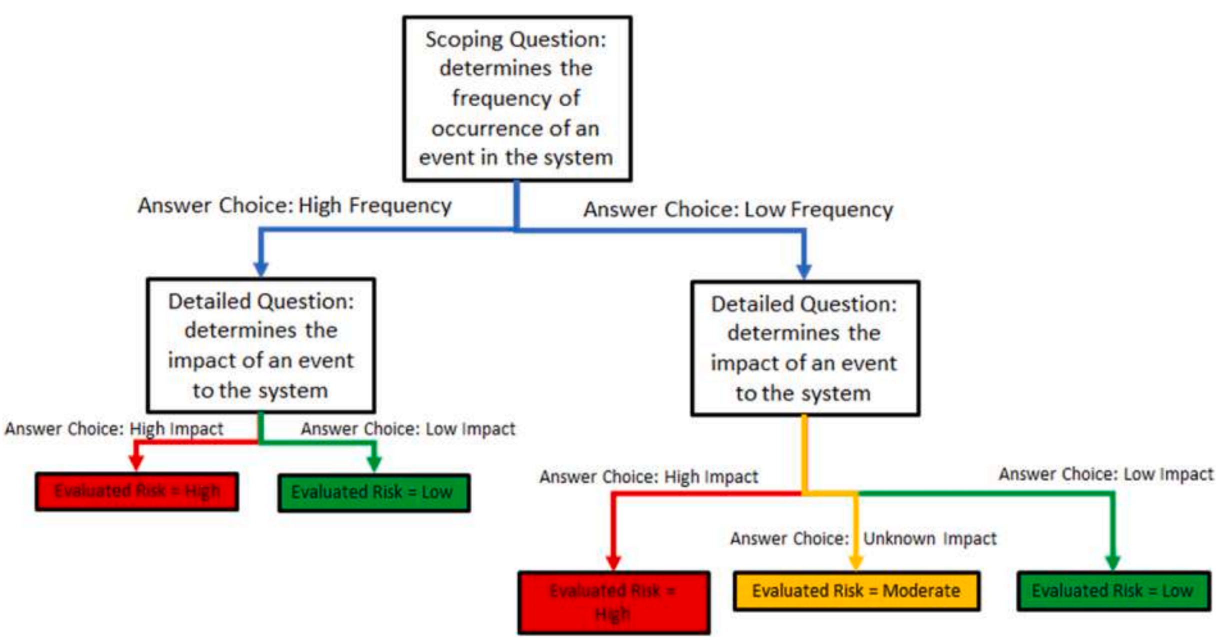

Fig. 2. General question logic to determine risk scores using likelihood and consequence indicators; both frequency and consequence can be found to have high, moderate, or low risk, this example shows a subset of possible outcomes. Total risk for a hazard is a product of frequency risk and consequence risk (total risk not shown here).

semi-quantitative risk matrix approach adapted from the Alberta Drinking Water Safety Plan approach (AESRD, 2013). CWRS researchers selected the risk matrix used for evaluation and First Nations community stakeholders provided insight into which answer choices should correspond to each level of risk during the review process. The matrix used to assign risk scores is shown in Fig. S3. Hazard events that were evaluated to have "unknown" risk were coded as moderate risk to account for the knowledge gap which itself presents a non-zero risk (Hokstad et al., 2009; Hrudey, 2012). The RAWA was programmed to assign a color system of red, yellow, green to demonstrate high, moderate, and low risk levels based on calculated risk scores. The definitions of high, moderate, and low risk levels are shown in Table 1.

We focused on the development of risk assessment questionnaires for both chlorine disinfection and distribution system hazards for this study because these water system components are shared by all six First Nations drinking water systems included in this study and represent areas where significant risks to safe drinking water are possible. The RAWA includes numerous additional system components, including a range of treatment technologies, source water hazards, etc. but the analysis of these surveys is beyond the scope of this work. The hazardous events included in the questionnaires were categorized as either maintenance, monitoring, or operations hazards. The hazardous events included in the chlorination and distribution questionnaires are shown in Tables 2A and 2B, respectively. Community characteristics are provided in Table 3.

The results from the RAWA were initially presented to stakeholders as a table. The results included the name of the hazard, the risk level assigned to the hazard, a description of the issue in the system and suggested activities to address the concerns. The information used to populate these fields came from CWRS researchers prior to review of the RAWA. Suggested activities were included to begin to discuss action items, a component of an improvement plan in water safety planning. However, after an initial review of the RAWA tool, we determined suggested activities and therefore action items are highly system specific. As a result, we have not included the discussion and co-development of suggested activities in this study although it is a critical component to future WSP success.

2.2. Preliminary engagement session and community selection

APC representatives selected the participating communities based on three primary water treatment system characteristics: source water type (surface water or groundwater), size of the water system based on population and water treatment ownership status (whether the treatment facility is owned and operated by a First Nations community or by a municipality under a municipal transfer agreement (MTA)). The community selection process also ensured that representation from the geographical regions across Atlantic Canada were included. Three First Nations water systems were selected in Nova Scotia (representing both mainland Mi'kmaq and Unama'ki), two in New Brunswick (representing both Wolastoqey and Mi'kmaq communities) and one in Prince Edward Island. Community names and other potential identifiers have not been included in this work to preserve anonymity in accordance with OCAP Principles (First Nations Information Governance Centre, 2021). In addition to direct community involvement throughout the research process, several reports from this study have been shared with and reviewed by the participating water system operators and stakeholders to ensure that the results of this project are being accurately communicated.

An engagement session was held in 2017 with water system stakeholders from First Nations water systems including water system operators, community-based water monitors (CBWM), and federal agency representatives (from Health Canada, First Nations and Inuit Health Branch (FNIHB), Indigenous Services Canada (ISC) and Indigenous and Northern Affairs Canada (INAC)), including environmental health officers (EHOs) who oversee the community-based water monitoring program in the participating communities. During the engagement session, the project goals were introduced, and the water safety planning process was explained. Federal representatives and EHOs were asked to provide feedback on risk assessment documentation and make recommendations for additional activities to be completed during the initial water system visits. Community engagement sessions were also held with First Nations communities and water system staff specifically to ensure that the First Nations Information Governance Centre information management principles of Ownership, Control, Access and Possession (OCAP) were upheld by the research team and to ensure the project was adhering to best practices for collaborative community-based research involving Indigenous populations (Black and McBean, 2017a, Bradford et al., 2017; Castleden et al., 2017; Kot et al., 2017; First Nations Information Governance Centre, 2021).

2.3. Co-develop and complete the RAWA with First Nations system stakeholders

The water operator of the participating facilities, the community-based water monitor for the communities (employed through FNIHB), and any secondary operators participated in the review and development of the risk questionnaires for both chlorination and distribution system hazard identification. The operators and monitors provided feedback and guidance on technical, cultural, and operational details of the both the questions and answers included in the RAWA. To facilitate collaboration, we visited the water treatment systems for a tour of the facilities and water systems to provide the research team with a better understanding of water system infrastructure and treatment capacity. During the visits, the operators and monitors completed the risk assessment questionnaires for their water systems through the RAWA interface. Operator and monitor feedback, guidance, and comments were used to revise the question language, content, and logic in the RAWA. All feedback and contributions were recorded by the research team and used to refine the RAWA to better define hazards and ascertain the risk levels present in First Nations systems. The RAWA results were used to produce risk reports for maintenance, monitoring, and operations hazards present in the chlorination and distribution system components.

2.4. Review of risk assessment report content and structure

Following the development of the RAWA and completion of the risk assessment questionnaires by the First Nations water system operators and monitors, the research team and First Nations stakeholders reviewed and refined the risk assessments. We collected participant responses through semi-structured interviews to elicit feedback. The questions used to structure the discussion are available in Table S1. The purpose of this discussion was to determine what components of the risk assessment reports were perceived as beneficial to water system management and what components needed revision. In addition, we attempted to understand how the RAWA could be adapted and leveraged as a long-term risk management strategy in these water systems.

Table 1
Definition of risk levels, risk scores, and risk assessment criteria used in the RAWA.

Risk Level	Risk Scores	Description of Risk (Risk Assessment)
Low	1–8	The product of likelihood and consequence indicates the hazard does not occur frequently or does not have a large impact on the system
Moderate	16–32	The product of likelihood and consequence indicates the hazard poses a moderate risk to the system and the production of safe drinking water
High	64–256	The product of likelihood and consequence indicates the hazard poses a significant risk to the system and the production of safe drinking water

Table 2
A & B. Hazardous events included in the RAWA.

(A) Chlorination hazards

Chlorination Hazardous Event	Category
Equipment Calibration	Maintenance
Equipment Failure	Maintenance
Equipment Replacement	Maintenance
Maintenance Plans	Maintenance
Compliance with Regulations	Monitoring
DBPs	Monitoring
Feedwater Conditions	Monitoring
Monitoring for High Chlorine	Monitoring
High Chlorine at Booster Stations	Monitoring
Monitoring for Low Chlorine	Monitoring
Chlorine Concentration	Monitoring
Chlorine Demand	Monitoring
Contact Time	Monitoring
pH Control	Monitoring
Low Chlorine at Booster Stations	Monitoring
Disinfection Failure Procedures	Operations
Backup Systems	Operations
Chemical Safety	Operations
Incorrect Dosing	Operations
Dosing Controls	Operations
Chlorine Supply	Operations
Chemical Supplier	Operations

(B) Distribution system hazards

Distribution System Hazardous Event	Category
Leaks in System	Maintenance
Pipe Breaks	Maintenance
Pipe Incidents	Maintenance
High Flow Entering Distribution System	Monitoring
Sediment Build Up	Monitoring
Biofilm Presence	Monitoring
Iron Precipitation	Monitoring
Manganese Precipitation	Monitoring
Chlorine Residual Degradation	Monitoring
Insufficient Water Available	Operations
Transmission Pump Failure	Operations
System Pressure Drops	Operations
Cross Connections	Operations
Flushing Procedures	Operations
Staff Training	Operations
Hygienic Sampling Procedures	Operations
Leak Isolation Procedures	Operations
Repair Materials	Operations
Bypasses	Operations

Table 3
Participating communities and key characteristics, including ownership status, source water, treatment and disinfection processes and number of stakeholders involved in the RAWA development.

Water System	Ownership Type	Source Water Type	Treatment Processes	Disinfection Process	Number of Stakeholders involved in co-development
CommunityA	Community owned and operated (non−MTA)	Groundwater	Disinfection	Chlorination	2
CommunityB	Community owned and operated (non−MTA)	Groundwater	Disinfection	Chlorination	1
CommunityC	Community owned and operated (non−MTA)	Groundwater	Disinfection	Chlorination Supplementary UV available	2
CommunityD	Municipal Transfer Agreement (MTA)	Surface water	Membrane Filtration and Disinfection	Chlorination	3
CommunityE	Community owned and operated (non−MTA)	Surface water	Membrane Filtration and Disinfection	Chlorination	3
CommunityF	Community owned and operated (non−MTA)	Ground water	Disinfection	Chlorination Supplementary UV available	1

This review process aimed to determine how a risk assessment strategy could support a WSP management approach in First Nations water systems and to identify potential challenges community driven WSP implementation could face. During the feedback sessions, we took hand-written notes to document responses. We digitized hand-written notes and then conducted a code and theme analysis (Creswell and Poth, 2012). The analysis focused on finding key ideas from stakeholder responses and condensing these ideas (codes) into themes to reveal core ideas expressed by stakeholders. We determined through consultation with the Dalhousie University Ethics Review Board that a full ethics application and review was not necessary to conduct this component of the research project as the operators and stakeholders were participating in their professional capacity.

3. Results

The quality and value of the output of the RAWA is entirely dependent on the appropriateness of the questions and the context and background of the unique position and history of First Nations water systems. The results of the RAWA tool development process are therefore, discussed first in this section to highlight the importance of co-development and the initial feedback First Nations stakeholders provided. The results of the RAWA questionnaires for the six participating communities are presented second in this section to characterize the types of information that were collected during the risk assessment process and show the variability in risk within and between participating communities. The thematic analysis is discussed last to examine the overall key considerations necessary for successful future examination of WSPs in First Nations.

3.1. RAWA tool development process

In general, operators and community-based water monitors viewed the RAWA as an added benefit for his or her water system. First Nations water experts were instrumental to revising and improving the RAWA; operators identified several key benefits as well as improvements to be made to the questionnaires. The results from the co-development of the RAWA and the overall themes uncovered in this study are present in Table 4 and discussed separately below.

3.1.1. Optimization of the risk assessments for First Nations systems through co-development

The co-development process with First Nation operators and monitors resulted in several key design features being included in the RAWA. The following elements of the RAWA tool were implemented following First Nations operator and water monitor collaboration and tool revision:

1. Inclusion of an overall risk score.
2. Development and presentation of suggested risk mitigation measures and actions.
3. Categorization of risk into maintenance, monitoring, and operational events to help understand risk type
4. Development of risk assessment questionnaire specifically for community-based water monitors
5. Use of the RAWA as a data repository for First Nations operational data storage and analytics

After reviewing the initial RAWA drafted by the research team, Operators indicated an overall risk score output from each risk assessment questionnaire and an overall risk score for the water system would be a useful feature. In the RAWA compiled by the research team, results were presented as a break-down of each possible hazard that could occur within an assessment (treatment process, distribution system, etc.) as shown in Figs. 4C and 5C. However, operators indicated that an overall risk score would improve their ability to communicate results to Chief and Council or to community members who do not work directly with the water system. Operators suggested the use of a gauge to visualize the overall risk score. The risk visualization approach initially developed by the authors for the RAWA is shown in Fig. 3A. Co-developed, First Nations informed risk visualizations shown in Fig. 3B demonstrate the importance of presenting risk visually. We elected to include both visualizations in the final RAWA to provide different ways of understanding the risk information at different levels of granularity. This

Table 4
Themes and supporting ideas produced from participant interviews and discussions.

	Theme	Supporting Ideas	Improvements to Tool
1	System specificity is critical to acceptance of risk-based management and WSP development	The questions provided are general enough to apply to many systems, but do not address the individual idiosyncrasies of a specific system	Add a comment section to each assessment to give each system the ability to adjust risk to better reflect each specific system
		Some systems are better equipped to meet water quality guidelines and the questions and hazards should reflect these crucial capacity differences	Revise questions in assessments based on operator and community water monitor feedback
2	The RAWA tool provides a centralized record keeping location	There is no current recordkeeping mechanism in many communities and the RAWA tool could provide a location to aggregate water system data and standard operating procedures	Provide several options for graphical visualizations and report styles an operator can generate to share the risk assessment results
		The RAWA could provide stakeholders with a method to develop standard operating procedures and management plans	Aggregate the results of the assessments by category (operations, maintenance, etc.) to aid in the generation of maintenance plans and standard operating procedures
		Having data centrally located could improve data integrity	Add a feature to allow operators and community water monitors to add water quality data to the assessment
3	The RAWA, and potentially WSP adoption, will facilitate communications among water system stakeholders	Several operators mentioned the utility of the WSP tool reporting functions, indicating these reports could provide critical evidence to Chief and Council where improvements are required	Generate a summary risk score for each community to communicate the overall system status to regional or federal agencies
		The WSP tool results can be used by regional or federal agencies to determine regional concerns that need to be addressed in First Nations water systems	
		In MTA systems, the WSP tool could provide a common platform by which operators from the municipality can connect with community-based water monitors	Develop an assessment specific to the community-based water monitor to gather knowledge from each stakeholder
4	Ability to interact with data from RAWA provides stakeholders with stronger data ownership	Increased visual representation of the results from the WSP tool would aid stakeholder understanding of the critical hazards in a water system	Provide several options for graphical visualizations and report styles an operator can generate to share the risk assessment results
		Operators expressed a desire to interact with	

(*continued on next page*)

Table 4 (*continued*)

	Theme	Supporting Ideas	Improvements to Tool
		RAWA results more to generate several different reports to communicate with other water system stakeholders	
		Having the risk assessments in a web application form provides operators with the ability to easily generate reports and keep centralized records	Add suggestions for improvements to the water system in the results reports generated from the tool to aid community decision-making and prioritization of risks to be addressed
5	There remain questions as to how stakeholders will address responsibilities and liabilities	Several stakeholders expressed concern with liability. If the operator uses the WSP tool, is the operator then liable for the information generated and decisions made from this data?	Create a login feature for each community that allows several stakeholders from a community to contribute to the WSP and complete the assessments
		In many First Nations systems, there are multiple operators. Will both operators be liable or responsible for completing the WSP?	Create a login feature which allows each operator to complete the assessments separately
		Many operators are trained to operate the water system but not certified. Does this impact the operator's ability to complete the WSP tool?	

feature could facilitate standardized reporting to regional or federal authorities to fulfill yearly risk management requirements (such as the annual performance inspections carried out by INAC).

Results at the end of each risk assessment were presented as a table (Fig. 3A) containing the hazard, the risk level identified through the assessment process, the category of hazard, potential control measures for the hazard, a description of any identified issues and general suggested activities a water system can perform to mitigate risk. Operators indicated the suggested mitigation activities column could be improved by tailoring the activities with specific recommendations to lower the identified risk level. First Nations stakeholders also suggested adding a feature allowing operators and monitors to add comments that can then be included in the detailed risk report to provide context for Chief and Council. This would provide clear explanations why hazardous events are identified as moderate or high risk and limit miscommunication between all water system stakeholders, providing better quality and communicable results.

Operators indicated characterizing hazards by category (maintenance, monitoring or operational events) was a useful way to view the results from the risk assessments. Categorization allowed an operator to quickly identify which sections of their water systems need immediate attention (high risk), which situations can be relegated to future improvement initiatives (moderate risk), and which areas are being well managed (low risk). For example, in Community E, maintenance issues in the chlorine disinfection systems are higher priority than monitoring or operations activities (Fig. 4B). Categorizing risk also provides another aggregation of hazards that can be used by regional or federal agencies to quickly evaluate where issues in a system exist prior to investigating individual hazards and aids in the understanding of patterns of risk across systems.

When completing the risk assessments, the research team actively tried to include both the water monitor and operator present at water system visits. However, we observed that the water monitor could not answer many of the operational questions posed in the chlorine disinfection assessment because their expertise centered on sampling in the distribution system. Specifically, in the case of the MTA community, the

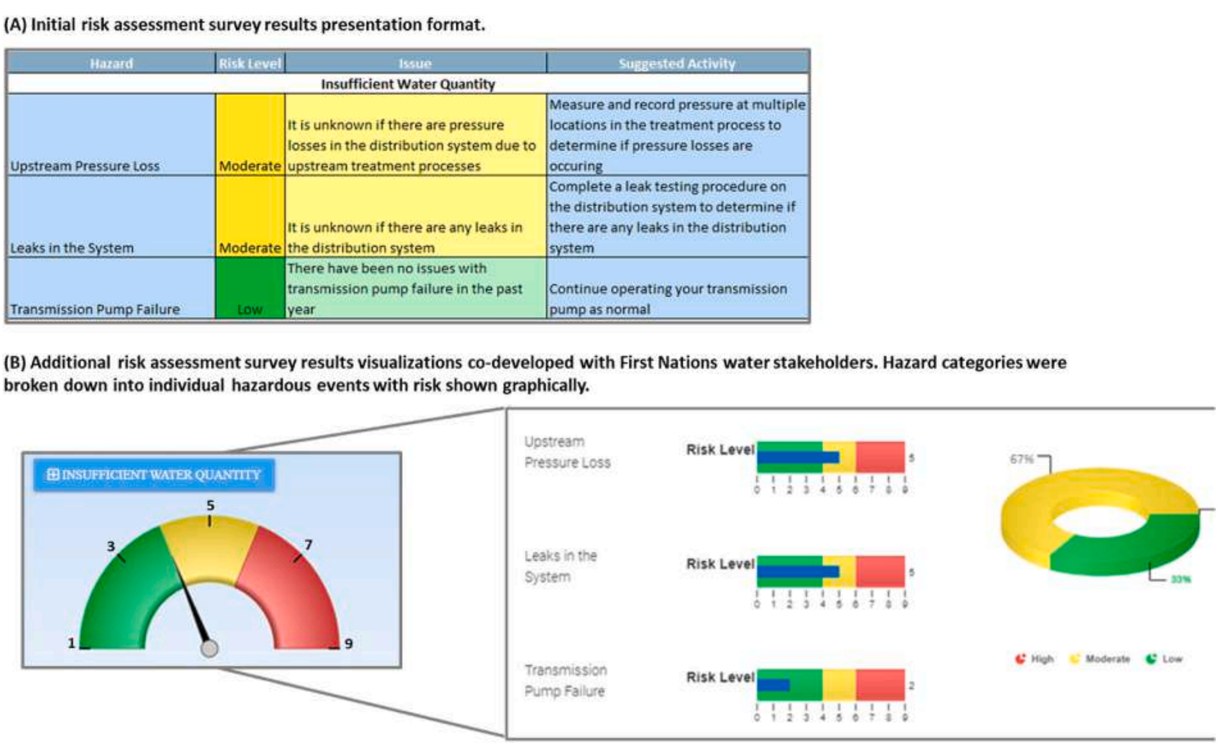

Fig. 3. The initial RAWA results format (A) and the redesigned risk visualization following First Nations stakeholder input (B).

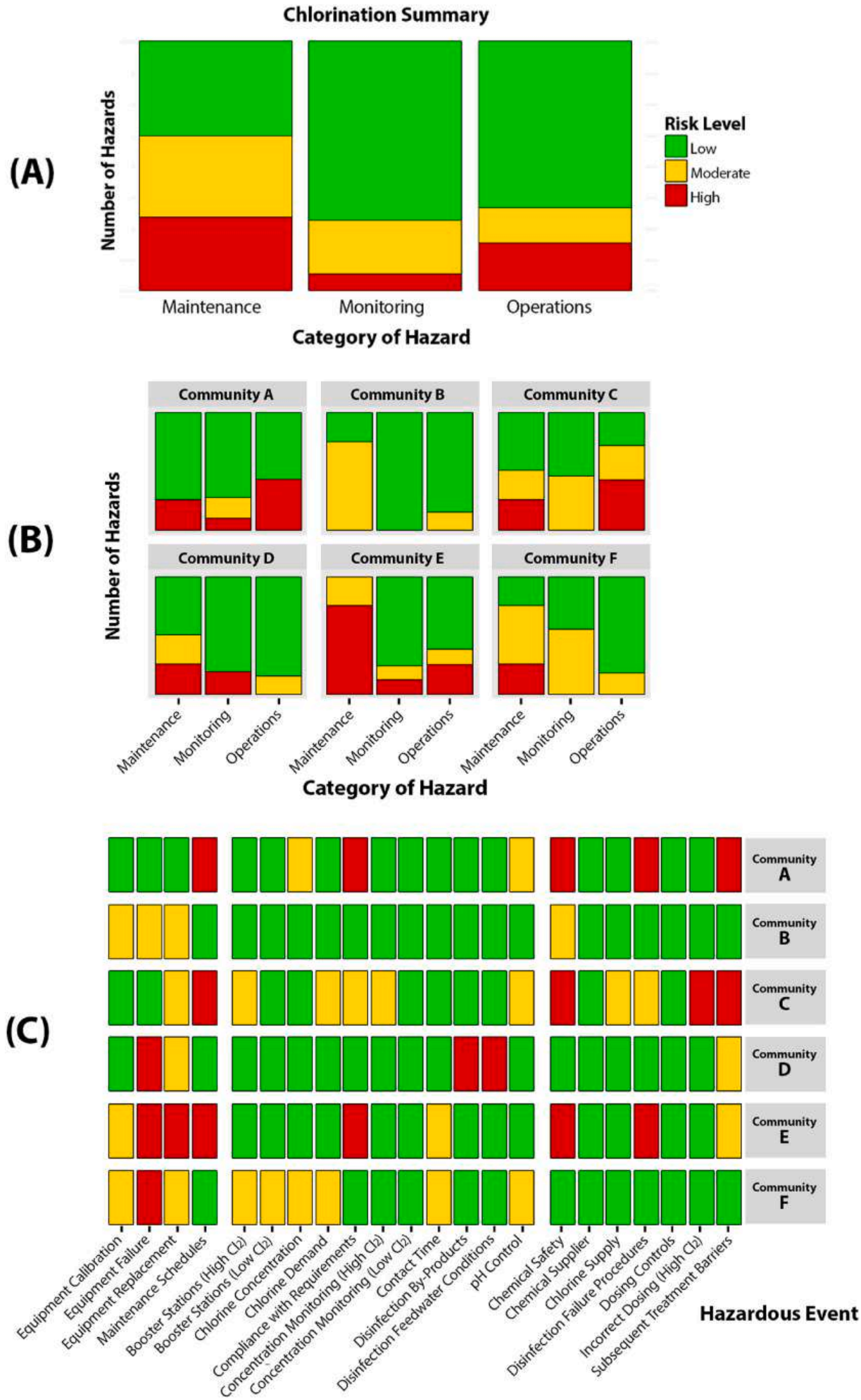

Fig. 4. Chlorine disinfection hazard evaluation results presented by (Panel A) category of hazard across all systems, (Panel B) by category of hazard for each system, and (Panel C) by specific water system and hazardous event.

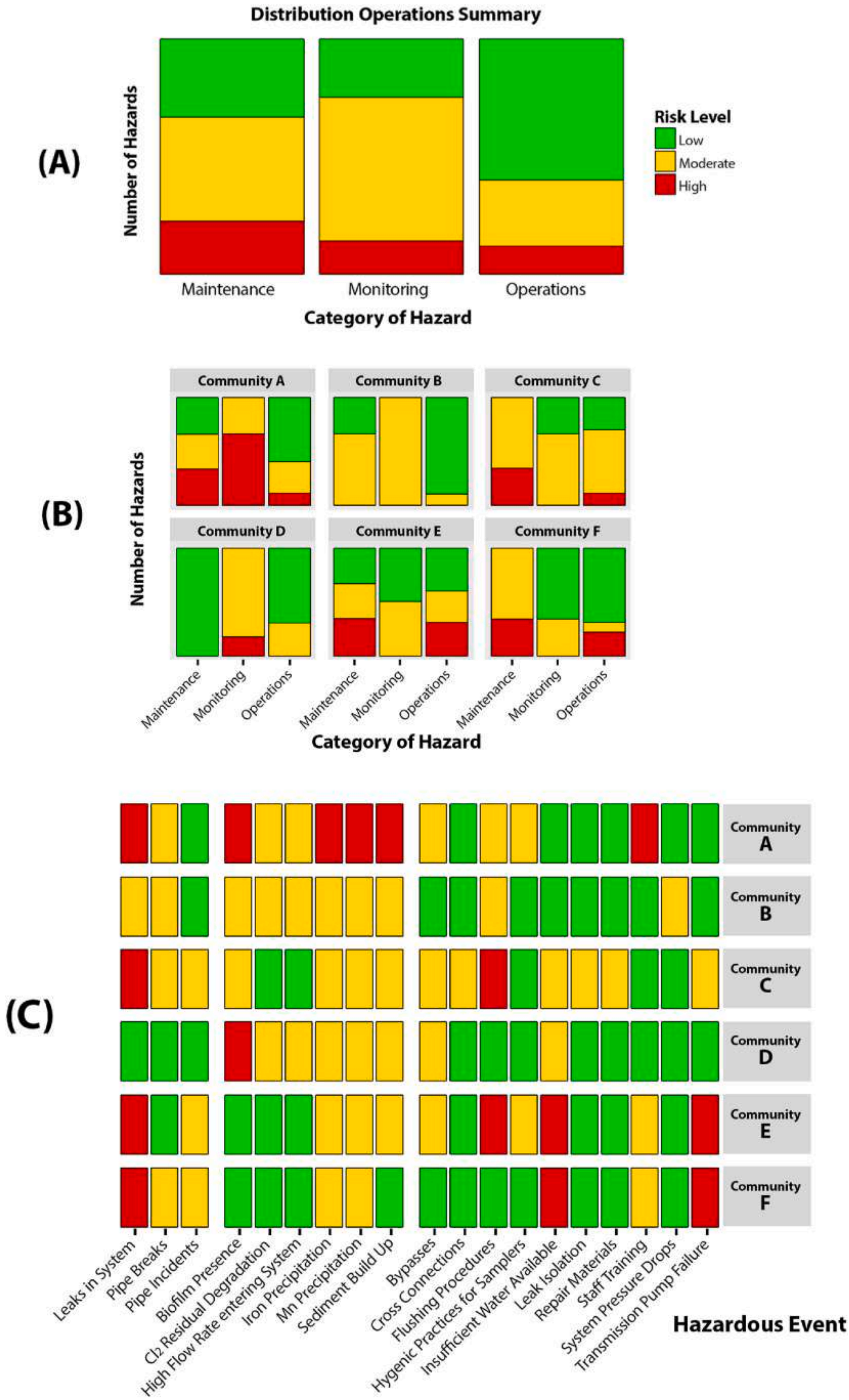

Fig. 5. Distribution system hazard evaluation results presented by (Panel A) category of hazard across all systems, (Panel B) by category of hazard for each system, and (Panel C) by specific water system and hazardous event.

monitor does not need to interact with the water operator on a daily or weekly basis. In three of the six water systems, the operator and the water monitor were the same person. This led to the suggestion of a new risk assessment questionnaire specifically for water monitors to better reflect their specific knowledge of the water system. For example, instead of asking about "iron precipitation" in the distribution system, the water monitor would be asked to check taps for red staining during regular sampling to determine if there are any indicators of iron presence. This distinction is critical in the risk identification process and serves to engage the water monitor in the risk assessment process.

Additionally, feedback centered on data sharing and ownership revealed the need to incorporate login and data management features to the RAWA. Because there are often several stakeholders involved in the acquisition and reporting of water quality data from First Nations communities, several operators mentioned the importance of the web-application platform as not only a data repository, but as a tool that provides a community with ownership of water quality data. Previously, many operators have not had digital records of water quality data collected on a daily basis and access to data reported to federal agencies is limited. As a result, many operators have had limited ability to examine data and evaluate trends in operation that could be very informative. A platform that provides data access, storage and manipulation and is owned by a First Nations community would increase the adoption of the web-based risk assessment tool developed for this study.

3.2. Understanding risk results from hazard evaluation

To best understand similarities and differences between risks in the chlorination treatment and distribution systems in each of the six communities, the results from the hazard assessment are presented with increasing degrees of granularity. Because data and information needs vary for different water system stakeholders, a range of figures were constructed to categorize and describe patterns of system risk. The risks presented in the subsequent figures are representative of a water system stakeholder's perception of risk and are not based on water quality data, but rather on an operator or manager's knowledge of the system. As a result, when we refer to "risk" in these results, we refer to perceived risk as opposed to absolute risk as determined numerically from water quality data.

3.2.1. Chlorine disinfection assessment

The results from the RAWA chlorine disinfection hazard evaluations are shown in Fig. 4: Panel (A) presents risk levels for all 6 communities aggregated by category of hazard (maintenance, monitoring, and operations), Panel (B) presents each community individually aggregated by the category of hazard and Panel (C) presents risk levels in each community for each individual hazard. Fig. 4A presents the proportion of each risk level in relation to the total number of hazards in that category; that is, the sum of each bar is 100%, with each bar having a different number of total hazards. Over all 6 communities, 14.4% of all chlorine related hazards were identified as high risk, 20.5% were identified as moderate risk and 65.2% were identified as low risk. Fig. 4A demonstrates that the majority of high-risk chlorine hazards were associated with maintenance concerns. A total of 29.2% of maintenance hazards were identified as high risk, with only 6.1% and 19.0% of monitoring hazards and operations hazards found to be high risk, respectively.

To understand the contributions from each water system to the total risk levels, results disaggregated by specific water system are shown in Fig. 4B. Community E has either moderate or high risk for each of the maintenance hazards (4 total maintenance hazards per water system). Only Community B has no moderate or high risks for monitoring hazards in the chlorine disinfection evaluation. Every water system has at least one moderate or high-risk hazardous event identified for a maintenance hazard and an operational hazard. By disaggregating the results by community, Panel B reveals which systems contribute the most moderate and high-risk levels to each category overall and provides stakeholders with information about which category of hazards contain the most moderate and high risks, effectively highlighting general areas of concern in a community.

To provide further granularity and detail into risk distribution within each community, Panel C disaggregates the results by individual hazard to help water system stakeholders identify where high and moderate risks exist in the chlorine disinfection system. From this disaggregated plot, it can be seen that individual systems are unique, with specific high-risk hazards in each system. Operator and monitor knowledge captured by the RAWA helps inform system-specific risks and threats. For example, in Community D, the two hazards associated with the formation of disinfection by-products ("Disinfection By-Products" and "Disinfection Feedwater Conditions") were identified as high risk. However, these hazards were not identified as high or moderate risk in any other community, suggesting disinfection by-product formation is a concern unique to Community D. Community C is the only community with high risk of overdose of chlorine to the water system. These examples highlight a key feature of the tile plot in Panel C: unique issues for specific water systems can be identified and therefore prioritized in an individual water system.

3.2.2. Distribution system assessment

The results from the RAWA distribution system hazard evaluations are shown in Fig. 5: Panel (A) presents risk levels for all 6 communities aggregated by category of hazard (maintenance, monitoring, and operations), Panel (B) presents each community individually aggregated by the category of hazard and Panel (C) presents risk levels in each community for each individual hazard. Fig. 5 highlights where community level and regional level concerns exist in First Nations distribution systems. When compared to the chlorine disinfection assessment in Fig. 4, overall, there are more moderate and high-risk hazards present in the distribution system. As with the chlorine risk results, maintenance represents the area with the largest number of high-risk hazards and indicates alternative approaches to maintenance funding and management will be important to mitigating risk and ensuring safe drinking water. Across all 6 water systems, 14% of the hazards were identified as high risk, 42.1% were identified as moderate risk and 43.9% were identified as low risk. Disaggregated by category, in Panel A, it is clear that monitoring is characterized by the largest number of moderate and high-risk hazardous events, indicating that water quality and operational monitoring within the distribution system is an area for improvement.

Panel B reveals that only Community D does not have high or moderate risks associated with maintenance hazards in the distribution system. There are no other communities with only low risk hazards in any other categories. For the monitoring category, there are several communities with predominantly moderate or high-risk hazards. This is partly due to the authors decision to code "unknown" risk as moderate risk. Monitoring questions in the distribution system assessment contained an answer choice "unknown" for parameters such as biofilm formation and iron and manganese precipitation. These parameters either cannot be monitored directly, are not easy to monitor, or are not parameters necessary for compliance (and therefore not measured by the community). Some water system operators and monitors identified monitoring hazards as low-risk if monitoring programs were in place, however, others indicated the hazard was low-risk without any record of monitoring for the parameters necessary to identify a hazard as low-risk. The RAWA tool, like any risk assessment process, is only as valid and robust as the quality of the data informing the assessment and all risk assessments are ultimately subjective judgements (Hrudey, 2012). Overall, Panel 4B shows a high degree of variability between community water systems, demonstrating key differences in system operations, monitoring and maintenance practices in the distribution system.

Fig. 5C, similar to Fig. 4C, highlights concerns specific to an individual water system. Notably, five of the six waters systems have identified either high or moderate-risk associated with the "Leaks in the

System" hazard. This result demonstrates there are patterns of hazards shared by many First Nations water systems; these hazards could be best addressed by a regional or federal authority. Other hazards with predominantly high and moderate risk across all six water systems include: "Iron Precipitation", "Manganese Precipitation", "Biofilm Buildup" and "Transmission Pump Failure". For hazards such as "Biofilm Buildup" several systems have moderate risk assigned to this hazard as a result of selecting "unknown" in the hazard evaluation for the distribution system. This is a hazard where risk could be addressed through a monitoring requirement or guideline generated by a regional or federal authority to measure and record water quality parameters to generate knowledge about this specific hazard. The "Transmission Pump Failure" hazard is an operational concern and risk could potentially be lowered by ensuring standard operating procedures are available and presented to operators to avoid this concern in the future. The risk assessment results, as presented in Panel C, therefore provide federal and regional stakeholders with a tool to identify common high and moderate risk hazards across water systems.

3.3. Thematic analysis

3.3.1. Understanding the necessary enabling environment for WSP implementation

Follow-up conversations with First Nations water stakeholders in the six participating water systems revealed five major themes addressing the possible implementation of community-driven risk-based management and future WSP adoption. Each theme is presented with the supporting ideas or comments from operators, community water monitors or regulatory agency stakeholders that generated the themes in Table 4. Identifying information for interviewees was removed from the responses to preserve anonymity and minimize competing interests between stakeholder groups during the co-development of this tool.

The identified themes provide critical insight into not only the needed improvements to the risk assessment tool, but they also elucidated necessary conditions that would enable the successful adoption of a broader WSP management approach. The research team used the information collected from the thematic analysis to refine the RAWA. All feedback made by water stakeholders has been incorporated into the tool. For example, in Theme 1, improving the RAWA to include features allowing for greater system specificity is a critical feature for better risk identification. The supporting ideas for Theme 1 indicate the importance of including features that allow First Nations system experts to explain and support the risk assessment results. This also could provide federal agencies with community-derived guidance to inform funding allocations based on risk mitigation strategies in specific water systems. Accuracy and specificity of the risk assessment process is critical and the supporting ideas for Theme 1 reflect the need to maintain the co-development process as iterations of the RAWA are created.

Theme 2 and Theme 3 reveal the utility of the co-developed RAWA as a communication and data storage vehicle. These two themes highlight the plurality of stakeholders present in First Nations water systems and how current issues with communication and record keeping could be alleviated by adding data management features to the RAWA. Many operators commented on the difficulty of documenting standard operating procedures and developing maintenance plans above and beyond the daily operational requirements of their job. Knowledge about the system is largely held by operational staff; there are standard operating procedures but these procedures, in addition to other key maintenance and monitoring information, are not formally documented in many of the water systems. As a result, when an operator needs to mitigate an incident, the incident report may not refer to documentation of the procedures the operator utilized to resolve the incident, which often results in speculation by external stakeholders that the system is not operating correctly. Several operators indicated the RAWA could provide the backbone for a larger data storage and management system to alleviate communication concerns between stakeholders and serve as a vehicle to document operating procedures and maintenance and emergency response plans informed by the risk assessment process.

Theme 4 reveals the importance of data ownership and the presentation of data between the stakeholders in the water systems. Multiple operators commented on the additional data ownership the co-developed RAWA could provide to a community. Currently, operators and monitors are providing this information to external stakeholders and reporting to databases that are not easy to access for day-to-day evaluations of water quality data. Having the ability to interact with the data from the risk assessments and generate reports immediately is a new asset to many operators and can be expanded in future iterations of the web application.

In this study specifically, we learned operator certification is required provincially, but federal recommendations for First Nations operator certification are guidelines not requirements (Black and McBean, 2017b). This gap in training and liability is presented in Theme5. Operators are provided with training opportunities annually in the Atlantic provinces by federal agencies, however, certification of operators remains a concern for legal reasons. By introducing a new framework such as a WSP, it introduces larger questions related to the governance structures and stakeholder responsibilities in the already complicated jurisdictional environment.

4. Discussion

4.1. A RAWA enables communication between water system stakeholders

This research revealed that a key benefit of a co-developed RAWA was the ability of the assessment tool to aggregate and organize risk information within and between First Nations water systems at various levels of granularity. the web-based nature of the tool facilitates the communication of system risk to the multitude of stakeholders interacting with the system. Communication improvements resulting from WSP implementation have been seen previously in case studies globally (Byleveld et al., 2008; Hasan et al., 2011; Gunnarsdóttir et al., 2012; Kot et al., 2015; Kayser et al., 2019; WHO & IWA, 2017; Tsoukalas and Tsitsifli, 2018). Water system stakeholders in First Nations include treatment facility operators, water quality monitors, Chief and Council, EHOs, Health Canada, INAC, FNIHB and other related agencies such as the APC. Often, these stakeholders are working to improve community water systems, but have different improvement policies and strategies and capital investment capabilities, leading to noticeable challenges aligning priorities across stakeholders (McCullough and Farahbaksh, 2012; Bradford et al., 2017; Castleden et al., 2017; Dunn et al., 2017). The RAWA was designed to help prioritize high-risk hazardous events, and operators and monitors viewed the risk assessment tool as an opportunity to communicate water system concerns to other stakeholders. The risk assessment questionnaire used in this study relied on operator expertise and the generated results were able to highlight operational concerns in the water system.

Using the RAWA as a communication tool was particularly valuable for the MTA water system where the operator is employed by the neighbouring municipality and the community-based water monitor is employed by FNIHB. The operator and the monitor actively communicate water quality results, but the monitor is not involved in the treatment or distribution of water, only sampling for water quality parameters at the endpoint of the system. The risk assessment tool results provided another way to encourage communication between operators and monitors about the hazardous events present across the entire water system. Until this study, the monitor in the MTA community had not considered the other components of the water supply system outside of designated sampling locations. The RAWA helped to provide a more holistic view of the system and opened new conversations about sampling location appropriateness. Communication amongst stakeholders, particularly governance agencies, has been identified as a critical component of successful WSP implementation (Hasan et al., 2011; Perrier et al., 2014; Amjad et al., 2016; Kayser et al., 2019), particularly important when adapting new methodologies to Indigenous communities (Bradford et al., 2017; Castleden et al., 2017). The findings of this research suggest that risk-based assessment tools, particularly a web-based application co-developed by First Nations water experts, could help in engaging and empowering communities to inform water governance decision-making through improved communication to system stakeholders.

4.2. Recommendations for future RAWA development and use

Throughout this study, we have highlighted the importance of co-development in the risk assessment step of the WSP implementation cycle. The RAWA was designed to cover the risk assessment step of WSP only, and we demonstrated the tool is able to identify key issues in a water system, as was verified by First Nations stakeholders in the co-development process. We noted the importance of system-specificity during the co-development process and feedback from First Nations stakeholders indicated system-specificity could be best integrated into the RAWA in the form of community-specific action items to address high-risk priorities. While this study did not focus on developing and reviewing action items, operators and monitors were keen to reflect on this component of the RAWA, stressing the importance of selecting proper action items for each community. The backbone of hazards created in this study can be used by different communities to generate an initial evaluation of perceived risks in a water system; we recommend that future iterations of the RAWA include system-specific action items to address particular local characteristics and operational practices in community water systems.

Furthermore, to maintain the system-specific elements of the WSP approach, changes to the RAWA tool need to be made to ensure the tool is flexible enough to encompass the variations in First Nations systems in the Atlantic region. The list of hazards generated by the CWRS team is relatively static and the generation of questions from these hazards relies upon researchers or water quality managers with sufficient experience in the water industry to think critically about risk. To ensure communities can update and expand the RAWA tool, more work needs to be done with communities to identify the best way to develop and refine the hazard lists and questions in the tool. This will ensure that the RAWA supports a WSP cycle that is continuously updated in line with recommendations from the WHO (Bartram et al., 2009). We also advocate for yearly reviews of the RAWA tool, including questions, action items and data visualizations by water system stakeholders, perhaps at yearly operator training events to keep the co-development process a key feature of the RAWA.

4.3. WSP development is influenced by current water governance realities

The RAWA developed in this study is a tool to help water system stakeholders fulfill Step 3 of the WSP process (Fig. S1) and the results obtained from completing the risk assessment component of the WSP cycle have larger implications for water safety planning in First Nations water systems. This research aimed to co-develop a risk assessment tool with First Nations to facilitate a bottom-up, community-driven management approach as a key first step to WSP implementation. A study of the Alberta drinking water safety plan implementation highlighted the importance of ensuring a WSP does not become a “top-down” or bureaucratic tool water systems are mandated to complete (Perrier et al., 2014). This concern is particularly important in Indigenous water systems where multiple stakeholders have influence in decision-making process. A study by Bradford et al. (2017) considered how the roles of governance and water policy practices affected Indigenous water systems. The study demonstrated the importance of using methodologies promoting “power with” dynamics between water systems and external stakeholders, instead of the traditional “power over” dynamic (Bradford et al., 2017). The feedback from First Nations stakeholders in our study highlights the importance of co-developing risk assessments, and by extension, management strategies such as WSPs. System specific nuances and concerns revealed through the co-development of the RAWA provided a critical lens through which to not only evaluate, but also to communicate risk. When considering the complete WSP cycle (Fig. S1), it is clear defining the WSP team and incorporating appropriate hazards is critical to the accuracy of the risk assessment and to the successful adoption of a risk management methodology. There is a defined need in literature to expand future studies to account for Indigenous viewpoints and appropriate implementation strategies (Castleden et al., 2017) and this study highlights the importance of co-development when considering new management strategies for First Nations water systems.

Despite numerous federal policies addressing drinking water quality in First Nations communities and the passing of the Safe Drinking Water for First Nations Act in 2013, access to safe drinking water is still an issue for many First Nations communities (Morrison et al., 2015; Black and McBean, 2017a). In 2017, there were twenty federal agencies operating under eleven legislative frameworks, each having separate but competing or overlapping mandates (Bradford et al., 2017). This has historically led to duplications of efforts to improve water systems (Bradford et al., 2017). Ultimately, competition for federal funding within communities can lead to suboptimal resource allocation for water systems (OAG, 2021). Given this reality, a community-driven WSP management approach has an advantage over current water governance regimes because it provides First Nations water stakeholders with an evidence-based risk framework to inform communication and action. A key output of the WSP process is a complete evaluation of risks in a water system, prioritized by using the risk matrix to assign risk levels (Bartram et al., 2009; WHO, 2012). The RAWA developed in this study equips the community with information about their water system that is separate from other evaluative practices performed by federal agencies. The tool transforms operator and monitor knowledge into constructive, system-specific, recommendations that can be shared with community leadership and federal agencies. Management features of the RAWA are a potential step towards developing community agency, capacity, and ownership of data, key characteristics that are currently lacking in First Nations water management approaches.

The development of a risk-based management approach could be a particularly important step for First Nations communities, because as a result of Western regulatory paradigms reactive management of hazards in drinking water systems is a normative behaviour in Canada. Focus has historically been placed on monitoring end of pipe water quality (Hrudey, 2013) and this strategy has been superimposed on First Nations, resulting in the adoption of and reliance on this reactionary approach. WSPs or similar processes have been widely implemented internationally since 2004 and are currently practiced in over 90 countries, including the UK, EU, Australia, New Zealand, and Iceland (WHO, 2017). The RAWA tool produced for this study could help support First Nations to develop their own WSP process informed by Indigenous world views and ways of knowing. The semi-structured interviews conducted in this research highlighted the importance of system and community specificity and revealed the current difficulties in effectively communicating urgent concerns to First Nations and non-First Nations stakeholders. These themes reflect, in part, the current regulatory and governance realities in First Nations systems. Management features of the RAWA may help to shift water system management from a reactionary to a proactive, risk-based approach. The web app structure provides operators and First Nation stakeholders with immediate results, and more importantly, action items to decrease risk in the system. Compared to monitoring end of pipe quality, this the tool provides the opportunity to develop a proactive, operator-informed method to manage risks.

While developing capacity and agency through a community-driven risk-based water management approach could help ensure the production of safe and sustainable drinking water, several case studies of WSP implementation point to the importance of strong supporting institutions and stakeholders, such as federal governments, to promote successful WSP utilization (Gunnarsdóttir et al., 2015; Amjad et al., 2016; Baum and Bartram, 2017; Kot et al., 2017). If First Nations led WSP implementation is to succeed, supporting institutions beyond the federal government will likely be needed, as the federal government does not currently require risk-based water management in Canada. Also, if WSPs were required federally, the WSP approach risks becoming another top-down, bureaucratic tool, instead of a useful new management framework for the provision of safe water (Perrier et al., 2014; Bradford et al., 2017; Castleden et al., 2017). However, there are several jurisdictions, such as Iceland, where federal regulations and WSPs co-exist and have been successful and could serve as guides for similar implementation for First Nations communities in Canada (Gunnarsdóttir et al., 2012, 2015). Community consultation and co-development throughout the WSP process are critical components to ensuring community-specificity, a bottom-up approach and continued active engagement.

This current study was conducted in collaboration with the APC, a First Nations Chief Secretariat focused on developing policy alternatives for issues affecting First Nations communities in Atlantic Canada, Quebec, and Maine, USA. During the time this research was conducted, the APC was establishing the Atlantic First Nations Water Authority (AFNWA), the first Indigenously owned and operated water authority in Canada (Indigenous Services Canada, 2020). Regional entities may provide a management solution for First Nations communities for the following reasons: (1) historical land and treaty agreements are regional, not federal, (2) regional authorities fulfill a liaison role between the federal government and First Nations communities and (3) communication in a regional capacity functions similarly to communication in a water utility serving many small systems. First Nations stakeholders involved in this study noted the value the RAWA could have for a regional entity like the AFNWA, as the tool could be used to effectively manage data collection, prioritize system improvements and review and alleviate operational challenges identified by operators and monitors.

5. Conclusion

The risk-based management tool, developed in this study, provides system specific, communicable results to help First Nations water systems prioritize improvements and mitigate risk. In this study, we co-developed a risk assessment web application with First Nations stakeholders, piloted the developed application and then examined the benefits and challenges of implementing the risk assessment as a component of the larger water safety planning methodology. The RAWA developed for this study identified key high-risk hazards not only at the individual water system level, but across systems as well. This outcome suggests the importance of the RAWA as a decision-making and policy-making tool for not only for communities, but also for regional and federal management efforts.

This study demonstrated the importance of co-development with First Nations water experts to generate a risk assessment web application that accounts for Indigenous perspectives. First Nations stakeholders provided a highly knowledgeable perspective that informed the improvement to the RAWA as a result, including the addition of an overall risk score, several visualizations of risk results, login features for data sharing and security and improved questions to assess risk. The identified improvements in conjunction with First Nation stakeholder commentary coalesced into five key themes: the need for system specificity, the potential data management capabilities of the RAWA, the utility of the RAWA as a communication tool, the important of data ownership and the remaining questions of responsibility and liability when implementing a new management methodology. These topics also represent areas for future research and co-development with First Nations water stakeholders.

