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Associations of maternal urinary arsenic concentrations during pregnancy with childhood cognitive abilities: The HOME study

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ABSTRACT

Arsenic exposure during pregnancy may increase the risk for intellectual deficits in children, but limited data exist from prospective epidemiologic studies, particularly at low arsenic exposure levels. We investigated the association between prenatal maternal urinary arsenic concentrations and childhood cognitive abilities in the Health Outcomes and Measures of the Environment (HOME) Study. We used anion exchange chromatography coupled with inductively coupled plasma mass spectrometry detection to measure arsenic species content in pregnant women's urine. The summation of inorganic arsenic (iAs), monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA) refers to ∑As. We assessed children's cognitive function ($n = 260$) longitudinally at 1-, 2-, and 3-years using Bayley Scales of Infant and Toddler Development, at 5 years using Wechsler Preschool and Primary Scale of Intelligence, and at 8 years using Wechsler Intelligence Scale for Children. We observed a modest decrease in mental development index and full-scale intelligence quotient at ages 3 and 5 years with each doubling of ∑As with estimated score (ß) differences and 95% confidence interval (CI) of −1.8 from −4.1 to 0.5 and −2.5 from −5.1 to 0.0, respectively. This trend was stronger and reached statistical significance among children whose mothers had lower iAs methylation capacity and low urinary arsenobetaine concentrations. Our findings suggest that arsenic exposure levels relevant to the general US population may affect children's cognitive abilities.

1. Introduction

Arsenic, which occurs in organic and inorganic forms, is ubiquitous (WHO, 2001). Inorganic arsenic (iAs) is an established cause of cancer of the lung, skin, and bladder (IARC, 2012). Also, evidence is growing that iAs is a risk factor for non-cancer health outcomes, such as diabetes and cardiovascular disease (IARC, 2012; Kapaj et al., 2006; Nachman et al., 2017; Ng et al., 2003; Sanchez et al., 2016; Tolins et al., 2014; Tsuji et al., 2015). Arsenic crosses the placenta and enters the fetus (Davis et al., 2014; Gilbert-Diamond et al., 2016; Gluckman et al., 2008; Hall et al., 2007; Punshon et al., 2015; Steinmaus et al., 2014; Vahter, 2008, 2009). Arsenic exposure during early brain development may result in impaired cognitive abilities that last throughout the life course (EFSA, 2009; Freire et al., 2018; Gluckman et al., 2008; Grandjean and Landrigan, 2014; Nachman et al., 2017; Signes-Pastor et al., 2017b; Tolins et al., 2014; Tsuji et al., 2015; Wasserman et al., 2014).

Several countries have established a maximum contaminant level (MCL) of 10 μg/L for arsenic in drinking water. Yet, several million

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people worldwide consume water with arsenic content above this MCL (Ayotte et al., 2017; EPA, 2001; US EPA, 2012; WHO, 2017, 2011). When arsenic exposure from water and occupation is low, diet becomes the major source (EFSA, 2009; Nachman et al., 2018). Food contains iAs along with several organic forms with variable toxic effects (Cubadda et al., 2016). A multistep process via the one-carbon cycle metabolizes the iAs in the liver. The metabolism cycle generates monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). Then, the human body excretes them in the urine within a few days along with unmetabolized iAs (Antonelli et al., 2014; Challenger, 1951; Jansen et al., 2016; Tseng, 2009). Hence, urinary arsenic concentration is a widely used biomarker of iAs exposure (Signes-Pastor et al., 2017b, 2017c) and the concentrations ratio of $\frac{MMA}{iAs}$ and $\frac{DMA}{MMA}$ reflects iAs methylation capacity (Niedzwiecki et al., 2014). The methylation capacity is considered the major iAs detoxification process (Niedzwiecki et al., 2014), and is regulated by the polymorphisms in *AS3MT* gene (Agusa et al., 2011; Jiang et al., 2018; López-Carrillo et al., 2014).

Previous prospective studies on arsenic exposure and childhood neurodevelopment include populations from Bangladesh (Hamadani et al., 2010, 2011; Rodrigues et al., 2016; Tofail et al., 2009; Vahter et al., 2020; Valeri et al., 2017; Wasserman et al., 2016), China (Liang et al., 2020; Wang et al., 2018), Mexico (Levin-Schwartz et al., 2019), Nepal (Parajuli et al., 2013, 2014, 2015), and Spain (Forns et al., 2014; Freire et al., 2018). Most published studies are from contaminated areas with water arsenic above the MCL and show inconsistent findings (Hamadani et al., 2010, 2011; Nahar et al., 2014a, 2014b; Parvez et al., 2011; Rodrigues et al., 2016; Rosado et al., 2007; Tofail et al., 2009; Vahter et al., 2020; Wasserman et al., 2004, 2007). Evidence regarding the effects of arsenic exposure on childhood neurodevelopment among populations with access to low arsenic drinking water is still scarce (Desai et al., 2018, 2020; Forns et al., 2014; Freire et al., 2018; Kordas et al., 2015; Liang et al., 2020; Signes-Pastor et al., 2019; Wasserman et al., 2014).

We hypothesized that higher prenatal arsenic exposure impairs childhood cognitive function in communities with low-level exposure. We also expect that a decreased iAs methylation capacity would exacerbate the toxic effect. To test our hypothesis, we measured maternal urinary arsenic species concentrations in pregnancy and calculated maternal iAs methylation capacity. Then, we evaluated their association with cognitive abilities in US children enrolled in Health Outcomes and Measures of the Environment, the HOME Study, a prospective birth cohort study.

2. Methods

2.1. Study participants

The HOME Study enrolled pregnant women from the greater metropolitan area of Cincinnati, Ohio between March 2003 and February 2006. The study was designed to investigate the effects of exposure to environmental toxicants on neurodevelopment and other health endpoints in children. Eligibility criteria for HOME Study mothers were i) being ≥18 years old; ii) living in a house built before 1978; iii) having no history of human immunodeficiency virus infection; and iv) not taking medication for seizures or thyroid disorders. Children completed multiple longitudinal follow-up visits through age 12. The visits included assessment of mental, psychomotor, and cognitive development, physical growth, and health conditions (Braun et al., 2017; Chen et al., 2014). The HOME Study enrolled 389 singleton infants and nine sets of twins (Braun et al., 2017); however, only singletons were included in this study. Among singletons ($n = 389$), 310 had pregnancy urinary arsenic concentrations (excluding 79) and 276 at least one cognitive assessment to age 8 years (excluding 34). We also excluded children with missing values in relevant covariates ($n = 16$). The statistical analysis included 260 children (Fig. S1). Mothers gave informed consent before enrollment in the study and at postnatal follow-up visits for their children's participation. The institutional review board for the Cincinnati Children's Hospital Medical Center and participating hospitals and clinics approved the HOME Study Protocols (i.e, 2015–6165 and 2015–6170).

2.2. Sample preparation and chemical analyses

We collected maternal urine samples at 16- and 26-week gestation; however, samples collected at 16-week gestation were only analyzed for arsenic speciation when the 26-week gestation urine samples had insufficient volume. Among the 310 participants with arsenic data, 298 and 12 had their urinary arsenic speciation measured in samples collected at 26- and 16-week gestation, respectively. The Trace Element Analysis Core (TEA) at Dartmouth College determined urinary arsenic speciation (Jackson, 2015; Signes-Pastor et al., 2020). TEA analyzed the urine samples with an Agilent LC 1260 equipped with a Thermo AS7, 2 × 250 mm column and a Thermo AG7, 2 × 50 mm guard column interfaced with an Agilent 8900 inductively coupled plasma mass spectrometry in oxygen reaction cell mode. Each urine samples batch included blanks and replicate samples of certified reference material. The urinary arsenic species included iAs (arsenite + arsenate), and the organic compounds MMA, DMA, and arsenobetaine (AsB). The average (standard deviation) recoveries for the certified reference material NIST 2669 level I ($n = 38$) were 109% (13), 121% (19), 106% (11), and 111% (32) for AsB, DMA, MMA, and iAs, respectively. The average (standard deviation) recoveries for the NIST 2669 level II ($n = 34$) were 102% (10), 97% (11), and 106% (20) for DMA, MMA, and iAs, respectively. The arsenic species limit of detection (LOD) was 0.5 μg/L for iAs, MMA, and DMA, and 0.1 μg/L for AsB. A kinetic Jaffe reaction measured the urine creatinine content (Lausen, 1972).

2.3. Cognitive assessment

Children's cognitive abilities were assessed at ages 1, 2, 3, 5, and 8 years by HOME Study examiners trained and certified by a developmental psychologist (**KY**). We administered the Bayley Scales of Infant and Toddler Development, 2nd edition (Bayley) Mental Development Index (MDI) at 1, 2, and 3 years of age. Intelligence was evaluated using Wechsler Preschool and Primary Scale of Intelligence, 3rd edition (WPPSI) and Wechsler Intelligence Scale for Children, 4th edition (WISC) Full-Scale Intelligence Quotient (FSIQ) at ages 5 and 8 years, respectively (Bayley, 1993; Wechsler, 2003, 2004). Examiners were blinded to the mother's urinary arsenic concentrations. The Bayley-MDI, WPPSI-FSIQ, and WISC-FSIQ are commonly used in research studies. They provide reliable and valid measures of cognitive function and are statistically equivalent to a population mean of 100 and a standard deviation of 15 (Jiang et al., 2018; Kordas et al., 2015; Parajuli et al., 2015; Tofail et al., 2009; Wasserman et al., 2011, 2018). Prior publications provide further details (Braun et al., 2017; Chen et al., 2014; Nellis and Gridley, 1994).

2.4. Statistical analyses

We calculated summary statistics for each variable: median (range and interquartile range) for continuous variables and relative and absolute frequencies for categorical variables. The LOD/$\sqrt{2}$ value was imputed for statistical analysis when maternal urinary arsenic species concentrations were <LOD (Hornung and Reed, 1990). Maternal sum of urinary arsenic ($\sum$As) was calculated as the summation of arsenate, arsenite, MMA, and DMA. The iAs refers to the summation of arsenate and arsenite, and the primary and secondary methylation indices ($PMI = \frac{MMA}{iAS}$ and $SMI = \frac{DMA}{MMA}$) were calculated as measures for iAs methylation capacity. Maternal urinary arsenic concentrations were positively skewed; thus, they were $\log_2$-transformed to reduce the

influence of extreme values in regression analyses. The MDI and FSIQ scores were normally distributed, and thus transformation was unnecessary.

The dose-response association between arsenic exposure and child cognitive function was evaluated using log_2-transformed maternal prenatal arsenic concentrations using generalized additive models (GAM) and using tertiles in regression analysis. We observed no strong evidence of non-linearity. Thus, we used linear mixed models to create the regression estimates of maternal urinary ∑As and methylation indices in pregnancy with children's cognitive function, using unstructured covariance to account for correlation across repeated measurements in the same child. To investigate the association between arsenic exposure and cognitive function at different ages, we included interaction terms between arsenic (continuous) and child age (categorical) in the models. The ∑As, iAs, PMI and SMI were investigated as independent variables in separate regression models. The regression analyses were also performed for each cognitive ability assessment approach individually (i.e., MDI at 1, 2 and 3 years, and FSIQ at 5 and 8 years, respectively).

We identified covariates based on *a priori* associations with exposures and outcomes observed in the literature, previous work investigating neurodevelopmental outcomes in the HOME Study, and the Directed Acyclic Graph using the DAGitty software (Fig. S2) (Desai et al., 2020; Kordas et al., 2015; Liang et al., 2020; Parajuli et al., 2015; Signes-Pastor et al., 2019; Textor et al., 2017; Vahter et al., 2020; Valeri et al., 2017; Wang et al., 2018; Wasserman et al., 2018). We adjusted the models for household income (categorical), maternal race (categorical), maternal age at delivery (continuous), maternal Intelligence Quotient (IQ) measured by Wechsler Abbreviated Scale of Intelligence (continuous), maternal pre-pregnancy body mass index (continuous), log_{10}-average serum cotinine in pregnancy based on two time point measurements as an indicator of tobacco smoke exposure, log_{10}-urinary creatinine (continuous), Home Observation for Measurement of the Environment score at 1 year - HOME score (continuous), and child sex (binary). Further details regarding covariates can be found in our prior publication (Braun et al., 2017). Models for PMI and SMI were further adjusted for maternal ∑As to account for the overall iAs exposure. Urinary AsB comes from direct ingestion of fish/seafood and does not pose a health risk; however, it is prone to iAs exposure misclassification when urinary arsenic speciation is not performed and total arsenic is used to measure the exposure (Jones et al., 2016; Navas-Acien et al., 2011; Signes-Pastor et al., 2017b, 2019). Here maternal urinary arsenic species concentrations were measured and ∑As excluding AsB was applied to estimate iAs exposure. Fish/seafood may also contain other complex organosenical compounds that are excreted as MMA and DMA after ingestion, thus we performed statistical models restricted to participants with urinary AsB concentrations <1 μg/L suggesting little, or no fish/seafood consumption (Navas-Acien et al., 2011; Signes-Pastor et al., 2020). In sensitivity analysis, we examined maternal blood lead concentration from 16 weeks of gestation as a potential confounder. We also explored the potential effect measure modification of the arsenic-MDI/FSIQ relations by child sex, maternal smoking (i.e., maternal serum cotinine ≥3 ng/mL indicating active smoker status (Benowitz et al., 2008)), and maternal whole blood folate (above/below median of 510 nmol/L). Associations with a nominal level of 0.05 was defined as statistically significant. All statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

The biochemical, socioeconomic, and anthropometric characteristics of participants included in the analysis (n = 260) did not differ from those who were excluded (n = 129) (Table S1). Most mothers were non-Hispanic white; 67% of them were within the range of 25–34 years of age. Over 80% of participants' household income was ≥$20,000/year and were not exposed to tobacco smoke based on serum cotinine levels during pregnancy. Only 9% of women had serum cotinine levels >3 ng/mL, indicative of active tobacco smoking (Benowitz et al., 2008; Braun et al., 2017). Among these women we observed an average (standard deviation) pregnancy serum cotinine concentration of 78 (86) ng/mL, whereas the remaining women (91%) had an average concentration of 0.11 (0.27) ng/mL.

The studied children included 46% males and 54% females. Maternal urinary ∑As had a median (interquartile range) of 3.63 (2.40–5.86) μg/L (Table 1). Maternal urinary MMA concentrations were <0.5 μg/L for almost all participants. Concentrations of urinary arsenic in the HOME Study participants were lower than that noted for women of 18–45 years from NHANES 2003-04 or 2005-06 cycles (Table 2) (NHANES, 2022). The average (standard deviation) scores for MDI and FSIQ were 94 (1), 89 (14), 94 (13), and 103 (15) and 103 (16) at 1, 2, 3, 5 and 8 years of age, respectively.

A modest, non-statistically significant, decrease in MDI and FSIQ was observed at ages 3 and 5 years with each doubling of ∑As with −1.8 points lower child MDI score (95% confidence interval (CI): -4.1, 0.5) and −2.5 points lower IQ score (95% CI: -5.1, 0.0), respectively (Fig. 1; Table S2). Stronger score reductions, but still not statistically significant, were observed for PMI with −2.2 points lower MDI (95% CI: -5.0, 0.6) and −2.6 points lower FSIQ (95% CI: -5.8, 0.5) compared to SMI with −1.1 points lower MDI (95% CI: -3.2, 0.9) and −1.2 points lower FSIQ (95% CI: -3.4, 1.0) assessed at children's 3 and 5 year of age, respectively (Fig. 1; Table S2). The estimates from the regression analyses for each cognitive ability assessment approach or timing had a similar pattern of results, though confidence intervals tended to be less precise, likely due to reduced statistical power in these analyses with smaller sample sizes (Table S3).

The overall pattern of results was also consistent among participants with maternal urinary AsB <1 μg/L (n = 167). The association of ∑As with MDI at 3 years was attenuated (ß = −1.5; 95% CI: -4.5, 1.5), whereas a doubling of ∑As was associated with a −4.1-point decrease in FSIQ score at 5 years (95% CI: -7.4, −0.7). Statistically significant decreases were observed in children's MDI at 3 years and FSIQ at 5 and 8 years with each doubling of PMI, with reductions of −4.5 points (95% CI: -7.9, −1.1), −6.3 points (95% CI: -10.2, −2.4), and −5.9 points (95% CI: -10.5, −1.3), respectively (Fig. 1; Table S2). However, differences were not observed with SMI (Fig. 1; Table S2).

In our population, we observed average (standard deviation)

Table 1
Maternal urinary arsenic concentrations (∑As) in pregnancy according to maternal and children's factors, HOME Study.

Characteristics	n (%)[a]	∑As (μg/L) Median (IQR)[b]
All participants	260 (100)	3.63 (2.40–5.86)
Maternal age at delivery (years)		
< 25	47 (18)	4.62 (2.82–6.39)
25-34	173 (67)	3.52 (2.43–5.56)
≥ 35	40 (15)	3.33 (1.78–6.60)
Maternal race/ethnicity		
Non-Hispanic white	185 (71)	3.16 (2.23–5.27)
Non-Hispanic black and others	75 (29)	5.17 (3.34–7.22)
Maternal education		
High school or less	42 (16)	5.59 (2.93–7.65)
Some college or 2-year degree	62 (24)	3.86 (2.82–5.26)
Bachelor's	92 (36)	3.18 (2.32–6.40)
Graduate or professional	64 (25)	3.20 (2.14–4.86)
Maternal marital status		
Married or living with partner	224 (86)	3.48 (2.32–5.63)
Not married and living alone	36 (14)	5.06 (3.10–6.95)
Household income		
< $20,000	41 (16)	5.28 (3.00–7.27)
$20,000–79,999	137 (53)	3.63 (2.54–5.43)
≥ $80,000	82 (32)	3.07 (2.14–5.86)
Child sex		
Male	119 (46)	3.74 (2.43–6.39)
Female	141 (54)	3.61 (2.40–5.63)

[a] At enrollment.
[b] Sum of iAs (arsenate + arsenite), MMA and DMA.

Table 2
Urinary arsenic species concentrations in the HOME Study pregnant women enrolled between March 2003, and February 2006 and in women of 18–45 years of age from NHANES 2003-04 and 2005-06 cycles.

Urinary Arsenic (μg/L)	NHANES 2003-04[a]	NHANES 2005-06[a]	HOME Study				
	$n = 436$	$n = 532$	$n = 260$				
	Median (95% CI)	Median (95% CI)	Median (95% CI)	25th percentile	75th percentile	% <LOD	LOD
∑As[b]	6.10 (5.7–7.10)	6.18 (5.41–7.17)	3.63 (3.19–4.06)	2.40	5.86	–	–
iAs[c]	1.50 (1.50–2.10)	1.56 (1.56–2.26)	0.87 (0.71–0.92)	0.71	1.06	–	–
DMA	3.80 (3.00–4.00)	3.73 (3.27–4.63)	2.27 (1.94–2.75)	1.13	4.27	8%	0.5
MMA	0.60 (0.60–1.10)	0.64 (0.64–1.10)	<0.5	<0.5	0.53	74%	0.5
AsB	0.90 (0.70–1.40)	2.06 (1.19–2.87)	0.53 (0.35–0.78)	<0.5	2.29	47%	0.5

[a] NHANES data (NHANES, 2022). The NHANES urinary arsenic concentrations descriptive statistics were calculated using the "survey" package in R version 4.0.3 to account for the sample weights. The NHANES 2003-04 cycle contains 418 (96.87%) arsenite, 407 (93.34%) arsenate, 287 (65.82%) monomethylarsonic acid (MMA), 57 (13.07%) dimethylarsinic acid (DMA), and 138 (31.65%) arsenobetaine (AsB) values below the limit of detection (<LOD). The NHANES 2005-06 cycle contains 520 (97.74%) arsenite, 509 (95.67%) arsenate, 375 (70.48%) MMA, 74 (13.90%) DMA, and 152 (28.57%) AsB values < LOD. [b]Sum of iAs, MMA, and DMA. [c]Sum of arsenate and arsenite.

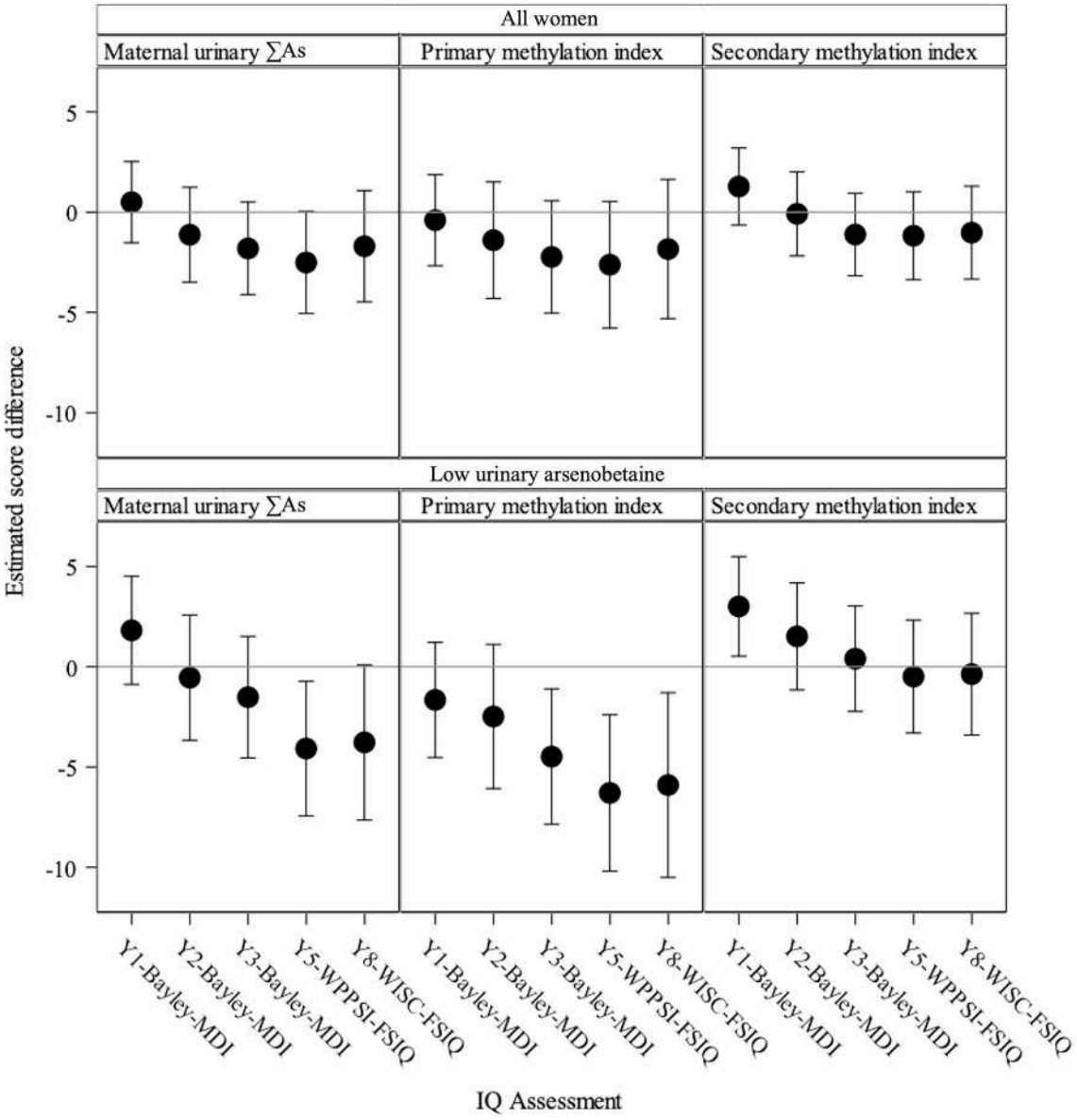

Fig. 1. Estimated beta coefficients and 95% CIs for child cognitive scores by a doubling increase in maternal prenatal arsenic concentrations (∑As), HOME Study among all women ($n = 260$) and among women with urinary arsenobetaine concentration <1 μg/L suggesting little, or no fish/seafood consumption ($n = 167$). All estimates are adjusted for household income, maternal race, maternal age at delivery, maternal intelligence quotient measured by Wechsler Abbreviated Scale of Intelligence, maternal pre-pregnancy body mass index (kg/m^2), $\log_{10}$-average serum cotinine in pregnancy (smoking), $\log_{10}$-urinary creatinine, HOME score, and child sex. Models for primary and secondary methylation indices are further adjusted for sum of maternal urinary arsenic concentrations (∑As).

maternal blood lead of 0.69 (0.31) μg/dL, and our sensitivity analyses showed that blood lead was weakly correlated with urinary ∑As (r = 0.13, *p*-value = 0.10), but did not correlate with urinary iAs, PMI or SMI (r < 0.08, *p*-value >0.18). The inclusion of maternal blood lead in the multivariable models did not change the regression coefficients for associations of any arsenic measure with MDI/FSIQ by >10% (Fig. S3). The analysis did not show evidence of effect measure modification of the associations of interest by child sex, maternal smoking, or maternal whole blood folate (data not shown).

4. Discussions

Fetal exposure to environmental toxicants such as arsenic may impact brain development with a marked effect throughout the lifespan (Grandjean and Landrigan, 2006, 2014). Oxidative stress, apoptosis, thiamine deficiency, and decreased acetyl cholinesterase activity are suggested arsenic-induced neurotoxic mechanisms (Ahmed et al., 2011; Mochizuki, 2019; Singh et al., 2011). Prior studies suggest that arsenic exposure associates with impaired cognitive abilities in populations living in water arsenic-contaminated regions (Hamadani et al., 2011; Nahar et al., 2014a, 2014b; Parvez et al., 2011; Rodrigues et al., 2016; Rosado et al., 2007; Vahter et al., 2020; Wasserman et al., 2004, 2007). However, the effects of arsenic neurotoxicity during vulnerable windows at levels relevant to the general US population and others, where public water arsenic concentrations are below 10 μg/L (EPA, 2001; US EPA, 2012; WHO, 2017, 2011), are not well established (Desai et al., 2018, 2020; Forns et al., 2014; Freire et al., 2018; Kordas et al., 2015; Liang et al., 2020; Signes-Pastor et al., 2019; Wasserman et al., 2014). While maternal urinary arsenic concentrations during pregnancy were relatively low in our study, they related to reduced cognitive scores during childhood. There was evidence that a lower maternal iAs methylation capacity may exacerbate the adverse effects.

In the present study, we did not observe a clear association between gestational arsenic exposure at levels relevant to the general US population and children's MDI at 1 and 2 years of age, but pregnancy urinary arsenic concentrations were associated with a reduction in MDI at 3 years, and FSIQ at 5 and 8 years of age. Other studies also reported that children ≥3 years of age showed impaired cognitive abilities related to prenatal exposure to toxicants such as mercury, polybrominated diphenyl ether (PBDEs), and chlorpyrifos, but not at earlier ages (Chen et al., 2014; Karagas et al., 2012; Rauh et al., 2006). While we were not able to consider these factors in our analysis, we do not anticipate they would be strongly associated with arsenic concentrations.

Although we did not observe associations between maternal urinary arsenic concentrations and cognitive abilities until age 3, some prior work from China (Liang et al., 2020; Wang et al., 2018), Nepal (Parajuli et al., 2013), and Bangladesh (Rodrigues et al., 2016; Valeri et al., 2017) found that gestational arsenic exposure at various levels may have an impact at earlier time points. In mother-infant pairs, cord blood arsenic concentrations related to a decrease in neonatal neurobehavioral scores (Wang et al., 2018) and increased risk of personal-social function at 6 months of age in China (Liang et al., 2020). Cord blood arsenic also related to reduced behavior responses and reflex scores at birth in Nepal (Parajuli et al., 2013), but the latter did not persist at 6 or 36 months of age (Parajuli et al., 2014, 2015). Studies from Bangladesh reported reduced IQ scores in 5-year-old children associated with urinary arsenic during pregnancy (Hamadani et al., 2011), but no relation with mental and psychomotor development indices at 18 months of age (Hamadani et al., 2010). Also, from Bangladesh, drinking water arsenic during pregnancy and cord blood and urine concentrations related to reduced cognitive function in children of ~3 (Rodrigues et al., 2016; Valeri et al., 2017) and ~10 (Vahter et al., 2020) years of age. However, another study from Bangladesh did not detect effects of gestational arsenic exposure assessed with maternal urinary arsenic on infants' problem-solving ability and motor development at 7 months (Tofail et al., 2009). Differences across neurodevelopmental domains, biological matrices used for exposure assessment, exposure levels, or participant characteristic across studies could in part explain these inconsistencies.

Among populations with lower levels of exposure, a study from Spain observed that detectable placenta arsenic concentrations were associated with impaired global and verbal executive abilities in children of 4-5-years of age (Freire et al., 2018). However, a prior study did not observe clear associations with maternal total urinary arsenic, which included AsB, and raises concerns of iAs exposure misclassification in this study (Forns et al., 2014). In the present study, we analyzed urinary arsenic species concentrations and calculated the summation of urinary iAs metabolites (i.e., iAs, MMA, and DMA excluding AsB) as a proxy for iAs exposure. In addition, we performed analysis restricted to women who were low consumers of fish/seafood (AsB <1 μg/L) (Navas-Acien et al., 2011; Signes-Pastor et al., 2020). In the above analysis, we observed stronger inverse associations of ∑As with FSIQ at 5 years and of PMI with MDI at 3 years and FSIQ at 5 and 8 years. Although, this sensitivity analysis was likely underpowered given the reduction in sample size, it suggests that accounting for the association of fish/-seafood consumption with arsenic exposure and neurodevelopment may be critically important for future research studies, especially among populations whose diets play a major role in arsenic exposure.

In this study, we found that a diminished iAs methylation capacity in mothers was inversely associated with child cognitive abilities. In humans, there is large inter-individual variation in methylation capacity of iAs and is characterized by the formation of DMA (60–70%) and MMA (10–20%) excreted along with unmetabolized iAs (10–30%) (Signes-Pastor et al., 2017a; Vahter, 2002). Altered profiles of urinary arsenic species in urine, which are genetically driven, appear to reflect differences in the efficacy of iAs metabolism (Agusa et al., 2011). In Taiwan, a stronger methylation capacity defined as higher urinary DMA% in 2-year-old children related to an increased cognitive and fine motor (Jiang et al., 2018). Thus, it is necessary to consider iAs methylation capacity when investigating the neurotoxicity of arsenic.

We did not have data on childhood exposure. However, prior studies suggest an inverse association between arsenic exposure during childhood and impaired neurodevelopment. Among ≤5-year-old children, urinary arsenic (median of 4.85 μg/L) related to a decreased in motor functions in Spain (Signes-Pastor et al., 2019). Urinary arsenic concentrations among 7-year-old children (median of 9.9 μg/L) were inversely associated with executive function in Uruguay (Desai et al., 2020), but not with the cognition (Desai et al., 2018; Kordas et al., 2015) in accordance with a recent study from China (Zhou et al., 2020). Reduced IQ and behavior scores were reported to be associated with children's biomarkers of arsenic exposure (e.g., blood, urine, nails, and hair) in Bangladesh (Hamadani et al., 2011; Nahar et al., 2014a, 2014b; Nahar and Inaoka, 2012; Vahter et al., 2020; Wasserman et al., 2011, 2016, 2018), India (Ghosh et al., 2017; Manju et al., 2017) and Mexico (Calderón et al., 2001; Roy et al., 2011). In the US, children consuming water arsenic ≥5 μg/L had lower IQ scores compared to those consuming water arsenic <5 μg/L (Wasserman et al., 2014). Several studies from China (Wang et al., 2007), India (Ehrenstein et al., 2007), Taiwan (Tsai et al., 2003), Bangladesh (Wasserman et al., 2004, 2007), and Mexico (Rocha-Amador et al., 2007) reported impaired cognitive ability associated with water arsenic exposure. A recent dose-response meta-analysis described a 0.08% decrease in IQ scale associated with each 1 μg/L increase in water arsenic concentration (Hasanvand et al., 2020). Studies from Italy (Lucchini et al., 2019) and Mexico (Rosado et al., 2007; Roy et al., 2011) found that proximity to industrial arsenic emissions may also affect children's cognitive abilities.

Exposure to environmental toxicants occur simultaneously as a mixture in real-life scenarios and their health impact may relate to the concentrations of each component of the mixture (Levin-Schwartz et al., 2019; Valeri et al., 2017; Wasserman et al., 2018). A negative effect of a mixture of arsenic, lead, and manganese assessed using cord blood concentrations, on children's cognitive abilities was reported in a

Bangladesh study (Valeri et al., 2017), and an additional study suggested that arsenic and cadmium exposures are the most important mixture components associated with a decrease in adolescent intelligence when applying the same flexible statistical methods (Wasserman et al., 2018). Other studies have applied multivariable-adjusted regression models to account for multiple exposures (Freire et al., 2018; Parajuli et al., 2015; Vahter et al., 2020). While little is known about the impact of multiple metal exposure, including arsenic, at relatively low levels on the development of cognitive abilities in childhood, in our study, maternal blood Pb concentrations did not appear to influence observed associations of arsenic with childhood cognition, but other neurotoxicants could confound or modify the effect of arsenic.

This study is based on a well-characterized US cohort (Braun et al., 2017) that counted on extensively trained research personnel to longitudinally assess children's cognitive abilities using established quality assurance/quality control (QA/QC) protocols (Bayley, 1993; Braun et al., 2017; Wechsler, 2003, 2004), and measured urinary arsenic species concentrations. While our findings are based on a modest sample size, we nevertheless observed that gestational exposure to arsenic may impair children's cognitive abilities, especially among older children whose mother had lower methylation capacity when adjusting for several potential confounding factors. Still, the effect of unknown factors or residual confounding, including from unknown or unmeasured co-exposures, remains a possibility. Our findings are among the first to suggest that even low-level arsenic exposure during vulnerable windows of growth and development may adversely impact children's congnitive abilities (Desai et al., 2020; Freire et al., 2018; Signes-Pastor et al., 2019; Wasserman et al., 2014). More prospective research is needed to confirm the relevant windows of exposure from gestation to early life on arsenic neurotoxicity at levels relevant to the general population and to evaluate cumulative exposures and mixture effects.

Credit authors contribution statement

Antonio J. Signes-Pastor (**AS**): Conceptualization, refinement of the statistical analytic plan, drafting of the manuscript, and critical review of the manuscript; Megan E. Romano (**MR**): Conceptualization, implementation of formal statistical analysis, drafting of the manuscript, and critical review of the manuscript; Brian Jackson (**BJ**): urine samples analysis and critical review of the manuscript; Joseph M. Braun (**JB**): refinement of the statistical analytic plan and critical review of the manuscript; Kimberly Yolton (**KY**): supervision of the neurodevelopmental tests and critical review of the manuscript; Aimin Chen (**AC**), Bruce Lanphear (**BL**), and Margaret Karagas (**MK**): Conceptualization, refinement of the statistical analytic plan, and critical review of the manuscript.

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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.2022.114009.

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Environmental release of non-Aroclor polychlorinated biphenyls by a silicone rubber production site did not lead to elevated plasma levels in the nearby population

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ABSTRACT

In 2019, high concentrations of the non-Aroclor PCB congeners 47, 51, and 68 were released by a silicone rubber production site in North-Rhine Westphalia, Germany. Local authorities announced a consumption alert for home-grown fruits and vegetables for the affected area which led to a great level of insecurity among the population regarding potential health effects. The aim of study was to determine the plasma levels of the non-Aroclor congeners and the six ndl-indicator congeners (PCB 28, 52, 101, 138, 153, 180) in children and women with child-bearing potential living close to the production site. Altogether n = 111 participants, with n = 73 female adults and n = 38 children were included in this analysis. For the non-Aroclor-PCBs as well as the lower-clorinated ndl-indicator PCBs 28, 52, and 101, the median plasma concentrations were below the LOQ. Only one adult showed an elevated PCB 47 value. In conclusion, no elevated plasma levels could be detected for PCB 47, 51, or 68 in the nearby population of the silicone-rubber production site. However, our study was highly important for risk characterisation as well as risk communication.

1. Introduction

For the first time in October 2018, residents from a small town in North-Rhine Westphalia, Germany, complained about the occurrence of white, pasty flakes in their gardens and the environment. Chemical analyses revealed high concentrations of the non-Aroclor PCB congeners 47, 51, and 68 in the flakes as well as in soil, air and bioindicators (Hombrecher et al., 2021). These tetrachlorinated PCBs are formed from thermal decomposition of bis(2,4-dichlorobenzoyl)peroxide (2,4-DCBP) that is used as initiator in the silicon rubber production process (Jan and Perdih, 1990; Perdih and Jan 1994). A local silicone rubber production plant was identified as the emitting source of these PCBs.

Concerning the congener distribution in dandelion, PCB 47 was the predominant congener among the three non-Aroclor PCBs, followed by PCB 68. For soil, highest PCB concentrations were detected in close vicinity to the production site (103 µg/kg soil). PCB 47 accounted for 70–80% to the sum of all congeners - being the predominant congener in soil, as well. Atmospheric air analyses revealed a concentration ranging from 2000 to 5000 pg/m^3 for the sum of the non-Aroclor PCBs in vicinity of the production site (Hombrecher et al., 2021). With the detection of the PCB emissions, the silicone rubber production site substituted the initiator 2,4-DCBP by at least 50% in 2020, leading to a significant decrease of environmental contamination. Repeated measures in the bio-indicator kale, for instance, detected a decrease of environmental contamination about 75% (Hombrecher et al., 2021).

PCBs are related to several adverse health effects. Dermatological, immunological, as well as metabolic abnormalities have been linked to a contamination with PCBs (Akahane et al., 2018; Fischbein et al. 1979, 1982; Haase et al., 2016; Safe 1993). In addition, the International Agency for Cancer Research (IARC) describes PCBs as carcinogenic to human (category 1) (International Agency for Research on Cancer (IARC), 2013). Usually, occupational and in particular nutritional exposure are the main routes for an uptake of the common Aroclor-PCB congeners. Consequently, local authorities announced a consumption alert for home-grown fruits and vegetables for the area around the silicon rubber production site. The occurrence of those flakes combined

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with the official consumption alert led to a high level of insecurity among the population. In collaboration with local health authorities, and the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (LANUV) we initiated a program in order to determine PCB plasma values in residents with focus on the non-Aroclor PCBs 47, 51, and 68, and to detect potential additional uptakes or limit value exceedances with health consequences.

For PCBs, the relevant limit value for the estimation of health effects at the environmental level is the human biomonitoring value (HBM-value) evaluated by the human biomonitoring commission of the German Environment Agency. The HBM-I value (human biomonitoring-I-value) describes the concentration below which health effects are not expected. The HBM-I-value is defined as the sum of ((PCB 138, 153, 180) x 2) and is defined as 3.5 μg/L (Umweltbundesamt, Kommission Human Biomonitoring, 2012). The HBM–II–value is set with 7.0 μg/L and corresponds to the concentration above which a health impairment is to be regarded as possible and relevant. Both HBM values apply to new-borns, infants as well as women with child-bearing potential. The aim of this program was to determine the plasma levels of the non-Aroclor congeners PCB 47, 51, and 68 as well as the six non-dioxine-like (ndl) indicator congeners PCB 28, 52, 101, 138, 153, and 180 in children and women with child-bearing potential living close to the silicon rubber production plant.

2. Materials and methods

The study took place in a local hospital in proximity to the residential area of the study population from October to November 2021. Inclusion criteria were an age between 6 and <18 years for all genders and a child bearing potential for participating adult women. A further inclusion criterion was a residency of at least five years close to the silicon rubber production plant where the consumption alert was announced. The study and the eligibility criteria for participation were officially announced by county authorities as well as the citizens' initiative.

EDTA-blood samples were taken via venepuncture. The (pseudonymized) blood samples were sent to our laboratory by courier and plasma was obtained by centrifugation of the blood samples (10 min at 850 *g*). The supernatant was transferred to pre-cleaned glass vials and stored at – 20 °C until analysis.

The analytical method for the determination of PCBs (including PCB 47, 68 and 51) in plasma has been described previously (12). Briefly, 2 ml of the plasma sample were deproteinized with formic acid. PCBs were then extracted with a solution of n-hexane containing the $^{13}C_{12}$-labelled internal standards ($^{13}C_{12}$-PCB 28, $^{13}C_{12}$-PCB 52, $^{13}C_{12}$-PCB 101, $^{13}C_{12}$-PCB 138, $^{13}C_{12}$-PCB 153 and $^{13}C_{12}$-PCB 180, 1 μg/L), purified on a silica gel column, eluted with petroleum benzene, carefully concentrated under nitrogen with 50 μL toluene as keeper and analysed by GC/EI-MS in Selected Ion Monitoring-Mode (SIM). For quantification of the non-Aroclor-PCBs, we have used $^{13}C_{12}$-PCB 52 as internal standard (see Figure S1). The limit of quantification (LOQ) – based on a signal to noise ratio of 6 – was set to 0.01 μg/L serum for all analytes investigated. We have prepared a matrix-matched calibration in bovine serum ranging from 0.04 to 3 μg/L. Glassware and reagents were carefully cleaned and blank values were included in each analytical series. For quality control, bovine serum was spiked with all analytes at a concentration of 0.4 μg/L and included in each analytical series. The Interday imprecision ranged from 2.9% to 5.1% for all analytes, with relative accuracy ranging from 95.5 to 104.2% for the different congeners. A section of the chromatogram of the processed plasma sample of a resident is shown in the Supplemental Files to this publication (Figure S1). The accuracy of our results is assured by the semi-annual successful participation in a proficiency test for the indicator-PCBs organised in Germany (www.g-equas.de). The results of our laboratory in this round robin in the last 12 years are summarized in the Supplemental Files to this manuscript. It has to be emphasized that the analytical staff was blinded concerning the proximity of the residents to the production site. The place of residence was revealed by the local authorities after the communication of the measurement values.

In addition, a questionnaire was handed out to the participants including information on occupational history, nutritional habits as well as current health complaints. Participants were asked, if and in which frequency they consumed home-grown fruit and vegetables. In addition, consumption of fish and seafood was queried. Further questions included the duration of staying outside in the garden or at a playground (for children). Statistical analyses were carried out using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Descriptive analyses were performed for all participants and categorized between children and adults. PCB plasma levels below the LOQ were set at LOQ/2.

This study was approved by the local ethics committee (EK 214/21) and all participants gave their written informed consent.

3. Results

Altogether n = 1520 persons in the county met the inclusion criteria. From those eligible persons, n = 149 filled out the questionnaire and n = 111 participated in the human biomonitoring analyses (n = 73 female adults and n = 38 children). Human biomonitoring data, demographic characteristics, and dietary habits can be found in Table 1.

Slightly more than every third participant (35.1%) stated, they would consume home-grown vegetables and fruits on a at least weekly basis. From those participants who answered they would never eat home-grown food, n = 11 commented they still avoid the consumption since the official consumption alert. 28.8% of all participants consumed fish and/or seafood on a weekly basis. An association between plasma PCB levels and consumption behaviour could not be detected.

For the non-Aroclor-PCBs as well as the lower-clorinated ndl-indicator PCBs 28, 52, and 101, the median plasma concentrations were below the LOQ. Only one adult showed an elevated PCB 47 value with a

Table 1
Demographics, dietary habits and PCB plasma levels of the six ndl-indicator congeners (PCB 28, 52, 138, 153, 180) and the non-Aroclor PCBs (PCB 51, 47, 68). Age- and sex-adjusted German reference values for all congeners can be found under the following link: https://link.springer.com/content/pdf/10.1007/s00103-016-2387-7.pdf (Umweltbundesamt, Kommission Human Biomonitoring, 2016)

Variables		All n = 111	Adults n = 73	Children n = 38
Sex female n (%)		94 (85)	73 (100)	21 (55)
Age median (min; max)		16 (6; 51)	36 (18; 51)	12 (6; 17)
Frequent consumption home-grown food n (%) (at least once/week)		39 (35.1)	25 (34.3)	14 (36.8)
Frequent consumption fish, seafood n (%) (at least once/week)		32 (28.8)	25 (34.3)	7 (18.4)
non-Aroclor PCBs median (max)	PCB 51	< LOQ (<LOQ)	< LOQ (<LOQ)	< LOQ (<LOQ)
	PCB 47	< LOQ (0.013)	< LOQ (0.013)	< LOQ (<LOQ)
	PCB 68	< LOQ (<LOQ)	< LOQ (<LOQ)	< LOQ (<LOQ)
ndl-indicator PCBs median (max)	PCB 28	< LOQ (0.03)	< LOQ (0.03)	< LOQ (0.01)
	PCB 52	< LOQ (0.01)	< LOQ (0.01)	< LOQ (<LOQ)
	PCB 101	< LOQ (<LOQ)	< LOQ (<LOQ)	< LOQ (<LOQ)
	PCB 138	0.06 (0.34)	0.07 (0.34)	0.04 (0.13)
	PCB 153	0.08 (0.44)	0.09 (0.44)	0.05 (0.22)
	PCB 180	0.05 (0.3)	0.06 (0.3)	0.03 (0.2)
Sum of ndl-indicator PCBs		0.2 (1.0)	0.24 (1.0)	0.14 (0.56)
PCB (138 + 153 + 180) x 2[a]		0.35 (2.0)	0.44 (2.0)	0.24 (1.1)

[a] HBM-I-value = 3.5 μg/L; HBM–II–value = 7 μg/L.

concentration of 0.013 µg/L. The higher-chlorinated PCBs (PCB 138, 153, 180) in the adults group were in median higher with 0.07 µg/L, 0.09 µg/L, and 0.06 µg/L, respectively, in comparison to the children with 0.04 µg/L, 0.05 µg/L, and 0.03 µg/L. All levels were within the reference range of the corresponding age-groups. Consequently, the HBM-I-value was not exceeded in any case. The age-dependency of the levels of the higher chlorinated PCBs is displayed in the Supplemental Material to this manuscript (Figure S1). Here, a clear age dependency can be derived, showing higher plasma concentrations of the PCBs 138, 153, and 180 with higher age.

Regarding the residency situation in terms of proximity to the production site, two persons were living less than 500 m next to the emitting source. One of those two participants had an elevated PCB 47 plasma level (Fig. 1). For no further participant, elevated non-Aroclor PCB levels could be determined.

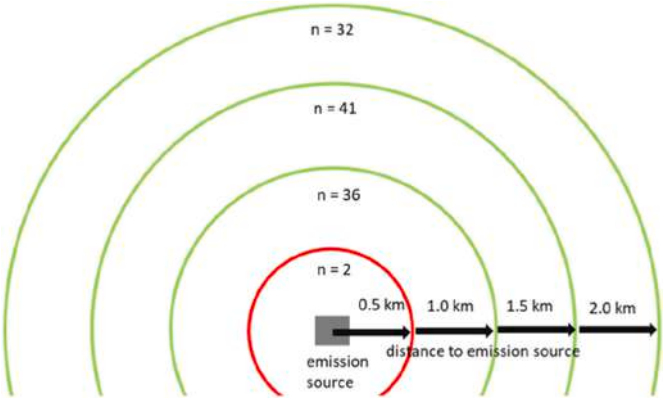

Fig. 1. Distance between silicon rubber production plant and housing of the study population. Red ring indicates elevated non-Aroclor PCB-level (PCB 47 plasma level elevated in one individual). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion and conclusion

Although the use of 2,4-DCBP in the silicone rubber production led to a considerable contamination of the surrounding environment (Hombrecher et al., 2021), no elevated plasma levels could be detected for PCB 47, 51, or 68 in the nearby population. Only one participant showed a slightly elevated PCB 47 plasma level above LOQ. This person was living closest to the production site with less than 500 m north of the site (in the main wind direction). Concerning dietary habits, this person did not eat any home-grown food or consumed fish more than once a week. PCB 47 was found to be the congener with the highest concentration also in environmental contamination measurements (Hombrecher et al., 2021), which is in accordance to our analyses. Close vicinity to the production site was the main contributor to environmental PCB contamination, as well. The housing of the other participant living less than 500 m next to the production site was southwest of the plant. The different directions of housing could explain the different PCB 47 plasma levels in terms of air transmission (wind direction), which has also been described by Hombrecher et al.

In contrast, occupational exposures in the silicone rubber industry frequently lead to elevated PCB plasma levels when 2,4-DCBP is used as initiator. In particular PCB 47 and 68 exceeding the LOQ of 0.01 µg/L were found to be elevated in the majority of German production workers in the silicone rubber industry with maximum PCB 47 plasma levels of 4.43 µg/L (Schettgen et al., 2022; Schettgen et al., 2022). The specific adverse health effects resulting from a non-Aroclor PCB contamination remain unclear. Neurotoxic effects due to metabolic activation are likely and have been described before (Fernandes et al., 2010; Hendriks et al., 2010).

Our study was highly important for risk characterisation as well as risk communication. As mentioned before, the residents were concerned about potential health effects caused by the PCB-containing flakes. Shortly after detection of the flakes, residents used social media to exchange about the current situation. Misinformation about potential health effects can cause great uncertainty within the residing population, although medical experts from governmental site as well as the local health authorities have commented in detail. In those situations, human biomonitoring can be used as an essential tool for risk assessment and communication.

Our study has clear strengths and limitations: a major strength is the sensitive and specific analytical method for these "unusual" PCB-congeners as well as the blinded study design that prevented any bias. However, our study has some important limitations that need to be addressed. First, our study population was small and may not be representative for the whole population of the affected area. Second, our biomonitoring analyses took place at the end of 2021. The flakes occurred initially in 2018, environmental contamination in bio-indicators was detected in 2019 with decreasing contamination levels in 2020. Shortly after the discovery of the environmental contamination caused by the silicone rubber production plant in 2019, the plant has gradually converted production to mainly use a chlorine-free, alternative initiator, leading to a large decrease in PCB-emissions. Thus, the study was conducted at a time-point when the exposure situation clearly has improved, which was mainly caused by the Covid-19-pandemic that meanwhile prevented the implementation of a human study. However, although there are currently no data available about plasma half-lives of PCB 47 and PCB 68 in humans, it can be expected to range from about 1 to 5 years with respect to previously published human data on similar congeners (Esser et al., 2021). It might be likely, that the population was contaminated and the non-Aroclor PCBs were already metabolized to other compounds, such as OH-PCBs or PCB sulfates (Quinete et al. 2016, 2017), and these compounds were not measured in this study. These important limitations need to be considered.

Author contribution statement

Andrea Kaifie, formal analysis, resources, writing (original draft, review and editing); André Esser, conceptualization, formal analysis, writing (review and editing); Patrick Ziegler, writing (review and editing); Thomas Kraus, conceptualization, resources, writing (review and editing); Knut Rauchfuss study design, supervision of the project and professional support; Thomas Schettgen, conceptualization, formal analysis, supervision, writing (review and editing). All authors read and approved the final manuscript.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://

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Global practices, geographic variation, and determinants of child feces disposal in 42 low- and middle-income countries: An analysis of standardized cross-sectional national surveys from 2016 – 2020

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ABSTRACT

Background: Despite considerable progress improving water and sanitation access globally, unsafe child feces disposal remains common in many low- and middle-income countries (LMICs), posing an important health risk. The present study characterizes the current prevalence of child feces disposal practices and child latrine use across low- and middle-income countries and investigates determinants associated with appropriate disposal practices.

Methods: Data for children ranging from 0 through 4 years of age were analyzed from standardized and nationally-representative surveys of 42 LMICs collected from 2016 to 2020 to assess child feces disposal practices. We report child feces disposal in three categories: disposal in any type of latrine, disposal in an improved latrine, and disposal through means other than in a latrine. Survey weighted multiple Poisson regression models were used to explore factors associated with these practices.

Results: Data on 403,036 children (weighted N = 191 million) demonstrated that a minority (40.3%) of children have their feces disposed of in a latrine of any kind, and just 29% have feces disposed of in an improved latrine. Prevalence varied considerably by country and region. In adjusted analyses, both child feces disposal in any latrine and disposal in an improved latrine increased with child age, higher intra-country relative wealth, and urban living, and decreased with breastfeeding and shared sanitation facilities. Disposal in improved latrines additionally increased with access to higher levels of service for drinking water and higher mother's education. Nevertheless, the role of facility access alone was insufficient, as only about half of children with household access to any latrine or improved latrines had their feces disposed of in these facilities. Child latrine use among households with latrine access was also low and highly variable across countries.

Conclusions: Children's feces in LMICs are infrequently disposed of in any latrine type, and even less frequently in improved latrines. In order to minimize health risks in LMICs, increased effort must be undertaken not just to increase sanitation coverage but to address these common barriers to safe child feces disposal and child latrine use.

1. Introduction

Poor access to sanitation in low- and middle-income countries (LMICs) is associated with a large burden of disease, including diarrheal disease, soil-transmitted helminth infections, schistosomiasis, trachoma, and child undernutrition (Freeman et al., 2017; Prüss-Ustün et al., 2019). However, even households with access to sanitation facilities often do not dispose of their young children's feces into their latrine when the child defecates elsewhere (Bauza et al., 2019b; Bauza and Guest, 2017; Majorin et al., 2017). Inadequate disposal of child feces presents a significant source of exposure and associated health risks. Young children often have underdeveloped immune systems and more frequent diarrheal disease which may lead to higher pathogen loads in their feces (Feachem et al., 1984; Walker, 2018). It is also common for young children to defecate inside or close to households, with past research identifying fecal contamination from young children's feces to be more common inside households than contamination from older children or adult's feces (Bauza et al., 2019a). As a result, susceptible

Abbreviations: DAL, Disposal of child's feces in any type of latrine; DIL, Disposal of child's feces in an improved latrine; CFD, Child feces disposal.

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children within the same or nearby households may be more likely to be exposed to feces from other young children, as children spend much time on the ground engaging in exploratory behaviors that include mouthing of hands, objects, and soil (Bauza et al., 2018; Kwong et al., 2016; Moya et al., 2004; Ngure et al., 2013). Consistent with this potential exposure route, past research has found unsafe child feces disposal to be associated with diarrhea (Majorin et al., 2019b), soil-transmitted helminth infection (Roy et al., 2011), environment enteric dysfunction (George et al., 2016), and stunting (Bauza and Guest, 2017) in children.

For children's feces to be safely managed, all points of potential exposure to pathogens from the feces must be blocked, including at the defecation and feces disposal sites as well as the material used for feces handling, child and caregiver hands, and the site and any materials used for anal cleansing (Bauza et al., 2020; Majorin et al., 2017). Despite the noted importance of many of these exposure points in the World Health Organization's *Guidelines on Sanitation and Health* (WHO 2018), international monitoring focuses exclusively on the disposal site of child feces. Historically, the WHO/UNICEF Joint Monitoring Program on Water, Sanitation and Hygiene (JMP) has defined "safe" child feces disposal as a child using a toilet facility or the child's feces being put into a latrine or buried, with the type of toilet facility not being considered. However, burial was later recommended against as a safe method of disposal following an expert consultation due in part to potential for contamination from buried feces to spread from animals or rain (Bain and Luyendijk, 2015). More recently, the JMP has updated what they consider to be "appropriate" disposal of child feces to include a child using an improved latrine or their feces being disposed of in an improved latrine or disposed with solid waste if that solid waste is stored, collected, and disposed of in a sanitary manner (UNICEF/WHO 2018).

Although some past studies have measured the scope or determinants of safe disposal of child feces, these studies are usually on a local or regional level within a specific country (Azage and Haile, 2015; Bauza et al., 2019b; Majorin et al., 2017; Sahiledengle, 2020) or region (Seidu et al., 2021). Moreover, no large multi-country studies have documented the scope and variation of child latrine use in LMICs, a behavior which also eliminates other sources of exposure that could be associated with defecation outside the latrine such as feces handling or contamination of the site of defecation. Overall, there is still limited evidence on the scope and determinants of safe CFD and child latrine use in LMICs on a global level based on recent data.

The objective of this research is to characterize the prevalence of different child feces disposal practices in LMICs and assess the personal, household, environmental, and community factors that are associated with safe disposal in a latrine. A secondary objective is to characterize the scope and variation of child latrine use across child age and countries. The knowledge from this study can help identify the scope and enabling factors to safe child feces disposal practices in LMICs.

2. Methods

2.1. Data sources

We analyzed data collected from households with young children from nationally representative surveys conducted within the past five years (2016–2020) in 42 LMICs within Sub-Saharan Africa, South Asia, East Asia and Pacific, Latin America and Caribbean, and Middle East and North Africa regions. This includes data from both Demographic and Health Survey (DHS) and Multiple Indicator Cluster Survey (MICS) datasets. All country surveys from LMICs in this time period which asked questions on child feces disposal (CFD) were included.

The DHS survey is administered by USAID in LMICs to women aged 15–49 in households selected by a stratified random sample designed to be representative of the population of the country and asks questions regarding household characteristics and women and children's health (Corsi et al., 2012). The DHS survey administers a CFD question regarding only the youngest child under 2 years old (except Afghanistan, India, and Myanmar, where the question is asked of the youngest child under 5 years old). The question on CFD is posed as: "The last time [name of child] passed stools, what was done to dispose of the stools?"(UNICEF/WHO, 2018). Possible responses include: 'child used toilet/latrine,' 'put/rinsed into toilet or latrine,' 'put/rinsed into drain or ditch,' 'thrown into garbage (solid waste),' 'buried,' 'left in the open,' or 'other.' The MICS survey is similarly designed and nationally representative, and is administered by UNICEF in sections to the head of household and women aged 15–49. While many questions cover all children up to 5 years old, the CFD question is posed regarding only children under 3 years old (Khan and Hancioglu, 2019). The question is posed in the same way as DHS.

2.2. Child feces disposal practice definitions

The Joint Monitoring Program on Water, Sanitation and Hygiene (JMP) guidelines categorizes sanitation facilities as 'improved' or 'unimproved'. 'Improved latrines' are "those designed to hygienically separate excreta from human contact, and include: flush/pour flush toilets connected to piped sewer systems, septic tanks or pit latrines; pit latrines with slabs (including ventilated pit latrines), and composting toilets"; unimproved latrines are pit latrines without a slab or platform, hanging latrines or bucket latrines (UNICEF/WHO, 2021). The JMP now employs additional rungs in its 'sanitation ladder' with 'open defecation' at the bottom, followed by 'unimproved latrines,' and improved latrines further categorized depending on whether they are shared ('limited'), unshared ('basic') or unshared with fecal waste safely disposed in-situ or treated offsite ('safely managed'). Data on fecal waste management classifications of improved latrines are unavailable for DHS datasets for the years covered by this analysis, so 'basic' and 'safely managed' are combined into a single category.

Child feces disposal was analyzed in two parallel ways, each as binary outcomes (Fig. 1). First, binary outcomes of disposal in any type of latrine (DAL = 'yes; ' defined by responses of 'used latrine,' or 'put/rinsed into latrine' to the child feces disposal question) were compared against disposal not in a latrine (DAL = 'no; ' defined by responses of 'put/rinsed into a drain or ditch,' 'thrown in garbage/solid waste,' 'buried,' 'left in open/not disposed of,' or 'other' to the child feces disposal question) (Analysis 1). As 'safely managed sanitation' requires as a starting point that feces be contained in an 'improved' latrine, the second analysis compared children whose feces are disposed of in an improved latrine (DIL = 'yes') against children whose feces were not disposal of in an improved latrine (DIL = 'no') (Analysis 2). For this purpose, disposal in improved latrines was defined by the respondent indicating that the last time the child defecated the child 'used latrine' or their feces were 'put/rinsed into latrine' *and* the respondent indicated that the household uses a latrine that met the 'safely managed,' 'basic,' or 'limited' definitions of 'improved' sanitation. Disposal not in improved latrines was defined by respondents indicating that the last time the child defecated the feces were 'put/rinsed into a drain or ditch,' 'thrown in garbage/solid waste,' 'buried,' 'left in open/not disposed of,' or 'other,' *or* if they used a latrine but the latrine in the household only met the JMP definition for an 'unimproved' latrine. As the included surveys did not allow us to verify if solid waste was stored, collected, and disposed of in a sanitary manner, we have classified the disposal of child feces with solid waste as inadequate disposal for this analysis, similar to other disposal options that were not in a latrine. Additionally, as safe disposal of child feces is a behavioral practice and is not simply a function of having access to a latrine, additional analyses were conducted to explore the extent to which the subset of households with access to any latrine (Analysis 3) and improved latrines (Analysis 4) reported using the same for the disposal of child feces.

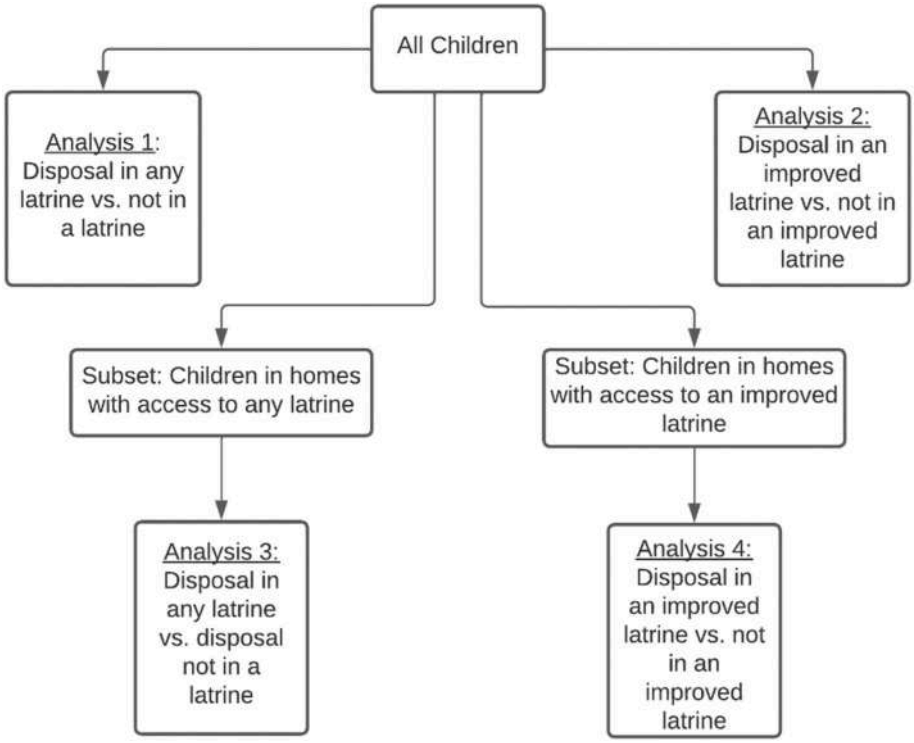

Fig. 1. Diagram of each binary analysis to investigate child feces disposal practices. Analyses include (1) whether disposal is in any latrine (DAL), (2) whether disposal is in an improved latrine (DIL), (3) DAL conditional on access to any latrine and (4) DIL conditional on access to an improved latrine.

2.3. Predictor variable definitions

The ages of children and mothers were recorded in months and converted to decimal years. The education level of mothers was grouped into three categories: less than primary, primary, and secondary or higher education levels. Whether or not the child is currently being breastfed was recorded. Intra-country wealth quintiles at the household level were calculated by DHS and MICS methodology from all households surveyed (i.e. not just from those included in this dataset). Urbanicity, defined in the original datasets as 'urban' or 'rural,' or 'camp,' was re-grouped to include the camp with rural designation due to sparse data (n = 446 and n = 1,084 children from Suriname and the State of Palestine, respectively). The number of other children under 5 years and total persons in the house was recorded in original datasets, and these variables were grouped as none versus 1 or more additional children (2 or more total children <5 years in the households), and less than five persons versus 6 or more, respectively, based on distributions of the data. Whether or not a latrine is shared amongst multiple families was included as a binary variable.

The quality of drinking water was included based on the JMP ladder grouped into four categories: a group of 'surface water' and 'unimproved' together (due to sparse surface water data), 'limited,' 'basic,' and 'safely managed' (UNICEF/WHO, 2018). The source of water for sanitation and hygiene was recorded only for MICS datasets, and therefore not included in the analysis.

2.4. Sample weighting

Due to the hierarchical cluster sampling procedures of the DHS and MICS protocols, the samples are weighted to be nationally representative (Corsi et al., 2012). DHS datasets weight data at the level of women respondents, and only ask about CFD for their youngest child under 2 years old (or under 5 years old in select countries). In MICS datasets, data are weighted at the level of the child (under 5 years old) while CFD questions were only asked regarding children 0–2 years old. Therefore, to compare weighted data across countries, weights were first denormalized by multiplying each weight by the UN estimated 2015 population of women aged 15–49 (for DHS) or children under 5 (for MICS), then dividing by the total number of surveyed women for that country (from DHS country-specific final reports), or the number of children (from MICS country-specific final reports) (Corsi et al., 2012). Survey weights were denormalized from each country prior to prevalence calculations, descriptive analyses, and statistical analyses to allow for pooled data interpretation.

2.5. Statistical analysis

All analyses were performed in STATA v16 (StataCorp LLC, College Station, TX, USA). Sample weighting and hierarchical cluster sampling was accounted for in our analysis by using the '*svyset*' command. Primary sampling units (PSU) were set by the survey design in each country, and the sampling strata were formed by combining the country, national region, and urban vs. rural designation. Descriptive univariate analyses accounting for survey design and weighting, including prevalence of outcome variables by country and region, were conducted using the denormalized weights.

Multivariate Poisson regression was used to assess potential determinants of child feces disposal practices. Estimates of prevalence ratios (PRs) for variables associated with child feces disposal practices (DAL and DIL, Analyses 1 and 2, respectively) were generated using survey-weighted multiple Poisson regression models using denormalized weights so data were representative and comparable across countries, and using clustering at the sampling strata (primary sampling unit) level. To account for general between-country differences, each country was adjusted for by treating it as a fixed factor in the model (results shown in supplement). To further explore factors associated with DAL and DIL after accounting for sanitation facility access alone, these models were run again after restricting to only observations with household access to any sanitation facility (for DAL as the response, Analysis 3) and access to an improved sanitation facility (for DIL as the

response, Analysis 4).

2.6. Sensitivity analyses

To explore how robust this analysis was, the model was run using a number of permutations. For all analyses except one, the denormalized weighting and survey clustering scheme of the main analysis was used. India accounted for roughly 40% of the observations, therefore we ran the analyses dropping India from the dataset to test if the results were overly influenced by this single country (New n = 217,607, 185,429 observations deleted). We also tested an alternative weighting scheme in which every country was given equal weight.

To standardize the population age between all datasets, we performed an analysis with all children older than 2-years-old dropped (thereby dropping observations of children 2 years old from MICS datasets and dropping children 2 and older for select DHS datasets which included information on children less than 5 years old; new n = 266,817, and 136,760 observations deleted). We also conducted an analysis in which we additionally standardized the household level sampling methodology between DHS and MICS datasets by including from MICS only the youngest child of each mother under 2 years old (new n = 357,865, and 47,204 observations deleted). We also accounted for possible correlation among observations from within the same household (MICS only) by adding an additional clustering term to the model at the household level.

Table 1
Description of the data sources used in the analysis. Sample sizes (unweighted and *denormalized* weights), the estimated total population of children in the country from UN projections of 2015[a], and the weighted composition of each country to the region and the overall dataset are displayed.

Region	Country	Survey Type	Year Survey Completed	N children surveyed	Weighted N children	2015 Child Population in Age Range[a]	Age Range (Years)	Weighted Percent by Region	Weighted Percent Total Dataset
East Asia and Pacific	Indonesia	DHS	2017	6,658	8,957,166	10,164,628	0–1	52.7	4.68
	Mongolia	MICS	2018	3,420	199,898	226,958	0–2	1.18	0.1
	Myanmar	DHS	2016	3,767	3,984,821	4,574,474	0–4	23.44	2.08
	Philippines	DHS	2017	3,766	3,795,277	4,663,949	0–1	22.33	1.99
	Timor-Leste	DHS	2016	2,692	60,685	64,277	0–1	0.36	0.03
	Total			**20,303**	**16,997,847**				**8.89**
Latin America and Caribbean	Costa Rica	MICS	2018	2,057	207,164	214,947	0–2	20.34	0.11
	Cuba	MICS	2019	2,870	345,900	387,010	0–2	33.97	0.18
	Haiti	DHS	2017	2,352	437,216	514,473	0–1	42.94	0.23
	Suriname	MICS	2018	2,371	27,987	31,972	0–2	2.75	0.01
	Total			**9,650**	**1,018,267**				**0.53**
Middle East and North Africa	Algeria	MICS	2019	8,623	2,645,683	2,849,428	0–2	40.29	1.38
	Iraq	MICS	2018	9,572	2,959,383	3,253,717	0–2	45.06	1.55
	State of Palestine	MICS	2020	3,906	412,594	416,280	0–2	6.28	0.22
	Tunisia	MICS	2018	1,883	549,640	633,660	0–2	8.37	0.29
	Total			23,984	6,567,300				3.43
South Asia	Afghanistan	DHS	2016	19,207	4,997,566	5,500,914	0–4	4.41	2.61
	Bangladesh	MICS	2019	13,570	8,069,534	8,811,102	0–2	7.12	4.22
	India	DHS	2016	185,429	86,410,488	118,983,308	0–4	76.23	45.2
	Maldives	DHS	2017	1,136	14,969	14,485	0–1	0.01	0.01
	Nepal	MICS	2016	3,719	1,554,700	1,682,271	0–2	1.37	0.81
	Pakistan	DHS	2018	4,477	12,310,060	10,710,158	0–1	10.86	6.44
	Total			**227,538**	**113,357,317**				**59.29**
Sub-Saharan Africa	Angola	DHS	2016	5629	2,303,263	2,166,002	0–1	4.33	1.2
	Benin	DHS	2018	5,265	821,713	725,532	0–1	1.54	0.43
	Burundi	DHS	2017	5,094	720,716	780,147	0–1	1.35	8.89
	Cameroon	DHS	2018	3,562	1,524,666	1,582,119	0–1	2.86	0.8
	Central African Republic	MICS	2019	4,992	402,799	444,428	0–2	0.76	0.21
	Chad	MICS	2019	11,824	1,452,347	1,632,666	0–2	2.73	0.76
	Democratic Republic of the Congo	MICS	2018	12,393	8,122,085	8,778,256	0–2	15.25	4.25
	Ethiopia	DHS	2016	3,914	6,365,234	6,451,717	0–1	11.95	3.33
	Ghana	MICS	2018	4,989	2,194,674	2,428,707	0–2	4.12	1.15
	Guinea	DHS	2018	2,825	951,202	793,358	0–1	1.79	0.5
	Lesotho	MICS	2018	1,652	112,509	152,151	0–2	0.21	0.06
	Madagascar	MICS	2018	7,462	2,091,566	2,263,462	0–2	3.93	1.09
	Malawi	DHS	2016	6,383	1,033,245	1,112,165	0–1	1.94	0.54
	Nigeria	DHS	2018	12,076	12,046,369	13,079,711	0–1	22.62	6.3
	Sao Tome and Principe	MICS	2019	1,051	17,504	18,756	0–2	0.03	0.01
	Senegal	DHS	2018	5,168	1,786,208	1,014,324	0–1	3.35	0.93
	Sierra Leone	DHS	2019	3,637	396,997	449,300	0–1	0.75	0.21
	South Africa	DHS	2016	1,223	2,160,530	2,318,621	0–1	4.06	1.13
	Tanzania	DHS	2016	4,035	3,601,115	3,609,576	0–1	6.76	1.88
	The Gambia	MICS	2018	5,530	200,969	227,019	0–2	0.38	0.11
	Uganda	DHS	2016	5,642	2,666,624	2,917,723	0–1	5.01	1.39
	Zambia	DHS	2019	3,804	1,028,679	1,141,807	0–1	1.93	0.54
	Zimbabwe	MICS	2019	3,411	1,252,703	1,393,489	0–2	2.35	0.66
	Total			**121,561**	**53,253,717**				**27.85**
Grand total				**403,036**	**191,194,448**	**229,179,047**			

[a] UN data population estimates for 2015 for children aged 0–2 years for MICS countries, for 0–1 years for DHS countries, except Afghanistan, Myanmar, and India, which were from 0 to 4 years.

3. Results

3.1. Sample demographic characteristics

The final sample for analysis included 42 countries and 403,036 children (N = 389,611 with sufficient information on sanitation included necessary for DIL definition). These were comprised of 24 DHS datasets (n = 297,741 children (73.78%)), and 18 MICS datasets (n = 105,836 children (26.22%)). Following denormalization, the weighted data represent more than 191 million observations, and are intended to be a representative sample of the more than 229 million children living in the LMICs included in this dataset (Table 1).

Overall the total dataset spanned five regions, with the majority of countries from Sub-Saharan Africa (23 out of 42). Among included children, there were slightly more male than female children (47.6% female). The mean age was 1.16 years, and 65.8% of children were currently breastfeeding at the time of the interview. 66.9% of children were from rural areas, with roughly 20% in each of five wealth quintiles. 51.7% of children were in a household with 2 or more children under 5 years old, and 55.7% were in households of 6 or more persons (see Table S1 for summary of weighted descriptive data). Mothers were an average of 27.7 years old, and 51.0% had secondary or higher education, with 20.5% attaining only primary and 28.5% attaining less than primary education. There was considerable variation in these demographic characteristics among countries (see Table S2 for country-specific socio-demographic data).

Sanitation and water access were moderate across the study population. In the overall dataset, 29.3% of children lived in households that practiced open defecation, while 12.9% had access to unimproved sanitation facilities, 13.0% had access to limited sanitation facilities, and 44.8% had access to basic or safely managed sanitation facilities, according to the JMP guidelines. 24.1% of children were from households that shared their sanitation facility. 13.6% of households had access to only surface or unimproved water, 17.8% had access to limited water, 24.7% had access to basic water, and 43.9% had access to safely managed water. There was also considerable variation in water and sanitation among countries (see Table S3 for country-specific data on sanitation and water).

Table 2
Prevalence (shown as percentages) of specific methods of child feces disposal (CFD) using denormalized weights by country, including prevalence of DAL (defined as final deposition of child feces into any latrine), and of DIL (defined as final deposition of child feces in a "limited," "basic" or "safely managed" sanitation facility based on JMP ladder).

	Survey-Reported Disposal Site							Latrine Disposal	
	Used latrine	Put/rinsed into latrine	Put/rinsed into drain or ditch	Thrown in garbage	Buried	Left in open/not disposed of	Other	DAL	DIL
Afghanistan	21.5	13.3	16.6	15.4	9.1	22.1	2.0	34.8	30.6
Algeria	13.9	4.3	1.8	78.9	0.2	0.2	0.8	18.1	17.4
Angola	2.5	25.0	0.0	56.1	3.9	8.2	4.3	27.5	22.7
Bangladesh	9.1	40.2	29.5	13.3	0.6	7.1	0.3	49.3	44.1
Benin	0.6	30.8	1.9	58.5	3.4	3.2	1.6	31.4	18.3
Burundi	0.9	72.8	6.4	6.5	4.7	3.6	5.1	73.7	37.3
Cameroon	1.4	63.6	9.1	21.1	1.1	3.6	0.1	65.1	36.9
Central African Republic	3.7	43.3	10.1	28.0	2.7	9.8	2.5	47	12.3
Chad	0.8	12.6	6.1	47.5	10.3	20.7	2.1	13.4	7.2
Costa Rica	16.2	4.6	1.7	75.2	1.6	0.1	0.6	20.8	20.3
Cuba	31.3	56.9	4.9	6.3	0.1	0.5	0.1	88.2	77.8
Democratic Republic of the Congo	1.9	57.5	14.1	15.5	3.9	4.1	3.0	59.4	20.7
Ethiopia	0.7	36.2	3.7	18.3	2.8	25.5	12.8	36.9	5.2
Ghana	2.5	20.7	7.4	54.6	7.1	3.9	3.9	23.1	17.0
Guinea	1.9	52.9	8.8	26.5	3.1	6.8	0.0	54.8	30.8
Haiti	0.4	63.5	4.6	21.4	3.0	5.5	1.6	63.9	37.2
India	22.0	12.7	5.3	14.2	1.5	43.7	0.5	34.8	28.4
Indonesia	7.6	38.0	14.6	32.8	3.3	0.6	3.1	45.6	38.5
Iraq	8.6	7.3	1.7	80.0	0.3	1.6	0.6	15.8	14.3
Lesotho	5.0	47.1	4.1	16.0	7.9	17.2	2.7	52.1	45.6
Madagascar	2.0	24.1	2.9	7.6	4.5	53.5	5.3	26.1	7.0
Malawi	3.3	80.2	7.9	4.1	2.0	2.1	0.5	83.5	69.1
Maldives	5.1	4.0	0.5	89.3	0.4	0.0	0.6	9.1	8.2
Mongolia	3.4	47.0	3.8	34.2	2.1	6.3	3.3	50.4	47.7
Myanmar	23.5	36.2	18.1	7.0	2.8	12.1	0.4	59.7	34.2
Nepal	19.9	50.5	3.1	15.0	0.1	10.5	1.0	70.3	68.8
Nigeria	1.8	53.1	8.0	30.5	1.6	4.5	0.5	54.9	31.8
Pakistan	4.0	31.7	15.0	44.3	0.3	4.4	0.3	35.7	32.0
Philippines	4.0	6.2	5.5	74.4	7.4	1.2	1.3	10.2	9.1
Sao Tome and Principe	8.4	8.4	11.0	33.3	7.1	28.0	3.8	16.8	14.5
Senegal	0.3	60.9	1.7	33.5	1.4	1.0	1.1	61.2	47.0
Sierra Leone	1.8	60.2	16.5	17.4	2.2	1.9	0.0	62.1	35.7
South Africa	4.1	11.6	4.3	77.0	2.2	0.4	0.4	15.7	10.0
State of Palestine	21.0	4.1	0.5	74.3	0.0	0.1	0.0	25.1	24.5
Suriname	6.8	5.3	2.7	80.1	2.4	1.6	1.0	12.1	11.6
Tanzania	1.1	66.8	6.1	9.1	4.0	6.3	6.6	67.9	21.8
The Gambia	5.1	72.7	4.5	16.2	0.8	0.6	0.1	77.8	44.3
Timor-Leste	9.6	15.8	5.2	20.0	2.5	46.9	0.1	25.5	19.1
Tunisia	11.1	4.0	2.1	81.0	0.3	0.8	0.8	15	14.8
Uganda	2.5	73.8	9.4	5.5	4.5	4.2	0.0	76.3	27.2
Zambia	1.4	72.3	8.8	8.9	4.9	0.9	2.8	73.7	39.3
Zimbabwe	3.9	60.2	3.7	9.2	16.0	5.6	1.3	64.2	54.3
Overall	**13.2**	**27.0**	**8.4**	**23.6**	**2.3**	**23.9**	**1.5**	**40.3**	**29.0**

3.2. Child feces disposal prevalence and scope

Disposal of feces in a latrine of any kind (DAL) was reported for 40.3% of children, while disposal of feces in a latrine meeting the 'improved' standard (DIL) was reported for only 29.0% of children (Table 2). There was wide variation in the prevalence of DAL across countries and regionally, as seen in Fig. 2 (Table 2; DIL shown in Fig. S1). Overall 13.2% of children used a latrine directly, while 27% of children had their feces deposited into a latrine (presumably by a caregiver), and 23.9% of children's feces were left in the open or not disposed of in any manner (Table 2). There was considerable variation among countries in child feces disposal practices.

3.3. Child latrine use by age

Child latrine use for defecation was generally low, but increased with child age. Among all children with access to a latrine, 24.5% were reported to use it directly, compared to 29.4% of children with access to a basic or safely managed latrine who were reported to use it directly. Direct latrine use increased from 9.4% among those <1-year-old to 55.5% among 4-year-olds for any latrine type, and from 12.1% among those <1-year-old to 57.9% among 4-year-olds for improved latrines (Fig. 3A). The positive trend of latrine use and age was consistent across countries. However, there was considerable variation among countries in both absolute values and strength of the trend. Among those with access to any latrine, prevalence ranged from a low of 2.8% of 2-year-old children directly using a latrine in Chad, to a high of 55.5% in the State of Palestine, followed by India which reported 38.9%. Among children with access to a basic or safely managed latrine, the proportion of direct use was slightly higher at all ages than use of any latrine, and showed the same increasing trend as children got older (Fig. 3B). There was also considerable variation in both absolute values and the strength of the trend (Fig. S2). There was little variation in access to facilities across age groups (Table S5).

3.4. Determinants of child feces disposal practices among all households

In the adjusted multiple Poisson regression model, the factors associated with disposal in any latrine (Analysis 1) were generally similar to those associated with disposal in an improved latrine (Analysis 2), with a few exceptions. Across both models, wealth was the strongest predictor of feces disposal practices, although the effect of wealth was higher in the improved disposal model. As compared the poorest quintile, each successive quintile from poorer to richest were each associated with a DAL increase of 8%, 19%, 31%, and 41%, respectively (aPR = 1.08, 1.05–1.11; aPR = 1.19, 1.16–1.23; aPR = 1.31, 1.27–1.35; aPR = 1.41, 1.36–1.46, Table 3). Similarly for improved disposal, each successive quintile were each associated with successively greater DIL increases compared to the poorest quintile, with the highest increase seen for the richest quintile (aPR = 5.48, 5.19–5.78, Table 3).

Other factors with stronger associations and effect sizes in at least one the two models were child age, urbanicity, current breastfeeding of the child, mother's education level, level of water access, and level of sanitation access. Older children were more likely to exhibit both DAL

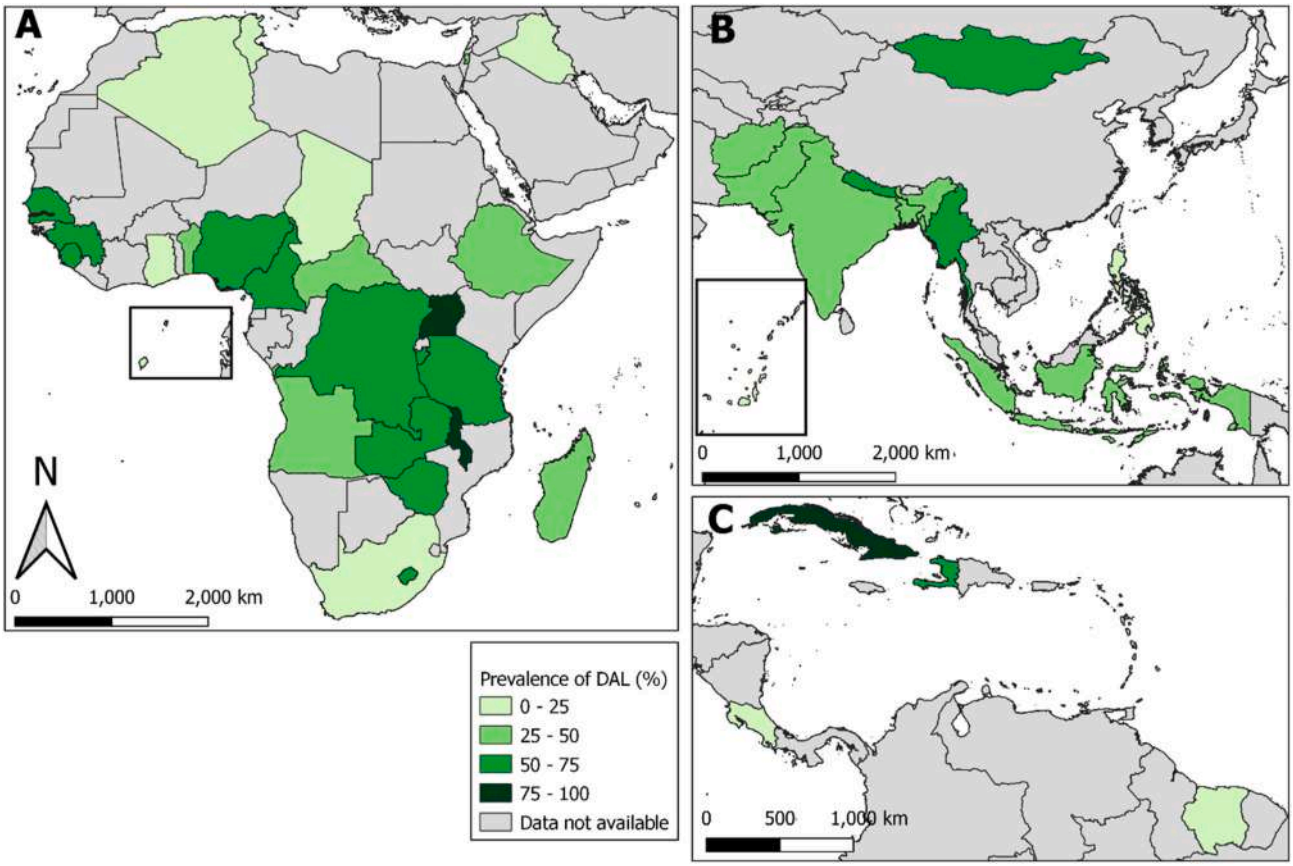

Fig. 2. Map of the prevalence of safe disposal of feces in a latrine of any kind (DAL) across LMICs included in the DHS and MICS surveys from 2016 to 2020. Grey countries did not have data included in this analysis, and darker green corresponds to higher prevalence of DAL. (A) depicts the countries included from WHO designated regions of Sub-Saharan Africa and North Africa and the Middle East, including an inset for Sao Tome and Principe. (B) depicts the regions of South Asia and East Asia and the Pacific, with an inset for the most populated islands of the Maldives. (C) depicts the countries included from Latin America and the Caribbean. A map of the prevalence of disposal in improved latrines (DIL) shown in the supplement (Fig. S1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

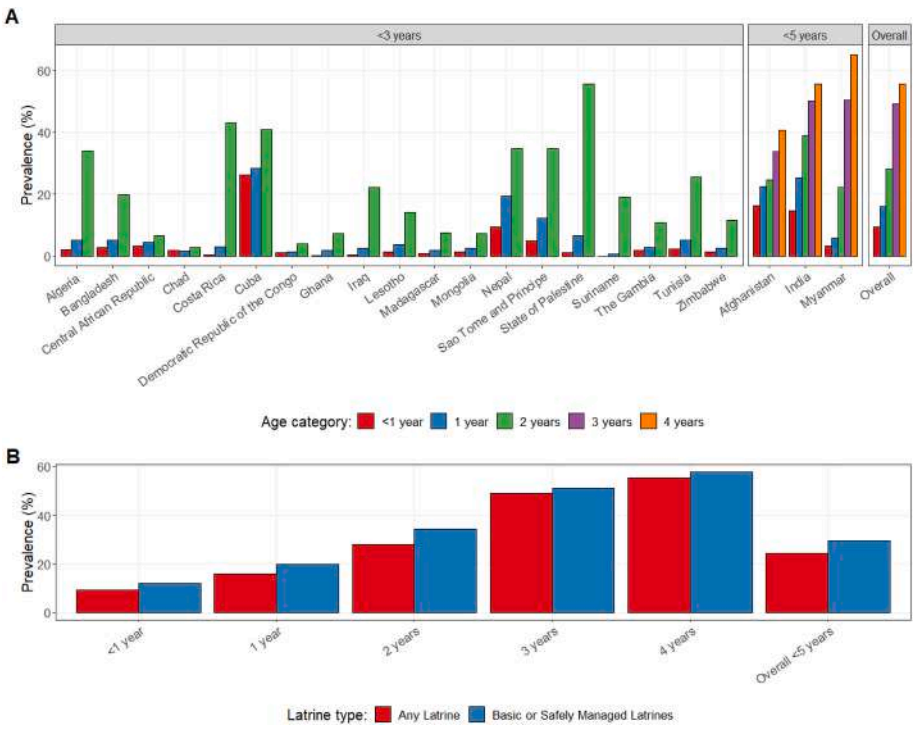

Fig. 3. Prevalence of direct latrine use among the child's age categories (in years) (A) for any latrine shown by country, and (B) comparing child latrine use in any latrine or a basic/safely managed latrine given access to either. Data for 3-year-old and 4-year-old children are only available from India, Myanmar, and Afghanistan. All prevalence values calculated as frequency among households with access to any or a basic or safely managed latrine, respectively. Only countries which asked the CFD question of children up through at least 2 years old were included (all MICS datasets and India, Myanmar, and Afghanistan from DHS). Percentages are shown using denormalized weights. All values are shown in Table S4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Adjusted prevalence ratios (aPR), 95% confidence interval (CI), and *p* values from multiple Poisson regression of predictor variables (reference categories in parentheses) on disposal in any latrine (DAL) and disposal in an improved latrine (DIL), accounting survey design and using de-normalized weighting, countries included as fixed effects (shown in Tables S6 and S7).

	Levels	Disposal in any latrine			Disposal in improved latrine		
Variable (referent)		aPR	95% CI	p	aPR	95% CI	p
Child Sex *(Female)*	*Male*	0.99	(0.98–1.001)	0.075	0.997	(0.98–1.014)	0.748
Child Age *(In years)*		**1.19**	**(1.18–1.2)**	**< 0.001**	**1.20**	**(1.19–1.22)**	**< 0.001**
Mother Age *(In years)*		**1.001**	**(1–1.002)**	**0.046**	**1.004**	**(1.003–1.006)**	**< 0.001**
Urbanicity *(Rural)*	*Urban*	**1.11**	**(1.083–1.13)**	**< 0.001**	**1.23**	**(1.2–1.27)**	**< 0.001**
Breastfeeding *(Not breastfeeding)*	*Yes*	**0.95**	**(0.93–0.96)**	**< 0.001**	**0.94**	**(0.92–0.96)**	**< 0.001**
Mother's Education *(Less than primary)*	*Primary*	1.02	(0.99–1.05)	0.13	**1.17**	**(1.12–1.209)**	**< 0.001**
	Secondary or higher	0.99	(0.97–1.02)	0.528	**1.18**	**(1.14–1.23)**	**< 0.001**
Number of children under 5 *(1)*	*2 or more*	**1.03**	**(1.01–1.05)**	**< 0.001**	**1.03**	**(1.01–1.05)**	**0.003**
Number of persons in the household *(<5)*	*6 or more*	0.99	(0.97–1)	0.054	**0.96**	**(0.94–0.98)**	**< 0.001**
JMP Water Ladder *(Surface and unimproved)*	*Limited*	**0.95**	**(0.91–0.99)**	**0.008**	**0.86**	**(0.8–0.92)**	**< 0.001**
	Basic	0.97	(0.94–1.01)	0.14	**1.09**	**(1.02–1.15)**	**0.006**
	Safely Managed	0.99	(0.98–1.05)	0.384	**1.38**	**(1.3–1.46)**	**< 0.001**
JMP Sanitation Ladder *(Open defecation)*	*Unimproved*	**1.18**	**(1.09–1.28)**	**< 0.001**	–	–	–
	Limited	**1.15**	**(1.06–1.25)**	**0.001**	–	–	–
	Basic/Safely Managed	**1.18**	**(1.1–1.27)**	**< 0.001**	–	–	–
Wealth quintiles *(Poorest 20%)*	*Poorer*	**1.08**	**(1.05–1.11)**	**< 0.001**	**2.12**	**(2.01–2.24)**	**< 0.001**
	Middle	**1.19**	**(1.16–1.23)**	**< 0.001**	**3.29**	**(3.12–3.46)**	**< 0.001**
	Richer	**1.31**	**(1.27–1.35)**	**< 0.001**	**4.59**	**(4.36–4.84)**	**< 0.001**
	Richest	**1.41**	**(1.36–1.46)**	**< 0.001**	**5.48**	**(5.19–5.78)**	**< 0.001**
Shared Latrine *(Not shared)*	*Shared*	**0.95**	**(0.91–0.98)**	**0.006**	–	–	–

Reference categories: Female, Rural, Non-breastfeeding, Less than primary education, 0–1 children under 5, 1–5 people in the household, Surface and unimproved, Open defecation, Poorest 20%, and Non-shared sanitation facility.

and DIL, with an increase in DAL of 19% and an increase in DIL of 20% associated with each year increase in age (aPR = 1.19, 95% CI: 1.18–1.2; aPR = 1.2, 95% CI: 1.19–1.22, respectively; Table 3). Living in an urban environment was associated with an 11% DAL increase over rural environments (aPR = 1.11, 95% CI: 1.08–1.13; Table 3), and a greater DIL increase of 23% over rural environments (aPR = 1.23, 95% CI: 1.2–1.27; Table 3). Being a child currently breastfeeding, regardless of other food consumed, was associated with a DAL decrease of 5% over children not breastfeeding (aPR = 0.95, 95% CI: 0.93–0.96; Table 3), and was associated with a similar DIL decrease of 6% over children not breastfeeding (aPR = 0.94, 95% CI: 0.92–0.96; Table 3). Mother's education level was not associated with DAL, but it was associated with DIL, with a DIL increase of 17% associated with primary education and an 18% increase associated with secondary or higher education as compared to less than primary education (aPR = 1.17, 95% CI: 1.12–1.21; aPR = 1.18, 95% CI: 1.14–1.23, respectively; Table 3). The household's water source was more strongly associated with DIL than DAL, such that compared to surface water and unimproved sources, limited water sources were associated with a 14% DIL reduction (aPR = 0.86, 95% CI: 0.8–0.92), basic water sources associated with a 9% DIL increase (aPR = 1.09, 95%

CI: 1.02–1.15), and safely managed water sources associated with a DIL increase of 38% (aPR = 1.38, 95% CI: 1.3–1.46; Table 3). Compared to open defecation, having household access to a sanitation facility classified as 'unimproved' was associated with a DAL increase of 18% (aPR = 1.18, 1.09–1.28; Table 3), 'limited' facility access was associated with a DAL increase of 15% (aPR = 1.15, 1.06–1.25; Table 3), and 'basic or safely managed' facility access was associated with a DAL increase of 18% (aPR = 1.39, 1.1–1.27; Table 3). However, sharing household sanitation facilities was associated with a DAL decrease of 5% as opposed to not sharing facilities (aPR = 0.95, 0.91–0.98; Table 3).

Other factors with weak associations or effect sizes included mother's age, number of children under five years in the household, and number of persons in the household. Child sex was not significantly associated with DIL or DAL (Table 3).

3.5. Determinants of child feces disposal practices among households with access to sanitation

Restricting analyses to only observations for children with household access to a latrine of any kind (new n = 2270, 885 observations, DAL as the response, Analysis 3) or an improved latrine (new n = 222,097 observations, DIL as the response, Analysis 4) indicated that only 52.2% of observations with household access to any latrine engaged in DAL, and among those with access to an improved facility, only 51.5% engaged in DIL. Household access to latrines of any kind and improved latrines were highly variable between countries, as were their respective uses for CFD conditional on household access (Fig. S3). The models indicated overall quantitatively similar results to the main analysis, especially for DAL (see Table S8). The magnitude of some associations with DIL differed, including a slight decrease in the association with urbanicity and DIL (from aPR = 1.23, 1.2–1.27, to aPR = 1.10, 1.07–1.12; Table S8), a decrease to non-significant association of mother's education and DIL (from 18% increases associated with primary and secondary or higher education compared to less than primary), and weaker but still significant positive associations between DIL and wealth quintiles (from over 200% increases to 13%, 26%, 39%, and 50% increases associated with each successive quintile compared to the poorest; Table S8). The direction of a single association changed: a switch from a 9% increase in DIL to an 8% decrease versus surface and unimproved water (Table S8) and a non-significant association between DIL with safely managed water (from a 38% increase versus surface and unimproved water; Table S8).

3.6. Sensitivity analyses

Results from sensitivity analyses indicate the main results were highly robust across the different permutations of the analysis (see Tables S9 and S10 for DAL and DIL, respectively). The main analysis and the sensitivity analysis model results using three different methods to control for the differences between DHS and MICS (i.e. dropping children over 2 years old, using only the youngest child under 2, and including a mother clustering term) were nearly identical to one another. The results of models that weighted countries equally or included all countries except India were similar to one another, and overall the results were similar to those of the main analysis, although the association between wealth quintiles and both DAL and DIL were weaker.

4. Discussion

This study used a large, nationally representative dataset from 42 LMICs to explore the scope of child feces disposal practices and determinants of proper disposal in a latrine. Results showed that proper disposal in a latrine was low overall, and not explained by latrine access alone but instead influenced by several factors. Child latrine use was also low and highly variable for children of the same age across countries, suggesting that children in many LMICs are likely developmentally ready to use the latrine at younger ages than initiated. These results can inform the scope and enabling factors to safe child feces disposal practices in LMICs.

4.1. Prevalence of child feces disposal practices

Across the LMICs surveyed, the prevalence of child feces disposal in a latrine was low, at only forty percent, and lower for disposal in improved latrines at only twenty-nine percent. Despite gains in WASH coverage over the past few decades (UNICEF/WHO, 2021), there are still many children (and their families) at risk of being exposed to pathogens from unsafe child feces disposal practices. There was considerable heterogeneity among countries in the prevalence of disposal of child feces in latrines. Among the LMICs surveyed, countries in central and eastern Africa showed the highest prevalence of DAL, though many of these same countries showed lower relative prevalence of DIL, likely due to lower levels of access to improved latrines (Table S3). North African countries and Afghanistan showed low DAL and DIL overall. Caribbean and Latin American coverage was limited, but showed a mix of both DAL and DIL prevalence in the region. Even within these regions, considerable variation was observed here and elsewhere (Seidu et al., 2021). While much of this variation may be due to differences in sanitation access and behavioral and normative factors around child feces disposal across countries and regions, solid waste infrastructure may also play a role in this variation. Wealthier countries with more developed solid waste infrastructure may enable more contained disposal of child feces with solid waste, making that disposal alternative more desirable to households than disposal in a latrine. However, sufficient information on solid waste infrastructure was not included in these datasets, so could not be assessed as part of this analysis.

The prevalence values of safe CFD from individual countries are similar to those reported elsewhere (Azage and Haile, 2015; Nkoka, 2020; Sahiledengle, 2020; Seidu et al., 2021). While few reports have aggregated many countries in this manner, the considerable between country variation in DAL and DIL prevalence observed here has been noted elsewhere as well (Seidu et al., 2021). This variation may reflect factors operating at national or community levels such as historical and cultural attitudes (Novotný et al., 2017), political and economic stability (Als et al., 2020), and differences in public health messaging and WASH infrastructure investment (Beardsley et al., 2021). While further work should explore inter-related effects of factors across national, community, and household scales, the factors in the present analysis focus only on household and individual level attributes common across countries.

Child feces management improvements may have an even greater impact in countries with a relatively larger proportion of their population under 5 years old, such as Afghanistan, Angola, Madagascar, and Ethiopia, where greater than 15% of the total population is under 5 years old (UN World Population Prospects, 2020). These countries each exhibited a prevalence of child feces disposal in any latrine less than 40%, and efforts in these large countries to increase adequate CFD behavior should be highlighted.

4.2. Factors associated with child feces disposal practices

Disposal of child feces in latrines increased with age, consistent with prior work (Nkoka, 2020; Sahiledengle, 2020; Seidu et al., 2021). Increased direct latrine use amongst older children may contribute to this, however a slightly larger effect was observed after restricting the ages to less than 2-years-old, meaning that even among 0–1 and 1-2-year-olds the effect holds. Some caregivers may believe their very young children's feces is not harmful (Majorin et al., 2014), making them less likely to dispose of this feces in latrines. This may also explain the observed negative association between current breastfeeding and disposal of child feces in latrines. The potential for older, ambulatory children to spread fecal pathogens through unsafe CFD can be greater

than in young children, so while this trend is positive, there is still considerable room for improvement, especially among older children under 5 years old. Early interventions may be critical in this effort to avoid potential habit forming among young children openly defecating (Bauza et al., 2020). Child sex was not associated with either DAL or DIL, consistent with other multinational studies (Seidu et al., 2021), however sex-specific considerations in public health interventions may still be warranted.

The positive associations between mother's age and DAL and DIL were extremely small, and coupled with other null findings (Azage and Haile, 2015), suggests this variable holds little relevance to public health. Interestingly, the mother's education was not associated with DAL, but there was an increase of DIL associated with mother's education level being anything greater than primary education. Improvements to safe CFD with mother's education is consistent with work in individual countries (Nkoka, 2020; Sahiledengle, 2020), and multiple country assessments (Beardsley et al., 2021). Educated mothers may be more likely to understand the importance of safe CFD practices for disease control (Dreibelbis et al., 2013; Mwambete and Joseph, 2010), and child caretakers (often women) are typically responsible for CFD, suggesting that expanding women's education could increase appropriate CFD as well as other beneficial health and well-being outcomes.

Urban living was associated with modest DAL and DIL increases over rural living, which has been observed in Ethiopia (Azage and Haile, 2015; Sahiledengle, 2020). Access to latrines, and improved latrines, tends to be greater in urban areas (UNICEF/WHO, 2021), and open defecation may be socially unacceptable in more urban areas.

The strongest factor associated with both DAL and DIL was the intra-country relative household wealth, which is frequently observed in studies of CFD (Azage and Haile, 2015; Sahiledengle, 2020), and may be related to either greater access to latrines themselves, or information regarding the safety, importance, and methods for safe CFD (Nkoka, 2020), or residual confounding (Majorin et al., 2019a).

Neither 'basic' nor 'safely managed' water sources were associated with a difference in DAL compared to 'surface water' or 'unimproved' sources, however they were associated with minor and modest DIL increases, respectively. This likely reflects that household access to these higher categories of improved water sources often accompanies access to improved sanitation. The minor reduction in DAL, and the stronger reduction in DIL associated with 'limited' water sources may reflect that 'limited' water sources are improved but more than 30 min round-trip from the home. Not having nearby access to water may accompany a lack of nearby access to sanitation facilities as well, resulting in a barrier to using those latrines for CFD, even if used by adults, as reported in one study in Odisha, India (Majorin et al., 2019a). Water access is also important for caregiver handwashing after CFD, cleaning any tools used to handle child feces, and flushing the latrine, if applicable. In this dataset, a quarter of households with safely managed water reported open defecation or unimproved latrines, implying there is still considerable disparity in sanitation after accounting for water source improvements. These results suggest that improvements to CFD will not *necessarily* come from improving water sources without also improving sanitation, but slight improvements may come from making access to water easier, which may accompany increasing sanitation facility access in cost effective ways. These findings are consistent with prior null associations from Ethiopia (Azage and Haile, 2015), and one study which found non-significant associations in Ethiopia, India, and Zambia (Beardsley et al., 2021).

4.3. Access to sanitation facilities

While over half of children in these LMICs were from households with access to improved latrines, access to sanitation facilities does not necessarily correspond to their use for CFD. Indeed, just half of those with access to a latrine actually dispose of their child's feces in it, and the same pattern holds for improved facilities, meaning there are other barriers or knowledge gaps to using latrines for disposing of child's fecal waste. DAL was modestly associated with access to a sanitation facility of any kind, however the specific type of facility (unimproved, limited, or basic/safely managed) does not appear to make a difference. Sanitation facilities higher on the JMP ladder do not appear to increase its use for CFD, as evidenced by the fact that the aPR's for each of the JMP sanitation ladder categories were almost exactly the same in our model, consistent with one study from Bangladesh (George et al., 2016), but in contrast to other studies which found that improved latrines were associated with increased safe CFD (Azage and Haile, 2015; Beardsley et al., 2021; Majorin et al., 2019a; Sahiledengle, 2020). These findings suggest that to increase safe CFD, expanding availability of any kind of latrine may be sufficient, as long as the latrine is able to contain the fecal waste.

When analyses were restricted to observations for children with household access to a latrine, the model of DAL was almost exactly the same, and DIL showed minor magnitude changes. These findings indicate that our analyses identify determinants of child feces disposal practices instead of latrine access, and underscore the notion that for CFD in any kind of latrine, the associated factors described here operate independently of access, and access alone is insufficient to drive CFD behavior. The two associations with DIL that changed in this sub-analysis were associations between DIL and a basic and safely managed water sources compared to surface and unimproved water, and the strength of the association of DIL with wealth quintiles. These patterns highlight that once improved latrine access is accounted for, water sources have little effect on CFD, and that even among those with improved latrine access, relative wealth is an important factor associated with CFD.

There was a slight reduction in DAL associated with facilities being shared, consistent with findings from Ghana and India (Majorin et al., 2019a; Ritter et al., 2018). This reduction may also be due to children being less likely to use shared facilities, which may sometimes be less clean than private latrines or have a fee associated with use. With respect to increasing CFD coverage, further cost-benefit analyses may be warranted to evaluate interventions seeking to improve sanitation facility coverage and the ratio of households to sanitation facilities built.

Flush toilet infrastructure and public sewage can be prohibitively expensive in rural and low-income regions, however in such settings basic and safely managed sanitation under JMP standards can be achieved using improved latrines. Even among the fifteen countries included here with greater than 50% of households having access to flush or pour-flush toilets, the mean use of such facilities for CFD was less than 35%, further indicating that facility access alone does not determine usage for CFD.

4.4. Child latrine use

Levels of child latrine use were low and highly variable across countries for children of the same age. Despite considerable variation in prevalence across countries, there was a positive trend of increasing direct latrine use with age within a country that was consistently observed. However, less than two-thirds of surveyed children with access to latrines are using them directly by 5 years old including only one-third of 2-year-olds, and more concerted efforts to improve this behavior are warranted to limit fecal contamination (Bauza et al., 2020), especially in sub-Saharan Africa. Latrines that are improved appear to increase this behavior slightly, however the direction of causality is unclear and latrine use among children with access to improved latrines was still low. The high variability of 2-year-old children using the latrine from 2.8% in Chad to a 55% in the State of Palestine, suggests that children are likely developmentally ready to use a latrine by this age, but there are likely other barriers to this behavior. Further research into the factors that influence this behavior are warranted, as well as interventions designed to reduce these barriers and enable younger child latrine training and use (Bauza et al., 2020; Sclar et al., 2022).

Currently, DHS only collects data on CFD practices for children under two, assuming that children's sanitation practices can be accounted for by the overall household sanitation practice (such as latrine use) once they are two years old. However, our analysis demonstrates that this is unlikely, and CFD practices for children that are two years and older are needed to better understand sanitation practices of a household and potential interventions to limit exposure to sanitation-related pathogens. This is especially critical amid calls for 'transformative WASH' interventions that 'radically reduce fecal contamination in the household environment' in order to realize health benefits (Pickering et al., 2019). While DHS used to collect CFD information for the youngest child under five years in a household, this was changed to the youngest children under two years with the release of the DHS-VII version of the survey without explanation. Our results present a strong case that DHS and MICS surveys should include all children under 5 years old when asking about CFD. While this may slightly increase survey time, there is great insight to be gained from richer data on children up to 5 years old, which can be used to better tailor behavioral and health interventions beyond just child feces management.

4.5. Sensitivity analyses

The similarity of results from the main analysis to the three models controlling for differences between DHS and MICS suggest that these discrepancies are minor, and more importantly that the trends in this analysis are robust. While the two weighting methods (denormalized and equal-country) provided qualitatively similar results, the denormalized weighting method used in the main analysis is more representative of the total population surveyed and therefore more representative of the target population of all children in LMICs. The analysis was robust to the over-representation of India in the dataset, as the model without India was very similar to the main results. One factor that decreased in magnitude was the association between intra-country relative wealth quintile and DAL and DIL, suggesting that while there were still strong associations with these wealth quintiles, the magnitude in the main dataset may be influenced by India, where wealth inequality is exceptionally high.

4.6. Limitations

Because this is a large pooled analysis, the exact values of the associations reported here may not exactly reflect those observed in a single country, but they benefit from greater generalizability to LMICs globally. The analysis is representative of millions of children from 42 different countries and multiple world regions, providing strong insight on common global trends. The cross-sectional nature of the study limits causal inference, but the broad scope again highlights global trends. Additionally, as child feces disposal practices were self-reported, this could have introduced reporting bias and potential overreporting of perceived desirable hygienic behaviors (Curtis et al., 1993; ManuN'ebo et al., 1997). If this reporting bias exists in our dataset, our estimates would be likely overestimate safe CFD, meaning the true levels could be even lower than our estimates.

Deposition of child feces via disposable diapers or some other methods into the garbage could be adequate for handling child feces, depending on solid waste infrastructure and storage and handling methods. However, such analysis is limited because only MICS records information on solid waste infrastructure, and is further limited by respondent knowledge. Therefore, the decision to classify solid waste disposal as failing to meet JMP 'improved' or 'appropriate' guidelines may have introduced differential mis-classification bias with respect to wealthier countries or areas with good solid waste management practices. The expense of disposable diapers suggests only wealthier families are likely to use them, and such infrastructure is often limited to wealthier countries or neighborhoods, and therefore potential misclassification would bias the effect of wealth toward the null. Solid waste disposal accounted for 23.6% of this dataset, and inadequate management of solid waste is common in LMICs, so the total bias was not likely very strong.

While children with disabilities may have difficulty using a latrine, information on child disability is extremely limited in DHS and MICS datasets, limiting our ability to discuss a possible role that disability may have in child feces disposal and latrine use practices.

5. Conclusions

The present analysis of a large nationally representative dataset for 42 LMICs demonstrates that adequate child feces disposal in a latrine and child latrine use were generally low and highly geographically variable. Only half of children with household access to any latrine or improved latrines had their feces disposed of in such facilities, indicating factors other than access drive child feces disposal behavior. Disposal of child feces in a latrine increased with child age, urban settings, higher income, and improved water or sanitation sources, and mother's education only was associated with increased disposal in improved latrines. These results were also highly robust across several sensitivity analyses. Additionally, few children had transitioned to latrine use by age two, indicating the importance of collecting CFD data for older children, particularly in nationally representative surveys like DHS. Overall, children's feces in LMICs are infrequently disposed of in any latrine type, and even less frequently in improved latrines. In order to minimize health risks in LMICs, increased effort must be undertaken not just to increase sanitation coverage but to address these common barriers to safe child feces disposal and child latrine use.

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Data availability

Data are freely available and accessible through written permission of DHS and MICS programs.

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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.2022.114024.

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Hot water plumbing in residences and office buildings have distinctive risk of *Legionella pneumophila* contamination

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ABSTRACT

Aim: To observe how *Legionella pneumophila*, the causative agent for legionellosis, can transmit through the hot water plumbing of residences and office buildings.

Method and results: Using qPCR, *L. pneumophila* and *L. pneumophila* Serogroup (Sg)1 were measured in hot water samples collected from 100 structures, consisting of 70 residences and 30 office buildings. The hot water samples collected from office buildings had a higher *L. pneumophila* detection frequency of 53% (16/30) than residences, with a 10^3 GU/L (median) concentration. An office building's age was not a statistically significant predictor of contamination, but its area (>100,000 sq. ft.) was, $P = <0.001$. Hot water samples collected at residences had a lower *L. pneumophila* detection frequency of 36% (25/70) than office buildings, with a 100 GU/L (median) concentration. A residence's age was a significant predictor of contamination, $P = 0.009$, but not its area. The water's secondary disinfectant type did not affect *L. pneumophila* detection frequency nor its concentration in residences, but the secondary disinfectant type did affect results in office buildings. *Legionella pneumophila's* highest detection frequencies were in samples collected in March–August for office buildings and in June–November for residences.

Conclusion: This study revealed that the built environment influences *L. pneumophila* transport and fate. Residential plumbing could be a potential "conduit" for *L. pneumophila* exposure from a source upstream of the hot water environment. Both old and newly built office buildings had an equal probability of *L. pneumophila* contamination. *Legionella*-related remediation efforts in office buildings (that contain commercial functions only) might not significantly improve a community's public health.

1. Introduction

Legionella spp. is an environmental bacterium that causes the human respiratory disease, legionellosis. Legionellosis is contracted by breathing or aspirating soil or water droplets contaminated with *Legionella*. Legionellosis is associated with two clinical manifestations: Legionnaires' disease (severe) and Pontiac fever (mild). Legionnaires' disease signs and symptoms are similar to pneumonia (Mandell et al., 2007).

In the United States, between 2000 and 2017, the Legionnaires' disease rate increased 5.5-fold from 0.42 to 2.29 per 100,000 persons (Barskey et al., 2020). In 2017, among the Legionnaires' disease cases reported to the Centers for Disease Control and Prevention (CDC) National Notifiable Disease Surveillance System (NNDSS), the case fatality rate was 7% (Barskey et al., 2020). Most Legionnaires' disease cases are reported in the Summer to Fall months. Lower case numbers are reported in the Winter to Spring (Barskey et al., 2022). In 2014 the estimated cost in the United States for Legionnaires' disease treatment was 402 million dollars (Collier et al., 2021).

In 1976, the United States' first recognized outbreak of Legionnaires' Disease occurred at Philadelphia's Bellevue-Stratford Grand Hotel (TIME, 1976). The bacteria, *L. pneumophila* had contaminated the water supply that the building used for its air conditioning cooling system, enabling exposure via aerosolization. Since the 1976 outbreak, many legionellosis cases and outbreaks have been caused by exposure to contaminated water aerosols in either a residence or office building setting (Garrison et al., 2016; Schumacher et al., 2020; Smith et al., 2019). In addition, many studies have detected *L. pneumophila* in hot water samples from residential and office buildings (Collins et al., 2017;

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Donohue et al., 2019a; Stout et al., 1992) (Buse et al., 2020; Donohue et al., 2019a, 2019b; Flannery et al., 2006; Moore et al., 2006; Pierre et al., 2019; Schwake et al., 2016).

In situations where there is a diagnosis of Legionnaires' disease, a residence or office building's hot water supply is often assumed to be the source of transmission (Garrison et al., 2016; Stout et al., 1992a; Wadowsky et al., 1982). The hot water environment provides a unique opportunity where a microbe can survive and multiply. The elevated temperature simultaneously increases a microbe's growth rate and decreases the amount of chemical disinfectant (e.g., chlorine or chloramine) in the water (Brazeau and Edwards, 2013; Cullom et al., 2020). The primary reason why a disinfectant is added to drinking water is to prevent microbial growth. Research by Jacangelo et al. (2002) and States et al. (1989); shows that the disinfectant type and its amount influence the likelihood of *L. pneumophila's* detection and growth. Since not all disinfectants have the same efficacy in preventing microbial growth, it is important to identify the factors (physical and chemical) that are associated with the risk of microbial contamination.

The objective of this study was to measure *L. pneumophila* contamination (detection frequency and concentration) in the hot water plumbing lines of three structure types: residences, apartments, and office buildings. A longitudinal sampling approach was utilized to determine if a structure had a sporadic (a single positive sample) or persistent (multiple positive samples) occurrence pattern. Additionally, a structure's age and area (sq. ft.) were assessed to determine if they were indicators of contamination. In addition to characterizing *L. pneumophila* incidence in the three structure types, the hot water's disinfectant residual was measured to identify the concentration at which efficacy was lost. Also, *L. pneumophila* environmental monthly detection rates by structure type were compared to CDC's legionellosis monthly case reporting data to determine if there are shared patterns. We hypothesize that a building's structural parameters could be risk factors for *L. pneumophila* contamination and the spread of legionellosis.

2. Materials and methods

Study Design. From January 2011 to October 2019, hot water samples from 100 taps in offices and residences were tested for *L. pneumophila* contamination. The offices and residences were in 31 states across the United States. The structures in this study were geographically dispersed, had no known *L. pneumophila* contamination, and were not associated with any legionellosis investigation. Only one tap at each structure was sampled at three independent time points within a year. There was an average three-month gap between sampling events. A total of 296 water samples were collected at 66 single-family homes (195 samples), four apartments (12 samples), and 30 office buildings (89 samples). Three single-family homes and one office building only had two sampling events due to the participant moving away from the structure. All sampling locations received public water. Secondary disinfectant usage was determined through the location's water quality report.

Following a standard operating procedure (SOP), participants collected water samples from a hot water tap at their structure. Briefly, the procedure required the participant to flush the line for 15 s, then collect the water in a 1-L high-density polypropylene (HDPP) bottle (Nalgene Inc., Rochester, NY). The 15-s water flush ensured that the water collected was from behind the hot-cold water interface in cases where a single faucet delivered both hot and cold water. Additionally, the 15-s flush also models how a person may collect a glass of water. The US Environmental Protection Agency's (EPA) drinking water guidelines are based on drinking water exposure modeling. Once the water sample was collected, it was packed in an insulated box with ice packs and returned overnight to the EPA laboratory. Upon arrival at the EPA laboratory, the water samples were immediately processed.

Residual Testing. Upon the sample arrival, the water's total chlorine concentration was measured colorimetrically using the N, N-diethyl-p-phenylenediamine (DPD) method (Hach Company, Loveland, CO). The manufacturer's instructions were followed. The color reaction was measured using a DR 3900 spectrophotometer (Hach Company).

Structure Characteristic: The year the structure was built and its square footage were obtained from publicly available property information databases. Square footage was used as a surrogate for building complexity and as an inference for the presence of a complex plumbing system (e.g., dead ends, pipe bends, and underutilized taps). The structure age was determined by subtracting the year of the last sample collection from the year of building construction.

Definitions**.** The following are the terms and definitions used in this paper. The word "occurrence" or "detection frequency" refers to any structure with at least one positive sample for *L. pneumophila.* The term "sporadic" indicates that only one of the three sampling events was positive for *L. pneumophila* or *L. pneumophila* Sg1. "Persistence" refers to the repeated detection of *L. pneumophila* or *L. pneumophila* Sg1 in two or more water samples from the same tap at a residence, apartment, or office building. The term "structure" refers to residences, apartments, and office buildings. A "residence" is defined as a structure (single-family homes or apartments) where activities related to home life (e.g., showering, sleeping, gardening, cooking) occur, be it a single-family home ($n = 66$) or a unit in an apartment complex ($n = 4$). The term "office building" ($n = 30$) was defined as a business place where office/professional or service transactions were performed as determined using the International Building Code (IBC) criteria (Council, 2000).

***Legionella pneumophila* and *L. pneumophila* Sg1 qPCR assays qPCR sample filtration.** One liter was vacuum filtered through a sterilized Whatman® Nucleopore™ Track-Etched Membrane, 47 mm, 0.4 μm polycarbonate membrane (Cytiva, Marlborough, MA). After filtration, the polycarbonate membrane filter was aseptically inserted into a sterile 2 mL O-ring screw cap microcentrifuge tube containing 0.30 ± 0.05 g of 0.1 mm sterile glass beads (BioSpec Products, Bartlesville, OK). The study generated 296 samples, not including method blanks, extraction blanks, and positive and negative controls. The samples were stored at −80 °C until their evaluation for *L. pneumophila* and *L. pneumophila* Sg1 presence.

qPCR DNA extraction. Details of the DNA extraction from membrane filters were previously published (Donohue et al., 2019b). Briefly, each polycarbonate membrane was mini-bead beaten in a bead beater (BioSpec Products, Bartlesville, OK) with 500 μL of Tissue and Cell Lysis Solution (Lucigen Corporation, Middleton, WI). After a 5 min cool down on the ice, the bottom of the tube was punctured with an 18 G needle (Becton, Dickinson and Company, Franklin Lakes, NJ). Next, the bead beating tube was inserted into a sterile 1.5 mL microcentrifuge tube (Eppendorf, Enfield, CT). The lysate was collected into the 1.5 mL microcentrifuge tube body by centrifugation at 3500 rpm for 5 min. Next, 2 μL of Proteinase K (50 μg/μL) (Lucigen Corporation) was added and followed by incubation at 65 °C in a water bath for 15 min. Next, 2 μL of RNase A (5 μg/μL) (Lucigen Corporation) was added to the mixture and incubated at 37 °C for 30 min. Subsequently, 350 μL of MPC Protein Precipitation Reagent (Lucigen Corporation) was added to precipitate the cellular proteins. The resulting supernatant was transferred to a sterile microcentrifuge tube containing an equal volume of ice-cold isopropanol (~−4 °C). The samples were inverted manually up to 40 times and centrifuged at 10,000×*g* for 10 min. The supernatant was poured off, and the resulting DNA pellets were washed with 500 μL of ice cold (~−4 °C) 70% ethanol. Samples were centrifuged, and the ethanol removed by pipet. The DNA pellet was air-dried for 15 min to remove residual ethanol. Then the DNA pellets were re-suspended in 50 μL of nuclease-free sterile water and stored at −80 °C until analyzed.

Assays and Conditions for qPCR. Two primer-probe sets were used to detect and quantify *L. pneumophila.* One primer-probe set targeted the *L. pneumophila* 16S rRNA gene (Lp16S), and the other set targeted the *L. pneumophila* Sg1 *wzm* gene (LpSg1) (Donohue et al., 2014; Merault et al., 2011). All DNA extracts were analyzed using the Lp16S which targets the 16S rRNA gene. Any DNA extract positive for *L. pneumophila*

Lp16S was also analyzed for the presence of *L. pneumophila* Sg1 using the LpSg1 primer-probe set. All primer-probes and qPCR conditions were previously published (Donohue et al., 2014). The Lp16S assay linearity (R^2), amplification efficiency (AE), and Limit of Quantification (LOQ) are $R^2 = 0.999$, AE = 0.96%, and LOQ = 100 GU/Rx. The LpSg1 assay linearity, amplification efficiency, and LOQ are $R^2 = 0.995$, AE = 0.96%, and LOQ = 100 GU/Rx. More assay details, qPCR conditions, and controls were previously published (Donohue et al., 2019b). In addition, specific information on the limit of detection, the limit of quantification, and sensitivity was previously published (Donohue et al., 2014).

Preparation of qPCR Standard/Positive Control. A previously published method was used to prepare the DNA standards for the qPCR method described below (Donohue et al., 2014).

qPCR Controls. Controls were included on each plate to ensure the integrity of the method and confidence in the results. Genomic DNA extracted from *L. pneumophila* Sg1 ATCC 33125™ (American Type Culture Collection; Manassas, VA) was used as a positive control for each assay. A serial dilution of seven concentrations ranging from 10^6 to a theoretical one genomic copy was made from this DNA. Negative controls included three non-template controls (NTC), where sterile water was used in place of the DNA extract (template).

Method blanks were prepared at the time of the water filtration. One hundred milliliters of sterile molecular grade water (5 Prime, Gaithersburg, MD) were vacuum filtered as described above, for every 10 samples filtered. If a method blank was positive, the set of samples corresponding with it was considered compromised, and the data discarded. Inhibition of the qPCR reaction was monitored using external controls for the *L. pneumophilia* and *L. pneumophilia* Sg1 assays. The external inhibition control was prepared as follows: all unknown samples were spiked with 1 μL of an exogenous control of 10,000 target gene copies extracted from *L. pneumophila* ATCC 33152™. A reaction was considered inhibited if the observed C_q value of the external or internal controls drifted ≥1.5 C_q units from the standard C_q value.

Interpretation of qPCR. An extract was considered *L. pneumophila* positive if both replicate's quantification cycle (Cq) values were <39. As stated earlier, an *L. pneumophila* positive sample was also tested for the presence of *L. pneumophila* Sg1. For the L. *pneumophila* Sg1 assay, both replicate Cq values needed to be < 39 to be considered positive. The 5 μL DNA aliquot added to each qPCR reaction was a 100 mL concentrate of the original volume collected. Therefore, each sample's DNA extract was analyzed in duplicate (2 × 100 mL or 200 mL of the original volume was tested). Performing the qPCR reaction in duplicate improves the assay's precision and verifies a positive detection.

Statistical Analysis. The Cq values were transformed using a standard curve into genomic units (GU). The average genomic unit per replicate was calculated for each sample with a Cq < 39. For positive taps, an average genomic target value was calculated. To determine if there was a statistically significant difference between detection frequencies between the different building types, the Fisher Exact test in SigmaPlot 14.0 (Systat Software Inc, San Jose, CA) was applied. The Chi-square (X^2), Fisher Exact test, and McNemar's tests were applied to the seasonal data analysis using SigmaPlot 14 (Systat Software Inc). The concentration data were checked for normality using the Shapiro-Wilk test. Mann-Whitney *U* test was used to determine statistical significance for the occurrence and persistence concentrations. A p-value of 0.05 or lower was considered significant.

3. Results

3.1. Water detection frequencies and concentrations by structure type

Water samples from the hot water plumbing were collected at 70 residences generating 207 samples, and at 30 office buildings generating 89 samples. Thirty-six percent (25/70) of residences had one or more water samples positive for *L. pneumophila*. *Legionella pneumophila* was detected in 53% (16/30) of the office buildings (Table 1).

Since this study used a longitudinal sampling design, the duration of *L. pneumophila* contamination was examined. Duration of contamination (periodicity) was divided into two categories: sporadic (one of three sampling events positive per structure) and persistent (two or more sampling events positive per structure). Of the 25 residences positive for *L. pneumophila*, 15 residences had only one positive sampling event (sporadic), and ten residences had multiple sampling events positive (persistent). Additionally, of the 25 residences positive for *L. pneumophila*, *L. pneumophila* Sg1 was only detected in 5 residences as a sporadic, single positive sample. Among the 16 *L. pneumophila* positive office buildings, five office buildings had only one positive sample. There were 11 office buildings that had persistent *L. pneumophila* detections. These 11 office buildings were also persistently positive for *L. pneumophila* Sg1.

Fig. 1 shows *L. pneumophila* concentration by structure type and

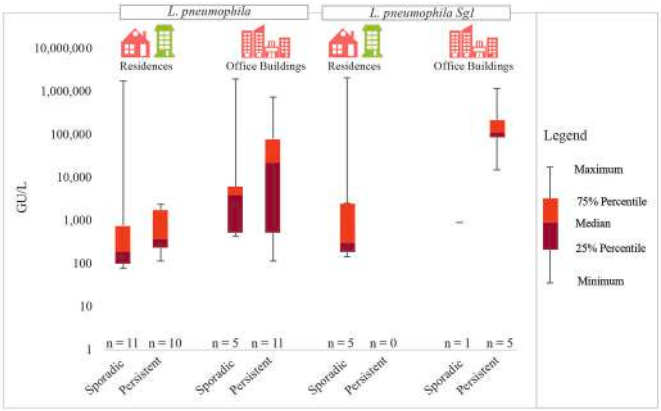

Fig. 1. *Legionella pneumophila (Lp)* and *L. pneumophila* Sg1 GU/L concentration by residences (single-family homes + apartments) and office buildings and by sporadic/persistent detections.

Table 1
Legionella pneumophila and *L. pneumophila* Sg1 detection rate, concentration, and percent positive by site.

Detection & Quantification	*L. pneumophila*			*L. pneumophila Sg1*		
	Residences	Office Buildings	P-Value	Residences	Office Buildings	P-Value
	n (%)	n (%)		n (%)	n (%)	
	70	30		70	30	
Detection Frequency						
No. of sites with one or more positive samples	25 (26)	16 (53)	Fisher Exact test: $P = 0.123$	5 (7)	6 (20)	Fisher Exact test: $P = 0.082$
No. of sites with no positive samples	45 (64)	14 (47)		65 (9)	24 (80)	
Concentration						
Median concentration GU/L	3.9×10^2	5.1×10^3	Mann-Whitney *U* test: $P = 0.007$	3.1×10^2	1.0×10^5	Mann-Whitney *U* test: $P = 0.177$
Average concentration GU/L	7.5×10^4	2.0×10^5		4.4×10^5	2.7×10^5	

occurrence pattern (sporadic or persistent). *Legionella pneumophila* concentrations were significantly lower in residences than in office buildings, with the median value of 3.9×10^2 genomic unit (GU)/L versus 5.1×10^3 GU/L, respectively (*U* test; $P = 0.007$) (Table 1).

Legionella pneumophila Sg1 did not persist in the residential hot water environment. However, there were some infrequent positive hot water samples where *L. pneumophila* Sg1 concentrations exceeded 10^5 GU/L. Without evidence of *L. pneumophila* Sg1 persisting in hot water, these infrequent detections could have come from the cold-water line or outside the residential setting. In contrast, an office building's water samples were repeatedly *L. pneumophila* Sg1 positive, with higher concentrations of 1.0×10^5 GU/L than residences 3.1×10^2 GU/L, *U* test: $P =< 0.177$ (Table 1). An office building's hot water infrastructure is more likely to harbor *L. pneumophila* Sg1 than a residence's hot water plumbing.

3.2. Structure's age and square footage

Fig. 2 shows *L. pneumophila* detection frequency in hot water by a structure's age and square footage (area). In this analysis, apartments were separated from single-family homes due to their larger size (10,000 to 100,000 sq. ft.) and their potential use of multiple hot water tanks or a hot water recirculating system. *Legionella pneumophila* detection frequency in newer single-family homes and office buildings was lower than in the older structures (Fig. 2A). Regardless of the type of structure (house or building), a structure older than 20 years had more persistent *L. pneumophila* detections than newer construction (<20 years old), Fisher Exact test: $P = 0.009$.

The size of a structure influenced *L. pneumophila* detection frequency (Fig. 2B). *Legionella pneumophila* detection frequency increased as the structure's area (size) increased. For example, the 1000 sq ft. single-family homes had a 27% (9/33) *L. pneumophila* detection frequency. In comparison, the 4000 sq ft. Homes had a 67% (4/6) detection frequency (Fisher-Exact test: $P = 0.348$). Interestingly, regardless of the single-family home's size, *L. pneumophila* was detected persistently in 15% of homes (indicating colonization), Fig. 2B.

The size of the office building's affected both *L. pneumophila* detection frequency and occurrence pattern. *Legionella pneumophila* detection frequency of the 10,000 sq. ft. or greater office buildings was 64% (11/17), with a 91% (10/11) likelihood of being colonized (persistent detections). In contrast, the *L. pneumophila* detection frequency of the 5000 sq. ft. or less office buildings was 43% (3/7), with only a 33% (1/3) likelihood of persistence. Interestingly residences and office buildings whose area was <5000 sq. ft. Have similar detection frequencies, 33% (21/63) and 43% (3/7), Fisher Exact test: $P = 0.712$. Even these structures' *L. pneumophila* persistence rates are similar at 13% (8/63) and 14% (1/7), Fisher Exact test: $P = 1.0$.

3.3. Role of secondary disinfectant by structure type

Table 2 shows *L. pneumophila* and *L. pneumophila* Sg1 positive samples by structure type (single-family homes, apartments, and office buildings) and the water's secondary disinfectant (chlorine or chloramine). Office buildings had the highest *L. pneumophila* detection frequency of 61% (11/18) among the structures with chlorine treated water. Apartments had the highest *L. pneumophila* detection frequency of 67% (2/3) among the structure with chloramine-treated water. Interestedly, *L. pneumophila* Sg1 detection frequency was similar across the structure types, and secondary disinfectants.

It is well established that a relationship exists between *L. pneumophila* concentration and secondary disinfectant residual concentration. Fig. 3 shows each structure type with positive *L. pneumophila* sample concentration by secondary disinfectant residual concentration. In the single-family home setting (Fig. 3A), *L. pneumophila* concentrations were

Table 2
Legionella pneumophila and *L. pneumophila* Sg1 detection frequency by structure and disinfectant type. The bolded text was the highest detection frequency.

Structure Type	No. of Total Sites	Species/Serogroup	Chlorine n (%)	Chloramine n (%)
Single-Family Home	N 66	 *L. pneumophila* positive *L. pneumophila* Sg1 positive	47 16 (34) 2 (4)	19 7 (37) 1 (5)
Apartment	n 4	 *L. pneumophila* positive *L. pneumophila* Sg1 positive	1 0 0	3 **2 (67)** **2 (67)**
Office Building	n 30	 *L. pneumophila* positive *L. pneumophila* Sg1 positive	18 **11 (61)** 4 (22)	12 5 (42) 2 (17)

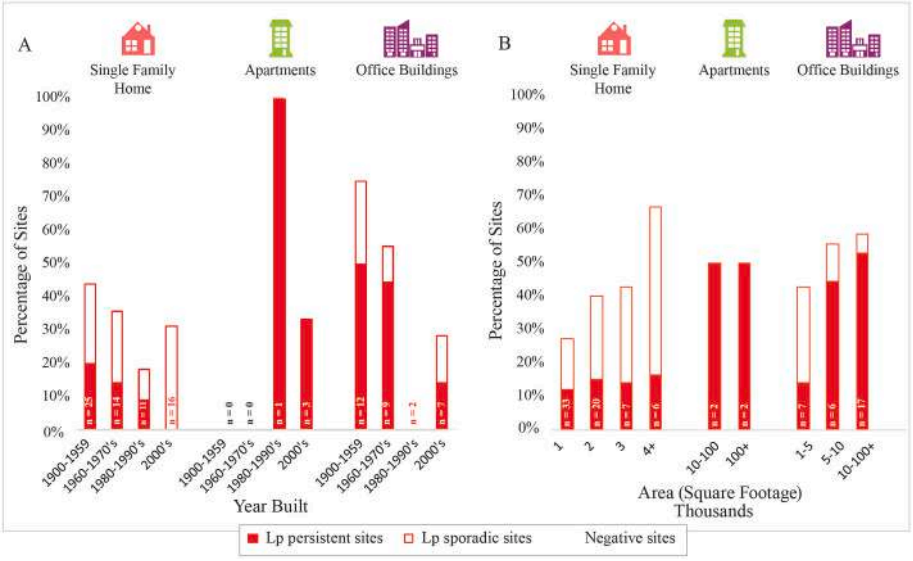

Fig. 2. *Legionella pneumophila* occurrence and persistence percentage by A) year built and by B) area (square footage) for each structure type.

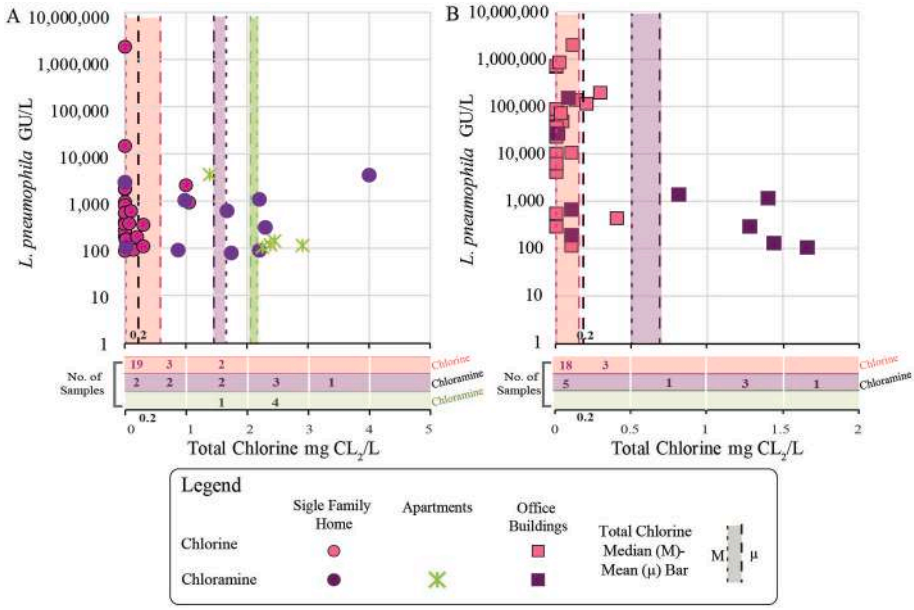

Fig. 3. Shows Legionella *pneumophila* concentration by total chlorine concentration for chlorine-and chloramine-treated water. A.) residences B.) office buildings. The table under the scatterplot is the number of samples in a specific total chlorine range (e.g., 0–0.2 mg Cl_2/L, 0.2–1.0 mg Cl_2/L, 1–2 mg Cl_2/L etc.). The black dotted line at 0.2 mg Cl_2/L is a reference point for the minimum residual amount at entry point into distribution. The vertical bar is the total chlorine median-mean range for a particular structure type (e.g., the pink bar is the total chlorine median-mean range detected in single-family homes with chlorinated water, the purple bar represents the single-family homes with chloraminated water, and the green bar represents apartments with chloraminated water). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

statistically similar for chlorine and chloramine treated water. However, as expected, the total chlorine concentrations were statistically different. *Legionella pneumophila* median concentration (densities) for chlorine and chloramine were 3.6×10^2 GU/L and 4.5×10^2 GU/L respectively, *U* test: $P = 0.710$; chlorine and chloramine residual median concentrations were 0 mg Cl_2/L and 1.7 mg Cl_2/L respectively; *U* test: $P = <0.001$.

In the office building setting (Fig. 3B), *L. pneumophila* positive samples in chlorine and chloraminated treated water were statistically different relative to concentration and disinfectant residual

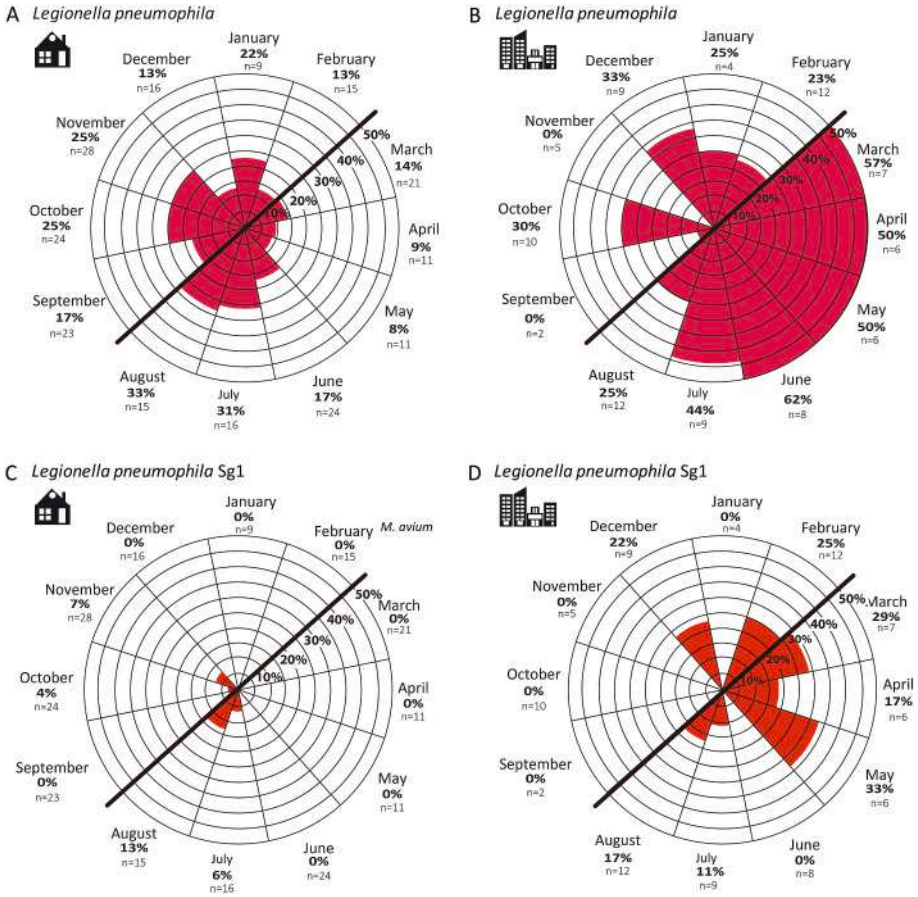

Fig. 4. Radial graphs show *L. pneumophila* and *L. pneumophila* Sg1 detection frequency by month for residences and office buildings. A) *L. pneumophila*-residential, B) *L. pneumophila*-office buildings, C.) *L. pneumophila* Sg1-residential, and D) *L. pneumophila* Sg1-office buildings.

concentrations. *L. pneumophila* median concentrations were 4.9×10^4 GU/L (chlorine) and 4.3×10^2 GU/L (chloramine), respectively; *U* test: $P = 0.006$. The office building's total chlorine median values were 0.0 mg Cl_2/L (chlorine) and 0.7 mg Cl_2/L (chloramine), respectively, *U* test; $P = 0.009$. In the office building setting, the disinfectant concentrations were lower than concentrations measured in single-family homes, especially the chloramine treated water, *t*-test (Welch's); $P = 0.027$. The lower total chlorine concentrations suggest that other factors, such as water age, and biofilm, possibly affect the *L. pneumophila* population count and the disinfectant decay.

3.4. Seasonal rates

Fig. 4 shows *L. pneumophila* and *L. pneumophila* Sg1 detection rates by the month of sampling for residences and office buildings. Monthly differences were observed in the frequency of *L. pneumophila* and *L. pneumophila* Sg1 detections (Fig. 4). In residences, water samples collected in August and November had the highest *L. pneumophila* detection frequency, 27% (4/15) and 28% (7/25), respectively (Fig. 4A). The *L. pneumophila* Sg1's highest detection frequency in residences was August, 13% (2/15) (Fig. 4C). In office buildings *L. pneumophila* highest detection frequency of 62% (5/8) were in samples collected in June (Fig. 4B). *Legionella pneumophila* Sg1's highest detection frequency of 40% (2/5) was in May (Fig. 4D).

Fig. 4 also demonstrates that there are seasons of higher *L. pneumophila* and *L. pneumophila* Sg1 detection frequencies than at other times of the year. In residences, Summer-Fall months (June–November) were significantly greater for *L. pneumophila* detections frequency of 23% (29/125) than Winter-Spring months (December–May) 12% (9/82), X^2: $P = 0.042$ or McNemar's Test: $P = <0.001$. In office buildings, *L. pneumophila's* highest detection frequency of 45% (21/47) was in the Spring-Summer months (March–August) compared to Fall-Winter months (September–Feb) 24% (10/42), Fisher Exact test: $P = 0.046$ or McNemar's Test: $P = 0.170$ (Fig. 4B).