This study revealed that First Nations water system stakeholders found value and benefit to collecting, prioritizing, and communicating information about hazards and risks in their systems and the co-development of tools and approaches could facilitate the adoption of water safety planning in communities. While a risk-based approach does not directly ameliorate the challenges associated with the current fragmented water governance in place for First Nations in Canada, the RAWA developed in this study helps First Nations communities gather their own data and knowledge in a way that can be readily communicated to other stakeholders. Challenges to risk management implementation remain, including the need for supporting institutions, the multiple federal and local stakeholders involved in First Nations water systems and a regulatory paradigm focused on reactive monitoring. A regional authority such as AFNWA could provide the support necessary to implement the RAWA and potentially WSPs in First Nations communities to address implementation challenges uncovered in this study. Risk management methodologies such as WSPs therefore have the potential to resolve communication concerns and provide a community-led approach to water management in the presence of properly formulated supporting institutions and governance strategies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113916.

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Comparing susceptibility and contagiousness in concurrent outbreaks with a non-VOC and the VOC SARS-CoV-2 variant B.1.1.7 in daycare centers in Hamburg, Germany

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A B S T R A C T

We describe two outbreaks of SARS-CoV-2 in daycare centers in the metropolitan area of Hamburg, Germany. The outbreaks occurred in rapid chronological succession, in neighborhoods with a very similar sociodemographic structure, thus allowing for cross-comparison of these events.

We combined classical and molecular epidemiologic investigation methods to study infection entry, spread within the facilities, and subsequent transmission of infections to households.

Epidemiologic and molecular evidence suggests a superspreading event with a non-variant of concern (non-VOC) SARS CoV-2 strain at the root of the first outbreak. The second outbreak involved two childcare facilities experiencing infection activity with the variant of concern (VOC) B.1.1.7 (Alpha). We show that the index cases in all outbreaks had been childcare workers, and that children contributed substantially to secondary transmission of SARS-CoV-2 infection from childcare facilities to households. The frequency of secondary transmissions in households originating from B.1.1.7-infected children was increased compared to children with non-VOC infections. Self-reported symptoms, particularly cough and rhinitis, occurred more frequently in B.1.1.7-infected children.

Especially in light of the rapidly spreading VOC B.1.617.2 (Delta), our data underline the notion that rigorous SARS-CoV-2 testing in combination with screening of contacts regardless of symptoms is an important measure to prevent SARS-CoV-2 infection of unvaccinated individuals in daycare centers and associated households.

1. Introduction

During the first wave of the SARS-CoV-2 pandemic in Germany, ranging from March until May 2020 , childcare facilities were closed in most federal states. In contrast, most of the facilities remained open during the second and beginning of the third waves, mid-October 2020 to February 2021 and February to May 2021, respectively, although infection rates among children and adolescents had substantially increased. Operation at reduced capacity, separation of children into smaller cohorts, and implementation of hygiene measures were

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undertaken to contain infections and keep facilities open. However, unlike in school settings, neither employees nor children were mandated to wear a mask in daycare centers. Nevertheless, the overall rate of daycare centers in Germany that had to be closed due to recognized infections increased from 1.7% by the end of January 2021 to a peak of 5.8% by the end of April 2021 (Autorengruppe, 2021). Considering the high incidence rates among children and adolescents in the second and third waves of the pandemic, it was controversially discussed how these age groups contribute to the SARS-CoV-2 infection dynamics.

To date, only a few case reports have been available, focusing on systematic studies on SARS-CoV-2 infection events in daycare centers (Autorengruppe, 2021). Moreover, some geographically limited studies were conducted (Ehrhardt et al., 2020; Haag et al., 2021; Hoch et al., 2021; Hoehl et al., 2021; Lübke et al., 2021; Thielecke et al., 2021). However, most of these surveillance studies were performed in periods with low overall incidence rates in Germany. Only a single study, describing three independent outbreaks with the VOC B.1.1.7, addresses the effect of newly emerging variants (Loenenbach et al., 2021). This study indicates that susceptibility as well as transmissibility of children and adults might converge with the emergence of new, more infectious variants. Given the variant B.1.617.2 and no available vaccination in children under 12 years of age, it is of significant interest to investigate transmissions in children and by children in households and reconsider hygiene measures in daycare centers. We report on three transmission clusters in daycare centers in Julin et al., 2021, occurring in the Hamburg metropolitan region. We compare transmission events, the number of infected individuals, and the development of symptoms in individuals experiencing a non-VOC SARS-CoV-2 outbreak and a VOC B.1.1.7 outbreak.

2. Methods

2.1. Sample collection

In light of the positive SARS-CoV-2 PCR test results of the index cases, nasopharyngeal samples were collected from every child and childcare worker in the respective daycare facility to find potentially associated infections. Serial testing procedures were performed to collect samples. Single samples were collected in medical practices. Index cases and all contact persons were contacted regularly by public health workers to ask for monitoring of symptoms. Household contacts were offered voluntary PCR tests. The qRT-PCRs of the SARS-CoV-2 specimens were performed in accredited laboratories in Hamburg, Germany.

2.2. qRT-PCR based SARS-CoV-2-testing

Samples were sent to the University Medical Center Hamburg-Eppendorf for independent SARS-CoV-2 qRT-PCR confirmation and PCR-genotyping (Corman et al., 2020; Puelles et al., 2020). PCR-genotyping for the SARS-CoV-2 variant of concern (VOC) B.1.1.7 was performed by using specific primer/probe (Brehm et al., 2021) sets for B.1.1.7 as published (Norz et al., 2021).

2.3. SARS-CoV-2 amplicon sequencing and bioinformatics analyses

Amplicon sequencing and bioinformatics analysis were performed as recently published (Brehm et al., 2021; Gunther et al., 2020). Library generation was performed using the CleanPlex SARS-CoV-2 Panel (Paragon Genomics, CA, USA). Merged reads were filtered for a minimum base quality of 20 and aligned to NC_045512.2 using minimap2 (Li, 2018) with default settings for short read alignment. Major variants (≥50% allelic frequency) were called using FreeBayes Bayesian haplotype caller v1.3.1 (Garrison and G, 2012) with ploidy and haplotype independent detection parameters to generate frequency-based calls for all variants supported by a minimum coverage of 10 (-K –F 0.5 -min-coverage 10). Resulting variants were annotated using ANNOVAR (Wang et al., 2010) and consensus sequences were generated using in-house scripts. Pangolin lineage and Nextstrain clade assignment of consensus sequences were performed using the pangolin (https://github.com/cov-lineages/pangolin) and Nextclade (https://github.com/nextstrain/nextclade) packages. Phylogenetic analysis and tree visualization were performed using Nextstrain which relies on maximum likelihood ancestral state reconstruction of discrete traits in combination with inferred probability distributions of ancestral state of each node (Hadfield et al., 2018). The sequences have been uploaded to GISAID (see Table S4 for accession numbers).

3. Results

The two outbreaks in daycare centers occurred in the same middle-class neighborhood involving three independent daycare centers, at a time when overall incidence rates were high and B.1.1.7 prevalence was increasing in the city of Hamburg (Fig. 1A). At the time of the outbreaks (end of January), the second wave was about to decline, and the 7-day incidence rates ranged between 70 and 100 cases per 100′000 individuals. Although children and adolescents were highly affected during the second and the third wave of the pandemic in Germany, 7-day incidence rates among children aged 0–5 years in Hamburg were still below the overall average (40–50 cases per 100′000 individuals in Germany). Restrictions in different areas of public life as well as for private meetings started by mid of December 2020 (Fig. 1B). Schools and daycare facilities worked under emergency conditions with reduced capacities.

3.1. Infection series in the three childcare facilities

The first case related to childcare facility 1 was a childcare worker who reported to health authorities with a positive PCR test result (C_t 17.7). The individual had been symptomatic and therefore had taken sick leave two days before the positive test result. Prior the occurrence of symptoms, the individual had been responsible for a group of children below the age of 3 in the nursery department, and additionally had supervised the dormitory for the entire daycare center's nursery. Consequently, a strict quarantine of 14 days was ordered for all children attending the nursery and close contacts among the childcare workers. At the time of the outbreak there was no possibility to shorten the quarantine through PCR testing. In the following days, two of the quarantined staff developed symptoms and tested positive. Rapid antigen tests were also performed on four additional childcare workers with three of them testing positive with results being confirmed by qRT-PCR. As these childcare workers took care of groups from the elementary cohort (children aged 3–6 years), a second quarantine was ordered. Within 8 days after the index case turned out to be SARS-CoV-2 positive, 8 out of 14 childcare workers and 22 out of 34 children tested positive (Fig. 2A). Due to the high infection rates (Facility Secondary Attack Rate (SAR) = 63,3%) the outbreak was considered a potential superspreading event. Because of the high number of positive PCR test results compared with previous outbreaks in childcare facilities, all contact persons of the second generation affected by the outbreak were offered voluntary PCR testing. This revealed several household transmissions originating from childcare workers as well as children. While childcare workers accounted for 5 infections of the second generation (2 parents and 3 children), children attending the daycare center infected a total of 15 household contacts (14 parents and one sibling). Shortly after the outbreak in F1, another SARS-CoV-2 outbreak was reported in a childcare center in the same city district. The index case of facility 2 (F2), a childcare worker taking care of children in the nursery, was tested positive by PCR three days after symptom onset while being on sick leave. PCR genotyping indicated the presence of characteristic mutations for the VOC B.1.1.7 (S:69–70:H–V: , S:501:N:Y) and the first outbreak of VOC B.1.1.7 in an educational institution in Hamburg. As a

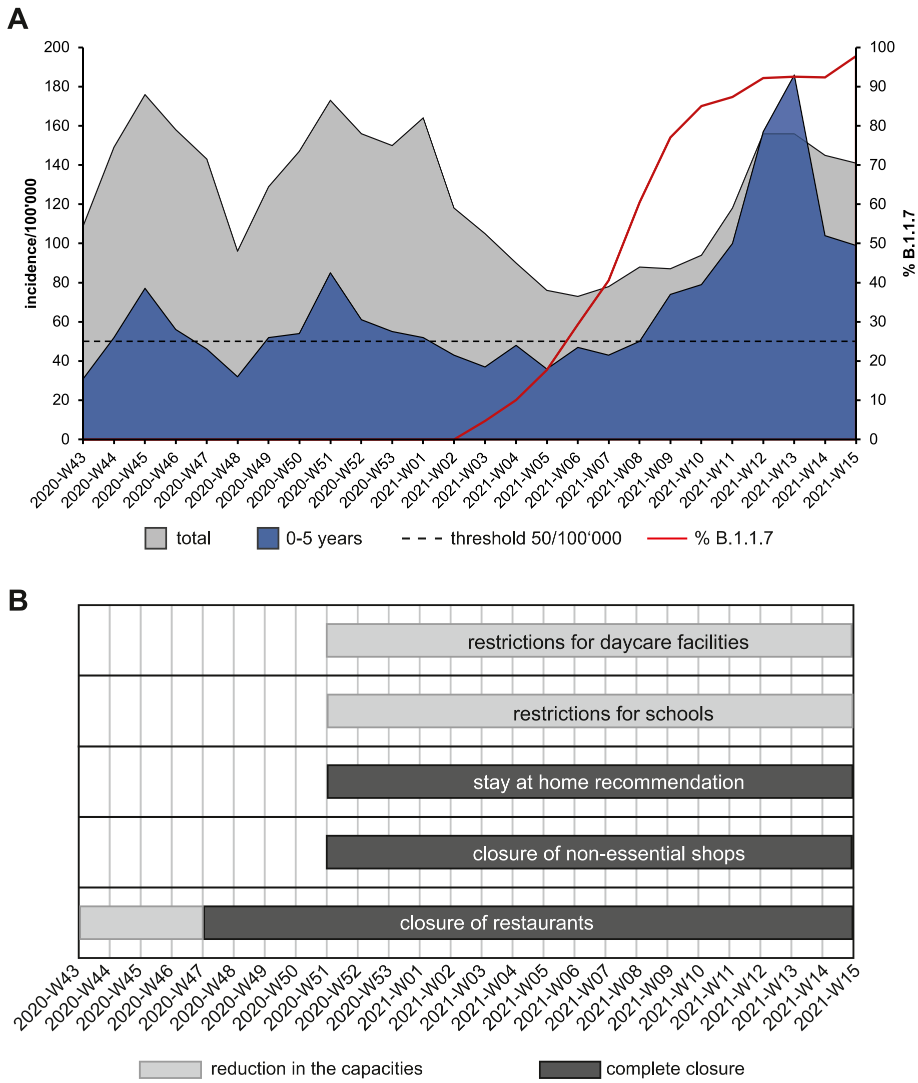

Fig. 1. A) SARS-CoV-2 7-day incidences (per 100′000 individuals), percentage of B.1.1.7 and hygiene measures (B) in the city of Hamburg, Germany, October 19, 2020–February 18, 2021. SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; W: calendar week.
(Data source: Hamburg Institute for Health and Environment).

consequence the whole daycare facility was quarantined. qRT-PCR revealed that due to strict separation between the nursery and elementary cohorts only contacts from the nursery cohort showed SARS-CoV-2 infections, while the elementary school remained unaffected. In total, 6 out of 14 childcare workers and 10 out of 71 children tested positive for SARS-CoV-2 by PCR (Facility SAR = 19.8%, Fig. 2B). The outbreak included infections in the households of children (11 parents, 2 siblings) and childcare workers, which occurred within 23 days after the index case turned out to be SARS-CoV-2 positive (one parent, one partner, and one child).

Due to infections in the household, the partner of one of F2's childcare workers, who is employed at F3, was infected and served as the index case at F3 only 2 days after the index case of facility 2. Consequently, a two-week quarantine was ordered for all employees and children during which 8 out of 42 children and 5 out of 17 employees tested positive (facility SAR = 23.3%, Fig. 2B). These 13 persons were responsible for 15 positively tested household contacts. Transmissions from children included 7 parents and 3 siblings while those from employees included 2 parents, 2 partners, and one child.

3.2. Viral genotypes in the three childcare facilities

Based on the assumption that the outbreak at F1 might have been a superspreading event and that outbreaks at F2 and F3 involved the VOC B.1.1.7, whole-genome sequencing (WGS) was performed. For F1 a total of 23 samples were available of which 20 yielded high-coverage genotypes (the index case and 19 contacts of the first generation (4 childcare workers and 15 children, Fig. 3A, F1-1 – F1-22)). Compared to the SARS-CoV-2 reference (NC_045512.2), all samples exhibit a set of 17 dominant mutations defining the index sequence (F1_1-A index). In three samples, F1_14-C, F1_20-A and F1_22-C, single nucleotide polymorphisms (SNPs) were detected with F1_14-C and F1_20-A showing 2 SNPs each with 70% and 100% variant frequency, respectively. F1_22-C contains one SNP with 10% variant frequency (Fig. 3A). The pangolin lineage of the 20 samples was determined as B.1.1. A total of 14 samples from childcare F2 and F3 were analyzed by WGS: 6 samples from childcare workers (4 and 2 for F2 and F3, respectively) and 8 samples from children (6 and 2 for F2 and F3, respectively). Index samples were not available for sequencing. Data analysis confirmed the presence of the VOC B.1.1.7 and identified an identical set of mutations shared by all samples (Fig. 3A), thereby confirming that the two outbreaks are directly linked.

To investigate whether similar genotypes could be identified beyond

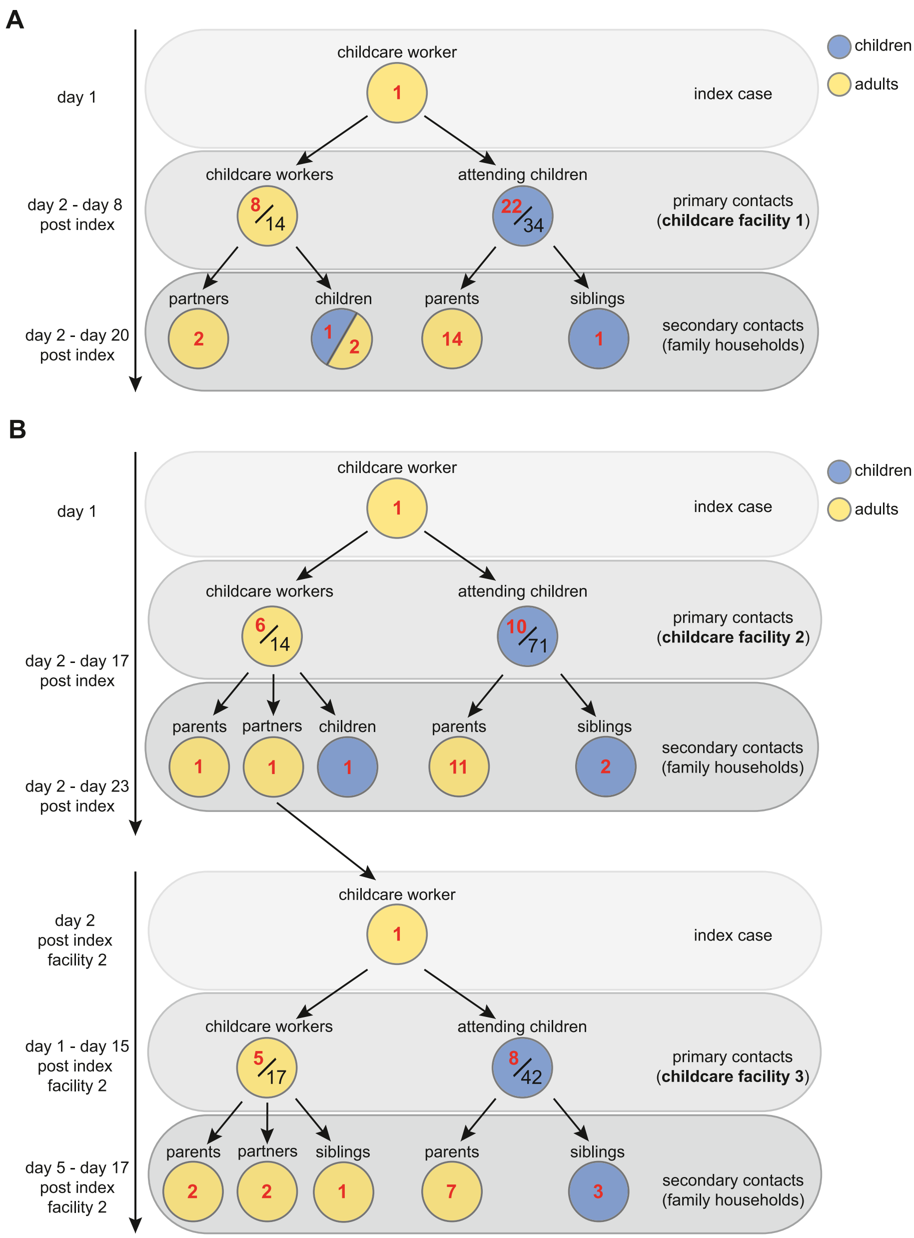

Fig. 2. Schematic overview of the infection series in the three childcare facilities in the city of Haag et al., 2021–February 2021. Red numbers indicate SARS-CoV-2 positive persons, while black numbers show the total number of potential contacts. (A) The infection series including index cases and contact persons of first and second generation are depicted for facility 1. (B) A schematic overview of the infection series including index cases and contact persons of first as well as second generation are depicted for facility 2 and 3.

the daycare centers, data were compared with sequences deposited in GISAID (gisaid.org), as well as with the dataset of the Hamburg-Surveillance program, an endeavor which systematically monitors SARS-CoV-2 variants in the Hamburg metropolitan area (Surveillance). The generated phylogenetic tree recapitulates the association of samples from F1 with B.1.1 and F2 and F3 with the variant B.1.1.7 (Fig. 3B). A zoom-in view reveals that three samples share the exact genotype of the outbreak at F1 (Fig. 3B). Interestingly, one of these samples was collected 10 days after the positive test of the index case and originated from a location very close (<1 km) to the daycare center. We performed the same analysis in the context of F2 and F3. Similarly, we identified three samples with the identical genotype as seen in the samples from F2 and F3 (Fig. 3C). In addition, we identified four samples that had 1-4 additional single nucleotide polymorphisms (SNPs) and two samples lacking one of the observed nucleotide exchanges. All samples with the prototypical outbreak genotype were collected in the city of Hamburg.

However, we were unable to trace any of the samples to the outbreaks. The fact that most of the closely related samples (with the exception of three samples with identical genotype to F1 sequences) show additional mutations and were found relatively far from the facilities suggests that all outbreaks were contained at the level of second generation contacts.

3.3. Comparison of symptoms and numbers of secondary contact persons

As SARS-CoV-2 variants differ between F1 (B.1.1, non-VOC) and F2/3 (B.1.1.7, VOC), we compared self-reported symptoms among the index cases and first-generation contacts.

We differentiated between children and childcare workers as well as between non-VOC and VOC facilities. In F1, fever, cough, and cold were the most frequently reported symptoms in both, children and childcare workers with 52% reported fever, 46% cough and 49% cold. While loss of taste/smell was also reported frequently among the childcare workers (77.8%), young children were rarely affected (4.5%). In general, children reported less symptoms with 5 children being symptom-free (Table 1, Table S1). Interestingly, among the individuals infected with B.1.1.7 in F2/3, the rate of reported symptoms was comparable among childcare workers and children (Table 1, S2 and S3). Fever was more

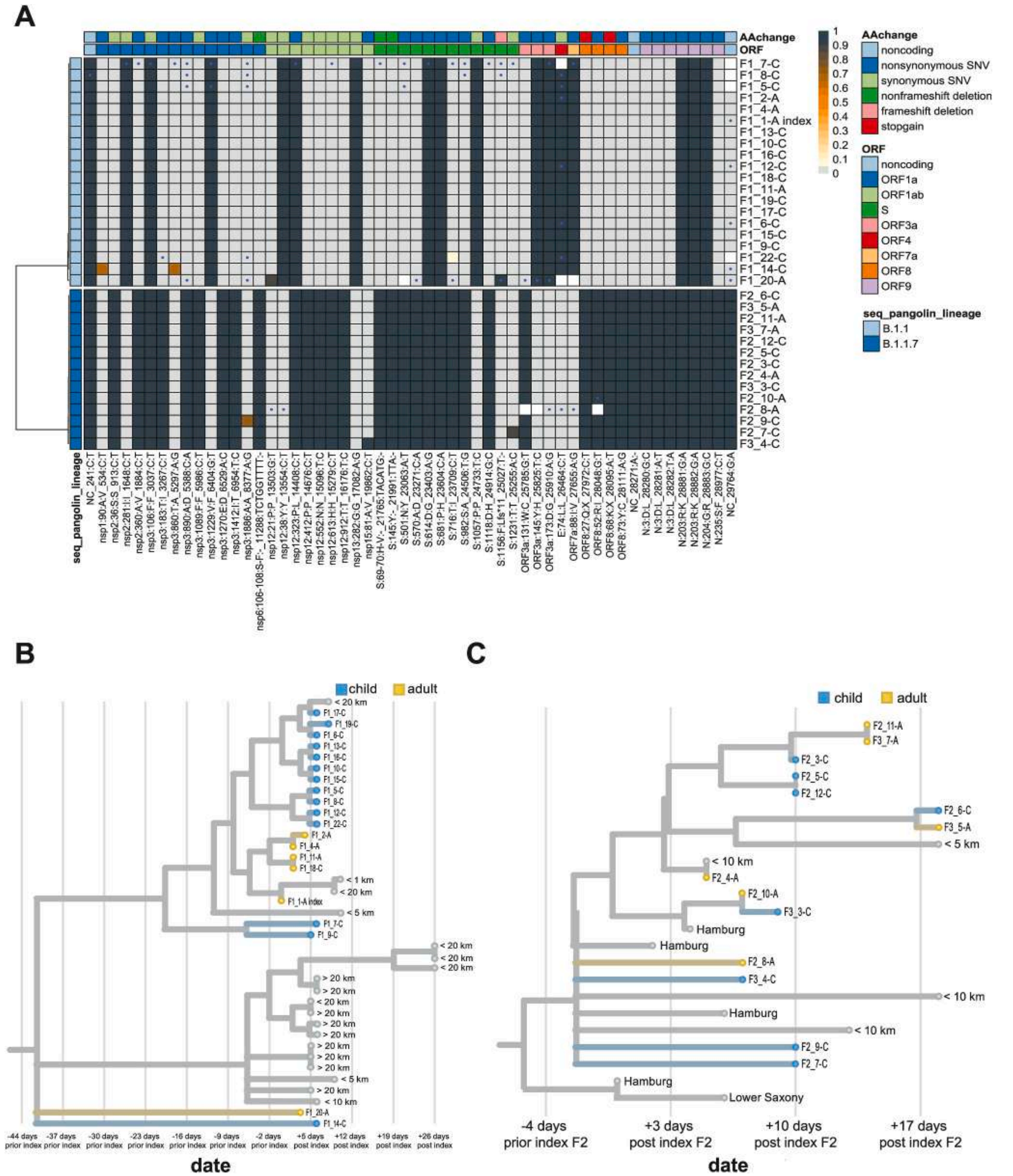

Fig. 3. Heatmap and phylogenetic tree representing the viral genotypes detected by SARS-CoV-2 whole-genome sequencing of samples from the three childcare facility outbreaks in Haag et al., 2021–February 2021. (A) The heatmap shows the position (left to right), the identity (lower row), and the frequency (color code) of substituted nucleotides identified by SARS-CoV-2 genome amplicon sequencing. The annotation to the pangolin lineage is depicted in the left column. Variants are relative to the reference strain initially found in Wuhan (NC_045512.2). White rectangles mark positions which were not covered by sequencing. Low coverage regions (<5 reads) are marked by blue dots. The open reading frame (ORF) as well as the type of amino acid change of the respective nucleotide position is highlighted as color code in the top rows of the heatmap. (B + C) Time-based phylogenetic tree highlighting the samples of the outbreaks in childcare facility 1 (B) and in childcare facilities 2 and 3 (C) in the context of German SARS-CoV-2 isolates. The phylogenetic tree was generated from the data of the childcare facility outbreaks, the Hamburg-Surveillance Database, and data available through GISAID (gisaid.org). The samples are color-coded by the allocation to children (blue) and childcare worker (yellow). The geographical distance of the related samples to the childcare facility is indicated as below 1, below 10 and below or above 20 km. AA: amino acid; A: adult; C: child; F: facility; km: kilometer; ORF: open reading frame; SNV: single nucleotide variant.

Table 1
Summary of self-reported symptoms of persons affected by the three childcare outbreaks (index and K1 contacts) in the city of Haag et al., 2021–February 2021. In the table, non-VOC/VOC infected children and non-VOC/VOC infected childcare workers are distinguished.
n: number of persons reporting the symptoms; %: percentage of people reporting symptoms; VOC: variant of concern. Percentage of asymptomatic non-VOC infected individuals: 15.2% (5/33), adults (1/11, 9.1%), children (5/22, 22.7%) and VOC-infected individuals: 11.8% (2/17) adults (1/7, 14.3%), children (1/10: 10%), see also Tables S1 and S2).

strain	group	fever		cough		cold		loss of taste/smell		shortness of breath		kidney pain		stay on ICU		fatigue	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
B.1.1	**children**	11	50.0	9	40.9	8	36.4	1	4.5	0	0	0	0	0	0	0	0
(non-VOC)	**childcare worker**	6	66.7	9	100	7	77.8	7	77.8	1	11.1	1	11.1	0	0	0	0
B.1.1.7	**children**	7	38.9	10	55.6	10	55.6	1	5.6	2	11.1	0	0	0	0	1	5.5
(VOC)	**childcare worker**	4	30.8	6	53.8	7	53.8	6	46.2	1	7.7	0	0	1	7.8	1	7.7

common in children (50%) than in childcare workers (16.7%), although overall rates were lower compared to F1 (35.3% vs. 52%). In F2 and F3, only two children and one childcare worker remained symptom-free.

We also analyzed SARS-CoV-2 household transmissions of the non-VOC and the VOC outbreaks. As the total number of second-generation contacts was unknown, SAR calculation was not possible. To compare non-VOC and VOC scenarios and detect potential differences between children and adults, we calculated the ratio of infected contact persons per condition (Table 2). While the ratios of infected second-generation contacts were comparable between non-VOC children (0.68) and non-VOC childcare workers (0.63), substantial, however not statistically significant, differences in the context of the VOC outbreaks were observed. The ratio of contacts infected by the VOC childcare workers was with 0.75 only slightly increased compared to the ratios of the non-VOC outbreak. In contrast, one VOC-affected child infected on average 1.3 household contacts indicating that with the occurrence of more infectious variants children contribute to SARS-CoV-2 transmissions more frequently.

4. Discussion

The study presented here provides the first comparative investigation of non-VOC- and VOC-induced SARS-CoV-2 outbreaks in childcare facilities. The fact, that the facilities were located within the same district of Hamburg, ministered to children with similar social-demographic background, and the outbreaks occurred within a short timeframe allow this comparison. Epidemiologic and molecular evidence including a SAR of 63.3%, suggests that the outbreak at F1 resulted from a superspreading event with the SARS-CoV-2 lineage B.1.1. In contrast, the outbreaks in F2/F3 represent a series of infection events with the SARS-CoV-2 VOC B.1.1.7. The SAR values (19.8%, F2 and 23.3%, F3) are lower, but are consistent with previous reports of B.1.1.7-associated outbreaks where SAR calculation ranged from 17 to 37% (Loenenbach et al., 2021).

Interestingly, childcare workers were identified as index cases in all three facilities. This is consistent with previous national and international reports of childcare center outbreaks, which all had originated from childcare workers (Hoehl et al., 2021; Lopez et al., 2020; Okarska-Napierala et al., 2021). However, these studies described outbreaks in the early phase of the pandemic, prior to the emergence of VOC SARS-CoV-2 strains. As children often remained asymptomatic during non-VOC SARS-CoV-2 infection, they might have been only sporadically identified as index cases (Davies et al., 2020; Laws et al., 2021). With the emergence of new, more infectious SARS-CoV-2 variants, such as B.1.1.7 or B.1.617.2, children may develop symptoms more frequently, potentially facilitating their identification as index cases. This observation is consistent with reports from the U.K. Office for National Statistics, which reported a higher proportion of persons who tested positive for the B.1.1.7 variant with symptoms compared with infected persons without the variant. Specifically, loss of taste and smell were reported less frequently and cough, sore throat, and myalgia were reported more frequently. However, two studies that examined the difference between B.1.1.7 infections and non-B.1.1.7 infections reported no significant difference in symptoms in adults or children (Graham et al., 2021; Meyer et al., 2021).

Although many childcare workers and children were affected by the SARS-CoV-2 outbreak at F1, infection of household contacts occurred only sporadically. On average, one child or childcare worker at the non-VOC facility was responsible for 0.68 and 0.63 household infections, respectively. While the average number of household infections originating from a VOC-infected childcare worker was 0.75, only slightly higher than that of non-VOC childcare workers, a VOC-infected child infected on average 1.3 household contacts. The observation of more frequent transmission in households among persons infected with B.1.1.7 is consistent with previous studies, including a prospective study from Norway that observed a significant increased SAR in households with VOC infected persons (Buchan et al., 2021; Julin et al., 2021). However, our values do not correspond to a SAR value since the total number of persons in the household was unknown in our reconstruction of these retrospective outbreaks. The finding that both, non-VOC- and VOC-infected children, were responsible for more household infections then their respective adult counterpart may be partly explained by the fact that small children cannot be easily separated. This finding is in contrast to previous studies describing lower household SAR of children compared to adults for infections with non-VOC strains (Wang et al., 2010). However, our results, together with data from three B.1.1.7 daycare center outbreaks (Loenenbach et al., 2021), suggest higher transmissibility of VOC SARS-CoV-2 strains by children compared with non-VOC strains. As the calculation of household SAR was not possible due to the non-prospective study design, our results are not directly comparable with previously published reports of childcare center

Table 2
Summary of secondary contact persons affected by the three childcare outbreaks in the city of Haag et al., 2021–February 2021. In the table, non-VOC/VOC children and non-VOC/VOC childcare workers are distinguished. The ratio of infected secondary contact person per non-VOC/VOC child/childcare worker is calculated by dividing the infected secondary contacts by the total number of infected persons. *Index case is not included.
VOC: variant of concern.

strain	group	facilty	total number of infected persons	infected K2 contacts	ratio
B.1.1 (non-VOC)	children	facility 1	22	15	0.68
	childcare worker	facility 1	8*	5	0.63
B.1.1.7 (VOC)	children	facility 2	10	13	1.3
		facility 3	8	10	1.25
		sum	18	23	1,28
	childcare worker	facility 2	6*	3	0.5
		facility 3	5*	5	1.0
		sum	11*	8	0.75

outbreaks and associated household infections (Madewell et al., 2020). Further limitation of this study are the self-reported symptoms of children and childcare workers and the lack of WGS from index cases in F2 and F3 and second-generation contacts. However, the epidemiologic investigation and the uniform genotypes of the available samples demonstrate a link between infection events. In addition, most second-generation infections could be linked to the outbreaks in the facilities based on their temporal classification.

Overall, our study provides new insights into the role of outbreaks in daycare centers during the SARS-CoV-2 pandemics. To our knowledge, it is the first study that, due to the timing of outbreaks and the geographical proximity of daycare centers, allows to compare VOC and non-VOC outbreaks. Altogether, revisiting hygiene measures should be reconsidered when new, more infectious variants such as B.1.1.7 and currently B.1.617.2 occur in daycare centers. Regular SARS-CoV-2 testing, obligation for childcare workers to wear a mask, segregation of children in small groups and, in case of an infected individual, screening of contacts regardless of symptoms might be an essential measure to prevent SARS-CoV-2 transmission in childcare facilities.

Declaration of competing interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113928.

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Concentrations and determinants of lead, mercury, cadmium, and arsenic in pooled donor breast milk in Spain

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ABSTRACT

Aim: To measure concentrations of lead (Pb), mercury (Hg), cadmium (Cd), and arsenic (As) in longitudinally collected donor breast milk samples and to determine associated factors.

Methods: Pb, Hg, Cd, and As concentrations were measured in 242 pooled breast milk samples from 83 donors to a Human Milk Bank in Spain, in 2015–2018, determining their association with the donors' sociodemographic profile, dietary and lifestyle habits, and post-partum time, among other factors, and with the nutritional characteristics of samples. Mixed-effect linear regression was used to identify predictors of Hg and As concentrations in breast milk and mixed-effect logistic regression to identify predictors of the presence of Pb and Cd.

Results: As was the element most frequently detected in milk samples (97.1%), followed by Hg (81.2%), Pb (50.6%), and Cd (38.0%). Their median breast milk concentrations were 1.49 μg/L, 0.26 μg/L, 0.14 μg/L, and <0.04 μg/L, respectively. Concentrations of As were higher in breast milk from primiparous donors, while Hg was higher in donors with a greater intake of fatty fish and meat and lower in samples collected after a longer post-partum time and with higher lactose content. Detection of Pb was higher among multiparous donors, those gaining weight since before pregnancy, and ex-smokers and was lower in samples collected more recently and from donors with greater intake of red meat and eggs. Cd detection was higher for donors with university education and those with greater intake of fried and canned food and more frequent use of hand cream and was lower for donors with greater bread intake.

Conclusions: These findings reveal relatively high As concentrations, moderate Hg concentrations, and low Pb and Cd concentrations in pooled donor breast milk. Several factors including post-partum time, parity, smoking habit, and the intake of certain food items were associated with the metal content of milk samples.

1. Introduction

The benefits for infants and mothers of breastfeeding are well documented (Labbok, 2001; Lawrence, 2000). Breast milk is the best source of nutrition for both full-term and preterm infants. It contains fats, carbohydrates, proteins, and other important dietary components, contributing to the growth, immunity, and development of the infant (Ballard and Morrow, 2013). Nevertheless, breast milk is also known to be a pathway for the maternal excretion of environmental chemicals, and there have been numerous reports worldwide on the presence of

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different types and concentrations of persistent organic pollutants (POPs) and toxic metals/metalloids due to past or current maternal exposure (Gil and Hernández, 2015; LaKind et al., 2018; Rebelo and Caldas, 2016).

Lead (Pb), mercury (Hg), cadmium (Cd), and arsenic (As) are widespread environmental metals that top the list of priority hazardous substances published by the Agency for Toxic Substances and Disease Registry (Agency for Toxic Substances and Disease Registry, 2019). Pb, Hg, As, and to a lesser degree Cd can readily pass through the placental barrier from the maternal bloodstream into the fetal circulation (Esteban-Vasallo et al., 2012) and can be excreted *via* breast milk after birth, with the amount transferred to the milk depending on their chemical form and distribution in maternal blood fractions (Gundacker and Zödl, 2005). Pb, Hg, Cd, and As are considered as persistent contaminants; however, unlike POPs, they do not bind to fat and therefore do not usually accumulate at higher concentrations in breast milk than in blood; hence, infants are likely exposed to higher levels of these metals before birth than during breastfeeding (Solomon and Weiss, 2002). Nonetheless, the metal content of breast milk is an important additional pathway of postnatal exposure and is likely to reflect intrauterine exposure (Solomon and Weiss, 2002) or even the lifetime exposure of the mother (LaKind et al., 2018). For instance, lactation can be accompanied by enhanced bone resorption due to the demand for calcium of nursing infants, which can mobilize Pb stored in maternal bone and contribute to the Pb content of breast milk (Ettinger et al., 2006). Pb and Hg are toxic for the reproductive system and the developing nervous system, Cd and As are known to be human carcinogens (Diamanti-Kandarakis et al., 2009; IARC, 2016), and all four metals are suspected endocrine disruptors (Diamanti-Kandarakis et al., 2009; Mendiola et al., 2011).

Pb and Hg have been the most investigated toxic metals in breast milk worldwide, with less research on the presence of As (Cherkani-Hassani et al., 2019; Rebelo and Caldas, 2016; Vollset et al., 2019). Breast milk concentrations of Cd are among the lowest reported (Cherkani-Hassani et al., 2017; Rebelo and Caldas, 2016) most likely because Cd-binding milk proteins have a greater affinity for calcium, which is abundant in breast milk (Vahter et al., 2002). Mothers who smoke or have a higher intake of certain food items (*e.g.*, fish) may be exposed to higher levels of Cd, Pb, and Hg (Bassil et al., 2018; Rebelo and Caldas, 2016; Vollset et al., 2019). With regard to As, higher breast milk concentrations were found in mothers from areas with elevated levels of As in drinking water (Samanta et al., 2007). Some studies have described a trend towards lower breast milk concentrations of toxic metals at later stages of lactation, but this has yet to be definitively established (Chao et al., 2014; García-Esquinas et al., 2011; Leotsinidis et al., 2005).

It is accepted that the benefits of breastfeeding generally outweigh the risks posed by the presence of environmental chemicals in the milk (Mead, 2008). However, it is important to improve knowledge on the exposure of infants to environmental chemicals and on changes in the exposure of mothers during lactation, particularly in the setting of the neonatal intensive care unit (NICU). However, only one recent study has been published on concentrations of toxic metals in donated breast milk given to hospitalized preterm newborns (Oliveira et al., 2020). Recent reports on toxic metals in breast milk from Spanish women are based on the analysis of Pb, Hg, and Cd in 100 samples collected at week 3 postpartum in Madrid (García-Esquinas et al., 2011) and of Pb, Hg, Cd, and As, among other trace elements, in 170 samples gathered in the city of Santiago de Compostela in Northern Spain (Mandiá et al., 2021). Our group recently reported the concentration profiles of various perfluoroalkyl substances (PFAS) and environmental phenols in donor breast milk from a Human Milk Bank in Granada, Southern Spain (Iribarne-Duran et al., unpublished results; Serrano et al., 2021). In the present study, we examined concentrations of Pb, Hg, Cd, and As and associated factors in longitudinally gathered breast milk samples from the same donors.

2. Materials and methods

2.1. Study population

During 2015–2018, 275 mature breast milk samples were obtained from 83 donors to the Regional Milk Bank of the Virgen de las Nieves University Hospital, Granada (Southern Spain) at different times postpartum (never before 2–3 weeks post-partum). All potential participants were invited to participate in this study. Exclusion criteria for the donors were previously described in detail (Serrano et al., 2021) and included: positive serology for HIV, syphilis, or hepatitis B or C; risk factor for sexual transmitted disease; transplantation in previous 6 months; current smoking or drug habit; and high consumption of alcohol (>20 g/day) or caffeine-containing drinks (>30 g/day). After providing their written informed consent, participating donors completed a questionnaire on socio-demographic characteristics and reproductive and lifestyle factors and donated milk samples for the analysis of environmental chemicals. The research protocol was approved by the Biomedical Research Ethics Committee of Granada. This study included 242 out of 275 milk samples from 83 donors with sufficient volume for the analysis of trace elements. Information on dietary habits and the use of personal care products (PCPs) was gathered for a sub-sample of 78 participants who provided a total of 228 samples.

2.2. Breast milk sample collection

Participating donors were asked by the milk bank to collect milk over a period of 1–4 consecutive weeks by manual expression and/or breast pump and to keep the samples frozen at −20 °C in their refrigerator until delivery to the bank. On arrival at the milk bank, samples were stored at −30 °C without ever breaking the cold chain. Immediately before pasteurization by the Holder method (within 2 weeks of arrival at the bank), samples collected from each donor were thawed and then pooled, obtaining an aliquot of 5–30 mL that was stored at −20 °C until analysis. The day of pasteurization was recorded as the donation date, and the interval between the first sample expressed by the mother and the donation date never exceeded 6 weeks. The median number of donations per woman was two, ranging from one to thirteen (four from 25% of participants and seven from 10%).

2.3. Sample preparation

First, 0.5 mL of milk sample was microwave digested in quartz vessels with 0.5 mL of HNO_3 (Suprapur, Merck, Darmstadt, Germany) using the Ethos UP system (Milestone, Shelton, CT, USA) programmed with 1800 W as maximum power and 210 °C as temperature limit (ramp time 20 min; hold time 15 min; cooling time 60 min). The digested solution was then transferred to a decontaminated tube for later analysis. Before their utilization, the quartz vessels were vigorously cleaned, soaked for 24 h in 10% HNO_3, thoroughly rinsed with Milli-Q® water, and dried at 80 °C for about 2 h. A certified reference material was used as quality control (ERM-BD151 Skimmed milk powder). Approximately 0.5 g of certified reference material was reconstituted with 4.5 mL of Milli-Q® water. The resulting liquid milk was then digested, as were all study samples. All samples were diluted 1:5 with 1% HCl (Suprapur, Merck).

2.4. Metal analysis

Quantification of Pb, Hg, Cd, and As concentrations in breast milk was performed by inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 8900 triple quadrupole ICP-MS (Agilent Technologies, Santa Clara, CA, USA) at the laboratory of the Department of Legal Medicine, Toxicology, and Physical Anthropology of the University of Granada (Spain). A calibration curve was prepared for each element in ultrapure water (Milli-Q) with 2% HNO_3 (Suprapur, Merck) and 1% HCl (Suprapur, Merck) using reference metal standard solutions (Agilent

Technologies) and analyzing blanks to correct the results. The instrument was tuned and performance parameters were checked before each analysis session. The quality of results was ensured by adding online multielement 400 μg/L internal standard solution with Sc, Ge, Ir and Rh to the samples. In addition, corresponding certified reference materials [National Institute of Standards and Technology NIST (USA) Trace Elements in Natural Water Standard Reference Material SRM 1640a and ERM-BD151 Skimmed milk powder] were reanalyzed together with a blank and an intermediate calibration standard every 12 samples. One in every twelve samples was also reanalyzed at the end of each session. Milk concentrations were expressed as μg/L. Limits of detection (LODs) were 0.10 μg/L for Pb, 0.05 μg/L for Hg, 0.04 μg/L for Cd, and 0.40 μg/L for As.

2.5. *Explanatory variables*

Information on potential explanatory variables was obtained from the questionnaires completed by donors on registration at the bank (donor selection questionnaire) and after inclusion in this study (research questionnaire). The research questionnaire was interviewer administered at the time of the first donation. The following data were gathered: age (continuous, years), university education (yes/no), occupation (unemployed/manual worker/non-manual worker), area of residence (urban/sub-urban/rural), living near agricultural land (yes/no), greenhouse (yes/no) or any industrial activity (yes/no), ex-smoker (yes/no), body mass index (BMI; underweight or normal/overweight or obese); parity (primiparous/multiparous), gestational length (continuous, weeks), birth weight (continuous, g), lifetime duration of breastfeeding (≤6/>6–12/>12–24/>24 months), weight gain during pregnancy (continuous, kg), weight change from before the most recent pregnancy (gain/loss/no change), presence of amalgam dental filling (yes/no), main source of drinking water (tap/bottled water), intake of coffee (1 cup per day/<1 cup per day) and alcoholic drinks (≥1 drink per month/<1 drink per month), average consumption frequency (servings [sv] per day or week) of seafood, fatty and lean fish, dairy products (yoghurt, milk, butter, cheese), red and cold meats, pulses, eggs, bread, chocolate, cereals, rice, pasta, fruit, raw and cooked vegetables, deep-fried food, and canned food (Table S1), and frequency of use of several PCP products (Table S2). PCPs were explored as potential determinants of breast milk metal concentrations because their utilization has been described as a potential source of exposure to toxic metals, including Pb and Cd (Mesko et al., 2020; Vahidinia et al., 2019). Data on dietary intake and the utilization of PCPs referred to the 12 months before the interview. None of the participating donors were pregnant during the period of donation. The post-partum time of the donation was calculated as the period between the date of the donation and the date of the most recent birth, categorized as ≤3, >3–6, >6–9, or >9 months. Cumulative lifetime breastfeeding was calculated by adding the aforementioned period to the lifetime breastfeeding time reported in the research questionnaire. The maternal age was updated at the time of each donation. Given that the transfer of metals into breast milk is produced by binding to proteins, the total protein content of the unpasteurized milk samples was examined as a potential explanatory variable, in addition to the total lipid, lactose, and caloric contents of samples.

2.6. *Statistical analysis*

In a descriptive analysis, concentrations of metals in individual samples (n = 242) and mean concentrations per donor (n = 83) were reported as medians, and 5, 25, 75, and 95 percentiles. Hg and As were detected in a large proportion of samples, and their values below the LOD were imputed as the LOD divided by the square root of two. Distributions of Hg and As were left-skewed and were therefore natural log (ln)-transformed to normalize data for analyses. Spearman correlation test was used to analyze associations between metal concentrations. Mixed-effect linear regression was used to examine predictors of Hg and As concentrations in breast milk. Given the high percentage of undetected values for Pb and Cd (49.5% and 62.0%, respectively), the odds of breast milk Pb and Cd concentrations above the LOD were assessed by using mixed-effect logistic regression. In mixed-effect models, the donor ID was treated as a random variable (cluster variable) to account for correlation between repeated measurements within subjects. A forward stepwise procedure was used to enter predictors (fixed variables) in the models. All variables listed in Tables 1, 2, S1, and S2 were tested as potential explanatory variables. Sensitivity analysis was performed by excluding outlier concentrations of As (n = 7) and Hg (n = 1) identified with studentized residuals >3. $P < 0.05$ was set to retain variables in the final model. Associations were expressed as exponentiated regression coefficients (exp[β]) or odds ratios (ORs) with 95% confidence intervals (CI). The “nlme” package in the statistical program R v.4.1.0 was used for statistical analyses (The R Project for Statistical Computing, https://www.r-project.org).

3. Results

Participating donors had a mean age of 33 years (range: 19–47 years), 61% had university education, 29% were manual workers, 42% resided in urban areas, 47% were ex-smokers, 31% were overweight or obese, and 10% had an amalgam dental filling (Table 1). At the time of the interview, 46% of donors were multiparous, the mean gestation time and newborn weight in their most recent pregnancy were 38 weeks and 2967 g, respectively, the mean weight gain during the pregnancy was 12 kg, with 53% donors gaining weight since before the pregnancy and 20% losing weight (Table 1). A full description of the dietary habits and PCP utilization of the participants is available in Serrano et al. (2021) and Supplementary material (Tables S1 and S2).

Milk samples were collected at a mean of 238 (range: 20–1513) days after the birth, and most (87%) of them were collected in 2015–2017; 25% of samples were collected after a lifetime breastfeeding time of >24 months and 28% after a time of ≤6 months. The mean protein content of

Table 1
Maternal characteristics and reproductive factors, n = 83 milk donors.

Variables	n (%)	Mean (range)
Maternal characteristics		
Age (years)		33 (19–47)
University education	51 (61.4)	
Occupation		
Unemployed	5 (6.2)	
Manual worker	24 (28.9)	
Non-manual worker	54 (65.1)	
Area of residence		
Rural	24 (28.9)	
Sub-urban	24 (28.9)	
Urban	35 (42.2)	
Living near agricultural land	40 (51.3)	
Living near greenhouse	14 (17.9)	
Living near industrial activity	61 (78.2)	
Ex-smoker	39 (47.0)	
Current BMI (kg/m^2)		23.67 (17.30–36.09)
Overweight/obese	26 (31.3)	
Amalgam dental filling	8 (9.6)	
Reproductive factors[a]		
Multiparous	38 (45.8)	
Length of gestation (weeks)		38 (26–41)
Birth weight (g)		2932 (840–4500)
Weight gain during pregnancy (kg)		12 (1–36)
Weight change from before pregnancy		
Weight loss	17 (20.5)	
Weight gain	44 (53.0)	
No weight change	22 (26.5)	

BMI: Body mass index.
[a] Reproductive data relative to the most recent pregnancy and before the first donation. None of the donors were or became pregnant during subsequent donations.

samples was 2.05 g/100 mL, their mean fat content was 3.83 g/100 mL, mean lactose content was 7.44 g/100 mL, and mean energy content was 68 kcal/100 mL (Table 2).