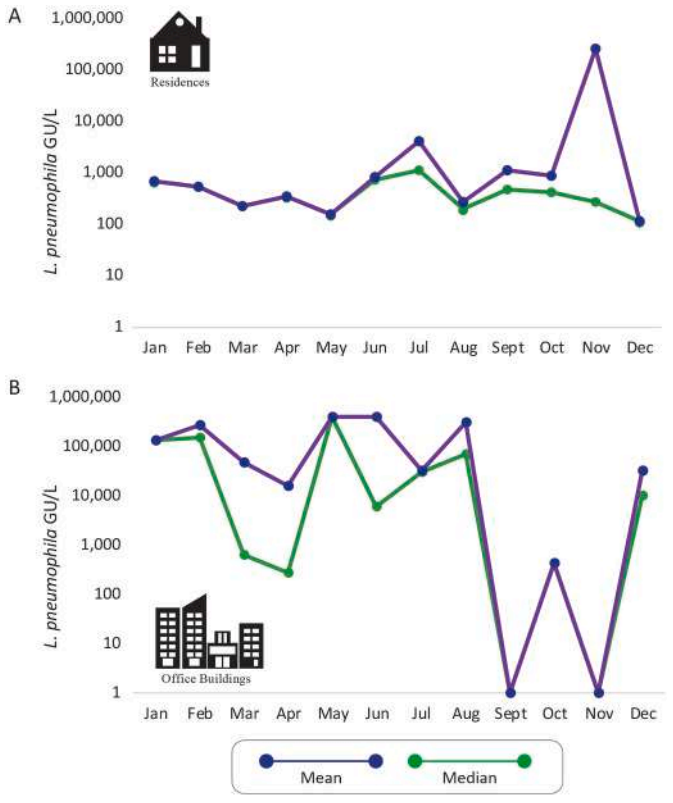

Fig. 5. *Legionella pneumophila* mean and median concentrations by month A) residences and B) office buildings.

Fig. 5 shows each month's positive samples' mean (dark purple) and median (green) concentrations. Depending on the month, *L. pneumophila* concentrations span a wide range of values, as observed in June–November for residences and February–August for office buildings. The mean and median concentration range did not change (zero to less than a 0.5 log difference) in January–June for residences and September–February for office buildings. These results indicate that time of year and the structure characteristic have different risk profiles. A monthly concentration analysis for *L. pneumophila* Sg1 was not performed because the dataset (n = 17) was too small to support statistical analysis.

3.5. Legionella pneumophila monthly occurrence (environmental) and monthly legionellosis cases (disease) comparison by structure type

Radial graphs in Fig. 4 show that each structure type experiences a different *L. pneumophila* seasonal pattern. These patterns were reminiscent of previously published monthly seasonal distribution of Legionnaires' disease cases (Barskey et al., 2022; Hicks et al., 2012). Fig. 6 compares the *L. pneumophila* monthly environmental occurrence pattern for residences (the orange line) to the CDC's NNDSS monthly percentage of legionellosis cases (the purple bars) for residences (Barskey et al., 2020; Hicks et al., 2012; Neil and Berkelman, 2008).

The similarity between the residential *L. pneumophila* detection frequency and legionellosis case reporting trends was noticeable in Fig. 6A. It suggests that most legionellosis cases potentially result from exposure via the residential setting. However, the legionellosis case rate and environmental occurrence comparison have limitations. The data source for the legionellosis case rates was an imperfect match with those for the residential settings. The data source that Neil and Berkelman (2008), Hicks et al. (2012), and Barskey et al. (2020) used included legionellosis cases from both "sporadic-community acquired," or travel-associated, and outbreak situations. Only 4% of their legionellosis cases were related to outbreaks, and 15–16% of cases were associated with travel exposure. Thus, it was fair to assume that the seasonal pattern reflected in the papers cited was a good representation of sporadic community-acquired legionellosis cases. Additionally, the years are not perfect matches. The reported legionellosis cases distribution was 2000–2009, and the environmental sampling timeframe was from 2011 to 2018. Therefore, the environmental divergence observed in April and October–November could be due to case pattern changes not observed in Hicks et al. (2012), 2000 to 2009 analysis. Nevertheless, despite the time frame differences, the overall patterns are comparable between environmental occurrence and clinical case reporting.

Additionally, we hypothesized that *L. pneumophila* occurrence trends in office buildings might have a relationship with outbreak-associated legionellosis cases. Legionellosis case data (first month) was extracted from the CDC's National Outbreak Reporting System (NORS) database-from 2009 to 2017; 147 water-related legionellosis outbreaks were reported, generating 635 confirmed legionellosis cases. Using the first month of a NORS's reported legionellosis case, an annual monthly percentage was calculated, Fig. 6B purple bars. The number of legionellosis cases associated with an outbreak was approximately 2–6 cases per outbreak. Approximately 18 water-related legionellosis outbreaks occur annually in the US. (CDC, 2020).

Fig. 6B compares this study's office building monthly *L. pneumophila* detection frequency to the NORS monthly percentage of legionellosis cases (first month). The outbreak data and office building monthly detection frequency data do not appear to be associated (Fig. 6B). The office building and NORS monthly detection frequency comparison in Fig. 6B have limitations. This study focused on the hot water in office buildings, whereas the NORS dataset includes water-related outbreaks from water fountains, cooling towers, and a building's potable water. The NORS dataset includes more water sources than just portable water from buildings.

Fig. 6. Compares this study's *Legionella pneumophila* monthly detection frequency (orange line) to the average percentage of legionellosis cases by month (purple bars). A) *Legionella pneumophila* residential monthly detection frequency compared to Hick et al. 2011 average percentage of legionellosis cases occurring annually by month and B.) compares *L. pneumophila* office buildings monthly detection frequency to CDC's NORs 2009–2017 (month of first reported legionellosis case) average percentage of legionellosis cases by month. The density of the purple bar color indicates legionellosis case percentages: light purple is <7% of cases, median purple is 7–10% of cases, and dark purple is >10% of legionellosis cases. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The legionellosis disease rate is increasing in the United States. Therefore, identifying at-risk locations where *L. pneumophila* control and prevention can be implemented is important. Identifying the structural characteristics (e.g., age, size, and disinfectant residual) that favor *L. pneumophila* contamination could help support the selection of strategies that could reduce contamination risk. Although the data set for this study is small, (66 single-family homes, 4 apartment complexes, and 30 office buildings), it includes structures that represent a variety of ages, sizes, and types at locations distributed across the continental United States. Thus, the data generated in this study can support hypothesis development, identify characteristics that favor the survival of *L. pneumophila* at locations where people live and work, and develop strategies to reduce colonization risk.

***Legionella pneumophila* in Residences.** When this project was initiated, the residential setting was hypothesized to be where most community-acquired legionellosis cases occur. Approximately 96% of reported legionellosis cases were considered community-acquired, involving situations where a single individual contracted the disease (Garrison et al., 2016). Unfortunately, these solitary cases rarely trigger a field investigation where the setting and source of *L. pneumophila* contamination are identified. Schumacher et al. (2020) provide a rare insight into community-acquired legionellosis cases. In their paper, a *L. pneumophila* Sg3 incident was initially thought to be connected to a Healthcare-Associated Infection (HAI). However, the field investigation revealed that the patients contracted Legionnaires Disease within their home from exposure to the bathroom shower or kitchen sink sprayer. A concentration of 0.05–2 CFU/mL was measured at these locations. Samples collected from all other potential exposure sites within the home and the associated healthcare centers were negative. Schumacher's study suggested that other *L. pneumophila* serogroups can cause Legionnaires Disease. The likelihood of other *L. pneumophila* serogroups in the residential setting is supported by this study and studies by Byrne et al. (2018); Collins et al. (2017); Lim et al. (2003). These studies show a higher recovery of *L. pneumophila* serogroups Sg3, Sg4, and Sg6 versus Sg1 in residential potable water.

Residential hot water plumbing in single-family homes is a much simpler design when compared to that in large office buildings. The water flow in the single-family home is unidirectional, although backflow events may occasionally occur. A water heater is used to raise the water temperature, and pipes of various materials take the water to areas of demand. A residence's smaller size contains less total pipe length, fewer risers, and dead ends than a multifunctional building complex. The homeowner's most important water maintenance task is replacing the hot water heater every 6 to 12 years—a far lower maintenance burden than what is required by an apartment or office building manager. Yet, despite a single-family home's design simplicity, older homes are prone to persistent *L. pneumophila* contamination.

Legionella pneumophila contamination risk in older residences suggests that other age-related risk factors such as biofilm development and pipe corrosion might be involved (Cullom et al., 2020; Lau and Ashbolt, 2009). Additionally, the age of the hot water heater was not asked of the participant. Therefore, a lack of routine home maintenance cannot be excluded as a contributor to risk. The Collins et al. (2017) shower study in the United Kingdom observed fewer *Legionella* positive samples in newer homes than in buildings 30 years old. They also reported that most *L. pneumophila* occurrences in houses were sporadic, with little evidence of plumbing colonization. Therefore, one can conclude that residences experienced transient pockets of contamination originating upstream of the hot-water system, such as a biofilm sloughing event. Both Cohn et al. (2015); Collins et al. (2017) observed circumstantial evidence that Legionella spp. Contamination can originate upstream of the hot-water supply.

The present study is one of the first to report on *L. pneumophila* residual concentrations within residential structures' hot water environment. An important insight observed in the residential setting was that, regardless of disinfectant type (chlorine or chloramine), *L. pneumophila* concentration was statistically the same. This observation indicates that irrespective of the secondary disinfectant type, there is still the possibility for *L. pneumophila* exposure and the potential for disease transmission in a residential setting. This finding is rational when combined with other data such as legionellosis cases and outbreaks reported in areas where the water's secondary disinfectant is chloramine (Holsinger et al., 2022; Rhoads et al., 2020; Tucker et al., 2018). More research is needed to explore further whether there is a relationship between residual disinfectant and L. pneumophila occurrence.

4.1. *Legionella pneumophila* in buildings

Buildings, especially those with many levels, are at risk for *L. pneumophila* contamination. This study adds to the body of evidence established by Buse et al. (2020); Donohue et al. (2019a); Flannery et al. (2006); (Garrison et al., 2016); Moore et al. (2006); Pierre et al. (2019), that report higher *Legionella* detections in buildings than single-family residences. For instance, Garrison et al.'s (2016) analysis of 25 legionellosis field investigations revealed that 93% (25/27) of outbreaks occurred in a "building setting," and 60% (15/25) of these outbreaks were due to deficiencies observed in the building's potable water supply network.

The present study indicates that older and newly built office buildings had an equal probability of *L. pneumophila* contamination. Several newspaper and journal articles by Stout et al. (2000), Kool et al. (1999), and Francois Watkins et al. (2017) linked a legionellosis case to new construction, specifically for healthcare buildings. These instances occurred when the building's plumbing was being tested months earlier before occupancy, and the water was left to stagnate before use.

A building's area (sq. ft.) appeared to influence *L. pneumophila* occurrence, especially *L. pneumophila* Sg1 occurrence. Buildings >100,000 sq. ft. (office buildings and apartments) had statistically significant higher *L. pneumophila* occurrence (X^2: $P = 0.016$) and *L. pneumophila* Sg1 persistence (X^2: $P = <0.001$) than offices < 100,000

sq. ft. This information is highly relevant for the United States, since there are approximately 32.6 million multifamily housing units and commercial buildings (US Census Bureau, 2012, 2020).

In studies by Flannery et al. (2006); Kool et al. (1999); Moore et al. (2006), chloramine was found to be an effective tool for removing *L. pneumophila* and *Legionella* spp. in buildings. However, recent research utilizing molecular techniques has shown that chloramine might only be suppressing *L. pneumophila* growth while promoting the growth of other *Legionella* spp. and opportunist pathogens (Donohue, 2021). Also, the Baron et al. (2015) case study on a chloramine treatment unit showed *Legionella* spp. growth during a shutdown period of service. As data continues to be published, it will be interesting to see how chloramine is evaluated as an overall disinfectant that protects against water-borne pathogens and microbial growth.

Over the last few decades, an office building's water age (i.e., the time the water spends in the building's plumbing before being used) has significantly increased. In addition, commercial buildings have become larger and multifunctional, requiring a more complex plumbing system. Increased water age was potentially an unintended consequence of the US 1992 Energy Act designed to identify ways to reduce energy consumption (EPACT92, 1992). Over the last twenty years, efforts to reduce energy consumption have accelerated. Smaller appliances and fixtures, low-flow and automatic faucets, and lower hot-water temperatures have impacted water use, potentially minimizing water flow and increasing water age. Research by Brazeau and Edwards (2013); Nguyen et al. (2012) demonstrate that 2–8 h water stagnation time could result in a 3–4 mg/L chlorine loss or 1.5–3.5 mg/L chloramine loss, dependent on the pipe material.

Water age can reduce water quality. Water quality degrades as the disinfectant residual decreases, potentially allowing for microbial colonization, biofilm development, and pipe corrosion products, e.g., rust, and iron oxides, to develop (Cullom et al., 2020; Rogers et al., 1994a, b). These physio-chemical and biological shifts in water quality could increase the survival and persistence of microbes and water-borne pathogens, such as Legionella, in the potable water supply (Buse et al., 2012; Cullom et al., 2020).

5. Conclusion

This study researched *L. pneumophila* occurrence and persistence in residences and offices to better understand the potential risk for microbial contamination and disease transmission associated with the building's structural characteristics. Although office buildings may be more prone to *L. pneumophila* contamination, they also have a smaller potential for disease transmission because fewer activities take place in office buildings whereby the occupants are exposed to water aerosols. The exception is healthcare and residential care centers, hotels, and multi-unit apartment complexes. This study suggests that *Legionella*-realated plumbing system remediation efforts in office buildings (that contain commercial functions only) might not significantly improve a community's public health.

Residential structures were not prone to *L. pneumophila* colonization, but they do have a much larger window for potential disease transmission. This was because many human activities are performed at higher frequencies and for longer durations in places where water aerosols are generated and inhaled by the occupants. This study revealed that residential plumbing could be a potential "conduit" for *L. pneumophila* exposure from a source upstream of the hot water environment. Thus, a residence may be the setting for a legionellosis case, even when it was not the source of the contamination.

Legionella pneumophila and other *Legionella* subspecies are environmental microbes that adapt and change in response to their environment. This study revealed that the built environment influences *L. pneumophila* transport and fate. More work is needed to evaluate if the trends and observations in this study are supported by future research with larger samples size and longitudinal analyses.

Disclaimer

The views expressed in this article are those of the author [s] and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. The United States Environmental Protection Agency, through its Office of Research and Development, funded and managed the research described here. Mention of trade names or office products does not constitute endorsement or recommendation of these materials for use.

Declaration of competing interest

Authors have no conflicts of interest to report.

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Influence of community-level sanitation coverage and population density on environmental fecal contamination and child health in a longitudinal cohort in rural Bangladesh

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ABSTRACT

Background: Household-level sanitation interventions have had limited effects on child health or environmental contamination, potentially due to low community coverage. Higher community-level coverage with safely managed sanitation can reduce opportunities for disease transmission.

Methods: We estimated associations between community sanitation coverage, environmental fecal contamination, and child health among 360 compounds in the control arm of the WASH Benefits trial in rural Bangladesh (NCT01590095). In each compound, we enumerated *E. coli* in environmental samples and recorded the 7-day prevalence of caregiver-reported diarrheal disease and acute respiratory infections (ARI) in children under five. We observed indicators of latrine access and quality among all neighboring compounds within 100 m of study compounds. We defined community coverage as the proportion of neighboring compounds with (1) at least one latrine, and (2) exclusively hygienic latrines (improved facility observed to safely contain feces), within both 50 m and 100 m of study compounds. We assessed effect modification by population density and season.

Results: Adjusted for confounders, study compounds surrounded by 100% coverage of at least one latrine per compound within 50 m had slightly lower $\log_{10}$ *E. coli* counts in stored water ($\Delta\log = -0.13$, 95% CI -0.26, −0.01), child hand rinses ($\Delta\log = -0.13$, 95% CI -0.24, −0.02), and caregiver hand rinses ($\Delta\log = -0.16$, 95% CI -0.29, −0.03) and marginally lower prevalence of diarrheal disease (prevalence ratio [PR] = 0.82, 95% CI 0.64, 1.04) and ARI (PR = 0.84, 95% CI 0.69, 1.03) compared to compounds surrounded by <100% coverage. Effects were similar but less pronounced at 100 m. At higher population densities, community latrine coverage was associated with larger reductions in *E. coli* on child and caregiver hands and prevalence of diarrheal disease. Coverage with exclusively hygienic latrines was not associated with any outcome.

Conclusion: Higher community sanitation coverage was associated with reduced fecal contamination and improved child health, with stronger effects at highly local scales (50m) and at high population densities. Our findings indicate that the relationship between community sanitation coverage, environmental contamination, and child health varies by definition of coverage, distance, and population density. This work highlights significant uncertainty around how to best measure sanitation coverage and the expected health effects of increasing sanitation coverage using a specific metric. Better understanding of community-level sanitation access is needed to inform policy for implementing sanitation systems that effectively protect community health.

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1. Introduction

Interventions to provide or promote on-site improved pit latrines have had limited effects on environmental fecal contamination (Sclar et al., 2016), diarrheal disease (Contreras and Eisenberg, 2020) and child growth (Humphrey et al., 2019; Luby et al., 2018; Null et al., 2018). One potential explanation for the null effects of many sanitation interventions is low community-level coverage achieved by most trials, whether by design or due to low intervention uptake (Pickering et al., 2019). Household-level access to an improved latrine is intended to prevent transmission of fecal-borne pathogens by separating household members from their own waste, but it does not account for transmission pathways that originate from outside the home, including environmental contamination from neighbors that do not have safely managed sanitation facilities. Humans, animals, and flies can carry fecal pathogens into the home environment from outside sources, and pathogens can infiltrate into surface- and groundwater sources from neighbors' open defecation or unhygienic latrines, contributing to transmission independent of household-level sanitation access (Julian, 2016; Knappett et al., 2011, 2012). Increasing the proportion of households in the community with safely managed sanitation facilities hypothetically could reduce transmission by limiting opportunities for pathogen spread from outside the home, as fewer people in the community are contributing uncontained feces. However, even high community-level sanitation coverage would leave several contamination sources unaddressed, such as free-roaming domestic animals (Baker et al., 2018; Zambrano et al., 2014), exposure to untreated fecal waste through irrigation, manure application, or uncontained fecal streams (Dickin et al., 2016), contaminated produce or other food obtained outside the home (Antwi-Agyei et al., 2015; Harris et al., 2018).

Existing evidence on the relationship between community-level sanitation coverage and individual health or environmental fecal contamination is mixed but mostly suggests that increased coverage is associated with improved child health. Observational studies have found that higher levels of community-level sanitation coverage is associated with improved child growth (Fuller et al., 2016; Hammer and Spears, 2016; Harris et al., 2017; Larsen et al., 2017; Vyas et al., 2016) and reduced diarrheal disease (Andrés et al., 2017; Komarulzaman et al., 2017; Larsen et al., 2017), anemia (Kmush et al., 2021; Larsen et al., 2017), active trachoma (Garn et al., 2018), infection with *Trichuris trichiura* (Oswald et al., 2019), neonatal mortality (Kmush et al., 2021), and environmental fecal contamination (Berendes et al., 2017, 2020, 2018). A meta-analysis of observational studies found that higher community-level sanitation coverage was associated with reduced diarrheal disease (Jung et al., 2017), and a mathematical model on enteric pathogen transmission found that the entire effect of a hypothetical sanitation intervention on infection rates was due to the indirect effects of community-level coverage, rather than household-level access to sanitation (Fuller and Eisenberg, 2016).

Other observational studies have found no association between community-level sanitation coverage and diarrheal disease (Harris et al., 2017), hookworm infection (Oswald et al., 2019), and environmental fecal contamination (Huda et al., 2019; Odagiri et al., 2016). A study on the spillover effects from a combined water, sanitation, and hygiene (WASH) intervention among nearby neighbors of intervention recipients found reduced *E. coli* in stored drinking water from tubewells but no difference in fecal contamination through other environmental pathways, helminth infections, diarrheal disease, or respiratory illness (Benjamin-Chung et al., 2018). A meta-analysis of intervention trials found no clear association between sanitation coverage and diarrheal disease, except among sewerage interventions (Contreras and Eisenberg, 2020; Wolf et al., 2018).

Most of the research on community-level sanitation measured coverage through surveys conducted among a subset of residents over large sampling areas. Few studies have comprehensively measured sanitation coverage for all households within the sampling area to capture both latrine presence and quality (Fuller et al., 2016; Harris et al., 2017; Huda et al., 2019), and no studies have assessed both child health and fecal contamination to assess if any health benefits of coverage are causally supported by reductions in contamination and to investigate which environmental pathways are most influenced by community-level sanitation coverage. The objective of this analysis was to estimate associations between community-level sanitation coverage within a proximate (50–100 m) radius of study participants, environmental fecal contamination along multiple pathways, and child health in a longitudinal study nested within a randomized controlled trial in rural Bangladesh (Luby et al., 2018). In addition, we analyzed population density and season as potential modifiers of the relationship between community-level sanitation coverage and each outcome.

2. Materials and methods

2.1. Study design

This study was conducted in a longitudinal cohort nested within the control arm of the WASH Benefits randomized controlled trial in rural Bangladesh (Luby et al., 2018). The trial enrolled multifamily compounds that included a pregnant woman in her first or second trimester. The household in which the pregnant woman lived was the target household. Enrolled compounds were grouped into clusters of 6–8 spatially contiguous compounds, and clusters were randomly assigned to one of six WASH intervention arms or into the control arm. We randomly selected 360 (of 696) compounds from the sanitation arm and 360 (of 1,382) compounds from the control arm of the parent trial to participate in a longitudinal substudy focused on environmental contamination. The present analysis includes data from the control arm of the substudy to capture sanitation conditions unaltered by the intervention. Participants provided written informed consent in Bengali. The study protocol was approved by human subjects committees at the icddr, b (PR-11063), University of California, Berkeley (2011–09−3652), and Stanford University (25863).

2.2. Data collection

Compounds participating in the substudy were visited eight times over 30 months. GPS coordinates were recorded at the entrance of the target household during the first visit. Samples were collected during each visit from various locations within the compound environment representing potential pathways of contamination from fecal sources. Stored drinking water samples and hand rinses from children and caregivers were collected at each visit. Samples of soil from the courtyard at the entrance of the target household and stored food for young children were collected during the third and fourth visits only. Samples were processed at the local field lab of the icddr,b on the same day as collection with IDEXX Quanti-Tray/2000 to enumerate the most probable number (MPN) of *E. coli*. Methods for sample collection and analysis have been detailed elsewhere (Contreras et al., 2021; Ercumen et al., 2018).

At each visit, field staff administered a survey that included caregiver-reported symptoms of diarrheal disease and acute respiratory infection (ARI) for all children under five years of age in the compound, sanitation behaviors (e.g., latrine use, open defecation by children), and presence and number of domestic animals (cattle, goats, sheep, pigs, poultry, dogs, and cats). Field staff also completed spot check observations of sanitary conditions, such as the type and hygienic condition of each latrine in the compound.

During the second visit, a community survey was conducted to measure population density and sanitation coverage within a 100 m radius of study compounds. Field staff identified all compounds within this range by walking 300 steps (approximately 100 m) in each direction away from the study compound. At each compound within this radius, they recorded the total number of people who lived in the compound,

the number of latrines in the compound, and GPS coordinates at the entrance of the compound and at each latrine. They observed and recorded the type and hygienic condition of each latrine, including where the latrine flushed to and whether feces were fully contained within a pit or septic tank.

2.3. Statistical methods

2.3.1. Outcome variables and parameters of interest

The primary outcomes of this study were i) counts of *E. coli* in environmental samples (stored drinking water, child hand rinse, mother hand rinse, soil, and child food), ii) diarrheal disease in children under five, and iii) ARI in children under five. *E. coli* counts were analyzed as a continuous variable representing the $\log_{10}$-transformed MPN of bacteria per unit of sample (per 100 mL of stored drinking water, per two hands for rinses, or per one dry gram of food/soil) and were analyzed separately by sample type. Samples without detectable levels of *E. coli* were assigned a value equal to half the lower detection limit. Diarrheal disease and ARI were operationalized as binary variables based on caregiver-reported symptoms for a seven-day recall period. Diarrheal disease was defined as passing three or more loose or watery stools or at least one stool with blood. ARI was defined as persistent cough, panting, wheezing, or difficulty breathing. Both child health outcomes were analyzed at the child level with observations pooled across sampling rounds, such that each child under five provided up to eight data points for each outcome. The parameters of interest for this analysis were i) $\log_{10}$ reductions in *E. coli* for each sample type and ii) prevalence ratios and prevalence differences for diarrheal disease and ARI, associated with different levels of community sanitation coverage.

2.3.2. Exposure variables

We aimed to capture multiple aspects of community-level sanitation coverage by using two exposure definitions (any latrine coverage and hygienic latrine coverage), operationalizing these exposures in both binary and continuous forms, and quantifying them within two different radii around study compounds (50 m and 100 m).

We defined "any latrine coverage" as the proportion of neighboring compounds with at least one latrine within the specified radius around each study compound. This definition identifies compounds that have no latrine access, while ignoring latrine quality within compounds with at least one latrine. Although compounds without their own latrines may use latrines in public locations (e.g. mosques) or in other compounds, we assume that lack of latrine access within the compound might indicate some degree of open defecation. We operationalized any latrine coverage as a binary indicator variable for whether 100% of compounds within the specified radius of the study compound had at least one latrine (100% vs. <100% coverage). This comparison reflects the impact of complete coverage with at least one latrine per compound and assumes that even one compound relying on open defecation can impact community contamination and disease transmission. We also aimed to assess the effect of any latrine coverage as a continuous variable (0%–100%). However, for most study compounds, 80–100% of neighboring compounds had at least one latrine. Therefore, we did not analyze a continuous form of any latrine coverage.

We defined "hygienic latrine coverage" as the proportion of neighboring compounds with exclusively hygienic latrines (i.e., at least one latrine in the compound and all latrines in the compound were hygienic) within the specified radius of each study compound. We defined "hygienic latrine" as an improved facility that does not drain into the environment and where feces are fully contained within the pit or septic tank, based on observations by field staff (UNICEF et al., 2019). This definition captures the role of latrine quality but does not differentiate between defecation in a non-hygienic latrine and open defecation. We operationalized hygienic latrine coverage as a binary indicator variable for whether 100% of compounds within the specified radius of the study compound had exclusively hygienic latrines (100% vs. <100% coverage). This comparison reflects the impact of complete coverage with high-quality latrines and assumes that even one non-hygienic latrine can lead to environmental contamination and pathogen spread. We also analyzed hygienic latrine coverage as a continuous variable (0%–100%) to assess the incremental effect of increasing coverage.

For both of these exposure definitions, we chose a maximum radius of 100 m from study compounds to reflect the upper range of the distance fecal pathogens have been shown to travel in the subsurface from pit latrines (Graham and Polizzotto, 2013). However, it is possible that neighbors' sanitation coverage impacts target households over shorter distances through other pathways, such as direct contact between household residents or animals. To assess the role of distance in our analysis, we used GPS data to quantify each exposure within 50 m and 100 m of study compounds.

2.3.3. Estimation strategy

Parameters were estimated through generalized linear models, with robust standard errors to account for data clustering and repeated measures. Log_{10} *E. coli* differences and prevalence differences were estimated using a Gaussian distribution (link = identity), and disease prevalence ratios were estimated using a binomial distribution (link = log). For binomial models that did not converge, a modified Poisson (link = log) distribution was used instead. For hygienic latrine coverage in its continuous form, we divided percent coverage by 10 so that model estimates reflect a 10 percentage point increase in coverage. We pre-specified smoothing spline regression with three knots; however, we found no differences between spline segments so instead modeled continuous hygienic latrine coverage without spline terms. We conducted both unadjusted and adjusted analyses. We pre-specified a list of potential confounders for each outcome based on plausible causal pathways and included all variables that were associated with the outcome in bivariate regression models ($p < 0.20$) as covariates in adjusted models. Potential covariates included indicators of socioeconomic status, hygienic latrine access, open defecation of young children, and the number of domestic animals by type in the study compound. The full list of potential covariates and included covariates for each outcome can be found in Supplementary Materials (Table S1).

We assessed effect modification by population density (defined as the number of people living within the specified radius of the study compound) and season (monsoon vs. dry season). Effect modification by season was not included in our pre-specified analysis plan; we added this analysis post hoc because we found stronger intervention effects from sanitation improvements in the parent trial during the monsoon season. We operationalized population density both as a continuous variable and in tertiles. We defined the monsoon season by year using daily rainfall data recorded by the Bangladesh Meteorological Department at three weather stations nearest the study region between 2014 and 2016 (Zaman, 2018). We calculated five-day rolling averages of daily rainfall at each station and defined the monsoon season for each year as the period between the first and last days with a five-day rolling average rainfall of 10 mm or greater at any station. Monsoon seasons were April 2-September 27, 2014, March 31-September 25, 2015, and March 30-October 30, 2016. We conducted subgroup analyses for each exposure-outcome relationship within each tertile of population density and within each season to qualitatively assess effect modification. We added an interaction term between each exposure variable and the potential effect-modifying variable (continuous population density or binary season) to adjusted models. We interpreted a p-value <0.2 on the interaction term as quantitative evidence of significant effect modification (Thiese et al., 2016). Soil and food samples were excluded from effect modification analyses due to sample size.

In primary models, we pooled outcomes measured across all eight sampling rounds. This analysis assumes that the community-level sanitation variables did not change significantly over time. As a sensitivity analysis, we restricted models of exposures within 100 m to outcomes measured during the second data collection round, when community-

level sanitation variables were measured. Soil and food samples were not collected during the second round and were not included in sensitivity analyses. In addition, we considered the role of spatial autocorrelation of outcomes in our analysis. We found evidence of spatial clustering of outcomes before analysis (results available in our pre-specified analysis plan: https://osf.io/6u7cn/) and therefore assessed spatial autocorrelation in residual error values for each study compound from adjusted continuous models using Moran's I. We found no evidence of residual spatial autocorrelation for any outcome (Table S2).

3. Results

Over eight sampling rounds, participants from 360 study compounds completed 2,679 data collection visits. We collected a total of 2,317 stored water samples, 2,621 child hand rinses, 2,656 caregiver hand rinses, 385 soil samples, and 273 stored food samples. Health data were reported for 867 individual children under five for a total of 4,712 child observations over eight rounds. Mean $\log_{10}$-transformed *E. coli* counts were 1.02 (standard deviation [sd] = 1.05) in stored water, 1.50 (sd = 1.01) in child hand rinses, 1.48 (sd = 1.02) in caregiver hand rinses, 5.21

Table 1
Baseline characteristics by sanitation coverage within 50 m and 100 m of study compounds.

	50 m Radius Around Study Compounds				100 m Radius Around Study Compounds			
	Proportion of Compounds with At Least One Latrine		Proportion of Compounds with Only Hygienic Latrines		Proportion of Compounds with At Least One Latrine		Proportion of Compounds with Only Hygienic Latrines	
	<100% coverage n = 81	100% coverage n = 261	<100% coverage n = 266	100% coverage n = 75	<100% coverage n = 151	100% coverage n = 207	<100% coverage n = 323	100% coverage n = 34
Measured at Baseline of Parent Trial								
Maternal years of education, median (sd)	5 (3.5)	7 (3.4)	6 (3.4)	7 (3.4)	5 (3.3)	7 (3.4)	6 (3.4)	8 (3.3)
Mother's age in years, median (sd)	23 (4.5)	23 (5.1)	23 (4.8)	23 (5.5)	23 (5.0)	23 (5.0)	23 (4.8)	20.5 (6.2)
Food insecurity, n (%)								
Food secure	54 (67)	183 (70)	181 (68)	55 (73)	99 (66)	148 (71)	219 (68)	27 (79)
Mildly food insecure	7 (9)	20 (8)	21 (8)	6 (8)	14 (9)	15 (7)	27 (8)	2 (6)
Moderately food insecure	19 (23)	48 (18)	54 (20)	13 (17)	34 (23)	37 (18)	66 (20)	5 (15)
Severely food insecure	1 (1)	10 (4)	10 (4)	1 (1)	4 (3)	7 (3)	11 (3)	0 (0)
Wealth, n (%)								
Quartile 1 (Least Wealth)	24 (30)	51 (20)	64 (24)	11 (15)	38 (25)	40 (19)	75 (23)	3 (9)
Quartile 2	24 (30)	67 (26)	73 (27)	18 (24)	43 (28)	52 (25)	89 (28)	6 (18)
Quartile 3	14 (17)	66 (25)	63 (24)	17 (23)	33 (22)	51 (25)	76 (24)	7 (21)
Quartile 4 (Most Wealth)	19 (23)	77 (30)	66 (25)	29 (39)	37 (25)	64 (31)	83 (26)	18 (53)
Number of children <18 in the target household, median (sd)	1 (1.2)	1 (1.2)	1 (1.1)	1 (1.2)	1 (1.2)	1 (1.2)	1 (1.2)	1 (1.4)
Number of individuals living in the target household, median (sd)	4 (2.5)	4 (2.1)	4 (2.3)	4 (1.9)	4 (2.2)	4 (2.2)	4 (2.2)	4.5 (2.0)
Distance in minutes to target household's primary drinking water source, median (sd)	0 (1.3)	0 (1.2)	0 (1.2)	0 (1.2)	0 (1.5)	0 (1.1)	0 (1.3)	0 (0.9)
Improved roof, n (%)	81 (100)	257 (98)	263 (99)	74 (99)	150 (99)	203 (98)	318 (98)	34 (100)
Improved floor, n (%)	2 (2)	39 (15)	24 (9)	17 (23)	14 (9)	28 (14)	32 (10)	10 (29)
Improved walls, n (%)	59 (73)	166 (64)	185 (70)	39 (52)	102 (68)	132 (64)	217 (67)	17 (50)
Measured at Baseline of Environmental Substudy								
Primary latrine used by target household is hygienic, n (%)	46 (61)	188 (75)	173 (68)	61 (85)	94 (67)	150 (74)	213 (69)	30 (88)
Open defecation by children <3, n (%)								
Daily	70 (86)	208 (80)	223 (84)	54 (72)	125 (83)	164 (79)	268 (83)	21 (62)
Occasionally	9 (11)	49 (19)	39 (15)	19 (25)	22 (15)	40 (19)	50 (15)	11 (32)
Never	2 (2)	4 (2)	4 (2)	2 (3)	4 (3)	3 (1)	5 (2)	2 (6)
Open defecation by children 3–8, n (%)								
Daily	23 (48)	41 (31)	58 (40)	6 (17)	34 (41)	32 (31)	65 (38)	1 (7)
Occasionally	6 (12)	27 (20)	23 (16)	9 (26)	14 (17)	19 (18)	31 (18)	2 (14)
Never	19 (40)	65 (49)	64 (44)	20 (57)	34 (41)	52 (50)	75 (44)	11 (79)
Number of cattle, n (%)								
None	24 (30)	78 (30)	82 (31)	19 (25)	44 (29)	63 (30)	94 (29)	12 (35)
Tertile 1 (1–2)	25 (31)	84 (32)	76 (29)	33 (44)	54 (36)	59 (29)	102 (32)	11 (32)
Tertile 2 (3–4)	13 (16)	51 (20)	53 (20)	11 (15)	22 (15)	45 (22)	60 (19)	7 (21)
Tertile 3 (5–57)	19 (23)	48 (18)	55 (21)	12 (16)	31 (21)	40 (19)	67 (21)	4 (12)
Number of poultry, n (%)								
None	9 (11)	23 (9)	26 (10)	6 (8)	14 (9)	19 (9)	27 (8)	5 (15)
Tertile 1 (1–10)	32 (40)	67 (26)	73 (27)	25 (33)	46 (30)	56 (27)	89 (28)	13 (38)
Tertile 2 (11–21)	20 (25)	84 (32)	82 (31)	22 (29)	46 (30)	64 (31)	100 (31)	10 (29)
Tertile 3 (22–132)	20 (25)	87 (33)	85 (32)	22 (29)	45 (30)	68 (33)	107 (33)	6 (18)
Number of goats and sheep, n (%)								
None	46 (57)	163 (62)	164 (62)	45 (60)	94 (62)	123 (59)	194 (60)	22 (65)
Tertile 1 (1–2)	20 (25)	59 (23)	58 (22)	20 (27)	30 (20)	54 (26)	77 (24)	7 (21)
Tertile 2 (3)	6 (7)	15 (6)	16 (6)	5 (7)	10 (7)	11 (5)	18 (6)	3 (9)
Tertile 3 (4–20)	9 (11)	24 (9)	28 (11)	5 (7)	17 (11)	19 (9)	34 (11)	2 (6)
Number of other animals, n (%)								
None	75 (93)	220 (84)	229 (86)	65 (87)	135 (89)	172 (83)	278 (86)	28 (82)
Tertile 1 (1)	2 (2)	20 (8)	18 (7)	4 (5)	9 (6)	13 (6)	21 (7)	1 (3)
Tertile 2 (2–3)	2 (2)	10 (4)	9 (3)	3 (4)	4 (3)	11 (5)	12 (4)	3 (9)
Tertile 3 (4–12)	2 (2)	11 (4)	10 (4)	3 (4)	3 (2)	11 (5)	12 (4)	2 (6)

(sd = 1.07) in soil, and 1.77 (sd = 1.36) in stored food. The overall prevalence of diarrheal disease and acute respiratory infection among children under five was 14.5% and 22.7%, respectively.

3.1. Community-level sanitation coverage

Of the 360 study compounds, 342 had neighboring compounds within 50 m (median n neighbors = 4, range 1–23) and 358 had neighbors within 100 m (median n neighbors = 10, range 1–43) who were captured by our community survey and included in the analysis of any latrine coverage. One study compound was missing data on the hygienic status of neighboring latrines, resulting in 341 and 357 study compounds for analysis of exclusively hygienic latrines within 50 and 100 m, respectively.

Among study compounds with neighbors present within the specified range, for 261 (76%) compounds, 100% of neighboring compounds within 50 m had at least one latrine and for 207 (58%) compounds, 100% of neighboring compounds within 100 m had at least one latrine (Table 1; Fig. 1). Within the 50 m radius, for 75 compounds (22%), 100% of neighbors had exclusively hygienic latrines (Fig. 1). Within the 100 m radius, for 34 compounds (10%), 100% of neighbors had exclusively hygienic latrines (Fig. 1; Table 1). Overall, enrolled compounds with 100% community-level sanitation coverage were more likely to be food secure, wealthier, use a hygienic latrine themselves, and report no open defecation for children aged 3–8 compared to those with <100% coverage (Table 1).

In adjusted analyses, compounds surrounded by 100% community-level "any latrine coverage" (i.e., all compounds within range having at least one latrine) within 50 m had slightly lower $\log_{10}$ *E.coli* counts in stored water (Fig. 2; Table S3; Δlog = −0.13, 95% CI -0.26, −0.01), child hand rinses (Δlog = −0.13, 95% CI -0.24, −0.02), and caregiver hand rinses (Δlog = −0.16, 95% CI -0.29, −0.03) and marginally lower prevalence of diarrheal disease (PR = 0.82, 95% CI 0.64, 1.04) and ARI (PR = 0.84, 95% CI 0.69, 1.03) compared to compounds surrounded by <100% coverage. At 100 m, 100% any latrine coverage was marginally associated with reduced $\log_{10}$ *E. coli* counts in caregiver hand rinses (Fig. 2; Table S3; Δlog = −0.10, 95% CI -0.21, 0.00) and reduced diarrheal disease (PR = 0.83, 95% CI 0.67, 1.02).

There were no associations between community-level "hygienic latrine coverage" (the proportion of compounds within range with only hygienic latrines) and any outcome after adjustment, including both binary and continuous forms of the exposure within a range of 50 m or 100 m (Fig. 3; Table 2; Table S4). There were marginally significant associations between hygienic latrine coverage in continuous form and lower *E. coli* counts in stored water at both 50 m and 100 m, but the magnitude of the associations was small (0.01 and 0.02 $\log_{10}$ reductions, respectively) (Table 2). Results were not significantly different for any exposure using outcome data from the second sampling round only, when the community coverage variables were measured (Tables S5–S7).

3.2. Effect modification

The median number of people living within 50 m of study compounds was 33 (tertiles = 0–21, 22–45, 46–159). The median number of people living within 100 m was 82 (tertiles = 0–62, 63–111, 112–354). Within 50 m of study compounds, population density modified the association between any latrine coverage and *E. coli* on caregiver hands (interaction p-value = 0.06) and diarrheal disease (interaction p-value = 0.15) (Fig. 4; Table S8). In the middle and highest population density tertiles, compounds surrounded by 100% any latrine coverage had approximately 0.25-$\log_{10}$ lower *E. coli* counts on child and caregiver hands than those surrounded by <100% coverage; there was no association in the lowest tertile. Similarly, in the highest tertile of population density, children in compounds surrounded by 100% any latrine coverage had 33% lower prevalence of diarrhea (PR = 0.67, 95% CI 0.47, 0.95). Also within 50 m, population density modified the association between 100% hygienic latrine coverage and all outcomes other than ARI (interaction p-values<0.2) (Fig. 4; Table S9). Qualitatively, being surrounded by 100% hygienic latrine coverage was associated with progressively larger reductions in *E. coli* counts and diarrhea

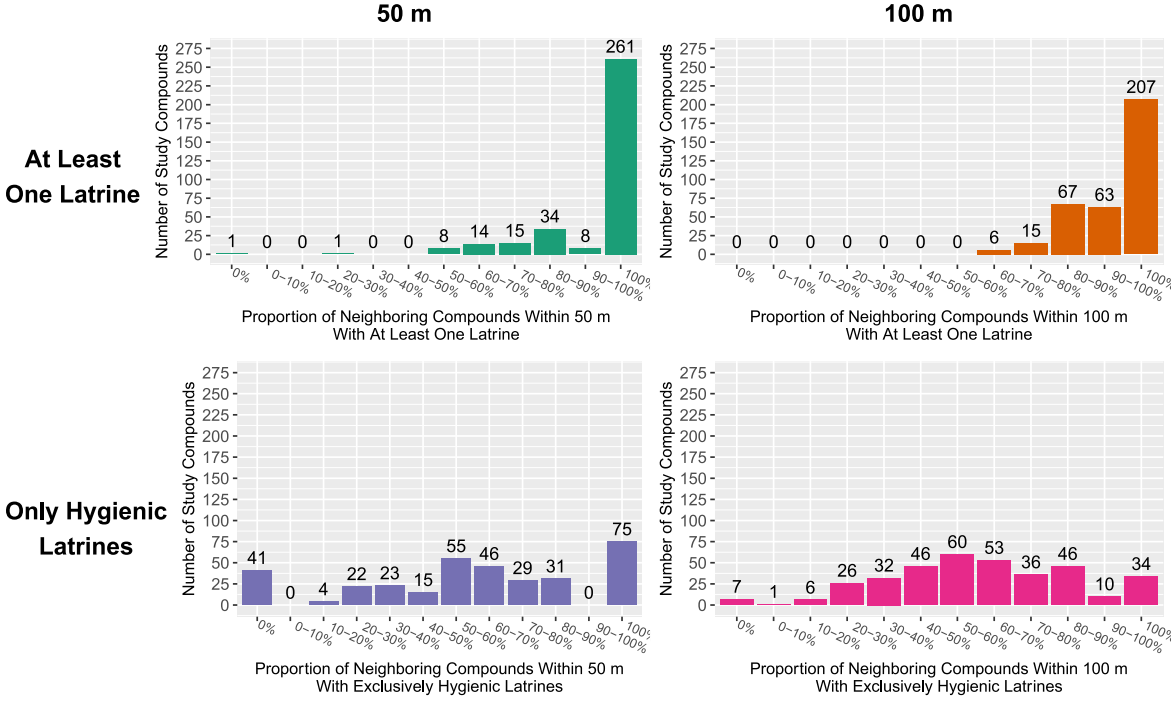

Fig. 1. Distribution of community-level any latrine coverage (the proportion of compounds within range with at least one latrine; top row) and community-level hygienic latrine coverage (the proportion of compounds within range with only hygienic latrines; bottom row). Distributions plotted within a radius of 50 m (left column) and 100 m (right column) around study compounds.

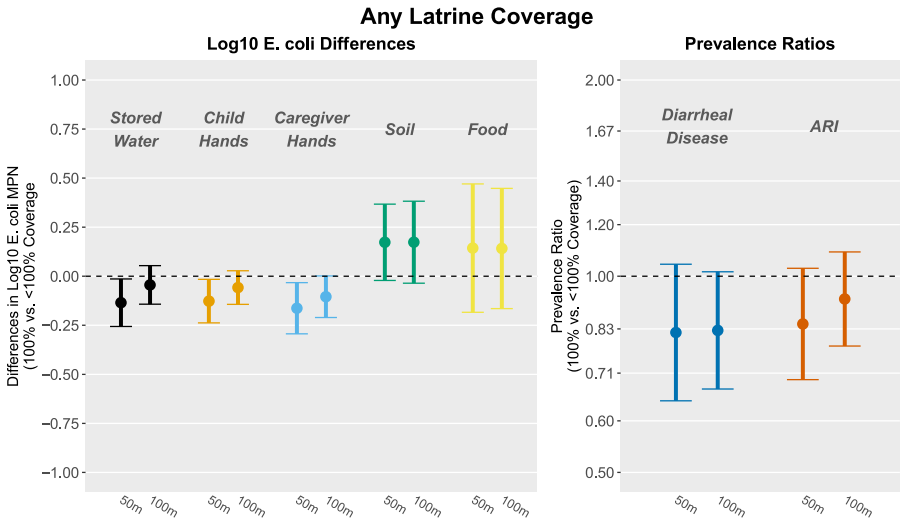

Fig. 2. Associations between study outcomes and community-level any latrine coverage (the proportion of compounds within range with at least one latrine) within 50 m (left within each outcome) and 100 m (right) of study compounds, modeled as binary variables. Estimates reflect $\log_{10}$ *E. coli* differences and diarrhea and ARI prevalence ratios comparing compounds surrounded by 100% vs. <100% coverage. All models were adjusted for relevant covariates (Table S1) and include robust standard errors.

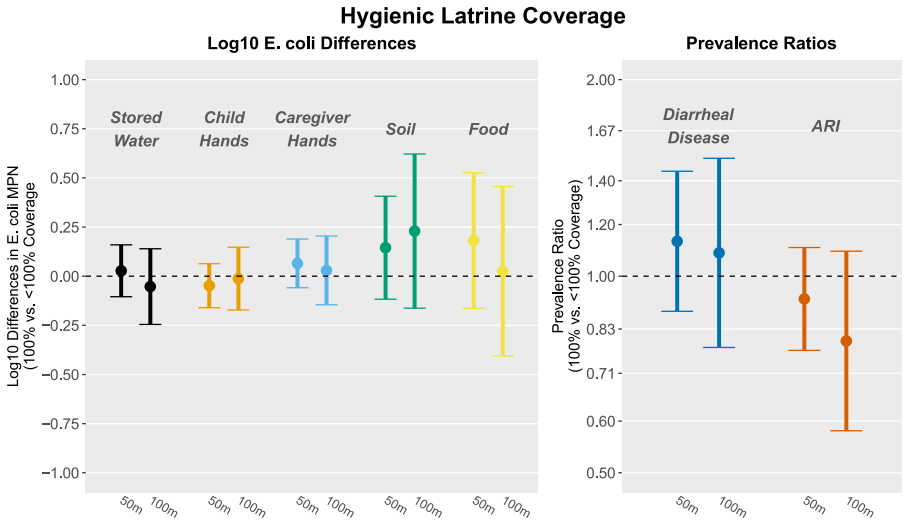

Fig. 3. Associations between study outcomes and community-level hygienic latrine coverage (the proportion of compounds within range with only hygienic latrines) within 50 m (left within each outcome) and 100 m (right) of study compounds, modeled as binary variables. Estimates reflect $\log_{10}$ *E. coli* differences and diarrhea and ARI prevalence ratios comparing compounds surrounded by 100% vs. <100% coverage. All models were adjusted for relevant covariates (Table S1) and include robust standard errors.

prevalence as population density increased but most subgroup estimates included the null. Results were generally similar using the 100 m radius (Fig. S1; Tables S10–S11) and for the continuous form of hygienic latrine coverage (Tables S12–S13).

Season did not modify any outcome association with having 100% any latrine coverage at 50 m or 100 m (Fig. 5; Table S14; Fig. S2; Table S16). Within 50 m of study compounds, season modified the association between having 100% hygienic latrine coverage and $\log_{10}$ *E. coli* counts on child hands and caregiver hands (in opposite directions) and the prevalence of diarrheal disease (Fig. 5; Table S15). During monsoon seasons, being surrounded by 100% hygienic latrine coverage within 50 m was associated with reduced *E. coli* contamination on child hands ($\Delta\log = -0.14$, 95% CI -0.27, 0.00) and increased contamination on caregiver hands ($\Delta\log = 0.16$, 95% CI 0.02, 0.30), but both magnitudes were small. Being surrounded by 100% hygienic latrine coverage was marginally associated with an increase in the prevalence of diarrheal disease (PR = 1.32, 95% CI 0.96, 1.81) in the monsoon season. Results were similar but weaker for the 100 m radius and for continuous hygienic latrine coverage (Fig. S2; Tables S17–S19).

4. Discussion

We found that 100% community-level coverage, with all neighboring compounds within 50 m of study compounds having at least one latrine, was associated with slightly lower counts of *E. coli* in stored drinking water and caregiver and child hand rinses (about 0.15-$\log_{10}$ lower MPN) and marginally associated with reduced prevalence of diarrheal disease and ARI (about 17% relative reduction) among study compounds. These associations were attenuated when we evaluated coverage within 100 m of study compounds. Community-level coverage with hygienic latrines was not associated with *E. coli* counts, diarrhea or ARI using either radius. Associations between coverage and *E. coli* on child or caregiver hands and diarrheal disease were consistently modified by population density, with coverage more strongly associated with outcomes for compounds in areas with higher population density. We found no strong evidence of effect modification by monsoon vs. dry seasons. Overall, our findings support a broad body of epidemiological evidence that community-level sanitation coverage can influence child health and environmental contamination.

We aimed to capture many potential definitions of community-level sanitation coverage, including the relevant sanitation metric and the

Table 2
Results for continuous exposure definition. Adjusted and unadjusted associations between study outcomes and community-level hygienic latrine coverage (the proportion of compounds within range with only hygienic latrines), modeled as a continuous exposure. Estimates reflect changes in study outcomes associated with a 10 percentage point increase in hygienic latrine coverage. Exposures were modeled for two different radii (50 m and 100 m) around study compounds. All models include robust standard errors.

	50 m				100 m			
	Unadjusted		Adjusted[a]		Unadjusted		Adjusted[a]	
	n	Estimate	*n*	Estimate	*n*	Estimate	*n*	Estimate
Log10 E. coli MPN Differences								
Stored Water	2204	−0.02 (−0.04, 0.00)*	2123	−0.01 (−0.03, 0.00)	2299	−0.04 (−0.06, −0.02)*	2214	−0.02 (−0.04, 0.00)
Child Hand Rinses	2488	−0.01 (−0.03, 0.00)	2443	0.00 (−0.02, 0.01)	2598	−0.02 (−0.04, 0.00)	2550	−0.01 (−0.03, 0.01)
Caregiver Hand Rinses	2522	0.00 (−0.02, 0.02)	2486	0.01 (−0.01, 0.03)	2633	−0.02 (−0.04, 0.01)	2594	0.00 (−0.02, 0.02)
Soil	364	−0.03 (−0.07, 0.00)*	359	0.02 (−0.01, 0.06)	382	−0.07 (−0.12, −0.03)*	377	0.01 (−0.04, 0.06)
Food	265	0.02 (−0.03, 0.07)	265	0.02 (−0.03, 0.07)	273	0.02 (−0.04, 0.07)	273	0.00 (−0.06, 0.06)
Prevalence Ratios								
Diarrheal Disease	4454	1.00 (0.96, 1.03)	4419	1.00 (0.97, 1.03)	4652	0.99 (0.94, 1.04)	4617	1.00 (0.96, 1.05)
Acute Respiratory Infection (ARI)	4457	0.99 (0.96, 1.01)	4422	1.00 (0.97, 1.02)	4655	0.99 (0.96, 1.02)	4620	1.01 (0.97, 1.04)
Prevalence Differences								
Diarrheal Disease	4454	0.00 (−0.01, 0.00)	4419	0.00 (0.00, 0.00)	4652	0.00 (−0.01, 0.01)	4617	0.00 (−0.01, 0.01)
Acute Respiratory Infection (ARI)	4457	0.00 (−0.01, 0.00)	4422	0.00 (−0.01, 0.01)	4655	0.00 (−0.01, 0.01)	4620	0.00 (−0.01, 0.01)

*Statistically significant at the 0.05 level.
[a] See Table S1 for full list of potential and selected covariates by outcome.

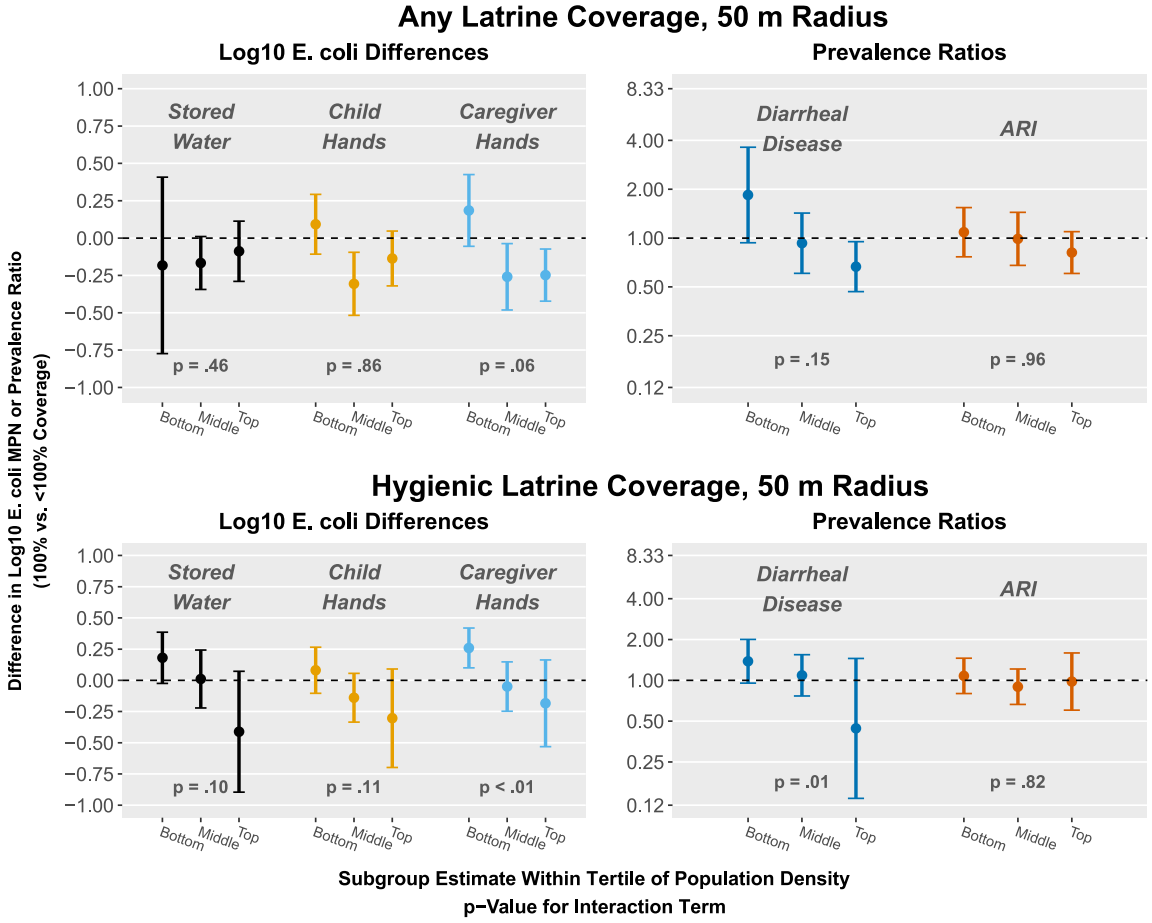

Fig. 4. Effect modification by population density on community-level any latrine coverage (the proportion of compounds within range with at least one latrine) and community hygienic latrine coverage (the proportion of compounds within range with only hygienic latrines) within 50 m of study compounds and study outcomes. Exposures were modeled as binary variables (100% vs. <100% coverage). Plots show subgroup estimates within tertiles of population density. P-values are for the interaction term between continuous population density and the exposure. All models are adjusted and include robust standard errors.

area defined by "community". Previous studies have measured community-level sanitation as the proportion of households within a given area with access to any latrine (Andrés et al., 2017; Harris et al., 2017; Larsen et al., 2017; Oswald et al., 2019), a basic latrine (Berendes et al., 2020), or an improved latrine (Andrés et al., 2017; Berendes et al., 2018; Fuller et al., 2016; Garn et al., 2018; Komarulzaman et al., 2017;

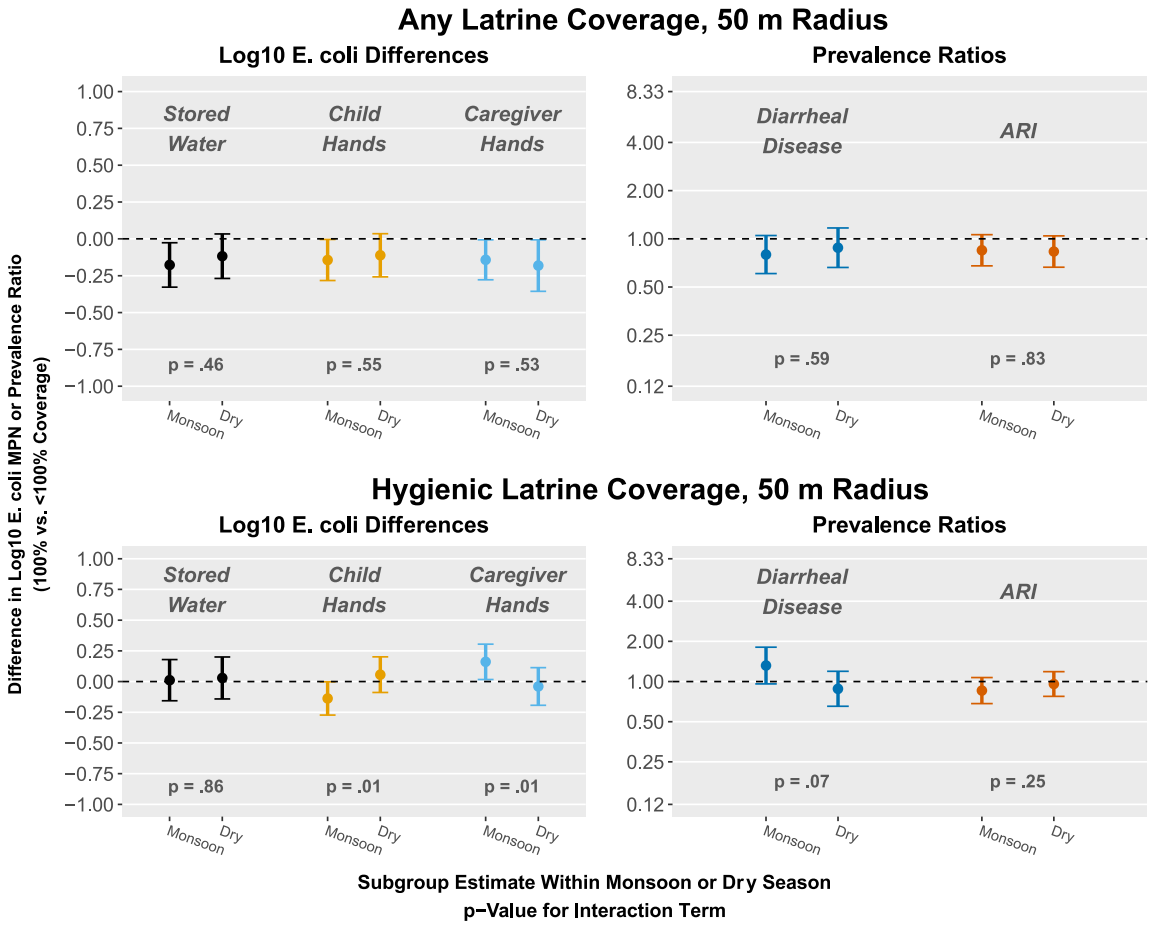

Fig. 5. Effect modification by season (monsoon vs. dry) on community-level any latrine coverage (the proportion of compounds within range with at least one latrine) and community hygienic latrine coverage (the proportion of compounds within range with only hygienic latrines) within 50 m of study compounds and study outcomes. Exposures were modeled as binary variables (100% vs. <100% coverage). Plots show subgroup estimates within each season. P-values are for the interaction term between season and the exposure. All models are adjusted and include robust standard errors.

Huda et al., 2019), with no significant differences in study results by latrine quality. In this study, we modeled both access to any type of latrine as well as access to hygienic latrines, which allowed us to assess the role of latrine quality in community-level coverage. Our definition for hygienic latrine captured both the type of latrine (improved facility) and whether it was observed to effectively isolate feces from the environment (not draining into the environment and feces well-contained in pit). For hygienic latrine coverage, we were able to assess the impact of reaching 100% community-level coverage as a binary exposure, as well as the impact of incremental increases in coverage as a continuous exposure. We were only able to assess any latrine coverage as a binary variable (100% vs. <100%) because most compounds within the specified radii had a latrine (Fig. 1). Our chosen exposure definitions of full community coverage with any latrine or with hygienic latrines capture the goals of many sanitation programs. In areas with lower or more variable latrine coverage, associations between complete coverage, fecal contamination, and child health might be stronger than what we measured here.

Although most associations we observed in our analysis were small, we found that 100% community-level coverage with any latrine was more important for fecal contamination and infectious disease transmission than 100% community-level coverage with exclusively hygienic latrines. This might indicate that access to any latrine may effectively reduce community contamination, for example by reducing open defecation. It is also possible that latrines classified as hygienic in our analysis did not sufficiently differ from unhygienic latrines in their ability to isolate fecal waste from the environment. For example, we did not collect data on pit emptying practices among neighboring compounds, and unsafe emptying or disposal practices could attenuate the benefits of community-level coverage with hygienic latrines. Among target households that reported emptying their latrine pit during the study period, 69% reported burying the pit contents, while 31% disposed of pit contents in a body of water or field. We also did not measure actual latrine use among neighbors and cannot ascertain whether the presence of a latrine or a hygienic latrine in the compound indicated exclusive use of these facilities for defecation. Among target households in our study, 77% reported exclusive latrine use among adults. Since these households were in the control arm of the parent trial and received no intervention, their practices are likely representative of neighboring compounds. Future research on community-level sanitation should aim to capture more nuanced dimensions of latrine use and pit emptying.

Definitions of "community" or "neighborhood" in previous literature have varied considerably, sometimes as entire villages or sampling clusters, which can encompass multiple villages (Berendes et al., 2017, 2020; Fuller and Eisenberg, 2016; Garn et al., 2018; Kmush et al., 2021; Komarulzaman et al., 2017; Larsen et al., 2017; Oswald et al., 2019; Vyas et al., 2016), and other times as circular areas around a target household defined by a set radius ranging between 20-1,000 m (Berendes et al., 2018; Fuller et al., 2016; Harris et al., 2017; Huda et al., 2019). We chose a maximum radius of 100 m in this study to focus on proximate fecal contamination amongst nearby neighbors and assessed

a smaller radius (50 m) to assess differences in transmission by distance. Our results were qualitatively similar between 50 m and 100 m exposures, although associations were generally stronger at 50 m, suggesting that the relevant distance for pathogen transport from latrines in this setting was captured within this smaller radius. Another study similarly found that community sanitation within 50 m but not 100 m was associated with fecal contamination (Berendes et al., 2018), while a different study found no association between community-level coverage within 20 m and fecal contamination (Huda et al., 2019). Most of the research on community-level sanitation has measured coverage over large sampling areas, and almost all these studies found that coverage was associated with improved child health. Pathogens introduced into the environment through unsafe sanitation may be carried by people, animals, food, and water as they travel across and between communities over large areas. Our analysis did not capture any potential roles of community-level sanitation coverage over larger scales. However, analyses at larger scales likewise fail to capture relevant associations within more narrowly defined communities (e.g., close-range neighbors) and may also miss heterogeneity in sanitation coverage within larger geographical units. Research on community-level sanitation coverage should more clearly differentiate between broadly defined communities comprised of entire villages, neighborhoods, or more, and narrowly defined communities of proximate neighbors. It is possible that sanitation coverage is important at each of these scales in unique ways and assessing coverage at only one scale might provide an incomplete picture and miss interactions across scales. Systems-based approaches to studying enteric disease transmission may be needed to simultaneously capture the role of community-level sanitation coverage across its numerous dimensions and their interactions (Eisenberg et al., 2012).

Population density is also relevant for understanding community-level sanitation. In our rural Bangladeshi setting, population density ranged from 0 to 354 individuals within 100 m (median = 82), and the importance of community-level sanitation coverage increased with increasing population density. In our analysis, compounds in relatively high-density areas whose neighbors within 50 m all had at least one latrine had up to 0.25-log lower *E. coli* counts on hands and 33% lower prevalence of child diarrhea. This relationship may be stronger in urban settings with higher population density, although generalizability to urban settings is limited due to differences in the types of sanitation infrastructure typically used in each setting. Population density should always be considered in measures of community-level sanitation coverage.

Among environmental samples, we found statistically significant associations between community-level coverage and *E.coli* counts in stored water and on child and caregiver hand rinses, but found no associations in soil or food samples. It is possible that our smaller sample size for soil and food samples, which were only collected during two of eight sampling rounds, was not sufficient to detect associations for these sample types. It is also possible that contamination of soil and food is more strongly connected to practices within the household (e.g., child defecation, animal feces management, food hygiene) than community-level sanitation. In contrast, contamination in drinking water might be connected to community sanitation through contamination occurring at the source (primarily tubewells in this setting) by groundwater infiltration or surface runoff into the wellhead. While domestic activities are an important determinant of hand contamination (Pickering et al., 2011), community-level sanitation might also impact hand contamination through person-to-person contact with community members. Overall, small magnitudes of the associations for water and hand rinses and small sample size for soil and food in our analysis preclude a robust understanding of the important pathways between community-level sanitation and household environmental contamination.

Associations between community-level sanitation and diarrheal disease were stronger than those for *E. coli* contamination, possibly because *E. coli* presence does not correlate strongly with pathogen contamination levels (Goddard et al., 2020). Community sanitation coverage may also improve health through pathways not captured in our study, such as reducing pathogen transmission by flies. Previous studies have found that community-level latrine coverage is associated with larger reductions in child diarrhea than household-level latrine access (Andrés et al., 2017; Harris et al., 2017; Komarulzaman et al., 2017). The sanitation intervention in the parent WASH Benefits trial that provided latrine upgrades to compounds was associated with a 39% relative reduction (PR = 0.61, 95% CI 0.46, 0.81) in child diarrhea compared to controls (Luby et al., 2018). In a previous analysis of our longitudinal substudy nested within the sanitation and control arms of the parent trial, we estimated a 19% relative reduction (PR = 0.81, 95% CI 0.66, 1.00) in diarrhea prevalence compared to controls (Contreras et al., 2022). The intervention also led to a 0.08-log reduction in *E. coli* counts in stored water and on child hands compared to controls, measured 1–3.5 years after implementation, but did not reduce *E. coli* in environmental samples at earlier sampling timepoints (Contreras et al., 2021; Ercumen et al., 2018). While the present observational analysis is susceptible to unmeasured confounding, our findings indicate some associations with community-level coverage that are comparable to or larger in size than the effects of the compound-level intervention, especially in areas with high population density.

One potential limitation of our study was the approximation of a 100 m radius based on ~300 steps from the study compound. If field staff did not travel a full 100 m, we may have missed neighbors that were within range. We used GPS data to exclude any neighboring compounds field staff visited that were beyond 100 m. In addition, our analysis was observational, and community-level sanitation coverage appeared highly associated with socioeconomic status. Although we adjusted for socioeconomic factors, including maternal education and wealth, it is possible that residual confounding was present. We analyzed several forms of the exposure variables, which increased the probability that one or more significant results were due to chance. However, the trends we observed are biologically plausible, such as more pronounced protective associations with increasing community-level coverage in high-density areas and within shorter pathogen transport distances. Also, our analysis was pre-specified (except effect modification by season) and highly powered to detect small reductions in *E. coli* counts with statistical precision due to the large number of samples collected and child observations made over eight data collection rounds. Generalizability of our findings to other settings may be limited due to the context-dependent designs of sanitation systems and hydrogeological features that can influence the environmental transport of pathogens, such as high groundwater table and a lithology dominated by alluvial sediments in our study area (Ahmed et al., 2004).

5. Conclusions

Overall, we found that latrine coverage within highly proximate areas was associated with reduced environmental fecal contamination and improved child health, especially where population density is relatively high in this rural setting. The associations between community-level sanitation coverage and environmental contamination and diarrhea were sensitive to how coverage was defined. There is no current consensus on the relevant scale over which community-level coverage influences fecal contamination or health, and few studies have assessed the role of population density. Continued work is needed to understand the complexities of pathogen transmission across multiple scales of community, which can inform policy for implementing transformative sanitation systems that effectively protect community health.

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Data statement

De-identified data used for this analysis will be made freely available on OSF upon publication (https://osf.io/6u7cn/).

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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.2022.114031.

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Maternal urinary organophosphate ester metabolite concentrations and glucose tolerance during pregnancy: The HOME Study☆,☆☆

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ABSTRACT

Background: Endocrine-disrupting chemicals may alter glucose homeostasis, especially during pregnancy. Biomonitoring studies suggest ubiquitous human exposure to organophosphate esters (OPEs), chemicals with endocrine-disrupting capabilities. Few studies have examined the association between maternal exposure to OPEs and blood glucose during pregnancy.

Methods: With data from 301 pregnant women in the Health Outcomes and Measures of the Environment (HOME) Study, a prospective pregnancy and birth cohort in Cincinnati, Ohio, USA, we examined whether OPE concentrations were associated with changes in blood glucose. We quantified four OPE metabolites in maternal spot urine samples collected at 16- and 26-weeks pregnancy. We extracted results from the glucose challenge test (GCT) and oral glucose tolerance test (OGTT) via medical chart review. Women with GCT $\geq$ 140 mg/dL or any abnormal values in OGTT ($\geq$ 95 mg/dL fasting glucose, $\geq$ 180 mg/dL 1-h glucose, $\geq$ 155 mg/dL 2-h glucose, $\geq$ 140 mg/dL 3-h glucose) were defined as having elevated glucose levels. We used linear regression and Bayesian Kernel Machine Regression (BKMR) to estimate the associations of individual OPE metabolites and OPE mixtures with blood glucose levels during pregnancy. We used modified Poisson regression to estimate the associations of OPE metabolite concentrations with elevated glucose levels. We further examined effect measure modification by maternal characteristics (age, pre-pregnancy body mass index [BMI], and race/ethnicity).

Results: Diphenyl phosphate (DPHP) had the highest geometric mean concentration of the urinary OPE metabolites (1.83 μg/L at 16 weeks, 1.24 μg/L at 26 weeks). Thirty women (10.0%) had elevated glucose levels. Individual OPE metabolites or their mixtures were not significantly associated with continuous GCT results. We did not observe effect measure modification by maternal age, pre-pregnancy BMI categories, or race/ethnicity. Compared with women in the 1st tertile of average DPHP of 16- and 26 weeks of pregnancy, women in the 3rd tertile tended to have a reduced risk of elevated glucose levels (RR = 0.41, 95% CI = 0.16–1.06, p for trend = 0.06).

Conclusion: In this cohort, maternal urinary OPE metabolite concentrations were weakly associated with blood glucose levels during pregnancy.

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☆☆ The study protocol was approved by the Institutional Review Board (IRB) at the Cincinnati Children's Hospital Medical Center (CCHMC). The Centers for Disease Control and Prevention (CDC) deferred to the CCHMC IRB as the IRB of record.

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1. Introduction

Since the phase out of polybrominated diphenyl ethers (PBDEs) and other organohalogen flame retardants, organophosphate esters (OPEs) have been used extensively as flame retardants and plasticizers in commercial and consumer products, such as furniture, children's products, electronics, and food packaging (Blum et al., 2019). Unlike PBDEs, which are classified as persistent organic pollutants (POPs), OPEs are less likely to accumulate in the environment based on their physical and chemical properties (Blum et al., 2019). However, growing evidence has shown that OPEs can persist in water and undergo long-range transport, acting like persistent mobile organic compounds (Blum et al., 2019; Zhang et al., 2016). OPEs have been widely detected in indoor air, house dust, and food products using OPE-plasticized film wrappers, (Blum et al., 2019; Carlsson et al., 1997; He et al., 2018; Hoffman et al., 2015). Humans can rapidly metabolize OPEs, so OPEs have relatively short half-lives (from hours to days) (Hoffman et al., 2014). Biomonitoring studies have shown that OPE exposure is ubiquitous in the general population, with higher concentrations found in pregnant women and children (Butt et al., 2014; Hoffman et al., 2014; Ospina et al., 2018; Percy et al., 2020). OPEs are endocrine disrupting chemicals that can alter metabolic pathways by interacting with peroxisome proliferator-activated receptors, estrogenic receptors, mineralocorticoid receptors, etc. (Belcher et al., 2014; Zhang et al., 2016).

Gestational diabetes mellitus (GDM) affects approximately 7% of pregnancies, although prevalence estimates vary in different populations (DeSisto et al., 2014; Eades et al., 2017; Gao et al., 2019; Lee et al., 2018; Preston et al., 2020). GDM, and even subclinical hyperglycemia during pregnancy, have been associated with a range of adverse pregnancy outcomes, such as preeclampsia, preterm birth, cesarean delivery, macrosomia, and neonatal hypoglycemia (Metzger et al., 2008; Metzger et al., 2019; Preston et al., 2020). Moreover, women with GDM have been reported to have higher risks of disordered glucose metabolism and cardiovascular diseases during long-term follow-up after pregnancy (Li et al., 2018; Lowe et al., 2018). Intrauterine exposure to untreated GDM has been associated with offspring insulin resistance and impaired glucose tolerance in childhood (Lowe et al., 2019). Recently, the contribution of endocrine-disrupting chemicals (EDCs) to metabolic disorders (e.g., diabetes, obesity, and non-alcoholic fatty liver disease) has been recognized. Specifically, some PBDEs, per- and polyfluoroalkyl substances (PFAS), phthalates, bisphenol A (BPA), and parabens have been positively associated with glucose levels during pregnancy and the risk of GDM. Certain GDM risk factors (e.g., maternal age, pre-pregnancy body mass index [BMI], race/ethnicity) may modify such associations (Bellavia et al., 2018, 2019; Li et al., 2019; Preston et al., 2020; Shaffer et al., 2019).

A growing number of experimental studies have investigated OPE exposure and glucose homeostasis. One animal study found that treatment with a mixture of OPEs (tricresyl phosphate [TCP], triphenyl phosphate [TPHP], and tris(1,3-dichloro-2-propyl) phosphate [TDCIPP]) altered circulating insulin and leptin in female mice and ghrelin in males despite no marked effect on glucose or insulin tolerance (Vail et al., 2020). Another study reported that exposure to tris (2-chloroethyl) phosphate (TCEP) triggered biochemical responses, such as altered glucose levels, in freshwater fish *Cirrhinus mrigala* (Sutha et al., 2020). In the presence of dissolved organic matter, exposure to tris (2-butoxyethyl) phosphate (TBOEP) led to a decrease in glucose while exposure to TCEP did not alter the metabolic response in *Daphnia Magna* (Kovacevic et al., 2018). Few human studies have examined the relationship between OPEs and glucose tolerance. A recent study using data from the 2011–2014 National Health and Nutrition Examination Survey (NHANES) reported the associations between certain urinary OPE metabolites and prediabetes as well as indices of glucose homeostasis in adolescents both in single- and multi-pollutant contexts, and such associations were sex-dependent (Luo et al., 2020). Nevertheless, to our best knowledge, no studies have examined whether gestational exposure to OPEs is associated with glucose levels.

In the present study, we evaluated the associations between maternal urinary OPE metabolites and maternal glucose tolerance in a well-established cohort of pregnant women. We further explored whether the associations differed across maternal age, pre-pregnancy BMI, and maternal race/ethnicity, which may act as effect modifiers of GDM risks (Preston et al., 2020). Additionally, we considered the mixture of OPE metabolites as the exposure to reflect a combined body burden. We hypothesized that maternal exposure to OPEs is associated with altered glucose homeostasis during pregnancy and such associations may be modified by maternal chronological age, pre-pregnancy BMI, and race/ethnicity.

2. Methods

2.1. Participants

From March 2003 to January 2006, pregnant women who met the following inclusion criteria were recruited to the Health Outcomes and Measures of the Environment (HOME) Study, a prospective pregnancy and birth cohort in Cincinnati, Ohio: living in the study region, < 19 weeks pregnant, ≥ 18 years old, residing in a home built in or before 1978, planning to continue prenatal care and deliver at the collaborating clinics and hospitals, planning to live in the greater Cincinnati area for the next year, and fluent in English. Pregnant women were excluded if: living in a mobile or trailer home, HIV-positive, taking medications for seizures or thyroid disorders, on radiation treatment or chemotherapy for cancer, or diagnosed with diabetes, bipolar disorder, or schizophrenia. Detailed information on the cohort profile has been published previously (Braun et al., 2017, 2020).

The present analysis included 301 women who delivered a live singleton infant without congenital abnormalities, provided at least one spot urine sample during pregnancy that was analyzed for OPE metabolites, underwent glucose challenge test (GCT; if subsequently indicated, undergoing oral glucose tolerance test [OGTT]) in the late second or early third trimester, and had their GCT tests after the first urine sample collection. The study protocol was approved by the Institutional Review Board (IRB) at the Cincinnati Children's Hospital Medical Center (CCHMC). The Centers for Disease Control and Prevention (CDC) deferred to the CCHMC IRB as the IRB of record.

2.2. Urinary OPE metabolites

Trained research staff instructed women how to collect their urine samples in polypropylene specimen cups at an average of 16 (15.9 ± 1.8) weeks and 26 (26.6 ± 2.0) weeks of pregnancy. We refrigerated samples up to 24 h before shipping them overnight on dry ice to the CDC's National Center for Environmental Health where they were stored at $-80\ ^{\circ}C$ until analysis.

OPEs are rapidly metabolized to diesters and eliminated in the urine, so these urinary metabolites are used as indicators of OPE exposure in population studies (Blum et al., 2019; Kosarac et al., 2016; Van den Eede et al., 2013). Four urinary OPE metabolites, bis(2-chloroethyl) phosphate (BCEP), bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), di-n-butyl phosphate (DNBP), and diphenyl phosphate (DPHP) were quantified using a previously described approach with modifications (Jayatilaka et al., 2017, 2019). Briefly, urine containing metabolite conjugates (200 μL) underwent enzymatic hydrolysis, followed by automated off-line solid-phase extraction to remove unwanted urinary components and to concentrate target metabolites. The sample extracts were dried, reconstituted and analyzed via isotope dilution high-performance liquid chromatography-tandem mass spectrometry (Jayatilaka et al., 2019). The limit of detection (LOD) for each metabolite was 0.1 μg/L. Additional information on analytical and quality control methods for urine samples in the HOME Study is presented elsewhere (Percy et al., 2020).

To account for inter-individual differences in urine dilution, we measured specific gravity with an Atago model PAL-10S handheld refractometer at CCHMC Schubert Research Clinic after the OPE measurements. We calculated specific gravity standardized concentrations with the following formula (MacPherson et al., 2018):

$$OPEmetaboliteSG_{std}(\mu g/L) = OPE_i(SG_m - 1)/(SG_i - 1)$$

where $OPEmetaboliteSG_{std}$ is the specific gravity standardized urinary OPE metabolite concentration, OPE_i is the measured analyte concentration, SG_i is the actual specific gravity of the sample, and SG_m is the median specific gravity of the cohort at each time point.

2.3. GDM screening test and outcome variables

Participants underwent the standard clinical screening for GDM at an average of 26.4 ± 2.0 weeks of pregnancy. Standard clinical screening for GDM includes two steps: first, participants underwent a 1-h non-fasting, 50-g oral GCT test; participants with GCT results ≥140 mg/dL after 1 h underwent subsequent 3-h fasting, 100-g OGTT (American Diabetes Association, 2008). The Carpenter and Coustan Criteria threshold was used to assess the OGTT results: abnormal levels defined as ≥ 95 mg/dL fasting glucose, ≥ 180 mg/dL 1-h glucose, ≥ 155 mg/dL 2-h glucose, and ≥ 140 mg/dL 3-h glucose (American Diabetes Association, 2008). The test results and GDM records were extracted via medical chart review by trained staff. Due to the limited number of GDM cases in the analytical sample (N = 15), we expanded our definition of abnormal glucose levels to include elevated, defined as failed GCT, requiring OGTT and having at least one abnormal value in the OGTT, or diagnosed with GDM in records.

2.4. Statistical analysis

We replaced OPE metabolite concentrations < LOD by the $LOD/\sqrt{2}$ for analysis purposes when the percentage of non-detectable concentrations was lower than 10% (Hornung and Reed, 1990), which included BDCIPP at 16 weeks pregnancy (4.3%), and DPHP at 16 weeks (1.0%) and 26 weeks (2.7%). We used multiple imputations for OPE metabolite concentrations < LOD from a truncated normal distribution when the percentage of non-detectable concentrations was between 10% and 30% (Lubin et al., 2004; Uh et al., 2008), including BCEP at 16 weeks (12.5%) and 26 weeks (17.2%) pregnancy, BDCIPP at 26 weeks pregnancy (11.1%), and DNBP at 16 weeks (15.5%) and 26 weeks (26.4%) pregnancy (Lubin et al., 2004; Uh et al., 2008). Given the association between birth weight and maternal glucose concentrations, we used birth weight as the dependent variable to indicate pregnancy outcomes in the multiple imputation models (Kc et al., 2015). We selected maternal age, race/ethnicity, education, household income, parity, infant sex, marital status, pre-pregnancy BMI, serum cotinine concentrations, and blood lead concentrations (a proxy for the indoor environment) as the auxiliary variables. We generated 20 imputed datasets and used Rubin's rule to pool the estimates from regression models (Hippel, 2018; Rubin, 1987). We did not impute values if they were non-reportable or the samples were missing (reasons for missing samples may include not collecting the sample, not enough sample for measurement, etc.).

We calculated summary statistics of the exposure and outcome variables. We applied t-tests, analysis of variance (ANOVA), and Chi-square tests to determine if there were difference in the exposure and outcome variables by maternal and child characteristics. We used linear mixed-effect models to calculate the intraclass correlation coefficients (ICCs) across the two-time points with crude and specific gravity standardized OPE metabolite concentrations. The cut-off points of ICCs to assess reproducibility are ≤ 0.4 (poor), 0.4–0.75 (fair to good), and ≥ 0.75 (excellent) (Percy et al., 2020; Rosner, 2015).

Specific gravity standardized OPE metabolite concentrations were modeled both continuously and in tertiles of their distributions, with the 1st tertile as the reference group. We $\log_{10}$-transformed continuous concentrations to reduce the influence of outliers. Plasma glucose concentrations from 1-h GCT were approximately normally distributed, so we left them untransformed for analyses.

We fitted separate general linear models to estimate individual associations of specific gravity standardized OPE metabolite concentrations at 16 weeks of pregnancy with continuous GCT results. Similarly, we examined the associations between the average of OPE metabolite concentrations at 16 and 26 weeks and continuous GCT results. A total of 95 participants (31.1%) had their GCT between 16 and 26 weeks urine sample collections, raising concerns of temporality of using the latter urinary OPE metabolite concentrations. Therefore, we created the average exposure variable as follows: if the GCT was conducted after the 26-week sample collection, the exposure variable would be the average of OPE metabolite concentrations at 16 and 26 weeks and participants missing either measurement were not included; if the GCT was conducted between 16 and 26 weeks, the exposure variable would be the concentrations at 16 weeks. Further, we used a modified Poisson regression with robust error variance to assess the individual associations of OPE metabolite concentrations with the two categories of glucose tolerance (Zou, 2004). When OPEs were modeled in tertiles, the linear trend was tested by assigning the median of each tertile as a continuous variable in the regression models (Greenland, 1995).

We also examined the associations of exposure to multiple OPEs and continuous glucose concentrations using Bayesian kernel machine regression (BKMR). BKMR uses a kernel function to allow for flexible models of the overall joint effect of the mixture, individual estimates of exposure-outcome associations, and potential interactions between exposures without the linear assumptions of exposure-response relationships (Bobb, 2017). Since BKMR is susceptible to extreme values, we excluded one observation with the 16-week DNBP concentration greater than 5 IQRs from the median.

A priori, we identified the following covariates as potential confounders from a directed acyclic graph (Supplementary Material Fig. 1): age at delivery, race/ethnicity, household income, education, marital status, infant sex, parity, pre-pregnancy BMI (categorized into three groups: underweight and normal-weight [<25 kg/m^2], overweight [25.0–29.9 kg/m^2], and obesity [≥ 30.0 kg/m^2]) (Rasmussen et al., 2009), serum cotinine concentrations at 16 weeks pregnancy, blood lead concentrations at 16 weeks, and gestational age at time of the GCT. Due to missing self-reported pre-pregnancy weight data (25%), pre-pregnancy BMI was calculated using a two-step process reported elsewhere (Romano et al., 2021). Other missing covariates were not imputed.

Previous studies have reported heterogeneous associations between EDCs and maternal glycemic status during pregnancy based on women's risk of GDM (Jensen et al., 2018; Preston et al., 2020; Shaffer et al., 2019). In a secondary analysis, we tested the cross-product interaction terms between continuous OPE metabolite concentrations and the following GDM risk factors as potential effect modifiers: age at delivery (<25, 25–34, and ≥35 years old), pre-pregnancy BMI categories (<25 kg/m^2, 25.0–29.9 kg/m^2, and ≥30.0 kg/m^2), and race/ethnicity (non-Hispanic white, non-Hispanic black and others). An interaction term with a p-value <0.1 was considered statistically significant and the models would be further stratified. Due to the limited number of women with elevated glucose levels, possible interaction may exist, but we were unable to stratify the modified Poisson regression models.

We conducted sensitivity analyses to test the robustness of our results. For general linear models, we excluded two participants with GCT results ≥200 mg/dL to eliminate the influence of outliers on the results. For BKMR models, we added back the observation with extreme 16-week DNBP concentration.

We performed data analysis using SAS (Version 9.4; SAS Institute Inc., Cary, NC, USA). We used R packages (mice and qgcomp) for left-truncated multiple imputation and bkmr for the mixture modeling (Bobb, 2017; Buuren and Groothuis-Oudshoorn, 2011; Keil, 2021; R

Core Team, 2021). We used the R package (ICC) to calculate 95% CIs of ICCs (Wolak et al., 2012).

3. Results

Study participants were predominantly non-Hispanic white (67.8%), aged between 25 and 34 years at delivery (62.8%), having a Bachelor's degree or higher education (77.4%), and with pre-pregnancy BMI below 30 kg/m^2 (80.4%) (Supplementary Material Table S1). The demographic and pregnancy characteristics were comparable between the original and the analytic population (Supplementary Material Table S1). More than 80% of the urine samples at 16 and 26 weeks had detectable OPE metabolites except for DNBP at 26 weeks (73.2%). DPHP had the highest geometric mean concentrations at 16 and 26 weeks of pregnancy (1.8 μg/L and 1.2 μg/L), followed by BDCIPP (0.8 μg/L and 0.6 μg/L), BCEP (0.6 μg/L and 0.5 μg/L), and DNBP (0.3 μg/L and 0.2 μg/L) (Table 2). Crude ICCs for urinary metabolites measured at the two time points ranged from 0.2 to 0.5, higher than the specific gravity standardized ICCs (0.2–0.4) (Table 3).

Urinary BCEP concentrations were higher among women who were not non-Hispanic white, not married or living alone, younger than age 25 years at delivery, with an annual family income < $40,000, or with pre-pregnancy BMI ≥30 kg/m^2. Women who had obesity tended to have higher BDCIPP concentrations in urine (Table 1). The average GCT result was 101.6 ± 28.8 mg/dL. Women who were not non-Hispanic white, not married or living alone, younger at delivery (age <25 years), and within the lower range of BMI (< 25 kg/m^2) had lower glucose concentrations. A total of 30 women (10.0%) had elevated glucose based on our definition, who were more often older than age 25 years at delivery (Table 1).

Overall, the associations between individual OPE metabolites and continuous GCT results were generally null using multivariable linear regression (Table 4). Similarly, increasing OPE concentrations were not associated with having elevated glucose levels using the modified Poisson regression (Table 5). When the OPE metabolites were modeled in tertiles, the associations either with the continuous glucose concentrations or with the elevated glucose levels largely remained null (Supplementary Material Tables S2–S5). However, compared to women in the 1st tertile of the average DPHP concentrations during pregnancy, women in the 3rd tertile tended to have a lower risk of elevated glucose levels (RR = 0.4, 95% CI: 0.2–1.1, p for trend = 0.06) while the association was not observed for the 2nd tertile (RR = 0.5, 95% CI: 0.2–1.2) (Supplementary Material Table S5).

The results of BKMR analyses that modeled OPE metabolites as a mixture were similar (Fig. 1). Fig. 1A shows the univariate exposure-response functions and 95% credible intervals for each urinary OPE metabolite at 16 weeks of pregnancy, holding all other OPE metabolites at their median concentrations. Fig. 1B represents the overall joint effect of the OPE metabolite mixture as the estimated difference in GCT results comparing concentrations of all OPE metabolites together in quantiles of their distributions to all OPE metabolites holding at their median concentrations. Similarly, Fig. 1C and D shows the results of the average

Table 1
Distribution of specific gravity standardized maternal urinary OPE metabolite concentrations at 16 weeks (μg/L), maternal serum cotinine (ng/ml) and blood lead levels (μg/dL), pregnancy glucose levels (mg/dL) from 50g glucose challenge test (GCT), and the number of women with elevated glucose levels by maternal and child characteristics (HOME Study, 2003–2006).

Categorical characteristics	N	BCEP (GM [GSD])	BDCIPP (GM [GSD])	DNBP (GM [GSD])	DPHP (GM [GSD])	Serum cotinine (GM [GSD])	Blood lead (GM [GSD])	Glucose levels (mean ± SD)	Elevated glucose levels N (%)
All participants	301	0.6 (3.2)	0.8 (2.6)	0.3 (2.0)	1.8 (2.6)	0.05 (20.70)	0.6 (1.5)	101.6 ± 28.8	30 (10.0)
Race/ethnicity									
Non-Hispanic White	204	0.5 (3.1) *	0.8 (2.6)	0.3 (2.1)	1.8 (2.6)	0.02 (12.24) *	0.6 (1.5) *	104.2 ± 29.1*	22 (10.8)
Non-Hispanic Black and others	97	0.8 (3.3) *	0.9 (2.5)	0.2 (1.9)	2.0 (2.5)	0.39 (19.82) *	0.8 (1.5.) *	96.0 ± 27.4*	8 (8.2)
Marital status									
Married/living with partner	243	0.6 (3.2) *	0.8 (2.6) *	0.3 (2.1)	1.8 (2.6)	0.02 (13.98) *	0.6 (1.5) *	103.2 ± 29.1*	27 (11.1)
Not married, living alone	58	0.8 (3.3) *	1.0 (2.5) *	0.3 (2.0)	2.1 (2.4)	0.76 (20.75) *	0.8 (1.4) *	94.7 ± 26.3*	3 (5.2)
Child Sex									
Male	136	0.5 (3.5)	0.7 (2.5) *	0.3 (2.2)	1.8 (2.6)	0.03 (20.14) *	0.6 (1.5)	100.7 ± 26.5	14 (10.3)
Female	165	0.6 (3.0)	0.9 (2.6) *	0.2 (2.0)	1.8 (2.6)	0.07 (20.49) *	0.6 (1.5)	102.3 ± 30.5	16 (9.7)
Maternal Age, years									
<25	59	0.9 (3.2)†	1.0 (2.6)	0.2 (2.1)	2.3 (2.4)	0.48 (16.29)†	0.7 (1.5)†	90.9 ± 22.1†	3 (5.1)
25-34	189	0.5 (3.1)†	0.8 (2.5)	0.3 (2.1)	1.8 (2.6)	0.03 (17.31)†	0.6 (1.5)†	101.3 ± 25.9†	17 (9.0)
≥35	53	0.5 (3.5)†	0.7 (2.8)	0.3 (1.9)	1.6 (2.8)	0.02 (15.12)†	0.7 (1.4)†	114.4 ± 38.5†	10 (18.9)
Maternal Education									
High school or less	68	0.8 (2.9)	1.0 (2.6)	0.3 (1.9)	2.1 (2.8)	1.24 (17.90)†	0.8 (1.4)†	94.9 ± 23.0	3 (4.4)
Some college/2 yr degree	67	0.6 (3.4)	0.8 (2.5)	0.3 (2.2)	1.8 (2.4)	0.08 (10.87)†	0.6 (1.5)†	102.6 ± 30.3	8 (11.9)
Bachelor's	99	0.5 (3.5)	0.7 (2.6)	0.3 (2.0)	1.7 (2.6)	0.01 (8.82)†	0.6 (1.5)†	104.7 ± 31.4	12 (12.1)
Graduate or professional	67	0.5 (2.9)	0.7 (2.5)	0.2 (2.2)	1.8 (2.6)	0.01 (6.93)†	0.6 (1.4)†	102.7 ± 28.0	7 (10.4)
Family Income									
<$40,000	104	0.8 (2.8)†	0.9 (2.6)	0.3 (2.0)	2.2 (2.6)†	0.45 (24.64)†	0.7 (1.5)†	97.5 ± 26.8	9 (8.6)
$40,000-$79,999	104	0.6 (3.9)†	0.8 (2.4)	0.3 (2.0)	1.6 (2.5)†	0.02 (10.20)†	0.6 (1.4)†	106.0 ± 30.4	14 (13.5)
≥$80,000	93	0.5 (2.8)†	0.7 (2.6)	0.2 (2.2)	1.8 (2.6)†	0.01 (6.54)†	0.6 (1.5)†	101.2 ± 28.3	7 (7.5)
Maternal pre-pregnancy BMI (kg/m^2)									
<25	162	0.5 (3.2)†	0.8 (2.6)†	0.2 (2.1)	1.8 (2.7)	0.03 (19.84)†	0.6 (1.5)	97.3 ± 26.7†	13 (8.0)
25-29	80	0.6 (3.4)†	0.7 (2.6)†	0.3 (2.1)	1.8 (2.5)	0.03 (15.21)†	0.6 (1.5)	104.0 ± 27.5†	7 (8.8)
≥30	59	1.0 (2.7)†	1.1 (2.4)†	0.3 (2.0)	2.0 (2.3)	0.21 (23.46)†	0.7 (1.4)	110.0 ± 33.8†	10 (17.0)
Parity									
0	134	0.6 (3.2)	0.7 (2.5)	0.2 (2.1)	1.7 (2.7)	0.03 (13.08)†	0.6 (1.5)	100.5 ± 25.7	12 (9.0)
1	98	0.6 (3.3)	0.8 (2.4)	0.3 (2.0)	1.8 (2.3)	0.06 (22.34)†	0.6 (1.5)	100.9 ± 28.7	9 (9.2)
2+	69	0.6 (3.2)	0.9 (2.9)	0.3 (1.9)	2.2 (2.6)	0.09 (36.82)†	0.7 (1.4)	104.5 ± 34.2	9 (13.0)

*P-value < 0.05 (two-sided p-values using *t*-test).†P-value < 0.05 (two-sided p-values using analysis of variance).

Table 2
Specific gravity standardized maternal urinary OPE metabolite concentrations (μg/L) (HOME Study, 2003–2006).

OPE metabolite	N	<LOD N (%)	Missing N	GM (GSD)	Percentiles 25th	50th	75th
16 weeks							
BCEP	298	38 (12.8%)	3	0.6 (3.2)	0.3	0.6	1.0
BDCIPP	296	13 (4.4%)	5	0.8 (2.6)	0.4	0.7	1.5
DNBP	298	47 (15.8%)	3	0.3 (2.0)	0.2	0.2	0.4
DPHP	300	3 (1.0%)	1	1.8 (2.6)	1.0	1.6	3.2
26 weeks							
BCEP	291	51 (17.5%)	10	0.5 (4.4)	0.2	0.5	1.1
BDCIPP	292	33 (11.3%)	9	0.6 (3.3)	0.3	0.6	1.1
DNBP	287	77 (26.8%)	14	0.2 (2.3)	0.1	0.2	0.3
DPHP	292	8 (2.7%)	9	1.2 (2.6)	0.6	1.2	2.1
Average of 16 and 26 weeks							
BCEP	288	–	–	0.7 (3.4)	0.3	0.6	1.2
BDCIPP	287	–	–	0.8 (2.6)	0.4	0.8	1.6
DNBP	284	–	–	0.3 (2.0)	0.2	0.2	0.4
DPHP	291	–	–	1.8 (2.3)	1.0	1.5	2.7

GM, geometric mean; GSD, geometric standard deviation. LOD, limit of detection. LOD was 0.1 μg/L for all OPE metabolites.
The average OPE metabolite concentrations were calculated as $(concentration\ at\ 16\ weeks + concentration\ at\ 26\ weeks)/2$

Table 3
Intra-class correlation coefficients (ICCs) of OPE urinary metabolites[a].

OPE metabolites	Crude ICC (95% CI)	Specific gravity standardized ICC (95% CI)
BCEP	0.5 (0.4, 0.6)	0.4 (0.3, 0.5)
BDCIPP	0.4 (0.3, 0.5)	0.4 (0.3, 0.5)
DNBP	0.3 (0.2, 0.4)	0.2 (0, 0.3)
DPHP	0.2 (0.1, 0.3)	0.2 (0, 0.3)

[a] Urinary concentrations were from samples collected at 16 weeks pregnancy and 26 weeks pregnancy.

OPE metabolite concentrations. We did not observe any nonlinear associations between urinary OPE metabolite concentrations and GCT results. The estimated posterior inclusion probabilities (PIP) for each OPE metabolite at 16 weeks of pregnancy were 0.20 (BCEP), 0.25 (BDCIPP), 0.38 (DNBP), and 0.20 (DPHP), like those for the average OPE metabolite concentrations: 0.22 (BCEP), 0.30 (BDCIPP), 0.32 (DNBP), and 0.23 (DPHP).

In the secondary analyses, none of the interaction p-values were statistically significant, suggesting no evidence of potential effect modification between continuous OPE metabolite concentrations and age, race/ethnicity, or pre-pregnancy BMI categories in our cohort (interaction p-values>0.1; Supplementary Material Table S6). Therefore, we did not further stratify the general linear models. In our sensitivity analyses, the results remained similar after excluding two participants with GCT results ≥200 mg/dL (Supplementary Material Table S7). Further, including the observation with extreme 16-week DNBP concentration did not significantly change the results of BKMR analyses (Supplementary Material Fig. S2).

Table 4
Differences in pregnancy glucose levels (mg/dL) from 50g glucose challenge test (GCT) per a $\log_{10}$ increase in maternal specific gravity standardized urinary OPE metabolite concentrations (μg/L).

OPE metabolite	Unadjusted N	β estimate (mg/dL)	95% CI	Adjusted[a] N	β estimate (mg/dL)	95% CI
16 weeks						
BCEP	296	−1.4	(-9.0, 6.1)	244	−0.5	(-8.1, 7.1)
BDCIPP	294	6.5	(-1.6, 14.5)	243	3.0	(-6.4, 12.4)
DNBP	296	0.4	(-10.5, 11.2)	244	−4.0	(-16.3, 8.4)
DPHP	298	−0.1	(-8.1, 7.9)	246	1.6	(-7.5, 10.8)
Average of 16 and 26 weeks[b]						
BCEP	288	−1.4	(-8.2, 5.5)	236	0.5	(-7.4, 8.4)
BDCIPP	287	7.4	(-0.6, 15.3)	236	5.2	(-4.1, 14.6)
DNBP	287	0.7	(-10.4, 11.7)	235	−3.0	(-15.6, 9.6)
DPHP	291	−4.8	(-13.5, 3.9)	239	−1.8	(-12.0, 8.4)

[a] Adjusted for maternal age at delivery, race, household income, education, marital status, child sex, parity, maternal pre-pregnancy BMI, serum cotinine concentrations, maternal blood lead levels, and gestational age at time of the GCT.
[b] The average OPE metabolite concentrations were calculated as $(concentrationat16weeks + concentrationat26\ weeks)/2$; when the 26-week sample was collected after GCT, the independent variables in the models were the concentrations at 16 weeks to hold temporality.

Table 5
Relative risk (RR) of elevated glucose levels per a $\log_{10}$ increase in maternal specific gravity standardized urinary OPE metabolite concentrations (μg/L).

OPE metabolite and timing of urine sample	Unadjusted N	RR	95% CI	Adjusted[a] N	RR	95% CI
16 weeks						
BCEP	298	1.1	(0.5, 2.4)	298	1.1	(0.6, 2.3)
BDCIPP	296	0.7	(0.3, 1.6)	296	0.6	(0.2, 1.5)
DNBP	298	1.2	(0.5, 2.9)	298	1.2	(0.5, 3.0)
DPHP	300	0.9	(0.3, 2.7)	300	0.9	(0.3, 2.9)
Average of 16 and 26 weeks[b]						
BCEP	290	0.9	(0.4, 2.1)	290	0.9	(0.4, 2.0)
BDCIPP	289	0.7	(0.3, 1.8)	289	0.6	(0.2, 1.6)
DNBP	289	1.5	(0.6, 3.6)	289	1.5	(0.6, 4.0)
DPHP	293	0.4	(0.1, 1.6)	293	0.4	(0.1, 1.8)

[a] Adjusted for maternal age at delivery, race, household income, education, marital status, child sex, parity, maternal pre-pregnancy BMI, serum cotinine concentrations, maternal blood lead levels, and gestational age at time of the GCT.
[b] The average OPE metabolite concentrations were calculated as $(concentrationat16weeks + concentrationat26\ weeks)/2$; when the 26-week sample was collected after GCT, the independent variables in the models were the concentrations at 16 weeks to hold temporality.

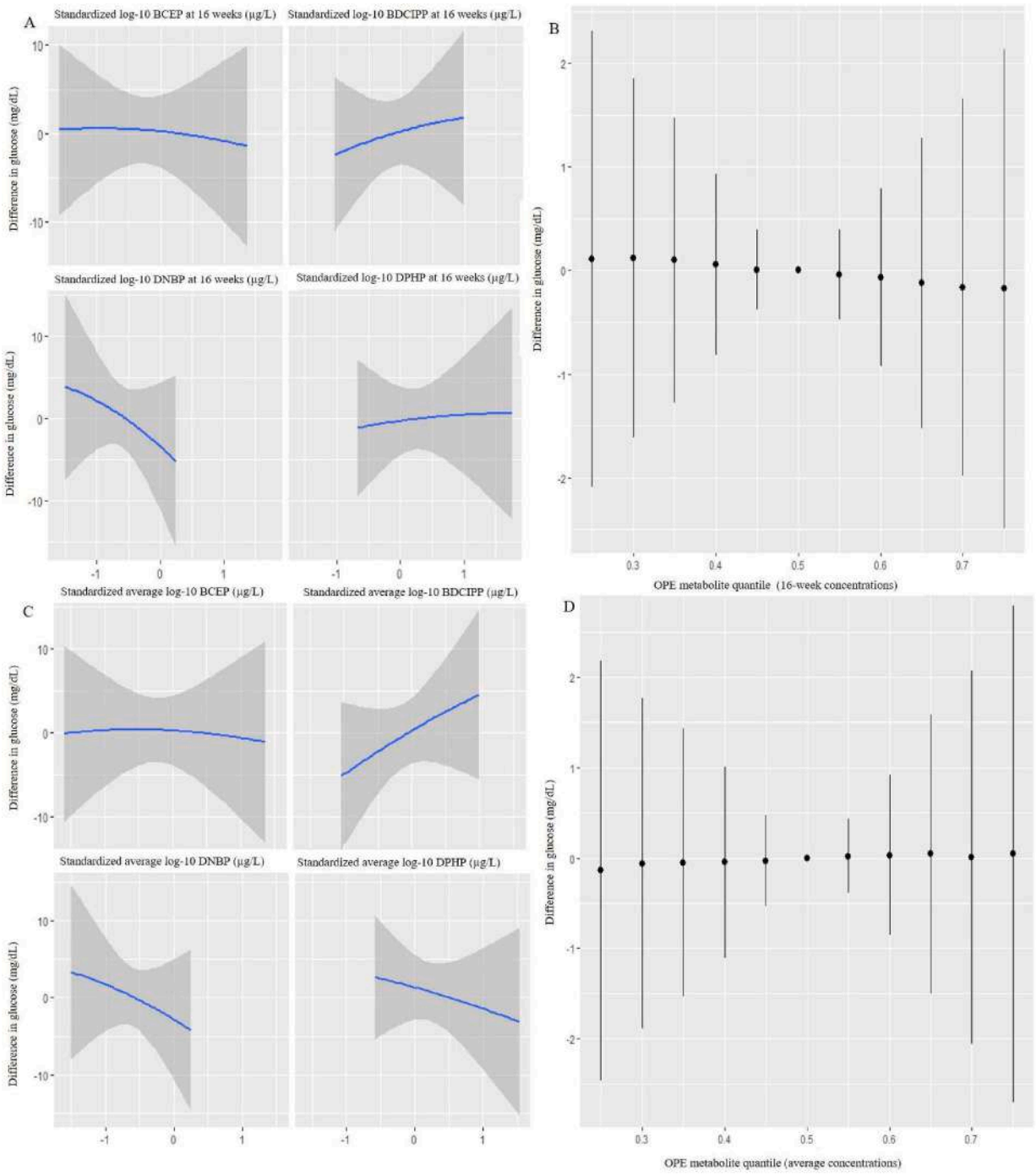

Fig. 1. Joint effects of the organophosphate ester (OPE) mixture on maternal glucose challenge test results estimated by Bayesian kernel machine regression (BKMR) adjusting for maternal age at delivery, race, household income, education, marital status, child sex, parity, maternal pre-pregnancy BMI, serum cotinine concentrations, maternal blood lead levels, and gestational age at time of the GCT.

A. Univariate exposure-response function and 95% credible intervals for OPE metabolite concentrations at 16 weeks of pregnancy, holding all other OPEs concentrations at the median.

B. Overall effect of the OPE mixture at 16 weeks of pregnancy, showing the estimated difference in glucose concentrations and 95% credible intervals when all OPE concentrations are held at a specified percentile compared to OPE concentrations held at the median.

C. Univariate exposure-response function and 95% credible intervals for the average of OPE metabolite concentrations at 16 and 26 weeks of pregnancy, holding all other OPEs concentrations at the median.

D. Overall effect of the average of OPE concentrations at 16 and 26 weeks of pregnancy, showing the estimated difference in glucose concentrations and 95% credible intervals when all OPE concentrations are held at a specified percentile compared to OPE concentrations held at the median.

The average OPE metabolite concentrations were calculated as [(*concentration at* 16 *weeks* + *concentration concentration at* 26 *weeks*)]/2.

When the 26-week sample was collected after GCT, the independent variables in the models were the concentrations at 16 weeks to hold temporality.

Abbreviations: BCEP, bis(2-chloroethyl) phosphate; BDCIPP, bis(1,3-dichloro-2-propyl) phosphate; DNBP, di-n-butyl phosphate; DPHP, diphenyl phosphate.

4. Discussion

In this study of pregnant women from a well-established, prospective pregnancy and birth cohort in the Greater Cincinnati Area, we did not observe any strong associations between urinary OPE metabolite concentrations and glucose levels from GCT results during pregnancy either when chemicals were assessed individually or as a mixture. Pregnant women in the highest tertile of the average DPHP concentrations tended to have a lower risk of elevated glucose levels, but only with borderline significance and wide 95% CIs, indicating the imprecision of the estimates.

Over the course of pregnancy, the mother's body undergoes numerous adaptations. One of the important metabolic adaptations is the shift of insulin sensitivity (Plows et al., 2018). In the 1st trimester, insulin sensitivity increases, and maternal glucose levels decrease; as pregnancy progresses, a mild state of insulin resistance results from multiple local and placental hormones, including estrogen, progesterone, cortisol, and placental growth hormones, and maternal blood glucose increases slightly during later pregnancy (Newbern and Freemark, 2011; Plows et al., 2018). To maintain glucose homeostasis, the mother's pancreatic β cells increase insulin secretion, and maternal insulin sensitivity returns to normal levels shortly after delivery (Johns et al., 2018). Inadequate compensations of insulin and other metabolic adaptations lead to GDM or subclinical hyperglycemia. The exact etiology of GDM is complex, involving both genetic and environmental factors (Johns et al., 2018).

Emerging evidence from epidemiological studies suggests that maternal exposure to some persistent organic pollutants (POPs) or their combinations at environmentally relevant concentrations could be associated with GDM. However, no consistent association of exposure to POPs and disturbed glucose metabolism has been confirmed for a single class of POPs, such as PBDEs, polychlorinated biphenyls (PCBs), and PFAS (Eslami et al., 2016; Liu et al., 2018; Preston et al., 2020; Rahman et al., 2019; Shapiro et al., 2016; Smarr et al., 2016; Valvi et al., 2017; Zhang et al., 2015). Recently, a significant positive association between BDE-28 and maternal glucose in single- and multi-pollutant statistical models was identified among HOME Study participants (Vuong et al., 2021). Other congeners of PBDEs, including BDE-47, BDE-153, BDE-154, BDE-183, have also been shown to disturb maternal glucose homeostasis and increase the risk of GDM in other populations (Eslami et al., 2016; Liu et al., 2018; Rahman et al., 2019; Zhang et al., 2015).

OPEs, one of the chemical classes that replaced PBDEs, have been shown to interact with various nuclear receptors and cause metabolic disruptions in experimental studies. For example, evidence suggested that exposure to the parent compounds of DPHP (TPHP and 2-ethylhexyl diphenyl phosphate), individually or in the mixture, could impair glucose homeostasis in earthworms, zebrafish, and mice (Du et al., 2016; Krumm et al., 2018; Walley et al., 2021; Wang et al., 2013; Yan et al., 2020). Walley et al. also reported sexually dimorphic effects of OPEs in mice. Prenatal exposure to the OPE mixture of TDCIPP (the parent compound of BDCIPP), TPHP, and TCP, increased fasting glucose in female offspring and altered glucose and insulin tolerance in males feeding on a high-fat diet (Walley et al., 2021). However, another study showed that exposure to the same mixture of OPEs did not affect glucose or insulin tolerance in either sex of adult mice, irrespective of diet (Vail et al., 2020). The inconsistent findings could be attributed to different exposure windows, lengths, or doses.

Population studies examining the association of OPE exposure with glucose regulation are lacking. Currently, only one cross-sectional study assessed the relationship between urinary OPE metabolite concentrations and glucose homeostasis in adolescents using data from the 2011–2014 NHANES (Luo et al., 2020). Luo et al. reported sex-specific associations. Specifically, they found a positive association between BDCIPP and prediabetes and 2 h-OGTT in girls, and consistent inverse associations for DNBP with prediabetes, fasting plasma glucose, 2 h-OGTT, fasting insulin, and homeostatic model assessment in boys (Luo et al., 2020). In our study, we identified null associations between BDCIPP or DNBP and maternal glucose levels. Instead, we observed a negative trend of elevated glucose across the tertiles of average DPHP concentrations. Future epidemiologic studies are needed to examine the association during pregnancy in different populations.

Our study has several strengths. This is the first prospective study examining pregnancy exposure to OPEs as it relates to glucose concentrations in the late 2nd or early 3rd trimester of pregnancy. The prospective design of the study with the repeated quantification of OPEs urinary concentrations during pregnancy reduces the risk of reverse causality. Also, with data on an extensive set of covariates, we can control for various potential confounders. Besides, by using BKMR, we captured the combined contribution of multiple OPEs, in addition to evaluating the contribution of four OPEs independently.

This study is also subject to several limitations. First, we could not assess the association between OPE metabolites and clinically diagnosed GDM because of the low number of GDM cases in our cohort. Instead, we only evaluated continuous and dichotomized glucose concentrations from the GDM screening tests. Second, our analysis was restricted to four OPEs; we did not evaluate other EDCs, such as PBDEs, PFAS, phthalates, parabens, and BPA, which could be correlated with the exposure to OPEs and associated with glucose status during pregnancy. Future research would benefit from examining the relationship between exposure to the mixture of different EDC classes and glucose homeostasis during pregnancy with a larger sample size. Additionally, despite having up to two exposure assessments during pregnancy, we did not collect maternal urine samples in the 1st trimester, so we were unable to investigate further trimester-specific associations. However, previous studies have suggested that the second trimester may be the particularly sensitive window regarding exposure to other EDCs (such as phthalates, parabens, and BPA) and maternal glucose concentrations (Bellavia et al., 2019; Chiu et al., 2017; James-Todd et al., 2016). Also, unlike PBDEs, OPEs are not biologically persistent and urinary concentrations of their biomarkers display moderate to high variability, so we cannot rule out the possibility of exposure misclassification (Percy et al., 2020). The results from the non-fasting GDM screening test could be influenced by the mother's diet (especially the timing or content of the last meal) and physical activity, on which we did not have information. We also did not have information on family history of diabetes or GDM history in previous pregnancies, which could be related to abnormal glycemic status in the current pregnancy. Finally, we were unable to assess insulin resistance directly due to the lack of insulin levels in the study sample.

In conclusion, we observed null associations between OPE metabolite concentrations in urine during pregnancy and plasma glucose concentrations from the GDM screening test. We did, however, find a suggestive negative trend between the tertiles of average DPHP concentrations and pregnancy glucose levels, but the estimates were imprecise. These findings cannot exclude the possibility that certain OPEs may have the ability to disturb glucose regulation during pregnancy. Given the widespread use of OPEs in daily products and the disease burden of GDM in pregnant women, future studies would benefit from in-depth investigations of the associations between OPEs and GDM using a larger sample size.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Use of trade names is for identification only and does not imply endorsement by the CDC, the Public Health Service, or the US Department of Health and Human Services.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114026.

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Nonylphenol (NP) exposure in Germany between 1991 and 2021: Urinary biomarker analyses in the German Environmental Specimen Bank (ESB)

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ABSTRACT

Nonylphenol (NP) is a high production volume chemical with a wide range of uses, e.g. in NP ethoxylates (NPEO). NP and NPEO have become ubiquitous in the environment and are considered of concern due to their general ecotoxicity and endocrine disrupting properties. However, knowledge on human exposure is scarce. In this study, we analyzed novel NP metabolites (OH-NP and oxo-NP) as robust biomarkers of exposure in 24h-urine samples from the German Environmental Specimen Bank (ESB). This enables us to reliably determine the individual NP body burden and to retrospectively evaluate NP exposure over the past 30 years. We analyzed 660 urine samples from eleven sampling years between 1991 and 2021. All samples were from young German adults between 20 and 29 years of age. OH-NP was quantifiable in all samples until 2017. In 2019 and 2021, the frequency of samples above the LOQ dropped to 90% and 77%, respectively. Median OH-NP concentrations significantly decreased from 4.32 μg/L in 1991 to 0.70 μg/L in 2021. OH-NP and oxo-NP levels correlated strongly, but oxo-NP concentrations and detections were considerably lower, in line with its known lower metabolic conversion. Reverse dosimetry back-calculated daily intakes (DI) of NP, based on OH-NP, decreased by almost a factor of four from medians of 0.16 μg/(kg bw*d) in 1991 to 0.04 μg/(kg bw*d) in 2021, respectively. The major drop took place only after 2012. This came as a surprise, because strict restrictions had been enacted much earlier in the EU, in 2003. All NP DIs were below the provisional tolerable daily intake of 5 μg/(kg bw*d) from the Danish Environmental Agency. DIs back-calculated from the ESB biomonitoring data agree well with calculations from food. This indicates to contaminated foodstuff as a major source of exposure. The time lag of regulatory restrictions to decreasing human exposure levels, the general lack of knowledge on exposure levels in susceptible populations such as children, and the ongoing worldwide use of NP underline the urgent need to continue monitoring NP exposures in Germany and worldwide. With these novel NP biomarkers, we provide a robust and sensitive tool for exposure and risk assessments, complementing environmental monitoring.

1. Introduction

Nonylphenol (NP) is a large-scale chemical and feedstock for the production of NP ethoxylates (NPEO), both of which have a long history of environmental pollution. After introduction in the 1930s, alkylphenol ethoxylates (APEO) (including NPEO as the predominantly used APEO) became increasingly popular as non-ionic detergents and their production volume reached approximately 360,000 metric tons APEO/year in 1975 in Europe and the United States with an estimated market share of approximately 20% of all synthetic surfactants (Stephanou and Giger, 1982; Wirth, 1975). APEOs were used as substitutes for other surfactants (such as branched alkylbenzene sulfonates) due to their preferential biodegradability (Stephanou and Giger, 1982), however, in the 1970s/1980s concern arose as adverse environmental effects of NP

Abbreviations: NP, nonylphenol; OH-NP, hydroxy-nonylphenol (mixture of isomers); oxo-NP, oxo-nonylphenol (mixture of isomers); TDI, tolerable daily intake; DI, daily intake; ESB, Environmental Specimen Bank.

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were observed (McLeese et al., 1980a, 1980b, 1981). In the following two decades, voluntary and legislative actions were enacted to reduce or ban the use of APEO (including NPEO) in certain applications in Europe (European Commission, 2002). In Germany, manufacturers agreed to a step-wise phase out of APEOs in domestic and industrial products between 1986 and 1992, including laundry detergents, domestic and industrial cleansers and agents for the manufacturing of textiles, leather & fur and paper. Even though complete phase out was not accomplished in Germany, APEO use in Germany was reduced by approximately 85% between 1986 and 1997 (Deutscher Bundestag, 2001; European Commission, 2002). To evaluate the effect of these voluntary actions, in the early 2000s the German Environment Agency retrospectively investigated NP concentrations in samples from the German Environmental Specimen Bank (ESB), including bream (*Abramis brama*) muscle tissue and zebra mussels (*Dreissena polymorpha*) from German rivers and blue mussels (common mussel, *Mytilus edulis*) from the North Sea and Baltic Sea (Günther et al., 2001; Wenzel et al., 2004). Overall, NP concentrations in these specimen in Germany considerably dropped in the 1980s and 1990s which might be attributed to the voluntary phase out of NPEO (Günther et al., 2001; Wenzel et al., 2004). However, in spite of strict regulatory measures and use restrictions on NP and NPEO since 2003 in the EU (The European Parliament and Council of the European Union, 2003), NP is still a high production volume chemical worldwide with a production and import volume of 10,000–100,000 t/year in the EU (as of 2021) (ECHA, 2021a).

Detrimental effects of NP on the environment have been reported for several species (Comber et al., 1993; Dwyer et al., 2005; Kuhn et al., 2001; Servos, 1999; Staples et al., 2004; Watanabe et al., 2017) and endocrine disrupting effects were found. These include, amongst others, *in vitro* estrogenicity (via yeast estrogen assay and MVLN transcriptional activation cell assay) and antiandrogenicity (e.g., via steroid 17α-monooxygenase inhibition and inhibition of androgen-induced AR transcriptional activity) as well as reduced serum testosterone *in vivo* in rats (Gabriel et al., 2008; Laurenzana et al., 2002; Lee et al., 2003; Preuss et al., 2006; Routledge and Sumpter, 1997). The ubiquitous presence of NP in the environment as well as in foodstuff (including dairy products, meat, fish, eggs, fruits and vegetables and various other foods) raised concerns for potential health risks also for humans (Al Rashed and Guenther, 2021; Guenther et al., 2002; Gyllenhammar et al., 2012; Lu et al., 2007). While environmental NP concentrations have been extensively investigated in numerous studies worldwide, there are still only few reliable and robust human exposure and human biomonitoring data. Similar to NP analysis in environmental matrices, NP analyses in human matrices is hampered by external contamination (Gries et al., 2019; Leng and Gries, 2017). This contamination of NP analyses specifically impacts urine analyses because of the rather low urinary excretion fraction of NP (Müller et al., 1998; Ringbeck et al., 2021a) (Calafat et al., 2005; Ye et al., 2007). We resolved this gridlock by investigating human NP metabolism and proposing the newly identified alkyl-oxidized urinary NP metabolites hydroxy-NP (OH-NP) and oxo-NP as novel exposure biomarkers for human biomonitoring (HBM) measurements. This approach circumvents the above limitations as these oxidized metabolites are unaffected by external contamination. Furthermore, OH-NP represents the major share of NP excreted in urine (Ringbeck et al., 2021a, 2021b). In this study we quantify these specific NP metabolites in human urine samples of the German ESB and thus retrospectively evaluate NP exposures between 1991 and 2021. The ESB collects and archives representative environmental samples including human matrices (e.g., urine, blood, and plasma) to monitor the overall chemical exposure in the German environment and the general population. With continuous ongoing sampling, the ESB enables the survey of long-term trends in the exposure profiles in Germany (Wiesmüller et al., 2007). Such trends have been investigated for numerous environmental pollutants and concerning chemicals. These include, i. a., phthalates and alternative plasticizers (Kasper-Sonnenberg et al., 2019; Koch et al., 2017; Lessmann et al., 2019; Schmidtkunz et al., 2019), parabens (Moos et al., 2015, 2017), bisphenol A (Koch et al., 2012), *N*-alkyl pyrrolidones (Ulrich et al., 2018), fragrances (Pluym et al., 2020; Scherer et al., 2021), glyphosate (Conrad et al., 2017), preservatives (Schettgen et al., 2020), and persistent polyhalogenated compounds (Fromme et al., 2020; Göckener et al., 2020). The temporal information obtained in the ESB is highly valuable for evaluating the impacts of chemical legislations on human exposures, or in the case of new chemicals, to follow their increase and propose regulatory action before exposures reach critical levels (Apel et al., 2020; Kolossa-Gehring et al., 2012, 2017; Lemke et al., 2021; Ougier et al., 2021). The results of this study will thus provide novel human biomarker-based exposure estimates for NP as the main representative of the class of alkylphenols and degradation product of its ethoxylates, describe potential time trends, and support the evaluation of the effectiveness of national and European legislative restrictions. Comparisons of calculated daily intakes with toxicological guidance values enable both a current and retrospective (over a 30-year-period) assessment of potential health risks advising potential future intervention measures.

2. Experimental

2.1. Study population

In this study, 660 urine samples from the German Environmental Specimen Bank (ESB), collected in 1991, 1995, 1999, 2003, 2006, 2009, 2012, 2015, 2017, 2019 and 2021 (60 per year; 30 males/30 females), were analyzed. 24-h urine samples were collected from students (20–29 years of age) from the University of Münster (Germany). Urine samples were aliquoted and stored at temperatures of approximately −150 °C (Lermen et al., 2014, 2019; Schröter-Kermani et al., 2016). These samples were shipped to the laboratory at the Institute for Prevention and Occupational Medicine (IPA, Bochum, Germany) and blinded and randomized for analysis. The 24-h urine volume and biometrical data including sex, body weight, body height, and age were recorded by the ESB and are shown in Table 1. Urinary creatinine was determined photometrically based on the Jaffe reaction (Lermen et al., 2019). Complete datasets were available for all parameters. For further information on the ESB, see (Kolossa-Gehring et al., 2012; Lermen et al., 2014; Schröter-Kermani et al., 2016; Wiesmüller et al., 2007). Collection of urine samples for the ESB has been approved by the Ethics Commission of the Medical Association of Westfalen-Lippe and the Medical Faculty of the Westfälische Wilhelms-Universität Münster, as well as the Ethics Commission of the Medical Association of the Saarland (since 2012). All volunteers gave written informed consent. This study was conducted in accordance with the Code of Ethics of the World Medical Association (1964 Declaration of Helsinki including its later amendments).

2.2. Quantification of oxidized NP metabolites in urine

Urinary alkyl-chain oxidized NP metabolites OH-NP and oxo-NP (each as mixtures of isomers) were analyzed as described in (Ringbeck et al., 2021b). In short, after the addition of internal standard (d_4-labeled OH-NP, used for quantification of both OH-NP and oxo-NP), the urine samples were incubated with β-glucuronidase/arylsulfatase from *Helix pomatia* for the hydrolysis of glucuronides (and sulfates, although negligible). After separation from cryophobic proteins by storing at −20 °C overnight and centrifugation, the processed urine samples were analyzed by online-SPE-LC-MS/MS. Considering the convention method character, some specifics shall be addressed here (for a detailed discussion see Ringbeck et al., 2021a,b). The mass transitions (ESI negative ion mode) of m/z 235 → 133 and m/z 235 → 117 were recorded for OH-NP for quantification and quality control, respectively, while for oxo-NP respective mass transitions were m/z 233 → 133 and m/z 233 → 117. The entirety of metabolite isomers of OH-NP and oxo-NP, respectively, was quantified by stable isotope dilution analysis using the

Table 1
Anthropometric characteristics of the study populations of the ESB between 1991 and 2021.

Year	Number of volunteers (male/female)	Age [years] Median (Min-Max)	Body height [cm] Median (Min-Max)	Body weight [kg] Median (Min-Max)	BMI [kg/m^2] Median (Min-Max)	Urinary creatinine [mg/L] Median (Min-Max)	24-h Urine volume [mL] Median (Mix-Max)
1991	60 (30/30)	24 (22–29)	176 (160–197)	67 (49–91)	21 (18–27)	1148 (402–3146)	1200 (400–2800)
1995	60 (30/30)	24 (20–29)	178 (159–194)	70 (52–100)	22 (18–30)	1091 (400–2748)	1530 (450–2600)
1999	60 (30/30)	24 (21–29)	176 (157–200)	67 (46–175)	22 (17–52)	1022 (275–2656)	1420 (550–4000)
2003	60 (30/30)	23 (20–28)	177 (157–200)	69 (49–105)	22 (18–34)	887 (230–3410)	1770 (410–3500)
2006	60 (30/30)	24 (20–29)	176 (158–195)	70 (47–95)	22 (18–32)	746 (290–2108)	1835 (719–4250)
2009	60 (30/30)	23 (20–28)	176 (163–198)	66 (50–102)	22 (18–34)	738 (271–2188)	2060 (540–3660)
2012	60 (30/30)	24 (20–29)	178 (160–192)	70 (51–92)	22 (18–31)	632 (205–2181)	2091 (574–3027)
2015	60 (30/30)	23 (20–29)	176 (157–196)	67 (49–95)	22 (18–26)	633 (146–2196)	1943 (271–4601)
2017	60 (30/30)	24 (20–29)	176 (159–196)	71 (46–110)	23 (16–34)	646 (165–2338)	2102 (561–3211)
2019	60 (30/30)	23 (20–29)	176 (158–193)	68 (47–101)	22 (18–32)	627 (199–2033)	2169 (807–4183)
2021	60 (30/30)	23 (20–28)	174 (160–198)	65 (50–94)	22 (17–28)	586 (262–1706)	2072 (1042–4918)
All	660	24 (20–29)	177 (157–200)	69 (46–175)	22 (16–52)	901 (146–3410)	1878 (271–4918)
Males	330	24 (20–29)	183 (165–200)	77 (52–175)	23 (18–52)	1045 (251–3410)	1921 (410–4918)
Females	330	23 (20–29)	170 (157–186)	62 (46–105)	21 (16–34)	757 (146–2748)	1836 (271–4601)

single-isomer standards 6OH-[4-NP$_9$] (4-(6-hydroxy-1,1-dimethylheptyl)phenol) and 6oxo-[4-NP$_9$] (4-(6-oxo-1,1-dimethylheptyl)phenol). Limits of quantification (LOQ) for the whole isomer peaks, determined in urine samples, were 0.5 µg/L for OH-NP (*m/z* 235 → 133) and 0.25 µg/L for oxo-NP (*m/z* 233 → 133). The characteristic "finger print" peaks of isomers provided additional means of quality control, besides measurement of quality control samples (concentrations: OH-NP: 6.1 µg/L, 34.4 µg/L, 291.6 µg/L; oxo-NP: 0.6 µg/L, 3.4 µg/L, 29.6 µg/L; measured every 60 samples, at least once per sample batch) and the concentration ratio of OH-NP to oxo-NP. Quality control material was prepared from native urine samples after known exposure to NP and includes the whole range of native NP metabolite isomers. For further information on the quantification approach, quality assurance and on chemicals and reagents used, see (Ringbeck et al., 2021b).

Results of this study are shown for the biomarker OH-NP (*m/z* 235 → 133) only. OH-NP is the major metabolite of NP and the most sensitive biomarker with mean excretion shares (F_{UE}) of 43.7 or 62.2%, depending on the mass transition used for quantification (Ringbeck et al., 2021a). Selection of the appropriate mass transition for quantification is crucial for obtaining comparable results since slightly different concentrations are obtained for each mass transition (both for OH-NP and oxo-NP) as a consequence of the quantitation approach and the quantitatively different MS^2 fragmentation behavior of individual isomers. This has been discussed in detail in previous publications (Ringbeck et al., 2021a, 2021b). Exposure estimation (in terms of daily intakes (DI)), however, is not affected by this as the individual F_{UE}s for each mass transition compensate for differences in analytical response and eventually the same DIs are obtained irrespective of the metabolite and mass transition chosen, as long as the corresponding F_{ue} is used for DI calculation, see (Ringbeck et al., 2021a). However, the use of OH-NP (*m/z* 235 → 133) as the most sensitive metric for NP exposure is advised and was used for the daily intake calculations.

2.3. Daily intake calculation and statistical analysis

Daily intakes (DI) were calculated based on the urinary OH-NP concentrations (*m/z* 235 → 133) and the respective 24-h urine volume of the study subject under consideration of the urinary excretion fraction (F_{UE}) of OH-NP (0.437 for *m/z* 235 → 133), the individual body weight and the molecular mass ratio of NP to OH-NP as described in Equation (1):

$$DI = \frac{c_{OH-NP} * V_{24h} * MW_{NP}}{bw * F_{UE} * MW_{OH-NP}} \quad (1)$$

With: DI = daily intake in µg/(kg bw*d); $c_{OH\text{-}NP}$ = urinary OH-NP concentration in µg/L; V_{24h} = 24-h urine volume in liter per day (L/d); $MW_{OH\text{-}NP}$ = Molecular weight of OH-NP (236.35 Da); bw = body weight in kg; F_{UE} = Urinary excretion fraction of OH-NP (0.437); MW_{NP} = Molecular weight of NP (220.35 Da).

Differences between study years were determined by the non-parametric Kruskal-Wallis ANOVA with post-hoc analysis for multiple group comparisons (the whole sample set as well as individual groups (sorted by year and sex) were not normally distributed in the Shapiro-Wilk test). Mann-Whitney-U test was used for sex comparison.

Exposure trends over the complete study period were analyzed by the non-parametric Wilcoxon rank sum and Jonckheere Terpstra tests by years of sampling for OH-NP, oxo-NP (in µg/L), daily intake (µg/(kg bw*d)) and the total OH-NP-concentration in 24-h urines (µg/24h). To assess the relationship between the daily intake and years of sampling and possible influencing factors, we calculated a multivariate linear regression model with the natural log-transformed DI as dependent variable and adjusted the model for years of sampling, sex, age and body mass index. The results are presented with the back-exponentiated parameter estimates (β and their corresponding 95% confidence interval) resulting in geometric mean ratios (gMR and 95% CI). We then calculated the average percent changes per one unit of increase [(gMR-1)*100%]. For the time trend analyses we used "SAS" 9.4 TS level 1M5

(©SAS Institute Inc., Cary, NC, USA). OriginPro 2020 (OriginLab Corporation, Northampton, MA, USA) was used for the Mann-Whitney-U test, Kruskal-Wallis ANOVA with post-hoc analysis and preparation of box-plots. For all analyses a p-value < 0.05 was considered statistically significant. Values below LOQ were set to 1/2*LOQ for further calculations and statistical analysis. Other figures were prepared and daily intake calculations were conducted using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

3. Results and discussion

3.1. Anthropometric description of the study population

Anthropometric characteristics of the study population are described in Table 1. The number of participants and sex distribution was the same in each study year (30 males, 30 females; total 60). Throughout the years, the median age (23–24 years), body height (174–178 cm), body weight (65–71 kg) and BMI (21–23 kg/m^2) were highly comparable and there was no temporal trend evident. The average 24-h urine volume almost doubled from 1991 (1200 mL) to 2021 (2072 mL), in line with medical recommendations for adequate fluid intake. Accordingly, in the same period the median urinary creatinine concentration decreased from 1150 mg/L to 590 mg/L by the same factor, caused by dilution with the higher urine volume (Lermen et al., 2019). While the median urine volume was comparable between males and females (1921 vs. 1836 mL), the average urinary creatinine concentration in males was considerably higher with 1050 mg/L compared to females with 760 mg/L, due to the higher muscle mass in males (Barr et al., 2005; Harper et al., 1977; Janssen et al., 2000). However, all of these potential dilution or creatinine related issues were avoided performing the final daily intake extrapolations and time trend investigations based on the 24-h urine volume (see section 3.3).

3.2. Urinary metabolite concentrations

Results of the analysis of the ESB samples are shown in Table 2 for the major urinary biomarker OH-NP (determined as described in section 2.2 using the convention method described in detail in Ringbeck et al., 2021b), both in µg/l and µg/g creatinine. Between 1991 and 2017, OH-NP could be detected in 100% of samples at concentrations above the LOQ of 0.5 µg/L. In 2019 and 2021, the fraction of samples above the LOQ decreased to 90% and 77%, respectively. Concentration data for oxo-NP are provided in the electronic supplement (Table S1). In line with the lower urinary excretion fraction of oxo-NP, urinary concentrations were considerably lower (factor 6–7) compared to OH-NP. The detection rate of oxo-NP decreased from 95% in 1991 to 48% in 2019 and to 17% in 2021 (see Table S1), underlining the decreasing trend in exposure observed with OH-NP.

Between 1991 and 2021, the median OH-NP concentrations in µg/L decreased by a factor of 6.2. The decrease was less pronounced (factor of 3.3) for the creatinine corrected OH-NP concentrations. This is a known effect for the annually collected 24-h urine samples of the ESB caused by increasing fluid intakes in the general population over the last decades (Lermen et al., 2019). This disparity becomes very visible by graphic boxplot display comparing the time trend in µg/L and µg/g creatinine (Fig. 1). Statistical time trend analyses over the complete study period

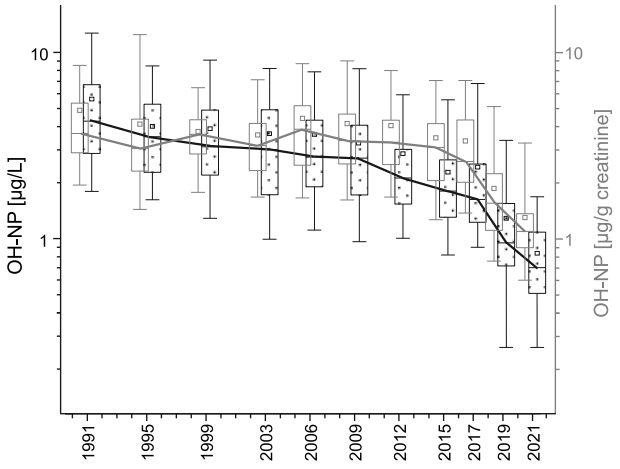

Fig. 1. Time-trend of OH-NP concentrations in µg/L (black lines with white dotted boxes) and after creatinine-adjustment (in µg/g creatinine; gray lines with gray boxes) in 24-h urine samples from the ESB between 1991 and 2021. Median values are connected with lines to highlight the trend. Values below LOQ were set to 1/2*LOQ. In the boxplots, median values, as well as interquartile ranges (IQR; P25–P75) are shown as gray boxes. Whiskers show the 5th and 95th percentiles. Minimum and maximum values are not shown for simplification. Boxes are slightly offset for better visibility; sampling years are explicitly labeled on the abscissa.

Table 2
Urinary OH-NP concentrations in µg/L and µg/g creatinine in 24-h urine samples from ESB between 1991 and 2021.

Value	Year	Min	P25	Median	GM (95th confidence interval)	P75	P95	Max
OH-NP [µg/L]	**1991**	1.14	2.87	**4.32**	4.52 (3.83–5.34)	6.78	11.48	28.22
	1995	0.56	2.27	**3.53**	3.48 (3.02–4.01)	5.37	8.23	12.39
	1999	1.07	2.19	**3.16**	3.28 (2.82–3.81)	4.93	7.78	14.25
	2003	0.90	1.71	**3.03**	2.98 (2.52–3.53)	5.00	7.80	10.30
	2006	0.85	1.90	**2.76**	2.91 (2.48–3.42)	4.33	7.63	21.56
	2009	0.70	1.70	**2.71**	2.65 (2.24–3.14)	4.11	7.73	10.84
	2012	0.90	1.53	**2.12**	2.22 (1.91–2.60)	3.03	5.47	26.36
	2015	0.70	1.30	**1.81**	1.92 (1.66–2.23)	2.71	5.42	8.58
	2017	0.63	1.22	**1.63**	1.94 (1.65–2.28)	2.52	6.12	8.32
	2021	<LOQ	0.51	**0.70**	0.66 (0.56–0.78)	1.09	1.56	2.44
OH-NP [µg/g creatinine]	**1991**	1.49	2.89	**3.67**	3.95 (3.44–4.54)	5.37	7.53	48.49
	1995	0.67	2.27	**3.05**	3.27 (2.78–3.84)	4.43	10.61	22.20
	1999	1.49	2.82	**3.64**	3.50 (3.19–3.84)	4.36	6.12	10.93
	2003	1.40	2.30	**3.17**	3.24 (2.89–3.63)	4.23	7.12	11.81
	2006	1.48	2.49	**3.88**	3.70 (3.22–4.26)	5.20	8.07	24.26
	2009	1.06	2.50	**3.37**	3.53 (3.08–4.05)	4.71	8.25	19.91
	2012	1.45	2.50	**3.30**	3.38 (2.96–3.87)	4.37	7.13	24.35
	2015	0.88	2.05	**3.10**	2.99 (2.61–3.43)	4.16	6.67	12.75
	2017	0.80	2.00	**2.60**	2.87 (2.50–3.30)	4.37	6.90	11.35
	2019	<LOQ	1.11	**1.56**	1.58 (1.38–1.82)	2.24	5.07	5.28
	2021	<LOQ	0.90	**1.10**	1.03 (0.89–1.19)	1.38	3.25	4.68

Min = minimum; GM = geometric mean; P25/P75/P95 = 25th/75th/95th percentile; Max = maximum; LOQ = limit of quantification.

showed significant downward trends for OH-NP (*m/z* 235 → 133; in µg/L), OH-NP (*m/z* 235 → 133; in µg/g creatinine) and total concentration of OH-NP in the 24-h urines (m/z 235 → 133, in µg/24h) ($p < 0.0001$ in both Wilcoxon rank sum and Jonckheere Terpstra tests), see section 3 in the supplement. Because ESB-samples are full 24-h urine samples, we could have also presented the data in amount of metabolite excreted per 24h, making any adjustment for urinary dilution unnecessary. However, then, the data would not be easily comparable with other HBM data from spot samples with concentrations classically expressed in µg/L and µg/g creatinine. Time trend analyses based on OH-NP in µg/24h are provided in the supplement and confirm the above observed time trends for the µg/L and µg/g creatinine data. Daily intake calculations in chapter 3.3. are based on the absolute amounts in the 24-h urine volume.

For quality control purposes, correlations between the concentrations of both biomarkers and their confirmatory mass transitions were investigated and concentration ratios were calculated (Fig. 2). The metabolite concentration ratio of OH-NP to oxo-NP of 6.9 was in line with the ratios observed in previous studies (Ringbeck et al., 2021a, 2021b, 2022) (Ringbeck et al., 2022 b). The metabolite ratio was unaffected by the sampling years investigated and did not differ between males and females (data not shown). The slightly lower ratio in the case of the current study might be explained by a matrix component observed at the mass-transition of oxo-NP (*m/z* 233 → 133), which was not chromatographically separated from the broad peak of the isomeric mixture of this metabolite. As can be seen from Fig. 2, middle column, potential interferences were restricted to oxo-NP and the mass transition *m/z* 233 → 133. Because we used oxo-NP, the minor oxidative metabolite of NP, for conformation only and not for reverse dosimetry exposure estimation, such potential interferences did not negatively influence the quantitative exposure estimates. The good correlations of OH-NP *m/z* 235 → 133 with the two other quality control mass transitions (*m/z* 235 → 117 for OH-NP and *m/z* 233 → 117 for oxo-NP), in conjunction with the characteristic peak shape of OH-NP (*m/z* 235 → 133), (Ringbeck et al., 2021a, 2021b), confirm the validity of the results obtained in this study.