As was the element most frequently detected in milk samples (97.1%), followed by Hg (81.2%), Pb (50.6%), and Cd (38.0%). The median concentrations (5th-95th percentiles) in breast milk were 1.49 μg/L (0.56–3.50 μg/L) for As, 0.26 μg/L (<0.05–1.17 μg/L) for Hg, 0.14 μg/L (<0.10–6.31 μg/L) for Pb, and <0.04 μg/L (<0.04–0.44 μg/L) for Cd (Table 3). Weak to moderate positive correlations were observed between Pb and Cd (Spearman coefficient, r = 0.52, p < 0.001), between Hg and As (r = 0.27; p < 0.001), between Hg and Cd (r = 0.20, p = 0.001), and between Hg and Pb (r = 0.15, p = 0.02).

Linear regression models showed that concentrations of As in breast milk decreased by 32% (($\exp^{\beta}$-1)*100) (95%CI = 8–49%) in samples collected in 2017 *versus* 2015 and by 22% (95%CI = 1–40%) in samples from multiparous *versus* primiparous donors (Table 4). For Hg, it was found that samples collected at >3–6 *versus* ≤3 months post-partum had 41% (95%CI = 17–58%) lower concentrations; in addition, Hg decreased by 38% (95%CI = 5–59%) per each unit increase in lactose content. In contrast, donors consuming 1 sv/week of fatty fish had 68% (95%CI = 6–166%) higher Hg concentrations compared to <1 sv/week; and those consuming 2 and > 2 sv/week of meat compared to ≤1 sv/week showed a more than 2-fold (95%CI = 34–477%) and 3-fold (95% CI = 87–523%) increase in Hg, respectively (Table 4).

In logistic models (Table 5), the odds of a Pb concentration above the LOD were lower for samples collected in 2017 (OR = 0.38, 95%CI = 0.16; 0.90) and 2018 (OR = 0.15, 95%CI = 0.04; 0.48) compared with 2015, and for samples from donors with an intake of ≥1 sv/week of red meat *versus* rarely/never (OR = 0.23, 95%CI = 0.10–0.50) and from those with an intake of 2 sv/week of eggs *versus* 1 sv/week (OR = 0.38, 95%CI = 0.17–0.84). The odds of Pb detection were higher for multiparous donors (OR = 4.56, 95%CI = 2.09–10.8), those showing an increase in weight since before the pregnancy (OR = 3.15, 95%CI = 1.10–9.54), and ex-smokers (OR = 1.95, 95%CI = 1.05–3.64). The odds of Cd > LOD were higher for donors with university education (OR = 3.27, 95%CI = 1.69–6.56), those with a higher intake of fried food (1 sv/week: OR = 2.94, 95%CI = 1.33–6.76; >1 sv/week: OR = 4.25, 95%CI = 1.85–10.3 *versus* <1 sv/week), those regularly consuming canned food (OR = 3.78, 95%CI = 1.65–9.33), and those using hand cream once a day *versus* less frequently (OR = 2.99, 95%CI = 1.28–7.20); and were lower for those with a greater bread intake (1 sv/day: OR = 0.18, 95%CI = 0.07–0.42; >1 sv/day: OR = 0.21, 95%CI = 0.08–0.51 *versus* <1 sv/day).

Table 2
Characteristics of pooled milk samples (n = 242).

Variables	n (%)	Mean (range)
Year of sample collection		
2015	70 (28.9)	
2016	79 (32.6)	
2017	61 (25.2)	
2018	31 (13.8)	
Post-partum time (days)		238 (20–1513)
Post-partum time (months)		
≤3	60 (24.8)	
>3-6	54 (22.3)	
>6-9	57 (23.6)	
>9	71 (29.3)	
Lifetime duration of breastfeeding (months)		17.2 (0.7–77.3)
≤6	67 (27.7)	
>6-12	68 (28.1)	
>12-24	46 (19.0)	
>24	61 (25.2)	
Nutritional parameters		
Total proteins (g/100 mL)		2.05 (0.20–96.0)
Total lipids (g/100 mL)		3.83 (1.05–33.0)
Lactose (g/100 mL)		7.44 (6.10–8.10)
Calories (kcal/100 mL)		68 (44–117)

Exclusion of As outliers led to an inverse association with lifetime breastfeeding, so that breastfeeding for >6–12 and > 12–24 months *versus* ≤6 months was associated with a significant decrease in As concentration of 21% (95%CI = 6–32%) and 22% (95%CI = 6–36%), respectively, while As concentrations were not influenced by the parity of donors after excluding outliers (Table S3). With respect to Hg, exclusion of one outlier in breast milk concentrations did not change the results (data not shown).

4. Discussion

This study is one of the first to provide information on toxic metal concentrations in breast milk from donors in Spain. As was present in almost all pools of milk, Hg in four out of five, Pb in one out of two, and Cd in one out of three. Results suggest a trend towards a decline in Hg concentrations over the lactation period and a decline in Pb concentrations between 2015 and 2018. In addition, smoking habit, the intake of certain food items, weight change since before the pregnancy, and schooling level, among other factors, emerged as significant predictors of breast milk metal concentrations, which were not associated with the total protein content of samples.

4.1. Arsenic

Concentrations of total As in breast milk from these Spanish donors were higher than concentrations in mature milk obtained from women in Germany (Sternowsky et al., 2002), Italy, Croatia, Slovenia (Miklavčič et al., 2013), Cyprus (Kunter et al., 2017), Sweden (Björklund et al., 2012), Japan, the USA (Carignan et al., 2015), Thailand (Chao et al., 2014), the United Arab Emirates (Abdulrazzaq et al., 2008). Total As concentrations were also higher than those found in 49 pre-concentrated and concentrated breast milk samples from a Brazilian milk bank (Oliveira et al., 2020). They were in the range of concentrations found in the milk of women from India (Samanta et al., 2007) and Bangladesh (Fängström et al., 2008; Islam et al., 2014) exposed to high levels of inorganic As in drinking water (Rebelo and Caldas, 2016), but they were lower than concentrations in primiparas from Lebanon (Bassil et al., 2018). Notably, the maximum As value in Spanish donors (56 μg/L) was higher than the maximum concentration found in milk from the highly exposed women in India (49 μg/L) (Samanta et al., 2007) and Bangladesh (8.9 and 19 μg/L) (Fängström et al., 2008; Islam et al., 2014). In addition, As concentrations in our study population were in the range of those found in the only Spanish study providing data on As in breast milk, which included 70 full-term and 100 preterm mothers recruited in Santiago de Compostela in 2018–2019 (mean = 1.37 μg/L) (Mandiá et al., 2021).

The main sources of As exposure are drinking water contaminated with inorganic As and the intake of seafood and rice, which can contain elevated concentrations of organic and inorganic As, respectively (Hughes et al., 2011). As in breast milk has previously been associated with the intake of fish/seafood (Bassil et al., 2018; Miklavčič et al., 2013) and rice/cereals (Bassil et al., 2018), but these associations were not found in the present study. Organic forms of As such as monomethyl As (MMA) and dimethyl As (DMA) are much less toxic than inorganic forms such as trivalent (AsIII) and pentavalent As (AsV), which have been classified as type 1 carcinogens (IARC, 2016). AsIII is the only form of As transported by the aquaglyceroporins present in mammary glands during lactation (Liu et al., 2004; Matsuzaki et al., 2005). It has been shown that the efficient maternal methylation of inorganic As into MMA and DMA leads to a lower excretion of As *via* breast milk, because MMA and DMA in blood plasma do not easily pass through the mammary glands. For these reasons, breast milk largely contains inorganic As, mainly as AsIII (Rebelo and Caldas, 2016). Rice and seafood consumption has been described as the major source of As exposure in Spain (Signes-Pastor et al., 2017). The specific source of exposure to As in the present donors remains unknown; however, previous studies showed

Table 3
Concentrations of metal(oid)s in breast milk (μg/L).

	LOD	% >LOD	Percentiles					Max.
			5	25	50	75	95	
Individual[a] sample concentrations (n = 242 samples)								
As	0.4	97.1	0.563	1.073	1.494	1.989	3.501	56.52
Hg	0.05	81.2	<0.05†	0.080	0.261	0.538	1.174	2.428
Pb	0.10	50.6	<0.10†	<0.10†	0.138	1.250	6.315	49.32
Cd	0.04	38.0	<0.04†	<0.04†	<0.04†	0.070	0.442	4.936
Mean concentrations per donor (n = 83 donors)								
As	0.4	–	0.596	1.160	1.660	1.981	7.582	52.17
Hg	0.05	–	<0.05†	0.151	0.277	0.529	1.134	1.515
Pb	0.10	–	<0.10†	<0.10†	0.220	1.138	6.013	24.70
Cd	0.04	–	<0.04†	<0.04†	<0.04†	0.093	0.480	1.860

LOD: Limit of detection.
[a] Pools of milk samples.

Table 4
Mixed-effects linear regression models for predictors of concentrations of As and Hg in pooled breast milk.

As (n = 242 samples from 83 donors)			Hg (n = 228 samples from 78 donors)		
Explanatory variables	Exp (β)	95%CI	Explanatory variables	Exp (β)	95%CI
Year of collection (ref: 2015)			Post-partum time (ref: ≤3 months)		
2016	0.78	0.59; 1.02*	>3–6 months	0.59	0.42; 0.83**
2017	0.68	0.51; 0.92**	>6–9 months	0.80	0.55; 1.15
2018	0.96	0.65; 1.42	>9 months	0.86	0.60; 1.23
Multiparous vs. primiparous	0.78	0.60; 0.99**	Lactose content (g/100 mL)	0.62	0.41; 0.95**
			Fatty fish intake (ref: < 1 sv/week)		
			1 sv/week	1.68	1.06; 2.66**
			>1 sv/week	1.40	0.77; 2.53
			Meat intake (ref: ≤1 sv/week)		
			2 sv/week	2.79	1.34; 5.77**
			>2 sv/week	3.42	1.87; 6.23***

***p < 0.001; **p < 0.05; *p < 0.10.

Table 5
Mixed-effects logistic regression models for predictors of Pb and Cd in pooled breast milk (n = 228 from 78 donors).

Detected Pb 113 out of 228 samples (49.6%) >LOD			**Detected Cd 88 out of 228 samples (38.6%) >LOD**		
Explanatory variables	**OR**	**95%CI**	**Explanatory variables**	**OR**	**95%CI**
Year of collection (ref: 2015)			University vs. lower schooling level	3.27	1.69; 6.56***
2016	0.56	0.23; 1.34	Bread intake (ref: <1 sv/day)		
2017	0.38	0.16; 0.90**	1 sv/day	0.18	0.07; 0.42***
2018	0.15	0.04; 0.48**	>1 sv/day	0.21	0.08; 0.51***
Multiparous vs. primiparous	4.56	2.09; 10.8***	Fried food intake (ref: <1 sv/week)		
Weight change (ref: no change)			1 sv/week	2.94	1.33; 6.76**
Weight gain	3.15	1.10; 9.54**	>1 sv/week	4.25	1.85; 10.3***
Weight loss	1.39	0.57; 3.40	Canned food intake (ever vs. never)	3.78	1.65; 9.33**
Ex-smoker vs. never smoker	1.95	1.05; 3.64**	Hand cream (ref: <once a day)		
Red meat intake (ref: never)			once a day	2.99	1.28; 7.20**
<1 sv/week	1.56	0.69; 3.53	>once a day	1.37	0.53; 3.47
≥1 sv/week	0.23	0.10; 0.50***			
Eggs intake (ref: 1 sv/week)					
2 sv/week	0.38	0.17; 0.84**			
>2 sv/week	1.32	0.56; 3.14			

LOD: Limit of detection.
***p < 0.001; **p < 0.05; *p < 0.01.

that the topsoil in Southeastern Spain (including Granada province) contains relatively high concentrations of As (Núñez et al., 2016), likely attributable to past usage of phosphate fertilizers. The high occurrence of As (mainly inorganic As) in the breast milk of donors is of particular concern, given that even low concentrations of As have been shown to impair cognitive function and increase the risk of cancer in infants and young children (Rodríguez-Barranco et al., 2016; Tyler and Allan, 2014). Nonetheless, it has been suggested that exclusively breastfed infants are exposed to lower concentrations of As than are non-breastfed infants (Carignan et al., 2015; Fängström et al., 2008), indicating that exclusive breastfeeding may protect the infant from As exposure. Therefore, it is imperative to implement preventive measures to eliminate or reduce the presence of As in breast milk and to closely monitor its concentration in nursing mothers.

The decrease in As concentrations observed in multiparous donors and those with longer lifetime breastfeeding may suggest the clearance of As during lactation, but As was not associated with the post-partum time. This is consistent with a study in Bangladesh that found no difference in As concentrations in milk samples collected at 1, 6, or 9 months post-partum (Islam et al., 2014). In Portuguese and Taiwanese women, As milk concentrations were significantly higher in colostrum than in mature milk (Almeida et al., 2008; Chao et al., 2014), but these results are not comparable to the present findings because colostrum, which may have a higher concentration of metal-binding proteins, was not collected. Overall, the transport mechanism of As *via* breast milk has not been fully elucidated, and the few available data on postnatal exposure to As from breast milk are not conclusive.

4.2. Mercury

Breast milk concentrations of total Hg reported in the literature vary widely among different regions, with the highest concentrations (up to 59 μg/L) found in the Brazilian Amazon (Rebelo and Caldas, 2016). In general, Hg concentrations in Spanish donors are comparable to those found in women from Austria (Gundacker et al., 2010), Croatia, Greece, Italy, Slovenia (Miklavčič et al., 2013; Valent et al., 2013), Sweden (Björnberg et al., 2005), Brazil (Oliveira et al., 2020), Japan (Iwai-Shimada et al., 2015; Sakamoto et al., 2012) and Iran (Behrooz et al., 2012; Okati et al., 2013), with mean/median concentrations ranging from 0.1 to 0.8 μg/L. The present concentrations are in the range of those observed in mature milk samples collected in 2003–2004 from Spanish women in Madrid (mean = 0.53 μg/L) (García-Esquinas et al., 2011) and more recently in Santiago de Compostela (mean = 0.31 μg/L) (Mandiá et al., 2021). They are lower than concentrations found in breast milk samples from the Faroe Islands (Needham et al., 2011), different Asian regions (China, India, Indonesia, Korea, Taiwan) (Bose-O'Reilly et al., 2008; Chien et al., 2006; Li et al., 2014; Orün et al., 2012; Vahidinia et al., 2019), the Middle East (Saudi Arabia, Iran, Turkey) (Al-Saleh et al., 2013; Orün et al., 2012; Vahidinia et al., 2019), Africa (Tanzania, Zimbabwe) (Bose-O'Reilly et al., 2008), and Latin America (Brazil, Mexico) (Cunha et al., 2013; Gaxiola-Robles et al., 2014; Santos-Silva et al., 2018; Vieira et al., 2013); however, they are higher than concentrations observed in samples from Cyprus (Kunter et al., 2017) and the United Arab Emirates (Abdulrazzaq et al., 2008).

A significant association was observed between the intake of 1 sv/week of fatty fish and a higher Hg concentration in breast milk, consistent with the findings of a large study of samples from Croatia, Greece, Italy, and Slovenia, which found an association between fish consumption and breast milk Hg concentrations (Miklavčič et al., 2013). Other studies of women with a high or relatively high consumption of fish also reported an association of fish/seafood intake with Hg concentrations in breast milk (García-Esquinas et al., 2011; Gaxiola-Robles et al., 2013; Grandjean et al., 1995; Iwai-Shimada et al., 2015; Vollset et al., 2019). In general, the intake of fish, particularly fatty fish, is the main source of exposure to methyl-Hg (MeHg), the most neurotoxic form of Hg (Gil and Gil, 2015). It has also been shown that when fish intake is high, around one-half of breast milk Hg is in the form of MeHg (Islam et al., 2014; Miklavčič et al., 2013; Valent et al., 2013) and the other half is in the form of inorganic Hg (Oskarsson et al., 1996). We were unable to distinguish between organic and inorganic Hg, but a significant amount of MeHg can be expected in the present milk samples because of the relatively frequent consumption of fish by the donors. This is a cause for concern, given that MeHg is almost completely absorbed by the gastrointestinal tract of infants and can readily cross the blood-brain barrier and affect neurological functions (Caserta et al., 2013; Grandjean and Landrigan, 2006), even at low doses (Karagas et al., 2012). The association of meat intake with Hg concentrations is less certain because of the limited information on Hg levels in land animals. Although Hg can also bioaccumulate in this type of animal, the meat is likely to contain low concentrations of Hg (Björnberg et al., 2005; Nawrocka et al., 2020; Vollset et al., 2019). The presence of amalgam fillings, a major source of elemental Hg exposure, was not associated with Hg excretion in these Spanish donors. Studies examining the association of amalgam fillings with Hg in breast milk have yielded conflicting results, with some showing a positive association (Björnberg et al., 2005; Vollset et al., 2019) and others finding no relationship between them (García-Esquinas et al., 2011; Gundacker et al., 2002).

The inverse association observed between post-partum time and Hg concentration suggests a depuration of this metal during lactation, especially in the first few months. However, Hg concentrations in breast milk from Iranian (N = 100) and Swedish (N = 20) women remained unchanged throughout lactation (Björnberg et al., 2005; Vahidinia et al., 2019), while García-Esquinas et al. (2011) reported non-significant decreases in Hg concentrations in milk from older and multiparous women in Spain and in those with a previous history of lactation, suggesting a possible clearance of Hg over their lifetime. A decrease in Hg concentrations over the lactation period can be explained by a reduction in the milk's content of proteins such as albumin and casein, which enable the transport of both inorganic and organic Hg (Sundberg et al., 1999). Indeed, the protein content of the present donor milk samples slightly decreased with longer post-partum time (data not shown). Moreover, MeHg is a lipophilic compound, so that accumulated body stores of MeHg would decline with longer breastfeeding time (Jain, 2013; LaKind et al., 2004). However, the depuration of lipophilic chemicals during lactation may also be influenced by the current exposure of the mother, the volume of milk consumed by the infant, and supplementation with formula or solid food, among other factors (LaKind et al., 2018). In addition, the reason for the inverse association between the lactose and Hg content of samples remains unclear.

4.3. *Lead*

Breast milk Pb concentrations in these Spanish donors are several times lower than concentrations described in studies published over the past two decades in Asia (Chao et al., 2014; Isaac et al., 2012; Li et al., 2000; Sharma and Pervez, 2005), the Middle East (Al-Saleh et al., 2003; Bassil et al., 2018; Vahidinia et al., 2019), South America (Counter et al., 2004; Marques et al., 2013; Oliveira et al., 2020), North America (Hanning et al., 2003; Sowers et al., 2002), and Africa (Adesiyan et al., 2011; Moussa, 2011), and they are similar to or in the lower range of those found in women from Japan (Sakamoto et al., 2012), Australia (Gulson et al., 2003), Mexico (Ettinger et al., 2004, 2006), and various European countries (Abballe et al., 2008; Almeida et al., 2008; Gundacker et al., 2002; Kunter et al., 2017; Leotsinidis et al., 2005). In comparison to other Spanish studies, Pb concentrations in our donors are much lower than concentrations in samples collected in 2003–2004 in Madrid (mean = 15.6 μg/L) (García-Esquinas et al., 2011) but comparable to those in samples recently collected in Galicia (mean = 0.30 μg/L) (Mandiá et al., 2021), indicating a decline in Pb exposure in Spain after the suppression of leaded gasoline in 2001 (RealDecreto403, 2000). In this line, a decreasing trend in breast milk Pb concentrations was observed in the present donors over the period under study (2015–2018).

Diet is considered the major source of Pb exposure for the general population, particularly the intake of vegetables and cereals (Martí-Cid et al., 2008), and breast milk Pb concentrations have been associated with the intake of potatoes in Spanish (García-Esquinas et al., 2011) and Lebanese mothers (Bassil et al., 2018). No food item was found to predict Pb excretion in the present milk samples, probably due to the low Pb concentrations, while the intake of red meat and eggs was associated with lower breast milk Pb. However, these results should be interpreted with caution, given that much of the Pb in breast milk comes from Pb stored in the bones and not from the exposure of mothers during lactation. Pb is also found in cigarette smoke (Bernhard et al., 2005) which may explain the higher odds of detectable Pb in breast milk from former smokers *versus* never smokers. Similar results were reported by the Lebanese and Spanish studies (Bassil et al., 2018; García-Esquinas et al., 2011; Mandiá et al., 2021).

Pb excreted into breast milk is mainly found in the casein fraction (Chao et al., 2014; Ettinger et al., 2006; Leotsinidis et al., 2005; Oskarsson et al., 1996) and, when bone Pb levels are not high, breast milk concentrations generally decline over the course of lactation due to the decrease in casein content (Chao et al., 2014; Ettinger et al., 2006; Leotsinidis et al., 2005). The lack of an association between Pb and post-partum time in the present study may be explained by the low Pb concentrations. The reason for the higher Pb concentrations found in multiparous donors is not clear; while a possible explanation for the higher concentrations in mothers gaining weight since before the pregnancy is that this weight gain would increase the release of Pb from bone deposits, because a low calcium intake has been previously related to obesity and weight gain (Lappe et al., 2017).

4.4. *Cadmium*

Most of the donors in our study had Cd below the LOD, and concentrations were far below the range of those reported for mothers worldwide (Cherkani-Hassani et al., 2017; Rebelo and Caldas, 2016; Oliveira et al., 2020), only being comparable to those described in a few European studies (Björklund et al., 2012; Kantol and Vartiainen, 2001; Vollset et al., 2019) and Iran (Vahidinia et al., 2019). In fact, the concentrations in our donors were several times lower than in previous Spanish studies, which reported mean concentrations of 1.31 μg/L (García-Esquinas et al., 2011) and 0.15 μg/L (Mandiá et al., 2021).

Maternal smoking has been associated with Cd concentrations in breast milk in previous studies (Bassil et al., 2018; García-Esquinas et al., 2011; Gundacker et al., 2007), but no significant association was observed between detectable Cd in breast milk and smoking habit, most likely due to the lack of current smokers and the low Cd concentrations among the donor mothers. Non-smokers are mainly exposed to Cd through their intake of foods such as cereals, tubers, green leafy vegetables, fruit, nuts, pulses, fish, and shellfish (Gundacker et al., 2007; Leotsinidis et al., 2005; Martí-Cid et al., 2008). In the present study, Cd concentrations were not associated with the intake of any of these food items but were higher in mothers with a university education which may be due to a higher intake of foods containing Cd. In line to the present findings, Vahidinia et al. (2019) found no association between low Cd concentrations in breast milk and the intake of vegetable and fruit. The positive associations with the intake of fried and canned food and the inverse association with bread intake should be interpreted with caution, because these novel findings may be affected by imprecision due to the low Cd concentrations. Finally, hand cream use was associated with Cd, and several studies have demonstrated the presence of toxic elements, including Cd, Pb, Hg, and As, in cosmetics products such as lipstick and eye cosmetics (Mesko et al., 2020). Toxic metals may be retained as impurities in the raw materials used in the cosmetics or released from the metallic devices used during their production (Bocca et al., 2014). However, further research is needed to elucidate the potential exposure to toxic metals from cosmetics and other PCPs.

4.5. Limitations and strengths

This study has a number of limitations. First, the milk donors are more homogeneous in terms of socioeconomic profile than are lactating women in general (*e.g.,* most donors had a university education and were non-manual workers); therefore, the study findings cannot be generalized to breastfeeding women in the general population. Second, only total Hg and As were determined, limiting the capacity to identify more specific sources of exposure. Third, bias may have resulted from a misreporting of dietary intake and other factors. It is also possible that their diet might have changed with respect to the 12 months before their first donation, the reference period for the dietary questionnaire. Nevertheless, misclassification is unlikely to be related to breast milk metal concentrations. Another limitation is that the large number of explanatory factors assessed may have generated some spurious statistically significant associations. Finally, the possibility of metal contamination cannot be ruled out, because no special provisions were made during the pooling or processing of the milk to avoid metal contamination.

Current knowledge on the presence of toxic metals in breast milk is generally based on a small number of samples; however, a large number were obtained longitudinally from donors in the present investigation, allowing exploration of the variation in concentrations over the lactation period. A further study strength is the assessment of pooled milk samples (over 1–4 weeks), given that the composition of breast milk and, therefore, its toxic metal concentrations, can change during a feeding session, at different times of day, and from day to day due to variations in the mother's dietary intake, among other factors. Hence, the evaluation of pooled samples may reduce the risk of exposure misclassification in comparison to the measurement of metal concentrations in spot breast milk samples.

It is important to note that the mere presence of the studied metals in breast milk does not necessarily imply a health risk for the breastfeeding infant. Nevertheless, breast milk donated to milk banks is supplied to highly vulnerable preterm infants, and preventive measures are required to avoid their exposure to metals from this source. Recommendations should be especially targeted at ensuring healthy habits in milk donors, including the maintenance of optimal calcium and iron intakes and the limited consumption of fatty fish during pregnancy and breastfeeding.

5. Conclusion

Toxic metals/metalloids such as Pb, Hg, Cd, and As continue to pose a public health threat worldwide. In this study, 97 and 81% of pooled donor breast milk samples had detectable concentrations of As and Hg, respectively, while 51 and 38% had detectable concentrations of Pb and Cd. Given the extreme vulnerability of preterm infants, it is essential to closely monitor concentrations of toxic metals in donor breast milk and to develop appropriate measures to reduce their exposure to these chemicals and avoid unnecessary risks.

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Declaration of competing interest

The authors declare no actual or potential competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113914.

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Cross-sectional associations between serum PFASs and inflammatory biomarkers in a population exposed to AFFF-contaminated drinking water

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ABSTRACT

Background: Per- and polyfluoroalkyl substances (PFASs) are widespread and persistent environmental contaminants. Exposure to several PFASs has been associated with altered immune function in humans, including autoimmune disease and impaired response to vaccination. However, changes to the profile of inflammatory biomarkers in adults exposed to PFASs has not been extensively described.
Objective: To estimate cross-sectional associations between serum PFASs and markers of inflammation among adults in a population exposed to aqueous film forming foam (AFFF)-contaminated drinking water.
Methods: We quantified concentrations of 48 PFASs in non-fasting serum samples from 212 non-smoking adults. In the same serum samples, we measured concentrations of ten pro- and anti-inflammatory cytokines. We restricted analysis to seven PFASs detected in >85% of participants and the following four cytokines detected in ≥30% of participants: interleukin [IL]-1β, IL-6, IL-10, and tumor necrosis factor [TNF]-α. We fit multiple linear regression or logistic regression models, adjusted for potential confounders, to estimate associations between concentrations of each PFAS and either continuous or categorical (above vs below limit of detection) concentrations of each cytokine. We additionally applied Bayesian kernel machine regression to describe the combined effect of the PFAS mixture on each cytokine outcome.
Results: Certain PFAS concentrations in this sample were elevated compared to a US nationally representative sample; median levels of PFHxS, ΣPFOS and ΣPFOA in this sample were 13.8, 2.1 and 1.7 times higher, respectively, than medians observed in the U.S. sample. Higher concentrations of multiple PFASs were significantly associated with lower odds of detectable IL-1β. Weaker associations were observed with other cytokines. In general, perfluoroalkyl carboxylic acids had inverse associations with TNF-α, whereas the perfluoroalkyl sulfonic acids showed positive associations.
Conclusions: We observed preliminary evidence of altered inflammatory profiles among adults with elevated serum concentrations of PFASs due to contaminated drinking water. Modifications to inflammatory pathways may be one mechanism by which PFAS exposures produce adverse health effects in humans, but this finding requires verification in longitudinal studies as well as phenotypic anchoring to immune function outcomes.

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1. Introduction

Per- and polyfluoroalkyl substances (PFASs) are a class of synthetic chemicals widely used in various commercial products and industrial processes since the 1950s because of their oil-, grease-, water-, stain- and heat-resistant properties (CDC 2017). PFASs are highly persistent in the environment and many can accumulate in the human body, with estimated elimination half-lives in humans as long as two to eight years for some of the longer chain (e.g., ≥6 carbons) PFASs (Bartell et al., 2010; Hu et al., 2016; Li et al., 2018; Olsen et al., 2007; Xu et al., 2020). Because of these properties and their widespread use, many different PFASs have been detected in the blood of most people (>99%) in the United States (U.S.) (CDC ATSDR, 2020).

Experimental studies provide evidence in support of immunotoxic effects of PFAS exposure. In several animal models, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) have been reported to alter adaptive and innate immune responses, including inflammation and cytokine production (DeWitt et al., 2012). Exposure to PFOA and PFOS in adult mice suppresses T-cell-dependent immunoglobulin M (IgM) production, one of the most sensitive predictive measures of immune function (DeWitt et al., 2012). The strength of these findings was confirmed by a systematic review of immunotoxicological evidence for PFOA and PFOS by the National Toxicology Program (NTP) and with support from epidemiological findings, the NTP determined that PFOA and PFOS were presumed immune hazards to humans (NTP 2016). The mechanism of action for immunotoxicity remains to be elucidated and results vary by species, strain, sex, and route of exposure. Changes in concentrations of circulating inflammatory cytokines may be one indicator of underlying immunotoxic effects of PFASs in humans. Additionally, disturbances to cytokine signaling may be involved in other adverse health effects of PFAS exposure, such as hepatotoxicity (Bassler et al., 2019).

In humans, there is evidence that PFAS exposure is associated with increased cholesterol levels, changes in liver enzymes, increased risk for kidney and testicular cancer, increased risk of high blood pressure and pre-eclampsia in pregnant women, and lower infant birth weight (ATSDR 2020). Of particular interest, these compounds also have been reported to impair immune system function in humans (ATSDR 2021). Epidemiological findings concerning PFAS immunotoxicity demonstrate suppressed vaccine responses across different populations and mixed results for findings of inappropriate immune stimulation such as allergy, asthma, and autoimmune disease across populations (ATSDR 2017, 2021; Costa et al., 2009; Steenland et al., 2010). However, multiple studies have indicated that exposure to elevated levels of PFASs is associated with a reduced humoral response to childhood immunizations with some evidence for reduced response to the influenza vaccine among adults (Grandjean et al. 2012, 2017; Granum et al., 2013; Kielsen et al., 2016; Looker et al., 2014; Mogensen et al., 2015; Stein et al. 2016a, 2016b). Additionally, in one large community-based study, exposure to PFOA was associated with increased odds of ulcerative colitis, an autoimmune disease (Steenland et al., 2013). In an occupationally exposed cohort, incidence of ulcerative colitis and rheumatoid arthritis was highest among individuals with the highest cumulative occupational and residential exposure to PFOA (Steenland et al., 2015).

We therefore aimed to study the association between PFAS concentrations in serum and circulating inflammatory cytokines in a human population with elevated PFAS exposure from contaminated drinking water. From 2013 to 2016, concentrations of PFOA and PFOS above the U.S. Environmental Protection Agency (EPA) health advisory level (70 ng/L) were detected in municipal water systems downstream of the Peterson Space Force Base, impacting approximately 80,000 people in Fountain Valley, Colorado (Barton et al., 2020). The source of the contamination was believed to be aqueous film-forming foams (AFFF) released from the nearby Space Force base after use in training exercises and in extinguishing fuel fires, with use dating back to the 1970s. Use of AFFF is common in the U.S., with over 533 civilian airports and 290 military bases reporting their use as of 2015 (Hu et al., 2016). Some AFFFs contain high concentrations of perfluorohexane sulfonate (PFHxS) and its precursors, which are less studied in humans compared to PFOA and PFOS. A recent animal study demonstrated that mixtures of AFFF-derived PFASs impair immune function in mice and that mixtures of PFASs may have more adverse effects than exposure to individual PFASs (McDonough et al., 2020b).

Each of the inflammatory biomarkers analyzed in this study have distinct roles in regulating the inflammatory process. While TNF-α, IL-6, and IL-1β are typically pro-inflammatory and IL-10 is generally anti-inflammatory, IL-6 can be either pro- or anti-inflammatory(Chatzantoni and Mouzaki 2006; Iyer and Cheng 2012; Kaneko et al., 2019; Scheller et al., 2011). TNF-α is often elevated during infection and higher circulating concentrations have been linked with insulin resistance and several autoimmune diseases(Chatzantoni and Mouzaki 2006; Nieto-Vazquez et al., 2008). Similarly, elevations in IL-6 and IL-1β are associated with autoimmune diseases, diabetes, and cancer (Kaneko et al., 2019; Robertson and Coveney 2021). IL-10 plays a role in controlling allergies and asthma, and IL-10 deficiency has been associated with inflammatory bowel disease and autoimmune disease (Iyer and Cheng 2012). IL-1β is a key pro-inflammatory mediator and as it is produced by many cell types, it may be a better marker of generalized immunosuppression than other cytokines. These biomarkers are useful in understanding immune system function and linked with autoimmune diseases. The goal of this study is to describe the cross-sectional associations between individual PFASs, and an AFFF-related mixture of PFASs, with circulating pro- and anti-inflammatory biomarkers.

2. Methods

2.1. Study population

The PFAS Assessment of Water and Resident Exposure (PFAS-AWARE) Study population, design and procedures are described in detail elsewhere (Barton et al., 2020). In brief, the PFAS-AWARE study included 220 non-smoking adults who lived in one of three PFAS-impacted water districts (Fountain, Security or Widefield) or whose water was provided by a PFAS-impacted private well in one of these areas ($n = 16$). In June 2018, all participants provided a 20-mL non-fasting blood sample and completed an exposure and health history questionnaire. For this analysis, seven participants were excluded because they did not live in the PFAS-impacted area for at least two years during the time of known contamination (2012–2015; prior to remediation efforts that began in fall of 2015 in Fountain), and one participant was excluded because they were undergoing dialysis treatment at the time of the blood draw, resulting in a final sample size of 212. For all analyses with TNF-α as the outcome, two individuals were excluded due to use of TNF-α altering medications as reported on the questionnaire. Medications considered to be TNF-α altering included: Remicade (infliximab), Enbrel (etanercept), Humira (adalimumab), Cimzia (certolizumab pegol), and Simponi (golimumab).

The study protocol was approved by the Colorado Multiple Institutional Review Board and all participants provided informed consent prior to study procedures.

2.2. Serum preparation

Venous blood was collected into BD vacutainer red top tubes (Becton Dickinson, New Jersey, USA) and within 30 min of collection, was centrifuged in a 5804R centrifuge (Eppendorf, Hamburg, Germany) for 15 min at 1300×*g* to separate serum. 500 μl serum aliquots were obtained and placed on dry ice (maximum time on dry ice was 54 h) until they were transported to the laboratory where they were stored at −80 °C until analysis.

2.3. Cytokine analysis

A panel of pro- and anti-inflammatory cytokines were selected based on toxicologic relevance and previously published literature (Bassler et al., 2019; Mitro et al., 2020; Stein et al., 2016a; Zota et al., 2018). Interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10), interferon gamma (IFNγ), granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-α) were initially measured in duplicate assays using the Cytokine Human Magnetic 10-plex panel for Luminex platform (Life Technologies Corporation, Maryland, USA). Due to low sensitivity of the Life Technologies assay, interleukins (IL-1β, IL-2, IL-6, and IL-10), IFNγ and TNF-α were measured in duplicate assays using human high sensitivity cytokine A Luminex performance assays (R&D, Minnesota, USA) to determine absolute cytokine concentrations.

2.4. Analysis of serum PFASs

Serum samples were analyzed at the Colorado School of Mines; detailed methods and details on quantitation are provided elsewhere (McDonough et al., 2021). In brief, samples were analyzed with online solid-phase extraction (SPE) using a SCIEX Exion high performance liquid chromatography (HPLC) system coupled to an X500R quadrupole-time-of-flight mass spectrometer (QTOF-MS). PFASs were quantified via negative electrospray ionization (ESI-) with SWATH® Data-Independent Acquisition using a suspect screening workflow (McDonough et al., 2020a). Quantitation was done for 48 targeted PFASs by isotope dilution. For PFASs with both branched and linear isomers detected (PFOS and PFHpS), total concentration of all isomers was measured and reported. Novel PFASs identified via suspect screening were measured semi-quantitatively by assigning response factors from structurally-related target compounds. Personnel conducting the laboratory analysis were not provided with any information about characteristics of the participant providing the specimen. Multiple analytical runs were used to analyze the sample sets resulting in variations in detection limits between runs.

2.5. Statistical analysis

All study data were input into and managed using REDCap (Research Electronic Data Capture) tools hosted at the University of Colorado Denver (Harris et al., 2009).

Summary statistics (median, range, percentiles) were computed for seven PFASs (perfluorooctanoic acid [PFOA], perfluorononanoic acid [PFNA], perfluorodecanoic acid [PFDA], perfluorohexanesulfonic acid [PFHxS], perfluoroheptanesulfonic acid [ΣPFHpS], perfluorooctanesulfonic acid [ΣPFOS], unsaturated perfluorooctanesulfonic acid [U-PFOS]) detected in >85% of participants. Distributions and outliers were also visually assessed via histograms and scatterplots, and pairwise correlations between PFASs were described using Spearman's rank correlation coefficient. All statistical analyses were conducted after substituting ½ the limit of detection (LOD) for PFAS values below the LOD (up to 15% of values) (U.S. Environmental Protection Agency, 2006). The LOD for each sample and analyte was specific to the sample batch.

Of the 10 cytokines measured, only those detected in ≥30% of participants (IL-1β, IL-6, IL-10, TNF-α) were included in this analysis. Cytokines with >15% of values below the LOD were not analyzed as continuous outcomes, but instead classified into two categories: at or above vs below the LOD. Substitution or imputation of greater than 30% of values has been shown to produce biased results with inaccurate measures of variance (Lubin et al., 2004). Pairwise correlations between cytokines were described using Spearman's rank correlation coefficient.

Associations between each of the seven PFASs and each of the four cytokines were analyzed separately using 1) linear regression (TNF-α only) and 2) logistic regression (IL-1β, IL-6, IL-10) where the outcome was binary (detected vs. not detected). In both linear and logistic regression models, PFASs were natural log-transformed to reduce the influence of outliers and modeled as continuous variables. All models were adjusted for age (continuous), race/ethnicity (binary: non-Hispanic white vs all others), smoking history (binary: former smokers vs never smokers), sex (binary), and continuous BMI (kg/m^2) a priori based on previous literature (Mitro et al., 2020; Olden and White 2005; Stein et al., 2016a; Timmermann et al., 2020; Williams et al., 2016; Zota et al., 2018). Further, as an exploratory analysis, we evaluated the potential for effect modification by the following variables by including product interaction terms in adjusted models: sex, race/ethnicity, and BMI (categorized into <25 kg/m^2 or ≥ 25 kg/m^2). The selected variables were evaluated as effector modifiers because of the following previous literature. Zota et al. observed different effects for several endocrine disrupting chemicals (including PFASs) and cytokines among participants with and without obesity (Zota et al., 2018). Endocrine disrupting chemicals have been observed to effect men and women differently in multiple settings including response to immunizations (Timmermann et al., 2020). Race/ethnicity was evaluated as an effect modifier due to current empirical literature suggesting that the lived experience of systemic racism may cause greater susceptibility of the effects of environmental exposures (Olden and White 2005; Williams et al., 2016). Stratified models are presented in the supplement for those outcomes where at least one PFAS-cytokine association had a statistically significant interaction term ($p < 0.05$).

To assess the appropriateness of linear regression, we explored the potential for non-linear associations between PFASs and cytokine outcomes by fitting generalized additive models (GAMs). GAMs were adjusted for the same covariates as the linear and logistic regression models described above (age, race/ethnicity, smoking history and sex). GAM results were then plotted for visual assessment of non-linearity. If the Akaike information criterion (AIC) for the GAM with a spline-transformed exposure term was less than the AIC for the corresponding untransformed model (indicating a better fit), and if visual inspection of plotted results confirmed a non-linear association (i.e., not driven by outliers), then multiple regression was performed with the PFAS exposure divided into quartiles and treated as a categorical predictor.

In addition to single pollutant models detailed above, Bayesian kernel machine regression (BKMR) was performed to assess the combined effect of the seven PFASs on each cytokine outcome (Bobb et al., 2015). Using BKMR allows for visualization of the exposure-response function while accounting for interactions between pollutants (Bobb et al., 2015). All PFAS concentrations were scaled and mean-centered for BKMR analyses and categorical covariates were included as binary variables (Bobb et al., 2015). As previously described, TNF-α was treated as a continuous outcome while IL-1β, IL-6, IL-10 were treated as binary outcomes. BKMR models for all four cytokines were adjusted for age, race/ethnicity, smoking history, BMI, and sex.

All statistical analyses were conducted using the statistical software R Studio Version 1.3.959 (RStudio Team 2020). The significance level for all statistical analyses was set at $p < 0.05$.

3. Results

3.1. Study population demographics and serum PFAS concentrations

The study population included in this analysis consisted of 212 adults that lived in the affected Fountain Valley water districts for at least two years between 2012 and 2015. As described in Table 1, the population was largely female (62%) with a median age of 61 years, 74% non-Hispanic white, largely overweight (44%) or obese (34%), and predominantly never smoking (61%). Slightly more participants were recruited from Security (45%), the water district nearest to the presumed source of contamination.

Summary statistics for the seven PFASs included in this analysis are presented in Table 2. PFOA, PFNA, PFHxS, ΣPFHpS, and ΣPFOS were detected in ≥98% of samples while U-PFOS and PFDA were detected in

Table 1
Select characteristics of study population.

Characteristic	Study Population (N = 212)
Sex	
Male	80 (37.7%)
Female	132 (62.3%)
Age (years)	
Mean (SD)	59.3 (14.6)
Median [Min, Max]	61 [22, 93]
Race/Ethnicity[a]	
Non-Hispanic White	157 (74.1%)
Other	55 (25.9%)
BMI[b]	
<25 kg/m^2	48 (22.6%)
25 kg/m^2 ≤ BMI <30 kg/m^2	93 (43.9%)
≥30 kg/m^2	71 (33.5%)
Smoking Status	
Never Smoker	130 (61.3%)
Former Smoker	82 (38.7%)
Water District (2012–2015)[c]	
Fountain	52 (24.5%)
Security	95 (44.8%)
Widefield	65 (30.7%)

Abbreviations: N, number of participants; SD, standard deviation; Min, minimum; Max, maximum; BMI, body mass index.
[b] The "Other" category includes individuals who identified as the following: Asian, Black or African, American Indian or Alaska Native, Other, and/or Hispanic. A binary grouping was made to avoid small cell sizes in these categories.
[a] BMI calculated from self-reported height and weight.
[c] Individuals on private wells were assigned a water district based on well location.

91% and 86% of samples, respectively. All frequently detected PFASs were targeted perfluoroalkyl acids (PFAAs) except for U-PFOS, a novel unsaturated analog of PFOS which was tentatively identified by high-resolution mass spectrometry. Concentrations of U-PFOS may represent total concentrations of multiple positional isomers. Identification and semi-quantitation of this novel PFAS are described in detail in McDonough et al., (2021).

Because of variability in the detection limits between multiple analytical runs, ranges of detection limits are presented. This PFHxS, ΣPFHpS and ΣPFOS dominated PFAS mixture is characteristic of exposure to AFFF contamination (Barton et al., 2020). Median concentrations (Table 2) observed in this study population were two times median levels measured through the U.S. National Health and Nutritional Examination Survey (NHANES) for PFOA and PFOS, and nearly 14 times the U.S. national median for PFHxS (U.S. Department of Health and Human Services Centers for Disease Control and Prevention, 2021).

Spearman's rank correlation analysis (r_s, Supplemental Table 1) shows that all PFASs were moderately to strongly correlated with one another (range r_s 0.22–0.93). The most strongly correlated PFASs were ΣPFHpS with U-PFOS ($r_s = 0.93$), ΣPFHpS with PFHxS ($r_s = 0.88$), PFHxS with PFOA ($r_s = 0.85$), and PFHxS with U-PFOS ($r_s = 0.85$). While still statistically significant, the weakest correlations observed were PFNA with PFHxS ($r_s = 0.26$), and PFDA with PFHxS ($r_s = 0.22$).

3.2. Inflammatory biomarkers

IL-1β, IL-2, IL-6, IL-10, IFNγ, and TNF-α were analyzed in all study participants, but only TNF-α, IL-1β, IL-6, and IL-10 were detected in ≥30% of participants. To assess the quality of results in each batch we used the coefficient of variation (CV), a measure of variance between replicate data points within an assay, and found CVs to be generally low (i.e., <10%) (Supplemental Table 2). Summary statistics are presented in Table 3, including the ranges of detection limits which varied across multiple runs. TNF-α was the only highly detected and normally distributed cytokine in this study population. Due to relatively low frequencies of detection (30–85%), we categorized concentrations of IL-1β, IL-6, and IL-10 as above versus below the limit of detection. Spearman's rank correlation analysis (r_s, Supplemental Table 3) shows that all cytokines were weakly correlated with one another (range r_s −0.15 – 0.43). The most strongly correlated cytokines were IL-10 with IL-6 ($r_s = 0.43$) while the weakest correlation was between IL-1β with IL-6 ($r_s = 0.02$).

3.2.1. TNF-α

As indicated in Table 4 and Fig. 1, while not statistically significant, all of the analyzed perfluoroalkyl carboxylic acids (PFCAs), including PFOA, PFNA, and PFDA, were inversely associated with TNF-α, whereas the perfluoroalkyl sulfonic acids (PFSAs), including PFHxS, ΣPFHpS, ΣPFOS, and U-PFOS, were positively associated with TNF-α. As a mixture, higher concentrations of all seven PFASs combined were associated with slightly higher TNF-α compared to the scenario where all PFASs were at their median value, although prediction intervals were wide and included the null value (Fig. 2). In other words, with increasing quantile of exposure, we observe that the effect of the seven PFAS mixture on TNF-α concentration is positive. This suggests that the effect of the mixture is heavily influenced by the sulfonates which also have a positive direction of association with TNF-α in the single pollutant models (Fig. 1).

In an exploratory analysis, we evaluated the potential for effect modification by sex, race/ethnicity, and BMI categories, by including a product interaction term with each PFAS and each potential modifier in fully adjusted models. Due to a small sample size, all results of interaction models should be interpreted with caution. We observed some significant interaction terms for PFAS-by-BMI category interaction terms, therefore we present results stratified by BMI category (<25 kg/m^2 or ≥ 25 kg/m^2) in Supplemental Table 4. PFHxS was significantly positively associated with TNF-α among participants with BMI <25 kg/

Table 2
Concentrations (ng/mL) of PFASs included in these analyses, among 212 participants in the PFAS-AWARE study.

PFAS	% Detected	DL[a]	Min	25th %	Median	75th %	Max	Median U.S.[b]
PFOA	100	0.01-0.1	0.18	2.2	3.3	5.6	17.2	1.57
PFNA	98	0.01-0.2	<DL	0.3	0.4	0.6	6.6	0.60
PFDA	86	0.01-0.2	<DL	0.07	0.1	0.2	1.9	0.10
PFHxS	100	0.11-1.0	0.9	7.7	16.6	33.7	164.4	1.2
ΣPFHpS	99	0.01-0.04	<DL	0.4	0.9	1.6	10.6	N/A
ΣPFOS	100	0.1–2.0	1.2	4.8	8.2	14.0	50.2	4.8
U-PFOS[c]	91	0.01-0.2	<DL	0.2	0.3	0.4	1.9	N/A

Abbreviations: PFASs, per and polyfluoroalkyl substances; N, number of participants; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFDA, perfluorodecanoic acid; PFHxS, perfluorohexansulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFOS, perfluorooctanesulfonic acid; U-PFOS, unsaturated perfluorooctanesulfonic acid; Min, minimum; Max, maximum; DL, detection limit; ng/mL, nanograms per milliliter.
[a] Multiple analytical runs were used to analyze sample sets, causing some run-to-run variation in detection limits. The range of detection limits for each compound is provided.
[b] Data from the 2015–2016 cycle of the US National Health and Nutrition Examination Survey (NHANES), https://www.cdc.gov/exposurereport/.
[c] Value is semi-quantitative.

Table 3
Concentrations (pg/mL) of cytokines included in these analyses, among 212 participants in the PFAS-AWARE study.

Cytokine	% Detected	DL[a] pg/mL	Min pg/mL	5th % pg/mL	25th % pg/mL	Median pg/mL	75th % pg/mL	95th % pg/mL	Max pg/mL
TNF-α	**100**	0.3-1.1	2.0	4.0	6.2	7.9	10.0	13.4	23.4
IL-1β	**31**	0.1-0.4	<DL	<DL	<DL	<DL	0.2	0.4	0.8
IL-6	**40**	0.5-1.3	<DL	<DL	<DL	<DL	1.3	3.6	32.2
IL-10	**35**	0.1-0.5	<DL	<DL	<DL	<DL	0.2	0.6	1.6

Abbreviations: N, number of participants; Min, minimum; Max, maximum; DL, detection limit; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; pg/mL, picograms per milliliter.
[a] Multiple analytical runs were used to analyze sample sets, causing some run-to-run variation in detection limits. The range of detection limits for each compound is provided.

Table 4
Associations between individual PFASs and TNF-α serum concentrations, estimates from single pollutant multiple linear regression models[a].

PFAS	N = 210[b]		
	Change in TNF-α per ln-unit increase in PFAS	95% CI	P-value
PFOA	−0.14	−0.73, 0.44	0.63
PFNA	−0.14	−0.81, 0.52	0.68
PFDA	−0.12	−0.47, 0.23	0.50
PFHxS	0.22	−0.21, 0.65	0.32
ΣPFHpS	0.08	−0.32, 0.49	0.68
ΣPFOS	0.15	−0.51, 0.81	0.65
U-PFOS[c]	0.25	−0.28, 0.78	0.35

Abbreviations: PFASs, per and polyfluoroalkyl substances; TNF-α, tumor necrosis factor alpha; N, number of participants; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFDA, perfluorodecanoic acid; PFHxS, perfluorohexansulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFOS, perfluorooctanesulfonic acid; U-PFOS, unsaturated perfluorooctanesulfonic acid; CI, confidence interval; BMI, body mass index.
[a] Models adjusted for age, BMI, sex, smoking history and race/ethnicity.
[b] Excludes two individuals who reported taking TNF-α altering medication.
[c] Value is semi-quantitative.

m^2 but not among participants with BMI ≥25 kg/m^2. A similar, but non-significant, trend was observed for PFHpS.

3.2.2. IL-1β

As indicated in panel 1 of Table 5 and Fig. 3, odds of detectable IL-1β were significantly reduced with a 1-ln-unit increase in PFOA (OR = 0.64 [95%CI: 0.41, 0.98]), PFDA (OR = 0.78 [95%CI: 0.91, 1.00]), and ΣPFHpS (OR = 0.70 [95%CI: 0.52, 0.94]). Consistent direction of association was noted in the models for PFNA, PFHxS, ΣPFOS and U-PFOS, though confidence intervals included the null. Assessing all seven PFASs as a mixture, there was a clear inverse linear pattern of association between the PFAS mixture and the odds of detectable IL-1β (Fig. 4).

In an exploratory analysis, there was some evidence for potential effect modification by race/ethnicity for the associations between PFOA and U-PFOS and the odds of detectable IL-1β (Supplemental Table 5). Odds of detecting IL-1β was inversely associated with PFOA and U-PFOS among those of race/ethnicity other than non-Hispanic white. Additionally, there was some evidence of potential effect modification by sex (Supplemental Table 6). Odds of detecting IL-1β were inversely associated with concentrations of PFOA, PFHxS, ΣPFHpS, and U-PFOS among male participants only.

3.2.3. IL-6

As indicated in panel 2 of Table 5 and Fig. 3, odds of detecting IL-6 were positively associated with certain PFAS concentrations but none of the findings were statistically significant. Assessing all seven PFASs as a mixture, odds of detecting IL-6 were positively associated with increasing exposure to the PFAS mixture (Fig. 4). In exploratory results, stratified by BMI category, positive associations between PFASs and odds of detecting IL-6 were observed among participants with BMI <25 kg/m^2 but not among participants with BMI ≥25 kg/m^2 (Supplemental Table 7).

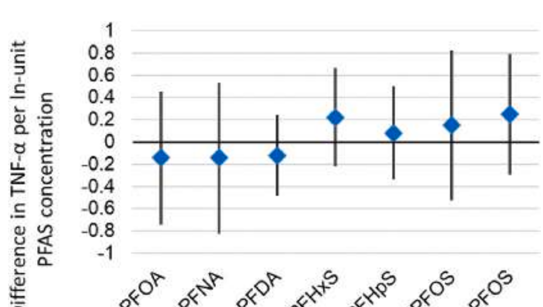

Fig. 1. Associations between individual PFASs and TNF-α serum concentrations, estimates from single pollutant multiple linear regression models. Models adjusted for age, sex, BMI, smoking history and race/ethnicity. Diamonds represent beta coefficients and bars represent 95% confidence intervals.

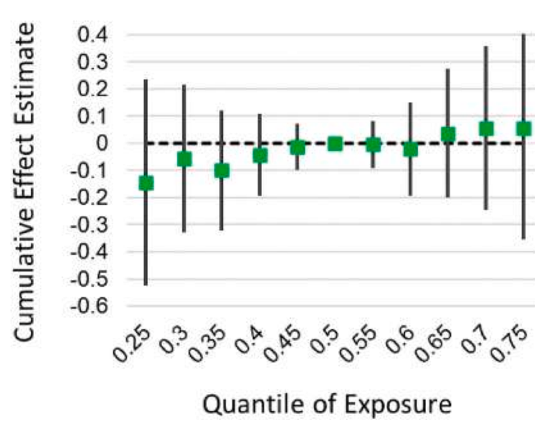

Fig. 2. Combined effect of seven PFASs on TNF-α estimated using Bayesian kernel machine regression, comparing the value of the exposure-response function when all PFASs are at a given quantile as compared to when all PFASs are at their median value. Models are adjusted for age, sex, BMI, smoking history and race/ethnicity.

Table 5
Associations between individual PFAS serum concentrations (ln-ng/mL) and the odds of detectable concentrations of inflammatory cytokines. Estimates from single pollutant multiple logistic regression models[a], among 212 participants in the PFAS-AWARE study.

PFAS	IL-1β			IL-6			IL-10		
	Odds Ratio	95% CI	P-Value	Odds Ratio	95% CI	P-Value	Odds Ratio	95% CI	P-Value
PFOA	**0.64**	**0.41, 0.98**	**0.05**	1.09	0.72, 1.69	0.68	0.81	0.53, 1.22	0.31
PFNA	0.69	0.41, 1.13	0.14	1.36	0.84, 2.27	0.22	0.78	0.48, 1.25	0.31
PFDA	**0.78**	**0.61, 1.00**	**0.05**	1.01	0.79, 1.31	0.93	0.97	0.76, 1.25	0.81
PFHxS	0.78	0.56, 1.07	0.12	0.99	0.73, 1.36	0.96	0.93	0.68, 1.27	0.65
ΣPFHpS	**0.70**	**0.51, 0.94**	**0.02**	1.19	0.88, 1.64	0.27	0.87	0.65, 1.15	0.33
ΣPFOS	0.78	0.48, 1.25	0.29	1.33	0.82, 2.16	0.25	0.79	0.49, 1.26	0.32
U–PFOS[b]	0.73	0.49, 1.07	0.11	1.07	0.73, 1.62	0.73	0.99	0.68, 1.47	0.97

Abbreviations: PFASs, per and polyfluoroalkyl substances; IL, interleukin; N, number of participants; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFDA, perfluorodecanoic acid; PFHxS, perfluorohexansulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFOS, perfluorooctanesulfonic acid; U-PFOS, unsaturated perfluorooctanesulfonic acid; CI, confidence interval; BMI, body mass index.

[a] Models adjusted for age, BMI, sex, smoking history and race/ethnicity.

[b] Value is semi-quantitative.

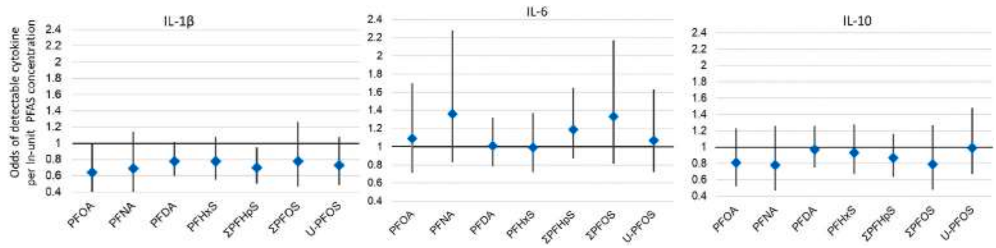

Fig. 3. Associations between individual PFAS serum concentrations (ln-ng/mL) and the odds of detectable concentrations of inflammatory cytokines. Estimates from single pollutant multiple linear regression models, among 212 participants in the PFAS-AWARE study. [a]Models adjusted for age, sex, BMI, smoking history and race/ethnicity.

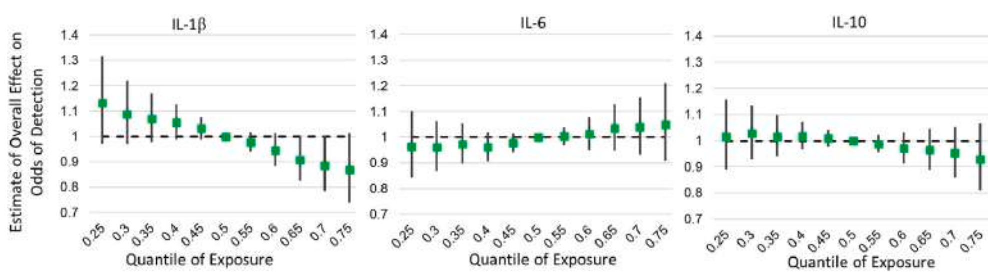

Fig. 4. Bayesian kernel machine regression results: combined effect of all seven PFASs on odds of cytokine detection, comparing the value of the exposure-response function when all PFASs are at a given quantile as compared to when all PFASs are at their median value. Models are adjusted for age, sex, BMI, smoking history and race/ethnicity, N = 212.

3.2.4. IL-10

As indicated in panel 3 of Table 5 and Fig. 3, odds of detecting IL-10 were weakly inversely associated with measured PFAS concentrations but confidence intervals were wide and included the null. When all seven PFASs were assessed as a mixture, there appeared to be a weakly inverse association with detectable IL-10 (Fig. 4). In exploratory results, stratified by BMI category, PFASs were positively associated with detectable IL-10 among healthy weight participants but inversely associated with detected PFASs among overweight participants, though confidence intervals were imprecise and included the null (Supplemental Table 8).

Because potentially non-linear associations between PFHxS and ΣPFOS and detection of IL-10 were observed in the GAMs, we conducted sensitivity analyses with these PFASs modeled as quartiles (Supplemental Table 9). Odds of detected IL-10 were highest in the third quartile of measured PFHxS concentrations compared to the first, with OR = 3.02 (95% CI: 1.26, 7.51). Odds of detecting IL-10 were highest in the second quartiles of measured ΣPFOS concentrations compared to the first but none of the exposure groups were significantly different from the lowest exposure group.

4. Discussion

In this study of adults highly exposed to PFASs via AFFF-contaminated drinking water, we found associations between PFASs and multiple circulating biomarkers of inflammation. Higher concentrations of PFOA, PFDA, and ΣPFHpS were significantly associated with lower odds of detectable IL-1β. We observed different patterns of association for each cytokine analyzed. Although confidence intervals were wide and included the null, PFAS concentrations were associated with lower odds of detectable IL-10, and with higher odds of detectable IL-6. In general, perfluoroalkyl carboxylic acids (PFOA, PFNA, and PFDA) had weakly inverse associations with TNF-α, while the perfluoroalkyl sulfonic acids (PFHxS, ΣPFHpS, ΣPFOS, and U-PFOS) showed positive associations.

While some of the existing human and animal data are conflicting regarding the directionality of effects of PFAS exposure on a range of immune endpoints, the weight of evidence is that exposure to well-studied perfluoroalkyl acids (PFAAs) (e.g., PFOA and PFOS) is immunosuppressive; both adaptive and innate immune responses are reduced (DeWitt et al., 2012). For example, several studies have demonstrated that immune responses to immunizations are reduced in populations that are highly exposed to PFASs (Grandjean et al. 2012, 2017; Granum et al., 2013; Kielsen et al., 2016; Mogensen et al., 2015; Stein et al., 2016b). Suppression of specific cytokines (in this case, IL-1β) could reflect an overall suppressive effect of PFAS exposure.

Autoimmune diseases have been previously linked with PFAS exposure in human studies. The largest cohort of PFOA-exposed individuals studied to date, the C8 Study, reported a probable link between PFOA and diagnosed high cholesterol, chronic kidney disease, ulcerative colitis, thyroid disease, testicular and kidney cancer, and pregnancy-induced hypertension and preeclampsia (C8 Science Panel, 2012). In the same population, other autoimmune outcomes including rheumatoid arthritis, Crohn's disease, type I diabetes, lupus, and multiple sclerosis were also evaluated, but were not found to be significantly more likely in more highly exposed participants (Steenland et al., 2013). In the subset of the cohort with occupational exposure to PFOA, both incident ulcerative colitis and rheumatoid arthritis were associated with PFOA exposure (Steenland et al., 2015). While the present study was too small to evaluate autoimmune disease outcomes, altered patterns of cytokine production may be involved in the pathogenesis of some of these diseases.

Few previous studies of PFAS exposure have evaluated circulating inflammatory biomarkers as outcomes. In a random sample of 200 participants from the C8 Study, several pro-inflammatory cytokines (including TNF-α, IL-6, IL-8, and IFN-γ) were measured along with biomarkers of liver disease, under the hypothesis that cytokine dysregulation was linked to liver injury (Bassler et al., 2019). The results supported this hypothesis, demonstrating that hepatocyte apoptosis was increased in individuals with higher PFAS serum concentrations, while TNF-α and IL-8 were downregulated. The authors concluded that hepatocyte apoptosis was mechanistically linked to decreased TNF-α (Bassler et al., 2019). Similarly, we observed weakly inverse associations between PFCAs and TNF-α, although confidence intervals were imprecise and included the null. IL-8 was not measured in our study and IFN-γ was only detected in two participants. A recent study in an elderly population identified decreased proteomic inflammatory markers associated with exposure to PFASs, another novel approach to measuring inflammation (Salihovic et al., 2020).

While this cohort did not include pregnant women, recent studies have demonstrated associations between PFASs and inflammation in pregnancy. In a cohort of women with overweight or obesity from the San Francisco Bay area, a doubling of ΣPFAS concentrations was associated with a 20.87% (3.46, 41.22) increase in IL-6 during pregnancy, with inconsistent and non-significant results for IL-10 and TNF-α (Zota et al., 2018). Similarly, in the Project Viva cohort study, pregnancy plasma concentrations of 2-(N-ethyl-perfluorooctane sulfonamido) acetic acid (EtFOSAA) and 2-(N-methyl-perfluorooctane sulfonamido) acetic acid (MeFOSAA) were associated with greater 3-year postpartum IL-6 (Mitro et al., 2020). In this study, IL-6 was 10.8% higher [95%CI: 3.3, 18.9] and 14.5% higher [95%CI: 5.7, 24.1] per doubling in EtFOSAA and MeFOSAA, respectively. The authors hypothesized that IL-6 may be upregulated in response to higher oxidative stress after PFAS exposure (Mitro et al., 2020).

Evidence from animal studies and cell culture models may be helpful in understanding the mechanism behind this observed association in a more controlled setting. For example, exposure to PFOA increased production of TNF- α, IL-1β, IL-6, and IL-8 in IgE-stimulated mast cells (RBL-2h3 cells) in culture (Lee et al., 2017). These results support the assumption that PFOA exacerbated mast cell-derived allergic inflammation via activation of NF-kB. In stimulated mast cells, PFDA and PFUnA exposure significantly increased IL-1β whereas none of the PFCAs (PFHpA, PFNA, PFDA, PFUnA) significantly increased IL-1β expression in unstimulated cells (Lee and Kim 2018). In vitro, in human bronchial epithelial cells, both PFOA and PFOS stimulated the release of IL-1β in virus stimulated cells (no effect observed in non-stimulated cells) at human relevant concentrations. Other PFASs did not induce cytokine release at the concentrations tested (Sorli et al., 2020). These findings reinforce that cytokine function and dysregulation is highly context dependent, and our results provide only a snapshot of a complex process of regulating inflammatory signaling.

Results from animal studies to date have been inconclusive regarding the influence of PFAS exposure on IL-1β. In mice exposed to PFOA via drinking water, IL-1β was significantly increased in the spleen but decreased with dose in the thymus (Son et al., 2009). TNF-α and IL-6 were significantly increased in the spleen at the highest administered dose but did not differ across exposure groups in the thymus (Son et al., 2009). These findings highlight that tissue-specific levels of cytokines can vary, making the interpretation of levels of cytokines in serum more challenging to interpret. By contrast, in zebrafish exposed to lower levels of PFOA, IL-1β expression in the spleen was significantly reduced at concentrations$\geq$0.01 mg/L. This study indicates that zebrafish may be more vulnerable than mice to the effects of PFOA exposure on cytokine production (Zhang et al., 2014). In another study of zebrafish, production of IL-1β after PFOA exposure was both time and dose dependent (Zhang et al., 2021). It is possible that the cytokine response to PFAS exposure in humans also depends on the dose and the duration of exposure, and on the specific mixture of PFASs received.

Our study has several limitations. This was a pilot study to establish blood concentrations of AFFF-related PFASs in a population with contaminated drinking water and may not be powered to detect associations of small magnitude or be generalizable to other, non-AFFF-exposed populations. As with any cross-sectional study, there is a potential for reverse causation. Additionally, we measured cytokines at a single time point, although concentrations in blood may vary substantially from day to day, introducing some degree of outcome measurement error. Both cytokines and serum PFAS concentrations can vary based on dietary intake (Aziz 2015; Roth et al., 2020; Seshasayee et al., 2021; van Bussel et al., 2011). The blood samples were non-fasting and cytokine concentrations may have been influenced by recent food intake (Aziz 2015; van Bussel et al., 2011), however we did not collect these data. While certain foods may be sources of PFAS exposure, we do not expect the fasting status of participants impacted serum PFAS concentrations due to their persistent nature (Sunderland et al., 2019). Further, while covariates were selected based on published literature, measures of socioeconomic status and typical food consumption were not collected and could result in residual confounding. We performed numerous statistical tests and therefore it is possible that some results reached statistical significance by chance.

Despite these limitations, measuring cytokines as a biomarker of inflammation is a minimally invasive and cost effective first step in evaluating the potential immunotoxic effects of exposure to AFFF-associated PFAS mixtures in humans. Strengths of our study include quantification of a large panel of PFASs and consideration of the combined effects of multiple PFASs in the exposure mixture using advanced statistical methods. Previous studies of cytokines have not been conducted in populations with AFFF exposure, so this study provides novel data on the potential immune-modifying effects of this exposure profile, with PFHxS as the predominant substance measured.

5. Conclusion

Modifications of inflammatory pathways may be one mechanism by which PFAS exposures produce adverse health effects in humans. We observed preliminary evidence of altered inflammatory profiles among adults with elevated serum concentrations of several AFFF-associated PFASs due to contaminated drinking water. Further studies are needed to examine whether altered cytokine profiles associated with this exposure mixture may be linked to increased risk of autoimmune disease or other evidence of immunotoxicity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113905.

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Determination of a guidance value for the communication of individual-level biomonitoring data for urinary arsenic

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ABSTRACT

Background: Available guidance values to interpret individual-level biomonitoring data (ILBD) for the sum of urinary inorganic-related arsenic species (SUIAS) are generally based on population statistical descriptors and not on a predetermined exposure level that should not be exceeded. The objective of this study was thus to propose a range of SUIAS concentrations, reflecting an exposure corresponding to WHO's provisional guideline value (PGV) for arsenic in drinking water (10 μg/L), within which an exposure-based biomonitoring guidance value can be identified. METHOD A comprehensive literature review was carried out in order to identify studies that were relevant to the determination of a guidance value. Drinking water arsenic exposure and urinary biomonitoring concentrations obtained from selected studies were used to conduct a structural equation modeling meta-analysis, from which regression coefficients were obtained to derive an interpretative guidance range. RESULTS Individuals exposed to the arsenic background level comparable to North American and European countries and to a water source contaminated at the WHO's PGV, would have, on average, urinary SUIAS between 9 and 20 μg/L, with the most probable value being 15 μg/L. To address the associated uncertainty, the final guidance value selection within this range may be based on a targeted sensitivity and specificity towards detecting overexposed individuals. Indeed, spans of sensitivity of 60–82%, and of specificity of 58–85%, were estimated for the proposed range based on drinking water exposure raw data from the literature. CONCLUSION The range of guidance values obtained appears suitable for interpreting and communicating ILBD in any population biomonitoring studies in which background exposure is comparable to the North American and European context. Before selecting a single value within the proposed range, it will be important for Public Health officials to assess the possible consequences of this selection on the management and communication of the biomonitoring results.

1. Introduction

Biomonitoring, which involves measuring contaminants concentrations in the human body through biomarkers, has become a commonly tool used in populational survey. The main goal of such biomonitoring surveys is to assess a population's environmental exposure to contaminants and to support subsequent health risk management (Angerer et al., 2007; Haines et al., 2011). Inorganic arsenic (iAs), a recognized human carcinogen, is often included in biomonitoring programs (Angerer et al., 2011; CDC, 2021; Health Canada, 2019; IARC, 2012).

Populations are exposed to iAs mainly from drinking water (DW) and some types of foods (e.g. rice, rice-based products, other grains and grain-based products and certain fruit juices and vegetables) (ATSDR, 2007; European Food Safety Authority (EFSA) et al., 2021; IARC, 2012; Joint F.A.O./WHO Expert Committee on Food Additives, 2011). The biomarker most commonly used to determine exposure to iAs is the sum of urinary inorganic-related arsenic species (SUIAS), which corresponds to the sum of the inorganic forms As (III) and As (V) and their metabolites DMA and MMA (Hsueh et al., 2002). In a health risk assessment perspective, the analysis of the SUIAS is considered more useful than

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measurements of total urinary arsenic. Indeed, this latter biomarker is strongly influenced by the presence of the much less toxic organic forms of As (eg: arsenobetaine ans arsenosugars) from the consumption of seafood (fish, shellfish and algae) (CDC, 2017; Navas-Acien et al., 2011).

For many biomarkers of toxicant exposure such as SUIAS, the paucity of available guidance values strongly challenges the communication to participants of biomonitoring campaigns of their individual results (Haines et al., 2011). This barrier is even more limitating in the case of iAs, in particular because the SUIAS data show a high interindividual variability and due to the premise of absence of threshold exposure for its carcinogenic effect (Concha et al., 2006; Health Canada, 2006; IARC, 2012; Lindberg et al., 2006). It results in missed opportunities to make potentially overexposed participants aware of their specific situation and to invite them to identify their individual sources of exposure and to control them when possible. This is a concern for the managers in charge of large biomonitoring campaigns since there is a need of follow-up with the study participants whom's individual results may be considered as truly alarming (Haines et al., 2011).

In the absence of appropriate individual overexposure guidance value for SUIAS, it is still possible to use statistical criteria to identify situations of overexposure (e.g. Population's 95th percentile value) (Angerer et al., 2011; Ewers et al., 1999; Garnier et al., 2021; Vogel et al., 2019). However, for a naturally occurring contaminant such as iAs, a double challenge needs to be addressed when applying this approach: on the one hand, not to trivialize biomarker levels that are considered "normal" from a statistical point of view, but which are still associated with an elevated exposure and, likely, corresponding health risk; and on the other hand, not to stigmatize behaviors based on traditional diets such as high rice or seafood consumption, the latter associated to undeniable nutritionnal benefits(Mozaffarian and Rimm, 2006).

Indeed, interpretation of SUIAS values is still complicated by the fact that, for a large proportion of individuals, iAs intakes come mainly from food sources whose iAs levels are more or less controlled depending on the jurisdiction, or even strongly associated with diets that are perceived as being particularly healthy (e.g. gluten free diets) (Bulka et al., 2017). Moreover, certain kinds of seafood such as finfish, algae and a few shellfish can contain DMA as well as arsenosugars that are partially metabolized to DMA (CDC, 2017; Navas-Acien et al., 2011; Taylor et al., 2017). The occasional consumption of these seafoods can therefore cause a punctual increase in urinary concentrations of this metabolite. This increase could in turn result into overestimating the true iAs exposure and trigger an unjustified shift in dietary habits (National Academy of Sciences, 1999).

Under these circumstances, SUIAS results from populations primarily exposed to iAs through consumption of contaminated water have the advantage of being more representative of its toxic potential. In addition, these biomonitoring results can be interpreted in relation to the exposure associated to WHO's provisional guideline value (PGV) for iAs of 10 μg/L (World Health Organization, 2003), which is applied as a standard in several jurisdictions (Canada, USA, European Union, among others) (Council Directive (EU), 1998; Health Canada, 2006; U.S. EPA, 2001). This PGV is set on the basis of the best possible compromise between the cancer risk and the lowest achievable concentration in water, taking current DW treatment techniques into account (World Health Organization, 2017).

The objective of this study was therefore to propose an approach for the determination of a guidance value for biomonitoring data of SUIAS that allows the identification of individual study participants that may likely have experienced an iAs overexposure for which action of reduction can reasonably be envisaged at the individual scale. More specifically, the study aimed to 1) identify a range of SUIAS concentrations which could indicate a possible iAs environmental overexposure for North American and European populations; 2) assess the sensitivity and specificity of the different values within this range in order to orient decision-makers on the *a priori* selection of a final guidance value that allows an appropriate identification of overexposed individuals.

2. Materials and methods

2.1. Framework

On the basis of the underlying premise that the WHO PGV in DW of 10 μg As/L constitutes a guideline that reflects an iAs exposure that is consensually considered as a level of which the exceedance should be avoided, the approach followed consists into predicting the range of SUIAS concentrations which would reflect 1) the consumption DW contaminated to or above the level of the PGV for As; and 2) the background exposure through the environmental, in particular, dietary sources of As in north American and European countries. Briefly, a literature review focusing on data on the relation between As exposure through DW and resulting SUIAS levels was conducted. A linear regression approach was applied on central tendencies values in order to obtain an equation linking relevant SUIAS to As concentration in DW. This equation was then used to compute a range of several possible SUIAS guidance values associated to a DW level of 10 μg/L, depending of the statistical descriptors considered in the equation terms. Finally, for the benefit of the decision-making process, sensitivity and specificity analyses were conducted with raw individual data on the guidance values derived in order to determine if individuals were correctly identified in function of the magnitude of their exposure through DW.

2.2. Selection of relevant studies

A literature search strategy was developed in order to identify publications presenting data on both biomonitored urinary concentrations and environmental exposure to As through DW. The last update of the literature research on the Ovid platform was on October 30th, 2020. Three concepts were used for the search strategy: (1) arsenic, (2) biomonitoring urine concentrations, and (3) DW concentrations. Studies that were selected needed to have measured individual As concentrations in the DW consumed (or presumably consumed) by the subjects participating in the study, as well as SUIAS concentrations for the studied population. After removal of duplicates, the relevance of the articles identified at this point was assessed after reviewing the title and abstract. Additional articles were also include following an analysis of their bibliography through a snowballing process based on the relevance of the titles. The texts of the resulting selections were analyzed to determined if they met the selection criteria (Table 1).

When the levels in DW are low, it is estimated that around 65%–80% of the exposure to iAs originates from the diet (European Food Safety Authority (EFSA) et al., 2021; Kurzius-Spencer et al., 2013; NRC, 2013). However, since diet varies greatly from one country to another, considerable variation in the urinary As background of different populations is observed. For example, the average background SUIAS concentration are generally less than 10 μg/L in European countries and approximately 50 μg/L in Japan (National Academy of Sciences, 1999). The populations of southern Asia are exposed to an even higher background level (>100 μg/L and as high as 2000 μg/L) owing to their high rice consumption and the irrigation of crops with heavily contaminated water, as well as the consumption of the water itself (Chakraborti et al., 2003; Hata et al., 2012; Sohel et al., 2010). This direct and indirect contribution of water to the exposure precludes the use of studies conducted in such context to characterize the relationship between water consumption and As excretion. For the purpose of the present study, only studies taking place in either North American or European countries were therefore considered, under the premise that their populations share roughly similar dietary habits and level of environmental exposure.

Previous studies have found significant positive correlations between DW As concentrations and SUIAS concentrations (Biggs et al., 1997; Calderon et al., 2013; Chakraborti et al., 2003; Hata et al., 2012; Laine

Table 1
Selection criteria of the studies retrieved from the literature research strategy.

Criteria	Inclusion	Complementary exclusion
Type of document	Peer-reviewed primary studies in English or French.	Editorials, reviews, conference papers, grey literature and preprints.
Study population	Studies taking place in North American and European countries. A minimun of 30 participants per studied population.	
Arsenic (As) environmental exposure	The study must present As concentrations in drinking water consumed (or presumably consumed) by the study subjects. The median or geometric mean of the As concentrations of the study population must reflect low to moderate As exposure (≤50 μg/L in the water samples)[A].	Study focusing on environmental exposure of other sources (air, soil, smoking, natural products, etc.).
As biomonitoring	The study must present urinary biomonitoring data. Urine As concentrations in spot samples, first morning urine samples, or urine collection for 12h or 24h are presented.	Biomonitoring matrices other than urine (blood, nails, hair, breast milk, etc.)
Speciation of urinary As	Urinary As must be presented as SUIAS	Data not expressed in SUIAS (i.e. As total).
Adjustment of urinary samples	Studies reporting unadjusted SUIAS, specific gravity adjusted or adjusted by osmolality.	Studies reporting only SUIAS concentrations corrected by creatinine[B].

A: The geometric mean or median were used since they are considered to be better central descriptors of the exposure than arithmetic mean.
B: Urinary samples corrected by creatinine were excluded since this correction might incorporate errors related to the impact of variations in muscle mass or malnutrition of participants (Middleton et al., 2016).

et al., 2015; Lindberg et al., 2006, 2008; Roychowdhury, 2010). However, authors have reported different ratios and equations depending on the levels of As in DW (Lindberg et al., 2006, 2008; Vahter et al., 2006). Moreover, it has been reported that at high DW As concentrations, methylation processes might be inhibited, leading to lower excretion rates of urinary As (Lindberg et al., 2008), and thus a modified relation between As concentrations in DW and urine. It was hence important to include studies reflecting only low to moderate exposures. The National Research Council (NRC, 2013) describes low As levels in DW as below 50 μg/L, moderate As levels as between 50 and 150 μg/L, and high As levels as above 150 μg/L. Therefore, only studies with a geometric mean or median of less than 50 μg/L in DW were retained. Yet, it was assumed that by using these central measures, most of the concentrations would be in the range of low to moderate exposure and maybe include only a few outliers reflecting higher exposure levels. Finally, the articles had to present at least one central measures (geometric or arithmetic mean, or median) for DW and SUIAS. If a study did not provide sufficient summary statistics in the article, the authors were contacted in order to obtain the missing information.

2.3. *Identification of the exposure groups of interest*

In the majority of the studies selected, the central measures of the As concentrations in DW and the SUIAS concentration of the entire studied population was used as a single, homogenous exposure group for the construction of the structural equation modeling (SEM) meta-analysis (see next section). In few studies however, specific exposure subgroups corresponding to measurements from different regions or towns, were identified in order to respect the domain of As concentrations in DW that this work was focusing on (see Results).

2.4. *Statistical analyses*

A regression model was fitted between the estimated effects of As concentrations. In order to consider both analytes variability, a structural equation modeling-based approach (SEM-based meta-analysis) was used to regress water on urine concentration (MetaSEM package) (Cheung, 2015). For this regression model, the arithmetic means had to be used as central measures together with their standard deviation as a way to account for their variability. These statistical parameters were required for the chosen meta-analysis described below, which allowed to weight the studies based on their sample size and standard deviations. Based on the Box-Cox transformation method developed by McGrath et al. (2020), missing arithmetic means and standards errors for both urine and water were estimated using available summary statistics (min, Q1, median, Q3 and max). The advantage of such an approach is that it works noticeably well for non-normal data, which often characterize environmental exposures.

As SEM is a covariance structure-based analysis, several steps were conducted before the linear regression procedure. First, using the DerSimonian-Laird meta-analytical approach (metacor package) (Laliberté, 2011), an estimation of the weighted average correlation coefficient was calculated based on the correlation coefficients of the selected studies. This estimated weighted coefficient (rho = 0.46) was applied to the studies lacking such study-specific rho (Figure S1). Next, estimated covariances were derived from the estimated correlation coefficient and standard deviations of the two analytes.

Regression coefficients, the slope β and the intercept α were estimated, along with their standard error and their 95% confidence intervals (CI) from the SEM-based meta-analysis. To derive the propose guidance values for SUIAS, a concentration of 10 μg/L DW was applied on the equation model built from the regression coefficients in order to define the central tendency of the guidance value. Then, a range of values were derived by taking into consideration the 95% CI of both the slope and the intercept, the later referring to the expected background As exposure from sources others than DW. Precisely, the range of guidance values were computed considering the lower and upper bounds of the slope plus the central value of the intercept, as well as the lower and upper bounds of both the slope and the intercept.

2.5. *Sensitivity vs specificity analysis*

In order to orient decision-makers on the selection, within the proposed range, of a final guidance value that will allow an accurate identification of overexposed individuals, sensitivity and specificity analyses were performed on a subset of raw data from studies in which north American populations are exposed to low to moderate concentrations of As in DW. The sensitivity analyses were performed to determine what percentage of the biomonitored participants experiencing a high As exposure through DW would have been accurately identified by referring to the values derived by the equation obtained from the SEM regression (true positive rate). The purpose of the specificity analysis was instead to determine the percentage of participants without a high exposure of As through DW, who would have rightfully been classified as “normally” exposed (true negative rate).

The raw data of three studies which measured As in DW and SUIAS concentrations were made available to us. These include two studies retained for the SEM based meta-analysis (one held in the United States (Calderon et al., 2013) and the other from Canada (Normandin et al., 2014), plus one other Canadian study (Gagnon et al., 2016), that was eligible until the ultimate step of selection was applied -see results). These datasets were screened to identify the participants that would allow to perform sensitivity (if exposed to DW concentrations ≥10 μg/L) and specificity (if exposed to DW concentrations <10 μg/L) analyses on a range of potential guidance values for SUIAS derived from the SEM-based meta-analysis.

Additionally, to the entire available dataset from these three studies, two different subsets of data were used from them in order to reflect exposure to organic As. These subsets implied 1) the exclusion of participants from the Calderon et al. (2013) study who reported having consumed seafood or fish 48 h prior to the urine sampling and 2) the exclusion of participants from the Gagnon et al. (2016) study that had detectable concentrations of arsenocholine or arsenobetaine in their urine. Finally, children from the Gagnon et al. (2016) study where excluded of the analysis to assess if their exclusion will impact the results of true positives and true negatives.

3. Results

3.1. Selected studies

A total of 1137 studies were initially identified from the literature search strategy. Excluding duplicates, revising the titles and abstract for relevancy, adding relevant reference by the snowballing approach and applying the inclusion criteria (see Table 1) to the resulting group of references allowed to reduce this number to 14 studies, from which only 11 could be used for the upcoming SEM meta-analysis. Indeed, the remaining 3 had either missing summary statistics or had a high proportion (>40%) of values below the detection limit (Fig. 1).

From the 11 references selected for the meta-analysis, nine were conducted in north America and two were conducted in Europe. The studies in North America included five from the United States (Calderon et al., 2013; Farzan et al., 2016; Gossai et al., 2017; Josyula et al., 2006; Rivera-Núñez et al., 2012), two from Mexico (Laine et al., 2015; Mendez et al., 2016), one from Canada (Normandin et al., 2014) and one study that took place in both the United States and Mexico (Roberge et al., 2012). For the studies in Europe, the first was conducted in the United Kingdom (Middleton et al., 2016), and the second in different regions of Hungary, Romania and Slovakia (Lindberg et al., 2006). Two articles had pregnant woman as the studied population, while the remaining nine articles concerned an adult population (Farzan et al., 2016; Laine et al., 2015). None of the articles that were retrieved from the literature research and met the selection criteria included children. More information on the selected studies and the summary statistics for the As exposure in DW and SUIAS concentration can be found in Supplementary Material (Table S1).

Seven of the 11 studies were considered as single homogenous exposure groups from which the arithmetic means of the As concentrations in water and the SUIAS concentrations of the entire studied population were used in SEM-based meta-analysis. Among the remaining ones, the article of Calderon et al. (2013) reported five exposure subgroups based on the As concentrations in DW. The last subgroup was excluded in order to respect the criteria of having a central measure of As exposure of less than 50 μg/L. Multiple exposure groups were used for the studies of Josyula et al. (2006), Lindberg et al. (2006), and Roberge et al. (2012). Two distinct exposure groups were identified from the study of Josyula et al. (2006), which reported As concentrations and relevant summary statistics for two communities in Arizona. For the study of Lindberg et al. (2006), three Hungarian, two Slovakian and two Romanian regions were considered each as different exposure groups. One Hungarian region also evaluated by Lindberg et al. (2006) was excluded since it did not respect the criteria of a minimum sample size of 30. Similarly, the article of Roberge et al. (2012) analyzed concentrations in eight communities in Arizona and northern Mexico, each of these communities was used as a separate exposure group for the meta-analysis (Table S2).

Overall, a total of 25 exposure groups, identified within the 11 selected articles, were used for the SEM meta-analysis (Fig. 2). The arithmetic means of the As concentrations in DW from the exposure groups range from 0.9 to 98.9 μg/L (up to 50 μg/L in geometric mean), the majority of exposure groups with a mean of less than 30 μg/L. The arithmetic means of the SUIAS concentration range from 3.1 to 76.3 μg/L, most of the means reporting urinary concentrations lower than 40 μg/L.

3.2. Regression and resulting range of possible guidance values for SUIAS

Table 2 presents the regression coefficients for the slope and the intercept, for the SEM regression applied in Fig. 2. The R^2 for the model ($R^2 = 0.81$) suggest that the model fits the data well within the domain of arithmetic mean DW concentrations investigated (0.9–98.9 μg/L).

The proposed guidance values for SUIAS derived from the resulting SEM meta-analysis (SUIAS $= 0.91x + 5.6$, where x is the DW As concentration in μg/L) are presented in Table 3. If a value of 10 μg/L for DW As is applied and combined with different statistical indicators (95% CI, best estimates) on the slope and intercept, a range of urinary SUIAS concentrations varying from 8.9 to 20.4 μg/L are derived, with the best estimate being 14.7 μg/L (Table 3). Specifically, using the model that relies only on the best estimate of the intercept is deemed to reflect the uncertainty around the impact of the exposure through DW, whereas using the 95% CI on the intercept reflects the additional uncertainty

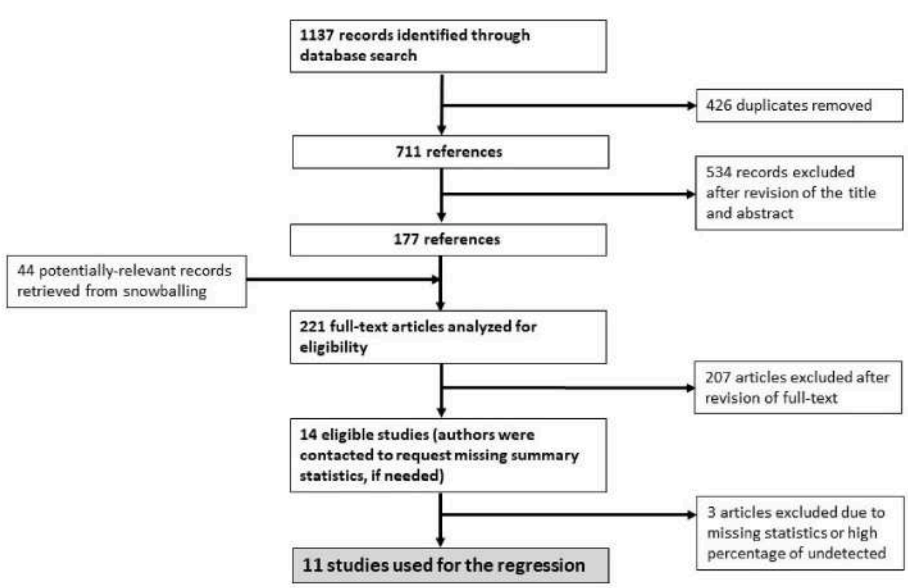

Fig. 1. Flow diagram of study selection from the literature search.

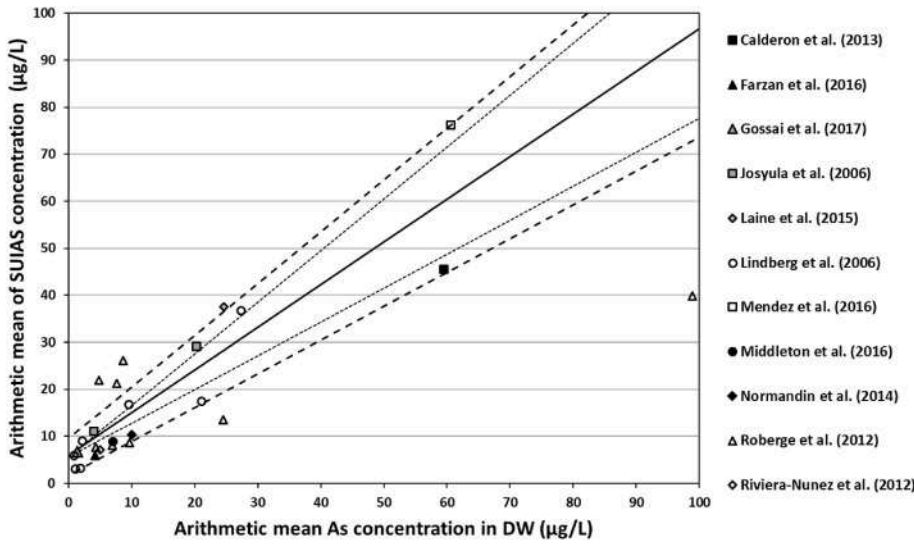

Fig. 2. Structural equation model (SEM) meta-analysis regression using the arithmetic means of arsenic (As) in drinking water (DW) (µg/L) and sum of urinary inorganic-related arsenic species (SUIAS) (µg/L) of 25 exposure groups identified within the 11 selected studies retrieved from the literature search. The continuous line represents the best estimate, the doted lines represents the 95% CI on the slope combined with the central value of the intercept, whereas the discontinuous lines reflects the combined consideration of the 95% CI for both the slope and intercept of the SEM regression.

Table 2
Structural equation modeling (SEM) meta-analysis that allows computing the sum of urinary inorganic-related arsenic species (SUIAS) concentrations (µg/L) as a function of drinking water arsenic concentrations (µg/L).

Slope		Intercept		Model R^{2A}
β coefficient (error)	95% CI	α coefficient (error)	95% CI	
0.91 (0.10)	0.72–1.1	5.6 (2.0)	1.7–9.5	0.81

A: For geometric mean/median concentrations of arsenic in drinking water of up to 50 µg/L, or arithmetic mean concentration of up to 100 µg/L.

Table 3
Proposed sum of urinary inorganic-related arsenic species (SUIAS) guidance values (µg/L) derived from the equation derived from SEM regression applied to an arsenic concentration in drinking water corresponding to the PGV of 10 µg/L. The best estimates as well as lower and upper bound values corresponding to the 95% CI of the slope and the intercept are presented.

Best estimate of both the slope and the intercept	95% CI slope + best estimate of the intercept		95% CI slope +95% CI intercept	
	Lower bound	Upper bound	Lower bound	Upper bound
14.7	12.8	16.6	8.9	20.4

resulting from other sources, including dietary exposure.

3.3. *Sensitivity and specificity of the proposed guidance values*

The sensitivity and specificity of the proposed guidance values (rounded to the closest µg/L), computed from the raw data of three studies (Calderon et al., 2013; Gagnon et al., 2016; Normandin et al., 2014), are presented in Table 4. When compiling the databases of the three studies and for a SUIAS guidance value of 15 µg/L, 69% of the participants that were exposed to a water sources with As at or more than 10 µg/L were correctly identified (true positive rate or sensitivity); the remaining 31% had a water source contaminated above the PGV but were not identified with the proposed guidance value (false negative). On the other hand, 77% of the participants that were not overexposed to As through DW presented a SUIAS concentration below the proposed guidance value and were therefore correctly categorized (true negative rate or specificity); the remaining 23% corresponds to participants with DW As concentrations of less than 10 µg/L that exhibited SUIAS concentrations higher than the guidance value and were therefore

Table 4
Sensitivity (true positive rate, %) and specificity (true negative rate, %) analysis of different proposed guidance values for sum of urinary inorganic-related arsenic species (SUIAS).

Study	outcome[a]	n[b]	Proposed guidance values[c] (µg/L)				
			9	13	15	17	20
Calderon et al. (2013)	sensitivity	776	87	80	77	74	69
	specificity	128	31	45	50	55	61
Calderon et al. (2013) [d]	sensitivity	555	87	81	77	74	70
	specificity	95	33	47	52	57	63
Gagnon et al. (2016)	sensitivity	158	55	36	31	25	18
	specificity	145	67	86	89	94	97
Gagnon et al. (2016) [e]	sensitivity	135	59	39	33	26	19
	specificity	125	68	86	88	93	97
Gagnon et al. (2016) [f]	sensitivity	85	49	33	27	22	15
	specificity	59	92	97	98	100	100
Normandin et al. (2014)	sensitivity	20	90	75	70	60	45
	specificity	86	83	92	95	98	99
Compilation of studies[g]	sensitivity	954	82	73	69	65	60
	specificity	359	58	73	77	81	85

[a] For the sensitivity analysis, it corresponds to the data when the As concentrations in drinking water is greater or equal to 10 µg/L. For the specificity analysis, it corresponds to the data when the As concentrations in drinking water were lower than 10 µg/L.
[b] Data set size.
[c] Rounded to 1 µg/L.
[d] Data set excluding participants that reported having eaten seafood or fish 48 h prior to the urine sampling.
[e] Data set excluding children (less than 18 years old).
[f] Data set excluding participants that had detectable concentrations of arsenocholine or arsenobetaine in their urine.
[g] Compilation of the complete data sets of the three studies: Calderon et al. (2013), Gagnon et al. (2016), Normandin et al. (2014).

incorrectly identified as overexposed based on DW concentrations (false positive). With a SUIAS guidance value of 9 µg/L the sensitivity reaches 82%; however, the specificity is lowered to 58%. While, when a SUIAS guidance value of 20 µg/L is used, there is a reduction of sensitivity to 60% and an increase of the specificity of 85%.

The results from the dataset of the study of Normandin et al. (2014) show higher sensitivity and specificity percentages than the dataset of Calderon et al. (2013) and Gagnon et al. (2016); with a candidate SUIAS guidance value of 15 µg/L the sensitivity is 70% and the specificity is 95%. The size of the dataset used for calculating the specificity of the participants from Normandin et al. (2014) study is only 20. For the database of Calderon et al. (2013), a slightly better specificity is observed when the participants self-reporting a consumption of seafood or fish 48 h prior to the urine sampling are excluded. For the database of Gagnon et al. (2016), the exclusion of children does not result in any significant changes for either the sensitivity or specificity. However, for the same study the exclusion of participants with detectable concentrations of arsenocholine and arsenobetaine in their urine, as an indicator of seafood or fish consumption, led to a significantly higher specificity.

4. Discussion

4.1. Originality

The objective of this study was to propose a range of potential SUIAS concentrations guidance values, reflecting a predetermined exposure level that should not be exceeded, that allows the *individual* identification of -and communication with- concerned biomonitoring study participants in North American and European populations, presumably sharing similar diets and environmental exposure. Rather than single guidance value, a range of values is proposed, more precisely 9–20 μg/L, with 15 μg/L being the best estimate. This range is accompanied by sensitivity and specificity analyses that allows for decision-makers and regulators to evaluate the implication, in terms of potentially false positive or negative *individual* results, of choosing *a priori* one or the other value among this range. In this regard, this constitutes a significant strength of this study and a primer, as to the best of the author's knowledge, such an analysis has never been performed for SUIAS, and this approach could be transposed to other contaminants or biological matrices.

Proposing a range of potential guidance values reflects the concern for accounting for underlying uncertainties associated to a determination process that is based on a relatively limited number of relevant studies and thus may not capture all the possible sources of variability in human As exposure and excretion (Concha et al., 2006; Lindberg et al., 2006; Samanta et al., 2007). Indeed, large interindividual variations in background As exposure may stem from differences in general dietary habits and specific food consumed in the days prior to the urine sampling (Cascio et al., 2011; Molin et al., 2012a). Besides, the heterogeneity of critical factors such as the DW consumption and As levels, the urinary excretion rates and the time elapsed between the onset of exposure and the collection of the urine sample is to be expected (Concha et al., 2006; Samanta et al., 2007). Notably, variations of urinary As concentrations by one order of magnitude have been observed between individuals from the same village or from the same family who get their water from a common source (Concha et al., 2006; Lindberg et al., 2006), suggesting wide variations in either the dietary sources of As exposure or the individual patterns of DW usage or consumption.

4.2. Interpretation

The range of guidance values proposed is based on studies implying individuals mostly exposed to As concentrations in water close to the WHO's PGV and whose background exposure, notably from dietary sources, is roughly similar to the North American and European population. While the uncertainty of the linear relationship derived from the data of selected descriptive studies increases above a water As concentration of 20 μg/L (see Fig. 1), its ability to predict the urinary concentrations reported in the literature for concentrations falling immediately on either side of the PGV of 10 μg/L of water is high (see Fig. 1).

The wide range for the proposed guidance values is attributed to the standard errors of the β and α coefficients for the slope and the intercept. In the SEM regression presented, the 95% CI for the consumption of DW with an As concentration of 10 μg/L, without any background noise, corresponds to urinary $\sum$As concentrations from 7.2 to 11 μg/L. The width of this range is rather the result of the CIs of the intercept (95% CI: 1.7–9.5), its best estimate being 5.6 μg/L. A high standard error on the intercept is to be expected, as it represents the background exposure, which can vary significantly as mentioned before. The best estimate of the intercept obtained from the SEM model is coherent with the background noise of the Canadian and American population and slightly higher than the German and French population measured through national biomonitoring surveys. The geometric means of SUIAS from the cycle 2 to 5 of the Canadian Health Measure Survey (CHMS) were between 4.3 and 5.4 μg/L (Health Canada, 2019). Similarly, the geometrical means of the American population from the National Health and Nutrition Examination Survey (NHANES) for the period of 2011–2016 were between 4.4 and 5.6 μg/L SUIAS (CDC, 2021). A German Environmental Survey in 1998 (GerES III) reported a geometrical mean of 3.9 μg/L for an adult population (Becker et al., 2003). While the French Nutrion & Health Survey (ENNS) conducted in 2006–2007 had a geometric mean of 3.8 μg/L for SUIAS (Frery et al., 2017). In this context, it is noteworthy that Hays et al. (2010) estimated a conversion factor of 24 μg/L in urine per ug/kg.day of external dose, such that an ingestion of 2 L per day of DW with 10 μg/L of iAs for a 70-kg adult would yield an external dose of 0.3 μg/kg.day and a corresponding SUIAS of 7.3 μg/L. Adding the 5.6 μg/L attributed to the background results in a total SUIAS of 12.9 μg/L, which is close to the best estimate value of 14.7 μg/L computed in Table 3.