3.3. Daily intakes and risk assessment

Reverse dosimetry calculated daily NP intakes are shown in Table 3 and Fig. 3. Between 1991 and 2021, median DIs of NP dropped almost by a factor of four. In the first two decades between 1991 and 2009, NP exposure was rather constant between 0.15 and 0.17 µg/(kg bw*d) (no significant differences in Kruskal-Wallis ANOVA with post-hoc analysis). However, from 2012 on, median DIs decreased considerably from 0.14 µg/(kg bw*d) to 0.04 µg/(kg bw*d) in 2021 with significant differences observed between median DIs of 2019 and 2021 each compared to the other sampling years from 1991 to 2017 ($p < 0.001$), but not between each other. Maximum Daily intakes decreased by a factor of ten from almost 1.7 µg/(kg bw*d) in 1991 to 0.18 µg/(kg bw*d) in 2021. Statistical time trend analyses over the complete study period showed significant downward trends for the DI ($p < 0.0001$ in both Wilcoxon rank sum and Jonckheere Terpstra tests), see section 3 in the supplement.

When adjusting the DI of OH-NP with years of sampling, sex, age and BMI (Table 4), again, the sampling years were significantly associated with the DI (gMR: 0.976; 95% CI: 0.971 to 0.981). The average change of the gMR was −2.37% (95% CI: -2.88% to -1.87%) per one year of increase. BMI was also significantly associated with the DI (gMR: 0.978; 95% CI: 0.963 to 0.995). Age and sex were not associated with the DI (gMR: 0.992; 95%CI: 0.971 to 1.013; gMR: 1.004; 95% CI: 0.913 to 1.104, respectively). Interactions between the included factors were not detected.

Daily intake levels were not significantly different between males and females (over all sampling years and individually for each sampling year) with overall median DIs of 0.124 and 0.127 µg/(kg bw*d), respectively. Accordingly, no considerable or significant sex differences were observed for OH-NP and oxo-NP concentrations (both with and without creatinine-adjustment) for the respective sampling years (see Supplement Tables S2–S5). This is in line with previous observations from our pilot study of German adults, as well as studies on Japanese, Thai, and Indonesian children and finding from other groups (Hou et al., 2015; Li et al., 2013; Ringbeck et al., 2021a, 2021b, 2022) (Ringbeck et al., 2022b). Significant, yet only minor sex differences have only been observed in Saudi Arabian children so far (Ringbeck et al., 2022b).

In the pilot study with urine samples of 32 German adults from the general population collected in 2014, a median DI of 0.11 µg/(kg bw*d) was derived (Ringbeck et al., 2021a, 2021b). This median DI is highly comparable to the median DI of 0.11 µg/(kg bw*d) of 2015 from the present study. Results from German adults in the ESB are also highly comparable to DIs reported for Japanese children (n = 180), sampled between 2012 and 2017 (Ringbeck et al., 2022). The median DIs in Japan showed a decline from 0.16 to 0.12 µg/(kg bw*d) between 2012 and 2017, similar to the decline in the ESB study from 0.14 to 0.11 µg/(kg bw*d) for the same years. In another study (Ringbeck et al., 2022b), the median DI of Thai children (n = 104), sampled in 2018, was 0.06 µg/(kg bw*d) which is about half the DI of German adults in this study (2017: 0.11 µg/(kg bw*d); 2019: 0.07 µg/(kg bw*d)) but in the same order of magnitude. Considerably higher DI were observed for Saudi (n = 109) and Indonesian (n = 89) children, sampled in 2017 and

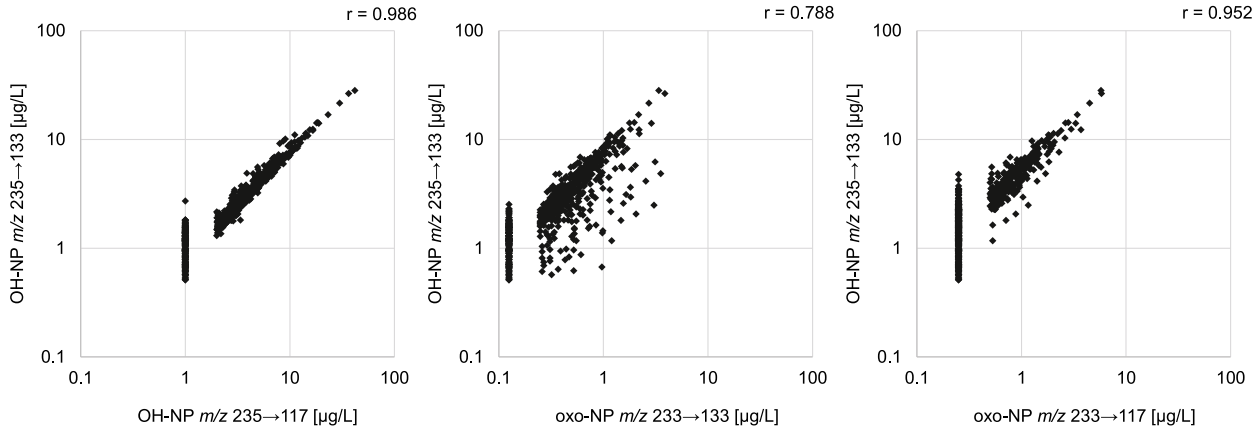

Fig. 2. Concentration ratios of OH-NP (*m/z* 235 → 133) to OH-NP (*m/z* 235 → 117), oxo-NP (*m/z* 233 → 133), and oxo-NP (*m/z* 233 → 117) as means of quality control. Samples with concentrations < LOQ were set to ½*LOQ. LOQs of OH-NP (*m/z* 235 → 117) and oxo-NP (*m/z* 233 → 117) were set to 2 µg/L and 0.5 µg/L, respectively.

Table 3
Estimated daily NP intakes in German adults between 1991 and 2021 based on 24-h urine samples from the ESB.

Value	Year	Min	P25	Median	GM (95th confidence interval)	P75	P95	Max
Daily intake [µg/(kg bw*d)]	**1991**	0.061	0.131	**0.164**	0.178 (0.154–0.207)	0.268	0.407	1.720
	1995	0.037	0.112	**0.149**	0.158 (0.136–0.185)	0.210	0.463	0.687
	1999	0.060	0.111	**0.151**	0.148 (0.132–0.165)	0.188	0.307	0.448
	2003	0.045	0.104	**0.153**	0.148 (0.130–0.169)	0.201	0.371	0.539
	2006	0.046	0.111	**0.173**	0.161 (0.140–0.186)	0.226	0.371	1.228
	2009	0.063	0.107	**0.156**	0.159 (0.138–0.183)	0.229	0.437	0.899
	2012	0.059	0.097	**0.136**	0.137 (0.120–0.157)	0.169	0.316	0.909
	2015	0.033	0.078	**0.106**	0.110 (0.096–0.127)	0.147	0.248	0.659
	2017	0.039	0.075	**0.108**	0.111 (0.096–0.128)	0.165	0.295	0.427
	2019	0.009	0.049	**0.071**	0.066 (0.056–0.077)	0.102	0.161	0.268
	2021	0.016	0.028	**0.043**	0.043 (0.037–0.049)	0.067	0.093	0.175
All	-	<LOQ	0.081	**0.125**	0.120 (0.114–0.126)	0.181	0.351	1.720
Males	-	<LOQ	0.078	**0.124**	0.119 (0.111–0.128)	0.183	0.342	1.228
Females	-	<LOQ	0.082	**0.127**	0.121 (0.112–0.130)	0.178	0.259	1.720

Min = minimum; GM = geometric mean; P25/P75/P95 = 25th/75th/95th percentile; Max = maximum; LOQ = limit of quantification.

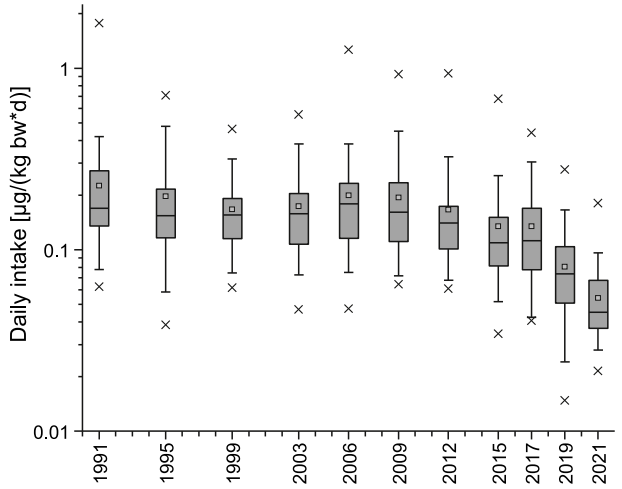

Fig. 3. Time trend of NP daily intakes in samples from the ESB in Germany between 1991 and 2021. Median values, as well as interquartile ranges (IQR; P25–P75) are shown as gray boxes. Whiskers show the 5th and 95th percentiles. Minimum and maximum values are shown as x's and mean values are shown as small squares.

Table 4
Multivariate linear regression analysis for the ln-transformed daily intake of OH-NP (µg/(kg bw*d)), adjusted for sampling year, sex, age and body mass index (BMI).

	gMR (95% CI)	% Change of the gMR per one unit of increase (95% CI)
Sampling year	0.976 (0.971; 0.981)[a]	−2.37 (−2.88; −1.87)
Sex (male = 1, female = 2)	1.004 (0.913; 1.104)[c]	0.44 (−8.66; 10.44)
Age (years)	0.992 (0.971; 1.013)[c]	−0.80 (−2.89; 1.34)
BMI (kg/m^2)	0.978 (0.963; 0.995)[b]	−2.16 (−3.75; −0.53)

a: $p < 0.001$; b: $p < 0.05$; c: $p > 0.1$; gMR: geometric mean ratio; 95% CI: 95% confidence interval.

2018, respectively, with median values of 0.36 µg/(kg bw*d) and 0.47 µg/(kg bw*d). These daily intakes are higher (approximately two to three times) than the median intakes found in the early years of this study (1991–2009). When compared to the respective sampling years, median DIs from Saudi Arabia and Indonesia exceed the 95th percentile of DIs in Germany (years 2017 and 2019). However, direct comparison of urinary metabolite levels and estimated daily intakes between adults and children should be made with caution because of possible differences in metabolism and corresponding urinary excretion fractions, which are currently available only for adults and are applied to children as an approximation. Thus, overall, there are indications to considerably declining time trends in NP exposure in Japan and Germany, but also to substantial regional differences. Some sub-populations (e.g. children) might experience higher NP exposures as it was shown for several other short-term chemicals such as phthalates or bisphenols (Wittassek et al., 2011; Becker et al., 2012). More population data is needed for a more robust picture.

We can also compare the above DIs derived by reverse dosimetry with DI estimates for the general German population derived from concentrations of NP residues in representative foods (including cereal products, dairy, meat, fish, fruits, vegetables, various types of processed food and many more) described by Guenther et al. (2002) and Al Rashed and Guenther (2021). Based on the average consumption of these foods they estimated DIs of 7.5 µg/day in 1999/2000 (Guenther et al., 2002) and 2.7 µg/day in 2018/2019 (Al Rashed and Guenther, 2021) (sampling years kindly provided by the authors in personal communications). Using the median body-weight from this study (69 kg) as a proxy, these results translate to DIs of 0.11 µg/(kg bw*d) and 0.04 µg/(kg bw*d), which are only slightly lower than the median DIs from our study of 0.15 µg/(kg bw*d) in 1999 and 0.07 µg/(kg bw*d) in 2019 (see Supplementary Fig. S1 for comparison). Considering the vastly different approaches in exposure estimation, their agreement is remarkable and supports the declining trend of NP exposure in Germany. The somewhat higher DIs estimated from HBM data compared to food residues indicate that foodstuff is a major source of NP exposure, but other exposure sources and pathways of uptake may also contribute to the overall NP exposure captured by the integral HBM approach (Angerer, 2012; Angerer et al., 2007). Also, uptake of NP-derivatives such as NPEO or tris (4-nonylphenyl) phosphite (TNPP) with potential metabolic breakdown to NP in the human body might not be captured by food analysis of NP alone. However, the contribution of these NP derivatives to the NP body burden is still uncharted territory and further research in this field is needed.

For risk assessment of NP exposure, the above DIs can be compared to the provisional tolerable daily intake (TDI) of 5 µg/(kg bw*d) derived by the Danish Environmental Protection Agency based on chronic toxicity in rats (Nielsen et al., 2000). The median hazard quotients (HQ = ratio of DI to TDI) in our study were between 0.01 (2021) and 0.04 (2006). The HQ of the maximum DI in our study (determined for 1991) was 0.4. Generally, HQ < 1 indicate to a reduced health risk but not

taking into account co-exposures to chemicals with a similar toxicity profile. However, NPs are currently reexamined under the European Chemicals Agency's Community Rolling Action Plan (CoRAP) with special focus on their potential endocrine disrupting potency (European Chemicals Agency, 2011). Recent multi-generational studies in mice indicate to adverse effects at levels substantially lower than previously assumed (Kim et al., 2019, 2020). Thus, the current margin of safety might diminish based on these findings. Furthermore, certain sub-populations (e.g., children) might have additional pathways and sources of NP exposure than the young German adults sampled in the ESB.

3.4. Impact of market regulations on NP exposure

In the 1980s, in many European countries, voluntary actions were taken to phase-out APEO including NPEO. In Germany, first steps were taken in 1986 and in the following eleven years national APEO production decreased by approximately 85% (Deutscher Bundestag, 2001; European Commission, 2002). These actions were accompanied by decreasing environmental NP concentrations in aquatic organisms (Günther et al., 2001; Wenzel et al., 2004). Interestingly, we did not observe a similar drop in urinary NP levels of the ESB during that time. Instead, levels seemed rather stable until 2009. Thus, either a major drop in urinary NP levels occurred before 1991 (six years after first voluntary actions were taken), or human NP exposures have not been affected by these early actions, or there is a considerable time lag between first actions and reduced human exposure. The voluntary APEO phase-out in Germany was followed by similar steps on a European level in 1995 and 2000, respectively (European Commission, 2002). After NP was classified as a 'priority hazardous substance' in the field of EU water policy in 2001 (The European Parliament and Council of the European Union, 2000, 2001), in 2003 comprehensive use restrictions of NP and NPEO in most domestic and industrial applications were enacted (The European Parliament and Council of the European Union, 2003), which were then implemented in German legislation in 2005 (Deutscher Bundestag, 2004). In 2012, NP was classified as a Substance of Very High Concern (SVHC) (ECHA, 2012). Interestingly, this is around the time human exposures started to drop in samples of the ESB. Given the long time period between the legal implementation of these restrictions in Germany (2005) and the beginning of the drop in NP exposure (2012), we cannot conclude with sufficient certainty that these legal regulations were responsible for the decline. An immediate effect of the SVHC classification in 2012 on the beginning of the decrease in NP exposure is unlikely, as this classification did not cause immediate legal actions. In 2021, the maximum permitted use of NPEO in textiles was further reduced to 0.01% and NPEO became subject to authorization under REACH annex XIV (European Commission, 2016; The European Parliament and Council of the European Union, 2006). The decrease in human NP exposures along with reduction measures at German and EU level are shown in Supplemental Fig. S1.

It remains to be seen whether the integration in REACH Annex XIV and the further restriction for NPEO in textiles will have a reducing effect on exposure. In this regard it is important to consider that other NP derivatives such as tris(4-nonylphenyl) phosphite (TNPP) are still used in several applications and articles of everyday life (such as food contact material or cosmetics) in the EU (European Commission, 2011, 2020). Furthermore, the impact of the various possible, yet largely unknown, uptake routes on total human NP exposure is still far from being understood.

4. Conclusions

With the retrospective analyses of the urine samples from the ESB we could show that NP body burdens in Germany are omnipresent in young adults, but are declining since 2012, after more than 20 years of stagnation. The omnipresence of NP exposure is reflected in the high detection frequencies of the major urinary NP metabolite OH-NP. The new approach of using this specific urinary biomarker for exposure assessment solves the decade-long problem of external contamination in direct NP analyses. The integral human biomonitoring approach also represents human exposure over all exposure sources and pathways, known or unknown. Our HBM-based daily intakes roughly confirm average daily NP intake predictions via NP residue levels in foodstuff. This points to foodstuff as a major source of exposure. However, foodstuff does not explain the full extent of internal NP exposure. Given the complexity of potential exposure pathways and various NP-derivatives (NPEO, TNPP) with multiple applications, the entirety of exposure sources and uptake routes of NP is still not fully elucidated. Also, both the environmental and (human) metabolic breakdown of the NP-derivatives to NP need further investigation. While previous monitoring surveys revealed temporal relation of NP residues in the environment with voluntary or legal actions on reducing or banning NP and NPEO in Germany or the EU, no immediate temporal relation between these actions on human NP exposure levels can be observed. Obviously, human exposure pathways as well as their lifestyle are more complex than those of breams and mussels are. Nevertheless, despite being unable to directly link exposure reductions to specific measures, human exposures have dropped significantly, especially in the last 10 years.

Now, the new HBM approach for NP needs to be applied to other, representative populations, such as the German Environmental Survey (GerES), to identify potentially more highly exposed or more susceptible sub-populations, such as children or pregnant women. The ongoing CoRAP re-evaluation of NP will show if the current reductions in NP exposure are sufficient, or if further exposure reductions need to be achieved for NP and its derivatives. Some Asian data indicate to ongoing, intensive use of NP, and consequently, margins of exposure are considerably smaller than in Germany.

Declaration of competing interest

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.2022.114010.

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Prenatal acute thermophysiological stress and spontaneous preterm birth in Western Australia, 2000–2015: A space-time-stratified case-crossover analysis

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ABSTRACT

Epidemiologic evidence on acute heat and cold stress and preterm birth (PTB) is inconsistent and based on ambient temperature rather than a thermophysiological index. The aim of this study was to use a spatiotemporal thermophysiological index (Universal Thermal Climate Index, UTCI) to investigate prenatal acute heat and cold stress exposures and spontaneous PTB. We conducted a space-time-stratified case-crossover analysis of 15,576 singleton live births with spontaneous PTB between January 1, 2000 and December 31, 2015 in Western Australia. The association between UTCI and spontaneous PTB was examined with distributed lag nonlinear models and conditional quasi-Poisson regression. Relative to the median UTCI, there was negligible evidence for associations at the lower range of exposures (1st to 25th percentiles). We found positive associations in the 95th and 99th percentiles, which increased with increasing days of heat stress in the first week of delivery. The relative risk (RR) and 95% confidence interval (CI) for the immediate (delivery day) and cumulative short-term (up to six preceding days) exposures to heat stress (99th percentile, 31.2 °C) relative to no thermal stress (median UTCI, 13.8 °C) were 1.01 (95% CI: 1.01, 1.02) and 1.05 (95% CI: 1.04, 1.06), respectively. Elevated effect estimates for heat stress were observed for the transition season, the year 2005–2009, male infants, women who smoked, unmarried, ≤ 19 years old, non-Caucasians, and high socioeconomic status. Effect estimates for cold stress (1st percentile, 0.7 °C) were highest in the transition season, during 2005–2009, and for married, non-Caucasian, and high socioeconomic status women. Acute heat stress was associated with an elevated risk of spontaneous PTB with sociodemographic vulnerability. Cold stress was associated with risk in a few vulnerable subgroups. Awareness and mitigation strategies such as hydration, reducing outdoor activities, affordable heating and cooling systems, and climate change governance may be beneficial. Further studies with the UTCI are required.

1. Introduction

Preterm birth (PTB) – birth before 37 completed weeks of gestation remains the leading cause of child mortality and long-term health morbidity, and is accompanied by sizeable economic burdens (Vogel et al., 2018). Analysis across 107 countries estimated a global rise in PTB from 9.8% in 2000 to 10.6% in 2014, an equivalent of 15 million live PTB (Chawanpaiboon et al., 2019). In Australia, the rate increased slightly from 8.4% in 2010 to 8.7% in 2017 (Morris et al., 2020). Most PTB cases are spontaneous, and the causes are multifactorial and heterogeneous (Cobo et al., 2020; Vogel et al., 2018). Despite the several well-known risk factors, the majority of PTB have unspecified causes and unclear biological mechanisms for appropriate prevention strategies (Cobo et al., 2020; Vogel et al., 2018). For instance, an individual participant meta-analysis of 4.1 million singleton births in five high-income countries reported that the aetiology of about 65% of PTB

Abbreviations: GoWA, Government of Western Australia; SA1, Statistical Area level 1; PTB, Preterm birth; UTCI, Universal Thermal Climate Index.

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could not be explained with a range of commonly reported risk factors (Ferrero et al., 2016). Recommendations included investigation of biological mechanisms and non-conventional risk factors (Ferrero et al., 2016; Vogel et al., 2018) such as environmental exposures.

Climate change continues to increase heat or cold extremes across the globe with potential impacts on health outcomes (IPCC, 2021). Emerging observational studies have indicated that prenatal exposure to extreme ambient temperatures (heat or cold stress) may contribute to the pathophysiology of PTB (Chersich et al., 2020). The hypothesised biological pathway is that thermal stress disrupts maternal thermoregulatory capacity and stimulates excessive immune-inflammatory activities prematurely, initiating labour and thereby leading to PTB (Green and Arck, 2020; Jee et al., 2021). However, the findings are disparate and have suggested both extreme heat and cold stress as risk factors (Cox et al., 2016; Li et al., 2018; Mathew et al., 2017), heat stress as a risk factor but 'protective' effect or no association with cold stress (Sun et al., 2019), and cold stress as a risk factor but 'protective' effect or no association for heat stress (Cheng et al., 2021; Liang et al., 2016; Yu et al., 2018). These differences may be attributed to heterogeneity in the study designs, geographic location, population characteristics, acclimatisation, adaptation, exposure assessment, and varied temperature metrics (Cheng et al., 2021; Chersich et al., 2020).

Most importantly, the existing literature is limited to the surrogate use of ambient temperature for heat or cold stress instead of the human thermophysiological index (Jendritzky et al., 2012; Vanos et al., 2020). The results have been criticised as unrealistic and physiologically less relevant for a better understanding of the associated health effects for appropriate interventions (Jendritzky et al., 2012; Staiger et al., 2019; Vanos et al., 2020). Four appropriate human thermophysiological indices were recently recommended (Staiger et al., 2019). These included Universal Thermal Climate Index (UTCI) which was reported in comprehensive comparative studies to be most suitable as it has high climatic sensitivity and best captures thermal stimuli similar to that of the human body, making it more thermophysiologically appropriate for medical and preventive medicine (Blazejczyk et al., 2012; Bröde et al., 2013; Jendritzky et al., 2012; Staiger et al., 2019). UTCI is a potential universal tool for monitoring the impacts of climate change on humans but it is underutilised in epidemiology and medical sciences until recently (Romaszko et al., 2022). Several recent studies are now using the UTCI in heatwave warning systems and medical or epidemiological fields as reviewed elsewhere (Krüger, 2021; Romaszko et al., 2022). Only one recent study has used UTCI derived with meteorological parameters from one synoptic meteorological station and investigated the association with preterm labour (Khodadadi et al., 2022), but no study has investigated PTB.

Here, we used spatiotemporal UTCI and conducted a space-time-stratified case-crossover analysis of the association between prenatal exposure to thermophysiological stress and spontaneous PTB in Western Australia over 16 years. We estimated the overall effects and the influence of sociodemographic vulnerabilities.

2. Materials and methods

Our analysis and reporting of results were informed by the REporting of studies Conducted using Observational Routinely collected health Data (RECORD) guidelines (Benchimol et al., 2015).

2.1. Study design and setting

A space-time-stratified case-crossover design was conducted (Wu et al., 2021). This is similar to the classic time-stratified case-crossover design, a case-only self-matched approach that compares the exposure at the time of the event ('case or hazard time') with related non-event periods ('control or referent times') (Maclure, 2017; Mostofsky et al., 2018). The classic time-stratified case-crossover design is applied for time-series data where all individuals have a shared area-level exposure (Armstrong et al., 2014; Mostofsky et al., 2018; Wu et al., 2021). However, the availability of space-time varying environmental exposure assessment led to the extension of this design into the so-called space--time-stratified case-crossover to accommodate the analysis of multiple space-time series datasets (Ragettli et al., 2017; Wu et al., 2021). The design has been applied previously for investigating acute effects (Lu et al., 2020, 2021; Nyadanu et al., 2022; Ragettli et al., 2017; Vicedo-Cabrera et al., 2020). Specifically, we matched the case and control times by a day of the week in the same calendar month and year within the same small spatial unit in the study location (Nyadanu et al., 2022; Ragettli et al., 2017; Wu et al., 2021). Thus, by design, the time-stratified case-crossover accounted for both measured and unmeasured individual-level characteristics and co-exposures that are short-term or time-invariant and controlled for long-term and seasonal trends (Mostofsky et al., 2018; Wu et al., 2021). The extension to space-time-stratified case-crossover further allowed for the analysis of multi-location time-series data, minimised exposure measurement bias and spatial confounding (Nyadanu et al., 2022; Ragettli et al., 2017; Vicedo-Cabrera et al., 2020; Wu et al., 2021).

This study was conducted for births between January 1, 2000 and December 31, 2015 in Western Australia. Western Australia is the largest state in Australia by area and covers 2.5 million km^2 areas with a population of 2.7 million as of March 31, 2021 (ABS, 2021). The state has diverse climatic conditions, ranging from temperate in the south-west, tropical in the north, and arid or semi-arid in the other parts.

2.2. Study population and case definition

We obtained de-identified data on births collected by the Midwives Notification System from the Western Australia Department of Health data linkage unit. The Midwives Notification System is a population-wide registry of all births with at least 20 weeks of gestation or at least a birth weight of 400 g if the gestational length is unknown (GoWA, Government of Western Australia, 2021). The data contained maternal and neonatal information. Maternal residential address at the time of delivery was available as the statistical area level 1(SA1), the second smallest geographical unit in Australia. This study included 4504 SA1s where eligible births were located. A total of 474,835 births were screened for eligibility. We included only singleton live births with spontaneous onset of labour and vaginal delivery at 20–36 weeks of gestation that had an SA1. The gestational age was estimated as the best clinical estimate from the perinatal records as the difference between the date of birth and start of pregnancy based on ultrasonography or the last menstrual period if ultrasound was not available. To eliminate the potential displacement of short-term effects by the reductions in the risk at longer periods, we considered a maximum lag of 21 days (Bhaskaran et al., 2013; Gasparrini et al., 2015; Khodadadi et al., 2022; Nyadanu et al., 2022). For this reason, we further excluded births within the first 20 days of the study period. Our final analytic sample included 15,576 spontaneous PTB (Fig. 1).

We extracted the available sociodemographic information to derive subgroups. Infant-related subgroups were based on sex (male or female) and gestational age (20–27, 28–31, and 32–36 weeks) (Blencowe et al., 2013). We further obtained the extreme ends of the PTB as periviable birth (20–26 weeks, the range of viability) (Catalano et al., 2019) and late PTB (34–36 weeks) (Blencowe et al., 2013). Maternal-related subgroups were age at birth delivery ($\leq$19, 20–34, and $\geq$35 years) (Wang et al., 2013, 2019), tobacco smoking status (non-smoker or smoker), marital status (married or unmarried), and race or ethnicity (Caucasian or non-Caucasian). We also categorised the season of birth into three (summer, December–February; winter, June–August; and transition, the remaining months that form autumn and spring) and year (2000–2004, 2005–2009, 2010–2015). The Index of Relative Socio-economic Disadvantage at a geographic area derived by the Australian Bureau of Statistics (ABS, 2018) was assigned to the maternal residence at the time of birth and categorised into quintiles in a previous study (Gebremedhin

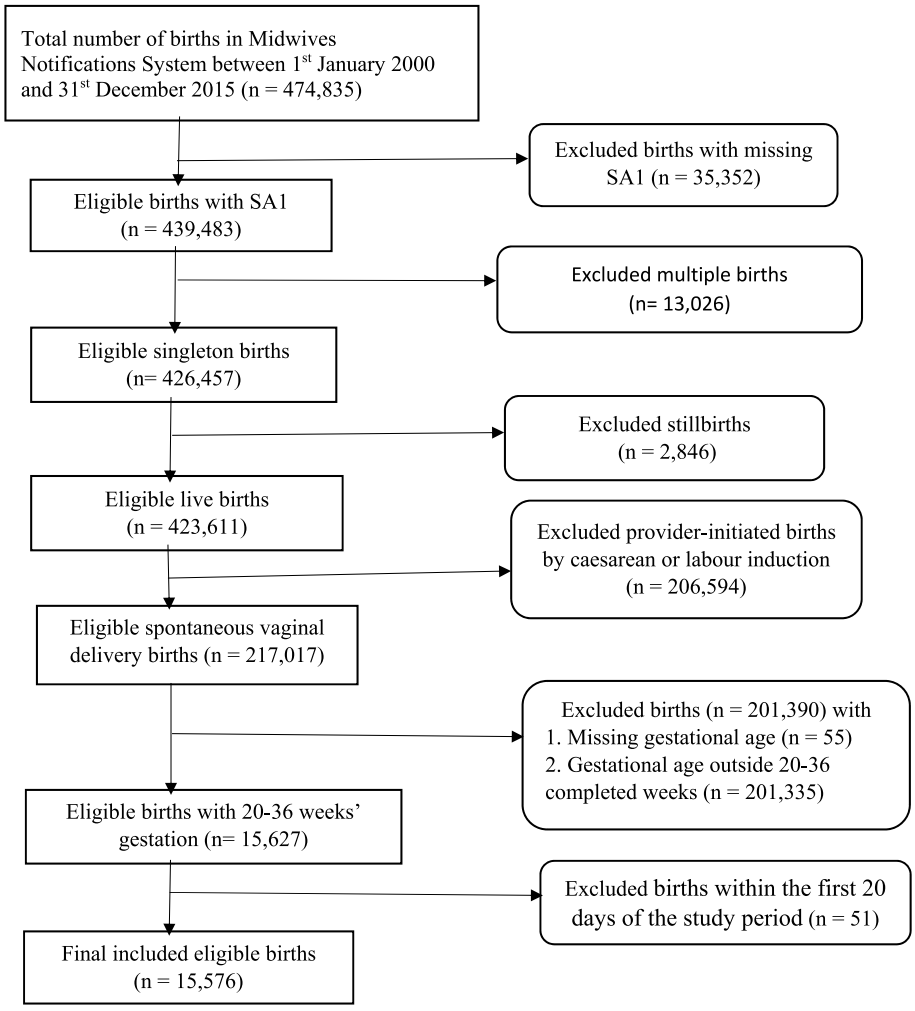

Fig. 1. Flow chart of the selection of the eligible spontaneous vaginal delivery preterm births included in this study, Western Australia, 2000–2015. Note, SA1; statistical area level 1.

et al., 2019). We derived two socioeconomic status (SES) subgroups from the quintiles as high (1st and 2nd quintiles) and low (3rd-5th quintiles) SES (Nyadanu et al., 2022).

2.3. Spatiotemporal Universal Thermal Climate Index exposure assessment

The UTCI is an equivalent air temperature (°C) that assesses the ambient thermal environment and accounts for heat transfer and exchange, both within the body and between the body surface and the ambient air layer (Blazejczyk et al., 2013; Jendritzky et al., 2012). UTCI is computed through a six-order polynomial equation with four input variables: air temperature and dew point temperature or relative humidity at 2 m above ground level, wind speed at 10 m above ground level, and mean radiant temperature (Blazejczyk et al., 2013; Bröde et al., 2012; Di Napoli et al., 2021). The mean radiant temperature is a measure of thermal-related comfort and includes non-meteorological variables such as metabolic rate and the thermal properties of clothing (Blazejczyk et al., 2013; Bröde et al., 2012; Jendritzky et al., 2012). We used the open-access UTCI dataset recently derived from the ERA5 reanalysis (Di Napoli et al., 2021). ERA5 is the 5th historical global gridded climate dataset of several climate variables produced by the European Centre for Medium-Range Weather Forecasts by merging the global climate model, measurements made near the Earth's surface at land stations, and satellite observations (Hersbach et al., 2020). A novel global dataset, ERA5-HEAT (Human thErmAl comforT) which contains the UTCI was produced from the ERA5 reanalysis climate dataset at a spatial resolution of 0.25° × 0.25° at an hourly level from 1979 to the present (Di Napoli et al., 2021). Details on UTCI calculation and assumptions were described elsewhere (Blazejczyk et al., 2013; Bröde et al., 2012; Di Napoli et al., 2021). We accessed the daily gridded UTCI of the 24-h averages from January 1, 2000 to December 31, 2015 across Australia. UTCI values were extracted at the SA1 level in Western Australia using ArcGIS software (version 10.8.1). UTCI has been used in several medical and epidemiologic studies (Romaszko et al., 2022).

2.4. Statistical analyses

2.4.1. Main and subgroup analyses

The analytical dataset was an SA1-level time-series of daily counts of spontaneous PTB and the corresponding daily UTCI exposures. To simultaneously investigate the immediate and cumulative risks, we combined a distributed lag non-linear model (DLNM) with conditional quasi-Poisson regression (Armstrong et al., 2014; Gasparrini et al., 2010). With the cross-basis term, the non-linear exposure–lag–response association was defined through natural cubic splines in both dimensions of the UTCI predictor and the lag days with 21 maximum lag days (Gasparrini et al., 2010; Khodadadi et al., 2022; Nyadanu et al., 2022). Spline knots were set at equally spaced values on the log scale of lags (Gasparrini et al., 2010). The selection of the optimum degrees of freedom (*df*) for UTCI predictor and lag days was based on the smallest Akaike Information Criterion (Gasparrini et al., 2010). This process

resulted in 2 and 3 *df* for the UTCI predictor and lags being selected, respectively. The model specification was given as

$$\log\left[E\left(Y_{t,s}\right)\right] = \alpha + cb(\text{UTCI}) + \text{holiday}, \quad \text{eliminate} = \text{factor} \quad (stratum) \qquad (1)$$

where α is the intercept; $Y_{t,s}$ is the observed number of spontaneous PTB at day *t* in spatial unit *s* (SA1); *cb* is the cross-basis function, the *holiday* is a binary indicator variable for public holidays, and *stratum* (introduced through the "eliminate" function in "gnm" package (Turner and Firth, 2020) was the conditional factor that defined the same day of the same week in the same calendar month of the same year at the same SA1.This analytical framework had been applied previously (Lu et al., 2020, 2021; Nyadanu et al., 2022; Ragettli et al., 2017; Vicedo-Cabrera et al., 2020; Wu et al., 2021).

With reference to the median UTCI, we estimated the relative risks (RRs) and 95% confidence intervals (CIs) at the 1st, 5th, 25th, 75th, 95th, and 99th percentiles of UTCI. Following previous reports (Basu et al., 2010; Cheng et al., 2021; Khodadadi et al., 2022; Nyadanu et al., 2022), we reported the RR (95% CI) for only the immediate effects of exposure on the day of PTB (lag 0) and cumulative effects from event day 0 up to the preceding day N (lag 0-N). The results of the individual lag days in distributed lag models could be biased by temporal collinearity or autocorrelation with potential erroneous findings (Basagaña and Barrera-Gómez, 2021; Bhaskaran et al., 2013). Additionally, labour could last more than one day, or the pregnant woman may not be admitted until a day following the thermal stress exposure (Basu et al., 2016). The acute immediate and cumulative effects up to the first six preceding days were reported. We also reported results for 0–13 and 0–21 lag days, representing second and third weeks, respectively as "long-term" exposures (Khodadadi et al., 2022; Nyadanu et al., 2022).

Potential effect modifications were investigated by performing subgroup analyses for each of the subgroups described earlier. The RRs (95% CIs) for the 1st and 99th percentiles, relative to the median UTCI were reported. Furthermore, the respective reference subgroups were used to compare the two RRs (95% CIs) for each subgroup by estimating the ratio of relative risks (RRRs) and the corresponding 95% CIs for both 1st and 99th percentiles of UTCI exposure for lag 0–6 for each subgroup with the Altman and Bland test of interaction effects (Altman and Bland, 2003; Hutchon, 2005).

We also estimated the attributed risk (AR) as the number of excesses per 10,000 singletons spontaneous PTB that could be attributable to immediate (lag 0) and cumulative (lag 0–6) heat stress exposure, relative to the median UTCI by following Ha et al. (2017) as

$$AR = I_u\,(RR - 1) \qquad (2)$$

where I_u is the background rate which was defined as the study-specific incidence rate and calculated from the eligible spontaneous vaginal delivery births (7.2%). This was also equivalent to the average of 2009–2015 state-wide singleton PTB incidence reported elsewhere (Newnham et al., 2017).

2.4.2. Sensitivity analyses

The robustness of the main analysis was checked by performing several sensitivity analyses for varying model conditions or assumptions. The *dfs* were changed to 3 for both UTCI predictor and lags and then to 3 for UTCI predictor and 4 for lags dimensions. Two separate reference values (the mean UTCI and the average of the standard 'no thermal stress' range, 17.5 °C) were also used. All analyses were performed with R statistical software (version 4.1.1) (R Core Team, 2021). The DLNM was fitted with the "dlnm" package (Gasparrini et al., 2010) and the conditional quasi-Poisson regression with the "gnm" package (Turner and Firth, 2020). We reported and interpreted the RR (95% CI) contextually without a 'statistical significance' threshold as recommended by the American Statistical Association (Wasserstein et al., 2019).

3. Results

3.1. Exposure and cohort characteristics

The standard UTCI has 10 thermophysiological stress categories where 9–26 °C is considered as *no thermal stress*, and values below and above this range are varied intensities of *cold thermal stress* and *heat thermal stress*, respectively (Blazejczyk et al., 2013; Di Napoli et al., 2021). The mean UTCI (standard deviation) and median (interquartile range) across the entire study period were 14.5 °C (6.7 °C) and 13.8 °C (9.2 °C), respectively and both were within the standard *no thermal stress* category. The 1st percentile (0.7 °C) and the 99th percentile (31.2 °C) were within the *slight cold stress* and *moderate heat stress* categories, respectively (Blazejczyk et al., 2013; Di Napoli et al., 2021). The UTCI distribution varied slightly among subgroups and the largest records were in summer (20.5 ± 5.3 °C) and 2010–2015 (15.1 ± 6.8 °C) (Table S1). Spontaneous PTB was fairly distributed across the seasons with half observed during the six months of transition season and approximately 25% each during the three months each of winter and summer. The prevalence of spontaneous PTB increased across the years. Most of the births were to women who had moderate PTB (86.6%), had male babies (56.2%), were non-smokers (75.8%), married (81.6%), aged 20–34 years (73.7%), Caucasian (71.6%), and low socioeconomic status, SES (64.7%) (Table 1).

3.2. Thermophysiological stress and risk of spontaneous PTB

The exposure-lag-response association for the short-term cumulative effects within a week showed changes from lower to greater risks across the exposures, relative to the median UTCI. The magnitude of effects began to decrease for exposures from the second week before birth (Fig. 2). Relative to the median UTCI, there was negligible change in the risk in the 1st to 25th percentiles for all exposure periods. However, strong positive associations were found in the 95th and 99th percentiles (heat stress) which increased with increasing cumulative heat stress episodes for the first week but were lower afterward. Specifically, for 99th percentile relative to median UTCI, immediate (lag 0 day) and

Table 1
The number of spontaneous PTB by year, season, type, and fetal and maternal sociodemographic characteristics in Western Australia, 2000–2015 (N = 15,576).

Variable	Characteristics	n (%)
Year	2000–2004	4162 (26.7)
	2005–2009	5101 (32.7)
	2010–2015	6313 (40.5)
Season	Transition	7793 (50.0)
	Winter	3963 (25.4)
	Summer	3820 (24.5)
PTB type	Extremely PTB (20–27 weeks)	889 (5.7)
	Very PTB (28–31 weeks)	1194 (7.7)
	Moderate PTB (32–36 weeks)	13,493 (86.6)
PTB type at extreme ends	Periviable birth (20–26 weeks)	709 (4.6)
	Late PTB (34–36 weeks)	11,905 (76.4)
Fetal sex	Male	8752 (56.2)
	Female	6824 (43.8)
Prenatal smoking	Non-smoker	11,805 (75.8)
	Smoker	3771 (24.2)
Marital status	Married/de facto	12,710 (81.6)
	Unmarried[a]	2866 (18.4)
Delivery age (years)	≤19	1196 (7.7)
	20–34	11,476 (73.7)
	≥35	2904 (18.6)
Race/ethnicity	Caucasian	11,155 (71.6)
	Non-Caucasian	4421 (28.4)
Socioeconomic status	High	5506 (35.3)
	Low	10,070 (64.7)

[a] Never married/separated/divorced/widowed/unknown. PTB, preterm birth.

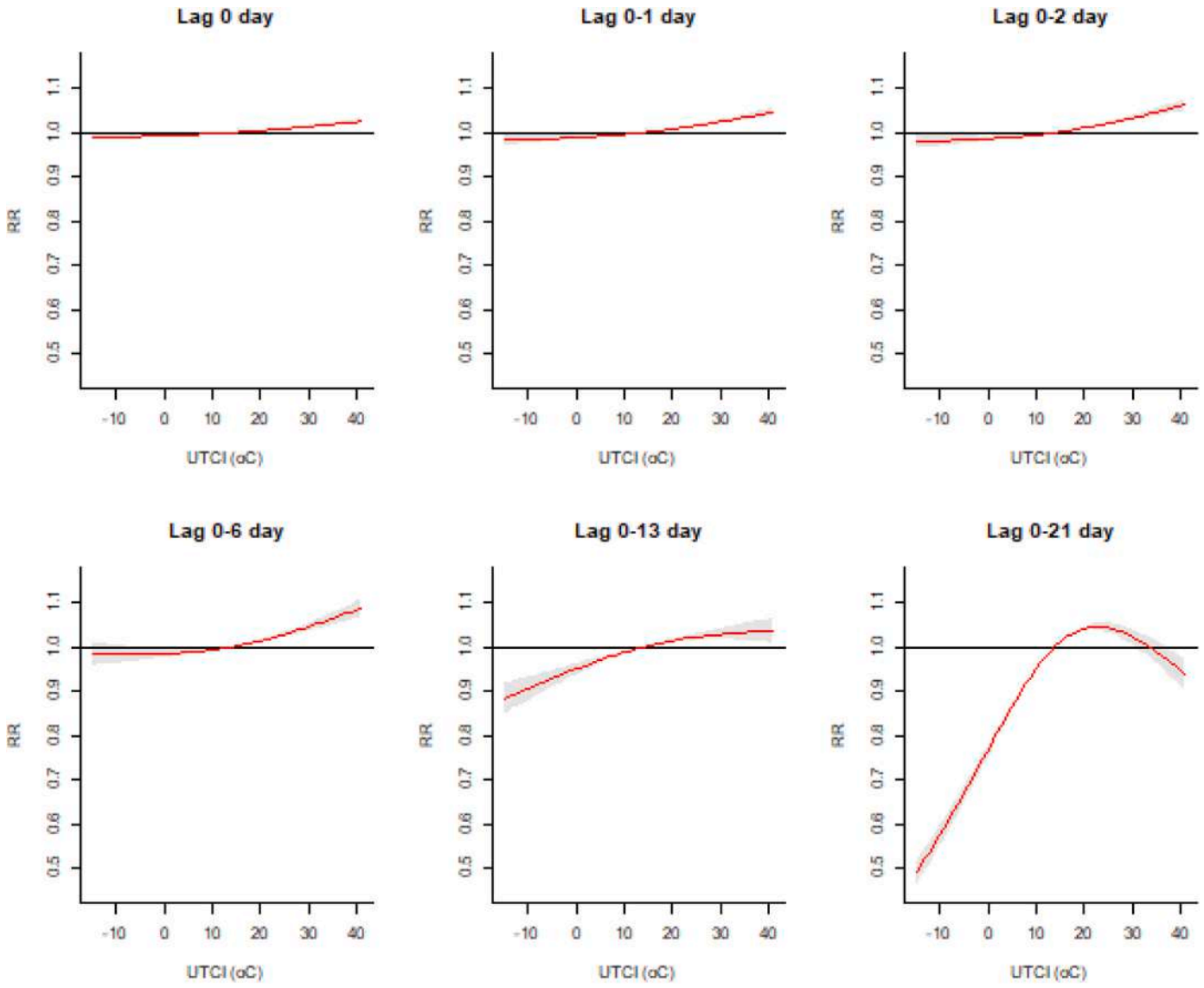

Fig. 2. Exposure-response curves of daily UTCI and cumulative relative risk of spontaneous PTB at different lag structures using median UTCI of 13.8 °C as reference. Solid red lines represent point estimates, and the whiskers represent 95% confidence intervals. Note: UTCI, Universal Thermal Climate Index in degree Celsius. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cumulative acute exposure (lag 0–6 day) risks were 1% (RR = 1.01, 95% CI: 1.01, 1.02) and 5% (RR = 1.05, 95% CI: 1.04, 1.06) greater, respectively (Table 2). Both cold and heat stress showed the most elevated risk during transition season for both immediate and cumulative acute effects but either lower or small positive associations during winter and summer (Table S2a and Table S2b). Cumulative acute exposure to both 1st and 99th percentiles relative to median UTCI showed lower effects during both winter and summer as compared to the transition season. This was as low as 18% lower effect in summer as compared to the transition season, for exposure to 99th percentile relative to median UTCI (RRR = 0.82, 95% CI: 0.80, 0.83) (Table 3). The risk was most elevated for the middle year 2005–2009 (Fig. S1).

Table 2
The cumulative relative risks of spontaneous PTB for different UTCI percentiles relative to the median (13.8 °C) in Western Australia, 2000–2015.

Lag days	1st (0.7 °C) RR (95% CI)	5th (4.2 °C) RR (95% CI)	25th (9.7 °C) RR (95% CI)	75th (18.9 °C) RR (95% CI)	95th (26.4 °C) RR (95% CI)	99th (31.2 °C) RR (95% CI)
0	0.99 (0.99, 1.00)	0.99 (0.99, 1.00)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	1.01 (1.01, 1.01)	1.01 (1.01, 1.02)
0–1	0.99 (0.99, 0.99	0.99 (0.99, 0.99)	1.00 (0.99, 1.00)	1.01 (1.01, 1.01)	1.02 (1.02, 1.02)	1.03 (1.02, 1.03)
0–2	0.99 (0.98, 0.99)	0.99 (0.99, 0.99)	0.99 (0.99, 1.00)	1.01 (1.01, 1.01)	1.02 (1.02, 1.03)	1.04 (1.03, 1.04)
0–3	0.99 (0.98, 0.99)	0.99 (0.98, 0.99)	0.99 (0.99, 1.00)	1.01 (1.01, 1.01)	1.03 (1.02, 1.03)	1.04 (1.04, 1.05)
0–4	0.99 (0.98, 0.99)	0.99 (0.98, 0.99)	0.99 (0.99, 1.00)	1.01 (1.01, 1.01)	1.03 (1.03, 1.04)	1.05 (1.04, 1.06)
0–5	0.99 (0.98, 0.99)	0.99 (0.98, 0.99)	0.99 (0.99, 1.00)	1.01 (1.01, 1.01)	1.03 (1.03, 1.04)	1.05 (1.04, 1.06)
0–6	0.99 (0.98, 0.99)	0.99 (0.98, 0.99)	0.99 (0.99, 1.00)	1.01 (1.01, 1.01)	1.03 (1.03, 1.04)	1.05 (1.04, 1.06)
0–13	0.95 (0.94, 0.96)	0.97 (0.96, 0.98)	0.99 (0.98, 0.99)	1.01 (1.01, 1.02)	1.02 (1.02, 1.03)	1.03 (1.02, 1.04)
0–21	0.78 (0.77, 0.80)	0.85 (0.84, 0.86)	0.95 (0.94, 0.95)	1.04 (1.03, 1.04)	1.04 (1.03, 1.05)	1.02 (1.00, 1.04)

Note: UTCI, Universal Thermal Climate Index in degree Celsius; PTB, preterm birth.

Table 3
The estimated interaction effects as ratio of relative risks (RRRs) and 95% confidence intervals (95% CI) of spontaneous preterm birth, relative to the indicated reference subgroup for acute cumulative exposure (lag 0–6) to 1st percentile of UTCI (cold stress) and 99th percentile of UTCI (heat stress) relative to median UTCI (no thermal stress) in Western Australia, 2000–2015.

Subgroup	1st percentile of UTCI RRR (95% CI)	99th percentile of UTCI RRR (95% CI)
Winter (ref Transition)	0.83 (0.81, 0.85)	0.87 (0.85, 0.89)
Summer (ref Transition)	0.90 (0.89, 0.92)	0.82 (0.80, 0.83)
Extremely PTB (ref Moderate PTB)	0.94 (0.93, 0.95)	1.50 (1.47, 1.52)
Very PTB (ref Moderate PTB)	1.35 (1.34, 1.36)	0.95 (0.94, 0.96)
Male (ref Female)	1.02 (1.01, 1.03)	1.15 (1.14, 1.17)
Smoker (ref Non-smoker)	0.91 (0.90, 0.92)	1.19 (1.17, 1.21)
Unmarried (ref Married)	0.57 (0.57, 0.58)	1.23 (1.21, 1.24)
non-Caucasian (ref Caucasian)	1.10 (1.09, 1.12)	1.07 (1.05, 1.08)
Low (ref High) SES	0.94 (0.93, 0.95)	0.89 (0.85, 0.94)
≤19 (ref 20–34) years	0.80 (0.79, 0.81)	1.46 (1.44, 1.47)
≥35 (ref 20–34) years	0.87 (0.87, 0.88)	1.01 (1.00, 1.02)

Note: UTCI, Universal Thermal Climate Index in degree Celsius; PTB, preterm birth; SES, Socioeconomic status.

Compared to no thermal stress, attributable risks indicated excesses of 11(95% CI: 9, 13) and 36 (95% CI: 29, 43) per 10,000 liveborn singletons with spontaneous PTB due to immediate (lag 0) and cumulative acute (lag 0–6) heat stress (99th percentile of UTCI) exposures, respectively. The attributable risk was not estimated for cold stress as it showed no association.

3.3. Thermophysiological stress and risk of spontaneous PTB in subgroups

Relative to median UTCI (no thermal stress), cold stress (1st percentile of UTCI) showed essentially no association for both extreme and moderate PTB but strong positive associations for very PTB while heat stress (99th percentile of UTCI) showed no association for very PTB but strong positive associations for both extremely PTB and moderate PTB (Table S3). Cumulative acute exposure (lag 0–6) showed 6% lower effect of cold stress exposure (RRR = 0.94, 95% CI: 0.93, 0.95) and a 50% higher effect of heat stress exposure (RRR = 1.50, 95% CI:1.47, 1.52) for extremely PTB as compared to moderate PTB. Conversely, cumulative acute exposure showed 35% higher effect of cold stress exposure (RRR = 1.35, 95% CI:1.34, 1.36) but 5% lower effect of heat stress exposure (RRR = 0.95, 95% CI: 0.94, 0.96) in very PTB as compared to moderate PTB (Table 3). The impact of the thermal stress was strong in the periviable births but essentially had no association with late PTB (Fig. S2).

Relative to no thermal stress, both thermal stress exposures, particularly heat stress showed sociodemographic disparities (Tables S3–S5). Specifically, cumulative acute exposure (lag 0–6) showed 15% higher effect of heat stress in male as compared to female infants (RRR = 1.15, 95% CI: 1.14, 1.17). As compared to non-smokers, mothers who smoked during pregnancy showed 19% higher effect for cumulative acute exposure to heat stress (RRR = 1.19, 95% CI: 1.17, 1.21). Cumulative acute exposure to heat stress showed 23% higher effect among unmarried as compared to married mothers (RRR = 1.23, 95% CI: 1.21, 1.24). Non-Caucasians experienced higher effect as compared to Caucasians and this was particularly stronger for cold stress exposure at 10% higher (RRR = 1.10, 95% CI: 1.09, 1.12) than heat stress exposure at 7% higher (RRR = 1.07, 95% CI: 1.05, 1.08). Cumulative acute exposures to both cold and heat stress showed small lower effect among mothers in low SES as compared to high SES residential areas. Compared to mothers aged 20–34 years old, cumulative acute exposure to heat stress showed 46% higher effect among mothers aged ≤19 years old (RRR = 1.46, 95% CI: 1.44, 1.47) and 1% higher effect among mothers aged ≥35 years old (RRR = 1.01, 95% CI: 1.00, 1.02) (Table 3).

The results of the sensitivity analyses for varying modelling assumptions and conditions were similar to the main results (Tables S6 and S7).

4. Discussion

4.1. Thermophysiological stress and risk of spontaneous PTB

Relative to the median UTCI (no thermal stress), we found no association with exposures to the first to 25th percentiles but strong positive associations were observed for the 95th and 99th percentiles for immediate and cumulative acute effects. The risk increased with increasing duration of heat stress exposure episodes and was strongest during transition seasons (spring and autumn) and 2005–2009. Assuming causality, attributable risk indicated that heat stress (99th percentiles) exposure relative to no thermal stress on the event day and cumulatively up to six preceding days could account for 11 (95% CI: 9, 13) and 36 (95% CI: 29, 43) excess cases per 10,000 spontaneous PTB, respectively.

Given that we used a human thermophysiological index as recently recommended (Staiger et al., 2019; Vanos et al., 2020) and applied elsewhere (Krüger, 2021; Romaszko et al., 2022), our findings are unique as compared to the previous findings that were based on ambient air temperature metrics (Chersich et al., 2020). Previous studies considered extremes of high and low-temperature thresholds (1st or 5th and 99th or 95th percentiles as compared to median) as heat and cold stress. Our findings were consistent with a study in Belgium and the USA that also found a greater risk for acute heat stress but a small lower risk or essentially no association for cold stress based on ambient temperature metrics (Cox et al., 2016; Sun et al., 2019). For example, the USA study of 32 million singleton births reported an RRs (95% CI) for PTB of 1.03 (95% CI: 1.02, 1.04) and 0.99 (95% CI: 0.98, 0.99) over the previous four days for heat and cold stress, respectively, relative to the median ambient temperature (Sun et al., 2019). Furthermore, the only available meta-analysis that pooled 21 studies found 1% greater odds of PTB (OR = 1.01, 95% CI: 1.01, 1.02) during high versus low-temperature exposure periods of <4 weeks which increased to 5% (OR = 1.05, 95% CI: 1.04, 1.05) after excluding two studies (outliers) (Chersich et al., 2020). There were, however, a few contradictory findings. Two time-series analyses on the Chinese population found greater risks for cold stress but a small lower risk or no association for heat stress in Shenzhen and Xuzhou (Cheng et al., 2021; Liang et al., 2016). Another Chinese study found a greater risk for both heat and cold stress for the immediate effect but no association for short-term cumulative effects in Guangzhou (He et al., 2016). Vicedo-Cabrera et al. found a greater risk for moderate heat but inconsistent associations for extreme cold and heat during the last one to four gestational weeks in Stockholm, Sweden (Vicedo-Cabrera et al., 2015). Specific to Australia, three previous studies examined the acute effect of ambient temperature on PTB (Jegasothy et al., 2022; Mathew et al., 2017; Wang et al., 2013). Matthew et al. found a greater risk of PTB that ranged from 2% up to 8.3% for 90th, 95th, and 99th percentiles of minimum and maximum summer temperatures relative to the median temperature on the day of delivery and up to 21 preceding days in Alice Springs, Central Australia (Mathew et al., 2017). Wang et al. analysed warm-season births in Brisbane, Queensland, and found the greatest hazard ratio of 2.00 (95% CI: 1.37, 2.91) for their highest heat stress, defined as a daily maximum temperature over the 98th percentile for four consecutive days in the last gestational week (Wang et al., 2013). The third study was conducted across New South Wales state with spatiotemporal exposure assessment and time-series analysis that reported the risk of spontaneous PTB at the 95th percentile of daily mean temperature (25 °C) relative to the median (17 °C). The results showed 3% greater risk (RR = 1.03, 95% CI: 1.01, 1.05) on day 0 (day of initial exposure, defined as one day before the event) and 16% greater risk (RR = 1.16, 95% CI: 1.08, 1.25) for the cumulative effect of exposure up to seven preceding days (Jegasothy et al., 2022). Our results were similar, although, the cumulative effect

estimate was greater than that of our study. This could be due to the one-day-delay exposure assessment, differences in population characteristics and climates, study design, and the use of ambient temperature. Given the geographical variability in climatic conditions and the influence of acclimatisation, adaptation, and mitigation strategies, even within a country or region, generalising location-specific findings to other parts is difficult and if necessary, should be done cautiously (Sexton et al., 2021; Vicedo-Cabrera et al., 2015). However, it is expected that there might be greater risks of PTB for heat stress than cold stress due to more severe heat stress episodes than cold stress across most regions in the world as the climate change crisis progresses (IPCC, 2021). Also, there could be better acclimatisation or easier adaptation to cold than heat stress (Sun et al., 2019).

4.2. Thermophysiological stress and risk of spontaneous PTB in subgroups

We found attenuation of risk in our latest period, similar to findings reported in Brisbane, Australia (Li et al., 2018). This may be attributed to thermal adaptation through acclimatisation or increasing mitigation responses such as the use of air conditioning (Barreca and Schaller, 2020), improved climate-specific clothing, thermal stress-resilient housing infrastructure, and improved healthcare system over the years (Adnan et al., 2022; Li et al., 2018; Sun et al., 2019). However, our observed elevated risk in the transition season as compared to other seasons could imply that pregnant women may not be able to quickly thermo-adapt when transitioning from high to low thermal stress or vice versa. It could also mean that pregnant women took more behavioural precautions such as reduced outdoor activities or increased use of heating or cooling systems during summer or winter seasons as compared with the transition season.

We observed lower risks of heat stress with increasing gestational age which was consistent with the previous findings (Avalos et al., 2017; Cox et al., 2016; He et al., 2016) and indicates a plausible causal link between prenatal heat stress and the shortening of gestational age (Barreca and Schaller, 2020; Strand et al., 2011). Basu et al., however, observed the strongest risk for near-term PTB in California, USA (Basu et al., 2010). We also observed that cold stress showed strong positive associations with very PTB but not for other types of PTB. A cold season analysis in California, USA, however, indicated the strongest odds of mean apparent temperature for near-term PTB (Avalos et al., 2017). Among the reasons stated earlier, the analytical design, exposure metrics, and climatic conditions could explain the differences. This requires further studies from other locations with a thermophysiological index. We found a stronger impact of heat stress in male neonates as compared with the female neonates similar to the largest cohort study conducted in the USA (Sun et al., 2019) but others reported otherwise (Avalos et al., 2017; Basu et al., 2010; Cox et al., 2016). However, it has been recognised extensively in the literature that male neonates are more vulnerable to pregnancy outcomes and the influence of environmental exposures (Al-Qaraghouli and Fang, 2017). As reported in a few previous studies, the comparatively higher-risk women for heat stress were women who smoked, unmarried, teenagers, and non-Caucasians (Basu et al., 2010; Mathew et al., 2017; Vilcins et al., 2021). These vulnerabilities are attributed to the level of outdoor activities, risky behaviours and lifestyle, poor antenatal care utilisation, resources for mitigation strategies, hereditary, and systemic racism (Adnan et al., 2022; Alson et al., 2021; Basu et al., 2010; Giudice et al., 2021; Mathew et al., 2017; Vilcins et al., 2021). Surprisingly, we observed a stronger risk of thermal stress for women that resided in the high SES areas but lower risk or no association for those in low SES areas. We used area-level SES as a proxy for individual SES which is known to produce misclassification bias to some extent (Wang et al., 2013). However, there are possible reasons for this finding. Women with low SES are more likely to be exposed to outdoor working conditions over long periods and lack cooling or heating systems at home (Adnan et al., 2022). Consequently, they are more likely to acclimatise to thermal stress as compared to women with high SES, resulting in the observed elevated risk in the high than low SES groups. Better individual-level indicators for SES such as occupation and further investigations are required. Given that climate change impacts are exacerbated by maternal sociodemographic and lifestyle factors, a better understanding and identification of higher-risk subpopulations is crucial for prioritised intervention (Ebi et al., 2021; Giudice et al., 2021).

Public health interventions and mitigation strategies may be required, particularly for the most vulnerable women. Examples include raising awareness and educating women to sufficiently hydrate and decrease outdoor activities during hot days, *greening* the environment to improve shade, provision of public shade structures, provision of affordable heating and cooling systems, and thermal stress warning systems that account for the human thermophysiology (Adnan et al., 2022; Giudice et al., 2021; Nyadanu et al., 2022).

4.3. Biological mechanisms

Several animal studies and clinical evidence have provided strong support for the pathophysiology of prenatal thermal stress exposure and PTB. Generally, any factor or exposure that initiates the breakdown of feto-maternal immune tolerance and excessive or premature activation of the inflammatory pathways causes uterine contractility, cervical ripening, and rupture of membranes which results in PTB (Di Renzo et al., 2018; Green and Arck, 2020). Heat or cold stress induces molecular and biochemical catalytic processes that cause oxidative damage, apoptosis, deregulate inflammatory production and abnormally high intracellular expression of heat shock proteins in the serum. These affect placental physiology and fetal development (particularly higher in sociodemographically vulnerable women) and cause implantation failure and feto-maternal complications such as pregnancy outcomes, including spontaneous PTB (Berestoviy et al., 2021; Collier et al., 2017; Di Renzo et al., 2018; Green and Arck, 2020; Jee et al., 2021). Heat stress also causes dehydration which reduces uterine blood flow and increases secretion of the pituitary antidiuretic hormone, prostaglandin, and oxytocin. These affect fetoplacental transport and induce spontaneous labour (Stan et al., 2002).

4.4. Strengths and limitations

Our study has several strengths. The novel study design and the modelling framework accounted for and substantially minimised both time-invariant and time-varying known and unknown confounding factors in the short-term periods, temporal autocorrelation, and spatial confounding (Armstrong et al., 2014; Gasparrini et al., 2010; Nyadanu et al., 2022; Wu et al., 2021). The space-time varying assessment of the UTCI exposure at the individual's residential microenvironment reduced exposure misclassification as compared to using ground-based monitoring stations that may be distant from the participants (Nazarian and Lee, 2021). To the best of our knowledge, this is the first study that used the available most suitable contemporary human thermophysiological index (UTCI) at a spatiotemporal resolution to examine the association between heat or cold stress and spontaneous PTB. This makes the findings more robust and physiologically relevant by combining knowledge from climate science, physiology, and epidemiology (Jendritzky et al., 2012; Romaszko et al., 2022; Staiger et al., 2019; Vanos et al., 2020). This was also the first study on this topic in Western Australia.

This study has some limitations, including our inability to account for indoor thermal environments (e.g., use of heating or cooling systems) and prenatal activity-time patterns. A prospective cohort with personalised activity-time exposure assessment using portable thermal sensors and indoor thermal environment assessments may help minimise some of these limitations. Given the space-time varying exposure assessment and acute exposure analysis, we expect any remaining exposure misclassification to be minimal and non-differential which would have rather attenuated the observed effect estimates towards the null (Sun

et al., 2019). We also lacked information on other relevant sociodemographic factors such as maternal occupation, education, illicit drug or alcohol use, and nutrition. As the primary aim in the present study was to investigate short-term associations between thermal stress and spontaneous PTB, future studies should investigate long-term effect across the entire pregnancy periods with the extended DLNM to identify other potential critical windows of susceptibility.

5. Conclusion

We find that prenatal exposure to acute heat but not cold stress relative to no thermal stress elevated the risk of spontaneous PTB. However, both heat and cold stresses elevated the risk in the more vulnerable subpopulations. Given the expected increasing events of climate change extremes in the coming years (IPCC, 2021) and the potential impacts on birth outcomes, we call on the public health officers, antenatal care providers, and obstetricians to help communicate the potential risk to pregnant women (Giudice et al., 2021). The provision of thermal adaptation or mitigation strategies and resources may help reduce the risk of spontaneous PTB, particularly for higher-risk pregnant women. In addition to an improved healthcare system, an appropriate climate change policy is required. Several comparative studies had indicated the suitability and relevance of thermophysiological metrics as compared to ambient temperature for medical and preventive medicine given that thermophysiological metrics capture the total thermal environment and human thermophysiological responses (Blazejczyk et al., 2012; Bröde et al., 2013; Kampmann et al., 2012; Staiger et al., 2019). Future studies should consider the four recently recommended appropriate human thermophysiological indices (Staiger et al., 2019), particularly UTCI which is now gaining high application in scientific research and recommendations among clinicians, epidemiologists, and specialists in public health and thermal stress management (Krüger, 2021; Nazarian and Lee, 2021; Romaszko et al., 2022; Staiger et al., 2019; Vanos et al., 2020).

Ethical approval

This study was approved by the Human Research Ethics Committees of the Western Australia Department of Health (#2016/51) and Curtin University (#HRE2020-0523). The participants' informed consent was waived, particularly due to the implausibility of obtaining retrospective consent for de-identified secondary data.

Data availability

The UTCI data is open access from the Copernicus Climate Data Store (https://doi.org/10.24381/cds.553b7518). The birth data cannot be made publicly available due to the data access agreement, but it can be requested from the Department of Health, Western Australia (https://ww2.health.wa.gov.au/Articles/J_M/Midwives-Notification-System).

Credit author contributions

SDN, GAT, BM, and GP: Conceptualisation, Methodology, Investigation, Writing—Critical Review and Editing, Project Administration. SDN: Data curation, Formal analysis, Writing—Original draft preparation. All authors have read and approved the final version of the manuscript.

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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.2022.114029.

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Prenatal ambient pesticide exposure and childhood retinoblastoma

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ABSTRACT

Background: Retinoblastoma is a rare tumor of the retina, most commonly found in young children. Due to the rarity of this childhood cancer, few studies have been able to examine prenatal pesticide exposure as a risk factor.
Objective: To examine the relationship between childhood retinoblastoma and prenatal exposure to pesticides through residential proximity to agricultural pesticide applications.
Methods: We conducted a population-based case-control study using cases aged 5 and younger identified from the California Cancer Registry, and controls randomly selected from California birth certificates. Frequency matching cases to controls by age resulted in 221 cases of unilateral retinoblastoma and 114 cases of bilateral retinoblastoma, totaling 335 cases and 123,166 controls. Based on addresses from birth certificates we employed Pesticide Use Reports and land use information within a geographic information system approach to individually assess exposures to specific pesticides within 4000 m of the residence reported on birth certificates. The associations between retinoblastoma (all types combined and stratified by laterality) and individual pesticides were expressed as odds ratios estimates obtained from unconditional logistic regression models including a single pesticide, and from a hierarchical logistic regression model including all pesticides.
Results: We found that exposures to acephate (OR: 1.70, 95% CI: 1.20, 2.41) and bromacil (OR: 1.87, 95% CI: 1.07, 3.26) were associated with increased risk for unilateral retinoblastoma. In addition to acephate, we found that pymetrozine (OR: 1.45, 95% CI: 1.00, 2.08) and kresoxim-methyl (OR: 1.60, 95% CI: 1.00, 2.56) were associated with retinoblastoma (all types combined).
Conclusion: Our findings suggest that certain types of prenatal ambient pesticide exposure from residing near agricultural fields may play a role in the development of childhood retinoblastoma.

1. Introduction

Retinoblastoma is a rare tumor of the retina, affecting an estimated 8000 children per year globally (Dimaras et al., 2015). Accounting for roughly 3% of all childhood cancers, it is the most common eye cancer in children (Yun et al., 2011). Though the survival rate is greater than 95% in high-income countries, survivors may face visual impairments, declines in neurocognitive development, and elevated risk for subsequent primary malignancies in adulthood (Yun et al., 2011; Dimaras et al., 2015; Willard et al., 2014; MacCarthy et al., 2009). In low-income countries, the tumor is associated with much lower survival (about 30%) likely due to later diagnosis and difficulty in accessing specialty care (Dimaras et al., 2010; Nyawira et al., 2013; Dean et al., 2014).

Retinoblastoma usually presents in children ages 5 and younger, as the result of biallelic mutation of the retinoblastoma tumor-suppressor (*RB1*) gene (Yun et al., 2011). Following a two-hit model of gene inactivation, retinoblastoma presents either unilaterally or bilaterally (Knudson, 1971). Most cases of bilateral retinoblastoma result from a germline mutation of the *RB1* gene (first hit) followed by a somatic mutation (second hit) post-conception. For bilateral retinoblastoma, this

Abbreviations: CI, confidence interval; CDWR, California Department of Water Resources; CNS, Central nervous system; EPA, Environmental Protection Agency; GIS, Geographic Information System; GRAPES, GIS-based Residential Ambient Pesticide Estimation System; OP, organophosphate; OR, odds ratio; PAN, Pesticide Action Network; PLSS, Public Land Survey System; PUR, Pesticide Use Reports; *RB1*, Retinoblastoma tumor suppressor; SES, socioeconomic status; SIR, standardized incidence ratio.

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first hit is acquired from a parent, in whom the mutation occurs in the germinal cells some time prior to conception (Omidakhsh et al., 2017). For unilateral retinoblastoma, which comprises 73% of all cases, both *RB1* mutations occur somatically in a retinal cell post-conception (Broaddus et al., 2009). This timeline, along with the early childhood occurrence, suggests that perinatal exposures are relevant in the etiology of retinoblastoma (Heck et al., 2015; Bunin et al., 1989). Furthermore, the difference in the timing of the *RB1* mutation between the subtypes suggests that pesticide exposures may impact unilateral and bilateral retinoblastoma cases differentially.

A number of environmental risk factors for retinoblastoma have been suggested, including air pollution, sunlight, X-rays, and various parental occupational exposures (Heck et al., 2013, 2015; Ghosh et al., 2013; Hooper, 1999; Jemal et al., 2000; Lombardi et al., 2013; Bunin et al., 1989, 1990; Abdolahi et al., 2013; Omidakhsh et al., 2021). Among these occupational exposures, pesticides have been associated with retinoblastoma risk albeit somewhat inconsistently. Some retinoblastoma studies focusing on parental pesticide exposure examined parents working as pesticide applicators or other agricultural workers, while others examined residential exposure from living on farms or using pesticides in homes and gardens (Flower et al., 2004; Rodvall et al., 2003; MacCarthy et al., 2009; Abdolahi et al., 2013; Fear et al., 1998; Pearce et al., 2006; Kristensen et al., 1996; Omidakhsh et al., 2017; Bunin et al., 1989).