A variation on the sensitivity and specificity percentage was expected between the three studies used for the exercise, among other due to the different selection criteria for the participants. The study of Normandin et al. (2014) had a more restrictive criteria, including only participants who reported drinking water directly from the source. The study of Gagnon et al. (2016) had an inclusion criterion of using the water at least for cooking; however, no direct water consumption was required. Participants with high As concentrations in DW were often aware of it, and they reported mainly only using DW for cooking. This lack of direct consumption of DW with high level of As could explain the low sensitivity found for this study. Lastly, the study of Calderon et al. (2013) included participants who did not reported drinking water from the source, or who had an onsite water treatment prior to consumption. There were two subsets used to evaluate the impact on the specificity when excluding participants who had consumed fish or seafood previous to the urine sample collection. The results show a very small increase on the specificity when excluding participants from the study of Calderon et al. (2013) who self-report the consumption through questionnaires. However, the exclusion of participants who had detectable arsenocholine or arsenobetaine in Gagnon et al. (2016) had a major increase on the true negative rate. This difference could be explained by the unreliability on the information gathered from administrated questionnaire versus a more precise determination by using arsenocholine and arsenobetaine that are biomarkers of recent consumption of fish and seafood (Molin et al., 2012b; Navas-Acien et al., 2011).

Based on Table 4, when a lower guidance value is applied the sensitivity increases and the specificity decreases. It is important to recall that no guidance value will offer a 100% sensitivity and specificity and that, owing to interindividual variabilities described above, there will always be a certain proportion of individuals significantly exposed to As but still falsely classified as not overly exposed. Conversely, the lower the specificity, the higher the percentage of participants that would be unnecessarily contacted, thus a higher guidance value would address this caveat. Similarly, individuals not exposed to contaminated water could be classified as being over-exposed owing to their exposure to another significant source (e.g. rice) or the cumulative effect of several secondary sources. For example, an average urinary As measurement of 15.81 μg/L was observed during a study conducted in the United Kingdom among Bangladeshi immigrants who consume rice on a daily basis (Cascio et al., 2011). Such case can represent an opportunity to remind people about the importance of a varied diet.

The choice of a specific guidance value within the range of concentrations proposed herein can depend on various considerations, including the need to balance ethical concern of non-maleficence with the requisite of identifying the individual experiencing an alarmingly high exposure (Haines et al., 2011). The *a priori* selection of a specific value could also depend on whether or not participants were instructed not to eat seafood 2 or 3 days before the sampling during the biomonitoring campaign. If such instruction was given, a lower value within the range could be justified. Information expected to be available (like DW sources or potential contamination and dietary habits) on the population being sampled could also guide the decision. The fact that the WHO'S DW guideline for As, used as a premise for the present work, corresponds to a cancer risk ($\approx 10^{-4}$) that already exceeds the risk considered negligible by many public health institutions could constitute an argument in favor of guidance value towards the lower end of that range. Thus, obtaining a large number of false positives can also be considered a lesser evil when this makes it possible to reach individuals belonging to subgroups for whom iAs exposure represents an increased risk, e.g. smokers (Chen et al., 2004; Lindberg et al., 2010; NRC, 2013). But conversely, it is of prime importance to avoid sending messages incompatible with public health goals. For instance, considering the undeniable nutritional benefits of fish consumption (Mozaffarian and Rimm, 2006) and knowing that a single meal of fish could increase SUIAS concentrations by 7.6 μg/L (Gagnon et al., 2016), it would therefore be justified to set the guidance value at the upper end of the proposed range of values, i.e. 20 μg/L. For the same reasons, it would not be advisable to instruct participants not to consume fish in the days preceding collection of the urine sample in order to avoid to give rise to unjustified concerns about potential risks associated with this type of food. On the other hand, it would be easier to interpret the results if participant were asked not to consume seafood in the two to three days preceding collection of the sample; a single meal of lobster or crab being sufficient to increase the excretion of SUIAS to 133.7 μg/L and 25.7 μg/L respectively (Gagnon et al., 2016).

4.3. Comparison with other studies and proposed interpretation values

The present range of values can be compared to other biological reference levels suggested or that can be deducted from literature data. Based on the data from the study of Biggs et al. (1997) conducted in several villages in the Antofagasta region in Chile, where all participants of a same village get their water supply from a single source and had practically no consumption of fish or seafood, a conversion factor of 0.97 was derived between the As concentration in DW and the SUIAS concentration (Smith et al., 2009). According to this conversion factor, the consumption of DW containing the MAC for As 10 μg/L would correspond to a urinary excretion of SUIAS of 9.7 μg/L. Thus, considering a background level of SUIAS of 1–5 μg/L, which are considered as a representative estimate of the background levels of North American and European populations (CDC, 2021; Frery et al., 2017; Health Canada, 2019), the proposed interpretation range for SUIAS concentration would be from 10.7 to 14.7. This range is coherent with the results presented herein. The study of Lindberg et al. (2006) in eastern European countries reports the following equation between As in DW and urine: $\ln \text{SUIAS} = 0.88 + 0.44(\text{DW\_As})^{1/2}$. The corresponding background concentration of SUIAS is 2.4 μg/L. With a DW As concentration of 10 μg/L, the urinary $\sum$As would be 9.7 μg/L. Both of the background exposure value and the predicted SUIAS are coherent with our results.

To the best of the authors' knowledge, biomonitoring guidance values, to communicate individual-level results, based on a predetermined exposure that should not be exceeded, are not available for SUIAS. Instead, some reference values (RV) are derived, which are statistical descriptors often corresponding to the 95th percentile of a representative population (Vogel et al., 2019). In Canada the 95th percentile for SUIAS is 20 μg/L (Faure et al., 2020; Health Canada, 2019). The German health authorities use a RV set at 15 μg/L urinary $\sum$As for both adults and children, which corresponds to the 95% CI of the population's 95th percentile value for individuals who did not consume fish 48 h prior to the sampling (Schulz et al., 2007; Wilhelm et al., 2004). Similarly, in France the 11 μg/L RV corresponds to the upper limit of the 95% CI of the 95th percentile of a representative adult population (18–74 years) that did not consumed fish 72 h prior to the sampling (Frery et al., 2017; Garnier et al., 2021). The overexposure of children is defined by exceeding both 11 μg/L and 10 μg/g creatine (Garnier et al., 2021). Although not derived in the same way, nor generally applied for the same purposes, it is still noteworthy that the proposed interpretation range recommended here is consistent with these RV, which describe the most exposed portion of the population.

Another guidance value used for the individual interpretation of biomonitoring SUIAS data is the biological exposure index (BEI), which is based on the continuous occupational As inhalation exposure (8 h per day, 5 days per week) to recommended limits in air. The current BEI for SUIAS proposed by the American Conference of Governmental Industrial Hygienists is set at 35 μg/L (American Conference of Governmental Industrial Hygienists, 2001). This value is a reference for occupational health, but it seems inappropriate for the general population since it is a very high concentration rarely found outside of occupational settings. Similarly, the biomonitoring equivalent (BE) is a pharmacokinetic extrapolation of a given health-based exposure guideline. For non-carcinogenic effect, Hays et al. (2010) determined a BE (based on a reference dose (RfD)) and a BE_{POD} (based on the RfD's point of departure) of respectively 6.4 and 19.3 μg SUIAS/L, suggesting that the putative guidance value chosen from the present work would only be borderline protective against such risk. With regard to carcinogenic effects, a BE is set at 1.4 μg SUIAS/L for a cancer risk of 10^{-4} derived from the evaluation from the Water Quality and Health Bureau of Health Canada (Hays et al., 2010). However, BEs were not designed for the individual communication of biomonitoring results, but rather for prioritizing the substances of concern or follow-up of biomonitoring programs at a populational level (Hays et al., 2007, 2010). Based on the BE for iAs cancer risk and given the geometric mean of SUIAS background concentrations (varying from 3.9 to 5.6 μg/L, see section 4.2) previously described for North American and European countries, actions for this chemical can be considered a high priority in these populations. By allowing to identify overexposed individuals in order to provide them information on how to reduce their exposure, the present work contributes in this regard. Thus, the BE and the putative guidance values proposed herein appear as complimentary tools for public health managers and decision-makers.

4.4. Limitations

As for every study, the present work presents some limitations that need to be acknowledged. First, applying a linear regression using individual data from the selected studies would have been preferable to using only central tendency data as used herein, since it would have better accounted for the variability of the study-specific observations, but the raw data from every selected study were not available. However, the SEM meta-analytical approach used appears as a reasonable alternative in such situation, as it allows weighting the contribution of both analyte concentrations for each exposure group in the regression setting as function of their variance. Therefore, it tends to give greater weight to the larger studies, allowing more precise coefficients of the final regression model. For comparison purpose, using a simple linear regression on the arithmetic means of the 11 selected studies have generated larger relative 95% CI on both the slope and, more so, on the intercept (Table S3), as well as an R^2 of 0.6 instead of the 0.81 value described above. Thus, the SEM approach used appears as an improved method as compared to if unweighted central tendency values had been used in the regression approach.

Second, there were differences between the inclusion criteria for the selection of the participants and often limited information was available about them. Few studies had information on the consumption of DW or the diet a few days prior to the sampling. Most of the studies did not require the participants to abstain from consuming fish or seafood before the collection of the urine samples. There are also some differences on the collection of urine sample (spot sample, morning sample, 12-h sample). However, these differences and lack of information could also be present when choosing a guidance value. It is, therefore, essential to reflect on the potential false positive or negative when deciding within the proposed range.

Another limitation is that no guidance value specific to young children or pregnant or nursing women is proposed. Pregnant women and infants appear to have higher methylation capacities than the general adult population (NRC, 2013; Tseng, 2009). However, the variations in methylation by age or gender are of little importance when exposure is measured based on the SUIAS. In fact, two of the studies included in the SEM analysis were focusing strictly on pregnant women (Farzan et al., 2016; Laine et al., 2015). The exclusion of these two studies did not change the results of the SEM-based meta-analysis (Table S4). Also, available CHMS data have shown that SUIAS concentration do not vary significantly between age groups (Health Canada, 2019), whereas the results from the sensitivity and specificity analysis performed herein did not show an impact when excluding children. Therefore, the need for age-specific guidance value is not warranted.

5. Conclusion

To the best of the authors' knowledge, this is the first study to propose a range of guidance values that allows an exposure-based communication of human biomonitoring data on urinary As at the individual level. Concretely, such guidance value could be used during biomonitoring surveys to determine whether participants should be informed of their individual result and receive information and general advice to help determine, and if necessary control, their sources of iAs exposure. The determination of such value addresses a need in environmental health since despite the many benefits of biomonitoring, few, if any, such interpretation values were available until now.

When selecting a single value from the range suggested in the present study, it will be important to consider the uncertainties mentioned above as well as the practical and ethical consequences that the determination of a guidance value close to the upper or lower limits of the proposed ranges could have on the management and communication of the results of biomonitoring results. The final choice of the retained guidance value, within the proposed ranges, depend on numerous risk management considerations that exceed the scope of this study.

Credit author statements

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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Evaluation of two-year recall of self-reported pesticide exposure among Ugandan smallholder farmers

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ABSTRACT

Objectives: To evaluate smallholder farmers' recall of pesticide use and exposure determinants over a two-year period in a low-income country context.
Methods: The Pesticide Use in Tropical Settings (PESTROP) study in Uganda consists of 302 smallholder farmers who were interviewed in 2017. In the same season in 2019, these farmers were re-questioned concerning pesticide use (e.g., use of active ingredients) and exposure information (e.g., crops, personal protective equipment [PPE], hygienic behaviours) they had previously provided. The extent of recall bias was assessed by comparing responses at follow-up in 2019 with practices and behaviours reported from the baseline interview in 2017.
Results: An 84% ($n = 255$) follow-up response rate was attained. We found instances of better recall (e.g., overall agreement >70% and Area Under the Curve (AUC) values > 0.7) for the use of some active ingredients, commonly used PPE items, and washing clothes after application, whereas only 13.3% could correctly recall their three major crops. We observed a trend where more individuals reported the use of active ingredients, while fewer reported the use of PPE items, two years later. In general, we found better agreement in the recall of years working with pesticides compared to hours per day or days per week in the field, with no apparent systematic over or under reporting by demographic characteristics.
Conclusions: While some of these findings provide consistency with those from high-income countries, more research is needed on recall in poorly educated agriculture communities in low- and middle-income settings to confirm these results.

What this paper adds.

What is already known about this subject?

Few studies have evaluated the recall of pesticide use in agricultural workers. These have generally found less agreement with recall when there are longer time intervals between assessments and more detailed questions used.

What are the new findings?

Smallholder farmers in Uganda could better recollect after a 2-year period the total number of years using pesticides, as well as certain active ingredients and personal protection equipment (PPE), compared to poorer recall of specific crops.

How might this impact on policy or clinical practice in the foreseeable future?

The use of pesticide products, PPE items, and years of pesticide use

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should inform exposure assessment, though additional research is needed to confirm these results with poorly educated agriculture communities in low- and middle-income settings.

1. Introduction

Global pesticide use has increased over the past 30 years, with approximately 4 million tonnes of active ingredient applied annually in recent years (Food and Agriculture Orga, 2020). The application of these pesticides in an occupational setting has been linked to an increasing range of cancer (Alavanja and Bonner, 2012), respiratory (Ye et al., 2013; Negatu et al., 2017; Hansen et al., 2021a), neurological (van der Mark et al., 2012; Negatu et al., 2018; Fuhrimann et al., 2021a), and other (Blair et al., 2014) adverse health outcomes.

A recent systematic review on occupational pesticide exposure studies published between 1993 and 2017 found that in nearly 1300 papers, approximately five times as many studies were based on indirect (e.g., self-reported) compared to direct (e.g., biomonitoring) exposure assessment methods (EAMs) (Ohlander et al., 2020). Moreover, from the 16 studies identified in that review from low-income country contexts, the majority (88%) used indirect exposure assessments. Direct EAMs, such as the collection of biomarkers, for example, are typically much more costly and resource intensive, presenting a particular challenge in low- and middle-income countries (LMICs) (Fuhrimann et al., 2020). The reliance on self-reported data in these settings is mainly due to a lack of spray records and other recording or monitoring systems, which could otherwise provide data on pesticide handling or user safety (Nanyunja et al., 2015). Hence, it is of utmost importance to better understand the reliability of self-reported data on recalled exposure relevant variables, such as pesticide use, PPE use, or hygienic behaviours.

Several factors can cloud recall ability in self-reported studies, such as a long interval between the event and reporting, higher complexity of information either in the content (e.g., recalling technical or detailed information) or wording of the question, and the triviality of events to the respondent (Stull et al., 2009). Unreliable reporting will result in non-differential misclassification of the exposure of individual study participants and will result in biases towards the null in studies where self-assessed exposure is associated with (self-reported) health outcomes. However, when the self-reported data is used to construct a job- or task-exposure matrix and the exposure is assigned at group level, the association between exposure and health outcome will result in little or no bias; albeit, the association will be less precise when the self-reporting is noisy (Armstrong, 1998). It is therefore important to understand the potential effect of recall bias in low-income contexts, where pesticide exposure is reported to be highest due to a lack in knowledge, attitude, and practices of safe pesticide use (Atuhaire et al., 2016) and the continuous use of highly hazardous pesticides (Jepson et al., 2020).

Few studies have validated self-reporting of pesticide use and relevant information on factors affecting exposure or examined the extent of recall bias in agricultural workers. Blair et al. (2002) compared recall of the use of specific pesticides, method of application, and time spent mixing pesticides over a one-year period in a sample of participants from Iowa, USA in the Agricultural Health Study (AHS). Hoppin et al. (2002) also used data from the AHS, comparing the reported starting decade of specific pesticide use to the registration year in the USA. Engel et al. (2001) examined recall of pesticide use in orchard workers in the USA within a ~20-year period and Lee et al. (2010) validated reported pesticide use in South Korean farmers over a 4-week period. Results of these studies suggest high concordance (>90%) in responses for ever having used pesticides, but indicate exact agreement could be as low as 40–50% for more detailed information, such as specific product or active ingredient used or frequency of application (Blair et al., 2002).

These studies represent a limited evidence base, with several published nearly two decades ago. The studies each used quite different intervals of recall, which provides a challenge to interpret and compare results of different exposure indicators. Also, this research did not examine recall of PPE use or hygiene practices and took place in just two countries (USA and South Korea); the implications are uncertain for LMICs, where workers may be poorly educated and illiterate, and where different pesticides, application methods, and personal protective equipment (PPE) may be used (Rother, 2018). For example, a study of smallholder farmers in Ethiopia found only 8% read and understood pesticide labels (Mengistie et al., 2017).

This manuscript is embedded in the "IMPRoving Exposure aSSessment methodologies for epidemiological studies on pesticides" (IMPRESS) project, which aims to improve understanding of the performance of pesticide EAMs used in epidemiological investigations, and to use this information to recommend enhancements in scientific practice for the future (Jones et al., 2020). In this paper, we assess the ability of Uganda's smallholder farmers to recall, over a two-year period, their working history related to pesticide exposure, including active ingredients used, PPE worn, and hygienic behaviour. The study group is a cohort of the "PESticide use in TROPical settings" (PESTROP) Project (Fuhrimann et al., 2019).

2. Methods

2.1. Study population

The PESTROP study cohort is located in Uganda, Wakiso District in the rural communities of Mende and Masulita. These smallholder farmers predominantly grow a mix of beans, maize, sweet potatoes, banana, cassava, coffee, tomatoes, and groundnuts on an average area of 3–4 acres (Staudacher et al., 2020; Diemer et al., 2020). Pesticide use in these communities has a potential to result in various negative health outcomes (Fuhrimann et al., 2021b, 2022; Hansen et al., 2020, 2021b). In the second cropping season of 2017 (October to December), 302 farmers were interviewed in a baseline study. Participants were informed of general results and provided personalized feedback on their pesticide exposure during an in-person group meeting. Exactly two years later, the same respondents were followed up under the IMPRESS study to assess their ability to recall information they had submitted at baseline, in addition to other exposure and health assessments (Fuhrimann et al., 2021b).

2.2. Data collection

We used a structured questionnaire designed to obtain insights on sociodemographic information (e.g., sex, age, education), practices of pesticide use, and corresponding protective behaviour. Staudacher et al. (2020) and the YouTube video 'Pestrop – An Ugandan Story' (https://www.youtube.com/watch?v=XhizHAtjyno) provide further details on the PESTROP cohort population and methodology at baseline. Only a few extraneous questions (for ethical and practical reasons) were excluded in the follow-up questionnaire (see Supplementary Material).

Trained research assistants administered via tablet the piloted and ethically approved tool through Open Data Kit (https://opendatakit.org). Interviews were conducted in the local dialect (Luganda) at locations of respondents' convenience, mainly at home and/or in the field. Urine samples were also collected prior to the reassessment from a subset of 86 interviewed farmers, as part of another IMPRESS study work package, which also involved questionnaire interviews.

The recall questionnaire in 2019 consisted of the following items, reflecting practices in the 12 months prior to the survey in 2017 as follows:

- Major crops (up to 3) on which they had worked;
- Average hours per day and days per week working in the field;
- Total years mixing or applying pesticides;

- Use (yes/no) of 15 different active ingredients (those most commonly reported at baseline), involving brand names of 53 pesticide products registered by Uganda's Ministry of Agriculture, Animal Industry and Fisheries;
- Use (yes/no) of 12 PPE items during pesticide application; and
- Typical time following pesticide application that they would bathe (e.g. showering, bathing) and change work clothes (i.e., next day, many hours later, few hours later, immediately).

We examined the effect of recall on semi-quantitative exposure-intensity scores based on the use of specific PPE items and hygienic behaviour (changing and showering after application). These characteristics represent available data in the current study from a subset of underlying exposure-modifying factors that were used to develop a context-specific pesticide exposure algorithm for applicators in LMICs who use handheld knapsack sprayers (Fuhrimann et al., 2020) (See Supplementary Material for exposure score calculations and individual inputs [PPE, CHANGE, and SHOWER]).

2.3. Data analysis

All data analysis was performed using Stata v16.

In the current paper, we compared recalled information in 2019 to interview responses in 2017. We assessed the recall of crops by determining the percentage who correctly identified their three major crops from 2017 (in any order), as well as the percentage who could identify any of their top three crops; we did not exclude farmers who worked with fewer than 3 crops. We calculated correlations to examine if this recall was related to the number of individual crops reported in 2019.

We examined the recall of average hours per day and days per week working in the field by calculating geometric mean ratios (GMRs) of the follow-up compared to baseline estimates (Goedhart et al., 2018). The number of years mixing or applying pesticides was ascertained by subtracting the age at the time of the follow-up survey, or the age when pesticide use ceased (if reported), by the reported age when pesticide use started. Covariates using categories defined by the approximate median values were included to assess any differences in estimation relative to reference groups by demographic and other characteristics, namely sex, age (<or ≥50 years), number of years working on a farm (<or ≥20 years), highest level of years of education attained (any primary school or completion of primary school/higher), farm size (<or ≥2 acres), total number of workers on the farm (<or ≥3 workers), and monthly household income (<or ≥27 USD). While no data were missing, workers who reported <1 full year of experience mixing or applying synthetic pesticides in 2017 either at baseline (n = 28) or follow-up (n = 24), were excluded from this specific analysis. We used the test of proportions to compare characteristics in the recall only subgroup with the full cohort.

We assessed the recollection of the use of active ingredients, PPE items, and practice of hygiene habits through sensitivity, specificity, overall agreement, prevalence ratio of follow-up compared to baseline reporting, and area under the curve (AUC). Sensitivity represents the number of correct affirmative responses (true positives/[true positives + false negatives]) and specificity indicates the number of correct negative responses (true negatives/[true negatives + false positives]). Overall agreement shows the percentage of all correct responses (i.e., both affirmative and negative). The prevalence ratio of follow-up compared to baseline reporting suggests whether there is generally higher (>1.0) or lower (<1.0) reporting at the group level at follow-up. The AUC is an indicator of how well responses can be distinguished and is an overall summary of sensitivity and specificity; AUC of values < 0.7, ≥0.7, ≥0.8, and ≥0.9 represent non-useful, fair, good, and excellent agreement, respectively (Carter et al., 2016). We performed further analysis by comparing the aggregation of active ingredients by the World Health Organization (WHO) hazard levels (World Health Organization, 2020) and type of application (i.e., fungicide, herbicide, insecticide). In addition, we examined recall of the more commonly used individual active ingredients in those who did and did not provide a urine sample for biomarker analysis prior to the follow-up interview and those who were and were not literate (i.e., could not read and write in any language) by comparing the AUC for each sub-group using the 'roccomp' command in Stata (Janssens and Martens, 2020). Also, we assessed the correlation between the use of specific active ingredients in 2019 (as reported by any days of use in 2019) with over-reporting (i.e., incorrectly reporting use in 2017).

We quantified the effect on exposure estimates due to recall discrepancies by comparing the output of Eq. S1 and S2 using the initial and recalled values (see Supplementary Material). We compared the Spearman correlation of the individual inputs (i.e., PPE, CHANGE, SHOWER scores) and median values of Eq. S(1) and Eq. S(2) at both time-points.

2.4. Ethical considerations

The Higher Degrees, Research and Ethics Committee of Makerere University School of Public Health approved this study (reference no. 719).

3. Results

A total of 255 individuals completed the survey in 2019, representing 84% participation of the original PESTROP cohort (Fig. S1). The mean age at follow-up of respondents was 50.4 years (standard deviation [SD] = 13.6), with a mean experience working on agricultural farms of 32.3 years (SD = 15.1). The mean size of farms and total number of workers per farm were 3.8 acres (SD = 3.6) and 4.0 workers (SD = 3.1), respectively. With the exception of a higher proportion of pesticide users, characteristics appeared comparable between those who participated in the second survey with those who did not (Table 1).

Approximately one third of participants could remember the first major crop they were growing at the time of the baseline survey. Although only 13.3% could recollect each of the top three crops they had been growing, 87.1% could remember at least one (Table 2). The median number of crops reported at follow-up was five (range = 1–14), which had little correlation with either the ability to recall any (r = 0.04) or all three (r = −0.02) major crops.

Agreement between the reported and recalled years working with pesticides was moderate to strongly positively correlated (rho = 0.64), with weaker positive correlations for reported and recalled days per week (rho = 0.31) and hours per day (rho = 0.39) working in the field. The overall GMRs of recalled years (0.94 [95% CI: 0.85–1.05]), days per week (1.01 [95% CI: 0.97–1.05)], and hours per day (1.06 [95% CI: 0.97–1.15]) did not indicate any systematic under or overestimation. There were no strong trends by demographic or farm subgroup in the recalled time spent either working with pesticides or working in the field (Table S2).

There were 205 (80.4%) and 184 (72.2%) respondents, respectively, who reported and recalled the use of any synthetic pesticides. The opposite pattern was observed in the reporting of any individual active ingredient, which was lower at baseline (n = 183; 71.8%) than recalled (n = 218; 85.5%). All but three of the 14 reportedly used active ingredients suggested some general level of overestimated recall, as evidenced by prevalence ratios of >1.0. Sensitivity, which ranged from 0% to 87.6% for active ingredients, was better for the more commonly used active ingredients; the three most reportedly used being glyphosate, cypermethrin, and mancozeb. By contrast, specificity (ranging from 44.8% to 100%) and overall agreement (59.8%–96.0%) tended to be lower for the more common pesticides (Table 3). AUC values were <0.7, except for mancozeb, 2,4-D, and dichlorvos. There were no observable differences in recall ability of working with common pesticides between farmers (n = 85) who provided a urine sample for biomarker analysis and those who did not, or between farmers who were literate compared to those who were illiterate (Table S3). Recall was similar (overall

Table 1
Descriptive characteristics of the recall only (n = 255) and full PESTROP cohorts (n = 302) at baseline.

Characteristic	Recall only n (%)	Full cohort N (%)	Test of proportions
Age (Range=19–92)			p = 0.314
<50 years	115 (45.1)	155 (51.3)	
≥50 years	140 (54.9)	147 (48.7)	
Sex			p = 0.912
Male	151 (59.2)	177 (58.6)	
Female	104 (40.8)	125 (41.4)	
Experience working on a farm (Range=2–73)			p = 0.963
2–29 years	110 (43.1)	131 (43.4)	
≥30 years	145 (56.9)	171 (56.2)	
Education			p = 0.909
Did not complete primary	85 (33.3)	98 (32.5)	
Completion of primary or higher	170 (66.7)	204 (67.5)	
Use of pesticides			p = 0.024
Yes	205 (80.4)	214 (70.9)	
No	50 (19.6)	84 (27.8)	
Missing	-	4 (1.3)	
Size of farm (Range=<1–20)			p = 0.651
≤2 acres	103 (40.4)	113 (37.4)	
>2 acres	152 (59.6)	189 (62.6)	
Number of workers on farm (Range1-27)			p = 0.647
≤3 workers	129 (50.6)	161 (53.3)	
>3 workers	126 (49.4)	141 (46.7)	
Literate			p = 0.971
Yes	229 (89.8)	271 (89.7)	
No	26 (10.2)	31 (10.3)	
Monthly household income (Range 3–1350)			p = 0.796
<40 USD	122 (47.8)	138 (46.3)	
≥40 USD	129 (50.6)	155 (52.0)	
Missing	4 (1.6)	5 (1.7)	
Provided urine sample			p = 0.497
Yes	85 (33.3)	86 (28.5)	
No	170 (66.7)	216 (71.5)	

Table 2
Recall of working with major crops (n = 255).

Crops at baseline	Agreement at follow-up n (%)
1st major crop	87 (34.1)
2nd major crop	44 (17.3)
3rd major crop	42 (16.5)
Any order	
All 3 crops	34 (13.3)
Any 2 crops	133 (51.8)
Any 1 crop	222 (87.1)

agreement = 69.3%–73.5%), but slightly better for fungicides (AUC>0.7), when grouped by types of product and also when grouped by WHO Hazard levels (overall agreement = 75.1%–79.8%) (Table S4). There were very weak to moderate positive correlations between over-reporting an active ingredient and its use in 2019 (Table S5).

The majority of the farmers only used basic PPE to cover their upper-body, legs, and feet. The three most common PPE items reported at baseline were gumboots (79.2%), long pants (72.9%), and long-sleeve shirts (61.6%), which were more reliably recalled (AUC>0.7). A small percentage of farmers reported using (any) protection for the eyes (n = 7; 2.8%), mouth (n = 21; 8.2%), or hands (n = 28; 11.0%), with specificity rates over 90% (Table S6 and Fig. 1). Regarding hygiene habits, less than half could correctly recall bathing or changing clothes immediately following pesticide use, while most could recollect whether they washed their own clothes (Table 4).

Spearman correlations were moderate for PPE scores (rho = 0.49), but weak for CHANGE (rho = 0.27) and SHOWER (rho = 0.17) scores. Median PPE scores (Eq. S(1)) at baseline and recall were each 0.73 (Interquartile range [IQR] = 0.64–0.91). The median recalled PPE-Hygiene Score (Eq. S(2)) was slightly higher (0.52; IQR = 0.40–0.64) compared to baseline (0.47; IQR = 0.35–0.59), with a weak correlation (rho = 0.31).

4. Discussion

We studied Uganda's smallholder farmers' ability to recall their previously reported use and pesticide exposure determinants over a two-year period. In general, we found better agreement in the recall of years compared to hours per day or days per week, with no consistent over or under reporting in this regard. We found instances of better recall for the use of common active ingredients and PPE items, as well as washing clothes. However, there was poor recall of specific crops, and we observed overall trends where more individuals reported the use of active ingredients, and fewer reported the use of PPE items, two years later. We are not aware of any other studies examining recall of pesticide applicators in LMICs; therefore, we discuss our results as they relate to the available research, including in higher income settings.

4.1. Major crops

Difficulty in recall of crop details may be relevant for a setting with two or more cropping seasons, such as in Uganda, during which activities could vary across the variety of up to 14 crops these smallholder farmers grew (Wollburg et al., 2020). Recall of specific crops did not appear to be influenced by the number of crops with which one worked; recall accuracy might be adversely affected from changing crops rather than the total number. Unfortunately, we did not have crop details for intervening years and we are not aware of other studies examining the

Table 3
Recall of the use of specific active ingredients.

Active Ingredient	Baseline Prevalence n (%)[a]	Recalled Prevalence n (%)[a]	Prevalence Ratio	Sensitivity (%)	Specificity (%)	Overall Agreement (%)	AUC
Glyphosate	145 (58.0)	185 (74.0)	1.28	87.6	44.8	69.6	0.66
Cypermethrin	110 (44.0)	128 (51.2)	1.16	71.8	65.0	68.0	0.68
Mancozeb	102 (40.5)	136 (54.0)	1.33	81.4	64.7	71.4	0.73
Profenofos	89 (35.7)	85 (34.1)	0.96	41.6	70.0	59.8	0.56
2,4-D	88 (35.3)	117 (47.0)	1.33	76.1	68.9	71.5	0.73
Dichlorvos	30 (12.1)	55 (22.1)	1.83	63.3	83.6	81.1	0.73
Dimethoate	26 (10.4)	36 (14.5)	1.38	38.5	88.3	83.1	0.63
Lambda-Cyhalothrin	24 (9.6)	36 (14.4)	1.50	41.7	88.5	84.0	0.65
Chlorpyrifos	17 (6.8)	27 (10.8)	1.59	29.4	90.5	86.4	0.60
Carbaryl	11 (4.4)	1 (0.4)	0.09	9.1	100	96.0	0.55
Permethrin	9 (3.6)	10 (4.0)	1.11	0	95.8	92.4	0.50
Carbofuran	8 (3.2)	6 (2.4)	0.75	12.5	97.9	95.2	0.55
Diazinon	8 (3.2)	31 (12.5)	3.88	12.5	87.6	85.1	0.50
Paraquat	7 (2.8)	27 (10.8)	3.86	42.9	90.1	88.8	0.66

[a] Excluding 'Not applicable' and 'don't know' responses.

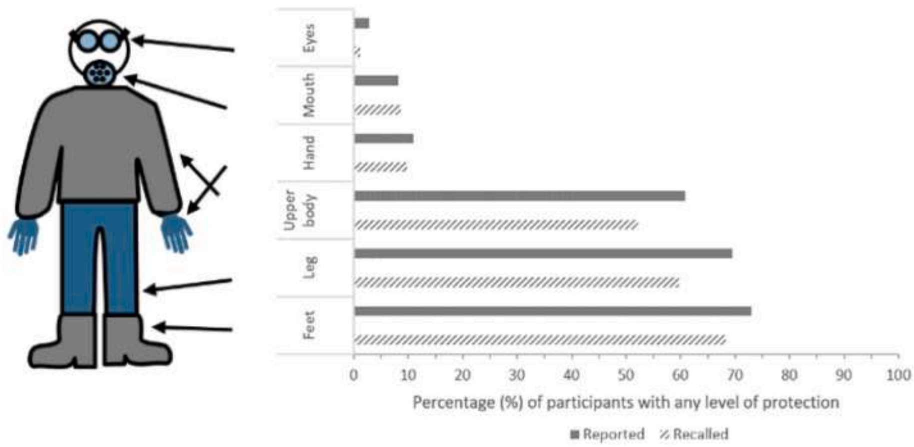

Fig. 1. The number of individuals reporting (in 2017) and recalling (in 2019) any level of protection for a given body part, based on PPE use.

Table 4
Recall of bathing and washing clothes after applying pesticides.

Behaviour	Baseline Prevalence n (%)[a]	Recalled Prevalence n (%)[a]	Prevalence Ratio	Sensitivity (%)	Specificity (%)	Overall Agreement (%)	AUC
Bathing immediately	90 (39.3)	73 (31.9)	0.81	40.0	73.4	60.3	0.57
Changing clothes immediately	92 (40.2)	80 (34.9)	0.87	47.8	73.7	63.3	0.61
Washing own clothes	127 (55.5)	130 (56.8)	1.02	78.7	70.6	75.1	0.75

[a] Excluding 'Not applicable' and 'don't know' responses.

recall of crops in similar settings. The poor recall of crops in this low-income smallholder farmer contexts does not support the use of crop exposure matrices (e.g., London and Myers, 1998; Miligi et al., 1993) when studying smallholders growing a great variety of crops, to infer contact with specific active ingredients.

4.2. Frequency

Similar to previous research in the USA and South Korea, we found better agreement in the estimated years, compared to days per week (Blair et al., 2002) or hours per day (Lee et al., 2010). A longer unit of recall for pesticide use, such as years, may be easier to remember as it would typically involve a static start date compared to the frequency of application or other work practices that might be more dynamic and change between agriculture seasons within a year or from year to year (Fadnes et al., 2009). The difficulty to recollect such practices also may be compounded by a general lack of record keeping or documentation by smallholder farmers in Uganda (Nanyunja et al., 2015). Nevertheless, if the exposure of interest is subject to change from year to year, shorter recall may be more relevant for epidemiology studies. The period of recall may be assisted by the salience of events (Kjellsson et al., 2014), which, for smallholder farmers, may include the relatively infrequent purchase of pesticides in the course of a year (Ngowi et al., 2007). In general, with the exception of the number of workers, we did not observe any differences in recalled duration by demographic or farm features.

4.3. Use of active ingredients

Previous research on recall ability of pesticide use has generally found better agreement for general questions, such as ever/never use of pesticides (Blair et al., 2002; Lee et al., 2010) or broad categories (Engel et al., 2001), compared to more detailed questions about the use of specific products or individual active ingredients. The observed sensitivity for insecticides, herbicides, and fungicides in the present study (79% or higher) is comparable to that identified in orchard workers in the USA (Engel et al., 2001). Contrary to that study, however, sensitivity in the current survey was also acceptable for individual active ingredients, (so long as it was used by >10% of participants at baseline). A potential explanation for this disparity could be the much longer time interval between the two interviews in (Lee et al., 2010) (>20 years) compared to our analysis (2 years).

While one of the earlier studies found little differential reporting of pesticides, except for some lower reporting of specific active ingredients (i.e., malathion, carbaryl, and dichlorodiphenyltrichloroethane) (Blair et al., 2002), another study found over reporting of herbicides and fungicides (Engel et al., 2001); the latter trend, which is more consistent with our results, might have been influenced by the expanded use of these products during the study period. Indeed, we found positive correlations between over-reporting and use of specific active ingredients at the time of follow-up. We also observed a non-statistically significant increase in the percentage who applied pesticides at the time of follow-up compared to baseline (75.3% vs 71.8%), based on reported annual application practices of individual active ingredients (data not shown). Additional involvement in the study, such as providing a urine sample, might have prompted more awareness of pesticide use, though we did not observe any such differences in recall ability of active ingredients. The absence of literacy skills might make it more difficult to be aware of the use of specific active ingredients, yet, again, we did not detect any differences. Another study of smallholder farmers found most (70%) never read pesticide labels (Mengistie et al., 2017); in these settings, pesticides may be repackaged and sold at lower prices, so specific active ingredients may not be clear (Staudacher et al., 2021). Ultimately, the smaller sample sizes for comparison groups would only have detected larger differences in recall between these subgroups.

4.4. PPE and other exposure determinants

The most common form of PPE worn (gumboots) was similar to that identified in a study of farmers in Tanzania (Lekei et al., 2014). The high percentages of overall agreement in responses observed in the current study is in part due to the low reporting of most items, as observed in other studies of farmers in Uganda (e.g., 12% wore gloves) (Oesterlund et al., 2014). The lack of a range of PPE items may be due to lower awareness of the importance of PPE, higher costs, or discomfort in hot and humid conditions (Lekei et al., 2014). For those not wearing the listed PPE items, typical work attire for this tropical setting often involves barefoot or sandals, short trousers, and a short-sleeved t-shirt. Sensitivity was lower (<50%) for washing and changing clothes immediately following the application of pesticides, with around 40% of participants reporting at baseline; these rates are much lower than those reported by pesticide applicators in Italy (>80%) (Riccò et al., 2018). Based on the low reported prevalence of some of these items, it does not appear subjects over-reported socially desirable behaviours (Moore and Rutherfurd, 2020). Information on the use of PPE should be provided via the agro input dealers and label information on the bottle. However, a recent mystery shopping survey suggested this information was unfortunately not consistently disseminated (Staudacher et al., 2021). Training is also organized, mostly by NGOs, though only reaching a small fraction of the farmers and with limited impact (Clausen et al., 2017).

4.5. Exposure algorithms

We found lower overall agreement with the recall of specific hygiene habits (i.e., bathing immediately, changing clothes immediately, and washing own clothes) compared to PPE use. This differs from recall in UK cohorts, where hygiene habits were recalled at least as well as PPE use (Mueller, Jones, Mohamed, Bennett, Harding, Povey, Basinas, Kromhout, van Tongeren, Fuhrimann, Galea). Due to this trend in the current study, we found better agreement in the PPE scores than the PPE-Hygiene scores when comparing reported and recalled data; the PPE-Hygiene scores based on recalled data suggested inflated exposure levels.

4.6. Strengths/limitations and future research

Our research represents the first study to examine the extent of recall of exposure determinants in pesticide users in a LMIC setting. Our results benefitted from a high response rate (84%), although respondents were more likely to use pesticides; users may be motivated to continue participation in research that is more relevant to their own behaviour. Interviews took place at the same time of the year to minimise any seasonal effects on reporting. We used the baseline self-reported data as a proxy for exposure in the prior year to which we compared recall from the follow-up survey two years later. A potential limitation is the accuracy of self-reporting to represent true exposures. In another component of the IMPRESS project, we will examine biomarkers of short-term exposure to active ingredients, which will help validate self-reported current exposures. While the results and extent of recall bias in the current context are encouraging, further research in other LMIC settings would help identify the most reliable and important exposure determinants for use in epidemiological studies of pesticide applicators. In addition, it would be useful to assess the accuracy of recall over longer periods and to investigate the effectiveness of tools to improve recall, such as better documentation of spraying records and other agricultural practices.

5. Conclusion

We performed the first study to assess recall on the use of pesticides and other exposure determinants in a LMIC context. Reporting practices using longer units of time (i.e., years) appeared to be more reliable than recalled hours per day or days per week, with no apparent systematic bias by different demographic or farm characteristics. In general, we found higher levels of agreement for the recall of PPE items and certain active ingredients, with difficulties remembering specific crop types. While some of these findings provide consistency with those from high-income countries, more research is needed on recall bias in poorly educated agriculture communities in low- and middle-income settings to confirm these results.

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Declaration of competing interest

No conflicts of interest are declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113911.

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Exposure to polycyclic aromatic hydrocarbons and volatile organic compounds is associated with a risk of obesity and diabetes mellitus among Korean adults: Korean National Environmental Health Survey (KoNEHS) 2015–2017

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ABSTRACT

Environmental pollutants have been known to increase the risks of not only respiratory and cardiovascular disease but also metabolic diseases such as obesity and diabetes mellitus (DM). Polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs) such as benzene and toluene are major constituents of environmental pollution. In the present study, we employed the population of the Korean National Environmental Health Survey (KoNEHS) Cycle 3 conducted between 2015 and 2017, and assessed the associations of urinary biomarkers for PAHs and VOCs exposure with obesity and DM. A total of 3787 adult participants were included and the urinary concentrations of four PAH metabolites and two VOC metabolites were measured. For correcting urine dilution, a covariate-adjusted standardization method was used.

The highest quartiles of urinary 2-hydroxynaphthalene (2-NAP) [OR (95% confidence interval (CI)) = 1.46 (1.13, 1.87)] and sum of PAH metabolites [OR (95% CI) = 1.45 (1.13, 1.87)] concentrations were associated with a higher risk of obesity [body mass index (BMI)$\geq$25 kg/m^2]. BMI was positively associated with urinary 2-NAP [β (95% CI) = 0.25 (0.09, 0.41), p = 0.003] and sum of PAH metabolites [β (95% CI) = 0.29 (0.08, 0.49), p = 0.006] concentrations. The risk of DM was increased with increasing quartile of 2-hydroxyfluorene (2-OHFlu) and trans, trans-muconic acid (t,t-MA) (p for trend<0.05 and < 0.001, respectively). The highest quartile of t,t-MA showed a significantly higher risk of DM [OR (95% CI) = 2.77 (1.74, 4.42)] and obesity [OR (95% CI) = 1.42 (1.06, 1.90)]. Urinary t,t,-MA level was positively associated with BMI [(β (95% CI) = 0.51 (0.31, 0.71), p < 0.001] and non-alcoholic fatty liver disease index [(β (95% CI) = 0.09 (0.06, 0.12), p < 0.001].

In conclusion, the benzene metabolites t,t-MA and PAH metabolite 2-OHFlu were associated with an increased risk of DM. Urinary biomarkers for PAHs and VOCs were positively associated with BMI in the Korean adult population. Further studies to validate these observations in other populations are warranted.

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1. Introduction

Obesity and diabetes mellitus (DM) are major metabolic diseases with increasing health burden worldwide. According to the World Health Organization, more than 1.9 billion adults (39% as of 2016) are overweight, and 422 million (8.5% as of 2014) have DM (WHO, 2016). In Korea, the prevalence of obesity in adults was 35.7% (45.4% for men and 26.5% for women) in 2018 (KSSO, 2019) and that of DM was 14.4% (approximately 5.02 million) in 2016 (Kim et al., 2019). Therefore, identification of modifiable risk factors for these diseases is an important public health challenge.

Chemical exposure has been recognized as a major risk factor for both obesity and DM. For example, consumer chemicals such as phthalates, bisphenols, and parabens, as well as persistent organic pollutants such as polychlorinated biphenyls, organochlorine pesticides, and polybrominated diphenyl ethers have been linked with these diseases among humans and are often termed as obesogen and diabetogen, respectively (Gore et al., 2015; Lind and Lind, 2018). Similar associations of polycyclic aromatic hydrocarbons (PAHs) and DM have been demonstrated in several studies including a few meta-analyses (Alshaarawy et al., 2014; Khosravipour and Khosravipour 2020; Ranjbar et al., 2015; Stallings-Smith et al., 2018; Yang et al., 2014). However, limited studies have been reported regarding obesity in adults (Muscogiuri et al., 2017; Ranjbar et al., 2015; Ribeiro et al., 2020). Furthermore, there is a lack of epidemiological studies on the association of volatile organic compounds (VOCs) with obesity or diabetes.

Although most of these chemicals are not intentionally manufactured for specific purposes, they are present in the air, food, or other media, naturally or as combustion byproducts, and can reach humans through multiple routes including inhalation, ingestion, or dermal contact. Accordingly, many existing national biomonitoring programs worldwide, e.g., the National Health and Nutrition Examination Survey (NHANES) of the United States (US), Canadian Health Measures Survey (CHMS), and Korean National Environmental Health Survey (KoNEHS), include these chemicals in the list of measured chemicals in humans (Health Canada, 2015; CDC, 2019; Choi et al., 2017). Urinary PAH and benzene metabolites have been detected in over 90% of the survey participants in all three countries (Health Canada, 2015; CDC, 2019; Choi et al., 2017). Regarding PAHs, while measured metabolites often differ, the detection levels were generally similar among Korea, the US, and Canada (Health Canada, 2015; CDC, 2019; Choi et al., 2017). In the case of benzene, the geometric mean concentration of trans, trans-muconic acid (t,t-MA), a benzene metabolite, was reported to be 58.8 μg/L in Koreans and 56 μg/L in Canadians (Health Canada, 2015; Choi et al., 2017); however, in the US, a direct comparison is not possible because other metabolites were measured (CDC, 2019).

PAHs are produced by incomplete combustion of organic materials such as meat, coal, oil, gas, wood, and tobacco (Moorthy et al., 2015; Zhang et al., 2016). For individuals without occupational exposure, main sources of PAH exposure include ambient air pollution (especially motor vehicle exhaust), smoke from wood or fossil fuel combustion, tobacco smoke, and cooking of foods (Ma and Harrad, 2015; CDC, 2019). Among the general population, it is proposed that PAHs may disrupt endocrine function, and involve in the etiology of DM (Alshaarawy et al., 2014) and obesity (Poursafa et al., 2017). To date, most reports on the metabolic disruption caused by PAHs are based on the observations of the national scale cross-sectional studies like US NHANES and CHMS (Alshaarawy et al., 2014; Bushnik et al., 2019; Ranjbar et al., 2015; Stallings-Smith et al., 2018).

VOCs include various organic chemicals, ubiquitously present both indoor and outdoor. Benzene and toluene are common constituents of polluted air and tobacco smoke, traffic exhausts, solvents, and adhesives (Mogel et al., 2011; Mohamed et al., 2002; Weisel, 2010). Adverse health effects of VOCs include irritation to the eyes, nose, and skin; headaches; loss of coordination; nausea; and damage to the liver, kidney, and central nervous system (United States Environmental Protection Agency,). However, adverse effects of non-occupational, long-term exposure to VOCs at low concentration have not been well investigated. Particularly, the cardiovascular and metabolic effects of VOCs such as benzene or toluene have been less studied.

In this study, we aimed to investigate the association of PAH and VOC exposure with obesity and DM, using national biomonitoring data from a representative adult Korean population. By examining multiple groups of chemicals in the association models, we intended to identify chemical risk factor candidates for both obesity and DM. Moreover, we applied a covariate-adjustment standardization method for urinary dilution adjustment to circumvent potential collider problem caused using urine creatinine (Cr) for this purpose. This study sough to elucidate chemical risk factors for obesity and DM among adults and promote further validation studies to confirm the associations found.

2. Materials and methods

2.1. Study population

This study included adult individuals from the general population (n = 3787, aged ≥19 years) who participated in the KoNEHS Cycle 3 (2015–2017). The KoNEHS is a cross-sectional biomonitoring program to determine the exposure level of Koreans to major environmental chemicals, which has been carried out in a 3-year interval since 2009. A two-stage proportionally stratified sampling design was adopted to recruit a nationally representative population (NIER, 2019). The KoNEHS included a questionnaire survey through face-to-face interviews, physical examination, and collection of biospecimen (NIER, 2019). Demographic and socioeconomic information, factors related to exposure to environmental chemicals, and medication history were investigated through questionnaires. Spot urine and blood samples were collected from the participants without consideration of fasting status. The study was approved by the Ethical Review Board of the National Institute of Environmental Research (NIER), Korea (IRB No. NIER-2016-Br-003-01).

Among the initially recruited adults (n = 3787), only those with available information on concentrations of urinary PAH and VOC metabolites were included in the present study. The number of adults with information on urinary chemicals varied: PAH metabolites except for 1-hydroxyphenanthrene (1-OHPhe) (n = 3751), all PAH metabolites (n = 3754), and VOC metabolites (n = 3777). For the measurement of HbA1c, a population subset (n = 1266) was randomly selected following age and sex stratifications. Ethical review regarding this measurement was exempted by the Institutional Review Board (IRB) of the Seoul National University (IRB No. E1911/002–008).

2.2. Measurement of urine PAH and VOC metabolites

Details on chemical analysis are described elsewhere (NIER, 2018). PAH metabolites including 1-hydroxypyrene (1-OHP), 2-hydroxynaphthalene (2-NAP), 1-OHPhe, and 2-hydroxyfluorene (2-OHFlu), were measured by gas chromatography–mass spectrometry. The method detection limits (MDLs) of 1-OHP, 2-NAP, 1-OHPhe, and 2-OHFlu were 0.015, 0.05, 0.04, and 0.04 μg/L, respectively.

VOC metabolites including trans, t,t-MA, and benzylmercapturic acid (BMA) were measured by a high-performance liquid chromatography–mass spectrometry. The MDLs of t,t-MA and BMA were 2.3 and 0.197 μg/L, respectively.

2.3. Measurement of health outcome markers

BMI was calculated as weight/height^2 (kg/m^2), and when a BMI was greater than 25 kg/m^2 was classified as obesity (WHO, 2000). Self-answered medication history for DM was available for all the participants and was used to define the DM cases among the study population. The KoNEHS did not distinguish between type 1 and 2 DM; however, given the low prevalence of type 1 DM in Korea (Park, 2006), those who reported the use of DM medication were assumed to have type 2 DM.