Due to the rarity of retinoblastoma, most of the previous studies have been underpowered and consequently limited from estimating risk separately for unilateral and bilateral disease. Furthermore, pesticide exposure information in these studies is only available in broad categories that assess all pesticides together or characterize them broadly by type (e.g. agrochemicals, insecticides). The different exposure definitions across prior studies may have also led to the inconsistent results. Here, we therefore aim to expand upon the literature by separately assessing risk for unilateral and bilateral retinoblastoma and use information on a large number of specific pesticides applied commercially on fields in California.

2. Materials and methods

2.1. Study population

Retinoblastoma cases (International Classification of Childhood Cancer code 050) (Steliarova-Foucher et al., 2005) aged 5 and younger were drawn from California Cancer Registry records of incident cancer diagnosed in 1988–2013. Case ascertainment in the California Cancer Registry is extensive. Since 1985, California state law has required that all cancers diagnosed in California be reported to the California Cancer Registry (California Cancer Registry, 2018). The state registry is internally validated by the California Department of Public Health and meets data standards for the National Program of Cancer Registries and the National Cancer Institute's Surveillance, Epidemiology, and End Results program. Cases were linked with birth certificates based on exact matches for name, date of birth, and social security number when available, using a probabilistic linkage program, which achieved an 89% matching success for all childhood cancer cases (n = 13,674). The 11% of cases that did not match with a birth certificate were likely not born in California (Urayama et al., 2009). Controls were randomly selected from California birth certificate data and 20:1 frequency-matched by birth year to all childhood cancer cases during the study period (n = 270,941). The median age at diagnosis for all retinoblastoma cases combined was 1 year old, with a median birth year of 2004 for both cases and controls included in this study. Unilateral retinoblastoma cases had a mean age at diagnosis of 14 months old, and bilateral retinoblastoma cases had a mean age at diagnosis of 5 months old.

Controls who died before the age of 6 (n = 1215), and observations with unknown sex (n = 3), gestational age less than 20 weeks (n = 680), or birth weight less than 500 g (n = 131) were excluded from the sample. Since exposure data is not available for most other states, observations with birth addresses outside of California were also excluded (n = 488). Inclusion criteria for the parent study was birth in California during the study period. For the present analysis, an additional inclusion criterion was residence in the study area, assessed by birth certificate address.

We limited analysis to birth years 1998–2011, since prior to 1998, full residential addresses were not available in the electronic birth certificate dataset. Furthermore, we restricted the sample of cases and controls to mothers living within 4000 m of a site to which at least one pesticide—among the more than 600 pesticides applied in California agriculture—was applied during their pregnancy assuming they lived at the address recorded on the birth record. This restriction is intended to limit exposure misclassification due to differences in exposure environments between children living in proximity to agriculture and children living in urban environments. Agricultural pesticide use is generally rare in urban areas, but non-agricultural commercial pesticide use such as home fumigation or applications to parks and roadways can nevertheless be extensive and in California, is only reported at the county level. This restriction also is likely to generate a rural study sample that is more homogeneous with regard to other unmeasured social and environmental factors such as parental occupation or air pollution exposures. Our resulting study population contained 221 cases of unilateral retinoblastoma and 114 cases of bilateral retinoblastoma, totaling to 335 cases and 123,166 controls.

2.2. Ethics approval and consent to participate

Ethics approval for the current study was obtained from the University of California Los Angeles, Office of the Human Subjects Research Protection Program and the California State Committee for the Protection of Human Subjects. As this study involved secondary analysis of existing registry data, patient consent was not required.

2.3. Pesticide exposure assessment

The California Department of Pesticide Regulation has mandated since 1990 that all agricultural use of pesticides be reported in the form of Pesticide Use Reports (PUR). These reports identify the quantities in which specific pesticides are applied, the sites and times at which these applications take place, as well as which crops are treated. Land-use data is collected through California's Public Land Survey System (PLSS), which records the exact locations of the crops that are reported in the PUR data. Our study's GIS-based Residential Ambient Pesticide Estimation System (GRAPES) maps the PUR data onto the PLSS grid according to land use information provided by the California Department of Water Resources (CDWR) and allows us to more precisely locate pesticide applications (Cockburn et al., 2011). CDWR provides digitally accessible land-use maps dating back to 1986; therefore, pesticide exposure information is available for the entire study period (California Department of Water Resources, 2022).

Regarding geocoding accuracy, 54% of residential addresses were geocoded via exact parcel centroid point, address range interpolation was used for 39% of the addresses, and 7% of the addresses were geocoded by USPS zip area centroid. To assess potential misclassification due to geocoding inaccuracy, we performed a sensitivity analysis that dichotomized results by geocoding quality type. Cases and controls were geocoded, blind to case-control status, using the Texas A&M geocoding service (Texas A&M Geoservices, 2019).

We defined exposure to each pesticide as ever pesticide exposure, to any of the 600+ PUR pesticides, during pregnancy within a 4000 m buffer of the maternal address listed on the birth certificate. Using date of last menstrual period and date of birth from the birth certificate, we were able to assess whether mothers were exposed to specific pesticides at any point during the pregnancy.

Out of all agricultural pesticides used in our study area, we selected 132 pesticides classified as possibly or probably carcinogenic by the

Environmental Protection Agency (EPA, 2012), and are reporting results for 58 of these pesticides to which a minimum of 20 cases were considered exposed during the study period.

2.4. Statistical analysis

Following the analytic strategy suggested by Momoli et al. (2010), we used unconditional logistic regression models to estimate odds ratios (OR) and 95% confidence intervals (CI) for associations between each of the selected pesticides and all types combined retinoblastoma. We further stratified analyses by laterality using the same controls and tested for heterogeneity between the two subtypes using methods developed by Wang et al. (2016). Based on previous literature, we adjusted all models for birth year, child's sex, maternal age, neighborhood socioeconomic status (SES), and maternal race/ethnicity (Heck et al., 2012; Omidakhsh et al., 2017; Bunin et al., 1989). Neighborhood SES was measured with an index developed by Yost et al. (2001), which summarizes the following census-group-level socioeconomic indicators into one 5-level variable: education, median household income, percent living 200% below poverty, percent blue-collar workers, percent older than 16 years without employment, median rent, and median house value. To account for individual SES, we also attempted adjusting for maternal education (Supplementary Table 1). Additionally, we tested the inclusion of maternal birthplace and paternal age in our model, which have been associated with retinoblastoma and may be associated with pesticide exposure (Heck et al., 2012). However, these variables did not change estimates by more than 5% and were not included in the final models. Maternal smoking may be related to retinoblastoma (Azary et al., 2016). In a subanalysis among the children for whom data were available, we additionally adjusted for maternal smoking, as ascertained by cotinine in neonatal dried blood spots (Supplementary Table 2). This did not change results and was left out of final models.

We used single-pesticide models to separately estimate the OR and 95% CI for each pesticide without adjusting for exposure to any of the other pesticides. In order to co-adjust for the other selected pesticide exposures and account for multiple comparisons, we built a multiple-pesticide model via a semi-Bayesian hierarchical logistic regression model with a pre-specified second-stage variance of 0.5, representing a 16-fold uncertainty about the 95% CI for the prior OR estimates. This semi-Bayesian approach assumes that individual pesticide effect estimates for retinoblastoma follow separate prior (or second-stage) normal distributions with separate group means for each subset (or class) to which each pesticide belongs. In other words, we assumed that pesticides belonging to a specific subset would have a common category-specific prior effect on retinoblastoma, and the semi-Bayesian hierarchical regression model would shrink the estimated individual pesticide effects within the subset towards their common category specific effect (group mean) to produce a weighted posterior effect estimate for each individual pesticide (Greenland, 1992, 1994).

We performed this analysis using two separate approaches for generating the pesticide subset categorization scheme. First, each pesticide was assumed to belong to its respective chemical class, as categorized by the Pesticide Action Network (PAN) database (Kegley et al., 2011): 2,6-dinitroaniline, amide, anilide, azole, chloroacetanilide, dicarboximide, halogenated organic, n-methyl carbamate, organochlorine, organophosphate (OP), pyrethroid, substituted benzene, triazine, or urea. Alternatively, we assumed that each pesticide belongs to subsets of pesticide applications that are highly correlated with each other in our controls. Specifically, we conducted factor analysis using principal components extraction with varimax rotation for exposures among control subjects to generate subsets of correlated pesticide applications in pregnancy based on factor loadings >0.60. Both approaches yielded similar posterior OR estimates; therefore, here we present results from the hierarchical logistic regression model based on the first assumption of the chemical classes. Analyses were conducted using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

Among our study population of 335 cases and 123,166 controls, there were 46 pesticides to which at least 20 unilateral cases were exposed, and 34 pesticides to which at least 20 bilateral cases were exposed. Overall, there were 58 pesticides examined, to which at least 20 total cases (all subtypes) were exposed. Table 1 shows the demographics of the study population. For both subtypes, retinoblastoma cases were more likely to be male than controls. A greater proportion of bilateral retinoblastoma occurred among children of Hispanic mothers and mothers over the age of 30.

Most of the pesticides that loaded onto the same factor had correlation coefficients ranging from 0.5 to 0.75, with the highest correlation between any two pesticides being 0.63. This correlation structure is depicted in greater detail by Lombardi et al. (2021). Our sensitivity analysis to assess exposure misclassification resulted in increased point estimates when observations were restricted to only those geocoded by exact parcel centroid—the highest resolution geocode type; and resulted in estimates that were closer to the null for observations with lower resolution geocodes.

Fig. 1 shows estimated posterior ORs for all retinoblastoma cases combined obtained from the multiple-pesticide model; sample sizes and percent exposed to each pesticide, along with estimated ORs from single-pesticide models are shown in Supplementary Table 3. Acephate was the pesticide to which the largest proportion (63%) of cases were exposed, while flonicamid was the pesticide to which the smallest proportion was exposed. We obtained elevated OR estimates for all retinoblastoma with exposure to acephate, pymetrozine, and oxythioquinox in single-pesticide models. When we adjusted for exposures to other pesticides in the hierarchical logistic regression model, acephate (OR: 1.59, 95% CI: 1.19, 2.12) remained positively associated with retinoblastoma, as did pymetrozine (OR: 1.45, 95% CI: 1.00, 2.08) and oxythioquinox (OR: 1.48, 95% CI: 0.97, 2.26) although the 95% CIs included null. Effect estimates for kresoxim-methyl (OR: 1.60, 95% CI: 1.00, 2.56) and captan (OR: 1.31, 95% CI: 0.95, 1.80) were shifted further away from the null in the hierarchical logistic regression model. We observed an inverse association between retinoblastoma and exposure to hydramethylnon (OR: 0.59, 95% CI: 0.35, 0.98) in the hierarchical logistic regression model for all retinoblastoma.

Fig. 2 presents associations between prenatal pesticide exposure and retinoblastoma, stratified by laterality. The effect estimates and distributions for unilateral and bilateral retinoblastoma are presented in Supplementary Table 4 and Supplementary Table 5, respectively. For the unilateral subtype, we observed elevated odds with exposure to acephate (OR: 1.70, 95% CI: 1.20, 2.41), in addition to several other elevated OR estimates in the single-pesticide models; however, only exposure to bromacil (OR: 1.87, 95% CI: 1.07, 3.26) showed an association in the multiple-pesticide adjusted model. We observed several additional elevated OR estimates with wide confidence intervals for bilateral retinoblastoma in the single-pesticide and multiple-pesticide adjusted models.

4. Discussion

In this case-control study, we observed elevated associations with childhood retinoblastoma for maternal ambient exposure to several pesticides during pregnancy. In analyses for all types of retinoblastoma, we estimated elevated odds with exposure to acephate, pymetrozine, and kresoxim-methyl. After stratifying by laterality, unilateral retinoblastoma was still associated with exposure to acephate and newly with bromacil. Similar to one of the previous studies examining prenatal pesticide exposure and retinoblastoma, our CIs for bilateral disease estimates were wider than those for unilateral retinoblastoma due to the smaller number of cases (Omidakhsh et al., 2017). We observed an elevated estimate for diazinon in the single-pesticide model for bilateral retinoblastoma; however, our results did not suggest any pesticide

Table 1
Demographic characteristics of children in California born in 1998–2011 exposed to at least one pesticide during pregnancy.

Characteristic	Retinoblastoma (all types) N = 335	Unilateral Retinoblastoma N = 221	Bilateral Retinoblastoma N = 114	Controls N = 123,166
Sex of Child, n (%)				
Male	187 (55.8)	121 (54.8)	66 (57.9)	62738 (50.9)
Female	148 (44.2)	100 (45.2)	48 (42.1)	60428 (49.1)
Maternal Race/Ethnicity, n (%)				
Hispanic	176 (52.5)	113 (51.1)	63 (55.3)	63230 (51.3)
Other†	73 (21.8)	53 (24.0)	20 (17.5)	23877 (19.4)
White non-Hispanic	86 (25.7)	55 (24.9)	31 (27.2)	36059 (29.3)
Maternal Age, n (%)				
19 or less	22 (6.6)	15 (6.8)	7 (6.1)	12038 (9.8)
20-24	73 (21.8)	54 (24.4)	19 (16.7)	28157 (22.9)
25-29	95 (28.4)	66 (29.9)	29 (25.4)	33096 (26.9)
30-34	88 (26.3)	51 (23.1)	37 (32.5)	29743 (24.1)
35 and older	57 (17.0)	35 (15.8)	22 (19.3)	20128 (16.3)
Missing				4
Census-based SES[a] index level, n (%)				
1 (lower SES)	89 (26.6)	59 (26.7)	30 (26.3)	32206 (26.2)
2	80 (23.9)	48 (21.7)	32 (28.1)	29472 (24.0)
3	72 (21.5)	54 (24.4)	18 (15.8)	24930 (20.3)
4	51 (15.2)	29 (13.1)	22 (19.3)	19625 (16.0)
5 (higher SES)	43 (12.8)	31 (14.0)	12 (10.5)	16788 (13.6)
Missing				145
Maternal Education, n (%)				
8 or less years	25 (7.7)	13 (6.0)	12 (11.1)	12849 (10.7)
9–11 years	65 (20.1)	39 (18.1)	26 (24.1)	22186 (18.5)
12 years	94 (29.0)	69 (31.9)	25 (23.1)	33176 (27.6)
13–15 years	72 (22.2)	54 (25.0)	18 (16.7)	25110 (20.9)
16 or more years	68 (21.0)	41 (19.0)	27 (25.0)	26854 (22.3)
Missing	11	5	6	2991
Maternal Smoking Status, n (%)[b]				
Yes	28 (12.2)	17 (14.4)	11 (9.8)	22 (5.9)
No	202 (87.8)	101 (85.6)	101 (90.2)	353 (94.1)

† Other races, in order of frequency among cases, included Filipino, Black, Vietnamese, Indian, Japanese, Cambodian, Chinese, Hmong, and American Indian.

[a] Neighborhood SES was measured with an index developed by Yost et al. (2001), which summarizes the following census-group-level socioeconomic indicators into one 5-level variable: education, median household income, percent living 200% below poverty, percent blue-collar workers, percent older than 16 years without employment, median rent, and median house value.

[b] Maternal smoking status was ascertained by cotinine presence in neonatal dried blood spots—this information was only available for a subset of 230 cases and 375 controls. The listed percentages for this variable use the type-specific totals within the subset as denominators.

associations in the multiple-pesticide model for the bilateral subtype. Collapsing the subtypes into an 'all retinoblastoma' category improved sample size but precluded analysis of potential exposure effect differences based on subtypes.

Many studies that previously linked pesticides to eye cancers have been underpowered or did not examine retinoblastoma, specifically (Rodvall et al., 2003; Fear et al., 1998; Kristensen et al., 1996). Moreover, exposure classification in these studies has been relatively crude, with pesticide exposure mostly being defined in terms of parental employment (Bunin et al., 1990; Abdolahi et al., 2013; Omidakhsh et al., 2021; Fear et al., 1998; Pearce et al., 2006). This study is the first, to our knowledge, to examine risk for childhood retinoblastoma with ambient exposure to specific pesticides during pregnancy.

For the pesticides found to be positively associated with retinoblastoma in our study, data on carcinogenicity is still limited. Pymetrozine and bromacil have both been found to cause skeletal malformations in rats and rabbits (EPA 1996, 2000). Early skeletal and ectodermal cells share genetic signaling networks, suggesting that skeletal malformation may be linked with abnormal neuroectodermal tissue (Richtsmeier and Flaherty, 2013). Retinoblastomas originate in the germinal neuroectodermal layer along with embryonal central nervous system (CNS) tumors and neuroblastomas (Gatta et al., 2012; Kohe et al., 2018; Ortega-García et al., 2011). This also suggests that retinoblastomas and CNS tumors may have overlapping etiologies. A growing number of studies have found associations between residential pesticide exposures and CNS tumors occurring in childhood (Van Maele-Fabry et al., 2017).

In a previous study by Pearson et al. both bromacil and kresoxim-methyl were associated with childhood CNS tumors (Pearson et al., 2016). Other studies found that kresoxim-methyl alters neural transcription, induces neuronal death, and facilitates oxidation in neuroblastoma cells (Regueiro et al., 2015; Flampouri et al., 2018; EPA, 2001).

Acephate, which showed a strong association with unilateral retinoblastoma, is an organophosphate (OP) insecticide that can cause brain cholinesterase inhibition in humans, overstimulating the nervous system (Maroni et al., 1990). OP metabolites have been found in cord blood and meconium samples from mothers and infants exposed during pregnancy, indicating vertical transmission of the exposure to the developing fetus (Ostrea et al., 2009). A study in mice also found that acephate induced chromosomal aberrations at sub-acute doses, with a dose-response effect; and the authors concluded that the pesticide may be considered a potential mutagen (Behera and Bhunya, 1989). Lastly, there is very little data available concerning the carcinogenicity of either oxythioquinox or captan to date; however, both can cause irreversible eye damage (EPA, 1999a, 1999b). Oxythioquinox was voluntarily removed from the market in 2001, prior to EPA completing human health risk assessment. Captan continues to be commonly used on edible crops and ornamental plants. At the present time, no safe level of exposure has been identified for these pesticides.

Our finding of a decreased OR estimate for hydramethylnon was unexpected. Due to the large number of pesticides being examined and the small number of exposed cases, it is possible that this finding is the result of chance. It is also possible that the decreased OR estimate is the result of pesticide correlation due to co-application with a reproductively highly toxic pesticide we did not investigate here that contributes to early loss of susceptible fetuses, as we pre-selected only for pesticides with potential carcinogenicity according to EPA.

Pesticide applications and exposures are highly correlated. For

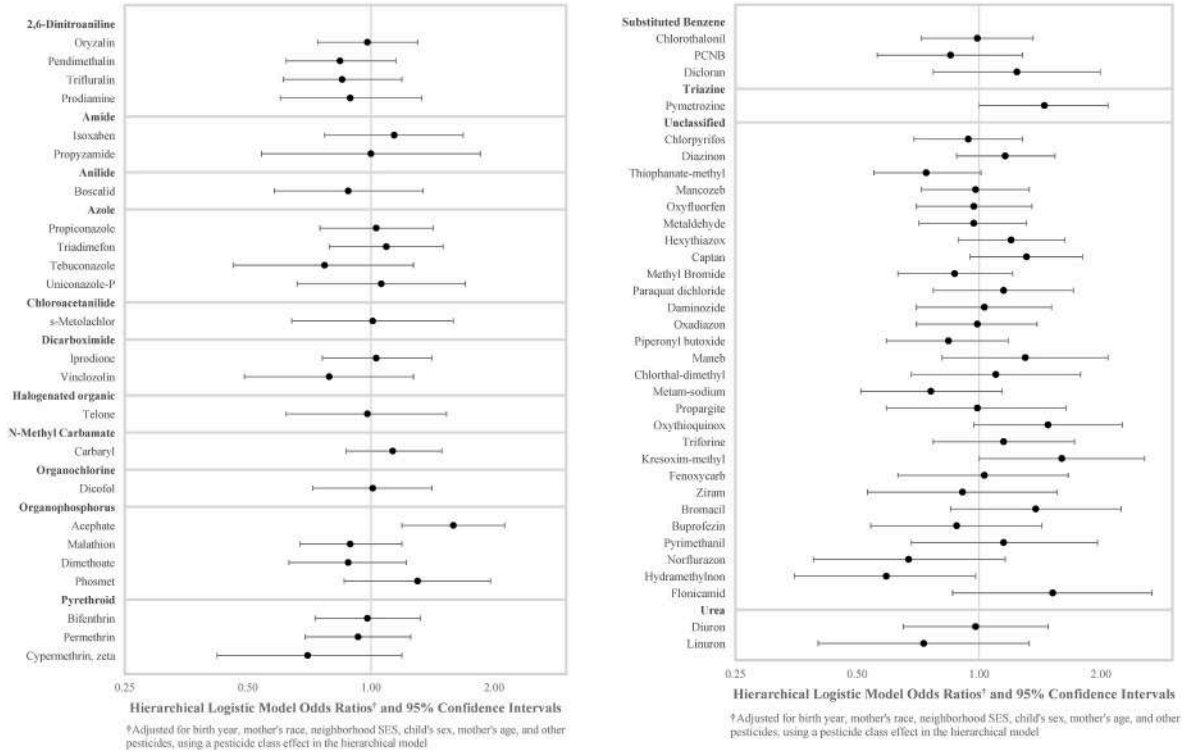

Fig. 1. Fig. 1. Estimated Odds Ratios and 95% Confidence Intervals from the Hierarchical Logistic Regression Model for all cases combined.

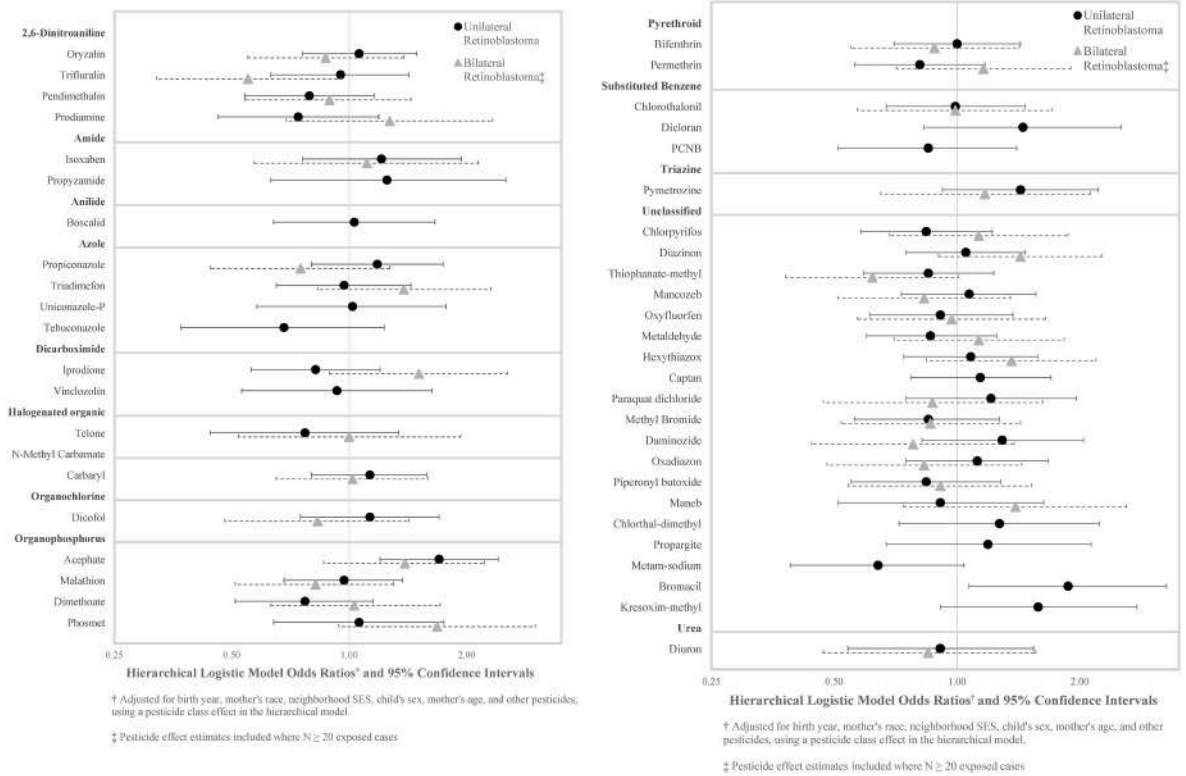

Fig. 2. Fig. 2. Estimated Odds Ratios and 95% Confidence Intervals from the Hierarchical Logistic Regression Model, stratified by laterality.

example, correlations in our data reflect commonly practiced co-applications, such as maneb with paraquat (Lombardi et al., 2021; Costello et al., 2009). We attempted to address these correlated exposure patterns by using a semi-Bayesian hierarchical logistic modelling approach.

The Agricultural Health Study observed an elevated point estimate with wide confidence intervals for retinoblastoma in children of the pesticide applicators enrolled in Iowa (SIR: 1.63, 95% CI: 0.41–6.53),

while another cohort study found observed a slightly decreased risk estimate with wide confidence intervals for eye cancers in such children (SIR: 0.76, 95% CI: 0.09–2.75) (Flower et al., 2004; Rodvall et al., 2003). Two other studies reported null effects for retinoblastoma (OR: 1.65, 95% CI: 0.51–5.33) and eye cancers (OR: 0.71, 95% CI: 0.15–2.08) with parental occupational pesticide exposure (Pearce et al., 2006; Fear et al., 1998). One of the few studies distinguishing between subtypes reported a null association between unilateral retinoblastoma and parental occupational exposure to agrochemicals (OR: 1.00, 95% CI: 0.51–1.96), while another reported a positive association between bilateral disease and paternal occupational pesticide exposure (OR: 2.12, 95% CI: 1.25–3.61) (MacCarthy et al., 2009; Abdolahi et al., 2013). Most of these studies assessed parental exposure based on occupation-industry group, which may misclassify pesticide exposures (Daniels et al., 2001). The only two previous retinoblastoma studies to report on residential pesticide use both suggested increased risks for unilateral disease with parental use of insecticides (OR: 2.80, 95% CI: 1.1–67); (OR: 2.70, 95% CI: 0.6–15.6) (Omidakhsh et al., 2017; Bunin et al., 1989). However, these studies also assessed exposures retrospectively from parental interviews, which may be biased due to differential exposure reporting between case parents and control parents (Rull et al., 2006).

While studies have linked residential proximity to agricultural pesticide applications with other childhood cancers, few have examined risks for retinoblastoma. A cohort study in Norway observed an increased risk estimate for eye cancers in children of farm residents (OR: 3.17, 95% CI: 0.93–10.9), but only two of the four exposed cases were retinoblastomas (Kristensen et al., 1996). A case-control study in Texas found no association between retinoblastoma and proximity to agricultural land use (OR: 0.90, 95% CI: 0.5–1.5) (Carozza et al., 2009).

Unilateral and bilateral retinoblastoma are both induced by inactivation of the *RB1* gene; however, the two subtypes are initiated by slightly different molecular genetic processes. Children with bilateral retinoblastoma have inherited a defective *RB1* allele from one parent, and the second *RB1* allele is inactivated during DNA replication of progenitor cells in the fetal retina. Unilateral retinoblastoma occurs when both *RB1* alleles are inactivated in the same retinal progenitor cell (Dyer, 2004). Since only a 'second hit' is required for disease to occur in children born with a defective *RB1* gene, approximately 90% of those who inherit the gene mutation develop retinoblastoma (Draper et al., 1992). The likelihood of both alleles being inactivated post-conception is much lower. This etiology is consistent with expecting bilateral cases to be affected by or susceptible to pesticide exposures differently from unilateral cases. Thus, it is not entirely surprising that pesticides associated with unilateral retinoblastoma may not also be associated with bilateral retinoblastoma as the underlying pathophysiological mechanisms differ; specifically, the elevated risks for bilateral retinoblastoma with prenatal exposure to pesticides correspond to a 'second hit' model (Knudson, 1971); while for unilateral retinoblastoma both hits occur post-conception.

Though the germline *RB1* mutation inherited by children who develop bilateral retinoblastoma may arise de novo in either parent preconceptionally, it most commonly arises in the father (Kato et al., 1994). Since we assigned exposure based on maternal address listed on the birth certificate, a potential limitation of this study is that we were not able to address preconception residential pesticide exposure affecting the parental germline, and we had no information on whether the parents cohabitated prior to or during the pregnancy. Also, birth certificate addresses may not be the same as prenatal residential addresses if mothers moved during pregnancy. These instances would likely result in exposure misclassification, with true exposures via the new residence being unaccounted for and pesticide applications near the previous residence being falsely classified as exposures. However, a previous sensitivity analysis by our group showed that pesticide exposure estimates did not vary substantially when full residential histories were available. When comparing use of birth certificate address to use of residential addresses from a public-record database for assignment of early life pesticide exposures, there was moderate to strong correlation between exposure assignments (Spearman correlation = 0.76–0.83) (Ling et al., 2019).

Exposure misclassification may also be introduced by geocoding inaccuracy. Our sensitivity analysis by geocoding accuracy suggests that the inclusion of data with lower resolution geocodes resulted in small biases towards the null. This result is to be expected, since exposures determined by lower resolution geocoding types, such as zip area centroid, may be less accurately classified than exposures determined with finer geocode accuracy. However, it would be inappropriate to exclude the observations with lower resolution geocodes from our analysis, as suggested by a study by Thompson et al. (2021).

Pregnant mothers may have also been exposed to pesticides through their occupation, which has been assessed in most of the cited previous literature on retinoblastoma and pesticide exposure. Unfortunately, parental occupation information was not available on California birth certificates. At the population level, between 6% and 10% of rural California residents are employed in 'agriculture, forestry, fishing, hunting, mining industries' (USDA Economic Research Service, 2007). Information regarding the proportion of this population that is employed in agriculture, specifically, is sparse. Nonetheless, the unavailability of occupational information on birth certificates precluded adjustment for potential occupational pesticide exposures in our analysis.

Despite these limitations, this study also has several strengths. Among the studies that have investigated retinoblastoma and pesticide exposure thus far, this population-based study has the largest number of cases yet. This allowed us to stratify by laterality in order to separately investigate risk factors for unilateral and bilateral retinoblastoma and resulted in fairly stable estimates, at least for the unilateral cases. Furthermore, our objective and spatially refined assessment of exposure using statewide reports allowed us to access information on individual pesticides that was not subject to recall bias. In addition to these strengths, our exposure assessment is validated by previous studies that have demonstrated pesticide volatilization as a potential mechanism of exposure. A study in Northern California found strong associations between regional agricultural pesticide application and outdoor air concentrations up to 5000 m away, even days after the original application (Harnly et al., 2005). Volatilized pesticides may also drift into homes and persist in dust, as suggested by associations observed between household pesticide dust concentrations and nearby agricultural application (Harnly et al., 2009).

5. Conclusions

In conclusion, this case-control study is among the first to focus on specific pesticide exposures as they individually relate to unilateral and bilateral retinoblastoma. Future studies are needed to also assess agriculturally common pesticide mixtures and mixture effects. The associations we observed between retinoblastoma and residential proximity to applications for specific pesticides that have previously raised concern as to their carcinogenic potential contribute to the growing body of knowledge concerning prenatal pesticide exposure and rare childhood cancers of the nervous system. Strategies for reducing exposure in those living near agricultural fields should be considered as a protective health measure.

Consent for publication

Not applicable.

Availability of data and materials

The datasets in the current study are used under the approval of the California Committee for the Protection of Human Subjects. Approvals would be necessary to share data.

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Authors' contributions

JEH and BR supervised and substantially conducted the conception, design, and interpretation of data, in addition to reviewing and editing the manuscript for the current study. ST performed the formal analyses, produced visualizations, and wrote the manuscript. MC oversaw geocoding methodology in addition to reviewing and editing the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have 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.2022.114025.

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Prenatal pyrethroid exposure and lung function among school-aged children

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ABSTRACT

Background: Previous epidemiological evidence mainly focused on the adverse effects of prenatal exposure to pyrethroid insecticides (PYRs) on respiratory health during childhood. It remains unclear whether the PYR exposures can also impact on children's lung function.
Objectives: To explore the potential effects of prenatal PYR exposures on lung function in a population of Chinese children.
Methods: This study included 233 mother-child dyads from the Laizhou Wan Birth Cohort (LWBC), Shandong province, northern China, between September 2010 and December 2013. Three metabolites of PYRs [3-phenoxybenzoic acid (3-PBA), and cis- and trans-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis-DCCA and trans-DCCA)] were measured using gas chromatography-mass spectrometry (GC-MS) in maternal urine samples collected at recruitment. Lung function was assessed with spirometry in children aged 6–8 years. Multivariable linear regression and generalized linear models (GLMs) assessed the associations of prenatal PYR exposures with lung function in children.
Results: Among the PYR metabolites, 3-PBA (81.5%) were most frequently detected, followed by trans-DCCA (55.4%) and cis-DCCA (21.9%). The 3-PBA concentration was associated with a 1% decrease in FEV_1/FVC in the highest quartiles of exposure compared to the lowest quartile, with a potential dose response association (p-trend = 0.085). Our findings provide a suggestive effect modification by sex, with girls being more susceptible than the boys (p-trend = 0.011). However, there were no associations between the trans-DCCA concentration and lung function parameters.
Conclusion: Prenatal 3-PBA concentrations were associated with a modest decrease in FEV_1/FVC among school-aged children, and the association was slightly more pronounced for the girls than for the boys.

1. Introduction

Pyrethroids are synthetic insecticides derived from the natural pyrethrins, universally used for controlling insect pests in agriculture and households (Burns and Pastoor. 2018; Saillenfait et al., 2015). Currently, PYRs account for approximately 30% of the worldwide insecticide market with increasing usage since restrictions have been placed on many of the organophosphate insecticides (U.S. EPA. 2017; Hossain et al., 2013). For general population, ingestion of contaminated food,

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Abbreviations

PYRs	Pyrethroid insecticides
3-PBA	3-phenoxybenzoic acid
cis-DCCA	cis-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid
trans-DCCA	trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid
FVC	Forced vital capacity
FEV_1	Forced expiratory volume in 1 s
$FEF_{25–75\%}$	Forced expiratory flow between 25% and 75% of FVC
PEF	Peak expiratory flow
LWBC	Laizhou Wan Birth Cohort
GC-MS	Gas chromatography-mass spectrometry
GLMs	Generalized linear models
GAMM	Generalized additive mixed model
ΣPYRs	the total PYRs
DAG	Directed Acyclic Graph
COPD	Chronic obstructive pulmonary disease
ISAAC	International Study of Asthma and Allergies in Childhood (ISAAC)
CHMS	Canadian Health Measures Survey
NHANES	National Health and Nutrition Examination Survey
95% CI	95% Confidence interval
Cr	Creatinine;
LOD	Limit of detection
BMI	Body mass index

dust, and water are the common routes, followed by skin absorption, air ingestion, and unintentional ingestion (Chrustek et al., 2018). Human exposure to PYRs was primarily assessed by measures of nonspecific urinary metabolites such as 3-phenoxybenzoic acid (3-PBA), and cis- and trans-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis-DCCA and trans-DCCA) (Starr et al., 2008; Kim et al., 2021). The accumulation and widespread application lead to environmental contamination and increased human exposure, raising concerns about their potential adverse effects on human health, in particular susceptible pregnant women and children (Ye et al., 2017; Saillenfait et al., 2015; Ding et al., 2015; Viel et al., 2015; Mamane et al., 2015; Buralli et al., 2020; Vrijheid et al., 2016).

In early stages of life, especially during the intrauterine development, immature lungs may be highly vulnerable to harmful effects of environmental factors including PYRs (Miller and Marty, 2010; Raanan et al., 2016; Abellan et al., 2019; Balte et al., 2017). Experimental evidence suggested that insecticide exposures could release proinflammatory mediators in lung cells, enhancing the airway hyper-responsiveness, and leading to abnormal alveolar maturation and reduced surfactant production (Diel et al., 2003; Fryer et al., 2004; Nishino et al., 2013; Pandher et al., 2021; Shaikh and Sethi, 2021). To date, only two epidemiologic studies have assessed the effects of PYRs and lung function in children (Ye et al., 2016a; Hu et al., 2021). Using the Canadian Health Measures Survey (CHMS) from 2007 to 2009, a cross-sectional study of 1997 subjects reported that higher the total PYRs (ΣPYRs) concentrations were associated with reduced forced expiratory volume in 1 s (FEV_1) and lower forced vital capacity (FVC) in children aged 6–19 years (Ye et al., 2016a). Another study using data from the US National Health and Nutrition Examination Survey (NHANES) 2007–2012 has provided evidence of a negative association between environmental 3-PBA concentration and lung function among children (Hu et al., 2021). However, these studies were limited by the cross-sectional design, and the indicative causal direction of the association could not be determined. As far as we are aware, no prospective, well-designed study focused on the prenatal exposure window to evaluate the impact of PYRs exposure on lung function.

Investigating the effects of prenatal exposure to PYRs on lung function is particularly important, because the impaired lung function during childhood not only poses a burden of adulthood morbidity but is also a risk factor for the development of chronic lung diseases (Fuchs et al., 2017; Løkke et al., 2006; Grant et al., 2020). Therefore, we explored the associations of prenatal PYRs exposure with lung function among school-aged children from a prospective cohort study conducted in Shandong, China. Because previous studies have found sex-specific effects of PYRs on health outcomes, we interrogated the interaction terms and stratified the analysis to assess whether sex modifies the associations between PYRs and lung function in children (Hwang et al., 2019; Andersen et al., 2021).

2. Methods

2.1. Study population

We used data from the Laizhou Wan Birth Cohort (LWBC), an ongoing prospective birth cohort conducted in Laizhou Wan (Bay) of Bohai Sea in Shandong province, northern China. Participant eligibility, recruitment, and follow-up have been described in detail elsewhere (Ding et al., 2017). Briefly, we recruited pregnant women from the Binhai Hospital, a local obstetric hospital, from September 2010 to December 2013. Eligible women were 18 years or older, were residing in the study area for the previous 3 consecutive years, were planning delivery in the collaborating hospital. Women were excluded from the study if they had assisted reproduction, multiple pregnancies, a previous diagnosis of diabetes, hypertension, HIV infection, or used illicit drugs.

A total of 773 mother-child dyads were recruited in the study, none of the women reported occupational exposure to pesticides during pregnancy. For the present study, the population was restricted to women who had the prenatal PYR concentrations measured, and their children had lung function data available at the age of 6–8 years old (n = 238). Among these, 5 women had no urinary creatinine levels available, and they were further excluded from the study. Finally, 233 mother-child dyads with complete data were included in the analyses.

The present study protocol was approved by the Medical Ethics Committee of Shanghai Children's Hospital, Shanghai Jiao Tong University School of Medicine (approval ID, 2019RY014).

2.2. Urinary PYR metabolites measurement

Spot urine specimens were provided by pregnant women during the admission for delivery, and then aliquoted and stored at −80 °C until PYR measurements. The concentrations of three urinary metabolites of PYRs, including 3PBA, cis-DCCA and trans-DCCA, were measured using gas chromatography-mass spectrometry (GC-MS) based on the slightly modified method of Kühn et al., (1996). The details of the procedure and quality control process have already been described in our previous paper (Ding et al., 2015; Hu et al. 2019, 2020).

The limit of detection (LOD) was 0.05 μg/L for 3-PBA, 0.23 μg/L for trans-DCCA, and 0.38 μg/L for cis-DCCA, respectively. The concentrations of PYR metabolites < LOD were imputed as LOD/ $\sqrt{2}$ in the analyses (Hornung and Reed 1990). The cis-DCCA was excluded from the analyses due to low detection frequency (21.9%). Urinary creatinine levels were measured using automatic chemical analyzer (7100 Automatic Analyzer, Hitachi, Japan) and was used to normalize PYR metabolite concentrations for urine dilution.

2.3. Lung function assessment

Lung function tests were conducted by specially trained medical staff using an electronic spirometer (Breath Home-A1, Hongxiang Co. Ltd, Guangzhou, China) according to the recommendations of the American Thoracic Society/European Respiratory Society guidelines (Graham et al., 2019; Ma et al., 2022; Han et al., 2018). Children with recent surgery of the chest or abdomen, having acute and/or chronic respiratory conditions, persistent cough, or who were taking medication for tuberculosis were excluded from the lung function testing. During the lung function testing procedure, each participant was instructed to maintain sitting position, wear a nose clip, and then breathe through the mouthpiece after at least 5 min of normal breathing in the room air. Three acceptable curves were performed by each participant and the best one was chosen. All testing procedures were reviewed and verified by two physicians specializing in pediatric spirometry. In the present study, we assessed the following five lung function parameters: forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), FEV_1: FVC ratio, forced expiratory flow between 25% and 75% of FVC ($FEF_{25–75\%}$), and peak expiratory flow rate (PEF).

2.4. Asthma and allergies questionnaires

Mothers also answered a detailed questionnaire about their child's health and respiratory symptoms on the same day the children performed the lung function tests. The definition of asthma and allergic diseases were based on the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire (Ellwood et al., 2005). In our study, the Chinese version of the ISAAC questionnaire was used, which has previously been validated (Chan et al., 2001). The diagnosis of asthma and allergic diseases were obtained from the questionnaire through the following questions: "Has your child ever been diagnosed with asthma by a doctor?", "Has your child ever been diagnosed with allergic rhinitis by a doctor?", and "Has your child ever been diagnosed with atopic dermatitis by a doctor?" If the answer of any of these questions was "yes", the child was regarded as having asthma and/or allergic disease.

2.5. Demographic covariates

At study entry, maternal age, education, smoking during pregnancy, and household income were collected from self-administered questionnaires. Maternal body mass index (BMI, kg/m^2) was calculated from self-reported height and pre-pregnancy weight.

Children's age was recorded at the time of the spirometry test. At the same time, weight and height were measured by the trained study staff.

2.6. Statistical analysis

Characteristics of the women and their children were summarized using mean ± standard deviation (SD), and percentage (%), as appropriate. Student's t-test, Mann-Whitney *U* test, and chi-square test were applied to compare the differences between the baseline population (n = 773) and the present study population (n = 233). The distributions of urinary PYR concentrations were examined using detection rates and selected percentiles. Because the values of the lung function had skewed distributions, all analyses were performed on log_{10}-transformed values of continuous variables.

Multivariable linear regression and generalized linear models (GLMs) were performed to estimate the associations of prenatal PYR concentrations with lung function in children, respectively. The 3-PBA concentration was first modeled as a continuous variable with log_{10}-transformation in multivariable linear regression models. Also, 3-PBA concentrations were also set as categorical variables and analyzed by GLMs using the low-exposure group as the reference group. Because 44% of trans-DCCA concentrations were < LOD, a three-level ordinal variable was defined: all samples with no detectable levels (<LOD) were assigned to the low-exposure group (reference group), and two equally sized groups were formed among the samples with detectable levels to form the median- and high-exposure groups. Tests for trend (*p*-trend) were performed using the categories representing the median concentrations of each corresponding quartile.

Stratified analyses were conducted to assess the potential effect modification by children's sex and tested for interaction by including a cross-product term (log_{10}-transformed exposure*sex). Interaction terms were considered suggestive of an interaction if the *p*-value < 0.10.

To confirm the robustness of associations in our results, we further conducted several sensitivity analyses. First, multiple imputations were performed to impute the values of PYR metabolites < LOD to avoid the potential bias caused by the replacement of the undetected measurements with a constant value of LOD/ $\sqrt{2}$ (Lubin et al., 2004). Second, we re-ran all models for the lung function measures by using z-scores and predicted values, according to the Global Lung Function Initiative (GLI) equations taking age, sex, and height into considerations (Chinn and Rona, 1992). Third, to exclude the possible effects of asthma and allergic diseases on the results, we also evaluated the relationships between 3-PBA concentrations and lung function in children without aforementioned diseases, and after adding asthma and allergic diseases as covariates in the linear model. Finally, since in many cases non-linear relationship was found between environmental exposures and health outcomes (Liang et al., 2020; Jiang et al., 2020), we chose a generalized additive mixed model (GAMM) to estimate smooth functional relationships between theses variables.

Potential confounders were identified for inclusion according to a priori knowledge (Ye et al., 2016a, 2016b; Hu et al., 2021; Raanan et al., 2016; Abellan et al., 2019) and using a Directed Acyclic Graph (DAG) that allowed to find the minimally sufficient adjustment set. The confounders included maternal age, pre-pregnancy BMI, education, monthly household income, and children's sex, age, BMI, height and passive smoking (Fig. S1).

All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL) and R studio (R Version 4.0.3, Package "mgcv"); $p < 0.05$ (two-tailed) was considered statistically significant.

3. Results

Women in the present analysis were on average 28 years old, with a mean pre-pregnancy BMI of 21.72 kg/m^2. Only one-fourth of women (25.8%) had an education attainment of bachelor's degree, and nearly one-third reported a monthly income ≥3000 CNY (31.8%). Almost two thirds of women (71.2%) never smoked, and 28.8% reported active or passive smoking during pregnancy. In comparison to the baseline population, the women in this study tended to have lower household monthly income and to be non-smokers. Regarding children's characteristics at birth, 52.4% were males and mean gestational age was 39.47 weeks (SD = 1.31). The mean birth weight, birth length, and head circumference were 3402.48 ± 48 g, 51.05 ± 2.58 cm, 33.42 ± 1.22 cm, respectively (Table 1).

Clinical and demographic characteristics of the 6–8 years old children were presented in Table 2. The mean (±SD) age, height and BMI at spirometry were 7.36 ± 0.58 years, 1.30 ± 0.07 m and 16.50 ± 2.76 kg/m^2, respectively. There was an almost equal proportion of boys (n = 122, 52.4%) and girls (n = 111, 47.6%). Compared to the boys, the girls exhibited a shorter height and lower BMI. One percent (n = 3) of children had a history of asthma. Approximately seven percent of children suffered from an allergic disease, with 4% (n = 10) having atopic dermatitis and 3% (n = 7) having allergic rhinitis. The mean (±SD) for FEV_1, FVC, FEV_1: FVC, $FEF_{25–75\%}$, and PEF were 1.62 ± 0.35 L, 1.75 ± 0.43 L, 93.33 ± 5.04%, 2.30 ± 0.49 L/s, 3.51 ± 0.89 L/s, respectively. Moreover, FVC, FEV_1 and PEF values were higher in boys than in girls.

Table 3 summarized the detection rates and distributions of metabolite concentrations of PYR in urine. For PYR metabolites, 3-PBA was the

Table 1
Comparison of basic demographics between the baseline and study population in Shandong, China.

Characteristics	Study population (n = 233)	Baseline population (n = 773)	*P*-value
	Mean ± SD or N (%)		
Maternal characteristic			
Maternal age (years)	28.41 ± 4.61	28.34 ± 4.50	0.852[a]
Pre-pregnancy BMI (kg/m^2)	21.72 ± 2.75	21.93 ± 3.25	0.764[a]
Monthly household income (CNY)			
≤3000 ($ 472.50)	159 (68.2)	461 (54.3)	0.025[b]
3000–5000 ($ 472.50–787.50)	55 (23.6)	239 (30.9)	
>5000 ($ 787.50)	19 (8.2)	73 (9.4)	
Maternal Education			
< Bachelor Degree	173 (74.2)	576 (74.5)	0.965[b]
Bachelor Degree	59 (25.3)	190 (24.6)	
> Bachelor Degree	1 (0.5)	7 (0.9)	
Smoking during pregnancy			
No	166 (71.2)	485 (62.7)	0.017[c]
Yes or live with	67 (28.8)	288 (37.3)	
Parity			
Primipara	162 (69.5)	519 (67.1)	0.526[c]
Multipara	71 (30.5)	252 (32.9)	
Children's characteristic			
Gestational age (weeks)	39.47 ± 1.31	39.47 ± 1.38	0.807[a]
Birth weight (g)	3402.48 ± 48	3419.47 ± 497.39	0.644[a]
Birth length (cm)	51.05 ± 2.58	50.85 ± 2.54	0.404[a]
Head circumference (cm)	33.42 ± 1.22	33.23 ± 1.42	0.063[a]
Sex			
Male	122 (52.4)	397 (51.4)	0.77[c]
Female	111 (47.6)	376 (48.6)	

[a] Means were compared by T-test.
[b] Mean rank was compared by Mann-Whitney *U* test.
[c] Proportions were compared by Pearson chi-square test.

Table 2
Demographic characteristics of children in the present study (n = 233).

	Total (n = 233)	Boys (n = 122)	Girls (n = 111)	*P*-value
Children characteristic	Mean ± SD			
Age at spirometry (years)	7.36 ± 0.58	7.42 ± 0.57	7.29 ± 0.56	0.083[a]
Height at spirometry (m)	1.30 ± 0.07	1.31 ± 0.07	1.28 ± 0.07	0.001[a]
BMI at spirometry (kg/m^2)	16.50 ± 2.76	16.85 ± 2.86	16.12 ± 2.60	0.042[a]
Atopic dermatitis				
Yes	10 (4.30)	4 (3.30)	6 (5.40)	0.424[c]
No	223 (95.70)	118 (96.70)	105 (94.60)	
Allergic rhinitis				
Yes	7 (3.00)	6 (4.90)	1 (0.90)	0.073[c]
No	226 (97.00)	116 (95.10)	110 (99.1)	
Asthma				
Yes	3 (1.30)	2 (1.60)	1 (0.90)	0.618[c]
No	230 (98.70)	120 (98.40)	110 (99.10)	
Lung Function				
FEV_1 (L)	1.62 ± 0.35	1.68 ± 0.33	1.56 ± 0.36	0.005[a]
FVC (L)	1.75 ± 0.43	1.82 ± 0.40	1.67 ± 0.46	0.010[a]
FEV_1/FVC (%)	93.33 ± 5.04	93.07 ± 5.52	93.61 ± 4.45	0.404[a]
$FEF_{25–75\%}$ (L/s)	2.30 ± 0.49	2.36 ± 0.50	2.23 ± 0.48	0.056[a]
PEF (L/s)	3.51 ± 0.89	3.64 ± 0.90	3.36 ± 0.87	0.015[a]

most frequently detected (81.5%), followed by lower detection rates for trans-DCCA (55.4%), and cis-DCCA (21.9%). The creatinine-adjusted median concentrations for 3-PBA and trans-DCCA were 0.94 and 0.72 μg/g, respectively.

Linear regression model assessment of the associations between urinary PYR metabolites and lung function were presented in Table 4. The 3-PBA concentration was associated with a 1% decrease in FEV_1/FVC in the highest quartiles of exposure compared to the lowest quartile, with a suggestive downward trend from the Q1 to Q4 (p-trend = 0.085). No associations were observed between other lung function parameters and urinary 3-PBA concentration. Furthermore, there were no associations between the trans-DCCA metabolites and lung function. To better visualize the analytical trends in the data, those results were also presented in Fig. S2.

The sensitivity analyses were conducted with lung function measurements using z-scores (Table S1) and the percent predicted values (Table S2). Under these analyses, the results remained robust. The associations between urinary PYR metabolites and lung function in children after the multiple imputation for undetected levels of PYR metabolites were almost identical to the main results (Table S3). After adding asthma and allergic diseases as covariates, similar inverse associations between 3-PBA concentrations and lung function were observed (Table S4). Results for the lung function did not change when restricted to children without asthma and allergic diseases (Table S5). In GAMM analyses (Fig. S3), we found no evidence for nonlinear relationship between prenatal 3-PBA exposures and lung function.

Effect modification by sex was examined by interaction terms and stratified analyses (Table 5 and Fig. 1). The inverse associations between 3-PBA concentrations and FEV_1/FVC among girls (β = -0.01; 95% CIs = −0.02, −0.002) were apparent and had a more consistently monotonic trend than corresponding estimates for boys (p-trend = 0.011), although the p-interaction was not statistically significant (p-interaction value = 0.181).

4. Discussion

In this prospective birth cohort study, multiple lung function parameters were measured to assess the health impact of prenatal PYR exposures. We found that prenatal 3-PBA concentration was associated with a modest decrease in FEV_1/FVC among children at 6–8 years old. There is slight effect modification by sex, with girls showing larger decreases in lung function than the boys of same age.

Our results are in agreement with the findings from the CHMS in Canada and from the NHANES, in US, indicating that exposure to PYRs has adverse effects on children's lung function development (Ye et al., 2016a; Hu et al., 2021). Specifically, a nationally representative cross-sectional study in Canada, of children and adolescents aged 6–19 years old reported that each 10-fold increase in total PYR metabolites (ΣPYR) concentration was associated with a statistically significant 17.4 mL reduction in FEV_1 in children aged 6–11 years (n = 1023), and a 37.1 mL reduction in FVC in adolescents aged 12–19 years (n = 974) (Ye et al., 2016a). Similarly, a recent cross-sectional NHANES study conducted among pediatric population analyzed 1174 children aged 6–17 years and reported that 3-PBA concentration was inversely associated with FEV_1, FVC, and PEF, with the strongest associations among 11–17 years old boys (Hu et al., 2021). Our prospective study supports and extends the results of previous epidemiological studies of PYRs exposure and lung function. Taken collectively, differences between the above-mentioned studies and our study may be related to variation in study design, confounder control, age of the participants, levels of exposure, and the use of different measures of pulmonary function.

Another specific feature of our study was the high concentration of 3-PBA, a general metabolite of PYRs (Ahn et al., 2011). This finding is consistent with data from the NHANES showing 3-PBA to be the most frequently detected metabolite, while either trans-DCCA or cis-DCCA had low frequencies of detection in children (Hu et al., 2021). In general, the median creatinine-adjusted 3-PBA concentration in our study (median 0.94 μg/g) was approximately 2 times of that in the NHANES study (median 0.46 μg/g). On the other hand, due to the differences in

Table 3
Urinary concentrations of pyrethroid (PYR) metabolites (n = 233).

	Detection rate	Not adjusted for creatinine (μg/L)				Creatinine adjusted (μg/g)			
Metabolites	N (%)	25th	50th	75th	95th	25th	50th	75th	95th
3-PBA	190 (81.5)	0.17	0.44	1.00	2.94	0.49	0.94	1.96	5.64
trans-DCCA	129 (55.4)	< LOD	0.17	0.72	2.90	< LOD	0.72	1.52	7.06
cis-DCCA	51 (21.9)	< LOD	< LOD	< LOD	1.45	< LOD	< LOD	< LOD	3.43

< LOD indicated less than the limit of detection; the LOD of 3-PBA, trans-DCCA and cis-DCCA was 0.05 μg/L, 0.23 μg/L, 0.38 μg/L respectively.

Table 4
Regression Coefficients (95% CI) for lung function by PYR concentrations in children aged 6–8 years in Shandong, China (n = 233).

	N	FEV_1 (L)	FVC (L)	FEV_1/FVC	$FEF_{25–75\%}$ (L/s)	PEF (L/s)
Exposure (μg/g)		Adjusted β (95% CI)[d]				
3-PBA						
Continuous[e]	233	0.01 (−0.01, 0.02)	0.01 (−0.01, 0.03)	−0.006 (−0.01, 0.01)	−0.02 (−0.04, 0.01)	−0.01 (−0.03, 0.02)
Q1 (≤0.49)	59	Reference	Reference	Reference	Reference	Reference
Q2 (0.50–0.94)	58	−0.003 (−0.03, 0.02)	0.002 (−0.02, 0.03)	−0.006 (-0.01, 0.003)	−0.03 (−0.06, 0.01)	−0.01 (−0.04, 0.02)
Q3 (0.95–1.96)	58	−0.01 (−0.03, 0.03)	−0.01 (−0.03, 0.02)	−0.002 (-0.01, 0.007)	−0.02 (−0.05, 0.01)	−0.02 (−0.04, 0.02)
Q4 (≥1.97)	58	0.01 (−0.01, 0.03)	0.02 (−0.01, 0.05)	**−0.01 (-0.02, -0.001)***	−0.03 (−0.06, 0.01)	−0.01 (−0.04, 0.02)
p for trend		0.570	0.259	0.085	0.210	0.406
trans-DCCA						
Continuous[e]	233	0.02 (−0.01, 0.03)	0.02 (−0.01, 0.04)	−0.002 (−0.01, 0.01)	0.01 (−0.02, 0.03)	0.01 (−0.01, 0.03)
Low (≤0.10)	104	Reference	Reference	Reference	Reference	Reference
Mid (0.23–0.64)	64	−0.001 (−0.02, 0.02)	−0.01 (−0.03, 0.02)	0.004 (−0.003, 0.01)	0.003 (−0.03,0.03)	−0.002 (−0.03, 0.02)
Hig (≥0.65)	65	0.02 (−0.003, 0.03)	0.01 (−0.01, 0.04)	0.001 (−0.01, 0.01)	0.02 (−0.01, 0.04)	0.01 (−0.01. 0.04)
p for trend		0.124	0.230	0.661	0.265	0.416

[d] Adjusted for maternal age, pre-pregnancy BMI, education, monthly household income, and children's age, sex, BMI, height, and passive smoking.
[e] Urine concentrations of PYR metabolites were adjusted for creatinine then log_{10}-transformed.

Table 5
95% CI for 3-PBA concentrations and lung function stratified by sex among children aged 6–8 years in Shandong, China (n = 233).

	N	FEV_1 (L)	FVC (L)	FEV_1/FVC	$FEF_{25–75\%}$ (L/s)	PEF (L/s)
3-PBA (μg/g)		Adjusted β (95% CI)[f]				
Boys						
Continuous[e]	122	−0.003 (−0.02, 0.02)	−0.007 (−0.02, 0.02)	−0.003 (−0.01, 0.01)	−0.01 (−0.04, 0.02)	0.002 (−0.02, 0.03)
Q1 (≤0.37)	31	Reference	Reference	Reference	Reference	Reference
Q2 (0.38–0.92)	30	0.002 (−0.02, 0.03)	0.01 (−0.02, 0.04)	−0.01 (−0.03, 0.002)	−0.05 (−0.09, −0.002)	−0.008 (−0.04, 0.03)
Q3 (0.93–2.05)	31	−0.01 (−0.04, 0.01)	−0.01 (−0.04, 0.02)	0.002 (−0.01, 0.02)	−0.002 (−0.05, 0.04)	0.01 (−0.03, 0.05)
Q4 (≥2.06)	30	−0.001 (−0.03, 0.03)	0.01 (−0.03, 0.04)	−0.007 (−0.02, 0.01)	−0.02 (−0.07, 0.03)	−0.006 (−0.04, 0.03)
p for trend		0.682	0.837	0.738	0.814	0.953
Girls						
Continuous[e]	111	0.01 (−0.01, 0.04)	0.02 (−0.004, 0.05)	**−0.01(-0.02, -0.003)***	−0.03 (−0.06, 0.004)	−0.02 (−0.05, 0.02)
Q1 (≤0.51)	30	Reference	Reference	Reference	Reference	Reference
Q2 (0.52–0.98)	26	−0.01 (−0.05, 0.02)	−0.01 (−0.05, 0.03)	−0.004 (−0.02, 0.01)	−0.03 (−0.08, 0.02)	−0.02 (−0.07, 0.02)
Q3 (0.99–1.94)	28	−0.004 (−0.04, 0.03)	0.004 (−0.03, 0.06)	−0.008 (−0.02, 0.002)	−0.04 (−0.09, 0.01)	−0.02 (−0.07, 0.03)
Q4 (≥1.95)	27	0.01 (−0.03, 0.05)	0.02 (−0.02, 0.06)	**−0.01 (-0.02, -0.002)***	−0.04 (−0.09, 0.01)	−0.02 (−0.07, 0.03)
p for trend		0.439	0.162	**0.011**	0.111	0.421
p-interaction value		0.531	0.297	0.181	0.143	0.712

[f] Adjusted for maternal age, pre-pregnancy BMI, education, monthly household income, and children's age, BMI, height, and passive smoking.
[e] Urine concentrations of PYR metabolites were adjusted for creatinine then log_{10}-transformed.

the assessment of the exposure (ΣPYR compared to one metabolite in our study), the findings from the CHMS study cannot be directly compared with our results (Ye et al., 2016a).

Spirometry is a frequently used tool for diagnosing and monitoring lung disease in children. Most guidelines suggest FEV_1/FVC (an airway obstruction measure) or FEF_{25-75} (a measure of small airway function) are more appropriate to assess the severity of lung disease in children (Bacharier et al., 2004; Spanier et al., 2014). In the present study, FEV_1/FVC, a measure of airway obstruction (Mori et al., 2011), was negatively associated with prenatal PYRs exposure among school-aged children. This is a particularly important finding given the increasing understanding about the importance of pulmonary decrements in causing long-term lung function decline and poor respiratory health (Bacharier et al., 2004; Paull et al. 2005). Several investigators have suggested that FEV_1/FVC may be more sensitive to identify individuals at risk of developing a progressive decline in lung function (Bui et al., 2017; Huang et al., 2019; Mathur, 2010). Thus, the FEV_1/FVC ratio may be a predictor of future risk to the children, as many studies have indicated that persistently reduced childhood lung function increases future risk for persistent asthma and possibly chronic obstructive pulmonary disease (COPD) (Sears et al., 2003; Qin et al., 2017). However, there is no clear evidence that the prevention, identification, and treatment of airway dysfunction in an individual is valuable in preventing lung damage. Further research will be needed to confirm this association between airway dysfunction and chronic lung diseases such as COPD and asthma.

The mechanisms underlying the associations of 3-PBA in pregnancy with reduced lung function in offspring are unknown. Interestingly, the major milestone of respiratory system development occurs during the second and third trimesters of pregnancy and continues until around 3

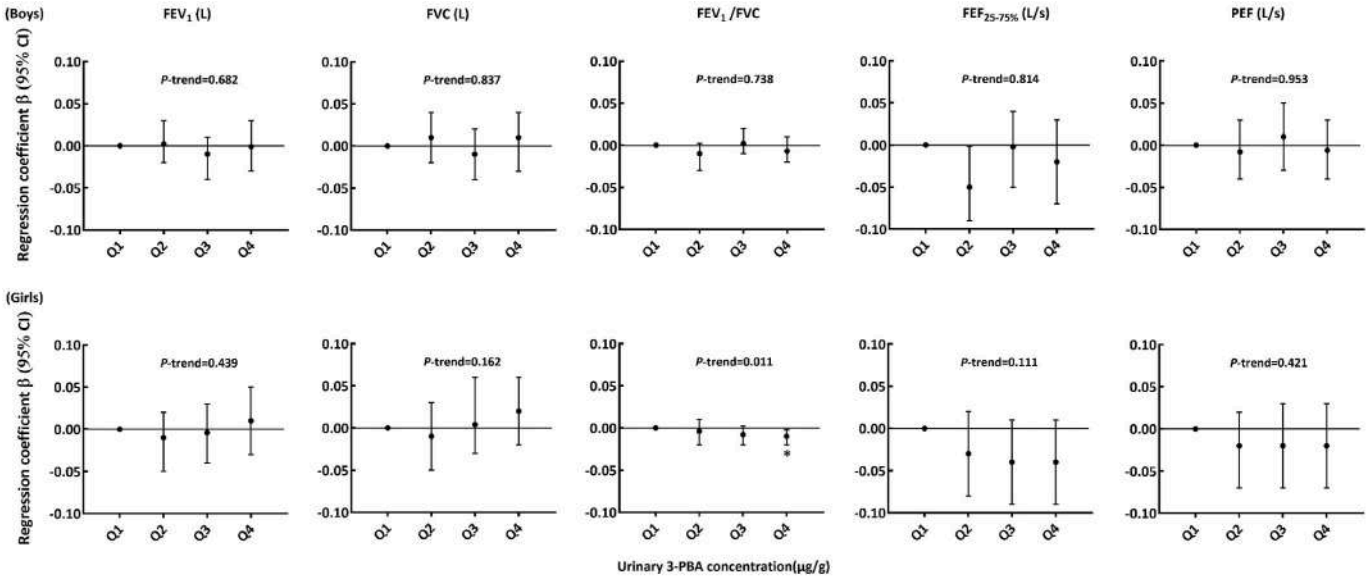

Fig. 1. 95% CI for 3-PBA concentrations and lung function stratified by sex among children aged 6–8 years in Shandong, China (n = 233).

years of age (Hislop et al., 2002). Therefore, it is biologically plausible that harmful conditions acting during this crucial period of lung development might have more relevant long-lasting pathophysiological consequences in the lung. In addition, 3-PBA induced disruption of interrelated systems-autonomic, neuroendocrine, and immune-during the perinatal development may contribute to increased vulnerability to airway inflammation and reactivity as well as reduced lung function in later childhood and even into adulthood (Shi et al., 2007; Morales et al., 2015). Additional research is warranted to more clearly elucidate the pathogenic mechanisms.

We also found that prenatal 3-PBA concentrations were associated with reduced lung function particularly among girls. This finding may be consistent with an estrogen effect of pyrethroids, since estrogens are known to possibly influence airway inflammation, are associated with increased risk of persistent lower lung function, and may play a role in chronic lung diseases (Miller and Marty, 2010; Saillenfait et al., 2015). Moreover, this sex-different effect could be explained, at least in part, by taking into account the relative differences in lung and airway size between boys and girls. On average, female lungs tend to be smaller and weigh less than those of males (Carey et al., 2007). Thus, the total number of alveoli and alveolar surface area are smaller for girls than for boys of a given age (Vidaillac et al., 2018). Inherent sex differences in lung anatomy affect the susceptibility to respiratory tract infections (Brooke-Hollidge et al., 2021). However, the mechanisms underlying the sex-specific effect and susceptibility to PYRs are not yet fully understood. Future studies of in utero and early life PYRs exposure and lung function should also investigate sex-specific effects, as this finding may provide insight into potential mechanisms of action.

The main strength of our study lies in its population-based prospective design, with information on relevant potential confounders available for a number of participants. Second, we used maternal PYR concentrations from samples collected in pregnancy as a proxy for early life in *utero* exposure. Furthermore, we benefited from objective assessment of lung function in children. We investigated the potential effects of prenatal PYR exposures and lung function among school-aged children, to identify the susceptible exposure window in early life. Third, we confirmed our findings by using multiple imputation to calculate the undetected PYR metabolites, which made our major results and conclusions less likely to suffer from the bias caused by simply imputing the undetected analytes with LOD/ $\sqrt{2}$. Finally, we conducted several sensitivity analyses including both stratified and GAMM models to examine the relationship of PYRs with lung function. The results exhibited similar patterns supporting the robustness of our findings.

The findings from our study should be interpreted in light of certain limitations. First, PYRs are rapidly metabolized and excreted and a single urine analysis is not likely to be as robust as multiple measures in assessing longer term exposure (Starr et al., 2008). The ability of urinary metabolites concentrations from a single spot urine sample to reflect the PYR exposure level throughout pregnancy is still unclear, despite previous studies suggesting that single urine measurement may adequately designate stable average exposure over months if a person stayed in the same microenvironment for that period (Wielgomas et al., 2013). Furthermore, it should also be noted that PYR metabolites detected in urine are not specific to the parent compound and may reflect the potentially low toxic preformed metabolites in the environment. In addition, our study only measured prenatal PYRs exposure and did not no estimate the postnatal exposure. In a recent air pollution exposome paper, the authors observed that prenatal but not postnatal exposure to benzene and NO_2 were associated with reduced FEV_1 in children, suggesting that the prenatal life is the most critical period for the impact of pollutant exposure (Morales et al., 2015). Third, differences were observed between mothers of children included in the study and those from the entire cohort: the mothers included in the study had a lower household monthly income and were more likely non-smokers. While these differences may have some effect on the generalizability of results, it should not affect their internal validity. Fourth, we could not perform stratified analysis for most of the effect modifiers, due to lower sample size of children with asthma. Their number was too low for precise risk estimation. Thus, future studies should provide new information regarding the influence of PYRs on lung function impairment in children with asthma. This stems for the low prevalence, of only 1.3% of asthma in our pediatric population, lower than the reported prevalence of 3% nationwide in China (National Cooperative Group on Childhood Asthma, 2013). Nonetheless, our prevalence data was comparable with the data from domestic cities such as Jinan (1.1%) and Nanjing (1.3%) (Wang et al. 2013, 2019). This study was based on data collected from a single hospital located in the Laizhou Wan (Bay) area from Shandong province, which although reflects the characteristics of school-aged children from this area, may affect the external validity of the findings. Finally, we only considered PYR exposures although the human is exposed to a wide range of environmental chemicals like organophosphate insecticides that can also potentially affect lung development (Raanan et al., 2016; Lin et al., 2018).

5. Conclusions

In conclusion, the findings of the present study suggested that prenatal 3-PBA concentration was associated with a modest decrease in FEV_1/FVC among school-aged children, and girls may be more susceptible than boys. Further studies in larger population are required to confirm our results.

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Contributors

PH and YZ are joint first authors, contributed to the data collection, data cleaning and had primary responsibility for writing the manuscript. GD and YG contributed to the analysis and conceived this study. AV, YT and YH contributed to the revision of the manuscript and interpretation of the findings. All authors critically reviewed the manuscript for important intellectual content.

Declaration of competing interest

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

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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.2022.114027.

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Proximity to livestock farms and COVID-19 in the Netherlands, 2020–2021

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ABSTRACT

Objectives: In the Netherlands, during the first phase of the COVID-19 epidemic, the hotspot of COVID-19 overlapped with the country's main livestock area, while in subsequent phases this distinct spatial pattern disappeared. Previous studies show that living near livestock farms influence human respiratory health and immunological responses. This study aimed to explore whether proximity to livestock was associated with SARS-CoV-2 infection.

Methods: The study population was the population of the Netherlands excluding the very strongly urbanised areas and border areas, on January 1, 2019 (12, 628, 244 individuals). The cases are the individuals reported with a laboratory-confirmed positive SARS-CoV-2 test with onset before January 1, 2022 (2, 223, 692 individuals). For each individual, we calculated distance to nearest livestock farm (cattle, goat, sheep, pig, poultry, horse, rabbit, mink). The associations between residential (6-digit postal-code) distance to the nearest livestock farm and individuals' SARS-CoV-2 status was studied with multilevel logistic regression models. Models were adjusted for individuals' age categories, the social status of the postal code area, particulate matter (PM_{10})- and nitrogen dioxide (NO_2)-concentrations. We analysed data for the entire period and population as well as separately for eight time periods (Jan–Mar, Apr–Jun, Jul–Sep and Oct–Dec in 2020 and 2021), four geographic areas of the Netherlands (north, east, west and south), and for five age categories (0–14, 15–24, 25–44, 45–64 and > 65 years).