For the one-third of the population randomly chosen following age and sex stratifications (n = 1266), HbA1c levels were measured in whole blood samples with ethylenediaminetetraacetic acid using Cobas c513 Analyzer and Tina-quant® HbA1c Gen. 3 reagents (Roche). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT) and triglyceride levels were measured in serum samples collected during a visit conducted by the standard KoNEHS protocol. Liver enzymes and triglycerides were analyzed using ADVIA 1800 (Siemens, USA). The associations of AST, ALT, and GGT with each chemical were analyzed individually and as a component of the hepatic steatosis index (HSI) and Framingham steatosis index (FSI). HSI and FSI were calculated using the following equations (Kotronen et al., 2009; Long et al., 2016):

HSI = 8 × alanine aminotransferase (ALT)/aspartate aminotransferase (AST) ratio + BMI (+2, if diabetes; +2, if woman)

FSI = −7.981 + 0.011 × age (years) − 0.146 × sex (woman = 1, men = 0) + 0.173 × BMI (kg/m^2) + 0.007 × triglycerides (mg/dL) + 0.593 × hypertension (yes = 1, no = 0) + 0.789 × diabetes (yes = 1, no = 0) + 1.1 × ALT/AST ratio ≥1.33 (yes = 1, no = 0).

2.4. Adjustment method for urinary dilution

To account for urinary dilution, a covariate-adjusted standardization was used, based on a previous study (O'Brien et al., 2016). For biomonitoring of PAHs and VOCs in urine, urinary chemicals measured are frequently adjusted for urinary dilution using urine Cr. Although urine Cr is widely used, it is known to be influenced by factors like muscle mass and therefore, body mass index (BMI) of an individual (Barr et al., 2005); thus, the use of urine Cr to adjust urinary dilution could cause a collider problem in an association model with obesity-related outcomes. The use of urine Cr for correction of urine dilution may therefore partly explain often discrepant directions of association reported previously for outcomes like obesity. To address this problem, urinary flow rate or a covariate-adjusted standardization has been suggested as a better alternative (Bulka et al., 2017).

For the covariate-adjusted standardization, first, the predicted urinary Cr level was estimated for each participant employing a regression model with the ln-transformed urinary Cr and several other important covariates, such as age, sex, and BMI, among the overall study population (n = 3787, for the results of linear regression analysis refer to Table S1). Then, a ratio was derived by dividing the measured Cr level by the predicted Cr level ($\widehat{\mathrm{Cr}}$) for a given individual urine. The urinary levels of chemicals were divided by the ratio (Cr/ $\widehat{\mathrm{Cr}}$, $\mathrm{Cr_{ratio}}$) to adjust for urinary dilution:

$$\text{Covariate}-\text{adjusted standardized chemical measure} = [\text{Chemical concentration}] \times \frac{\widehat{Cr}}{Cr}$$

In addition, a conventional Cr adjustment was also applied, and the results of association studies were compared. For Cr adjustment, urinary concentrations of chemicals (μg/L) were divided by urine Cr levels (g/L) to yield Cr-adjusted concentrations of chemicals (μg/g).

2.5. Statistical analysis

For the non-detects, when the detection frequency was greater than 65%, the MDL divided by $\sqrt{2}$ was used as a proxy value. Survey-weighted logistic regression models were used to derive the odds ratios (ORs) of urinary chemicals for the risk of obesity and DM. Urinary chemical concentrations were divided into quartiles and were modeled in the logistic regression for each outcome. The covariates that were adjusted in the statistical models were chosen based on previous studies which reported associations of urinary chemicals with obesity or DM, or their risk factors (Bertoglia et al., 2017; Corbasson et al., 2016; Duan et al., 2019).

These covariates included age (years), sex (male and female), BMI (kg/m^2), cigarette smoking (never smoker, former smoker, and current smoker), alcohol drinking (never drinker and drinker), education (<elementary school, middle school, high school, and college or above), and exercise (no, low-intensity, moderate-intensity exercise) in the model with DM as an outcome. All covariates except BMI were also included in the model with obesity as an outcome.

Regarding sensitivity analyses, the following tests were conducted. For obesity, we performed additional analysis using two different obesity criteria (BMI≥27 kg/m^2 and ≥30 kg/m^2). In addition, HbA1c was chosen as a DM outcome and was added in the linear regression models as a continuous variable, after excluding the participants with self-reported use of DM medication. Due to right skewness, urinary chemicals, urinary Cr, and HbA1c were natural log (ln)-transformed. Weights of HbA1c (subpopulation) have not been developed. Lastly, we obtained two previous KoNEHS data of Cycles 1 and 2, and compared

Table 1
Demographic characteristics of study participants.

	All participants	Male	Female
N (weighted %)	3778 (100)	1646 (49.7)	2132 (50.3)
Age (years)			
19–29	281 (17.7)	136 (9.4)	145 (8.3)
30–39	541 (18.0)	224 (9.3)	317 (8.7)
40–49	620 (20.7)	276 (10.5)	344 (10.2)
50–59	883 (19.9)	356 (10.0)	527 (9.9)
60–69	934 (12.6)	414 (6.1)	520 (6.5)
70–86	519 (11.1)	240 (4.4)	279 (6.7)
BMI (kg/m^2)			
<18.5	90 (3.1)	29 (1.3)	61 (1.9)
18.5–23	1166 (33.7)	424 (12.9)	742 (20.7)
23–25	960 (25.4)	437 (13.4)	523 (12.0)
≥25	1562 (37.8)	756 (22.0)	806 (15.8)
Cigarette smoking			
Never smoker	2416 (62.0)	393 (14.5)	2023 (47.5)
Former smoker	759 (18.5)	711 (17.3)	48 (1.2)
Current smoker	603 (19.5)	542 (17.9)	61 (1.6)
Alcohol drinking			
Never drinker	761 (15.3)	144 (3.4)	617 (11.9)
Drinker	3017 (84.7)	1502 (46.3)	1515 (38.4)
Education			
<Elementary school	719 (11.2)	207 (3.2)	512 (8.0)
Middle school	548 (9.7)	229 (4.3)	319 (5.4)
High school	1183 (29.8)	536 (14.8)	647 (15.1)
College or above	1328 (49.3)	674 (27.5)	654 (21.8)
Exercise			
No	2081 (55.6)	861 (26.0)	1220 (29.7)
Low intensity	296 (7.4)	130 (3.7)	166 (3.7)
Moderate intensity	1401 (37.0)	655 (20.0)	746 (17.0)
Diabetes*			
No diabetes	3441 (94.0)	1450 (45.3)	1948 (47.4)
Diabetes	337 (6.0)	196 (4.3)	184 (2.9)

Low intensity exercise: Exercise without sweating Moderate exercise: Exercise above low intensity.
* DM via self-answered medication history.

Table 2
Distributions of urinary PAH and VOC metabolites among participants (unit: μg/L).

	DF (%)	Total		BMI <25 kg/m^2		BMI ≥25 kg/m^2		No Diabetes		Diabetes	
		n	Median (25th, 75th)	n	Median (25th, 75th)	n	Median (25th, 75th)	n	Median (25th, 75th)	n	Median (25th, 75th)
PAH metabolites											
1-OHP	71.6	3754	0.20 (<MDL, 0.43)	2201	0.20 (<MDL, 0.42)	1553	0.20 (<MDL, 0.45)	3419	0.20 (<MDL, 0.44)	335	0.18 (<MDL, 0.39)
2-NAP	98.7	3754	2.42 (1.23, 6.16)	2201	2.33 (1.17, 5.39)	1553	2.64 (1.36, 7.24)	3419	2.43 (1.24, 6.12)	335	2.14 (1.14, 6.73)
1-OHPhe	67.5	3751	0.13 (<MDL, 0.30)	2200	0.13 (<MDL, 0.29)	1551	0.15 (<MDL, 0.33)	3416	0.13 (<MDL, 0.30)	335	0.16 (<MDL, 0.35)
2-OHFlu	85.9	3754	0.38 (0.16, 0.78)	2201	0.37 (0.14, 0.75)	1553	0.40 (0.18, 0.82)	3419	0.38 (0.16, 0.78)	335	0.44 (0.17, 0.84)
VOC metabolites											
t,t,-MA	99.7	3777	88.5 (41.4, 184.5)	2215	83.7 (38.4, 180.5)	1562	95.9 (46.7, 194.3)	3440	88.84 (41.10, 183.99)	337	82.78 (43.90, 208.86)
BMA	99.6	3777	4.74 (2.42, 8.95)	2216	4.56 (2.23, 8.77)	1561	4.99 (2.66, 9.16)	3440	4.72 (2.40, 8.88)	337	5.28 (2.69, 10.38)

DF, detection frequency.
NA, not available (below MDL).
Weights were adjusted.

Table 3
ORs (95% CIs) for obesity (BMI ≥25 kg/m^2) according to quartiles of PAH and VOC metabolites.

		OR (95% CI)		
		Total	Male	Female
PAH metabolites				
1-OHP	Q1	Ref	Ref	Ref
	Q2	0.84 (0.65, 1.10)	1.07 (0.71, 1.62)	0.90 (0.65, 1.26)
	Q3	0.86 (0.67, 1.11)	0.96 (0.59, 1.55)	1.02 (0.73, 1.42)
	Q4	0.80 (0.60, 1.07)	0.82 (0.52, 1.30)	0.90 (0.65, 1.24)
2-NAP	Q1	Ref	Ref	Ref
	Q2	1.20 (0.95, 1.53)	1.26 (0.88, 1.82)	1.24 (0.90, 1.71)
	Q3	**1.56 (1.21, 2.01)**	1.21 (0.81, 1.80)	1.21 (0.86, 1.71)
	Q4	**1.45 (1.13, 1.87)**	1.25 (0.81, 1.93)	**2.07 (1.55, 2.78)**
1-OHPhe	Q1	Ref	Ref	Ref
	Q2	0.83 (0.65, 1.06)	**0.53 (0.37, 0.76)**	1.26 (0.90, 1.78)
	Q3	0.99 (0.76, 1.29)	0.90 (0.62, 1.31)	1.08 (0.76, 1.55)
	Q4	0.84 (0.67, 1.07)	**0.67 (0.47, 0.94)**	1.23 (0.87, 1.73)
2-OHFlu	Q1	Ref	Ref	Ref
	Q2	1.09 (0.80, 1.49)	0.95 (0.61, 1.48)	1.01 (0.74, 1.38)
	Q3	1.17 (0.88, 1.56)	1.26 (0.84, 1.89)	1.26 (0.85, 1.88)
	Q4	1.18 (0.85, 1.65)	1.09 (0.69, 1.72)	1.25 (0.88, 1.78)
∑PAH metabolites	Q1	Ref	Ref	Ref
	Q2	1.25 (0.97, 1.62)	1.30 (0.88, 1.92)	1.13 (0.78, 1.62)
	Q3	**1.43 (1.10, 1.86)**	1.11 (0.74, 1.68)	**1.55 (1.11, 2.16)**
	Q4	**1.44 (1.08, 1.91)**	1.13 (0.71, 1.80)	**1.88 (1.36, 2.59)**
VOC metabolites				
t,t,-MA	Q1	Ref	Ref	Ref
	Q2	1.12 (0.88, 1.43)	1.39 (0.99, 1.96)	1.18 (0.82, 1.68)
	Q3	1.25 (0.97, 1.62)	1.28 (0.85, 1.95)	**1.50 (1.10, 2.04)**
	Q4	**1.42 (1.06, 1.90)**	**1.67 (1.11, 2.51)**	1.37 (0.94, 2.01)
BMA	Q1	Ref	Ref	Ref
	Q2	1.07 (0.85, 1.36)	**1.44 (1.01, 2.06)**	0.80 (0.57, 1.13)
	Q3	1.22 (0.98, 1.53)	1.30 (0.88, 1.91)	1.23 (0.89, 1.72)
	Q4	0.97 (0.75, 1.26)	1.04 (0.69, 1.56)	0.98 (0.72, 1.34)

Age, sex, cigarette smoking, alcohol drinking, education, and exercise were included as covariates.
For all urinary chemicals, covariate-adjusted standardization was applied.
Bold numbers indicate that 95% CIs of ORs did not include one.
∑PAH metabolites indicates sum of PAH metabolites.

the results with those found in the present population. All analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Demographic characteristics and distribution of measured chemicals

The mean age of the participants was 53 years, and 49.7% of them were men. Of the total participants, 6.0% (N = 337) were classified as diabetic and 37.8% were obese (Table 1). The detection frequencies (DF) of PAH metabolites ranged between 67.5% and 98.7% (Table 2). The median (interquartile range; IQR) of the urinary concentrations for 1-

Table 4
Linear regression for BMI and HbA1c with the levels of PAH and VOC metabolites.

	BMI			HbA1c[a]		
	n	β (95% CI)	p-value	n	β (95% CI)	p-value
1-OHP	3754	−0.01 (−0.11, 0.09)	0.850	1168	0.000 (−0.003, 0.003)	0.832
2-NAP	3754	**0.25 (0.09, 0.41)**	**0.003**	1168	0.005 (0.000, 0.009)	0.062
1-OHPhe	3751	0.05 (−0.07, 0.18)	0.397	1168	0.000 (−0.005, 0.004)	0.848
2-OHFlu	3754	0.14 (−0.02, 0.29)	0.081	1168	−0.001 (−0.006, 0.003)	0.556
∑PAH metabolites	3751	**0.29 (0.08, 0.49)**	**0.006**	1168	0.004 (−0.001, 0.010)	0.139
t,t,-MA	3777	**0.51 (0.31, 0.71)**	**<0.001**	1177	**0.007 (0.001, 0.013)**	**0.014**
BMA	3777	**0.21 (0.02, 0.41)**	**0.034**	1177	0.006 (−0.001, 0.012)	0.072

For all urinary chemicals, covariate-adjusted standardization was applied.
All urinary concentrations of chemicals and HbA1c were ln-transformed.
∑PAH metabolites indicates sum of PAH metabolites.
[a] This analysis was performed after exclusion of participants with self-reported use of DM medication.

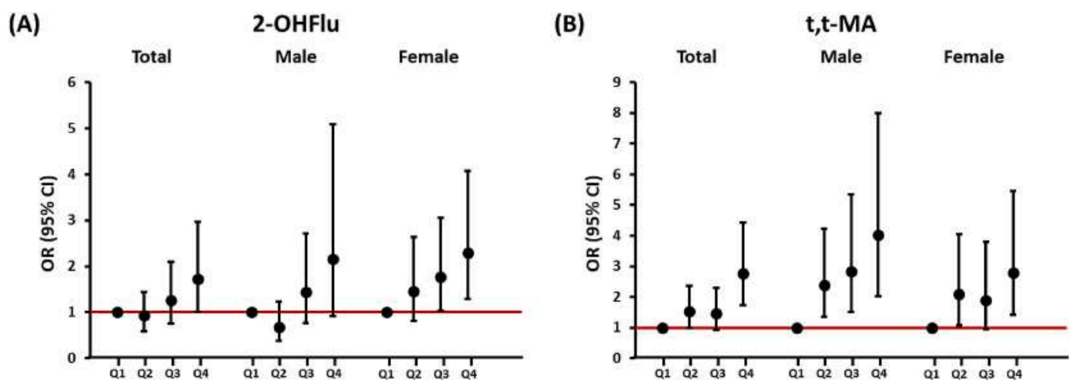

Fig. 1. ORs (95% CIs) for prevalent diabetes according to quartiles of 2-OHFlu and t,t-MA. All p-values for trend were <0.05 except for 2-OHFlu in male.

OHP, 2-NAP, 1-OHPhe, and 2-OHFlu was 0.20 (<MDL, 0.43) μg/L, 2.42 (1.23, 6.16) μg/L, 0.13 (<MDL, 0.30) μg/L, and 0.38 (0.16, 0.78) μg/L, respectively. For VOC metabolites, DFs were much higher (DF > 99%, Table 2). The median (IQR) concentrations of t,t-MA and BMA in urine were 88.5 (41.4, 184.5) μg/L and 4.74 (2.42, 8.95) μg/L, respectively.

3.2. Association with obesity

Following the covariate-adjusted standardization, the highest quartiles of urinary 2-NAP [OR (95% confidence interval (CI) = 1.45 (1.13, 1.87)] and t,t-MA [OR (95% CI) = 1.42 (1.06, 1.90)] concentrations were positively associated with higher risk of obesity (BMI ≥25 kg/m^2) (Table 3). In addition, the sum of PAH metabolites showed a significant association [OR (95% CI) = 1.44 (1.08, 1.91)]. In contrast, t,t,-MA showed significant association with obesity regardless of sex. These observations were different from those detected following Cr adjustments (Table S2). With the conventional Cr adjustment of the urinary chemical data, several significant associations were null and often showed negative associations with obesity risk, e.g., the highest quartiles of urinary 1-OHP [OR (95% CI) = 0.72 (0.54, 0.96)] and BMA [OR (95% CI) = 0.68 (0.52, 0.89)] (Table S2).

Regarding sensitivity analyses, when a BMI of 30 kg/m^2 or higher was used as the obesity criterion, the odds ratio of obesity became greater as the quartile of 2-NAP and the sum of PAH metabolites increased (Table S3). However, the association of t,t,-MA with obesity was not significant when the more stringent obesity criterion was applied. In Cycle 1, the 2nd and 3rd quartiles of urinary 2-NAP were positively associated with higher obesity odds [OR (95% CI) = 1.49 (1.25, 1.78) and 1.47 (1.21, 1.78), respectively] (Table S4). The increased obesity odds in the highest quartile of t,t-MA was the same with those observed in both Cycle 1 [OR (95% CI) = 1.35 (1.07, 1.69)] and 2 [OR (95% CI) = 1.66 (1.33, 2.06)] (Table S4).

The findings from the regression analysis showed generally similar results (Table 4). Following the covariate-adjusted standardization, BMI was positively associated with urinary 2-NAP [β (95% CI) = 0.25 (0.09, 0.41), p = 0.003], sum of PAH metabolites [β (95% CI) = 0.29 (0.08, 0.49), p = 0.006], t,t,-MA [β (95% CI) = 0.51 (0.31, 0.71), p < 0.001], and BMA [β (95% CI) = 0.21 (0.02, 0.41), p = 0.034].

3.3. Association with DM

The risk of DM was increased with an increasing quartile of 2-OHFlu and t,t-MA (p for trend = 0.039 and < 0.001, respectively, Fig. 1). Compared to the lowest quartile, the participants among the highest quartile of t,t,-MA showed significant higher risk of DM [OR (95% CI) = 2.77 (1.74, 4.42)] (Table 5). Interestingly, there was a positive relationship (β = 0.007; 95% CI, 0.001, 0.013, p = 0.014, Table 4) between t,t-MA and HbA1c levels among the participants without DM. The associations observed between urinary chemicals and DM were consistent regardless of adjustment methods for urinary dilution (Tables 5 and S6).

In previous KoNEHS cycles, similar patterns of associations were observed for 2-OHFlu and t,t-MA; in Cycle 2, the 3rd quartile of 2-OHFlu showed increased DM odds compared to the lowest quartile [OR (95% CI) = 1.49 (1.02, 2.17)] (Table S5). The highest quartile of t,t-MA showed the same pattern of increased DM odds [OR (95% CI) = 1.83 (1.21, 2.78)] (Table S5).

3.4. Association with liver function

Urinary 1-OHP, sum of PAH metabolites, and t,t,-MA concentrations were positively associated with serum AST concentrations (Table 6). Similarly, urinary 1-OHP and t,t,-MA concentrations were positively associated with serum ALT concentrations. Urinary 1-OHP and 2-OHFlu concentrations were positively associated with serum gamma-glutamyl transpeptidase (GGT) concentrations. Urinary t,t,-MA was positively associated with the index of non-alcoholic fatty liver disease (NAFLD),

Table 5
ORs (95% CIs) for prevalent diabetes according to quartiles of PAH and VOC metabolites.

		OR (95% CI)		
		Total	Male	Female
PAH metabolites				
1-OHP	Q1	Ref.	Ref.	Ref.
	Q2	1.21 (0.79, 1.84)	1.23 (0.67, 2.27)	1.31 (0.73, 2.33)
	Q3	1.09 (0.70, 1.72)	1.33 (0.68, 2.60)	1.30 (0.75, 2.25)
	Q4	1.11 (0.68, 1.80)	1.04 (0.52, 2.07)	1.37 (0.74, 2.52)
2-NAP	Q1	Ref.	Ref.	Ref.
	Q2	**0.64 (0.41, 0.97)**	0.68 (0.34, 1.34)	**0.51 (0.28, 0.93)**
	Q3	0.90 (0.57, 1.43)	0.98 (0.48, 1.97)	0.96 (0.54, 1.69)
	Q4	**0.57 (0.36, 0.89)**	0.74 (0.35, 1.57)	0.73 (0.43, 1.26)
1-OHPhe	Q1	Ref.	Ref.	Ref.
	Q2	0.81 (0.49, 1.32)	0.74 (0.36, 1.55)	1.12 (0.58, 2.13)
	Q3	1.00 (0.59, 1.68)	0.89 (0.43, 1.87)	1.45 (0.76, 2.77)
	Q4	1.03 (0.63, 1.70)	1.03 (0.55, 1.91)	1.10 (0.61, 1.97)
2-OHFlu	Q1	Ref.	Ref.	Ref.
	Q2	0.93 (0.59, 1.44)	0.68 (0.37, 1.24)	1.46 (0.81, 2.64)
	Q3	1.26 (0.76, 2.10)	1.45 (0.77, 2.71)	**1.77 (1.03, 3.06)**
	Q4	**1.73 (1.00, 2.97)**	2.15 (0.91, 5.08)	**2.29 (1.29, 4.07)**
∑PAH metabolites	Q1	Ref.	Ref.	Ref.
	Q2	1.02 (0.67, 1.55)	0.94 (0.56, 1.58)	0.72 (0.40, 1.28)
	Q3	0.95 (0.63, 1.43)	0.94 (0.43, 2.05)	1.09 (0.64, 1.88)
	Q4	0.91 (0.53, 1.58)	0.80 (0.39, 1.64)	0.81 (0.47, 1.40)
VOC metabolites				
t,t,-MA	Q1	Ref.	Ref.	Ref.
	Q2	1.52 (0.98, 2.36)	**2.38 (1.34, 4.22)**	**2.10 (1.09, 4.03)**
	Q3	1.46 (0.92, 2.30)	**2.83 (1.50, 5.34)**	1.90 (0.95, 3.80)
	Q4	**2.77 (1.74, 4.42)**	**4.02 (2.02, 8.00)**	**2.79 (1.43, 5.45)**
BMA	Q1	Ref.	Ref.	Ref.
	Q2	1.00 (0.64, 1.58)	0.84 (0.40, 1.78)	1.51 (0.87, 2.62)
	Q3	0.93 (0.56, 1.54)	0.77 (0.43, 1.40)	0.95 (0.49, 1.87)
	Q4	1.28 (0.84, 1.96)	1.09 (0.61, 1.97)	1.60 (0.92, 2.78)

Age, sex, BMI, cigarette smoking, alcohol drinking, education level, and exercise were included as covariates.
For all urinary chemicals, covariate-adjusted standardization was applied.
Bold numbers indicate that 95% CIs of ORs did not include one.
∑PAH metabolites indicates sum of PAH metabolites.

and FSI, (Table 6).

4. Discussion

In this study, based on evidence from a representative adult Korean population, we found that the exposure to several PAHs and VOCs was significantly associated with increased risk of obesity and DM. The results of the current study clearly show the importance of chemical exposure as a modifiable risk factor for these diseases whose prevalence has increased rapidly worldwide.

4.1. Association with obesity

In this study, urinary 2-NAP and sum of PAH metabolites were associated with higher risk of obesity. Similarly, in Cycle 1, the 2nd and 3rd quartiles of urinary 2-NAP were positively associated with higher obesity odds. These results are consistent with those of previous studies that have shown the positive association between PAH exposure and body weight outcomes (Table 7). The underlying mechanisms of PAH-related obesity have not been elucidated yet (Ruiz-Hernandez et al., 2015). One proposed mechanism is their ability to bind nuclear receptors and other transcription factors, thereby influencing consequent gene expression. The peroxisome proliferator-activated receptor (PPAR)γ is a major regulator of adipogenesis and PAHs act as ligands for the PPARγ nuclear receptor. Moreover, they have been shown to cause obesogenic effects accompanied by altered methylation of PPARγ or PPARγ target genes (Stel and Legler, 2015). PAH could also play a role as an endocrine disruptor and induce various cell signaling pathways, such as the mitogen-activated protein kinase pathway, and affect the cell cycle, DNA damage control regulatory mechanisms, apoptosis, autophagy, and immune and inflammatory responses (Zhang et al., 2016). However, there is no direct evidence that exposure to PAH induces obesity, and it is not certain whether all PAHs have the similar effects on obesity; therefore, further studies on the relationship between PAH and obesity are needed.

Significant association between t,t-MA, a benzene metabolite, and obesity observed in this study has seldom been reported in epidemiologic studies. It is noteworthy that two previous KoNEHS cycles also showed significant association between t,t-MA and obesity (Table S4). Benzene is one of the most widely used solvents in industries and main air pollutants both indoor and outdoor (Bahadar et al., 2014). Most of the studies on benzene exposure are based on occupational exposure and their effects on the development of hematologic malignancies such as acute myeloid and acute nonlymphocytic leukemias (ATSDR, 2007; Galbraith et al., 2010). Regarding its metabolic effects, only a couple of studies have been reported: The urine muconic acid were significantly associated with metabolic syndrome in analysis from the KoNEHS Cycle 2 (2012–2014) (Shim et al., 2019). In addition, urinary t,t-MA levels have been found to be associated with the Framingham Risk Score but not obesity in individuals with dyslipidemia, as assessed in 210 individuals with mild to high cardiovascular disease risk (Abplanalp et al., 2017). However, the association of t,t-MA with obesity was insignificant with a more stringent obesity criterion (BMI $\geq$30 kg/m^2). Inconsistent direction of association may in part be attributed to the small number people with BMI $\geq$30 kg/m^2 in this study population. It is necessary to verify the effects and mechanisms of benzene exposure on obesity through further studies.

4.2. Association with DM

The present finding of an association between urinary 2-OHFlu and DM is consistent with previous studies that have shown positive association between PAH exposure and DM (Table 7). In a meta-analysis regarding the association between PAHs and diabetes, the authors have reported the highest likelihood of diabetes associated with 2-OHFlu (Khosravipour and Khosravipour, 2020). PAHs are known to increase oxidative stress and inflammation and act as an endocrine disruptor (Alshaarawy et al., 2013; Khalil et al., 2010; Zhang et al., 2016). Through these actions, these chemicals may induce insulin resistance and further develop DM. However, since there is no study to evaluate directly whether PAHs could cause insulin resistance or disrupt the associated signaling pathways, mechanistical studies are warranted.

In the current population, 2-NAP was inversely associated with DM. This observation is perplexing and warrants explanation. One possible explanation may be that naphthalene is a PPARγ agonist and that PPARγ agonist is used as a treatment for diabetes, promoting adipocyte differentiation and resulting in increase in subcutaneous fat and weight gain,

Table 6
Linear regression results for liver enzymes with urinary PAH and VOC metabolites.

	n	AST		ALT		GGT		FSI		HSI	
		β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value	β (95% CI)	p-value
1-OHP	3714	**0.014 (0.007, 0.021)**	**<0.001**	**0.011 (0.000, 0.022)**	**0.042**	**0.019 (0.001, 0.037)**	**0.043**	0.005 (−0.018, 0.028)	0.652	−0.028 (−0.086, 0.031)	0.352
2-NAP	3714	0.010 (0.000, 0.021)	0.055	0.004 (−0.012, 0.019)	0.627	0.019 (−0.009, 0.047)	0.18	−0.006 (−0.050, 0.039)	0.799	−0.072 (−0.160, 0.015)	0.105
1-OHPhe	3711	−0.004 (−0.019, 0.010)	0.544	−0.010 (−0.027, 0.008)	0.277	0.015 (−0.005, 0.036)	0.131	0.019 (−0.010, 0.048)	0.206	−0.069 (−0.146, 0.009)	0.084
2-OHFlu	3714	0.010 (−0.001, 0.022)	0.072	0.009 (−0.007, 0.026)	0.255	**0.024 (0.000, 0.048)**	**0.046**	0.005 (−0.032, 0.042)	0.792	0.018 (−0.067, 0.104)	0.673
∑PAH metabolites	3711	**0.015 (0.002, 0.028)**	**0.027**	0.007 (−0.013, 0.027)	0.512	0.031 (−0.003, 0.064)	0.075	0.003 (−0.052, 0.058)	0.912	−0.085 (−0.196, 0.025)	0.130
t,t,-MA	3737	**0.020 (0.003, 0.036)**	**0.019**	**0.028 (0.005, 0.050)**	**0.016**	0.010 (−0.020, 0.040)	0.521	**0.089 (0.056, 0.123)**	**<0.001**	0.095 (−0.017, 0.208)	0.097
BMA	3737	0.006 (−0.007, 0.019)	0.375	−0.002 (−0.024, 0.019)	0.828	−0.006 (−0.038, 0.025)	0.694	0.010 (−0.034, 0.054)	0.647	−0.050 (−0.172, 0.073)	0.426

For all urinary chemicals, covariate-adjusted standardization was applied.
All urinary concentrations of chemicals, ALT, AST, and GGT were ln-transformed.
Bold numbers indicate significant results.

Table 7
Association between PAHs and metabolic disorders in human studies.

References	Data Source	n	Chemicals	Association with DM or obesity
Present study	KoNEHS 2015–2017	3781	2-NAP 2-OHFlu 1-OHPhe 1-OHP	2-NAP, sum of PAHs: higher risk of obesity 2-NAP, 2-OHFlu, sum of PAHs: positively associated with BMI 2-OHFlu: higher risk of DM 2-NAP: lower risk of DM
Stallings-Smith et al. (2018) (Stallings-Smith et al., 2018)	NHANES 2005–2014	8664	1-NAP, 2-NAP 2-OHFlu, 3-OHFlu, 9-OHFlu 1-OHPhe, 2-OHPhe, 3-OHPhe, 1-OHP	2-NAP, 2-OHFlu, 9-OHFlu, 2-OHPhe, and a summed variable of all low molecular weight PAHs showed a positive association with diabetes
Ranjbar et al. (2015) (Ranjbar et al., 2015)	NHANES 2001–2008	4765	1-NAP, 2-NAP 2-OHFlu, 3-OHFlu, 1-OHPhe, 2-OHPhe, 3-OHPhe 1-OHP	2-OHPhe: positively associated with obesity 1-NAP: lower risk of obesity 2-NAP, 2-OHFlu, 3-OHFlu, 2-NAP: greater likelihood of MetS 1-NAP, 2-NAP, 2-OHPhe, 1-OHP: greater likelihood of T2DM 1-NAP, 2-NAP, 2-OHFlu, 3-OHFlu, 2-OHPhe: greater likelihood of dyslipidemia 2-NAP, 2-OHPhe: positively associated with hypertension
Alshaarawy et al. (2014) (Alshaarawy et al., 2014)	NHANES 2001–2006, merged	2769	1-NAP, 2-NAP 2-OHFlu, 3-OHFlu, 1-OHPhe, 2-OHPhe, 3-OHPhe, 1-OHP	positive association between urinary biomarkers of 1 and 2-NAP, 2-OHFlu, 2-OHPhe and summed low molecular weight (LMW) PAH biomarkers, and diabetes mellitus
Yang et a1., 2014 (Yang et al., 2014)	General Chinese population (Wuhan community)	2824	1-NAP, 2-NAP 2-OHFlu, 9-OHFlu 1-OHPhe, 2-OHPhe, 3-OHPhe, 4-OHPhe, 9-OHPhe 6-hydroxychrysene 3-hydroxybenzo[a]pyrene	2-OHFlu, 1-OHPhe, 2-OHPhe, and 4-OHPhe were associated with elevated risk of diabetes
Bushnik et al. (2019) (Bushnik et al., 2019)	Canadian Health Measures Survey 2009–2015 children aged 3–18 years	3667	Naphthalene (sum of 1-NAP and 2-NAP) Fluorene (sum of 2-OHFlu, 3-OHFlu, and 9-OHFlu) Phenanthrene (sum of 1-OHPhe, 2-OHPhe, 3-OHPhe, 4-OHPhe, and 9-OHPhe) Pyrene (1-OHP)	BMI, WC, and WHR were positively associated with total PAH and naphthalene metabolites in the total population aged 3–18.

KoNEHS, Korean National Environmental Health Survey; NHANES, National Health and nutrition examination survey.
NAP, hydroxynaphthalene; OHFlu, hydroxyfluorene; OHPhe, hydroxyphenanthrene; OHP, hydroxypyrene.
MetS, metabolic syndrome; WC, waist circumferences; WHR, waist-to -hip ratio.

but improves insulin sensitivity, reducing blood glucose levels (Kim and Ahn 2004). Although no study has shown the effects of naphthalene or 2-NAP on insulin secretion or as PPARγ agonist, PAHs have been known as ligands for the PPARγ nuclear receptor (Jin et al., 2014; Stel and Legler 2015; Yan et al., 2014). On the other hand, the possibility of a chance finding cannot be ruled out. A sensitivity analysis on previous KoNEHS cycles showed that the association between 2-NAP and DM varied depending on the cycle, i.e., negative in Cycle 3 (the present population), positive in Cycle 2, but null in Cycle 1 (Table S5). However, different directions of association across the cycles could also be due to the difference in exposure levels (Table S7). The concentrations of most chemicals were significantly different by cycle, i.e., the highest in the Cycle 3, except for 2-NAP which was the highest in Cycle 1. Further researches, such as whether the results of this study can be reproduced in other populations or whether naphthalene has a PPARγ agonistic effect in preclinical experiments, are required to confirm the effects of naphthalene on DM.

The association of t,t-MA levels with DM observed in this study can be supported by the reported association of benzene exposure with insulin resistance in elderly adults (Choi et al., 2014), as well as in children and adolescents (Amin et al., 2018). Moreover, in this study, urinary t,t-MA levels were found to be positively related to HbA1c in non-diabetic participants. During metabolic process of benzene, reactive oxygen species such as superoxide anion, hydroxy radicals, and hydrogen peroxide are generated (Caro and Cederbaum, 2004; Shen et al., 1996); thus this oxidative stress and inflammation could involve in the development of insulin resistance and T2DM. Abplanalp et al. demonstrated that benzene exposure could induce glucose intolerance and insulin resistance in C57BL/6 mice by diminishing insulin-stimulated Akt phosphorylation in the liver and skeletal muscle and by increasing nuclear factor kappa B phosphorylation (Abplanalp et al., 2019). These changes were reversed by treatment with superoxide dismutase mimetic, proving oxidative stress induced by benzene increased insulin resistance *in vivo*. Moreover, in the present study, t,t,-MA levels found to be positively associated with serum ALT, AST and FSI. Benzene-associated NAFLD can be a cause of insulin resistance; thus, it can be linked to DM. Few epidemiological studies have reported the relationship between benzene exposure and DM so far; however, the results of this study and previous papers suggest that exposure to benzene may contribute to the increased risk of DM incidence. Therefore, it is necessary to evaluate the effects of benzene exposure on DM incidence in various ethnic and age groups. In addition, the association of other VOCs such as acrolein, formaldehyde, and vinyl chloride warrants further investigation.

4.3. Importance of adjusting method for urine dilution

The results of association studies presented here were based on the covariate-adjusted standardization for urine dilution. While the DM outcome was not influenced by the type of adjusting method for urine dilution, i.e., covariate- and Cr-adjusted standardization, the association with obesity was different. Following the covariate-adjusted standardization, urinary 2-NAP and t,t-MA concentrations were positively associated with obesity, but with Cr adjustment, both metabolites showed a null association. With Cr adjustment, urinary 1-OHP and BMA were associated with decreased risk of obesity (Table S2). Because urinary Cr can be influenced by muscle mass, and BMI may be correlated with the urinary Cr level, dilution adjustment using urinary Cr may act as collider when BMI is a target outcome (Bulka et al., 2017; Sala et al., 2005). A previous study has also shown that the association between arsenic exposure and BMI could be better assessed using a covariate-adjusted standardization method compared to urine Cr adjustment (Bulka et al., 2017). In fact, as previously discussed, we could confirm the significant association between urinary Cr and BMI among the present study participants, i.e., higher urine Cr in obese participants (Lee et al., 2020).

Specific gravity (SG) has also been widely used to adjust urinary dilution. SG is less influenced by demographic factors such as age, sex, and muscle mass than urine Cr (Sauve et al., 2015; Suwazono et al., 2005; Wang et al., 2015). However, as urine SG is influenced by the amount of urine solutes excreted (Sauve et al., 2015), urine SG tends to be greater in people with proteinuria and glycosuria (Chadha et al., 2001). In our previous study, urine SG was positively correlated with HbA1c and tended to be higher among patients with DM (Lee et al., 2020). In contrast, the Cr-ratio that was employed for CAS, did not show any significant differences by DM status. Therefore, for association models between urinary chemicals and DM-related outcomes, CAS was regarded as a more appropriate method for correcting urine dilution and circumventing a potential collider problem.

4.4. Limitations and strengths

This study has several limitations. As a cross-sectional study, the associations observed in the present study cannot indicate causal relationship. Second, the use of single spot-urine measurements may not represent long-term exposure. The fact that this study did not consider the fasting status of the subjects and hence may also increase the variability of the urinary metabolite levels is another limitation. Lastly, the identification of DM patients via self-answered medication history might have been underestimated. According to the Diabetes Fact Sheet in Korea 2020, about 30% of people did not recognize themselves as DM patients even though they had diabetes (Jung et al., 2021). DM awareness rate tended to be higher among th e old and among female Korean adults (Jung et al., 2021), and therefore, age or gender may have biased the observed associations.

However, this study has several strengths. First, some associations observed in the present study were supported by mechanistic *in vitro* studies. Second, a large number of subjects ($n = 3778$), representing the general Korean population, employed in the current study is a merit, which may provide reasonable, if not reliable, estimates of the association even considering the variability of the urinary concentrations of the measured metabolites. It has been previously suggested that high variability of urinary PAH metabolites might be overcome with an appropriate sample size (Li et al., 2010). Moreover, the sensitivity analyses with different obesity criteria, measured DM criteria, and previous KoNEHS cycles that generally support the present findings provide additional lines of evidence for the tested associations.

5. Conclusion

In the present study, we identified major environmental pollutants as potential chemical risk factors for obesity and DM based on a representative adult Korean population that participated in the KoNEHS Cycle 3: PAH metabolite 2-OHFlu and benzene metabolite t,t-MA were associated with increased DM risk in Korean adults. Urinary levels of PAHs and benzene metabolites were positively associated with BMI. Further studies to validate these observations in other populations are warranted.

Declaration of competing interest

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113886.

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Glyphosate and AMPA exposure in relation to markers of biological aging in an adult population-based study

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A R T I C L E I N F O

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A B S T R A C T

Background/Aim: Glyphosate, a broad-spectrum herbicide, and its main metabolite aminomethylphosphonic acid (AMPA) are persistent in the environment. Studies showed associations between glyphosate or AMPA exposure and several adverse cellular processes, including metabolic alterations and oxidative stress.
Objective: To determine the association between glyphosate and AMPA exposure and biomarkers of biological aging.
Methods: We examined glyphosate and AMPA exposure, mtDNA content and leukocyte telomere length in 181 adults, included in the third cycle of the Flemish Environment and Health Study (FLEHSIII). DNA was isolated from leukocytes and the relative mtDNA content and telomere length were determined using qPCR. Urinary glyphosate and AMPA concentrations were measured by Gas Chromatography-Tandem Mass Spectrometry (GC-MS-MS). We used multiple linear regression models to associate mtDNA content and leukocyte telomere length with glyphosate or AMPA exposure while adjusting for confounding variables.
Results: A doubling in urinary AMPA concentration was associated with 5.19% (95% CI: 0.49 to 10.11; $p = 0.03$) longer leukocyte telomere length, while no association was observed with urinary glyphosate concentration. No association between mtDNA content and urinary glyphosate nor AMPA levels was observed.
Conclusions: This study showed that AMPA exposure may be associated with telomere biology in adults.

1. Introduction

Glyphosate (N-[phosphonomethyl]-glycine) is a broad-spectrum, non-selective herbicide used in agricultural formulations worldwide. Glyphosate is the active ingredient in the commercial formulation Roundup®, which was first sold by Monsanto in 1974. From 1974 to

; AHS, Agricultural Health Study; AMPA, Aminomethylphosphonic acid; BUND, Bund für Umwelt undNaturschutz Deutschland; FLEHS, Flemish Environment and Health Study; G-EQUAS, German External Quality Assessment Scheme; GC-MS-MS, Gas Chromatography-Tandem Mass Spectrometry; IARC, International Agency for Research on Cancer; IQR, Interquartile range (25th - 75th percentile); LLOQ, Lower limit of quantitation; NHANES, National Health and Nutrition Examination Survey; PCB, Polychlorinated biphenyl; POP, Persistent organic pollutant; ROS, Reactive oxygen species.

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2014, agricultural use of glyphosate rose 300-fold, while non-agricultural use increased 43-fold. Following the introduction of genetically modified herbicide-tolerant crops in 1996, agricultural applications of glyphosate boomed (Benbrook, 2016). Glyphosate's main degradation metabolite is AMPA, which is formed by microbial degradation in the soil (Agostini et al., 2020). Both glyphosate and AMPA are known to persist in the environment and can be found in house dust (Curwin et al., 2005), soil, air, surface water, and groundwater (Bai and Ogbourne, 2016; IARC, 2017). Humans are mainly indirectly exposed through contaminated food, posing a potential long-term threat to human health. Glyphosate is poorly metabolised – and exclusively to AMPA – in humans and is mainly excreted in urine (Agostini et al., 2020). A recent study (Connolly et al., 2020) emphasized the importance of human biomonitoring studies as a measure of internal exposure. In their comprehensive review, they reported 21 studies using human biomonitoring to assess urinary glyphosate and/or AMPA concentrations and found glyphosate to be omnipresent in the environment. Furthermore, other studies confirmed that urinary glyphosate levels are good exposure markers, since glyphosate does not accumulate and is insufficiently metabolised in humans (Agostini et al., 2020; Zoller et al., 2020). The recent finding that glyphosate's excretion fraction in urine is 1% (Zoller et al., 2020) suggested that the back-calculated oral dose would be 20 times higher than previously assumed (Connolly et al., 2020).

Several epidemiological studies reported associations between glyphosate exposure and different health disorders, including cancer (Leon et al., 2019), respiratory diseases (Hoppin et al., 2008), chronic kidney disease (Gunatilake et al., 2019), and neurological diseases (von Ehrenstein et al., 2019), as well as metabolic alterations and oxidative stress (Agostini et al., 2020; Meftaul et al., 2020). The toxicity of AMPA is ambiguous as it was reported to be similar or less compared to glyphosate (Moore et al., 2012) and in contrast, due to its longer persistence in the environment, some studies demonstrated that environmental AMPA toxicity was higher than that of glyphosate (Daouk et al., 2013; Guilherme et al., 2014). Although several studies focused on the effect of glyphosate and AMPA exposure on human health (Agostini et al., 2020), including disturbance of the oxidative balance and DNA damage, the association of glyphosate or AMPA exposure on biomarkers of aging such as mitochondrial DNA (mtDNA) content and telomere length is poorly investigated.

Mitochondria are intracellular organelles responsible for energy production via oxidative phosphorylation. In addition, they also play a role in apoptosis and reactive oxygen species (ROS) production (Martens and Nawrot, 2016). Mitochondria are especially vulnerable to oxidative damage since they are the main intracellular source as well as targets of ROS. mtDNA lacks several protective structures, like chromatin, histones, and DNA repair mechanisms, resulting in a high mutation rate (Janssen et al., 2012). This high mutation rate was associated with changes in mtDNA content (Kauppila et al., 2017; Vriens et al., 2019). Several studies reported a lower mtDNA content with chronological aging (Chistiakov et al., 2014; Knez et al., 2016; Seo et al., 2010). Telomeres are ribonucleoprotein structures consisting of 5′-TTAGGG-3′ tandem repeats positioned at the end of chromosomes, forming protection from degradation, ensuring genome stability, and preventing loss of genetic information (Blackburn, 1991). During each mitotic cycle, telomeres shorten due to the inability of DNA polymerase to preserve the length of the 3′ overhang, which is known as the end-replication problem (Fragkiadaki et al., 2020). Telomere length is maintained by telomerase, which is an enzyme responsible for adding the 5′-TTAGGG-3′ tandem repeats to the ends of chromosomes, and is mainly active in germ cells, stem cells, and immortalized cells (Martens and Nawrot, 2016). Both mtDNA content and telomere length can be affected by various lifestyle factors and are considered to be biomarkers of biological aging. Accumulating evidence linked these biomarkers to age-related diseases (Chistiakov et al., 2014; Sahin and DePinho, 2012; Seo et al., 2010). Exposure to environmental pollutants, like glyphosate and AMPA, may be associated with the onset of age-related diseases (Vriens et al., 2019). Therefore, in this study we investigated the association between glyphosate and AMPA exposure and markers of biological aging in a study population of the Flemish Environment and Health Study (FLEHS).

2. Materials and methods

2.1. Study population

This study was part of the third cycle of the FLEHS, which collects data on human environmental exposures as well as human biological samples in Flanders, Belgium. Inclusion criteria were: i) age between 50 and 65 years, ii) living at least 10 years in Flanders, iii) not having active cancer treatment or renal pathologies, and iv) being able to fill out an extensive questionnaire in Dutch. In total, 209 participants were included in this study. More details of the recruiting procedure are available in the supplemental information. After excluding 28 subjects due to missing data (telomere length: $n = 11$, mtDNA content: $n = 9$, AMPA concentration: $n = 2$, other covariates: $n = 6$), data were analysed for 181 adults.

The medical ethical committee of the University Hospital of Antwerp and the University of Antwerp approved the study (Belgian registration number: B300201419834). Informed consent was obtained from each individual for study participation. This study has been carried out according to the Helsinki declaration.

2.2. Data and sample collection

Data obtained by self-administered questionnaires included information on lifestyle factors and socioeconomic status. Smoking status was defined as never smoked, former smoker or current smoker and alcohol consumption as never, less than monthly, less than weekly or weekly consumption. Socioeconomic status was based on the highest household educational level, coded low (maximum lower grade of secondary school), middle (secondary school) or high (college or university). BMI was calculated as kg/m^2. To correct for urinary concentration, glyphosate and AMPA concentrations were normalized to the urine specific gravity using the following formula: exposure marker* [(1.024–1)/(urine specific gravity – 1)]. Urine samples were collected in metal-free polyethylene containers. Blood samples were collected in EDTA Vacutainer Blood Collection Tubes and immediately centrifuged for serum collection. Samples were stored within 24 h at −80 °C until further use.

2.3. Handling of measurements below quantitation limits

Exposure measurements with observations below the lower limit of quantitation (LLOQ) were treated with Censored Likelihood Multiple Imputation by using the *lodi* package in R. A range of 20–100 imputations was suggested, since a high number of imputations may cause simulation error (van Buuren, 2018). Therefore, we opted to carry out 20 imputations. In addition, to test the robustness of our results, the AMPA concentration was 15% trimmed to account for the measurements below the LLOQ, according to the Guidelines for Data Quality Assessment (version QA00) of the Environmental Protection Agency (EPA). Also, both single imputation by fixed value (i.e., LLOQ/2) and random imputation using values between 0 and LLOQ were investigated. Glyphosate and AMPA concentrations were also investigated as binary variables, coded 0 when below the LLOQ and 1 when above the LLOQ.

2.4. Measurement of mitochondrial DNA content and telomere length

Mitochondrial DNA (mtDNA) content (Janssen et al., 2012) and telomere length (Martens et al., 2016) were measured as described elsewhere. Briefly, DNA was isolated from the buffy coat, containing leukocytes, using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The relative mtDNA content was measured by determining the ratio of two mitochondrial gene copy numbers (MT-ND1 and MTF3212/R3319) to a single copy nuclear control gene (RPLP0). Relative telomere length was determined by the ratio of the telomere sequence to the RPLP0 control gene, proportional to the mean telomere length of the study population. Both mtDNA content and telomere length were measured using the 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). The full protocol is available in the supplemental information.

2.5. Measurement of glyphosate and AMPA

Samples were shipped on dry ice and stored at −18 °C until further use. Urinary glyphosate and AMPA concentrations were measured according to the procedure described elsewhere (Alferness and Iwata, 1994) with some modifications (Hoppe, 2013). Briefly, 100 μL urine and 50 μL of the working solution of the internal standard were transferred to screw-capped glass tubes containing 1 mL of acetonitrile. After evaporation to dryness in a vacuum centrifuge for derivatization, 0.5 mL of 2,2,2-trifluoroethanol and 1 mL of freezing cold (−40 °C) trifluoroacetic anhydride were added to the residue. The mixture was heated to 85 °C for 1 h. After cooling, the solution was cautiously evaporated at 80–85 °C without a stream of air or nitrogen. After cooling, the oily residue was dissolved in 200 μL of acetonitrile and measured by Gas Chromatography-Tandem Mass Spectrometry (GC-MS-MS). The urine samples were distributed across 11 runs. Each run included seven calibration standards, a blank and a calibration verification at 1 μg/L. Two quality control samples with known glyphosate/AMPA spiked concentration and a real-life urine sample of an exposed person were included. The LLOQ for both glyphosate and AMPA was based on the lowest calibration standard of 0.1 μg/L. Details of GC-MS-MS analysis and method validation were reported elsewhere (Conrad et al., 2017). With the presented method, the contracted laboratory successfully participated in the recent three rounds of the German External Quality Assessment Scheme (G-EQUAS) for glyphosate measurements in urine. The target levels of the control material ranged from 0.42 to 3.73 μg/L and the analysed concentrations deviated from 1.1 to 7.1%, confirming the accuracy of the method. Reference values can be obtained at https://app.g-equas.de/web/info (G-EQUAS 64–66). Currently, G-EQUAS only includes glyphosate, not AMPA.

2.6. Statistical analysis

Data management and statistical analysis were done using RStudio software (version 1.1.463), R version 3.6.3. Glyphosate and AMPA concentration, as well as telomere length and mtDNA content were log10 transformed to normalize their distribution. The association between glyphosate and AMPA in urine and markers of biological aging (i.e., mtDNA content and telomere length) was explored using multiple linear regression models adjusted for the following *a priori* selected covariates: sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season of sampling, and urine specific gravity. Models exploring the association between glyphosate and AMPA exposure and mtDNA content were additionally adjusted for platelet count (amount/μL). Since DNA was extracted from leukocytes, we additionally adjusted for leukocyte count (amount/μL) in sensitivity analyses. Glyphosate exposure has been linked with chronic kidney disease, hence the additional adjustment for cystatin C (mg/L) and alfa-1-microglobulin (mg/L) in sensitivity analyses. For descriptive purposes, continuous variables (i.e., age, BMI, urine specific gravity, mtDNA content, and telomere length) were presented as means ± standard deviation (SD) and categorical variables (i.e., sex, smoking status, alcohol consumption, socioeconomic status, and season) as numbers (frequency in percentage). All reported *p*-values were considered significant when $p < 0.05$. Estimates were provided as the % difference (95% CI) of mtDNA content or telomere length from a doubling in glyphosate or AMPA concentration.

3. Results

3.1. Population characteristics

The 181 participating adults (51.9% women) were 58.2 ± 4.0 (SD) years old and had a mean BMI of 25.5 ± 4.2 kg/m^2. The majority never smoked (45.9%), while 40.9% were former smokers. Most of the participants consumed alcohol on a weekly basis (64.1%) and had a high socioeconomic status based on the highest obtained diploma in the household (55.8%) (Table 1). 57.5% of glyphosate and 41.4% of AMPA measurements were below the LLOQ. The geometric mean for the urinary glyphosate and AMPA concentration was 0.11 μg/L (IQR: 0.05–0.20) and 0.16 μg/L (IQR: 0.08–0.33), respectively (Fig. 1). The raw analytical data for the urinary glyphosate and AMPA concentrations are shown in Table 2. Urinary glyphosate and AMPA concentrations were positively correlated (R = 0.55; $p < 0.0001$), as well as leukocyte mtDNA content and telomere length (R = 0.56; $p < 0.0001$) (Supplementary Fig. 1). The population characteristics of the trimmed population are shown in Supplementary Table 1.

3.2. Association of urinary glyphosate or AMPA concentration and markers of biological aging

After adjustment for sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season of sampling, and urine specific gravity, a doubling in glyphosate concentration was not associated with leukocyte telomere length (p = 0.15), however the urinary concentration of the glyphosate metabolite AMPA was associated with a 5.19% (95% CI: 0.49 to 10.11; p = 0.03) longer leukocyte telomere length (Table 3). No associations were found between AMPA or glyphosate concentrations and mtDNA content.

In sensitivity analyses (Table 4) for the association between AMPA exposure and telomere length, additionally adjusting for leukocyte count and cystatin C and alfa-1-microglobulin did not affect the experimental outcome. Using the trimmed population, single imputation, and random imputation, the association between urinary AMPA concentration and telomere length remained significant (p = 0.01, p = 0.045, p = 0.046 respectively; Supplementary Table 2). Also, when using AMPA concentration as a binary variable, the results remained the same (p = 0.03; Supplementary Table 3).

Table 1
Study population characteristics (n = 181).

Characteristic	Mean ± SD or *n* (%)
Age (years)	58.2 ± 4.0
Sex (female)	94 (51.9)
BMI	25.5 ± 4.2
Smoking status	
Never	83 (45.8)
Former smoker	74 (40.9)
Current smoker	24 (13.3)
Alcohol consumption	
Never	16 (8.8)
< Monthly	34 (18.8)
< Weekly	15 (8.3)
Weekly	116 (64.1)
Socioeconomic status	
Low	38 (21.0)
Middle	42 (23.2)
High	101 (55.8)
Season of sampling	
Winter	-
Spring	36 (19.9)
Summer	69 (38.1)
Autumn	76 (42.0)
Urine specific gravity	1.0 ± 0.01
Leukocyte count	6970 ± 1630

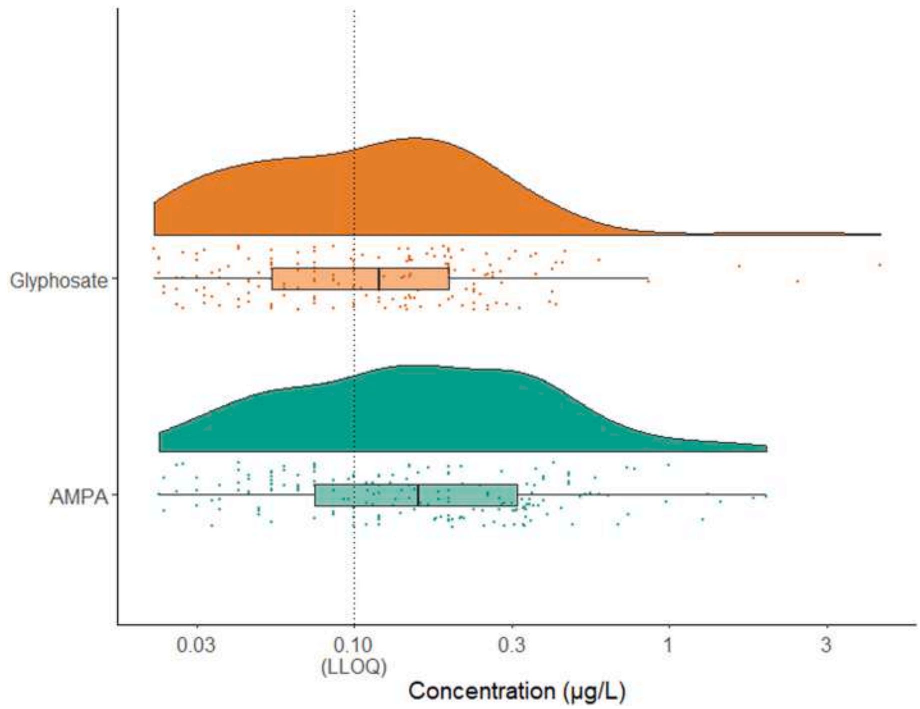

Fig. 1. Raincloud plot for glyphosate and AMPA concentration (adjusted for urine specific gravity) (n = 181). The geometric mean for the urinary glyphosate and AMPA concentration was 0.11 µg/L (IQR: 0.05–0.20) and 0.16 µg/L (IQR: 0.08–0.33), respectively. The x-axis shows log10 transformed values. The dotted line represents the LLOQ.

Table 2
Raw analytical data for the urinary glyphosate and AMPA concentrations. Data are shown for the 50th, 75th, 90th, and 95th percentile, as well as the maximum concentration.

	50th	75th	90th	95th	Max
Glyphosate (µg/L)	<LLOQ	0.13	0.22	0.31	3.72
AMPA (µg/L)	0.10	0.18	0.34	0.40	1.50

Table 3
The association between glyphosate and AMPA exposure and markers of biological aging (n = 181). Estimates were provided as the % difference (95% CI) of telomere length or mtDNA content from a doubling in glyphosate or AMPA concentration. Models were adjusted for sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season, and urine specific gravity. mtDNA content has been additionally adjusted for platelet count.

	% difference (95% CI)	*p*-value
Telomere length		
Glyphosate	3.31 (−1.17 to 8.07)	0.15
AMPA	5.19 (0.49–10.11)	0.03
mtDNA content		
Glyphosate	2.38 (−4.34 to 9.51)	0.50
AMPA	4.32 (−3.07 to 12.19)	0.26

4. Discussion

The key point of our study is that urinary AMPA levels are associated with longer leukocyte telomere length in adults. To our knowledge, the present study is the first to investigate the association between glyphosate and AMPA exposure and biomarkers of aging in a population-based set-up.

Glyphosate and AMPA are persistent in the environment and present in food sources. Remarkably, only 21 human biomonitoring studies measuring glyphosate levels (and even fewer for AMPA levels) in urine have been published up till 2020 (Connolly et al., 2020). In our population-based study, urinary AMPA levels were significantly associated with leukocyte telomere length while this was not the case for urinary glyphosate levels. AMPA is more persistent in the environment than glyphosate, with an environmental half-life time of 151 days, ranging from 76 to 240 days depending on environmental conditions like temperature and soil moisture (Domínguez et al., 2016; Silva et al., 2018). In contrast, the biological half-life time of glyphosate in urine was within 9 h (Connolly et al., 2019; Zoller et al., 2020). The degradation of both glyphosate and AMPA is faster at warm and moist conditions (Bento et al., 2017). In a study investigating the presence of glyphosate and AMPA in agricultural top soils of the European Union, glyphosate and AMPA were present in 21% and 42% of the tested soil samples, respectively (Silva et al., 2018). Notably, it was suggested that the majority of urinary AMPA concentrations in human biomonitoring studies originated from exposure to AMPA itself (i.e., via food and water residues or from degradation of glyphosate in the soil) as opposed to the degradation of glyphosate in the body (Connolly et al., 2020). These findings might explain why we observed an association between telomere length and AMPA and not with glyphosate exposure. In our population, the mean urinary glyphosate and AMPA concentration was 0.11 µg/L and 0.16 µg/L, respectively. In 2013, the German Federal Institute for risk assessment (BfR) evaluated a Europe-wide investigation of glyphosate residues in human urine initiated by the German 'Bund für

Table 4
Sensitivity analysis for the association between AMPA concentration and telomere length (model 1). Estimates were provided as the % difference (95% CI) of telomere length from a doubling in AMPA concentration. Models were adjusted for sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season, and urine specific gravity.

	n	% difference (95% CI)	*p*-value
Additionally adjusted for leukocyte count	181	0.91 (0.06–5.19)	0.047
Additionally adjusted for cystatin C and alfa-1-microglobulin	179	1.82 (0.03–3.67)	0.046

Umwelt und Naturschutz Deutschland' (BUND). They analysed 182 urine samples from 18 European countries to determine glyphosate and AMPA residues (BfR, 2013; Hoppe, 2013). The mean urinary glyphosate concentrations ranged from 0.09 μg/L in Bulgaria, Macedonia, and Switzerland to 0.82 μg/L in Malta, with an overall mean of 0.21 μg/L. The mean urinary AMPA concentration ranged from 0.08 μg/L in Macedonia and Switzerland to 0.40 μg/L in Malta, with an overall mean of 0.18 μg/L. Their results indicated that there are large regional and individual differences. Compared with their findings, the urinary concentrations for both glyphosate and AMPA were below average in our population. In a retrospective analysis from 2001 to 2015 analysing 399 urine samples, 31.8% and 40.1% of the samples contained glyphosate and AMPA concentrations at or above the LLOQ, respectively. Both the highest measured concentrations for urinary glyphosate and AMPA were observed in 2013: 2.80 μg/L and 1.88 μg/L, respectively (Conrad et al., 2017). Up to 2020, the highest reported urinary concentration of non-occupational exposure to glyphosate was 9.4 μg/L (Curwin et al., 2007). In addition, occupational exposure to glyphosate showed a less than 10-fold increase in urinary glyphosate concentrations, with an average range from 1.35 μg/L to 3.2 μg/L (Connolly et al., 2020). In occupationally exposed workers in China, the maximum concentration of urinary glyphosate was 17.202 mg/L, while the highest measured urinary AMPA concentration was 2.73 mg/L (Zhang et al., 2020). .

Longer telomere length should provide prolonged cell survival, which in turn increases the chance of accumulation of cancer-causing mutations (Aviv et al., 2017; Noy, 2009; The Telomeres Mendelian Randomization, 2017). Several studies described the association between longer telomere length and an increased risk of different cancers such as non-Hodgkin lymphoma (Lan et al., 2009), lung cancer (Seow et al., 2014), and melanoma (Han et al., 2009). In addition, other studies reported an increase in telomere length in relation to an environmental exposure: i) the study of the National Health and Nutrition Examination Survey (NHANES) reported an age-independent association between exposures to polychlorinated biphenyls (PCBs) and longer leukocyte telomere length (Scinicariello and Buser, 2015), ii) another study (Shin et al., 2010) described higher leukocyte telomere length after exposure to low doses of persistent organic pollutants (POPs), and iii) urinary antimony and mercury exposure were positively associated with leukocyte telomere length (Vriens et al., 2019). Similar to glyphosate and AMPA, these chemicals are persistent in the environment and present in food sources. The Agricultural Health Study (AHS), a large prospective adult cohort study in the USA, published several studies investigating the association between glyphosate exposure and the risk of cancer. They found a relative risk of 2.6 for the development of multiple myeloma after exposure to glyphosate (De Roos et al., 2005). Another study (Flower et al., 2004) reported that the risk for cancer development was increased in children of glyphosate applicators, compared with children of the general population. However, the IARC (Humans, 2017) noted that both studies had limited power due to missing data on covariates or to study rare diseases like childhood cancer. In addition, case-control studies in Sweden (Eriksson et al., 2008), the USA (De Roos et al., 2003), and Canada (McDuffie et al., 2001) demonstrated an increased risk for the development of non-Hodgkin lymphoma. Using a mice model, a positive trend in the incidence of renal tubule carcinoma and hemangiosarcoma in male mice was reported following glyphosate exposure (Guyton et al., 2015; Humans, 2017). An almost twofold increase in the incidence of mammary gland tumours in female rats who received glyphosate-based formulations through drinking-water was found, compared to control animals (Séralini et al., 2014). Since there was limited evidence in human studies and sufficient evidence in animal studies, the International Agency for Research on Cancer (IARC) classified glyphosate as probably carcinogenic to humans (Group 2A) (Humans, 2017). As enabling replicative immortality in cells is a hallmark of cancer development (Hanahan and Weinberg, 2011), our results suggest a possible underlying mechanism for tumour development related to AMPA exposure. Based on our findings, further investigating the association between telomeres and AMPA exposure in large cohorts is warranted. In addition, *in vitro* studies could further unravel the telomere-related mechanisms through which glyphosate and AMPA exert their potential carcinogenic effects.

Inflammation, adjusted detoxification mechanisms or oxidative stress are possible underlying mechanisms not only linked to telomere biology (Aubert and Lansdorp, 2008; Martens and Nawrot, 2016) but also to glyphosate exposure. Firstly, pollutants may induce acute inflammation (Pandey et al., 2019) in which immune cells proliferate rapidly (Dioni et al., 2011). Both naïve and memory B cells were reported to be capable of upregulating telomerase activity *in vitro* in response to activation signals (Weng et al., 1997). Thus leukocyte telomere length may not only be altered with aging, but also as a consequence of the activation and differentiation of immune cells (Hodes et al., 2002). Several studies using rodent models, investigated the effect of glyphosate exposure on the inflammation process. Another study (Kumar et al., 2014) demonstrated airway inflammation in mice after exposure to both farm air samples containing glyphosate and glyphosate alone. A dose-dependent adverse inflammatory effect after short-term exposure with Roundup® was reported in liver and adipose tissues of rats (Pandey et al., 2019). Using pregnant rats, changes in the expression of genes related to oxidative stress and inflammation after perinatal glyphosate-based herbicide exposure were reported (de Souza et al., 2019). Secondly, several studies described that glyphosate suppressed the activity of various cytochrome P450 (CYP450) enzymes (Samsel and Seneff, 2013). These enzymes are involved in the detoxification of xenobiotics and can produce carcinogenic metabolites (Pande et al., 2008). Another study (Eshkoor et al., 2013) reported that the *CYP1A2* gene polymorphism generated ROS, which causes DNA damage and carcinogenesis in cells. Thirdly, oxidative stress induced by glyphosate exposure in various tissues of rats has been broadly described (Cattani et al., 2014; Larsen et al., 2012; Turkmen et al., 2019). Glyphosate induced an increase in lipid peroxidation in pregnant rats (Beuret et al., 2005), as well as decreased glutathione levels in rats (Cattani et al., 2014) and enhanced H_2O_2 production in *C. elegans* (Bailey et al., 2018).

Recently, multiple studies investigated the toxicity of glyphosate using different human cell lines, both in healthy and tumour cells (Koller et al., 2012; Li et al., 2013; Mesnage et al., 2013). Specifically stem cells have been used for toxicity testing, for understanding cell proliferation and differentiation, and for investigating their role in the aging process (Kang and Trosko, 2010). Notably, one of the mechanisms responsible for cellular effects might be stem cell dysfunction due to telomere interference (Fragkiadaki et al., 2020; Ganguly et al., 2017).

This study has the following strengths and limitations. Our population-based study was part of FLEHS III and thus is representative for the middle-aged population living in Flanders. Missing data due to observations <LLOQ is a common obstacle in environmental epidemiological studies, by which our study was affected as AMPA had 41.4% and glyphosate 57.5% measurements <LLOQ. The IQR for both glyphosate and AMPA concentration is going below the LLOQ. We therefore performed four steps to treat the imputation of these observations. First, we followed the Guidelines for Data Quality Assessment (version QA00) of the Environmental Protection Agency (EPA). For AMPA we used 'trimmed mean', as 15% trimming may be a good estimator of the population mean in environmental data. Therefore, we trimmed 15% in the tails of our observations in the sensitivity analysis. In addition, we also performed a sensitivity analysis classifying the observations as detect/nondetect (i.e., binary). Both sensitivity analyses did not influence our results. Secondly, we performed a single imputation by replacement of fixed values (i.e., LLOQ/2) and found a significant association between AMPA exposure and telomere length. Thirdly, we applied a random imputation method using values between 0 and LLOQ (Bernhardt et al., 2015; HBM4EU, 2019; Pleil, 2016). This resulted in similar estimates and p-values. Lastly, we performed censored likelihood

multiple imputation. The multiple imputation method has an advantage over the previous methods, as it provides a variance/confidence interval that can better understand the differences between different imputations (Den Hond et al., 2015). Trimming, classification, single, random, and multiple imputation performed similarly with respect to the significance of the models and the estimation in general, indicating that the association between AMPA exposure and telomere length is robust. For investigating the association of glyphosate and AMPA exposure, leukocyte telomere length was used. Although these telomeres are shorter compared with other tissues due to their high replication capacity, telomere length within individuals in different tissues were highly correlated (Friedrich et al., 2000; Martens and Nawrot, 2016). To correct for changes in the leukocyte cell proportions, we additionally adjusted our models for leukocyte count. Recently more cases of chronic kidney disease have been reported in agricultural communities. This has been linked with occupational exposure to glyphosate among Sri Lankan farmers (Gunarathna et al., 2018; Jayasumana et al., 2015). However, in our study, additionally adjusting for cystatin C and alpha-1-microglobulin, indicators for renal function (Murty et al., 2013; Penders and Delanghe, 2004), did not affect our results.

5. Conclusion

Here, we found that urinary AMPA concentrations are associated with longer leukocyte telomere length in adults. To further understand the exact mechanism of glyphosate and AMPA toxicity on telomere length, more epidemiological as well as *in vitro* studies are required.

Author contributions

The FLEHS study was carried out by the Flemish Centre for Expertise on Environment and Health, in which TSN, WB, LB, EDH, VN, NVL, DC and GS have a coordinating role. TSN, MP, and CC designed the research hypothesis. DM and BGJ performed the mtDNA and telomere experiments. KS and EW critically revised the manuscript. CC analysed the data and interpreted the results. CC and MP drafted the article. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113895.

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Health-related economic benefits of universal access to piped water in Arctic communities: Estimates for the Yukon-Kuskokwim Delta region of Alaska

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ABSTRACT

This paper presents estimates of the potential health-related economic benefits of providing universal access to in-home water and sanitation services to households in rural Alaska. In particular, we use data on disease incidence rates, health care costs, and local estimates of the impact of piped water on disease reduction to estimate the potential health-related economic benefits of providing universal access to piped water in the Yukon Kuskokwim (Y.K.) Delta region of Alaska. We include estimates of avoided treatment and diagnosis costs as well as private benefits associated with reduced morbidity and mortality associated with improved access to in-home piped water. To our knowledge, these are the first estimates of the economic benefits of improved access to water and sanitation in rural Alaska and the Arctic. Our analysis suggests increased access to in-home piped water in the region may yield substantial reductions in direct medical expenses incurred by public agencies and families, as well as reductions in time and travel costs associated with improved health outcomes. These benefits, along with the array of health and non-health-related benefits not included in our analysis, may provide new impetus to expanding access to high-quality water and sanitation services in the region.

1. Introduction

With the adoption of the Sustainable Development Goals (SDGs), the international community has committed to the ambitious goal of achieving universal access to safe and affordable drinking water for all by 2030 (U.N. General Assembly, 2015). Unlike previous global initiatives to improve human well-being, the SDGs' focus on universal access brings the experience of marginalized populations in high-income countries into global focus. Recent global experience with COVID-19 has also underscored the importance of ensuring universal access to water, sanitation, and hygiene to combat the spread of infectious diseases. Though often not included in national or global discourse on water and sanitation, many Arctic and sub-Artic communities lack access to in-home water and sanitation services (Eichelberger, 2010, 2016, 2018; Daley et al., 2014; Sarkar et al., 2015; Berner et al., 2016; Thomas et al., 2016; Wilson et al., 2019). This is also true for communities in rural Alaska, where only 85% of households have in-home piped water and sanitation services.[1]

Globally, it can be difficult to make an economic case for providing rural, low-income communities access to high-quality water and sanitation services. These communities are often dispersed, expensive to serve, and have limited financial resources to pay for piped services.

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Similar conditions exist in rural Alaska. Indeed, communities in rural Alaska are particularly expensive to serve given their remote locations and extreme climate and weather conditions. In 2012, the capital costs to construct piped water systems for unserved communities in Alaska was estimated to be $300 million (Thomas et al., 2016a). Unlike communities without access to in-home water and sanitation services in many low- and middle-income countries, many households in rural Alaska have access to high-quality healthcare through the Alaska Tribal Health System. Thus, while households may have a limited ability to pay for piped water and sanitation services, there may be considerable public benefits associated with avoided healthcare costs.

This paper presents estimates of the potential health-related economic benefits of providing universal access to in-home water and sanitation services to households in rural Alaska. These estimates are important for beginning to assess the economic rationale for improving access to water and sanitation services in the region. We use novel, region-specific data on disease incidence rates, health care costs, and local estimates of the impact of piped water on disease reduction to estimate the potential health-related economic benefits of providing universal access to piped water in the Yukon Kuskokwim (Y.K.) Delta region of Alaska. We include estimates of avoided treatment and diagnosis costs as well as private benefits associated with reduced morbidity associated with improved access to in-home piped water. We also estimate the economic benefits associated with potential reductions in mortality associated with increased access to in-home piped water. To our knowledge, these are the first estimates of the economic benefits of improved access to water and sanitation in rural Alaska and the Arctic.

2. Background and literature

Over 99% of households in the United States have complete plumbing[2] in their home. However, according to the most recent census, an estimated 1.5 million individuals lack access to piped water and sanitation services (U.S. Census Bureau, 2016). American Indian and Alaska Native people represent the largest demographic of households without access to complete plumbing (Gasteyer et al., 2016; Deitz and Meehan, 2019). Alaska ranks last among states with respect to the fraction of the population without access to complete plumbing.

The Y.K. Delta region is located in the western part of Alaska and covers approximately 75,000 square miles (Fig. 1). Approximately 56% of households in the region had access to piped water in 2018, though only 48% had an active account with a local service provider. Nine percent of households had active accounts for "hauled service," in which water is delivered by truck or small vehicle to a storage tank in the house. Households considered unserved by piped or hauled service typically use five-gallon buckets (i.e., honey buckets) as toilets that they empty manually and "self-haul" water from a central community water point or natural source. Many self-haul communities in the region have a central facility for showers and laundry (i.e., washeteria). However, these facilities are not always available when residents need them and are unaffordable for some households.

Recent research in Alaska suggests that households with inadequate access to water and sanitation services face higher rates of diarrhea, pneumonia, respiratory syncytial virus (RSV), methicillin-resistant staphylococcus aureus (MRSA), and infant hospitalization than households with in-home water and sanitation services (Gessner, 2008; Hennessy et al., 2008; Thomas et al., 2016; Singleton et al., 2018). In addition to these health-related effects, households without in-home water and sanitation services must invest time hauling water, disposing of their waste, and coping with the stress of uncertain access and quality of water sources (Eichelberger, 2010, 2016, 2019).

These health disparities likely contribute to significant healthcare costs, both public and private, particularly in remote communities. More than 60,000 people live in remote communities in Alaska that are accessible only by plane year-round or boat or snowmobile in the summer and winter, respectively. So-called "hub" communities, such as Bethel in the YKHC service region (Fig. 1), have larger populations than smaller surrounding communities and serve as the centers for health care and transportation services in their respective region (Sherry, 2004).

Community clinics are usually the first point of health services for residents of smaller communities, either through in-person visits with doctors and health aides or telemedicine services. Patients may then be referred to either the larger health center in the hub community or the Alaska Native Medical Center (ANMC) in Anchorage, which provides specialty care, surgeries and hospital care for all eligible Alaska Native/American Indian patients in Alaska. The remoteness of both hubs and communities necessitates air travel to access many health services. Often, a family member accompanies patients – particularly if the patient is a minor. The costs of travel (which include airfare, food, and lodging) may be borne by the individual, Medicare, or in some instances, the tribal government.

Evaluations have not been conducted to identify the causal impact of in-home water and sanitation services on improving health and non-health-related outcomes for households in rural Alaskan communities. Nevertheless, comparisons of communities with and without access to in-home water and sanitation services and before and after data from pilot interventions suggest that in-home water and sanitation services can yield health improvements. For example, using health data from 2000 to 2004, Hennessy et al. (2008) show that hospitalization rates for pneumonia, RSV, and skin infections were higher in regions where households lacked access to high-quality water and sanitation services. Similarly, from 2007 to 2013 a study of the impact of the installation of piped water in four communities in southwest Alaska showed a 38% reduction in clinic visits per year associated with gastrointestinal illness, a 16% decrease in clinic visits per year for respiratory illness, and a 20% reduction in clinic visits for skin infections (Thomas et al., 2016). Reductions in respiratory illness were also documented by Gessner (2008), who found an association with access to piped water and sanitation services and decreased incidence of pediatric lower respiratory tract infections. More recently, Mosites et al. (2020a) used clinic and hospital records data to document that increased access to piped water was associated with reductions in MRSA, pneumonia, RSV, other respiratory illnesses, and non-MRSA skin infections.

3. Materials and methods

Water and sanitation interventions can yield a range of health and non-health-related benefits. Health-related benefits associated with improved access to high-quality water and sanitation services include reductions in morbidity and mortality associated with waterborne and water-washed diseases[3] and improvements in dietary choices and mental health associated with improved water security. Public benefits associated with reductions in morbidity include reduced treatment and diagnosis costs incurred by the public sector. Private benefits associated with reduced morbidity include avoided privately-borne treatment and diagnosis costs, lost time and productivity of individuals and caregivers, acute and chronic pain and suffering. Non-health related private benefits associated with water and sanitation interventions include reduced coping costs (i.e., costs associated with the collection, storage, and

[2] The U.S. Census Bureau defines complete plumbing as the presence of hot and cold running water, a flush toilet, and an indoor bathtub or shower (Deitz and Meehan, 2019). Households with complete plumbing according to the U.S. Census do not necessarily have access to safe and affordable water and sanitation services.

[3] Waterborne diseases are those that are transmitted through contaminated drinking water. Water-washed diseases are infections caused by poor personal hygiene resulting from inadequate water availability.

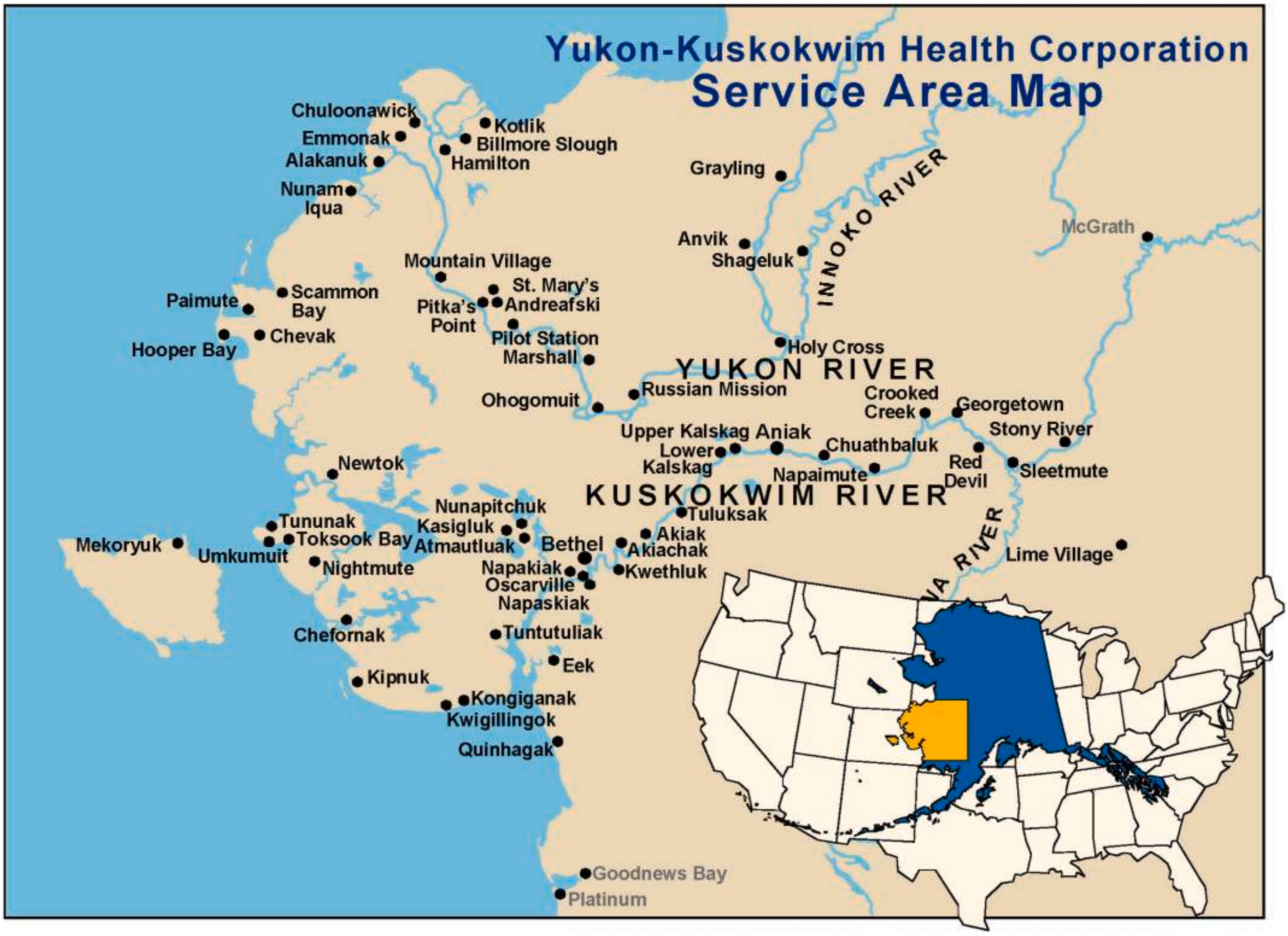

Fig. 1. Map of the Yukon-Kuskokwim Delta region and Yukon-Kuskokwim Health Corporation service area.

treatment of water), aesthetic improvements (e.g., improved social standing and convenience), and improved school attendance among children (Whittington et al., 2009, 2012; Whittington and Pattanayak, 2015).

This study estimates the potential health-related benefits of providing universal access to piped water in 48 communities in the Y.K. Delta region of Alaska. In particular, we estimate the health-related economic benefits associated with transitioning communities from their current level of access to water and sanitation services to universal access. We focus specifically on the health-related economic benefits associated with the reduction in waterborne and water-washed diseases. We do not attempt to estimate the health-related benefits associated with improved dietary choices or mental health, nor the non-health-related benefits associated with improved water and sanitation services because the data required to estimate these benefits in the region credibly were not available.

3.1. Modeling approach

The economic benefits associated with reduced morbidity are estimated by determining the avoided cost of illness associated with waterborne and water-washed disease incidence. This includes both avoided treatment and diagnosis costs as well as avoided travel and time costs for patients and caregivers. Following Mosites et al. (2020a), which examines the health impacts of improved access to water and sanitation services in the Y.K. Delta region, we examine the potential benefits associated with reductions in diarrhea, pneumonia, RSV, MRSA, other respiratory infections, and other skin infections.

The model begins by estimating the number of cases of each disease avoided using Equation (1).

$$\Delta Cases_{jk,t} = Pop_{j,t} * Inc_{jk,t} * \Delta Cov^{water}_{j,t} * \varepsilon^{water}_{k} \qquad \text{Eq. 1}$$

Where $\Delta Cases_{jk,t}$ is the annual number of cases of disease k avoided in community j at time t, $Pop_{j,t}$ is the population in community j at time t, $Inc_{jk,t}$ is the baseline incidence of disease k in community j at time t, $\Delta Cov^{water}_{j,t}$ is the percent change in the number of households with access to piped water in community j from time t-1 to t, and ε^{water}_{k} is the elasticity of disease reduction of access to piped water for disease k (i.e., the effectiveness of increased access to piped water in reducing disease k).

We focus specifically on changes in access to piped water for three reasons. First, households with access to piped water in the Y.K. Delta region typically have complete plumbing and sanitation service that separates human waste from individuals (e.g., piped sewer, septic tank, or hauled sewerage service). Second, credible estimates for the independent impact of access to sanitation do not exist for Alaska. Third, Mosites et al. (2020b) found that increased access to vehicle hauled water service was associated with only small reductions in two of the six disease categories considered (skin and respiratory infections).

Cases of waterborne and water-washed illnesses can have varying levels of severity and require different types of medical care. The model considers the avoided treatment and diagnosis costs associated with outpatient visits, inpatient visits, and patients transferred to the Alaska Native Medical Center (ANMC) in Anchorage, AK for medical care for each of the illnesses considered. Equation (2) describes how treatment and diagnosis costs are calculated in the model.

$$\Delta C^{TD}_{morb,t} = \sum_{jk}\left[\Delta Cases_{jk,t} * \left(f^{out}_k * TD^{out}_k + f^{in}_k * TD^{in}_k + f^{trans}_k * TD^{trans}_k\right)\right] \quad 2$$

Where $\Delta C^{TD}_{morb,t}$ is the annual avoided treatment and diagnosis costs in the study region at time t, $\Delta Cases_{jk,t}$ is defined as in Equation (1), f^{out}_k is the fraction of cases of disease k that require outpatient treatment, and TD^{out}_k are the treatment and diagnosis costs associated with outpatient care for disease k. f^{in}_k, TD^{in}_k, f^{trans}_k, TD^{trans}_k are analogously defined for inpatient and transfer patients, respectively.

When an individual contracts a waterborne or water-washed illness, they may go to a local health clinic to obtain medical care, may need to travel to obtain inpatient care or may need to be transferred via medical evacuation for more advanced treatment. A family member often accompanies patients who travel for medical care. Equation (3) describes how travel costs are calculated in the model.

$$\Delta C^{trav}_{morb,t} = \sum_{jk}\left[\Delta Cases_{jk,t} * \left(f^{out}_k * Trav^{out}_k + f^{in}_k * Trav^{in}_k + f^{trans}_k * Trav^{trans}_k\right)\right] \quad 3$$

Where $\Delta C^{trav}_{morb,t}$ is the annual avoided travel costs associated with improved access to water piped water, the f_k terms are defined as in Equation (2), and the $Trav_k$ terms are the travel costs associated with outpatient, inpatient, and transfer medical care for disease k, respectively. The travel costs associated with each level of medical care are a function of the number of caregivers, type and mode of travel (e.g., local travel, commercial plane, medical evacuation flight), duration of stay for inpatient and transfer medical care for each type of disease, and meals and lodging costs for caregivers who accompany patients for care received over several days.

In addition to the public and private costs associated with this travel, there is an economic cost associated with the time sick individuals and their caregivers spend seeking and obtaining medical treatment. We use the economic concept of the shadow value of time to estimate the avoided time losses associated with improved access to water (Equation (4)).

$$\Delta C^{time}_{morb,t} = \sum_{jk}\left[\Delta Cases_{jk,t} * \mathrm{T}_{j,t} * \left(f^{out}_k * Time^{out}_k + f^{in}_k * Time^{in}_k + f^{trans}_k * Time^{trans}_k\right)\right] \quad 4$$

Where $\Delta C^{time}_{morb,t}$ is the economic value of the annual avoided time losses in year t, $\mathrm{T}_{j,t}$ is the shadow value of time in community j at time t, the f_k terms are defined as in Equation (3), and the $Time_k$ terms represent the amount of time sick individuals and their caregivers spend seeking and obtaining care for outpatient, inpatient, and transfer medical care for disease k. Using a standard benefit-cost approach, the shadow value of time is assumed to be a fraction of the local wage rate. The specific assumptions used to estimate the shadow value of time for each community are described below.

The annual health-related benefits of morbidity reduction are calculated by summing the avoided treatment and diagnosis costs ($\Delta C^{TD}_{morb,t}$) from Equation (2), the avoided travel costs ($\Delta C^{trav}_{morb,t}$) from Equation (3), and the economic value of the avoided time losses ($\Delta C^{trav}_{morb,t}$) from Equation (4).

In addition to health-related benefits associated with reduced morbidity, water and sanitation interventions can lead to reductions in mortality associated with waterborne and water-washed disease. We examine this as an extension to our main modeling results. The potential benefits associated with mortality reduction are estimated using the economic concept of the value of statistical life (VSL). The VSL is a measure of how individuals value small changes in mortality risk. Estimates of the VSL are typically obtained from studies that examine how large groups of individuals make trade-offs between income (wages) and mortality risk. In this study, we use estimates of the VSL recommended by the United States federal government to estimate the benefits associated with reductions in mortality (See Section 4 for more detail). Equation (5) describes how the model estimates the economic benefits associated with mortality reduction.

$$\Delta C_{mort,t} = \sum_{jk}\left[\Delta Cases_{jk,t} * \mathrm{CFR}_k * VSL_t\right] \quad 5$$

Where $\Delta C_{mort,t}$ are the economic benefits of avoided mortality in year t, $\Delta Cases_{jk,t}$ is defined as in Equation (1), CFR_k is the case fatality rate for disease k, and VSL_t is the value of statistical life at time t.

There is considerable variation and uncertainty in several of the parameters included in the model, including the disease incidence rates, treatment and diagnosis costs, the impact of piped water on improved health outcomes, and case fatality rates for the diseases in the Y.K. Delta region. As a result, we conduct probabilistic sensitivity analysis using Monte Carlo simulations to examine the sensitivity of our results to modeling assumptions. In particular, we assign a distribution to parameters in the model and use 10,000 draws of parameter values to estimate the range of potential benefits associated with universal access to piped water in the study region. Table S1 provided in Supplemental Information presents information on the distributions and parameter values used in the Monte Carlo simulations.

3.2. Data and assumptions

This analysis considers 48 communities in the Y.K. Delta region of Alaska. This study received ethical approval from the Alaska Area Institutional Review Board (project number 1203772–3). Additional approvals and privacy consultations were obtained from the Alaska Native Tribal Health Consortium (ANTHC), Southcentral Foundation (SCF), and the Yukon-Kuskokwim Health Corporation (YKHC).

Community-level population and socio-economic data were obtained from the 2016 American Community Survey of the United States Census (Table S2 provided in Supplemental Information). The YKHC Office of Environmental Health and Engineering provided community-level data on water and sanitation service coverage. These data indicate the percent of households in each community billed for piped or hauled water services from 2013 to 2015. (See Supplemental Information for additional information on the data and assumptions used in the model.)

Households in communities in the Y.K. Delta region have varying degrees of access to piped water service. The benefits of expanding access to piped water service in the region would be overstated if we assumed that communities went from no access to piped water service to full (100%) access. Thus, the model estimates the economic benefits of bringing communities from their current level of access to piped water service to 100% access.

Following Mosites et al. (2020a), disease categorizations and years of interest were defined to be consistent with previous work in rural Alaska (Hennessy et al., 2008). Data on cases of each disease and disease category were obtained from the YKHC electronic health record system, Records and Verification Electronic Network (RAVEN). The de-identified dataset included information on visit date, residence location, and diagnosis code for all inpatient and outpatient visits using specified International Classification of Diseases, 9th and 10th Revisions (ICD-9/10) codes to capture respiratory, skin, and gastrointestinal infections of interest for the years 2013, 2014, and 2015. In the base case of the model, we assume that baseline disease incidence rates are the same in each community (Table 1). Our sensitivity analysis considers incidence rates that vary by the percent of the population in each community with piped water service.

Values for the elasticity of disease reduction of access to piped water (ε^{water}_k in Equation (1)) for the diseases considered were obtained from Mosites et al. (2020a), which examined the association between access to piped water and disease reduction in rural Alaska (Table 1). The data

Table 1
Summary of assumptions related to disease incidence, effectiveness of piped water service, treatment and diagnosis costs and mortality risk reduction.

Parameter	Units	MRSA[a]	Pneumonia/ influenza	Respiratory infection (other)	RSV[b]	Skin infection (other)
Disease incidence and elasticity of disease reduction						
Disease incidence ($Inc_{jk,t}$)	cases/10,000/year	56	1,314	11,479	14	3,032
Elasticity of disease reduction (ε_k^{water})	% disease reduction/% increase in coverage	−0.8%	−0.4%	−0.2%	−0.6%	−0.4%
Treatment and diagnosis costs						
Outpatient (TD_k^{out})	USD/case	603	566	280	963	366
Inpatient (YKHC)						
Fraction inpatient (f_k^{in})	% of cases	64%	2%	0%	16%	1%
Stay duration	days/case	4.8	4.5	5.1	2.7	7.6
Costs (TD_k^{in})	USD/case	13,077	12,146	10,716	7,546	15,989
Inpatient (ANMC)						
Fraction transferred (f_k^{trans})	% of cases	5%	2%	0%	11%	1%
Stay duration	days/case	8.2	11.8	3.3	6	3.8
Costs (TD_k^{trans})	USD/case	103,029	161,363	38,847	204,061	44,092
Mortality						
Case fatality rates (CFR_k)	deaths/100 cases (%)	15%	9%	0.04%	0.04%	0%
Value of mortality risk reduction (VSL_t)	\$/death avoided	9,400,000	9,400,000	9,400,000	9,400,000	9,400,000

[a] MRSA = Methicillin-resistant staphylococcus aureus.
[b] RSV = Respiratory syncytial virus.

used to identify these associations were from the same years considered in this analysis (the most recent data available) and include the Y.K. Delta region.

Cost of illness studies typically use self-reported medical expenditures to estimate avoided medical expenditures (Alberini and Krupnick, 2000; Poulos et al., 2012). This study uses illness-specific treatment and diagnosis costs from YKHC and ANTHC's medical billing systems. The model considers outpatient, inpatient, and transfer patient care separately. Data on the fraction of outpatient, inpatient, and transfer patients for each disease considered were obtained from YKHC's RAVEN system. Data on treatment and diagnosis costs for inpatient and outpatient care and duration of stay for inpatient care for each disease were also obtained from YKHC's RAVEN system. Data on treatment and diagnosis costs and duration of stay for transfer patients were obtained from Alaska Native Medical Center's electronic medical record system, where patients requiring more advanced care typically receive treatment (Table 1 and Table S3 in Supplemental Information).

Alaska-specific case fatality rates for the diseases considered are not available. Thus, for the modeling extension that considers mortality risk reduction, case fatality rates were obtained from the literature to estimate the number of deaths avoided (Table 1 and Table S1 in Supplemental Information). To value mortality risk reduction, we use the VSL recommended by the United States Environmental Protection Agency (\$9.4 million per death avoided) in our base case analysis (Robinson et al., 2019). We vary this in our sensitivity analysis using values recommended across United States federal agencies.

We assume that the costs of local travel to obtain outpatient care are \$10 per case. Rural health clinics in the Y.K. Delta region do not have the capacity to provide inpatient care. Thus, we assume that patients that require inpatient care must travel to Bethel. We obtained quotes for commercial round trip airfare from each community to Bethel during two periods (summer and winter) to estimate the travel costs associated with inpatient care. In our base case, we assume that round trip airfare associated with inpatient care is \$1000 per person. Transfer patients were assumed to require medical evacuation. The cost of medical evacuations was obtained from YKHC and was assumed to be \$14,000 per trip (Table S1 in Supplementary Information).

Our estimates of travel costs include the cost of meals and lodging for those requiring inpatient and transfer medical care and their caregivers. We use the United States Department of Defense reimbursement rates for lodging and meals in Bethel and Anchorage for inpatient and transfer patient care, respectively. Data on duration of stay for inpatient and transfer patients were obtained from YKHC's RAVEN system and ANMC's electronic medical record system, respectively (Table 1 and Table S1 in Supplemental Information).

The time required to obtain medical care includes travel time as well as time receiving care. Travel time for outpatient care was estimated from the travel times for commercial airfare quotes noted above. Travel time for medical evacuations was assumed to be 1 h. The time spent obtaining care was assumed to be 1 h for outpatient care. Disease and treatment-specific durations of care are summarized in Table S3 in Supplemental Information.

We monetize the value of time associated with time dedicated to obtaining medical care using the shadow value of time. In our base case, we assume that the shadow value of time is 50% of local wages (Whittington and Cook, 2019; Boardman et al., 2018). In our base case, we estimate local hourly wages using the mean earnings per household for each community obtained from the 2016 American Community Survey and assumptions about the number of earning individuals per household, the number of workdays per year, and work hours per day as described in Table S1 in Supplemental Information. Our sensitivity analysis also calculates the shadow value of time using mean income in lieu of mean earnings (Table S1 in Supplemental Information).

4. Results and discussion

The average population for the communities in our sample was 412 (Table 2). Nearly all individuals in the communities included in our analysis are Alaska Native people (Table 2). Communities in our sample had a mean household size of 4.1 and an average mean income of \$47,394 (U.S. Census Bureau, 2016). According to YKHC administrative records, approximately 44% of households had access to piped water service in the region. However, the average fraction of households in each community with complete plumbing and access to piped water was 46% and 38%, respectively.

Mosites et al. (2020a) found that increased access to piped water yielded statistically significant reductions in pneumonia, RSV, other respiratory illnesses, MRSA, and other skin infections. In the study region, illnesses requiring outpatient and inpatient care were higher among children under five than individuals older than five. We estimate that transitioning communities in the Y.K. Delta region from their current level of access to piped water service to full coverage would avoid 4,

Table 2
Socio-economic profile and access to water and sanitation services among 48 communities in the YK Delta region.

	Units	Mean	Minimum	Maximum	Standard Deviation
Population	individuals	412	3	1,194	282
Alaska Native	%	95%	70%	100%	6%
Household size	individual/hh	4.1	2.1	6.0	0.9
Mean household income	$/hh	47,394	32,321	66,658	7,476
Households with complete plumbing[a]	%	46%	0%	87%	28%
Households with piped water service[a]	%	38%	0%	98%	37%

[a] Discrepancies may exist due to different data sources.

619 cases per year of the illnesses considered in this analysis (Table 3). Nearly 70% of the cases avoided were respiratory illnesses (pneumonia, RSV, and other respiratory illness). Aside from MRSA, the vast majority of cases avoided were cases requiring outpatient care. Approximately 65% of MRSA cases avoided were those requiring inpatient care.

Avoided treatment and diagnosis costs constitute the largest share of the economic benefits associated with morbidity reduction. Overall, providing universal access to piped water in the Y.K. Delta would yield an estimated $5.6 million annual reduction in medical treatment and diagnosis costs (Table 3). The fraction of avoided treatment and diagnosis costs associated with outpatient, inpatient, and transfer patient care varies by illness considered. For example, avoided treatment and diagnosis costs associated with outpatient care constitute a large fraction of the total avoided treatment and diagnosis costs for both "respiratory infection (other)" and "skin infection (other)" (Fig. 2). Conversely, for pneumonia and RSV, the majority of morbidity-related benefits are attributable to reductions in treatment and diagnosis costs for patients that must be transferred to ANMC for medical care. Although pneumonia accounts for only 13% of the total cases of illness avoided, reductions in pneumonia account for approximately 50% of the avoided medical treatment and diagnosis costs.

Treatment and diagnosis costs constitute 80% of the estimated economic benefits associated with morbidity reductions that accompany a transition to universal access to in-home piped water service in the Y.K. Delta region (Fig. 3). We estimate that universal access to piped water in the Y.K. Delta region would yield approximately $1.2 million in avoided health-related travel costs per year. As noted above, this includes both transportation costs as well as the cost of meals and lodging for patients and caregivers for those requiring inpatient or transfer patient medical care. The economic value of the time saved by avoided illness is estimated to be approximately $56,000 per year.

As an extension of our primary analysis, we consider the economic benefits associated with potential mortality reductions associated with universal access to in-home piped water service in the study region. The case fatality rates for the illnesses included in our analysis are relatively low. We estimate that providing universal access to piped water in the Y.K. Delta region would result in 62 avoided deaths per year. The majority of avoided deaths were associated with simulated reductions in cases of pneumonia. Using a central value of $9.4 million for the VSL, the economic benefits associated with mortality risk reduction are approximately $586 million per year.