Results: Over the period 2020–2021, individuals' SARS-CoV-2 status was associated with living closer to livestock farms. This association increased from an Odds Ratio (OR) of 1.01 (95% Confidence Interval [CI] 1.01–1.02) for patients living at a distance of 751–1000 m to a farm to an OR of 1.04 (95% CI 1.04–1.04), 1.07 (95% CI 1.06–1.07) and 1.11 (95% CI 1.10–1.12) for patients living in the more proximate 501–750 m, 251–500m and 0–250 m zones around farms, all relative to patients living further than 1000 m around farms. This association was observed in three out of four quarters of the year in both 2020 and 2021, and in all studied geographic areas and age groups.

Conclusions: In this exploratory study with individual SARS-CoV-2 notification data and high-resolution spatial data associations were found between living near livestock farms and individuals' SARS-CoV-2 status in the Netherlands. Verification of the results in other countries is warranted, as well as investigations into possible underlying exposures and mechanisms.

1. Introduction

The first case of COVID-19 in the Netherlands, early 2020, was living in the province of Noord-Brabant in the south of the Netherlands. In the subsequent weeks, it became apparent that COVID-19 incidence remained elevated and largely concentrated in the southeast of the Netherlands (Fig. S1 panel A). The early COVID-19 hotspots were in part explained by the multiple introduction events involving infected persons

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who had returned from February holidays spent in northern Italy and Austria, with increased spread by intensive local Carnival celebrations at the end of February. The southeast of the Netherlands is however a region with a high density of livestock farms, including pig, poultry, mink, cattle, goats and others (Fig. 1, Fig. S1 panel C,D), and had also been the epicentre of the large goat-related Q fever pneumonia epidemic, running from 2007 to 2010 (Fig. S1 panel B). This triggered societal discussion about a possible relation between COVID-19 and livestock farming.

After the first epidemic wave, and when testing upon symptoms became available to the general public on June 1, 2020, the initial hotspot in the southeast of the Netherlands was no longer visible. The changing geographical focal points for SARS-CoV-2 over different periods illustrates the complexity of studying environmental risk factors for an infectious disease that spreads through human-to-human transmission in human networks, driven by behaviour and other human factors. The changing spatial transmission patterns also show that if exposure to livestock plays a role, it will most likely play a minor role relative to other factors driving the transmission. However, questions on a possible association between exposure to livestock and COVID-19 remain, and plausible mechanisms do exist. For instance, previous studies learnt that residential proximity to livestock farms and exposure to (parts of) micro-organisms, endotoxins, and ammonia emitted from livestock farms, was associated with various positive and negative health effects, including modulated immune responses, increased risk of pneumonia (Kalkowska et al., 2018; Klous et al., 2018; Post et al., 2019), reduced lung function (Borlée et al., 2017), mortality from respiratory diseases (Simões et al., 2022), and a lower prevalence of asthma and COPD (Borlée et al., 2015; de Rooij et al., 2019; Post et al., 2021; Smit et al., 2014). Although the mechanisms underlying these associations are not yet fully understood, possibly, similar exposures and mechanisms also influence the probability to acquire SARS-CoV-2, or the probability to develop symptoms and therefore to be tested.

In this exploratory study, we investigated whether residential proximity to livestock farms was associated with individuals' SARS-CoV-2

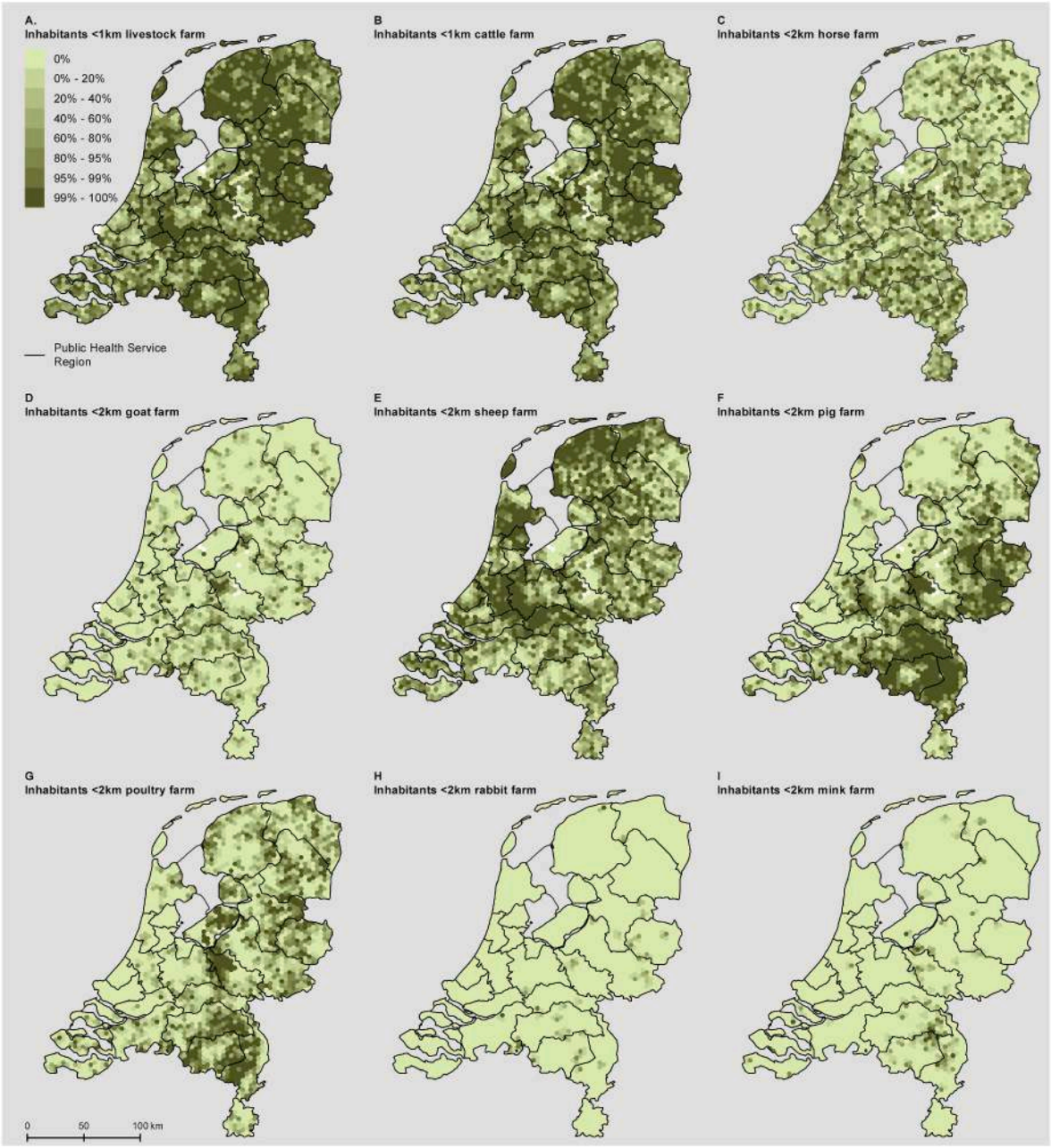

Fig. 1. Nine-Panel plot illustrating the spatial patterns of home address distances to the livestock farm types included in this study.

status in the period 2020–2021, and whether results were consistent across geographic regions, time periods, and age categories.

2. Materials and methods

2.1. Study population

Our study was based on data on the Dutch population on January 1, 2019 and notified SARS-CoV-2 infected patients with an estimated symptom onset during 2020–2021.

2.1.1. Patients

Laboratory-confirmed SARS-CoV-2 is mandatory notifiable in the Netherlands. We included all notified patients with disease onset, a positive test result, or a notification date before January 1, 2022 in this study. For this, on February 4, 2022, data were extracted from the national database at the National Institute for Public Health and the Environment, to which all 25 Public Health Services in the Netherlands report the laboratory-confirmed SARS-CoV-2 cases. Patient data included age, self-reported date of disease symptoms onset and the six-digit postal code of the residential address. Six-digit postal-code areas comprised on average about eighteen residential addresses. In case date of disease onset was not registered, the date of the laboratory test result or else the date of notification to the Public Health Service was used as proxy. Patients with disease onset in 2022 or for whom the database lacked information on postal code or age were excluded.

2.1.2. General population

The study population was based on the digital population on January 1, 2019 of 436,748 six-digit postal-code areas from all 355 municipalities of the Netherlands. To be able to compare patients to the general population taking into account age, a synthetic study population per six-digit postal-code area was constructed with a spatially-representative age distribution. This synthetic population was created, because individual-level data including age and address is not publicly available due to privacy issues. The synthetic population consisted of an attributed number of individuals per six-digit postal code for five age-groups (0–14, 15–24, 25–44, 45–64 years, and 65 years and older). These numbers had to be attributed based on neighborhood data, which is a part of a municipality that is seen as homogeneously based on historical or urban planning characteristics, and the smallest area for which the age distribution is publicly available. Attribution was done using Hamilton's method (Kohler and Zeh, 2012). Population statistics were retrieved from Statistics Netherlands, which annually provides publicly available statistical data on municipalities, districts, and neighbourhoods (Prins, 2000). The Netherlands had 13,379 neighbourhoods on January 1, 2019 with an average population of about 1,300 inhabitants.

To combine the patient populations with the total synthetic population, the number of notified patients per combination of six-digit postal code and age group were removed from the total synthetic number of inhabitants to obtain the number of non-patients per combination of six-digit postal code and age group. In case this procedure led to a negative number of non-patients for the combination, the number of non-patients was set to zero.

2.1.3. Distance to livestock farms

The distances of the centroid of address locations per six-digit postal-code area to the nearest livestock farm were calculated with ArcGIS 10.6 (ESRI [Environmental Systems Research Institute], 2011), based on information about locations of farms according to the national agricultural census of April 1, 2018 (re: horses, pigs, and poultry); the identification and registration data of July 1, 2019 (re: cattle, goats, and sheep); a list of active farm locations from the Netherlands Food and Consumer Product Safety Authority from June 15, 2020 (re: mink and rabbits). Only farms with a minimum number of animals were included, as in previous studies (e.g. Borlée et al., 2015; Post et al., 2021; Smit et al., 2014): cattle farms (at least 5 animals), pig farms (≥25), poultry farms (≥200), goat farms (≥50), sheep farms (≥50), horse farms (≥20), rabbit farms (≥200), and mink farms (≥200). The distances to livestock farms of any type were based on the minimum Euclidean distance of the six-digit postal-code centroid to the closest farms of any type.

Based on the distances to various types of livestock farms, we defined exposure bands of 0–250, 251–500, 501–750 and 751–1000 m, with >1000 m as reference category, for livestock farms of any type. Farm types were each included in separate analyses. No analyses were performed including multiple farm types at once, due to expected multicollinearity issues, and because only a small selected population was expected to live within 10 km of each of the farm types.

2.3. Contextual variables

2.3.1. Air pollution

Since ambient air pollution was indicated as a possible risk factor for COVID-19, we included air pollutants with the known largest health impact in the Netherland: particulate matter (PM_{10}) and nitrogen dioxide (NO_2). The modelled annual concentration of PM_{10} and NO_2 for 2019 was assessed for each six-digit postal code by linking maps yielding 1×1 km^2 grids of the concentrations to all residential addresses in the Netherlands on January 1, 2019, then averaging the concentrations per six-digit postal-code area. These were included as continuous variables in the statistical models. The PM_{10} and NO_2 concentrations were calculated with the Operational Priority Substances (OPS) dispersion model, which takes into account dispersion, transport, chemical conversion, deposition, and the meteorological conditions in 2019 (Sauter et al., 2018; Van Jaarsveld and De Leeuw, 1993). Source data for this OPS model were the 2018 emissions reported to the Netherlands Pollutant Release and Transfer Register (Wever et al., 2020) and emissions from neighbouring countries (EMEP/CEIP, 2020). NO_2 levels were derived from the modelled NO_x concentration using an empirical relationship between measured NO_x and NO_2 concentrations (van de Kassteele and Velders, 2006; Velders et al., 2014). The concentrations of particulate matter and NO_2 were calibrated against results from Air Quality Monitoring Networks at 35–45 rural and urban background locations in the Netherlands (number depends on the contaminant). The modelled ambient concentrations represented the average of spatial background concentrations with a resolution of about 1×1 km^2 (Velders et al., 2020).

3.3.22.3.2. Social status

To adjust for contextual confounding due to social status, we used a social status score at the four-digit postal-code level (on average, 1,987 residential addresses), derived most recently for 2017 by the Netherlands Institute for Social Research. This score is based on income level, unemployment rate, and education level (Knol, 1998), and was standardised with an average of zero and a standard deviation of one. A low social status for a postal code was indicated by a low score. The social status scores were applied to all six-digit postal-codes in the four-digit postal-code area (on average, 109 six-digit postal code areas per four-digit area) to be included as continuous variable in the statistical models.

2.3.3. Urbanisation

The degrees of urbanisation of the six-digit postal-code areas were based on statistical data for 2018 from Statistics Netherlands pertaining to 500 x 500-m squares. This indicator was based on the average address density within a radius of 1 km divided into five categories: very strongly urbanised (≥2500 addresses per km^2), highly urbanised (1500–2499 addresses per km^2), moderately urbanised (1000–1499 addresses per km^2), low-urbanised (500-999 addresses per km^2) to non-urban (<500 addresses per km^2).

2.3.4. Excluded postal code areas

The populations living in very strongly urbanised areas (≥2500 addresses per km^2) were excluded from the statistical analyses because the population in these areas tends not to live in proximity to livestock farms, and to exclude risk factors associated with living in these areas. The populations living in six-digit postal codes known to include a nursing home were excluded because of possible data quality issues with regard to the number of cases and non-cases in nursing homes. Locations of livestock farms in Belgium and Germany were not known, therefore, persons living in six-digit postal codes within two km of the border with Belgium or Germany were excluded.

2.4. Statistical analyses

2.4.1. Models

To estimate associations (odds ratio OR and 95% confidence interval CI) between SARS-CoV-2 status and distance to livestock farms, we applied logistic regression models using a random effect for the regional catchment areas of the 25 Public Health Services in the Netherlands. Included covariates were age category, social status score, and air pollution (PM_{10} and NO_2).

Data management was carried out in Stata version 16 (StataCorp, 2019) and in R version 4.0.1 (R Core Team, 2022). Statistical analyses were performed with R, using glmer function of the lme4 package for the multilevel regression analyses (Bates et al., 2017).

2.4.2. Stratifications by epidemic phases, geographic regions and age categories

In addition to analyses of cumulative positive SARS-Cov-2 tests for the entire study period in all studied postal code areas, subsets of the data were explored. This was done to assess whether obtained results were robust over space, time, age groups, and urbanicity levels, and not mainly driven by differences in testing behaviour or virus exposure due to e.g. changing testing policies, triage in hospitals, virus variants, immunity build-up, and the levels of community transmission at the time when social distancing measures were implemented.

Separate analyses were performed for each of eight phases: four quarters of each studied year (January–March, April–June, July–September and October–December in 2020 and in 2021). Each individual notified with a positive SARS-CoV-2 test was assigned to a phase based on the date of symptom onset. In the prior phases they were treated as population control while in subsequent phases they were treated as immune and therefore excluded.

And separate analyses were performed for five age classes (0–14, 15–24, 25–44, 45–64 years, and 65 years and older), the four geographic regions in The Netherlands (North, East, West and South), according to the NUTS (Nomenclature of territorial units for statistics) level 1 classification in the European Union as depicted in Fig. S2.

2.4.3. Sensitivity analyses

To assess as sensitivity analysis whether inclusion of the covariates affected the outcomes, we performed the analyses without all covariates, without the age categories, without the social score, without the air pollution variables, and with only age as covariate. To assess if nonlinear relations between SARS-CoV-2 status and covariates may have affected the outcomes, we performed three sensitivity analyses using categories based on quintiles for social scores, PM_{10} and NO_2 concentrations. Two additional sensitivity analyses were performed to evaluate whether the removal of border areas and areas with a nursing home influenced the results. Separate analyses were performed for the four degrees of urbanisation (highly urbanised, moderately urbanised, low-urbanised, and non-urban) and for the very strongly urbanised areas that had been excluded from the main study population. An additional analysis was limited to the more rural areas by excluding the highly urbanised areas. To assess if the lack of widely available testing during the first two quarters of 2020 affected the results, we performed the analyses for the period July 2020–December 2021.

Finally, to assess exploratory whether obtained results were driven by particular livestock species, we performed analyses separately per farm type: cattle farms (at least 5 animals), pig farms (≥25), poultry farms (≥200), goat farms (≥50), sheep farms (≥50), horse farms (≥20), rabbit farms (≥200), and mink farms (≥200). For livestock farms of any type and for each farm type, we excluded populations living over 10 km. Next, we defined quintile exposure bands for each farm type, resulting in an equal distribution of the study populations across the distance bands (Table S1). Case data for the entire study period were used, except for analyses on mink farms. Due to SARS-CoV-2 infections in mink farms that were first detected in April 2020, all mink were culled between June and December 2020. Therefore, the analyses on mink farms were performed separately for the first quarter of 2020 (before culling) and for the remaining months of 2020, before mink farming was prohibited in January 2021.

2.4.4. Privacy

Dutch Civil Code allows the use of health records for statistics or research in the field of public health under strict conditions. All data management and statistical analyses were carried out within the National Institute for Public Health and the Environment. COVID-19 is listed as a notifiable disease and SARS-CoV-2 is listed as a notifiable causative agent in law. No informed consent from patients nor approval by a medical ethics committee is obligatory for registry-based health studies of this type.

3. Results

3.1. Population characteristics

As of January 1, 2019, the total Dutch population consisted of 17, 278, 309 inhabitants. The merger of population data with the individual patient data and exclusion of postal code areas with a missing social status and with nursing home presence, the very strongly urbanised areas and the 2 km border zone with Belgium or Germany led to a synthetic study population of 12, 628, 244 individuals with full data on exposure at residential address and potential confounders. The majority of exclusions was due to living in a very strongly urbanised area (87%), followed by border areas (10%).

The cumulative number of notified individuals with a positive SARS-CoV-2 tests on the date of database assessment (Feb 4, 2022) with disease onset in 2020 or 2021 was 3,190,258, of which 3,121,352 were individuals' first infections. Among these, 3,089,123 had available data on age and a valid postal code and could be merged with the population data. After exclusion of areas with a missing social score, presence of a nursing home, very strongly urbanised areas and the 2 km border zone with Belgium or Germany, 2,223,692 cases remained and were included in the study.

Fig. S2 provides a map of the included postal code areas. Fig. S3 provides an epicurve of the notified positive SARS-CoV-2 tests in the study population, and Fig. S4 shows how these cases are distributed spatially during the eight phases that we distinguished in this study.

The characteristics of the study population, including the distributions of the distances to the nearest livestock farms, are depicted in Table 1. Over the period 2020–2021, individuals notified with a positive SARS-CoV-2 test were younger than the total study population. Further, notified SARS-CoV-2 cases lived less often in region North, in postal-code areas with on average higher air pollution levels and similar social status scores, and more often in the closer distance bands to livestock in comparison to the total study population. Of the total study population, 38.5% lived within 1 km (Table 1) and all lived within 6.5 km from the closest livestock farm.

Table 1
Characteristics of the study population in rural areas in the Netherlands.

Characteristic	Total population[a]	Individuals notified with a positive SARS-CoV-2 test and symptom onset before 1 January 2022[a,b]
n	12,628,244	2,223,692
Age category:		
0-14 (%)	16.5	14.9
15-24 (%)	11.9	17.2
25-44 (%)	22.6	27.9
45-64 (%)	29.0	28.3
65 and older (%)	20.0	11.6
Distance to the nearest livestock farm:		
>1 km (%)	38.5	38.0
751–1000 m (%)	16.3	16.1
501–750 m (%)	19.1	19.2
251–500 m (%)	17.1	17.4
0–250 m (%)	8.9	9.3
Ambient air pollution:		
Annual average concentration of PM_{10} in 2019:		
Mean (μg/m^3)	17.2	17.3
5-percentile (μg/m^3)	14.6	14.7
25-percentile (μg/m^3)	16.3	16.5
75-percentile (μg/m^3)	18.2	18.2
95-percentile (μg/m^3)	18.9	19.0
Annual average concentration of NO_2 in 2019:		
Mean (μg/m^3)	16.1	16,3
5-percentile (μg/m^3)	10.2	10.6
25-percentile (μg/m^3)	13.6	14.0
75-percentile (μg/m^3)	18.2	18.4
95-percentile (μg/m^3)	22.5	22.7
Social status postal code:		
Mean score	0.002	0.004
5-percentile (low)	−2.05	−2.00
25-percentile	−0.57	−0.53
75-percentile	0.72	0.73
95-percentile (high)	1.59	1.67
Region:		
North (%)	11.6	8.8
East (%)	25.3	24.7
West (%)	40.3	41.9
South (%)	22.7	24.6
Urbanisation degree postal code:		
Highly urbanised (1500–2499 addresses per km^2) (%)	33.3	33.1
Moderately urbanised (1000–1499 addresses per km^2) (%)	22.6	22.3
Low-urbanised (500-999 addresses per km^2) (%)	22.5	22.8
Non-urban (<500 addresses per km^2) (%)	21.7	21.8

[a] Excluding those living in in a postal code area with a missing social score, with a nursing home, in very strongly urbanised areas, or within 2 km from the border with Belgium or Germany.

[b] Individuals with a notified positive SARS-CoV-2 test and with estimated symptom onset in 2020 or 2021, excluding those without available data on age and 6-digit postal code area.

3.2. Statistical analyses

3.2.1. Distance to livestock farms

People living close to livestock farms had a higher probability of being notified with SARS-CoV-2. Expressed in ORs, there was a trend from an OR of 1.11 (1.10–1.12) in the 0–250 m distance band to 1.07 (1.06–1.07) in 251–500 m, to 1.04 (1.04–1.04) in 501–750 m, and to 1.01 (1.01–1.02) in the 751–1000 m distance band compared to > 1000 m. Analyses per region, per age group and per phase of the epidemic show similar results, except for the third quarters (July–September) of both studied years (2020 and 2021), the quarters that included periods with the lowest incidences (Table 2).

3.2.2. Sensitivity analyses

Exclusion of covariates, inclusion of covariates as categorical instead of continuous variables, and the inclusion of border areas or areas with a nursing home did not affect the observed associations (Table S2). Also analyses for the period July 1, 2020–December 31, 2021 and analyses excluding of the highly urbanised areas gave similar results. Stratification by degree of urbanisation resulted in similar pattens albeit with lower ORs. Within the very strongly urbanised areas, which were excluded from the study, no associations were seen except one OR slightly below 1 in the 251–500 m distance band.

The use of quintile distance bands for the distance to livestock up to a maximum of 10 km resulted in ORs of 1.09 (1.08–1.09) for the quintile of people closest to farms, to 1.04 (1.03–1.04), 1.01 (1.01–1.02) and 1.00 (1.00–1.01), versus 1 for the reference band (Table 3). Results for cattle, goat, pig, poultry and rabbit farms were similar in size and pattern of the highest ORs for people living the closest to farms with ORs gradually decreasing along the larger distances to farms. For sheep farms, ORs followed a similar pattern but were somewhat lower. Results for mink farms showed a similar pattern, but ORs were different in size for the two studied periods: OR 1.25 (1.15–1.36) for January–March 2020 (prior to the culling of mink) and OR 1.03 (1.02–1.05) for April–December 2020 (during culling until the ban on mink farms) for people living 0–3.55 km from the closest mink farm. Results for horse farms were an exception, with all ORs smaller than 1 and without any pattern along the distances (Table 3).

4. Discussion

Livestock farm proximity enhanced the probability of individuals to be notified with a positive SARS-CoV-2 test. Results were similar across regions and age groups, and for six out of eight studied time periods. The same association between livestock proximity and SARS-CoV-2 status was observed for the first, second and fourth quarter of 2020 and 2021 but not for the July–September periods, when the incidence was lowest.

Sensitivity analyses per livestock species showed comparable results across farms of any type, cattle, goat, pig, poultry and rabbit farms. For sheep farms, ORs were somewhat lower, possibly related to sheep grazing at locations other than the farm itself. Exposure misclassification is likely to be more limited for other ruminants. Cattle commonly graze during part of the year, but less than sheep, while most goats remain within the farm. For horses, results were not in line with the other species, with ORs below 1. As for sheep, farm location may not be a good proxy for the location of horses. Also, these 'farms' often included horse riding schools, while many other riding school locations were not available to our study, making results for horses hard to interpret. Analyses for mink resulted in higher ORs for the first quarter of 2020, before the culling of mink due to SARS-CoV-2 infections in mink. For the three subsequent periods, during culling and until mink farming in the Netherlands completely stopped by the end of 2020, ORs were reduced. The coherence of results across species suggest that the observed associations are not driven by one particular farm type, despite that in the analyses per species, no adjustments were made for the proximity to other species.

When limiting the population to the more rural areas (<1500 addresses/km^2) ORs remained similar. But when stratifying analyses by urbanisation level, ORs lowered in all strata. This could point at residual confounding by urbanisation level. Studies have pointed at population density as a driver of SARS-CoV-2 transmission (e.g. Smith et al., 2021), but also physical and mental health morbidity, social neighborhood factors like cohesion and physical exposures like air pollution are correlated with the degree of urbanisation (Zock et al., 2018). Possibly, address density acts as a proxy for unknown mechanisms that may affect

Table 2
Odds ratios (95% confidence interval) for categories of distance to nearest livestock farm (0–250, 251–500, 501–750, 751–1000 m, and over 1000 m) for being notified with a positive SARS-CoV-2 test. Results are for the Netherlands and for various subsets (eight quarters, four geographic regions, and five age groups). Excluded from the analyses are residential addresses in areas with a missing social score, with presence of a nursing home, in very strongly urbanised areas, or within 2000 m of the border of Germany or Belgium.

Dataset	Category	n	cases[a]	Distance of residential address to the nearest livestock farm				
				0–250 m	251–500 m	501–750 m	751-1000 m	>1000 m
				OR (95% CI)[b]	OR (95% CI)[b]	OR (95% CI)[b]	OR (95% CI)[b]	ref[b]
The Netherlands		12,628,244	2,223,692	1.11 (1.10–1.12)***	1.07 (1.06–1.07)***	1.04 (1.04–1.04)***	1.01 (1.01–1.02)***	1
Subsets								
Year 2020	Jan–Mar	12,628,244	14,252	1.08 (1.02–1.16)*	1.05 (1.00–1.10).	1.07 (1.02–1.12)**	1.05 (0.99–1.10).	1
	Apr–Jun	12,613,992	16,548	1.07 (1.01–1.14)*	1.03 (0.99–1.08)	1.04 (1.00–1.09).	1.02 (0.98–1.07)	1
	Jul–Sep	12,597,444	54,983	0.93 (0.89–0.96)***	0.99 (0.96–1.01)	0.98 (0.95–1.00)*	0.97 (0.95–1.00)*	1
	Oct–Dec	12,542,461	484,173	1.11 (1.09–1.12)***	1.07 (1.06–1.08)***	1.05 (1.04–1.06)***	1.03 (1.02–1.04)***	1
Year 2021	Jan–Mar	12,058,288	342,653	1.16 (1.15–1.18)***	1.11 (1.09–1.12)***	1.07 (1.06–1.08)***	1.03 (1.02–1.04)***	1
	Apr–Jun	11,715,635	275,374	1.09 (1.08–1.11)***	1.04 (1.03–1.06)***	1.02 (1.01–1.03)**	1.00 (0.98–1.01)	1
	Jul–Sep	11,440,261	197,605	0.99 (0.98–1.01)	1.01 (1.00–1.02)	0.99 (0.98–1.00).	0.99 (0.97–1.00)*	1
	Oct–Dec	11,242,656	838,104	1.11 (1.10–1.12)***	1.06 (1.06–1.07)***	1.04 (1.03–1.05)***	1.01 (1.00–1.02)*	1
Region[c]	West	5,090,497	931,404	1.10 (1.09–1.11)***	1.05 (1.04–1.06)***	1.04 (1.03–1.05)***	1.01 (1.00–1.01)	1
	East	3,201,038	549,839	1.17 (1.16–1.18)***	1.12 (1.11–1.13)***	1.05 (1.04–1.06)***	1.02 (1.01–1.03)***	1
	South	2,871,271	546,639	1.04 (1.02–1.05)***	1.04 (1.03–1.05)***	1.01 (1.00–1.02)*	1.02 (1.01–1.03)***	1
	North	1,465,438	195,810	1.11 (1.10–1.13)***	1.05 (1.04–1.07)***	1.08 (1.06–1.09)***	1.04 (1.02–1.05)***	1
Age group	0-14 yo	2,078,950	330,215	1.05 (1.04–1.07)***	1.07 (1.06–1.08)***	1.04 (1.03–1.05)***	1.00 (0.99–1.01)	1
	15-24 yo	1,502,784	383,341	1.17 (1.16–1.19)***	1.07 (1.06–1.08)***	1.04 (1.03–1.05)***	1.02 (1.01–1.03)***	1
	25-44 yo	2,854,256	621,282	1.10 (1.08–1.11)***	1.08 (1.07–1.09)***	1.06 (1.05–1.07)***	1.02 (1.01–1.03)***	1
	45-64 yo	3,667,725	629,873	1.11 (1.10–1.12)***	1.07 (1.06–1.08)***	1.04 (1.03–1.05)***	1.02 (1.01–1.02)***	1
	≥65 yo	2,524,529	258,981	1.13 (1.11–1.15)***	1.04 (1.02–1.05)***	1.01 (1.00–1.02)	1.01 (1.00–1.02)	1

$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[a] Individuals with a notified positive SARS-CoV-2 test and with estimated symptom onset before January 1, 2022, excluding those without available data on age and 6-digit postal code area.

[b] Model with 25 regional catchment areas of Public Health Services as random effect adjusted for age category, social status of the four-digit postal-code area, and annual average concentration of PM_{10} and NO_2 in 2019 of the six-digit postal-code area.

[c] Regions according to NUTS 1.

Table 3
Odds ratios (95% confidence interval) for quintiles of distance to nearest livestock farm and for distance to different farm types for being notified with a positive SARS-CoV-2 test. Results are for the Netherlands and excluded from the analyses are residential addresses with a distance of more than 10 km from the respective type of farm, in areas with a missing social score, with presence of a nursing home, in very strongly urbanised areas, or within 2000 m of the border of Germany or Belgium.

Type of farm	n	cases	Distance of residential address to the nearest livestock farm[b]				
			Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5
			OR (95% CI)[c]	OR (95% CI)[c]	OR (95% CI)[c]	OR (95% CI)[c]	Ref[c]
Any livestock	12,628,244	2,223,692	1.09 (1.09–1.10)***	1.05 (1.04–1.05)***	1.02 (1.02–1.03)***	1.01 (1.00–1.01)**	1
Cattle	12,628,244	2,223,692	1.10 (1.09–1.10)***	1.04 (1.03–1.04)***	1.01 (1.01–1.02)***	1.01 (1.00–1.01)*	1
Goat	11,419,526	2,012,574	1.06 (1.06–1.07)***	1.03 (1.02–1.03)***	1.04 (1.03–1.04)***	1.01 (1.01–1.02)***	1
Sheep	12,592,187	2,217,800	1.04 (1.03–1.04)***	1.01 (1.01–1.02)***	1.00 (0.99–1.00)	0.99 (0.99–1.00)**	1
Horse	12,583,312	2,216,261	0.98 (0.98–0.99)***	0.98 (0.97–0.98)***	0.97 (0.97–0.98)***	0.98 (0.97–0.98)***	1
Pig	11,469,245	2,026,060	1.11 (1.10–1.12)***	1.07 (1.06–1.07)***	1.05 (1.04–1.05)***	1.04 (1.03–1.04)***	1
Poultry	10,843,208	1,890,968	1.10 (1.09–1.11)***	1.05 (1.04–1.05)***	1.02 (1.02–1.03)***	1.00 (1.00–1.01)	1
Rabbit	2,636,600	493,186	1.07 (1.05–1.08)***	1.05 (1.04–1.07)***	0.99 (0.98–1.00)	1.00 (0.99–1.01)	1
Mink quarter 1[d]	3,983,911	5,663	1.25 (1.15–1.36)***	1.05 (0.96–1.14)	1.09 (1.00–1.19).	1.01 (0.93–1.11)	1
Mink quarter 2–4[d]	3,983,911	176,119	1.03 (1.02–1.05)***	1.05 (1.03–1.07)***	1.00 (0.98–1.02)	1.00 (0.98–1.02)	1

$p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[a] Individuals with a notified positive SARS-CoV-2 test and with estimated symptom onset before January 1, 2022, excluding those without available data on age and 6-digit postal code area.

[b] The upper and lower bounds of the quintiles are specified per type of farm in Table S1.

[c] Model with 25 regional catchment areas of Public Health Services as random effect adjusted for age category, social status of the four-digit postal-code area, and annual average concentration of PM_{10} and NO_2 in 2019 of the six-digit postal-code area.

[d] Quarter 1: January–March 2020, before culling of mink; quarter 2–4: April–December 2020, during culling of mink and before mink farming was prohibited.

individuals' COVID-19 status that are correlated with the level of urbanisation or rurality but are not directly related to livestock.

When comparing ORs across regions, ORs are lower in region South, a region that includes areas with high farm densities, and where the initial COVID-19 hotspot occurred that triggered unrest about livestock farming and COVID-19. While this finding should not be over interpreted, as it was primarily intended as part of a sensitivity analyses, this could be a topic for in depth follow up investigations. Future studies could also take into account combined effects of proximity to multiple farms and farm size or other farm characteristics.

Ambient air pollution has been associated with SARS-CoV-2 incidence in the Netherlands (Andree, 2020; Cole et al., 2020) and in several other countries among others Canada (Stieb et al., 2020), USA (Sidell et al., 2022), Italy (De Angelis et al., 2021) and Germany (Prinz and Richter, 2022), mostly in ecological settings. We therefore included ambient PM_{10} and NO_2 concentrations as covariates. Livestock production is one of the sources that contributes to air pollution, in particular to particulate matter (PM) concentrations, so the associations

with distance to livestock farms may have been over-adjusted. However, sensitivity analyses without PM_{10} and NO_2 gave similar results (Table S2) suggesting that overadjustment due to general air pollution is not an issue. Using modelled livestock-specific PM concentrations to better disentangle the different PM fractions, and to also account for example for the presence of multiple farms simultaneously as in Post et al. (2021), could be a refinement in follow-up research.

The results of our study are in line with previously reported associations between proximity to livestock farms and various health outcomes, including lower respiratory infections, where multiple livestock species have been implicated (Freidl et al., 2017; Kalkowska et al., 2018; Klous et al., 2018; Post et al., 2019; Poulsen et al., 2018; Simões et al., 2022; Smit et al., 2012). Hypotheses about underlying biological and physical mechanisms have been proposed. For instance, persons living in livestock areas having an enhanced responsiveness to livestock specific particulate matter (PM) including microbial contaminated PM, or Bio-PM triggering innate immune responses, possibly contributing to airway diseases (Liu et al., 2019; Poole and Romberger, 2012; Sahlander et al., 2012). Possibly, a similar mechanism might enhance the risk of SARS-CoV-2 infection (Diamond and Kanneganti, 2022). But this requires further investigation. Also, health conditions associated with exposure to livestock, such as a reduced lung function, may lead to more severe symptoms upon a SARS-CoV-2 infection, and possibly a higher inclination to be tested. Information on hospitalisations and deaths was not included in this study, but can be used in follow up studies. There is speculation that ambient particulate matter could transport virus particles and therefore increase SARS-CoV-2 transmission (for example Bontempi, 2020; Setti et al., 2020). However, we investigated proximity to livestock explicitly, while including ambient PM_{10} concentrations as covariate. Since multiple sources contribute to PM_{10} concentrations, and sensitivity analyses without air pollutants as covariates resulted in almost identical results, it seems unlikely PM_{10} in itself would be the main explanation of the observed patterns for livestock proximity.

Of the types of farms included in this study, only mink farms have been shown to be infected by humans with SARS-CoV-2 (ECDC, 2020; Enserink, 2020; Oreshkova et al., 2020). Whole genome sequences provided evidence of mink-to-human transmission following genetic evolution in the animals (Oude Munnink et al., 2020). But spill-back of a mink sequence into the community, as occurred in Denmark (Hammer et al., 2021), was not observed in the Netherlands (Oude Munnink et al., 2020) so this route is unlikely to explain our study results for mink farms. In our study, we found similar results for multiple time periods and regions, also in absence of mink. This means that mink were not the main driver of the study outcomes. A similar reasoning applies to the former goat-related Q fever epidemic, of which the main affected area overlapped with the initial COVID-19 hotspot (van Gageldonk-Lafeber et al., 2021; Weehuizen et al., 2022). The associations that we found were not limited to the former Q fever areas.

In recent years, outbreaks of animal coronaviruses have occurred in e.g. pigs (porcine epidemic diarrhoea virus: PEDV) (Dortmans et al., 2018), poultry (de Wit et al., 2021) and horses (equine coronavirus) (Zhao et al., 2019), and many other animal coronaviruses are endemic in the Netherlands and worldwide and present in the environment (Decaro et al., 2020; Khamassi Khbou et al., 2021). One consideration is whether these animal coronaviruses, when inhaled and present on mucus, could result in false positive SARS-CoV-2 tests specifically in people around livestock farms. However, this possible explanation of our study results seems very unlikely as we expect this would have been noticed given ongoing whole genome sequencing activities worldwide.

Our study was able to use individual patient data and the six-digit postal-code of the address of SARS-CoV-2 cases, avoiding some of the inherent limitations of studies that rely on publicly available information at higher aggregation levels (Heederik et al., 2020; Villeneuve and Goldberg, 2020). However, we could not control for individual factors such as comorbidities, household income and education level.

The main challenge of the study was to avoid possible interference by local, under-the-radar, virus introductions and spread. During the start of the epidemic, SARS-CoV-2 was introduced unevenly frequent across the country, for example by persons returning from February 2020 holidays, and locally amplified by carnival celebrations, but data to reconstruct such spread across networks are sparse. The areas with the highest level of transmission at the moment of the implementation of control measures (lockdown) may have happened to coincide with, in this case, intensive livestock production. Other factors that may have influenced the dynamics of the epidemic include weather conditions (e.g. rainfall, temperature) fluctuating over time across the seasons (Smith et al., 2021), immunity build up in the population and upcoming new variants. When exposure to the virus is unknown, risk estimates for the incidence may be biased and may change as an epidemic progresses (Koopman et al., 1991; Villeneuve and Goldberg, 2022).

While all reported SARS-CoV-2 cases were available to this study, not all infected individuals were tested or reported. Testing policies varied and changed substantially across settings (e.g. for healthcare workers, children, people in nursing homes) and over time (restrictive at first, broader later). Also, people living closer to testing facilities, for instance in the cities, were more prone to be tested than those living further away (Statistics Netherlands, 2021). This was especially relevant during the initial months after opening of testing streets in June 2020. One could speculate that together with lower case numbers, possibly due to summer weather conditions, distance to the testing streets in the third quarter of 2020 may result in OR's smaller than 1 for that period. The effect of testing can be seen in the epicurves in Fig. S3, which show that particularly in the first phases of the pandemic testing capacity was very limited. It is unknown if selective underdiagnosis and underreporting affected our results, since it is not known yet if the underreporting is related to proximity to livestock farms.

To address the issues related to virus transmission and underreporting, we performed the analyses for several phases over a period of two years, for several regions, and in various sensitivity analyses. Results for proximity to livestock were consistent, however with ORs turning lower or below 1 during the third quarter of the year, and with ORs turning lower in analyses stratified by urbanisation level.

5. Conclusions

This study suggests that proximity to livestock was associated with individuals' probability of being notified with a positive SARS-CoV-2 test in 2020–2021 in the Netherlands. This result adds to a range of other respiratory health effects that have been found to be associated with proximity to livestock farms. As mechanisms underlying these effects are only limitedly understood, while a considerable proportion of the Dutch population lives in the proximity of a livestock farm, more research regarding possible biological and physical explanations and their interactions is warranted. Moreover, better insight in a potential relation between SARS-CoV-2 infections and proximity to livestock farms requires international replication and verification, as well as follow-up observational studies with more advanced methods to account for individual risk characteristics such as comorbidities, exposure to multiple farms, and for the underling human behaviour and transmission and probabilities of testing and reporting.

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Declaration of interest

None.

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Appendix A. Supplementary data

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Urinary glyphosate kinetics after occupational exposure

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ABSTRACT

Glyphosate-surfactant herbicides are the most used and imported herbicide in Thailand. Urinary biomonitoring is a very important tool for evaluating glyphosate exposures and its adverse health effects. However, the data for glyphosate toxicokinetics, especially in Asian populations, is relatively limited. The majority of farmers in Thailand have long term experience with glyphosate use, but they generally follow poor safety practices, including insufficient or incorrect use of personal protective equipment during pesticide handling activities. Therefore, this study aimed to determine the toxicokinetics of glyphosate and its metabolite in urine among maize farmers from the northern region of Thailand. The effects of personal protective equipment usage, as well as farmer behavior during work, on urinary glyphosate levels were also studied. Full-voided spot urine samples were collected over the exposure assessment period (0–72 h). Urinary glyphosate levels were determined by liquid chromatography tandem mass spectrometry. The maximum concentration in urine (uC_{max}), the time of peak glyphosate levels in urine (uT_{max}), and the urinary elimination half-life ($ut_{1/2}$) were analyzed using the PKSolver program. The median of uC_{max} were 27.9, 29.2 and 17.1 μg/g creatinine in a one-time spray group, a two-time spray group Day 1 and a two-time spray group Day 2, respectively. The uT_{max} was 11.0 h in both study groups. The median of elimination $ut_{1/2}$ in the one-time and the two-time spray group were 7.0 and 18.1 h, respectively. Although these estimated urinary elimination half-lives may have been impacted by the variation in exposure doses among the participants, it provides the first urinary toxicokinetic data of glyphosate among the Asian population. The toxicokinetic information could be used to increase knowledge and awareness amongst farmers, particularly to minimize the risk of exposure to glyphosate and reduce possible adverse health effects from using pesticide.

1. Introduction

Glyphosate (N-[phosphonomethyl]glycine) is the most commonly used herbicide worldwide, including in Thailand (Laohaudomchok et al., 2021; Maggi et al., 2020). Although it has been suggested that glyphosate has low toxicity in mammals (Tarazona et al., 2017), some recent studies report that glyphosate exposure may be associated with hepatic disease, renal disease, Alzheimer's disease, and Parkinson's disease (Eriguchi et al., 2019; Jayasumana et al., 2015; Samsel and Seneff, 2015; Zhang et al., 2017). Furthermore, glyphosate has also been classified as part of "Group 2A Probably carcinogenic to humans" by the International Agency for Research on Cancer indicating that glyphosate exposure may increase the risk of cancer (IARC, 2016). Humans can be exposed to glyphosate via dermal, oral, and inhalation routes when the herbicide is applied in the field (Connolly et al., 2019a). The European Food Safety Authority (EFSA) proposed that the Acceptable Operator Exposure Level (AOEL), the maximum amount of active pesticide ingredient to which the operator may be exposed without any adverse health effects, was 0.1 mg/kg bw/day (EFSA, 2015; Niemann et al., 2015).

Biomonitoring is an essential tool to determine glyphosate exposure and its subsequent health effects. Biomonitoring involves the measurement of glyphosate and its metabolite (aminomethylphosphonic, AMPA) in biological specimens, such as blood and urine. Most studies performed urinary biomonitoring of glyphosate because collection of urine samples is more practical and less invasive than blood samples (Gillezeau et al.,

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2019). In addition, urinary glyphosate concentration has been used as a marker for exposure measurement and it is a valid indicator of potential health risks in humans (Niemann et al., 2015; Zoller et al., 2020).

Understanding the glyphosate toxicokinetics can provide information for the risk assessment and can be used to derive policy or safety instructions for farmers. To the best of our knowledge, only a few studies reported the elimination toxicokinetics of glyphosate in farmers, most of which were conducted in Caucasian populations (Connolly et al., 2018b, 2019a, 2019b; Zoller et al., 2020). Recently, Zoller et al. reported a half-life of 9 h for glyphosate after oral dose (Zoller et al., 2020). A study in horticulturists in Ireland showed the half-life in the range of 5.5–10 h (Connolly et al., 2019a). Moreover, the study from Sweden stated that the excretion half-life of glyphosate was 6–9 h for the rapid phase and 18–33 h for the slower phase (Faniband et al., 2021). The toxicokinetic data in Asian populations is rather limited. Therefore, this study was designed to determine the toxicokinetics of glyphosate and its AMPA metabolite in urine among Thai farmers who have regularly and intensively used glyphosate.

2. Materials and methods

2.1. Ethical approval and informed consent

This study obtained ethical approval from the Research Ethics Committee of the Faculty of Medicine, Chiang Mai University (Study code: FOR-2562-06349/Research ID: 6349 and Study code: FOR-2563-06996/Research ID: 6996). Participants were considered eligible for the study if they met the following inclusion criteria: (1) has actively been using glyphosate-based herbicides during the study period as a sprayer, and (2) never been diagnosed with kidney disease and diabetes. All the participants provided written consent prior to enrolling in the study.

2.2. Study site and study population

This study was conducted at Long District, Phrae Province, Thailand (Fig. 1). Only pesticide sprayers who sprayed their crops with glyphosate-based herbicides, were recruited into the study. Each respondent had face-to-face interview with a trained researcher regarding his/her demographic status, personal protective equipment (PPE) usage and work characteristics. The data collection included: (1) sprayer characteristics (e.g., sex, age, body mass index, and smoking habit), (2) types of herbicide used, (3) time of exposure/spraying (e.g., length of time working with herbicide and length of glyphosate-based herbicide exposure in a day), (4) types of spraying equipment used, (5) agricultural tasks on the farm (including mixing and loading herbicide, handling the equipment, and cleaning and collecting the equipment), (6) behaviors and PPE use during or after spraying tasks (including using masks or respirators, wearing rubber gloves, wearing rubber boots, wearing goggles/face shields, consuming food/drink during spraying task, and washing hands after spraying task). Furthermore, all the participants were requested to provide details of herbicide volumes and the frequency of spraying tasks (one or two-time spray) during the study period (0–72 h). The one-time spray defined as farmers who sprayed herbicide single time at Day 1. The two-time spray defined as farmers who sprayed herbicide the first task at Day 1 and the second task at Day 2 (Fig. 1).

2.3. Urine sample collection

The participants were asked to collect individual full urinary void spot samples (before glyphosate-based herbicide spraying, and up to 48 or 72 h after spraying) using provided plastic bottles. The written and graphical instructions regarding how to collect their urine samples, as well as the storage procedure (in ice boxes or at 4 °C) were provided during the study period. Participants who gave at least four spot urine samples as follows were selected for the toxicokinetic analysis: (1) the morning urine before the first spraying task (time 0), (2) the urine within 6 h after the first spraying task (time 0–6), (3) the urine between 6 and 12 h after the first spraying task (time 12–18), (4) the urine between 18 and 24 h after the first spraying task (time 18–24), (5) the urine between 24 and 30 h after the first spraying task (time 24–30), (6) the urine between 30 and 36 h after the first spraying task (time 30–36), (7) the urine between 36 and 42 h after the first spraying task (time 36–42), (8) the urine between 42 and 48 h after the first spraying task (time 42–48), and (9) the urine between 48 and 54 h after the first spraying task (time 48–54) or 48–72 h after the first spraying task (time 48–72). The eliminated glyphosate concentration in urine were plotted every 6 h in order

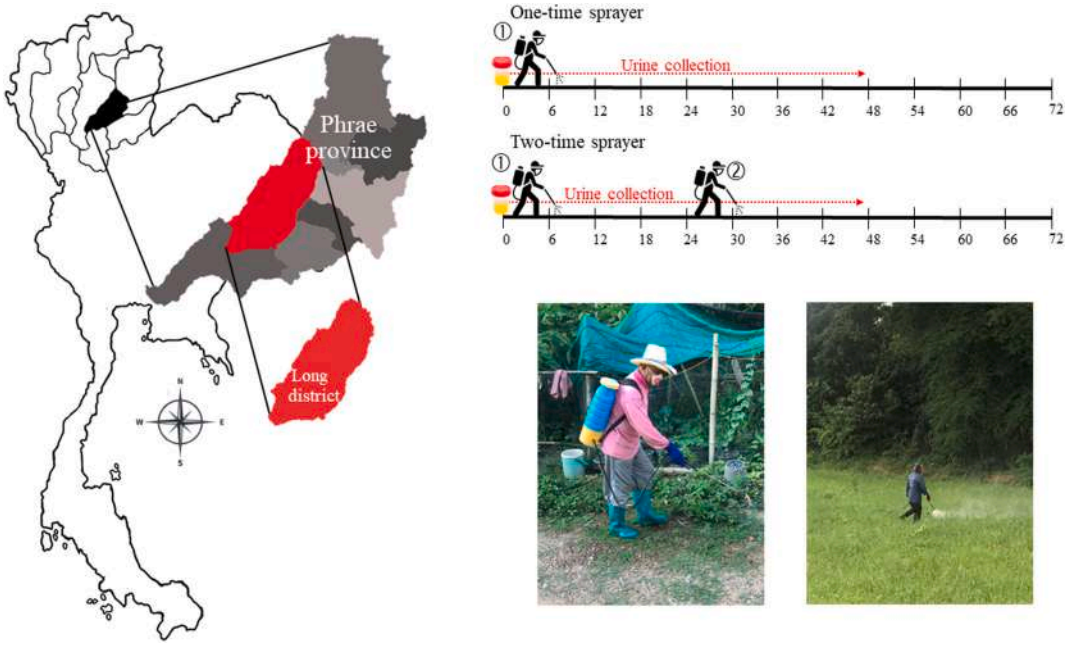

Fig. 1. Study site: Long District, Phrae Province, Thailand. A farmer with a manual lever-operated knapsack sprayer (left) or with a high-pressure lance sprayer (right). A diagram for urine sample collections in one- and two-time spray groups.

to demonstrated the concentration-time urine profiles.

Following the urine sample collection, the urine volume of each spot urine sample was then measured and recorded using volumetric cylinders. The volumetric cylinders were cleaned twice with distilled water between samples to avoid sample cross-contamination (Connolly et al., 2018a). The urine sample was then transferred into 15 mL polypropylene centrifuge tubes and labelled with the subject code, date, and time. All the samples were kept on dry ice for transportation to the Toxicology Laboratory, Faculty of Medicine, Chiang Mai University, for further analysis.

2.4. Cumulative herbicide exposure intensity index

The cumulative intensity of herbicide exposure in each participant calculated using a modified formula of Dosemeci et al. (2002). Factors relating to the intensity of herbicide exposure including mixing status, repair status (cleaning or fixing or any activities related to equipment maintenance during study period), application method (e.g., backpack, hand spray), use of PPE (e.g., gloves, respirators, face shields, boots), duration of exposure, and frequency were used for calculation of an estimate level of herbicide exposure.

$$\text{Exposure intensity index (EII)} = (\text{Mixing status} + \text{Application method} + \text{Repair status}) \times \text{PPE}$$

where

1) Mixing status refers to never mixing (score 0) and mixed (score 9)
2) Application method refers to does not apply (score 0), aerial aircraft (score 1), distribute tablets (score 1), application in furrow (score 2), boom tractors (score 3), backpack (score 8), and hand spray (score 9)
3) Repair status refers to does not repair (score 0) and repair (score 2)
4) Personal protective equipment refers to four groups of PPE categories that are identified considering combinations of PPE used (Table 1) and then the score for each category [PPE-0 (score 1.0), PPE-1 (score 0.8), PPE-2 (score 0.7), PPE-3 (score 0.6), PPE-1 and PPE-2 (score 0.5), PPE-1 and PPE-3 (score 0.4), PPE-2 and PPE-3 (score 0.3), PPE-1 and PPE-2 and PPE-3 (score 0.1)].

A cumulative herbicide exposure intensity index was subsequently calculated as follows:

$$\text{Cumulative herbicide exposure intensity index} = \text{EII} \times \text{duration} \times \text{frequency}$$

where

1) EII is the exposure intensity index
2) Duration is the total time period that participant contacted to the herbicide within one application
3) Frequency is one-time spraying (sprayed glyphosate only one time within 24 h) or two-time spraying (sprayed glyphosate more than one time within two consecutive days or within 48 h)

2.5. Standard and reagents

All chemicals were of analytical grade unless stated otherwise. Glyphosate (Lot number: G126126), AMPA (Lot number: G138076), 1,2–$^{13}C_2^{15}N$ glyphosate (Lot number: H155725WA) and $^{13}C^{15}N$ AMPA (Lot number: G126050WA) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Heptafluorobutyric anhydride (Lot number: BCBZ4404) was acquired from Sigma-Aldrich (St Louis, MO, USA). HPLC-grade acetonitrile (ACN) was obtained from J.T. Baker (Shanghai, China).

Table 1
The groups of PPE categories for EII calculation.

Categories	Description
PPE-0	Never used PPE
PPE-1	Face shields or goggles, fabric/leather gloves, other protective clothing
PPE-2	Cartridge respirators or gas masks, disposable outer clothing
PPE-3	Chemically resistant rubber gloves

EII, exposure intensity index; PPE, personal protective equipment.

2.6. Urinary glyphosate analysis

The analytical validated method reported by Jaikwang et al. was adopted for glyphosate and AMPA analysis (Jaikwang et al., 2020) using liquid chromatography–tandem mass spectrometry (LC–MS/MS); Agilent 1290 Infinity HPLC system coupled with an Agilent 6460 triple quadrupole mass spectrometer and electrospray ionization (Agilent Technologies, Inc., Palo Alto, CA, USA). Briefly, the chromatographic separation was achieved by a Gemini C6-Phenyl analytical column, with the gradient elution of 15 mmol/L heptafluorobutyric anhydride in water and acetonitrile. The sample was prepared by mixing a 100 μL of urine sample with 100 μL of internal standards solution in water (containing 50 μg/L of 1,2–$^{13}C_2^{15}N$ glyphosate and $^{13}C^{15}N$ AMPA). The mixture was then filtered through a 0.2 μm nylon membrane filter before being injected (injection volume of 10 μL) into the LC–MS/MS. The quality control samples at the concentration of 15, 50 and 150 μg/L were analyzed with studied samples to ensure the accuracy and precision of the analysis. The analytical limit of quantification (LOQ) of this method was 5 μg/L and the limit of detection (LOD) was 2.5 μg/L for both glyphosate and AMPA (Jaikwang et al., 2020). The creatinine concentrations were analyzed using the Alkaline Picrate method (Blaszkewicz and Liesenhoff-Henze, 2012) and were used for standardization of glyphosate and AMPA concentrations. For the accuracy of the kinetic measurement, samples with the urinary glyphosate concentrations lower than the LOQ level were excluded from further analysis.

2.7. Toxicokinetic and statistical analysis

Participants provided a minimum of four spot urine samples including at least two time points after the peak glyphosate levels in urine were detected for both groups, for the toxicokinetic analysis. The two-time sprayers must have the peak exposure on the day two after spraying as well as at least two samples after the peak sample on day two. Examples of the urinary concentration-time profiles included in this study are shown in Fig. 2.

The PKSolver program (Zhang et al., 2010) with a non-compartmental model was used to calculate the urinary elimination half-life ($ut_{1/2}$). The maximum concentration in urine (uC_{max}) is the highest urinary glyphosate concentration in urine over the study period. The time of maximum glyphosate concentration in urine or peak time ($uT_{max.}$) was the time to at which the body excreted the maximum urinary concentration (uC_{max}) (Fig. 2).

In short, area under curve from 0 to last time t were calculated using the linear trapezoidal method. Terminal elimination slope was automatically estimated using the regression with the largest adjusted R^2 where the regressions are performed based on the last three data points. For a double-peak concentration–time profile in two-time spray group, a set of secondary parameters such as T_{max1}, C_{max1}, T_{max2} and C_{max2} were manually defined the initial value of parameters before running model estimation.

Amount of glyphosate excretion in urine were calculated and presented as μg of glyphosate in total urine volume over 72 h. Pre-task concentration in both groups (time 0 and 24 h) were also presented to compare baseline concentrations between one-time and two-time sprayers.

Statistical analysis was performed using the SPSS for Windows, Version 16.0. (Chicago, SPSS Inc; 2007) and the GraphPad Prism version 8.3.0 for windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Descriptive statistics (mean ± standard deviation (SD), median or frequency (percentage), as appropriate) were used to

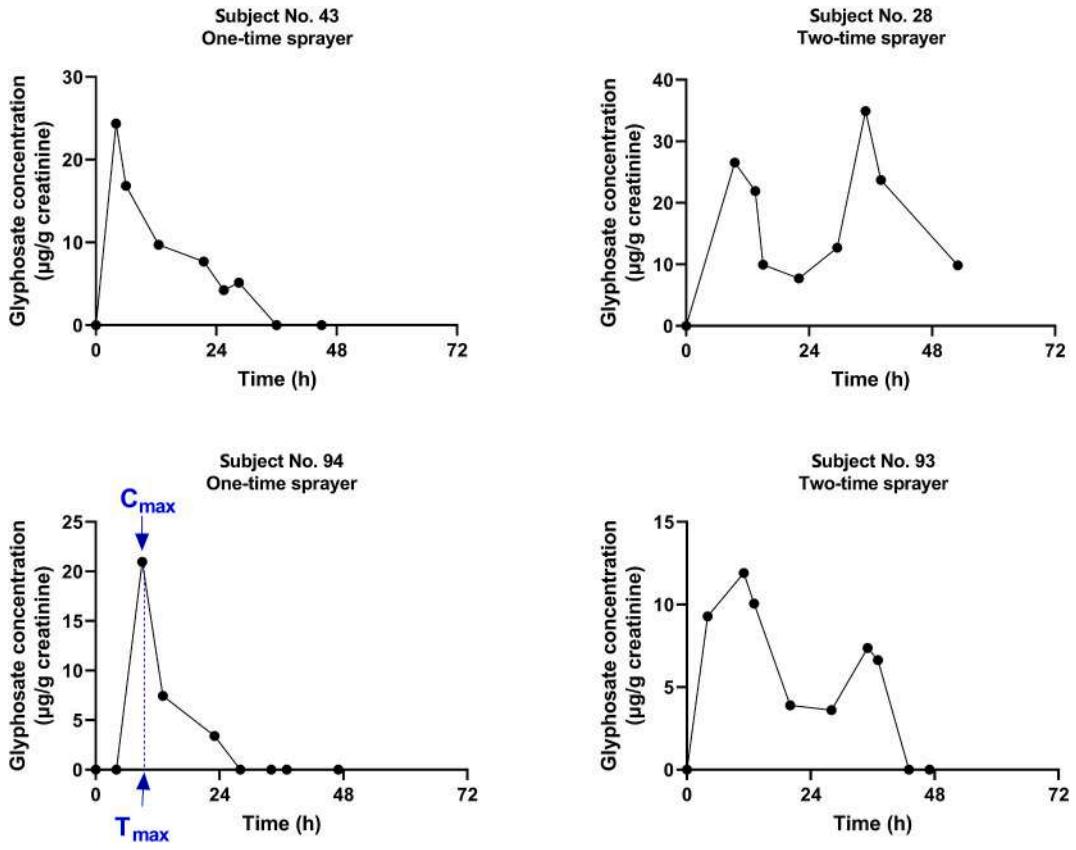

Fig. 2. Characteristics of urinary concentration-time profiles selected for the toxicokinetic study.

describe the basic features of the data in this study. Urinary glyphosate concentrations were presented as unadjusted for creatinine (µg/L) and creatinine-adjusted concentrations (µg/g creatinine). Mann-Whitney *U* test was employed to compare the statistical difference between one-time and two-time spray groups. Multiple linear regression analysis was applied to determine any associations between independent variables or confounding factors (one-time or two-time spraying, BMI, cumulative herbicide exposure intensity index, have food/drink during work and glyphosate volume) and the urinary glyphosate toxicokinetic parameters.

3. Results

In this study, 200 samples from 59 participants had glyphosate concentrations above the LOQ (5 µg/L) as shown in Table 2, while none were quantifiable for AMPA. Out of 59 participants, only 25 participants (13 participants from one-time spray group and 12 participants from two-time spray group) met the inclusion criteria for the toxicokinetic study as described in section 2.7. Participant and work characteristics are demonstrated in Table 3.

Toxicokinetic parameters are shown in Table 4. The uC_{max} and uT_{max} values between the one-time spray group and the two-time spray group were not statistically different (p-value = 0.810 and 0.639,

Table 2
Urinary glyphosate concentrations in one-time spray participants (n = 29) and two-time spray participants (n = 30).

	Number of urine samples (number of participants)	Glyphosate concentration (µg/L)		Creatinine-adjusted glyphosate concentration (µg/g creatinine)	
		Mean ± SD	Range	Mean ± SD	Range
One-time spray	80 (29)	27.4 ± 30.1	5.1 to 172.3	15.0 ± 14.6	1.7 to 65.8
Two-time spray	120 (30)	32.7 ± 41.5	5.5 to 296.4	19.3 ± 26.1	1.8 to 170.4

Table 3
Participant and work characteristics (n = 25).

Participant and work characteristics	One-time spray (n = 13)	Two-time spray (n = 12)
Sex		
Male	8 (61.5%)	9 (75.0%)
Female	5 (38.5%)	3 (25.0%)
Age (years)	50 ± 9 (39–66)	54 ± 9 (37–73)
Drinking Alcohol	12 (92.3%)	9 (75.0%)
Smoking	3 (23.1%)	9 (75.0%)
Time and exposure		
Length of time working with glyphosate-based herbicides (years)	32 ± 10 (11–46)	34.8 ± 13.3 (9–58)
Length of glyphosate-based herbicide exposure in a day (h/day)	3.8 ± 2 (1–8)	7.1 ± 2.6 (3–10)
Volume of glyphosate (L)	6.1 ± 6 (2–24)	8.6 ± 3.0 (3–14)
Equipment		
High-pressure lance sprayer	12 (92.3%)	12 (100%)
Manual lever-operated knapsack sprayer	1 (3%)	–
Behaviors		
Mix and load glyphosate	10 (76.9%)	11 (91.7%)
Handle the equipment	10 (76.9%)	12 (100%)
Clean and collect the equipment	12 (92.3%)	11 (91.7%)
Use masks or respirators	3 (23.1%)	7 (58.3%)
Wear rubber gloves	4 (30.8%)	10 (83.3%)
Wear rubber boots	13 (100%)	12 (100%)
Wear goggles or face shields	1 (7.7%)	4 (33.3%)
Have food or drink during spraying task	7 (53.8%)	9 (75.0%)
Wash hands after spraying task	7 (53.8%)	10 (83.3%)

Table 4
Urinary glyphosate toxicokinetic parameters (n = 25).

Variable	Glyphosate concentration				Creatinine-adjusted glyphosate concentration				
	n	Mean ± SD		Median (Range)		n	Mean ± SD		Median (Range)	
One-time spray										
$ut_{1/2}$ (h) (time 0–48 h)	13	9.9 ± 6.1		7.4 (5.4–23.3)		13	9.9 ± 7.1		7.0 (3.9–26.5)	
uC_{max} (μg/L or μg/g creatinine)	13	46.2 ± 26.7		43.1 (16.2–98.6)		13	32.0 ± 16.8		27.9 (11.8–65.8)	
uT_{max} (h)	13	12.9 ± 11.2		9.0 (3.5–32.0)		13	13.4 ± 10.1		11.0 (3.5–32.0)	
Two-time spray										
$ut_{1/2}$ (h) (time 0–72 h)	12	24.4 ± 14.9		23.9 (5.4–49.2)		12	23.5 ± 17.8		18.1 (4.4–67.5)	
uC_{max} (μg/L or μg/g creatinine)	12	Day 1 45.2 ± 8.9	Day 2 30.2 ± 28.6	Day 1 29.6 (13.7–134.8)	Day 2 19.5 (3.2–103.4)	12	Day 1 36.2 ± 25.9	Day 2 30.0 ± 46.1	Day 1 29.2 (10.3–96.2)	Day 2 17.1 (1.8–170.4)
uT_{max} (h)	12	Day 1 10.5 ± 5.1	Day 2 11.3 ± 7.8	Day 1 10.0 (3.0–21.5)	Day 2 9.5 (1.0–24.0)	12	Day 1 10.5 ± 5.1	Day 2 11.3 ± 7.8	Day 1 10.0 (3.0–21.5)	Day 2 9.5 (1.0–24.0)

respectively). The medians of elimination $ut_{1/2}$ in the two-time spray group (18.1 h, time 0–72 h) and in the one-time spray group (7.0 h, time 0–48 h) were not compared due to the two exposures in the two-time spray group and the effect of enterohepatic circulation (Table 4). However, the sample size used for this estimation is relatively low, therefore, more subjects should be included in a future study to minimize an unexpected uncertainty for the toxicokinetic study. Moreover, the accumulative elimination concentrations were demonstrated every 6 h as shown in Table 5. The average concentration at 30–36 h in the two-time spray group was noticeably higher than that in the one-time spray group.

As there are outliers in the data set, the median and interquartile range are used to summarize a typical value and the variability. The median accumulative glyphosate amounts over 72 h and interquartile range are demonstrated in Fig. 3 as subsets of the different collection period.

Pre-task concentrations of glyphosate and the excreted glyphosate amounts over the collection period (μg glyphosate/72 h-urine sample) are shown in Table 6. For the two-time spray group, the concentrations of glyphosate of the pre-work task on the second day (10.63 μg/g creatinine) were notably higher than the first day (less than 0.05 μg/g creatinine). This could be a consequence of an internal accumulation of glyphosate from day-one (Fig. 3) leading to high concentration of pre-task dose on day-two.

Detectable data from the eligible participants was used for the multiple linear regression analysis in order to investigate whether famer behaviors and work routine have impact on the kinetic parameters as shown in Table 7. The regression analysis demonstrated that if participants were exposed to glyphosate herbicide more than one time, the $ut_{1/2}$ would be expanded to approximately 23 h (B = 22.90; p-value = 0.01). An increase in glyphosate volume would slightly prolong the uT_{max} for approximately 1 h. However, BMI, cumulative herbicide exposure intensity index, have food/drink during spraying task, and glyphosate volume, did not impact uT_{max} and uC_{max} on Day 2 of the two-time sprayers.

Table 5
Accumulative glyphosate concentration (μg/g creatinine) at different time points.

Time (h)	One-time spray Mean ± SD	Two-time sprays Mean ± SD
0	1.6 ± 4.6	4.2 ± 7.8
0–6	23.7 ± 30.5	28.3 ± 31.7
6–12	21.5 ± 18.0	17.1 ± 12.3
12–18	11.5 ± 10.3	15.8 ± 15.3
18–24	6.4 ± 5.5	14.9 ± 18.3
24–30	7.2 ± 12.8	27.4 ± 52.3
30–36	9.7 ± 20.0*	16.9 ± 15.6*
36–42	5.9 ± 8.8	7.9 ± 8.5
42–48	3.1 ± 3.6	7.1 ± 8.9
48–54	5.4 ± 7.0	3.6 ± 5.1
54–60	7.2 ± 10.2	17.4 ± 8.7
60–66	–	13.9 ± 0.0

* Significant difference: Mann-Whitney *U* test.

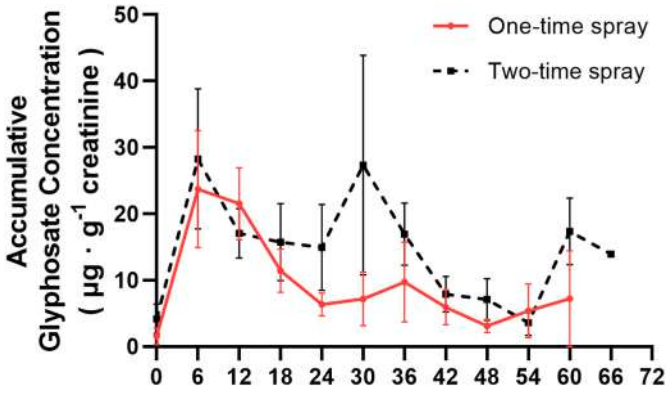

Fig. 3. The median and interquartile ranges of the accumulative urinary glyphosate concentrations at the different time points.

Table 6
The concentrations of glyphosate before work tasks and amounts of glyphosate over 48 h.

Variable	Glyphosate concentration Pre-task 1st day (μg/g creatinine)	Glyphosate concentration Pre-task 2nd day (μg/g creatinine)	Glyphosate amounts (μg glyphosate/72 h-urine volume)
	Median (range)	Median (range)	Median (range)
One-time spray	<0.05 (0–49.93)	5.03 (0–34.18)	71.47 (31.57–279.60)
Two-time sprays	<0.05* (0–24.33)	10.63* (0–170.40) *p = 0.0137	101.50 (38.27–424.60)

* Mann-Whitney *U* test (Pre-work task day one vs Pre-work task day two).

4. Discussion

In this study, the average urinary glyphosate concentrations in one-time spray participants (n = 29) and two-time spray participants (n = 30); were 27.4 ± 30.1 and 32.7 ± 41.5 μg/L or 15.0 ± 14.6 and 19.3 ± 26.1 μg/g creatinine, respectively (Table 2).

These other studies had urinary glyphosate concentrations 46.9 ±

Table 7
Factors involving in the urinary toxicokinetic parameters (n = 25).

Variable	uC_{max} (Day 1)			uT_{max} (Day 1)			$ut_{1/2}$			48-h glyphosate amount		
	B	SE	*P*-value	B	SE	*P*-value	B	SE	*P*-value	B	SE	*P*-value
One-/Two -time spray	1.92	11.95	0.87	−2.16	3.76	0.57	**22.90**	**7.18**	**0.01**	52.45	59.69	0.39
Body mass index (kg/m^2)	0.76	1.53	0.62	0.57	0.48	0.25	0.34	0.92	0.72	10.65	7.66	0.18
Cumulative herbicide exposure intensity index	0.10	0.09	0.28	−0.01	0.03	0.73	−0.12	0.06	0.05	0.05	0.46	0.92
Have food/drink during spraying task	−13.45	9.57	0.18	−5.63	3.01	0.08	2.66	5.75	0.65	−3.52	47.79	0.94
Volume of glyphosate (L/day)	−0.73	1.13	0.53	**1.02**	**0.36**	**0.01**	0.16	0.68	0.82	−1.38	5.64	0.81

SE is standard error, Bold indicate statistic significant data.