There is considerable variation and uncertainty associated with many of the parameters in the model. Using the distributional assumptions described in the Supplemental Information, Monte Carlo simulations indicate that the 5th and 95th percentiles of cases avoided per year are 3,208 and 6,025, respectively. The 5th and 95th percentiles of the health-related benefits associated with reductions in morbidity are $3,396,653 and $14,419,099 per year, respectively. When mortality reductions are included, the 5th and 95th percentiles of the total health-related economic benefits are $376 million and $1.7 billion per year, respectively (Table S4 in Supplementary Information).

The estimates of the economic benefits associated with mortality reductions are particularly sensitive to assumptions about the magnitude of the value of mortality risk reduction (the VSL), the case fatality rate for pneumonia/influenza, and the impact of piped water on the reduction of pneumonia/influenza cases. These parameters accounted for 46%, 24%, and 20%, respectively, of the variation in the benefits associated with mortality risk reduction in our Monte Carlo simulation.

Fig. 4 highlights the uncertainty in our estimates of avoided treatment and diagnosis costs as well as the economic benefits associated with reduced morbidity accompanying a transition to universal access to in-home piped water in the region. Our estimates of the treatment and diagnosis costs are most sensitive to assumptions about the cost of transfer patient care for pneumonia/influenza and the impact of piped water on reducing pneumonia/influenza cases. In particular, the cost of transfer patient care for influenza accounted for 58% of the variation in total treatment and diagnosis costs in the Monte Carlo simulations.

The results of this study suggest that providing universal access to piped water in the Y.K. Delta region of Alaska may yield considerable

Table 3
Summary of annual health-related economic benefits associated with universal access to piped water in the YK Delta region.

	Units	MRSA[a]	Pneumonia/influenza	Respiratory infection (other)	RSV[b]	Skin infection (other)	TOTAL
Cases Avoided	**no./yr**	**51**	**594**	**2,594**	**9**	**1,370**	**4,619**
Outpatient	*no./yr*	*16*	*567*	*2,587*	*7*	*1,346*	*4,521*
Inpatient	*no./yr*	*32*	*14*	*5*	*2*	*18*	*71*
Transfer	*no./yr*	*3*	*14*	*3*	*1*	*7*	*27*
Treatment & diagnosis costs avoided	**USD/yr**	**$715,773**	**$2,690,611**	**$879,937**	**$239,237**	**$1,079,681**	**$5,605,238**
Outpatient	*USD/yr*	*$9428*	*$320,627*	*$723,547*	*$6,604*	*$492,703*	*$1,552,910*
Inpatient	*USD/yr*	*$423,758*	*$165,900*	*$55,603*	*$11,750*	*$284,856*	*$941,868*
Transfer	*USD/yr*	*$282,588*	*$2,204,084*	*$100,786*	*$220,882*	*$302,121*	*$3,110,460*
Health travel costs avoided	**USD/yr**	**$171,141**	**$522,266**	**$145,851**	**$39,148**	**$286,950**	**$1,165,356**
Outpatient	*USD/yr*	*$313*	*$11,331*	*$51,734*	*$137*	*$26,915*	*$90,430*
Inpatient	*USD/yr*	*$79,070*	*$33,328*	*$12,661*	*$3,800*	*$43,469*	*$172,328*
Transfer	*USD/yr*	*$91,758*	*$477,607*	*$81,456*	*$35,212*	*$216,566*	*$902,598*
Health time costs avoided	**USD/yr**	**$10,810**	**$15,433**	**$13,628**	**$702**	**$15,484**	**$56,056**
Outpatient	*USD/yr*	*$69*	*$2,512*	*$11,469*	*$30*	*$5,967*	*$20,047*
Inpatient	*USD/yr*	*$9,425*	*$3,755*	*$1,592*	*$279*	*$7,836*	*$22,886*
Transfer	*USD/yr*	*$1,315*	*$9,166*	*$568*	*$392*	*$1,682*	*$13,123*
TOTAL MORBIDITY COSTS AVOIDED	USD/yr	**$897,723**	**$3,228,310**	**$1,039,415**	**$279,086**	**$1,382,115**	**$6,826,650**

[a] MRSA = Methicillin-resistant staphylococcus aureus.
[b] RSV = Respiratory syncytial virus.

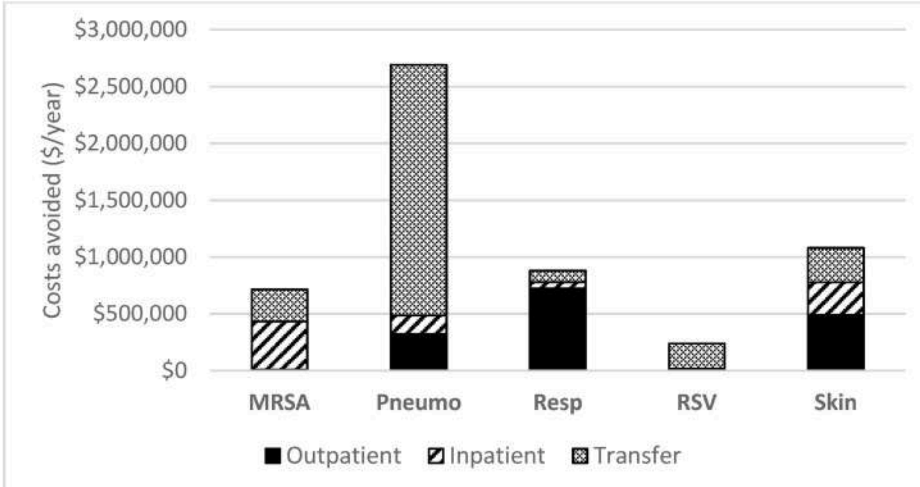

Fig. 2. Summary of treatment and diagnosis costs avoided.

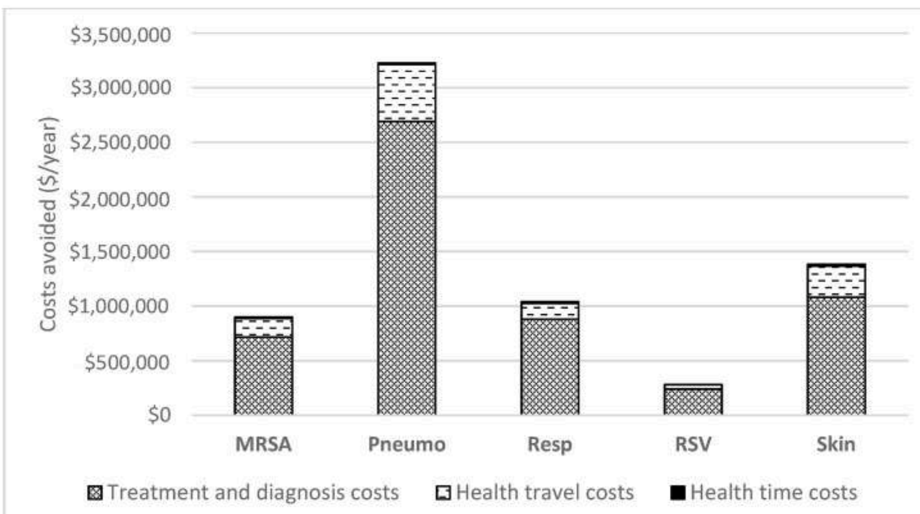

Fig. 3. Summary of total morbidity costs avoided.

health-related economic benefits. In our base case, we find that universal access to piped water in the Y.K. Delta could result in 4,619 avoided cases of illness per year or 41 cases per 100 persons without access to piped water per year. Under our base case assumptions, the health-related economic benefits associated with waterborne and water-washed illness reductions are estimated to be approximately $7 million per year. The majority of these benefits are associated with avoided treatment and diagnosis costs. However, increased access to in-home piped water also yields considerable reductions in health-related time and travel costs. There is considerable uncertainty associated with the potential reductions in mortality associated with expanding access to in-home piped water in the region. However, small reductions in mortality can yield substantial economic benefits. Thus, careful attention should be paid to documenting the extent to which increased access to water and sanitation in the region can reduce mortality.

We caution that the estimates presented in this study represent a lower bound for the economic benefits associated with universal access to water and sanitation in rural Alaskan communities. The estimates presented in this study are conservative because they rely on clinically diagnosed disease and thus likely underestimate the disease incidence and other non-clinical outcomes associated with inadequate access to water and sanitation services in the region. For example, our estimates do not include the impact of increased access to piped water on improved oral health (Atkins et al., 2016; Klejka et al., 2011) nor the health impacts of reduced consumption of sugar-sweetened beverages (Mosites et al., 2020b; Elwan et al., 2016), both of which can have significant impacts on health sequelae over the life course.

Additionally, our estimates only include health-related economic benefits associated with reductions in waterborne and water-washed diseases. They do not include the benefits associated with reduced coping costs, i.e., costs households incur to collect, store, and treat water and dispose of waste. This consists of the physical burden of collecting water, the economic value of time and resources spent collecting and treating water, the capital costs associated with storing water in the home, and time emptying honey buckets. Household-level data on coping costs in rural Alaska do not exist. This is an important area for future research.

Our estimates also do not include potential aesthetic benefits associated with improved access to water and sanitation services (increased social standing, improved smell within homes, reduced fear and dread associated with illness, etc.), nor potential benefits associated with improved school attendance among students. Our estimates also do not include potential improvements in mental health (such as those related to reduced stress and uncertainty of access) that may accompany

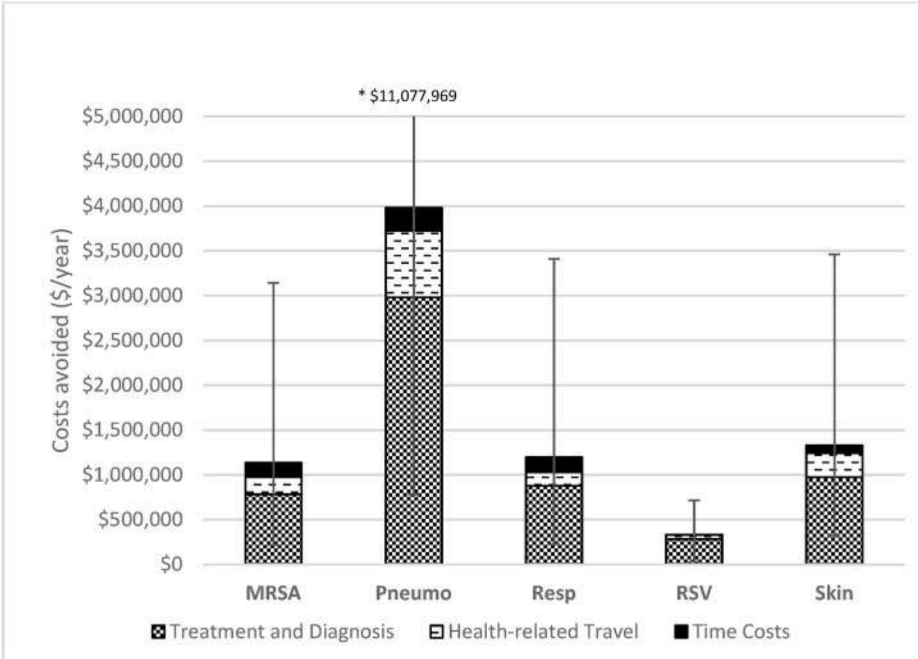

Fig. 4. Mean morbidity cost estimates with 5th and 95th percentiles from the Monte Carlo simulations (10,000 model runs).

improved water security (Eichelberger, 2010, 2016, 2019). Finally, our estimates do not include additional economic benefits to the broader public associated with knowing that Alaska Native people have access to safe water and sanitation services (Carson et al., 2020). Thus, while we document substantial health-related economic benefits associated with providing universal access to water and sanitation in the Y.K. Delta, the total benefits are likely larger than our estimates suggest.

In addition to the caveats above, our analysis has several limitations. First, our estimates are based on the relationship between increased access to in-home piped water and reduced incidence of waterborne and water-washed diseases identified in Mosites et al. (2020a). These associations were not causally identified and thus may overestimate the impact of access to in-home piped water on improved health outcomes. Second, our estimates are sensitive to uncertainty in several parameters in our model. Estimates of the economic benefits of mortality risk reduction are particularly sensitive to assumptions about the case fatality rate for pneumonia/influenza and the effectiveness of piped water on reducing pneumonia/influenza cases. This is likely attributable to the fact that the model combines pneumonia and influenza and Alaska-specific case fatality rates for the diseases considered were not available. Estimates of treatment and diagnosis costs are also sensitive to assumptions about transfer patient care for pneumonia/influenza. Region-specific case fatality rates for the diseases considered and better estimates of the impact of piped water on reducing the incidence of pneumonia/influenza would provide a more accurate estimate of the potential economic benefits of providing universal access to in-home piped water in the region.

Finally, this analysis considers a portion of the benefits side of the benefit-cost ledger. This highlights two important areas for future research. First, additional work is required to estimate the economic value of the full suite of health and non-health-related benefits associated with universal access to water and sanitation services in the region. Second, data on the cost of providing universal access to piped water in the region were not available. Previous estimates for rural Alaska indicated that it would cost $300 million to provide piped water service to unserved communities in rural Alaska and $400 million to upgrade and expand failing systems (Thomas et al., 2016a). Estimating the cost of providing universal access to piped water service in the region is an important area for future research.

5. Conclusion

Overall, our estimates suggest there may be considerable economic benefits associated with improving access to in-home piped water in the Y.K. Delta region and rural Alaska more broadly. In particular, we find that increased access to in-home piped water in the region may yield substantial reductions in direct medical expenses incurred by public agencies and families, as well as reductions in time and travel costs associated with improved health outcomes. This, along with the array of health and non-health-related benefits not included in our analysis, may provide new impetus to expanding access to high-quality water and sanitation services in this region and other rural arctic communities. Providing access to piped water service in rural Arctic communities is, however, both difficult and expensive. Additional work is required to better quantify the full suite of the economic benefits and the costs associated with achieving universal access to piped water in the region. Even if specific projects do not yield positive net benefits, there are potentially strong non-economic justifications for expanding access to high-quality water and sanitation services in the region. Indeed, recent global experience with COVID-19 highlights the critical importance of good hygiene and sanitation and underscores the importance of meeting the SDG aspiration of ensuring safe and affordable water and sanitation for all.

Competing interests declarations

The authors are employed by the organizations listed in the author affiliations. The authors have no additional competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113915.

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Increased preterm birth following maternal wildfire smoke exposure in Brazil

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ABSTRACT

Preterm birth (PTB) complications are the leading cause of death among children under 5 years of age, responsible for approximately 1 million deaths in 2015, according to the World Health Organization. Those infants born prematurely who survived the first 5 years, studies suggest that these infants are more likely to experience a range of poor health outcomes during childhood and even adulthood. Wildfire smoke has been suggested as a type of air pollution source with high toxicity for reproductive health. In this study, we estimated the association between preterm birth and wildfire periods in Brazil, a country included in the list of the 10 nations with the greatest number of preterm birth and also considered as a very fire-prone region. We applied a time-stratified case-crossover study design using conditional logistic regression models to estimate the odds ratio for preterm birth associated with wildfire-related prenatal $PM_{2.5}$, during different windows of exposure, including trimesters 1–3. After adjusting for several confounders (other air pollutants, demographics, meteorological variables, and spatiotemporal terms), we found that wildfire smoke exposure during pregnancy may be associated with preterm birth in Brazil. Southeast was the region with the highest increase in the odds of PTB (OR:1.41 (95%CI: 1.31–1.51) when the exposure occurred in the first trimester. In the North, exposure to $PM_{2.5}$ during wildfire periods in the second trimester of pregnancy was associated with increased odds of PTB (OR:1.05 (95%CI: 1.01–1.09) in preterm birth when the exposure occurred in the second trimester. This study suggests that wildfire smoke exposure during pregnancy may increase the risk for preterm birth in Brazil. This should be of great concern to the public health authorities, obstetricians, and policymakers.

1. Introduction

Air pollution exposure has been associated with critical neonatal morbidities, including low birth weight (Bell et al., 2008; Veras et al., 2010), birth defects (Girguis et al., 2016; Ritz et al., 2002), and preterm birth (Liu et al., 2019; Yorifuji et al., 2011). These associations may differ by air pollution source such as traffic emissions, industrial emissions, and biomass burning (Morello-Frosch et al., 2010). Studies have shown that distinct air pollutants/sources may play different roles during the various stages of fetal development (Šrám et al., 2005). Wildfire smoke has been suggested as a type of air pollution source with high toxicity for reproductive health, including birth weight and preterm birth (Reid et al., 2016). Fine particulate matter ($PM_{2.5}$) is the major pollutant emitted by wildfires. In the U.S., according to the National Emissions Inventory (NEI), in 2014 wildfires represented more than 20% of total $PM_{2.5}$ emissions annually (EPA 2014).

Among the pregnancy outcomes, a large body of epidemiological literature has indicated preterm birth (according to the World Health Organization, defined as a live birth that occurs before 37 completed weeks of pregnancy) as an important public health concern. Estimates show that preterm birth has risen annually, indicating a global preterm birth rate of about 11% in 2010, which affected approximately 15 million newborns (Kinney et al., 2012). One of the main consequences of preterm birth is that an infant born premature is more likely to

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experience a range of poor health outcomes during childhood and even adulthood (Liu et al., 2016). The latest report from the United Nations Inter-Agency Group for Child Mortality Estimation (UN IGME) estimates that 1 million children die every year due to preterm birth before the age of 5 years (UN IGME 2020). The later adult chronic medical conditions associated with premature delivery include diabetes (Crump et al., 2020), hypertension (Jones et al., 2019), cerebral palsy (Van Haastert et al., 2011), and asthma (Jaakkola et al., 2006). Therefore, the identification of any modifiable risk factors is of great importance.

In 2010, there were approximately 279,300 (±9% of all births in 2010) preterm births in Brazil, classifying the country as one of the ten countries with the highest numbers of preterm births worldwide (Blencowe et al., 2012). In 2020, there were more than 5 million wildfire records in Brazil, representing 56% of all wildfire occurrences in South America (http://queimadas.dgi.inpe.br/queimadas/bdqueimadas). About 12–16% of global wildfire-related particulate emissions occur across Brazil (Reddington et al., 2015). In this study, we examined the association between preterm birth and wildfire periods in a country included in the list of the 10 nations with the greatest number of preterm birth and considered as a very fire-prone region – Brazil.

2. Materials and methods

2.1. Birth data

We accessed birth data from the Ministry of Health in Brazil for the period between 1 January 2001 and 31 December 2018. We selected only the birth records of mothers aged between 18 and 45 years old and infants born before the 37th week of gestation. The WHO considers the 37th week of gestation as the standard cutoff used to classify preterm birth. As a result of this subset, there were a total of 190,911 records of preterm birth. The birth records included the following information: birth date, mother's age, mother's race (categorized as white, black, and indigenous), gestational age (categorized as the number of weeks of gestation), date of the last menstrual period, sex of the infants, and mother's home municipality. There are 5572 municipalities in Brazil, which are grouped in five regions: North, Northeast, Midwest, Southeast, and South. In Appendix 1 we show the spatial distribution of all municipalities and regions in Brazil. We also provide in Appendix a map illustrating the spatial distribution of preterm births in Brazil.

2.2. Exposure and covariates data

Wildfire data were accessed from the National Institute of Spatial Research of Brazil – *Instituto Nacional de Pesquisas Espaciais - INPE* (http://queimadas.dgi.inpe.br/queimadas/). The data comprise the date, latitude, and longitude of each wildfire record and were derived from seven satellite observations, including NOAA-18, NOAA-19, METOP-B, MODIS (NASA TERRA and AQUA), VIIRS (NPP-Suomi and NOAA-20), GOES-16, and MSG-3. We accounted for all wildfire records in Brazil captured by these satellites during the period 2000 through 2018. A wildfire record indicates the existence (on a certain day) of a fire at an image resolution element (pixel), which ranges from 375 m × 375 m to 5 km × 4 km, depending on the satellite. The instruments on the satellites can identify fires as small as 1 m wide and 30 m long. We merged the wildfire data with the health data, based on summing the daily wildfire records within each municipality in Brazil.

We also accounted for ambient air pollution and meteorological variables. For ambient air pollution, we considered daily $PM_{2.5}$ (μg/m^3), CO (ppb), NO_2 (ppb), and O_3 (ppb) concentrations. The data was accessed from the Environmental Information System for Health (http://queimadas.dgi.inpe.br/queimadas/sisam/v2/dados/download/). This is an environmental database system developed by INPE - National Institute of Spatial Research in Brazil. The INPE obtained daily concentrations of all these four pollutants from the Copernicus Atmosphere Monitoring Service (CAMS)-Reanalysis for the period between 2000 and 2018. The monitoring by these satellites instruments has a spatial resolution of 0.125° (approximately 12.5 km) and a temporal resolution of 6 h, including daily measurements for 00, 06, 12, and 18 UTC (Universal Time Coordinated). We calculated the daily mean temporal resolution for each pollutant from 2000 to 2018 and the mean concentration within each Brazilian municipality.

The meteorological data were collected by satellite-based remote sensors and were accessed from the same Brazilian database system as for air pollution data - Environmental Information System for Health. Weather data include surface temperature (°C), humidity (%), wind speed (m/s), wind direction (°), and precipitation (mm/day). Temperature, humidity, wind speed, and wind direction were derived from Era-Interim reanalyses, with a spatial resolution of 0.125° and temporal resolution of 6 h. This reanalysis was performed by the European Centre for Medium-Range Weather Forecast (ECMWF). Precipitation data was accessed from the Climate Prediction Center (CPC) and the National Ocean and Atmospheric Administration (NOAA). This data has an original spatial resolution of 0.50° (approximately 50 km), with interpolation to 12.5 km, and a temporal resolution of 6 h. We accounted for daily mean values of weather variables within each Brazilian municipality.

The validation for the CAMS global model is reported by Inness et al. (2018). Specifically, for the $PM_{2.5}$, the exposure variable in our study, it is evaluated with ground observations of the Aerosol Robotic Network (AERONET). About 400 AERONET stations worldwide measure spectral Aerosol Optical Depth (AOD) with ground-based sun photometers. Among those AERONET stations, about 27 stations are in Brazil. The validation by Inness et al. (2018) estimated a mean bias and standard deviations from the data provided by the satellite's instruments (included in the CAMS model for aerosols) relative to AERONET data. In South America, the data from satellite's instruments are slightly smaller, with an approximate bias of -0.006 ± 0.128. Another investigation shows that CAMs estimates in South America have a root mean square error – RMSE (compared with AERONET stations) of 0.268 (Gueymard and Yang 2020). Other studies have shown that AERONET observation sites in South America has significant representativity for AOD measured by Moderate Resolution Imaging Spectroradiometer (MODIS), aboard TERRA and AQUA satellites (Hoelzemann et al., 2009). Note that MODIS is an instrument included in the CAMS model. This association between AERONET data and AOD from MODIS is significant during the biomass burning seasons in South America, which the R^2 (coefficient of determination) for most of the AERONET stations in Brazil was higher than 0.85 (Hoelzemann et al., 2009).

2.3. Exposure assessment

We defined three windows of exposure, representing the three segments of pregnancy, including the first trimester (week 1 to week 12), second trimester (week 13 to week 28), and the third trimester (week 29 to week 37) based on date of birth and gestational age at birth. For each trimester, we estimated the average of daily estimated wildfire exposure, pollutant concentrations ($PM_{2.5}$, CO, NO_2, and O_3) and meteorological variables.

Then, we defined the "wildfire wave" concept to capture periods with high wildfire occurrences, which allows us to estimate the health effects of strong episodes of wildfire-related air pollution. We defined a wildfire wave as any average value of wildfire records and $PM_{2.5}$ concentration in the trimesters that exceeded the 90th percentile of the time series between 2001 and 2018. This process was performed by the Brazilian region. Note that here the 90th percentile was selected as the cut point for defining an extreme air pollution event. We accounted for $PM_{2.5}$ given the substantial concentration of $PM_{2.5}$ emitted by wildfires (as we mentioned in the introduction section). We estimated the range of percentile values for $PM_{2.5}$ and wildfire (supplementary materials). We observed that in most of the regions in Brazil and trimesters of exposure, the wildfire records existed only above the 90th percentile of the time series between 2001 and 2018. For example, in the Midwest, the 90th percentile for wildfires in the first trimester was nine wildfire records, while for the percentiles ≤80th, there were no wildfire records. We also observed that the 90th percentile cut-off values for $PM_{2.5}$ included extreme levels of $PM_{2.5}$, which allows our results to be helpful for the public health authorities. For example, in the first trimester of exposure, the 90th percentile for $PM_{2.5}$ was estimated in 77.89 μg/m^3, 90.49 μg/m^3, 41.13 μg/m^3, 53.96 μg/m^3, and 54.66 μg/m^3, for the Midwest, North, Northeast, South, and Southeast regions, respectively (supplementary materials). Note that all these cut-off values were much higher than the World Health Organization 24-h air quality standard for $PM_{2.5}$ (25 μg/m^3). In addition, we highlight that the concept of wildfire wave defined in our study is similar to the concepts of extreme air pollution events from wildfires defined in previous studies (Johnston et al., 2011; Liu et al., 2017).

Finally, we created a binary indicator variable for case (1)/control (0), which compares the exposure (wildfire waves) in each trimester of the infants born before 37 completed weeks of pregnancy (preterm birth, case) with the exposure on non-event trimesters (no wildfire waves, control). Note that i) the concept of "wildfire wave" captures trimesters with high wildfire occurrences; ii) the cases were defined as 1, which represents mothers exposed to wildfire waves during their pregnancy; and iii) the controls were defined as 0, which represents mothers not exposed to wildfire waves during their pregnancy. The exposure assessment used in our study is illustrated in Fig. 1.

2.4. Statistical analysis

We applied a time-stratified case-crossover study design using conditional logistic regression models. This study design is based on a binary indicator variable for case and control, as we described in the previous section.

We used a time-stratified sampling to select days of birth, which were matched for the day of the week, month, and calendar year of each birth record. Note that this study design will group the infants according to their period of exposure. We chose to conduct a matched analysis because the wildfire-related air pollution exposure is an episodic event (it is a triggering event). Also, the matching approach incorporates some advantages. First, assuming that the matching periods were close in time, the approach reduces the effects of confounding related to the seasonal trend by controlling for time-dependent risk factors, including the day of the week, season, and long-term trends by matching. Also, preterm births were defined as their own controls, allowing for control of all individual-level potential confounders represented by mothers' information (e.g., socioeconomic status, smoking history, pre-existing medical conditions) by design, except for ones that change rapidly. Note that in our study design we considered preterm birth as a consequence of a gradual crossover, meaning that it is plausible that several weeks of exposure are needed to manifest the effect. We highlight that the use of trimester exposure can be used to compare the weekly averages with the trimester averages. If they are similar, we can assume that there are no bias and trimester exposures are more clinically relevant.

We used the conditional logistic regression model to estimate the odds ratio (OR) for preterm birth associated with $PM_{2.5}$ during wildfire waves compared with the background. We adjusted the model for $PM_{2.5}$, CO, NO_2, O_3, meteorological variables (temperature, relative humidity, precipitation, wind speed, wind direction), spatial term (state, latitude/longitude of the municipality), and temporal term (year of birth). This model was performed in R, using the statistical package Survival ("clogit" function). The model was given by:

$$logit(PB) = \beta_{0i} + \beta_1 PM_{2.5} + \ldots + \beta_k Confounding_k$$

where *PB* is the probability of preterm birth; β_{0i} is the contribution to the logit of all terms constant within the ith matching set; β_1 to β_k are the regression coefficients that represent log-odds (interpretable in exponent form, e^{β}) for the exposure and all confounding variables (listed above).

In the primary analysis, we applied the conditional logistic model described above for each Brazilian region and trimester. We performed this subgroup analysis by region to capture the regional heterogeneity of

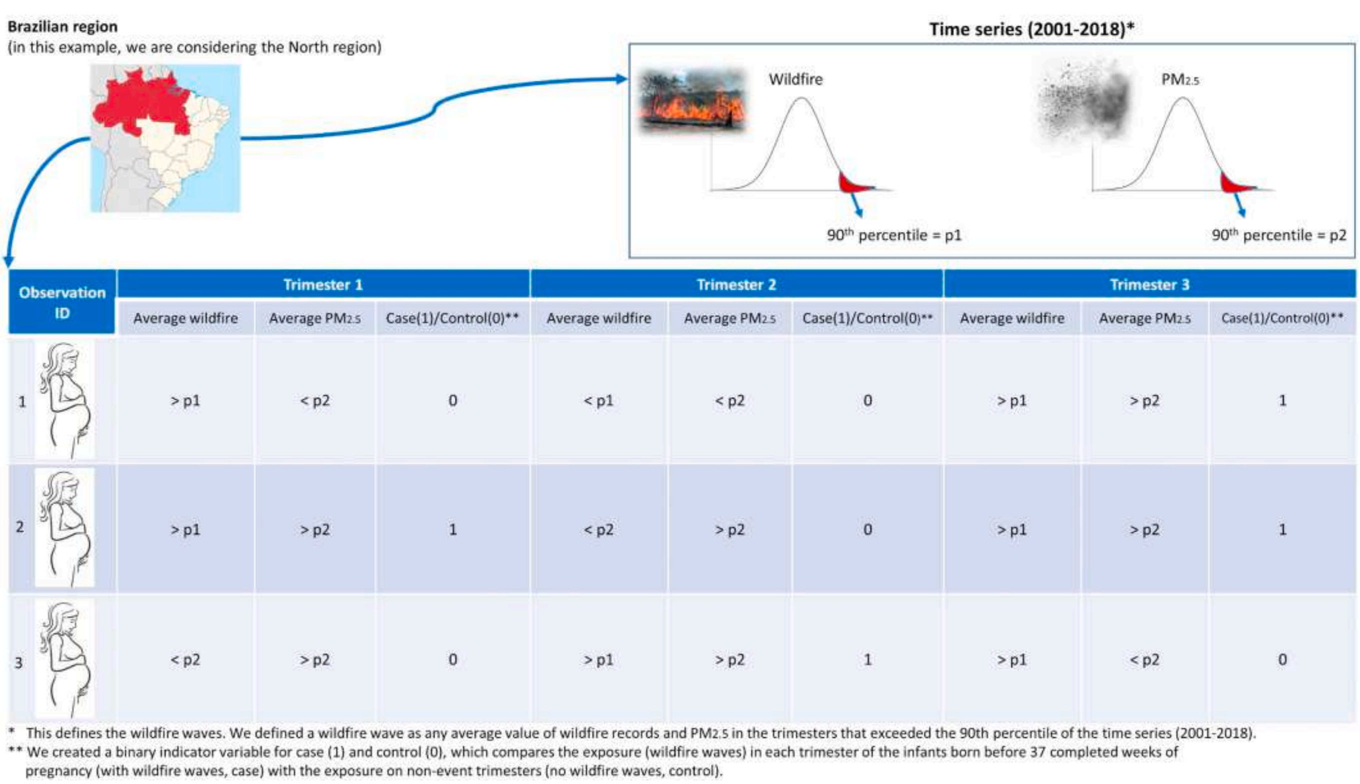

Fig. 1. Exposure assessment.

landscape in Brazil (e.g., Amazon Forest, Atlantic Forest, Pantanal, etc.), which is strongly correlated with wildfire occurrences.

Finally, after the primary analysis, we conducted sensitivity analysis by stratifying the analyses by sex, race, and gestation week of delivery (<22 weeks, 22–27 weeks, 28–31 weeks, and 32–36 weeks).

3. Results

3.1. Preterm birth characteristics

Table 1 provides the descriptive characteristics of preterm births in the study population, which included a total of 190,911 premature infants in Brazil between 2001 and 2018. Southeast is the region with the highest number of infants (89,242), representing 47% of the study population. In contrast, North and Midwest were the regions with the lowest number of infants, both representing about 7%. Regarding the infant sex, males were slightly higher than females (overall, 51% boys and 49% girls), except in the Southeast, where there was a slightly higher number of girls. Whites were the most predominant type of race, varying between 26 and 21% among the regions. As the gestational week, overall, about 60% of the infants in our study population (in all regions) were born during the 22–27 week (Table 1).

Table 1
Descriptive characteristics of preterm birth in Brazil, 2001–2018.

Region	Subgroup	n (%)
North	Infant sex: Male	6575 (51.2)
	Infant sex: Female	6227 (48.5)
	Gestational week: < 22 weeks	849 (6.6)
	Gestational week: 22–27 weeks	7333 (57.1)
	Gestational week: 28–31 weeks	3130 (24.4)
	Gestational week: 32–36 weeks	1528 (11.9)
	Maternal race: white	4035 (31.4)
	Maternal race: black	217 (1.7)
	Maternal race: indigenous	204 (1.6)
		12,840 (6.7)[1]
Northeast	Infant sex: Male	24,592 (50.3)
	Infant sex: Female	24,065 (49.2)
	Gestational week: < 22 weeks	4119 (8.4)
	Gestational week: 22–27 weeks	27,487 (56.2)
	Gestational week: 28–31 weeks	12,895 (26.4)
	Gestational week: 32–36 weeks	4377 (9.0)
	Maternal race: white	13,587 (27.8)
	Maternal race: black	1815 (3.7)
	Maternal race: indigenous	110 (0.2)
		48,878 (26.6)[1]
Midwest	Infant sex: Male	6974 (50.1)
	Infant sex: Female	6905 (49.6)
	Gestational week: < 22 weeks	937 (6.7)
	Gestational week: 22–27 weeks	8411 (60.4)
	Gestational week: 28–31 weeks	3794 (27.2)
	Gestational week: 32–36 weeks	788 (5.7)
	Maternal race: white	3680 (26.4)
	Maternal race: black	351 (2.5)
	Maternal race: indigenous	90 (0.6)
		13,930 (7.3)[1]
Southeast	Infant sex: Male	44,322 (49.7)
	Infant sex: Female	44,708 (50.1)
	Gestational week: < 22 weeks	4646 (5.2)
	Gestational week: 22–27 weeks	54,170 (60.7)
	Gestational week: 28–31 weeks	25,462 (28.5)
	Gestational week: 32–36 weeks	4964 (5.6)
	Maternal race: white	25,434 (28.5)
	Maternal race: black	4980 (5.6)
	Maternal race: indigenous	94 (0.1)
		89,242 (46.7)[1]
South	Infant sex: Male	12,822 (49.3)
	Infant sex: Female	13,159 (50.6)
	Gestational week: < 22 weeks	1285 (4.9)
	Gestational week: 22–27 weeks	16,726 (64.3)
	Gestational week: 28–31 weeks	6803 (26.1)
	Gestational week: 32–36 weeks	1207 (4.6)
	Maternal race: white	8200 (31.5)
	Maternal race: black	1115 (4.3)
	Maternal race: indigenous	64 (0.2)
		26,021 (13.6)[1]

Notes: the percentage was based on the proportion of observations by region. (1) these percentages, specifically, were based on the proportion of observations considering the whole Brazil (all study sample).

3.2. Exposure characteristics

Table 2 shows summary statistics for wildfire, air pollution, and meteorological variables. The North region was estimated with the highest wildfire records, with an average value varying between 16 and 22 wildfire occurrences over the trimesters. Also, north was the region with the highest ambient $PM_{2.5}$, with an average value between 38 and 40 μg/m^3 over the trimesters (throughout the entire study period).

3.3. Association between wildfire $PM_{2.5}$ and preterm birth

Results of the primary analysis stratified by region and trimester of exposure are shown in Fig. 2. Southeast was the region with the highest increase in the odds of preterm birth (OR:1.41 (95%CI: 1.31–1.51) when the exposure occurred in the first trimester. In the North, exposure to $PM_{2.5}$ during wildfire periods in the second trimester of pregnancy was associated with increased odds of PTB (OR:1.05 (95%CI: 1.01–1.09). In the Midwest, the highest increase in the odds of preterm birth was during the first trimester of exposure (OR:1.04 (95%CI: 1.01–1.07). In the South, the second trimester was estimated with the highest increased odds of preterm birth (OR:1.06 (95%CI: 1.04–1.07). Northeast was the only region in which we were unable to run the analysis because the models did not converge due to significant groups of ties, which a large number of health events (defined as cases) is out of a large number of subjects (case plus control days). This issue was observed for the first and third trimesters in the North region.

Fig. 3 shows the results from sensitivity analysis stratified by sex and race – only white and black. For the indigenous group, we found the same issue as the Northeast, in which the model did not converge. This issue related to the feasibility of the analysis was observed in numerous subgroup analyses, including all the analyses for the regions Northeast and North. The sensitivity analysis by gestational week (<22 weeks, 22–27 weeks, 28–31 weeks, and 32–36 weeks) is shown in the Appendix.

4. Discussion

Overall, our findings support the hypothesized association between wildfire periods during pregnancy and preterm birth in Brazil. These findings are consistent with the very few prior studies on preterm birth maternal exposure to wildfire. To our knowledge, the study by Rangel and Vogl (2019) was the only one to date focusing on this topic for the Brazilian population. In this previous study in Brazil, the authors also found that wildfire exposures in the 1–3 trimesters were associated with decreases in gestational length. Different from our study, Rangel and Vogl (2019) accounted only for the population in the city of São Paulo (located in the Southeast region). They quantified the decreased number of weeks associated with wildfire exposure and found that the highest association is when the exposure occurred in the second and the third trimester. In our study, the first and the third trimester were suggested with the highest association in the Southeast region.

In other countries, the scarce literature persists. We found only one study looking at wildfire smoke exposure and preterm birth in Colorado, United States, between 2007 and 2015 (Abdo et al., 2019). The authors reported that the exposure to wildfire smoke $PM_{2.5}$ during the second trimester was associated with an increase in the odds of preterm birth (OR:1.13 (95%CI: 1.08–1.17) in preterm birth. During the first and third trimesters, the associations were not statistically significant (Abdo et al., 2019).

A limited number of studies focusing on the impacts of wildfire-related air pollution on preterm birth, whereas numerous researchers

Table 2
Summary statistics for wildfire, air pollution, and weather by region, 2001–2018.

Region	Variable	First trimester				Second trimester				Third trimester			
		Min.	Mean	SD	Max.	Min.	Mean	SD	Max.	Min.	Mean	SD	Max.
North	Wildfire records	0	16.55	122.53	4702.00	0.00	19.41	164.70	6256.00	0.00	22.02	209.00	12,813
	$PM_{2.5}$ (μg/m^3)	2.93	40.28	37.48	306.21	2.58	39.52	38.15	311.96	2.54	39.81	39.46	273.29
	Temperature (°C)	23.07	26.31	0.96	29.34	23.07	26.35	0.97	29.48	22.87	26.40	1.02	29.41
	Relative humidity (%)	43.35	84.86	7.81	95.25	39.07	84.66	8.10	95.44	43.13	84.65	8.20	95.45
	Wind speed (m/s)	1.02	1.97	0.51	6.24	1.06	1.98	0.51	6.26	1.05	1.98	0.51	5.83
	Wind direction (°)	40.19	106.03	40.89	251.84	41.05	106.28	41.39	269.39	40.06	106.62	42.18	253.50
	Preciptation (mm/day)	0.00	5.81	3.54	24.93	0.00	5.86	3.65	28.30	0.00	5.99	3.67	22.11
Northeast	Wildfire records	0	1.54	14.56	1020.00	0.00	1.77	16.86	987.00	0.00	2.01	18.81	979.00
	$PM_{2.5}$ (μg/m^3)	0.69	17.27	22.66	192.25	0.71	17.55	23.01	224.91	0.72	17.87	22.98	206.49
	Temperature (°C)	19.69	25.91	1.66	31.70	19.59	25.93	1.68	31.53	19.61	26.01	1.68	31.70
	Relative humidity (%)	40.64	76.28	8.54	92.66	39.62	75.92	8.74	92.91	39.71	75.63	8.93	92.84
	Wind speed (m/s)	1.41	4.01	1.11	8.51	1.41	4.04	1.13	8.15	1.40	4.03	1.13	8.29
	Wind direction (°)	49.03	111.81	20.36	180.98	45.71	111.30	20.51	184.69	44.46	110.48	20.32	189.90
	Preciptation (mm/day)	0.00	3.61	2.97	17.91	0.00	3.46	2.81	16.94	0.00	3.38	2.83	15.98
Midwest	Wildfire records	0	10.55	90.43	4040.00	0.00	11.09	94.04	5564.00	0.00	12.56	116.88	5350
	$PM_{2.5}$ (μg/m^3)	3.60	30.55	39.13	347.35	3.28	29.05	37.94	306.30	2.70	27.72	35.57	356.24
	Temperature (°C)	17.46	24.04	1.84	30.11	18.14	24.06	1.80	29.52	18.21	24.13	1.83	29.73
	Relative humidity (%)	41.88	66.96	10.36	95.00	42.21	66.73	10.40	93.54	38.73	66.49	10.63	94.01
	Wind speed (m/s)	1.34	2.57	0.38	3.76	1.17	2.57	0.39	3.69	1.19	2.57	0.38	3.86
	Wind direction (°)	91.53	141.66	28.52	265.89	95.69	141.30	28.17	266.19	90.53	141.92	28.37	284.66
	Preciptation (mm/day)	0.00	3.86	2.90	15.99	0.00	3.80	2.90	13.66	0.00	3.80	2.91	14.51
Southeast	Wildfire records	0	0.80	4.63	275.00	0.00	0.85	4.23	214.00	0.00	0.89	4.78	216.00
	$PM_{2.5}$ (μg/m^3)	1.95	26.28	20.71	104.47	1.70	27.24	22.19	112.14	1.56	27.22	22.33	113.03
	Temperature (°C)	14.96	21.73	2.39	27.59	15.30	21.77	2.33	27.78	15.19	21.94	2.31	27.81
	Relative humidity (%)	44.34	76.94	6.12	88.10	45.28	76.91	6.17	88.06	45.78	77.01	6.32	87.78
	Wind speed (m/s)	1.64	2.70	0.48	6.07	1.56	2.72	0.48	6.05	1.59	2.72	0.49	5.95
	Wind direction (°)	68.45	146.16	25.01	217.36	70.19	145.18	24.95	217.54	64.81	146.06	25.06	221.50
	Preciptation (mm/day)	0.00	3.75	2.42	13.90	0.00	3.77	2.43	13.90	0.00	3.92	2.44	13.90
South	Wildfire records	0	1.00	7.37	335.00	0.00	1.03	7.49	355.00	0.00	1.08	7.60	331.00
	$PM_{2.5}$ (μg/m^3)	4.41	26.36	20.23	153.06	4.28	24.53	19.56	162.82	4.33	22.03	16.98	167.54
	Temperature (°C)	10.91	19.42	3.31	26.55	11.49	19.60	3.20	26.84	11.60	19.77	3.14	27.26
	Relative humidity (%)	59.18	79.38	4.58	88.79	58.35	79.40	4.64	88.44	58.61	79.43	4.64	88.15
	Wind speed (m/s)	1.77	3.10	0.75	6.86	1.72	3.09	0.75	7.33	1.70	3.09	0.75	7.12
	Wind direction (°)	95.32	150.35	20.60	224.29	96.61	149.52	20.01	231.19	98.09	149.68	20.15	227.03
	Preciptation (mm/day)	0.90	4.37	1.61	14.73	0.89	4.43	1.65	14.80	0.26	4.45	1.61	14.14

Note: minimum (Min.), Standard Deviation (SD), maximum (Max.).

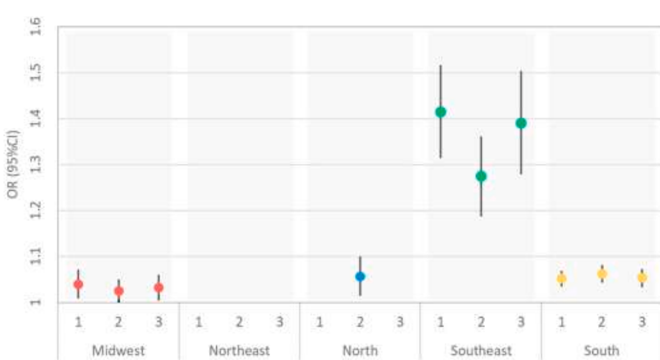

Fig. 2. Regional Odds Ratios (OR) for $PM_{2.5}$ during wildfire waves (and 95% CI) for preterm birth in the trimesters 1, 2, and 3. Note 1: numbers in the x-axis indicate the trimesters. Note 2: The analyses for Northeast (trimesters 1, 2, and 3) and North (trimesters 1 and 3) did not converge due to significant groups of ties, which a large number of health events (defined as cases) is out of a large number of subjects (case plus control days).

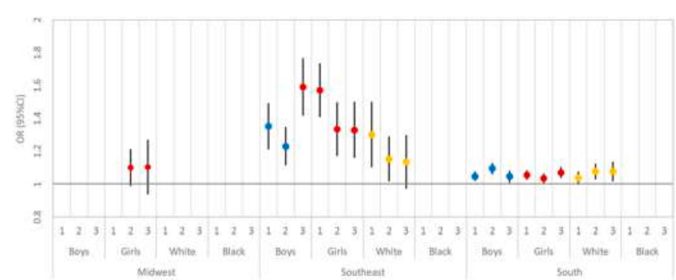

Fig. 3. Regional Odds Ratios (OR) for $PM_{2.5}$ during wildfire waves (and 95% CI) for preterm birth in the trimesters 1, 2, and 3, stratified by sex and race. Note 1: x-axis indicates the trimesters. Note 2: The results for North and Northeast were omitted because the analyses did not converge due to significant groups of ties, which a large number of health events (defined as cases) is out of a large number of subjects (case plus control days). Also, the blank results shown here (e.g., Midwest stratified by boys, white and black) represent this issue related to the feasibility of the analysis.

have investigated the impact of ambient $PM_{2.5}$ on preterm birth without specifying the wildfires as a specific air pollution source. For example, a global study has shown that the number of $PM_{2.5}$-associated preterm births was estimated as 2.7 million in 2010 (Malley et al., 2017). In Zhejiang Province, China, maternal exposure to the high concentration of $PM_{2.5}$ (tertile 3) was strongly associated with a risk of preterm birth (OR = 1.76, 95% CI: 1.35–2.29) (Sun et al., 2019). Note that in our study we defined wildfire waves based on days when wildfire records and $PM_{2.5}$ concentrations exceeded the 90th percentile. Another study focused on an assessment of critical exposure windows in the relationship between air pollution and preterm birth (in Atlanta, United States) reports that the third trimester (around week 30) is the most crucial exposure window (Chang et al., 2014).

Toxicological investigations indicate different potential mechanisms via which wildfire-related air pollution might contribute to preterm delivery. Some evidence indicates that ambient air pollution may decrease placenta exchange of nutrients and oxygen (Nordenvall and Sandstedt 1990), resulting in adverse fetal development, increasing the risk of preterm delivery (Clemens et al., 2017; Knottnerus et al., 1990).

Other studies suggest an effect in the mother's body that creates vulnerability to their fetuses. Women during pregnancy have their alveolar ventilation rate increased by about 50%, resulting in an increased uptake of inhaled air pollutants (Hackley et al., 2007). The presence of air particulate in the bloodstream of pregnant women can lead to oxidative stress, pulmonary inflammation, and placental circulation, which potentially may result in premature contractions and membrane rupture (Wick et al., 2010). This mechanism related to the respiratory system of pregnant women must be considered in the context of the toxicity of $PM_{2.5}$. A recent study has found evidence that wildfire particulate matter may be more toxic than equal doses of ambient $PM_{2.5}$ (Aguilera et al., 2021). The authors show that wildfire-specific $PM_{2.5}$ may increases respiratory hospitalizations by 10%, while non-wildfire $PM_{2.5}$ was associated with an increase of 1.3% (Aguilera et al., 2021).

The results from our analysis should be considered in the context of some limitations. First, we must take into account for misclassification error related to the location of the wildfire and the predicted concentration of $PM_{2.5.}$ These variables were estimated by satellite remote sensing, which may result in some exposure measurement error. Second, the findings found in our analysis only suggests an association between exposure to wildfire waves and preterm birth. Our results do not indicate the cause-effect between wildfire exposure and preterm birth. Third, although we accounted for a matching study design that controls for confounding factors as the mothers are defined as their controls, we still have to consider some residual confounding error. Finally, there is the limitation related to the definition of "wildfire waves". The wildfire concept is based on the 90th percentile cut-off values for $PM_{2.5}$ and wildfire records, which allows us to estimate the health effects of strong episodes of wildfire-related air pollution. However, we should be cautious in interpreting our results at a national scale, given that each region had a different cut-of value for $PM_{2.5}$ and wildfire records. For example, in the first trimester of exposure, the 90th percentile for PM2.5 was estimated in 77.89 μg/m3, 90.49 μg/m3, 41.13 μg/m3, 53.96 μg/m3, and 54.66 μg/m3, for the Midwest, North, Northeast, South, and Southeast regions, respectively (supplementary materials). Although the different cut-off values across regions, we can assume that all the values exceeded the WHO 24-h air quality standard for $PM_{2.5}$ (25 $\mu g/m^3$).

Our findings also have some strengths. To our knowledge, it is the first nationwide study in Brazil at the municipality scale, with a study period of 18 years. Second, we accounted for a strong quantitative approach that reduces the effects of confounding related to the seasonal trend by controlling for time-dependent/independent risk factors. Third, our modeling approach estimates the exposure variable based on extreme episodes of wildfire, defined as wildfire waves. This allowed us to capture days with elevated concentration, periodic, and short-lived characteristics of wildfire $PM_{2.5}$. Finally, we were able to control for important confounders and assess effect modification by some variables, such as individual SES. There is evidence that some confounders used in our study have an important effect on the relationship between maternal air pollution and preterm birth, such as maternal age (Han et al., 2018) and the air pollutants - CO, O_3, and NO_2 (Ritz et al., 2007).

5. Conclusions

This study shows that wildfire periods during pregnancy is associated with the risk for preterm birth in Brazil. This should be of great concern to the public health authorities, obstetricians, and policymakers with the objective of improving quality of children health.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113901.

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