1.3 μg/g creatinine in participants using manual pump backpack sprayers (Bootsikeaw et al., 2021). However, the glyphosate concentrations found in Thai farmers were much higher than those demonstrated in the studies from Ireland, United States, and Sri Lanka. These other studies revealed the urinary glyphosate concentrations ranging from 0.29 μg/L to 10.66 μg/L (Connolly et al., 2017, 2018a; Jayasumana et al., 2015; Mesnage et al., 2012). The high levels of urinary glyphosate detected in our study could be a result of the volumes of glyphosate use, activities, farmer behaviors, and PPE usage. Most Thai farmers usually wore protective clothing such as long-sleeved shirts and pants made from cotton or cotton-like fabrics. These types of woven fabrics have less protective capacity for pesticide penetration prevention in comparison with other types of fabrics, for example Tychem® C coveralls (Naksata et al., 2020). The Tyvek suit worn by the Irish workers (Connolly et al., 2018b) is more protective than woven fabric (Naksata et al., 2020).

As the urinary AMPA levels were lower than the LOQ levels, it was not possible to estimate the toxicokinetic parameters of each participant. The small amount of AMPA in our study is consistent with several studies reviewed by Connolly et al., in 2020, which summarized that urinary AMPA levels had a tendency to be lower than the urinary glyphosate levels. (Connolly et al., 2020a). Zoller et al. proposed that glyphosate was unlikely to transform to AMPA and that the AMPA detected in urine could be a result of the ingestion of AMPA via food residues (Zoller et al., 2020). Huch et al. suggested that the glyphosate biotransformation to AMPA in the gut would be imperceptible in humans. Furthermore, only 0.2–0.3% of AMPA converted from glyphosate and excreted in urine (Huch et al., 2021; Zoller et al., 2020). In addition, the studies in animals confirmed that only small levels of AMPA were detected after oral administration (EFSA, 2015). The urinary half-life of glyphosate for the one-time and two-time spray groups in this study were approximately 7 and 18 h, respectively. This may imply that glyphosate will not be able to detect in urine within approximately 1.5 days (3% remaining in urine) in a one-time spray participant. The half-life of the two-time spray group was significantly longer than the one-time spray group due to multiple/repeated exposure. Farmers, who used glyphosate on consecutive days or twice in one day during farm work, would have detectable glyphosate in urine up to approximately 5 days (3% remaining in urine) after exposure. However, these estimated half-lives could be affected by individual doses and times of exposure. In this study, the exact herbicide amount or concentration used or concentration in blood during spraying were not determined. Future study should include these parameters into account in order to eliminate any inaccurate on kinetic analysis.

Even if this present study used the non-compartmental model for kinetic analysis, the urinary half-life of one-time spray participants in our study was comparable to previous studies demonstrating the half-life of 7.2 h (Connolly et al., 2019b) and 9.05 h (Zoller et al., 2020). The study from Sweden indicated that urinary glyphosate excretion is likely to be a first-order kinetics with a two-phase excretion (Faniband et al., 2021). The first-order apparent half-life (creatinine adjusted) was approximately 3.9–10.0 h in the rapid phase and the slower phase half-life presented in the Swedish study was 16–35 h after an oral dose equivalent to 50% of the ADI for glyphosate (Faniband et al., 2021).

Zoller et al. determined the fraction of glyphosate and AMPA excretion in urine after consuming food with glyphosate residue (Zoller et al., 2020). Based on their results, glyphosate excretion data are best fitted with a one-compartment model, first-order absorption, and elimination kinetics with the uT_{max} of 5.62 h (median) (Zoller et al., 2020).

Connolly et al. reported the uT_{max} in horticulturists between 1 and 3 h after the end of work shift (Connolly et al., 2018a). On the other hand, the uT_{max} in our study on Thai farmers ranged between 12 and 14 h which was much longer than that previous reports (Connolly et al., 2019b; Zoller et al., 2020). A magnitude higher of uT_{max} could be attributed to the exposure duration, repeated exposures and spraying higher quantities of glyphosate-based herbicides. The multiple linear regression analysis from our study revealed that farmers who used high volumes of glyphosate trended to have longer uT_{max}.

The factors that affect the half-life were analyzed in this study. The linear regression analysis revealed that farmers who repeated use of glyphosate over 48 h would have the $ut_{1/2}$ longer by approximately 23 h as shown in Table 7. Considering this finding, the following reasons may explain the delay of glyphosate elimination. First, the farmers had been recurrent exposed to glyphosate from two spraying events, accordingly, the glyphosate took longer time to clear the repeated doses from the body. Additionally, the extended urinary glyphosate elimination could be a consequence of a cumulative dose from multiple glyphosate exposure over the spraying periods (Arcury et al., 2010). As reported by Shalat et al. (2003), the inadvertent ingestion of pesticide from hand-to-mouth events could contribute to an elevation of urinary pesticide levels (Shalat et al., 2003). Having food/drink during the spray task have no effects on the $ut_{1/2}$, thus, the inadvertent ingestion via ingestion parameters may not play a part in prolonging the $ut_{1/2}$ in our study. However, the inadvertent ingestion can also occur from smoking while working, wiping face due to sweating or adjusting the PPE during spraying. These activities could potentially contribute to total body burden and may be an exposure route of importance, and should be taken in to account for glyphosate elimination kinetic interpretation (Cherrie et al., 2006).

The frequency of spraying, body mass index, cumulative herbicide exposure intensity index, having food/drink during spraying task and volume of glyphosate used did not show to have any impact on the excreted glyphosate amounts over 48 h. This suggested that the glyphosate consistency excreted into urine over 48 h. Zoller et al. reported that the glyphosate excretion was completed within 10–20 h after oral route and the excretion of all of the participants reached a plateau after 45 h (Zoller et al., 2020).

Fig. 3 illustrated that the glyphosate concentration gradually decreased over 72 h with two peak of glyphosate concentrations for the two-time sprayers. Dermal absorption or reabsorption of the herbicide from hands could cause the moderate decline of the second excretion phase. Connolly et al. investigated whether dermal and inadvertent ingestion exposure contribute to the urinary glyphosate concentration in amenity horticultural workers (Connolly et al., 2019a). They revealed that the workers not only received glyphosate from inhalation glyphosate droplets during work but also from contamination on hands and gloves leading reabsorption and inadvertent ingestion of this herbicide after working task. The information demonstrated in our present study together with the previous studies confirmed that hygiene and PPE uses

are necessary for risk protection when working with glyphosate-surfactant herbicides. In Thailand even if most farmers have been educated that they should use PPE while working in the field, they are still not aware of the dangers of persistent exposure of glyphosate.

According to the linear regression (Table 7), food and drinking during task did not have any impacts on glyphosate concentrations. Moreover, the pre-task glyphosate concentrations (Table 6) were relatively low. This indicates that glyphosate exposure via ingestion route is unlikely involving in detected urinary glyphosate concentrations.

This current study has some significant limitations. Firstly, a spot urine sampling probably underestimates the exposure dose if participants did not collect all the voided urine specimens. Moreover, although all participants were educated and trained how to collect their urine samples, they might not be aware of importance of correct urine sampling procedure and how improper collection could affect the analytical results. However, only one out of twenty-five participants (4%) could not follow the procedure, this may slightly affect the analysis in this study.

Secondly, these workers perform pesticide application tasks on a daily basis. Thus, there is the possibility of a delayed dermal absorption due to the skin reservoir effect from a dose exposed several days before the urine sampling took place, leading to high concentrations in urine at day one. Thirdly, there were only two detectable urinary samples after the peak due to the detection limit of the analytical method and the timeline of the study design. Even though the half-life estimation in our study was comparable to previous studies but it may probably be less precise. Moreover, the LOQ (5 μg/L) of our study is much higher than presented in other studies (ranging from 0.05 to 0.5 μg/L) (Connolly et al., 2017, 2020b; Zoller et al., 2020). Subsequently, a low frequency of glyphosate detection in our study leading to a smaller sample size for kinetic study.

5. Conclusion

This study is the first study to report on the urinary toxicokinetic parameters in occupational exposure to glyphosate in an Asian population. The elimination half-life in urine was 7 h for the one-time spray group and 18 h for the two-time spray group. The accumulation of glyphosate-based herbicide exposure impacted the uT_{max} indicating that the farmer behavior during work, PPE usage, and time/dose of exposure had an impact on the kinetics. This information could be applied to emphasize the importance of safety procedures when glyphosate-based-herbicide is used. The farmers should increase their awareness and should pay more attention to their PPE usage to reduce any subsequent adverse effects. As glyphosate has become a primary herbicide used in Thailand after paraquat is banned, more study on the adverse effects of using glyphosate products should be prioritized. The urinary glyphosate data can be helpful as a non-invasive sampling strategy for studying the health effects of glyphosate exposure and risk assessment.

Authors' contributions

KW conceived the present idea of this manuscript. KW, SK, RS and NK involved in planning and supervised the work. KK, UI, SK and KW collected and managed samples. KK and UI interviewed the participants. KK and KW carried out the LC-MS/MS experiments. KK, NK, ST and KW analyzed toxicokinetic parameters. KK, KW, SK and NK interpreted the results and data analysis. KK, NK, and KW wrote an original draft of the manuscript. All authors have read, edited, and agreed to the published final version of the manuscript.

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Useful molecular tools for facing next pandemic events: Effective sample preparation and improved RT-PCR for highly sensitive detection of SARS-CoV-2 in wastewater environment

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ABSTRACT

Viral pandemics can be inevitable in the next future. Considering SARS-CoV-2 pandemics as an example, there seems to be a need to develop a surveillance system able to monitor the presence of potential pathogenic agents. The sewage and wastewater environments demonstrated to be suitable targets for such kind of analysis. In addition, it is important to have reliable molecular diagnostic tools and also to develop a robust detection strategy. In this study, an effective sample preparation procedure was selected from four options and combined with a newly developed improved RT-PCR. First, a model viral system was constructed, containing a fragment of the SARS-CoV-2 gene encoding for the Spike protein. The encapsidated *S* RNA mimic (ESRM) was based on the plum pox virus (PPV) genome with the inserted targeted gene fragment. ESRM was used for seeding wastewater samples in order to evaluate the viral recovery of four different viral RNA concentration/extraction methods. The efficiency of individual approaches was assessed by the use of a quantitative reverse transcription PCR (qRT-PCR) and by a one-step single-tube nested quantitative reverse transcription PCR (OSN-qRT-PCR). For the detection of viruses in wastewater samples with low viral loads, OSN-qRT-PCR assay produced the most satisfactory results and the highest sensitivity.

1. Introduction

The COVID-19 pandemic has shown the importance to have reliable and robust detection methods for attesting the presence of the pathogenic agents and therefore to organize the suitable countermeasures. It became evident that monitoring of anthropized environments such as wastewater can be considered a valuable surveillance tool that consequently can help health operators to follow the pandemic trend and manage it in an effective way (Zhu et al., 2021).

It is necessary to have various molecular tools that can simulate a real situation and can be used to create robust detection methods. Various attempts were already performed by other authors to detect SARS-CoV-2 from wastewater samples and to evaluate several available procedures using model samples. A common characteristic of some efforts was the use of surrogates for SARS-CoV-2, such as murine hepatitis virus, bovine coronavirus or feline calicivirus (Ahmed et al., 2020; Barril et al., 2021; LaTurner et al., 2021). Another option was the use of naked plasmids harboring SARS-CoV-2 sequences or the use of a commercial reference material (Sapula et al., 2021). As an important step, various approaches to concentration of SARS-CoV-2 from wastewater samples were evaluated, including polyethylene glycol (PEG) precipitation, ultracentrifugation or filtration methods (La Rosa et al., 2020a; LaTurner et al., 2021). Then, the concentrated viral RNA could be extracted by various methods, allowing it to be amplified by a specific real-time reverse transcription PCR assay. Such assays were oriented to viral surrogates or to molecular systems of SARS-CoV-2 (Bivins et al., 2021; Tran et al., 2021). The described strategies were applied to evaluate the recovery rates of viral concentration and RNA extraction

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methods of wastewater samples based on quantification of viral RNA by quantitative reverse transcription PCR assays (qRT-PCR). Among the potential detection methods, digital RT-PCR (dRT-PCR) or, in particular, droplet-based digital RT-PCR (ddRT-PCR) is suitable for the analysis of samples with low viral load (Yu et al., 2020). However, digital PCR is a low-throughput method with sensitivity comparable to RT-PCR.

Potential SARS-CoV-2 positive control for optimizing diagnostic procedures could involve specifically produced encapsidated RNA mimics and some examples of this strategy have already been published (Chan et al., 2020; Peyret et al., 2022). Plant viruses are non-infectious and non-toxic for humans; thus, they provide a suitable tool for this purpose. Incorporation of partial SARS CoV-2 sequence in the genome of a plant RNA virus enables its autonomous replication and encapsidation *in vivo*, resulting in the production of RNA mimics with stability comparable to native SARS-CoV-2 particles. Moreover, propagation of viruses in plants is efficient and cheap and their purification is relatively fast and simple, based mainly on ultracentrifugation (Bhat and Rao, 2020).

Produced encapsidated mimics could be used for artificial contamination of various kinds of samples and for testing various detection methods, including qRT-PCR. In order to increase the detection sensitivity of a qRT-PCR assay, the creation and optimization of a nested PCR system could be a useful alternative. Single-tube nested real-time PCR approaches have been frequently used to improve the detection of various pathogen agents and components in food and clinical samples including SARS-CoV-2 (Minarovičová et al., 2011; Costa et al., 2012; Wang et al., 2020; Yip et al., 2020). Wang et al. (2020) in their work demonstrated that clinical samples with low viral load could also be detected and quantified by the one-step single-tube nested quantitative reverse transcription PCR (OSN-qRT-PCR).

The schematic diagram in Fig. 1 describes the two PCR assays. OSN-qRT-PCR is based on the application of two pairs of primers (outer and inner) in one reaction tube, with different annealing temperatures (difference about 10 °C) to control the first and second rounds of PCR. The outer primers have higher melting temperature should anneal during the first 10 cycles of PCR. The inner primers should anneal to the products of the former PCR round at a lower annealing temperature during the next 40 PCR cycles, with the final detection of the fluorescence signal produced by the specific labelled probe.

Contrary to ddRT-PCR, the operation of the OSN-qRT-PCR method is the same as qRT-PCR and there is no need of extra professional training. Besides, the OSN-qRT-PCR assay is feasible in any qPCR-instrument-equipped laboratory. The cost of OSN-qRT-PCR is lower than ddRT-PCR and the turn-around time of OSN-qRT-PCR is shorter (2 h) than that of ddRT-PCR (3–4 h) though a bit longer than that of qRT-PCR (about 1 h and 30 min).

Our aim was to find a reliable and robust qRT-PCR detection method for SARS-CoV-2 in wastewater by designing a laboratory analysis system, able to mimic real conditions. Such analysis system can provide a model for future development of detection procedures for viral pandemics. For this reason, in this study we compared various strategies for the detection of SARS-CoV-2 in wastewater samples using different molecular tools: i) an encapsidated *S* RNA mimic (ESRM) based on the plum pox virus (PPV) genome bearing a fragment of SARS-CoV-2 Spike protein-coding sequence, ii) four different viral RNA extraction/concentration methods, iii) the application of two different detection assays: a conventional qRT-PCR and OSN-qRT-PCR.

2. Materials and methods

2.1. Construction of the encapsidated S RNA mimic (ESRM)

The viral vector pAD-agro consists of a full-length cDNA of the strain PPV-Rec (Predajňa et al., 2012) cloned in the commercial plasmid pCambia 1304 (Abcam, Cambridge, UK) with deleted β-glucuronidase gene. The cloning cassette comprising an *Eag*I/*Kpn*I linker and sites recognized by viral protease are inserted in the PPV polyprotein-coding region between the replicase (*NIb*) and capsid protein (*CP*) genes (Fig. S1). Biological safety was ensured by mutagenesis of the DAG motif within the CP-coding region, essential for aphid transmission (Blanc et al., 1997; Kamencayová and Šubr, 2012).

The 447 nt long fragment of the SARS CoV-2 *S* gene was amplified by PCR from cDNA of the isolate hCoV-19/Slovakia/SK-BMC5/2020 (GISAID.org accession ID EPI_ISL_417,879). PCR amplification was performed using EX Taq DNA Polymerase (Takara, Shiga, Japan) under the following conditions: initial denaturation at 94 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 56 °C for 20 s, elongation at 72 °C for 30 s and a final extension at 72 °C for 3 min. Used primers are specified in Table 1. The amplified product was inserted into *Kpn*I-digested pAD-agro using In-fusion HD Cloning Kit (Takara) and transformed into *E. coli* JM109. Plasmid DNA was isolated by QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and verified by sequencing. The resultant plasmid construct was electroporated into *Agrobacterium tumefaciens* EHA105. Agrobacteria from an overnight culture were sedimented by centrifugation (16000 g for 1 min) and resuspended in 10 mM 2-(N-morpholino)ethanesulfonic acid pH 5.6, 10 mM $MgCl_2$, 200 μM acetosyringone to reach final OD_{600} of ~0.1. The suspension was incubated at room temperature for 2 h and subsequently infiltrated into several leaves of 3–4 weeks old *Nicotiana benthamiana* by a needleless syringe (20 μl per plant). The plants were cultivated under controlled conditions (temperature 20–22 °C, 12 h light/dark photoperiod). Symptoms of viral infection were evaluated visually, the presence of PPV in plant tissues was confirmed by Western blotting using a specific polyclonal antibody (Šubr and Matisová, 1999), followed by

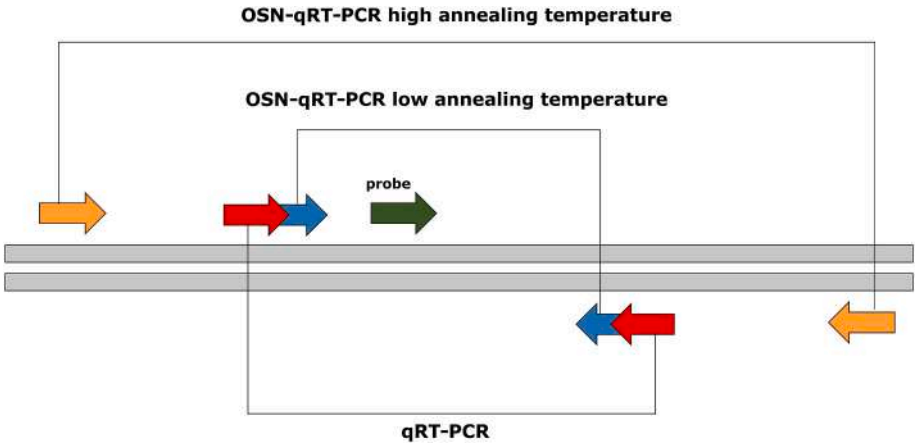

Fig. 1. Schematic diagram of qRT-PCR and OSN-qRT-PCR assays.

Table 1
Oligonucleotides used in this study.

Purpose	Primer/probe name	Sequence (5' – 3')	Amplicon size	Reference
amplification of SARS CoV-2 *S* gene fragment	IF-CoS For	[a]ATC AGG CCG GCC GGG GTA CCA TTG GCA AAA TTC AAG ACT CAC	447 bp	This work
	IF-CoS Rev	[a]GTG CAC AAC AAC GTT GGT ACC AGG AGC AGT TGT GAA GTT C		
recombinant analysis	NCuniFor	GAG GCA ATT TGT GCT TCA ATG G	1226 bp	Šubr et al. (2010)
	NCuniRev	CGC TTA ACT CCT TCA TAC CAA G		
qRT-PCR	HOT_Spike_Fw	AGT GCA AAT TGA TAG GTT GATC	88 bp	This work
	HOT_Spike_Rv	TCT GAT TTC TGC AGC TCT AAT TA		
OSN-qRT-PCR	LANL_May4.1_Fw	CRC GTC TTG ACA ARG TTG AGG CT	155 bp	https://covid19.edgebioinformatics.org/#/assayValidation
	LANL_May4.1_Rv	TAC ACA CTC TGA CAT TTT AST AGC AGC		
OSN-qRT-PCR	Inner_Spike_Fw	AGT GCA AAT TGA TAG GTT G	85 bp	This work
	Inner_Spike_Rv	GAT TTC TGC AGC TCT AAT TA		
qRT-PCR OSN-qRT-PCR	P-LANL_4.1	FAM-GGC AGA CTT CAA AGT TTG CA-BHQ1	probe	This work

[a] Sequence of the vector adjacent to the cloning site (for hybridisation at in-fusion cloning) is underlined.

further analysis by RT-PCR. Total leaf RNA was isolated using Nucleospin RNA Plant kit (Macherey-Nagel, Düren, Germany) and cDNA was prepared using random hexamer primers and AMV reverse transcriptase (Promega, Madison, Wisconsin, USA). RT-PCR was performed using primers NCuniFor/NCuniRev (Šubr et al., 2010) spanning the cloning cassette of pAD-agro (Fig. S1, Table 1). Electrophoretic analysis and sequencing of amplification products enabled verification of the inserted fragment.

The virus was purified two weeks after infiltration according to Laín et al. (1988) with certain modifications. The protocol included extraction from plant tissues with two volumes (2 mL per gram of tissue) of 18 mM McIlvain citrate-phosphate buffer pH 7 with 0.2% thioglycolic acid, 10 mM sodium diethyldithiocarbamate, 0.5 M urea, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride and 1/3 volume of chloroform, followed by phase separation by centrifugation at 1520*g* for 30 min and ultracentrifugation of the water phase at 57000 g for 2 h. The sediment was resuspended in 100 mM sodium borate pH 8.2 with 10 mM EDTA and clarified by low-speed centrifugation (1520 g for 15 min). Sucrose was added to the solution to the final concentration of 20% and another round of ultracentrifugation (57000 g for 2 h) was performed. The purified virus serving as ESRM was obtained by resuspension of the sediment in a small volume of 10 mM Tris-HCl pH 8 with 1 mM EDTA and clarification by centrifugation (16000 g for 5 min). The concentration of purified PPV was estimated by UV spectrophotometry, using an extinction coefficient of 2.4 mL/mg/cm (Purcifull, 1966) and concentration of encapsidated RNA calculated as 5% of that value (Hollings and Brunt, 1981) to be 8.1 x 10^8 genomic copies/mL (GC/mL). The final product was stored in aliquots at −20 °C until used.

2.2. Wastewater sample preparation

A sample of untreated wastewater influent was collected from a wastewater treatment plant treating municipal wastewater in Kysucké Nové Mesto (Slovakia). The wastewater sample was kept at 4 °C for 24 h and it had these basic characteristics: Chemical Oxygen Demand (COD) 750 mg/L; N 139 mg/L; Suspended Solids (SS) 312 mg/L; pH 8.14; T 17 °C. The wastewater was negative for SARS-CoV-2 by both qRT-PCR and OSN-qRT-PCR coupled to Method A (section 2.3.1.) with NucleoSpin RNA Virus Kit (Macherey-Nagel). ESRM was added to untreated wastewater at 1% (v/v) to a final concentration of 8.1 × 10^6 GC/mL and subjected to particular virus concentration/extraction protocols. In order to assess the detection limit of the two best concentration/extraction methods, the above samples were serially diluted to concentrations from 8.1 × 10^4 GC/mL to 8.1 × 10^2 GC/mL of ESRM and tested in triplicate.

2.3. Virus concentration and RNA extraction methods

The virus particles were concentrated by using four methods (A-D) from the wastewater influent, which was artificially contaminated as shown in Fig. 2.

2.3.1. Method A

Method A employed polyethyleneglycol (PEG) precipitation, which is commonly used to concentrate viruses from water matrices (Warish et al., 2020). In this study, we used protocol described by Wu et al. (2020) with some modifications. Artificially contaminated wastewater samples with ESRM (50 ml; wastewater/ESRM) were clarified from particulate biomass by centrifugation for 30 min at 4500 g and 4 °C (Medema et al., 2020). Meanwhile, a 50 mL Falcon tube with 4 g polyethyleneglycol 8000 (PEG 8000; Sigma-Aldrich, Saint Louis, Missouri, USA) and 0.9 g sodium chloride was prepared. After centrifugation, 40 mL of the supernatant was carefully transferred to the Falcon tube with PEG solution. The sample was inverted several times in hand during approximately 15 min at room temperature and permanent agitation and pelleted by centrifugation for 45 min at 12000g and 4 °C without brake. After centrifugation, the supernatant was removed by decantation via the opposing side of the pellet. The tube with the pellet was returned to the centrifuge again and centrifuged at 12,000 *g* for 5 min and 4 °C with a brake intensity set to 3 (of 9). The supernatant was carefully removed, and the pellet was dissolved in 800 μl of TRI Reagent (solution for RNA isolation; Molecular Research Center, Cincinnati, Ohio, USA) by vortexing for 15 s. After centrifugation at 2000*g* for 10 s the supernatant was transferred to a clean 1.5 mL microtube and used for RNA extraction.

Two different kits for rapid preparation of highly pure viral nucleic acids from wastewater samples were used: NucleoSpin RNA Virus Kit by Macherey-Nagel and AllPrep PowerViral DNA/RNA Kit by Qiagen.

NucleoSpin RNA Virus Kit was used for the isolation of viral RNA from concentrated wastewater samples according to the manufacturer's instructions.

Alternatively, AllPrep PowerViral DNA/RNA Kit (Qiagen) was used for isolation of viral RNA. The manufacturer's instructions were followed, but the volume of the elution buffer was reduced to 50 μl.

Extracted RNA was used immediately for qRT-PCR and OSN-qRT-PCR or stored at −70 °C.

2.3.2. Method B

Method B was based on ultracentrifugation, which is frequently used to concentrate viruses from wastewater (Fumian et al., 2010). In this study, we used the protocol published by Ahmed et al., (2020) with some modifications. The wastewater/ESRM sample (20 ml) were poured to polycarbonate ultracentrifuge bottles (# 355,618, Beckman Coulter,

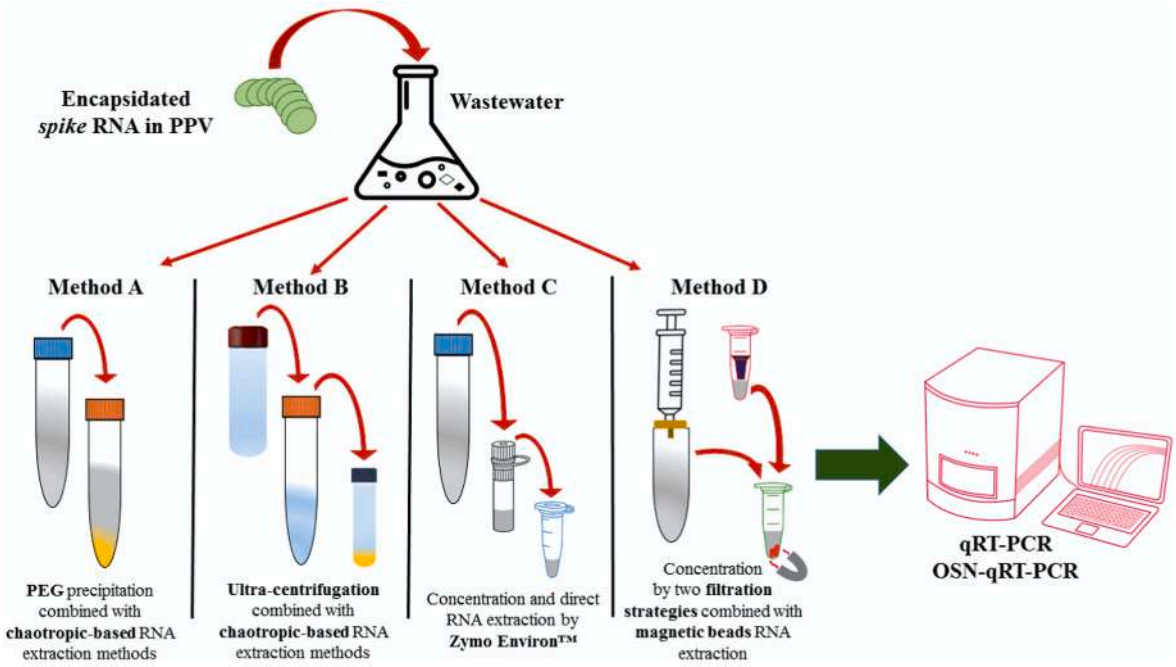

Fig. 2. Illustration of concentration/extraction methods evaluated in this study.

Indianapolis, Indiana, USA) and subjected to ultracentrifugation at 100000g for 1 h at 4 °C (Type 70 Ti rotor, Beckman Coulter, # 337,922) in an Optima XPN-90 Ultracentrifuge (Beckman Coulter, # A94468). The supernatant was removed, and the pellet was resuspended in 3.5 mL of 0.25 N glycine buffer (pH 9.5). Then, the sample was incubated on ice for 30 min and 3 mL of 2x phosphate buffered saline (PBS; pH 7.2) for neutralization was added. The sample was centrifuged at 12000g for 15 min at 4 °C. After that, the virus particles were recovered by ultracentrifugation again in polycarbonate ultracentrifuge bottles (Beckman Coulter, # 355,603) at 100000*g* for 1 h at 4 °C using a Type 90 Ti rotor (Beckman Coulter, # 355,530) in an Optima XPN-90 Ultracentrifuge. The pellet was resuspended in 400 µl of PBS (pH 7.2) and transferred to a microtube (1.5 mL) for RNA extraction. RNA was extracted using the two kits (NucleoSpin RNA Virus Kit, AllPrep PowerViral DNA/RNA Kit) as described above.

2.3.3. Method C

Method C applied commercial Zymo Environ Water RNA kit (Zymo Research, CA, USA). It included viral enrichment (from 4 ml), sample homogenization and RNA purification. We followed the protocol of producer with exclusion that elution volume was increased to 30 µl. RNA was used for qRT-PCR and OSN-qRT-PCR immediately or stored at −70 °C.

2.3.4. Method D

NucleoMag DNA/RNA Water Kit (Macherey-Nagel) designed for the isolation of microbial DNA/RNA from water was applied on filter-concentrated samples. The procedure is based on reversible adsorption of nucleic acids to paramagnetic beads under appropriate buffer conditions. The manufacturer's instructions for the extraction of RNA from water samples were followed with slight modifications. The filtration step was performed with two different membrane types. A 0.2 µm pores cellulose ester membrane (Whatman, Little Chalfont, UK) was after filtering wastewater/ESRM samples (10 ml) rolled into a cylinder and inserted into a Bead Tube with lysis buffer.

Alternatively, Vivaclear Mini 0.8 µm polyethersulphone (PES) centrifugal filters by Sartorius (Göttingen, Germany) were after wastewater/ESRM application (500 µl) centrifuged at 2000g for 10 min. The clarified filtrate was transferred to Bead Tube (Qiagen) with lysis buffer.

Samples from both filtration procedures were agitated by Vortex-Genie (Scientific Industries Inc., Bohemia, New York, USA) for 5 min. Then the protocol for NucleoMag DNA/RNA Water kit was followed. RNA was eluted using 50 µl of RNAse-free water for each sample. RNA was used for qRT-PCR and OSN-qRT-PCR immediately or stored at −70 °C.

2.4. Primer design

Primer sets and probe were designed using the software Primer3Plus (https://dev.primer3plus.com/index.html) They were oriented to SARS-CoV-2 *Spike* sequence between the primers LANL_MAY-4.1_Fw/LANL_MAY-4.1_Rv of the assay LANL-SARS-CoV-2.May4.1 reported on the webpage: https://covid19.edgebioinformatics.org/#/assayValidation. The oligonucleotide sequences and the characteristics of corresponding PCR assays are described in Table 1.

2.5. qRT-PCR assay

Quantitative RT-PCR assays were performed in 20 µL of total reaction volume. Each reaction tube comprised 5 µL of RNA, 2x of Luna Universal Probe One-Step qRT-PCR Kit, 20x Luna Warm Start RT Enzyme Mix (New England Biolabs, Ipswich, Massachusetts, USA), 10 µM of primers HOT_Spike_Fw/HOT_Spike_Rv and 10 µM of probe P_LANL_4.1 (Table 1). After vortexing and centrifugation, the reaction tube was transferred to QuantStudio 1 Real-Time PCR System (ThermoFisher, Waltham, Massachusetts, USA). The qRT-PCR amplification consisted of following steps: 55 °C for 10 min, 95 °C for 1 min, 45 cycles of 95 °C for 10 s, 60 °C for 1 min with the collection of fluorescence signal at the end of each cycle. Each run contained positive and negative controls. Data were collected and analysed using the software QuantStudio Design and Analysis Software v1.5.2 (ThermoFisher). Treshold cycle values (Ct) were calculated using the software at the automatic threshold setting. The fluorescence signal showed a typical S-shaped amplification curve and samples with $C_t \leq 38$ were considered positive.

2.6. OSN-qRT-PCR assay

For OSN-qRT-PCR amplification, the mix was identical as for qRT-

PCR with exception of primers. Outer primers LANL_MAY_4.1_Fw/LANL_MAY_4.1_Rv and inner primers Inner_Spike_Fw/Inner_Spike_Rv were added, each to 10 μM (Table 1). The OSN-qRT-PCR amplification contained the following steps: 55 °C for 10 min, 95 °C for 1 min, 10 cycles of 95 °C for 15 s, 64 °C for 30 s, 72 °C for 40 s, followed by 40 cycles of 95 °C for 15 s and 55 °C for 30 s with the collection of fluorescence signal at the end of each cycle. The fluorescence signal showed a typical S-shaped amplification curve and samples with $C_t \leq 30$ were considered positive. RNA isolated from ESRM by NucleoSpin RNA Virus Kit was used as a standard for the sensitivity analysis of qRT-PCR/OSN-qRT-PCR assay. A standard curve was generated using 10-fold serial dilutions (10^{-1} to 10^{-9}) of ESRM RNA, and RNase-free water was used as a negative control. All the amplification reactions were run in triplicates in two independent assays.

2.7. Recovery rate

ESRM recovery rate of the four concentration/extractions methods (A-D) were calculated based on the gene copies quantified by qRT-PCR and OSN-qRT-PCR as follows:

Recovery rate (%) = (GC recovered in concentrated wastewater/GC seeded) × 100. The mean and standard deviation for each concentration method and two quantification assays were calculated.

2.8. Statistical analysis

The one-way analysis of variance (ANOVA) was used to determine whether there was a difference in ESRM recovery among the concentration techniques tested. qRT-PCR and OSN-qRT-PCR assays were compared using a Pearson's correlation test.

3. Results

3.1. Production of encapsidated RNA mimic

The prepared modified PPV was capable of replication and systemic spread in infiltrated plants. Typical disease symptoms (mosaic, vein clearing and leaf distortion) were observed approximately 7 days post infection (dpi) and subsequent Western blot analysis showed the virus accumulation similar to wild-type PPV (Fig. S2). The sequence analysis of RT-PCR products verified the presence of inserted *S* gene fragment, confirming successful production of ESRM *in planta*.

The plants were harvested 14 dpi and used for ESRM purification. In addition to major intact PPV CP, the purified ESRM contained also its partially degraded products, as demonstrated by immunoblotting analysis (Fig. S2). Partial proteolysis commonly occurs in course of potyvirus purification, affecting mainly both CP termini. Similar to mild trypsinolysis, this process has no effect on infectivity or stability of virions in purified samples (Shukla et al., 1988). The yields of ESRM reached 70–80 μg per gram of fresh green mass, corresponding approximately to 4 μg/g of RNA.

3.2. qRT-PCR and OSN-qRT-PCR assays

Two qRT-PCR assays were developed: a conventional qRT-PCR and a one-step single-tube nested qRT-PCR (OSN-qRT-PCR). The C_t values and standard deviations obtained from dilutions of ESRM's RNA up to 10^{-9} are presented in Table 2. It was observed a C_t difference of 3.291 and 3.246 between $\log_{10}$ dilutions for qRT-PCR and OSN-qRT-PCR, respectively. Based on the C_t values a linear curve (Fig. 3A and B) was created by regression analysis where score R^2 was 1 and 0.999, with efficiency 101.316% and 103.287% for qRT-PCR and OSN-qRT-PCR, respectively. C_t values of qRT-PCR and OSN-qRT-PCR had a strong correlation (R^2 value 0.985). The linear regression of the two groups had a good goodness-of-fit ($R^2 = 0.971$) with a regression equation y = 0.9188x - 5.8258 (Fig. 3C). The ALOQ (assay limit of quantification, i.e. lowest

Table 2
The C_t values and standard deviations obtained from dilutions of ESRM's RNA.

DILUTION	QRT-PCR		OSN-QRT-PCR	
	C_T	SD	C_T	SD
UNDILUTED	10.93	0.08	–	–
10^{-1}	14.11	0.12	5.75	0.05
10^{-2}	17.46	0.02	9.06	0.13
10^{-3}	20.68	0.10	12.33	0.09
10^{-4}	23.86	0.22	15.40	0.24
10^{-5}	27.00	0.16	19.03	0.26
10^{-6}	30.40	0.12	22.21	0.12
10^{-7}	33.83	0.31	24.98	0.18
10^{-8}	37.33	0.27	27.35	0.31
10^{-9}	–	–	30.42	0.34
EFFICIENCY (%)	**101.316**		**103.287**	
SLOPE	**−3.291**		**−3.246**	
r^2	**1**		**0.999**	

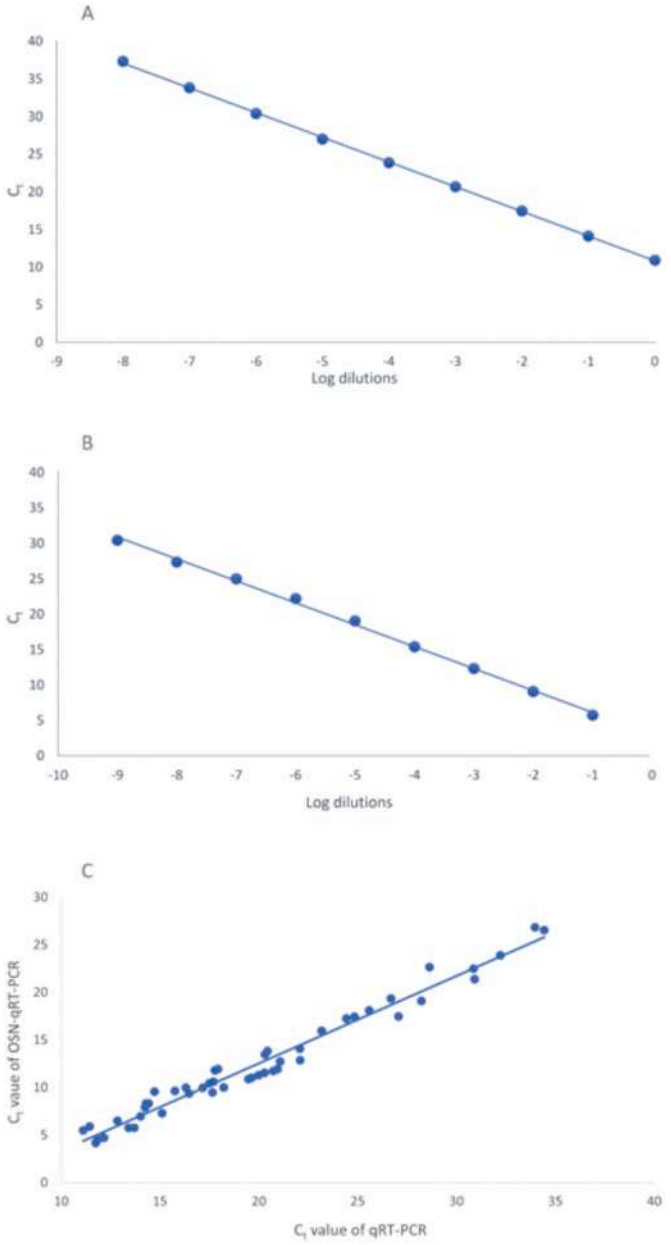

Fig. 3. The standard curves of A) qRT-PCR; B) OSN-qRT-PCR; C) C_t values of qRT-PCR and OSN-qRT-PCR analysed by linear regression and correlation analysis.

copy number detected in 100% of assays) for OSN-qRT-PCR was 1 copy/reaction and for qRT-PCR was 10-fold lower (10 copies/reaction).

3.3. Recovery of ESRM

The mean number of gene copies of ESRM recovered and recovery rates by qRT-PCR and OSN-qRT-PCR assay for the four concentration/extraction methods are shown in Table 3. The best performance was demonstrated by Method A using NucleoSpin RNA Virus Kit. The strikingly worst performance was assessed by Method C. Method A and Method B, which were based on different principles, both perfmormed better when combined with NucleoSpin RNA Virus Kit by (Macherey-Nagel) than with AllPrep PowerViral DNA/RNA Kit (Qiagen).

3.4. Limit of detection (LOD) for concentration/extraction methods

Concentration methods A and B and RNA extraction by NucleoSpin RNA Virus Kit performed better regarding recovery than the other evaluated methods. To determine LOD of ESRM by these two approaches, three serial dilutions of ESRM were analysed in triplicate. The minimum amount of ESRM detected by qRT-PCR for Method A was equivalent to 1.03×10^3 GC/mL and for Method B 6.75×10^3 GC/mL (Table 4). The lowest LOD was noted by OSN-qRT-PCR for Method A, 6.7×10^1 GC/mL, and for Method B it was 9.54×10^2 GC/mL. When the wastewater was seeded with ESRM at a concentration higher than 8.1×10^2 GC/mL, PEG precipitation revealed a mean recovery of 8.27% detected by OSN-qRT-PCR.

4. Discussion

Wastewater-based epidemiology (WBE) is currently being utilized to monitor the dissemination of SARS-CoV-2 (Sapula et al., 2021). However, due to the high chemical and biological complexity of wastewater, analysis may be skewed by low viral recovery, poorly reproducible results, or both (Shi et al., 2017). In addition, both particulate and dissolved constituents inherently present in wastewater get concentrated along with the target virus and can hinder the detection of viruses thus affecting the viral recovery yield of the concentration method

Table 3
Recovery of ESRM achieved by 4 concentration/extraction methods (A-D) from artificially contaminated wastewater.

Concentration/extraction method	Concentration (GC/mL)[a] of recovered ESRM[b]		ESRM recovery rate (%)[a]	
	qRT-PCR	OSN-qRT-PCR	qRT-PCR	OSN-qRT-PCR
Method A + NucleoSpin RNA Virus Kit	$3.13 \times 10^6 \pm 6.46 \times 10^5$	$3.92 \times 10^6 \pm 5.44 \times 10^5$	38.61 ± 7.98	48.39 ± 6.71
Method A + AllPrep PowerViral DNA/RNA Kit	$2.80 \times 10^6 \pm 7.12 \times 10^5$	$3.53 \times 10^6 \pm 8.01 \times 10^5$	34.51 ± 8.79	43.61 ± 9.89
Method B + NucleoSpin RNA Virus Kit	$2.17 \times 10^6 \pm 6.80 \times 10^5$	$2.89 \times 10^6 \pm 9.86 \times 10^5$	26.81 ± 8.4	35.67 ± 12.18
Method B + AllPrep PowerViral DNA/RNA Kit	$1.74 \times 10^6 \pm 9.44 \times 10^5$	$2.40 \times 10^6 \pm 9.10 \times 10^5$	21.52 ± 11.65	29.61 ± 11.23
Method C	$4.98 \times 10^5 \pm 1.09 \times 10^6$	$9.98 \times 10^5 \pm 1.43 \times 10^6$	6.15 ± 13.5	11.09 ± 17.62
Method D (first type)	$1.28 \times 10^6 \pm 1.39 \times 10^6$	$2.07 \times 10^6 \pm 1.36 \times 10^6$	15.84 ± 17.11	25.52 ± 16.84
Method D (second type)	$1.11 \times 10^6 \pm 1.18 \times 10^6$	$1.81 \times 10^6 \pm 1.22 \times 10^6$	13.64 ± 14.6	22.32 ± 15.01

[a] Values of mean ± standard deviation are presented.
[b] 8.1×10^6 ESRM seeded (GC/mL).

Table 4
Recovery and recovery rate of ESRM achieved by PEG precipitation or ultracentrifugation.
*Values of mean ± standard deviation are presented.

Concentration/extraction method	ESRM seeded (GC/mL)	Concentration (GC/mL)* of recovered ESRM		ESRM recovery rate (%)*	
		qRT-PCR	OSN-qRT-PCR	qRT-PCR	OSN-qRT-PCR
Method A + NucleoSpin RNA Virus Kit	8.1×10^4	$1.52 \times 10^4 \pm 5.76 \times 10^3$	$2.91 \times 10^4 \pm 8.72 \times 10^3$	18.77 ± 7.11	35.93 ± 10.77
	8.1×10^3	$1.03 \times 10^3 \pm 8.41 \times 10^2$	$1.57 \times 10^3 \pm 1.1 \times 10^3$	12.72 ± 10.38	19.38 ± 13.58
	8.1×10^2	0	$6.7 \times 10^1 \pm 1.15 \times 10^2$	0	8.27 ± 14.20
Method B + NucleoSpin RNA Virus Kit	8.1×10^4	$6.75 \times 10^3 \pm 9.82 \times 10^3$	$8.75 \times 10^3 \pm 1.21 \times 10^4$	8.33 ± 12.12	10.80 ± 14.94
	8.1×10^3	0	$9.54 \times 10^2 \pm 8.82 \times 10^2$	0	11.78 ± 10.89
	8.1×10^2	0	0	0	0

(Michael-Kordatou et al., 2020). Therefore, it is necessary to evaluate various aspects in order to select an efficient method to concentrate, and subsequently detect, SARS-CoV-2 from wastewater, which has been identified as a key research need for WBE (Kitajima et al., 2020; La Rosa et al., 2020b).

In this study, several molecular tools were evaluated in order to be prepared for use in potential future pandemics events. The first step of our evaluation strategy was the construction of ESRM based on the PPV genome bearing the fragment of SARS-CoV-2 Spike protein-coding sequence. In this way RNA is encapsidated which means that is better protected and stabilized. Although, ESRM has a different size comparing to SARS-CoV-2 this should not affect concentration, extraction and detection. ESRM was then used to assess the viral RNA recovery rate of four concentration/extraction methods utilizing two different PCR assays: a conventional qRT-PCR and a newly developed one-step single-tube nested real-time PCR (OSN-qRT-PCR). Since the composition of wastewater lays in a narrow range, the effectiveness of the detection method should not be much different. If the sample of wastewater is very alkalic or very acidic, additional neutralizing step may be necessary.

The different physical and chemical mechanisms involved in the tested concentration methods led to different degrees of recovery of ESRM. All four tested concentration methods led to detection of ESRM RNA at higher concentrations. Since we seeded the wastewater with known concentrations of ESRM, we were able to calculate the full process recovery rate (loss of ESRM through concentration and extraction methods) using two assays. We calculated recovery rate percentage, using qRT-PCR and OSN-qRT-PCR, at 8.1×10^6 GC/mL of ESRM seeded in wastewater samples. The other virus particles, present in wastewater, should not influence recovery and detection of ESRM unless they are in a big excess. The detection method should not be affected by any high concentration of competing virus particles (Hong et al., 2021).

The concentration methods used different physical and chemical mechanisms to concentrate SARS-CoV-2. The PEG method concentrates virus particles by precipitation of virus particles upon addition of polyethylene glycol and sodium chloride. Although there is uncertainty in the exact mechanism, virus precipitation is believed to occur similarly to precipitation of proteins by PEG, where water molecules are drawn

from the solution to hydrate PEG molecules, thereby increasing the effective protein concentration, leading to insolubility and precipitation of proteins after reaching saturation (Ingham, 1990; Yamamoto et al., 1970; LaTurner et al., 2021). The combination of PEG precipitation with NucleoSpin RNA Virus Kit displayed the highest recovery rate in our study. A similar recovery rate of 44.0 ± 27.7% was reported previously by Ahmed et al. (2020) in a study with enveloped MHV virus. Barril et al. (2021) achieved a higher mean recovery (62.2%) with this method but using it with a non-enveloped feline calicivirus (FCV).

Ultracentrifugation has been used for decades to concentrate viruses from environmental matrices. It has been reported that ultracentrifugation at 100,000 *g* is required to pellet most macromolecules and viruses (Ammersbach and Bienzle, 2011). In this study, good recovery rates were achieved using concentration by ultracentrifugation, RNA extraction by NucleoSpin RNA Virus Kit and detection by qRT-PCR or OSN-qRT-PCR. The determined recovery rates were comparable to the 33.5% reported by Ahmed et al. (2020). Fumian et al. (2010) reported that ultracentrifugation (100,000 *g* for 1 h at 4 °C) had a mean recovery of 47% (range of 34–60%) of non-enveloped rotavirus A from wastewater samples. This method also involves discarding supernatant a few times, which may have resulted in loss of virus. Nevertheless, the ultracentrifugation method may not be suitable for WBE studies because it requires expensive specialized centrifuges (Ahmed et al., 2020).

When comparing RNA extraction kits after PEG precipitation or ultrafiltration, NucleoSpin RNA Virus Kit performed better than Qiagen All Prep PowerViral DNA/RNA Kit in this study.

Our results are somewhat different from those of O'Brien et al. (2021), who tested 4 commercial kits for RNA extraction from wastewater and reported that pellet-based RNA extraction kits that included inhibitors removal and RNA preservation step yielded the most consistent, timely and accurate results. In that study, the most effective and efficient kit was Zymo Environ Water RNA. However, in our study Method C, which included Zymo Environ Water RNA Kit, had low recovery rates of only 6.15 ± 13.5% and 11.09 ± 17.62% when combined with qRT-PCR and OSN-qRT-PCR, respectively.

Method D that used NucleoMag DNA/RNA Water Kit with a concentration step by microfiltration or by PES centrifugal filtration was used for the first time in this study to the best of our knowledge. This concentration method was found moderately effective, allowing to recover 13.64–25.52% of ESRM from a small volume of wastewater.

For the two best concentration methods (PEG precipitation and ultracentrifugation), we determined also recovery rate at ESRM seeding levels from 8.1×10^4 to 8.1×10^2 GC/mL. At 8.1×10^4 GC/mL, the greatest recovery rate was determined, which illustrated that the concentration of virus particles in wastewater may influence the performance of the method. Similar results were reported in a recent study where greater recovery was observed, for bacteriophage φ6 surrogate, at the highest seeding level (Sangsanont et al., 2022). It was stated that the greater is the SARS-CoV-2 RNA concentration in a wastewater sample, the greater is the recovery and downstream detection probability (Ahmed et al., 2022).

In qRT-PCR, a standard curve is constructed to convert the threshold cycle values (C_t) value to virus titers. In a qualitative approach, a negative result is indicated by a lack of the typical amplification curve by a C_t value higher than a limit usually translated to number of gene copies per volume unit (Zhu et al., 2021). In this study, qRT-PCR and OSN-qRT-PCR assays for the quantification of ESRM were performed using the Luna Universal Probe One-Step qRT-PCR Kit. Selection of this chemical system was based on Sapula et al. (2021) who reported that this master mix had a higher detection efficiency for SARS-CoV-2 compared to TaqPath 1-Step Multiplex Master Mix (No ROX).

When ESRM RNA seeding levels were $<8.1 \times 10^3$ GC/mL, amplification was observed only by OSN-qRT-PCR assay after concentration by PEG precipitation and extraction by NucleoSpin RNA Virus Kit. PEG precipitation showed LOD of 8.1×10^2 for OSN-qRT-PCR and 8.1×10^3 for qRT-PCR. These results evidenced that OSN-qRT-PCR had lower LOD with PEG precipitation compared to the study by Barril et al. (2021), who used just normal qRT-PCR with non-enveloped FCV. LOD with ultracentrifugation was by an order of magnitude higher with each of OSN-qRT-PCR or qRT-PCR. The standard curve at various ESRM levels showed that the OSN-qRT-PCR method is highly sensitive and that OSN-qRT-PCR is able to increase the rate of positive detection of the virus. LOD of OSN-qRT-PCR was at a level of 1 copy/reaction, which is the theoretical optimum, while that of qRT-PCR was 10 copies/reaction. Similar results were published by Wang et al. (2020) with recombinant plasmids. Moreover, Pearson's correlation and linear regression between qRT-PCR and OSN-qRT-PCR results revealed a strong correlation, indicating that the presence of the external amplification step (10 PCR cycles) of the OSN-qRT-PCR assay did not affect the stability of the consequent inner PCR (40 PCR cycles) and that the C_t value still showed regularity similar to qRT-PCR. In our study, for the first time, OSN-qRT-PCR was evaluated as a reliable detection and quantification method for the analysis of wastewater samples.

5. Conclusion

The construction of ESRM and artificial contamination of wastewater with it was useful for optimization of detection of a virus in wastewater. Among the tested concentration/extraction approaches, the PEG precipitation method coupled to chaotropic solid-phase extraction achieved the highest recovery rate of ESRM. OSN-qRT-PCR represented an improvement in detection sensitivity as it had an LOD by an order of magnitude lower than qRT-PCR. Highly efficient concentration/extraction combined with highly sensitive detection can be a valuable molecular tool for detecting viruses in wastewater samples with low viral loads. In the future, the analysis of wastewater by advanced sensitive molecular approaches can play an important role in the surveillance of a range of human pathogenic viruses.

CRediT authorship contribution statement

Magdalena Rusková: Investigation, Methodology, Software, Visualisation, Writing - original draft, Writing - review & Editing. **Mária Bučková**: Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualisation, Writing - original draft. **Adam Achs:** Investigation, Methodology, Visualisation, Writing - original draft. **Andrea Puškárová:** Investigation, Methodology, Software, Visualisation, Writing - original draft. **Jer-Horng Wu:** Resources, Writing – Review & Editing, Funding acquisition. **Tomáš Kuchta:** Conceptualisation, Writing – Review & Editing, Supervision. **Zdeno Šubr:** Methodology, Validation, Visualisation, Writing- original draft, Writing – Review & Editing, Supervision. **Domenico Pangallo:** Conceptualisation, Resources, Writing- original draft, Writing – Review & Editing, Validation, Visualisation, Supervision, Project administration, Funding acquisition.

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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.

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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.2022.114017.

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Using Artificial Intelligence to extract information on pathogen characteristics from scientific publications

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ABSTRACT

Health risk assessment of environmental exposure to pathogens requires complete and up to date knowledge. With the rapid growth of scientific publications and the protocolization of literature reviews, an automated approach based on Artificial Intelligence (AI) techniques could help extract meaningful information from the literature and make literature reviews more efficient. The objective of this research was to determine whether it is feasible to extract both qualitative and quantitative information from scientific publications about the waterborne pathogen *Legionella* on PubMed, using Deep Learning and Natural Language Processing techniques. The model effectively extracted the qualitative and quantitative characteristics with high precision, recall and F-score of 0.91, 0.80, and 0.85 respectively. The AI extraction yielded results that were comparable to manual information extraction. Overall, AI could reliably extract both qualitative and quantitative information about *Legionella* from scientific literature. Our study paved the way for a better understanding of the information extraction processes and is a first step towards harnessing AI to collect meaningful information on pathogen characteristics from environmental microbiology publications.

1. Introduction

Human exposure to pathogens in the environment poses risks to public health (Hrudey and Hrudey, 2004). Health risk assessments are used to prevent or manage these risks and support decisions, for example on safe system design or emergency response. Exposure assessment is a first step in which knowledge about pathogen characteristics and their exposure routes are combined to estimate the exposure of the population to pathogens. With the fast-growing rate of scientific publications, such information is contained in a constantly increasing volume of text and journal articles. The conventional way is to generate review papers and meta-analyses to collate the published information, analyze the body of information in a comprehensive and integrated manner, and conduct such meta-analyses in an increasingly structured framework (Page et al., 2021). This process is time-consuming, labor-intensive and requires an expert that knows where to look and what to search for. The increasing rate of those publications has created a need for more efficient and extensive methods to collect all meaningful information for health risk assessment from various sources.

In recent years, automated approaches using Artificial Intelligence (AI) have been explored to systematically extract structured information from the ever-expanding body of scientific publications. Experts and curators in the field of biomedical sciences have been using AI and in particular Information Extraction (IE) techniques to extract information from Electronic Health Records (EHR) and Randomized Control Trials (RCT) (Cohen and Hersh, 2005; Meystre et al., 2008). Using text mining techniques (and consequently IE), Machine Learning (ML) and Natural Language Processing (NLP), experts extract information related to study characteristics such as disease-drug associations from EHR and RCT (Chen et al., 2008; Chung and Coiera, 2007; Kang et al., 2019; Uzuner et al., 2010). Kiritchenko et al. (2010), provided ExaCT, an IE system that extracts 21 key trial characteristics from publications and helps curators review and collect information from RCT (using a user interface). Their approach was based on ML using a Support Vector Machine (SVM) model for their sentence classification as well as rule-based techniques to extract exact values from segments within a text. A similar approach was adopted by Patrick and Li (2010), who used a multistage ML-based method with 2 different statistical classifiers namely SVM and Conditional Random Fields (CRF) and rule-based methods, they achieved an almost-optimal result (relative to other participants) for automated extraction of medication information from clinical notes. Although in the field of biomedical sciences, using such techniques (AI, IE, ML, and NLP) to extract information from text

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Abbreviations

AI	Artificial Intelligence
BERT	Bidirectional Encoder Representations from Transformers
CRF	Conditional Random Fields
DL	Deep Learning
EHR	Electronic Health Records
IE	Information Extraction
IK	Information Keywords
ML	Machine Learning
NER	Named Entity Recognition
NLP	Natural Language Processing
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RCT	Randomized Control Trials
Regex	Regular expressions
RNN	Recurrent Neural Networks
SVM	Support Vector Machine

documents has become a well-established approach; the development of similar applications in the field of environmental microbiology is still lagging and more complex because of the arbitrary and diverse form and structure in which the information is contained in case studies, reviews, and publications. The desired information is more scattered and complex compared to the structured information often contained in RCT and EHR. The less structured organization of the information requires an improved AI system that unravels the complexity of words and sentences by "understanding" and capturing the syntactic and semantic context of their surrounding words prior to the classification task.

This study aimed to evaluate the feasibility and performance of using an IE model to extract both qualitative and quantitative information about the waterborne pathogen *Legionella* from scientific publications. *Legionella* was selected since it is frequently associated with outbreaks via different water sources, many (types of) publications are available, and scientists and experts would like to have as much high quality information as possible to support decision making (van Heijnsbergen et al., 2015; Walser et al., 2014) and risk assessment (Papadakis et al., 2018).

To capture the information on *Legionella* as it is arbitrarily expressed in scientific literature, Deep Learning approach was developed in this study (instead of using the conventional classifiers used in ML), coupled with a rule-based technique. The quality of the extracted qualitative and quantitative information on *Legionella* was assessed using the evaluation metrics of precision, recall and F-score (Kiritchenko et al., 2010), along with a comparison between the system extraction and a human (manual) extraction.

2. Materials and method

2.1. Information keywords

The desired information (hereafter referred to as "information keywords") about *Legionella* was selected as general, explicit, and reproducible (waterborne) pathogen characteristics of both a qualitative and a quantitative nature (Table 1).

2.2. Selection of publications

50 peer-reviewed scientific publications about *Legionella* were manually selected from the search engine PubMed and used for the implementation of the IE task. We specifically aimed to extract information from peer-reviewed scientific publications, since this better warrants the quality of the text that we use for data extraction. The type of selected publications includes both scientific reviews and case studies on waterborne outbreaks, covering the different aspects of research on *Legionella*. A systematic review of the literature was performed adopting the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Liberati et al., 2009). The selection of publications was made considering their relevance to *Legionella* as well as their maximum possible reference to the desired Information Keywords (IK). The list of selected publications, the search terms, along with the flow diagram that describes the search process and the exclusion criteria can be found in the supplementary material.

Table 1
The desired extracted information (Information Keywords) from scientific publications regarding the waterborne pathogen Legionella. Incubation period is quantitative information whereas the rest information keywords are qualitative.

Information keywords	Description
Incubation period	The time elapsed between exposure to a pathogenic organism and symptom onset
Symptoms	The change in normal functions of a person indicating the presence of a disease
Clinical manifestations	The medical conditions of a patient after infection by the pathogen
Sources of exposure	Places or objects that spread the pathogen
Route of transmission	Route via which an individual became exposed to the pathogen
Environmental habitat	The environment/water system in which the pathogen grows
Species	Unit of classification and taxonomic rank of an organism

2.3. Template filling of the information

Template filling is an efficient approach (especially when the content of a text document describes an event or a situation) to extract information in a comprehensive, structured form. The process of template filling includes identifying and locating predefined entities and filling in their template slots. Table 2 depicts an example of template filling. The algorithm behind the template filling should be able to fill in the slots for both qualitative and quantitative information. However, not every slot can always be filled since it is possible that some IK might not be addressed in the text document. The IK vary in terms of their structure. Some consist of straightforward information such as "incubation period", and others, such as "Route of transmission" or "Environmental habitat" consist of lengthy, more vague, and free text information.

2.4. Information extraction task

2.4.1. Labeling and training the data

The first step of the IE task was to manually label the scientific publications. The labeling of data is part of the custom-trained NER model that requires a token-level classification, and it helps assess

Table 2
Example of template filling extracting information from a scientific publication.

Information keywords	Results
Species	*Legionella pneumophila*
Incubation period (days)	2–14
Symptoms	Headache, myalgia, asthenia, anorexia, fever, cough, chills, dyspnea, arthralgia
Route of transmission	Inhalation, micro aspiration, direct contact with surgical wounds
Environmental habitat	Aquatic habitats, water distribution systems
Clinical manifestation	Legionnaires' disease, atypical pneumonia, Pontiac fever
Source of exposure	Water supply, infectious aerosols, cooling towers, hot tubs, potting soil

whether a specific world within a sentence is relevant to a specific IK. Relevant words are those who are assigned to one of the IK labels, whereas irrelevant tokens are those who have no meaning to the labeling process and are assigned the label "O".[1] Fig. 1 serves as an example of the labeling process.

Next, the training and classification of labeled data was necessary so that the system will learn to correctly assign the right labels to words within sentences. This step was implemented using Python programming language (Van Rossum and Drake Jr, 1995) and the Spacy library (Honnibal, M., & Montani, 2017). The selection of Spacy library was made mainly because this tool is suitable for NLP tasks utilizing word embedding methods as well as Recurrent Neural Networks (RNN) for multiclass classification.

2.5. Overall architecture

Fitting the overall architecture into a general workflow resulted in the following process (Fig. 2).

2.5.1. Text pre-processing

Although scientific publications come in various document standards and formats, the 50 selected scientific publications were extracted from the PubMed search engine in a PDF format. The first step of the pre-processing process was the conversion of PDF files to text files so that they can be recognized and processed as raw data. Next, all the sections from the text documents that are irrelevant to the IE task were removed automatically. That includes references, editors' notes, and acknowledgments. It was decided that the summary of publications should also be excluded since the contained information can be found in the remaining sections of the text. To detect these sections ("References", "Acknowledgements", and "Summary") we assumed a consistency in the way the headings were expressed in the scientific publications before applying a rule-based keyword matching technique to filter them out. The cleaning process also included the conversion of all uppercase letters to lowercase, and removal of punctuation. The last step was the tokenization of words to facilitate the labeling process as well as the implementation of the model itself.

2.5.2. Rule-based techniques

For the IK "incubation period", regex pattern-matching was selected using a specific module embedded in Python (Kuchling, 2002). The information is in numeric form and follows a certain pattern in the text (e.g. "*the incubation period was 2 to 14 days", "the incubation ranges between 2 to 14 days prior to symptom onset*"). After isolating the sentences containing the word "incubation" from the text, a set of regular expressions was applied to every sentence for the extraction of digits or a range of digits that correspond to the number of days of the incubation period. For IK "symptoms" and "species", a pool parsing technique was adopted. Since the results of these 2 IK are finite and known, a pool with all the potential symptoms and species associated with *Legionella* was created. Then, during parsing of unseen text, several n-grams were matched each time to the pools to determine if any of the potential symptoms and species of the pool can also be found in the text document of interest. For the creation of the symptoms and species pool, all the potential symptoms and species (both pathogenic and non-pathogenic) associated with *Legionella* and Legionnaire's disease were collected after exploring the literature.

2.5.3. Supervised technique

For the remaining of IK, a supervised technique was used since the information to be extracted was neither confined within a finite set nor could be represented in a certain pattern of strings (as in the case of IK "incubation period", "symptoms", and "species"). The extraction of such information was therefore only possible by understanding the semantic pattern and relationship of the tokens[2] within a text document. Specifically, a custom-trained NER model using word embedding and RNNs was implemented. During the training process, after embedding the tokens (words) into a sequence of vectors (numerical representation of text), bidirectional RNNs were used to take the semantic context into consideration by encoding the vectors into a context-sensitive sentence matrix. Next, to improve the power of the model the system used an attention mechanism where the previously produced matrix was reduced to a sentence vector by selecting the most "appropriate" information (after applying weights to every token based on their importance). In the last step, after all text was converted to a single vector, the system was able to predict the classes of every token. This four-step formula named: "Embed, encode, attend, predict" is the fundamental approach adopted in Spacy library for NER and more documentation can be found in Honnibal (2016).

2.5.4. Post-processing of results

After the supervised and rule-based techniques had completed their task, the extracted information filled the slots of a pre-defined template comprised of the desired IK. The extracted information might consist of repeated words or words that have the same semantic meaning but differ in the length of characters in the text. For example, the slot of IK "Clinical Manifestation" may have both "Legionnaires Disease" and "Legionnaire's disease" in the template. Although the semantic meaning is the same, the two extracted sequences differ slightly (apostrophe). Therefore, to avoid extracting duplicate information, we used the Levenshtein distance, a string metric that measures the pattern similarity -or to put it differently- the differences between words and/or sequences of words (Levenshtein, 1966). Using the Levenshtein Python C extension module, the system decided whether or not to keep the extracted similar words in the template (Necas, D., Ohtamaa, M., Haapala, 2014).

2.6. Evaluation of the performance

The last step was the evaluation of the model output. To get an unbiased performance of the model, a 5-fold cross-validation method was implemented. After the system was trained by feeding it with 40 text documents (80% of total publications), the NER model was tested by using a set of 10 "unseen" testing data (20% of total publications). This process was repeated 5 times, each time with a separate set of training and testing data. For every iteration, the manually labeled values were compared with the predicted values for every IK in a so-called confusion matrix. Next, the evaluation metrics of precision, recall, and F-score were calculated to describe the performance of the model for that particular fold of data, and the metrics of all the folds were averaged to get the overall performance of the model.

The analytic approach of precision, recall, and F-score was adopted (Kiritchenko et al., 2010) and it was applied both to the system and to every IK separately after averaging the values through every fold (5 iterations). When it comes to classification tasks, precision is a metric that quantifies the number of correct positive predictions from all returned positive predictions. It is therefore the number of true positives divided by the number of true positives plus false positives (Equation (1)).

$$Precision = \frac{TP}{TP + FP} \tag{1}$$

Recall, on the other hand, is a metric that quantifies the number of correct positive predictions made of all positive predictions that could have been made by the system. Specifically, it is the number of true

[1] The choice of the word "O" is a default option and it means that all the words irrelevant to the IK are automatically assigned to the label "O".

[2] Tokenization: In a sequence of characters within a text document, tokenization is the process of chopping up the sequence into pieces (words), named tokens (Webster and Kit, 1992).

TOKENS	Legionella	spp	are	ubiquitous	in	aquatic	habitats	and	water	distribution	systems.	The	symptoms	of	LD	are	fever	cough	and	chills.
LABELS	O	O	O	O	O	ENV. HABITAT	ENV. HABITAT	O	ENV. HABITAT	ENV. HABITAT	ENV. HABITAT	O	O	O	CLIN. MANIFEST	O	SYMPTOMS	SYMPTOMS	O	SYMPTOMS

Fig. 1. Example of the labeling process. The labels "Env. Habitat", "Clin. Manifestation", and "Symptoms" are assigned to their respective words, whereas the remaining irrelevant words have been assigned to the label "O".

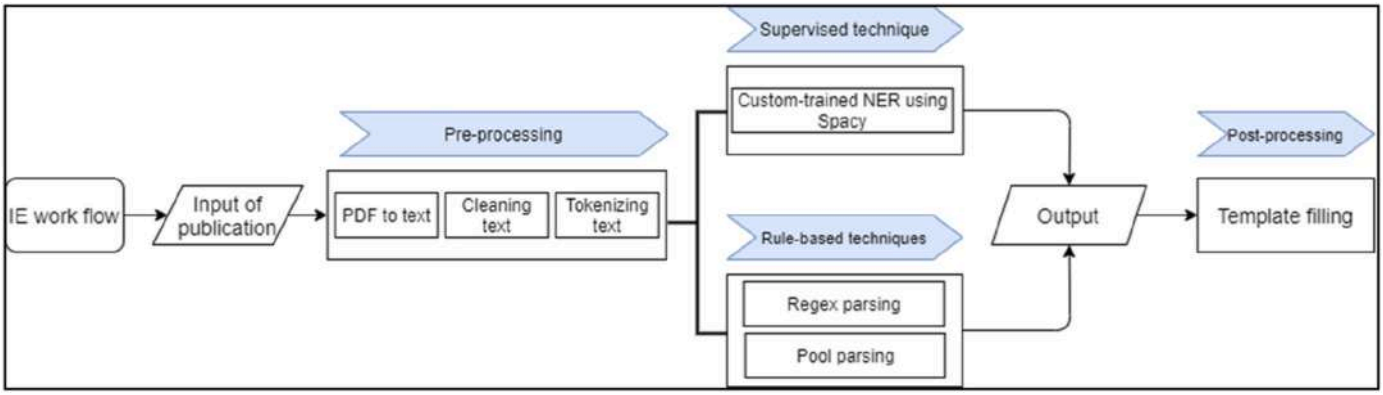

Fig. 2. The workflow of the IE task starts with the input of publication. Next, the publication gets converted to text, cleaned, and tokenized as part of the pre-processing step. The next part includes the supervised and rule-based techniques for the extraction of information. Finally, the output of this process gets filled in a template as part of the post-processing step. More can be found in chapters 2.5.1 -2.5.4.

positives divided by the number of true positives plus false negatives (Equation (2)).

$$Recall = \frac{TP}{TP + FN} \tag{2}$$

The F-score (Equation (3)) is the harmonic mean of precision and recall. It is a way to combine both analytic metrics into a single score that captures both properties (Olson and Delen, 2008).

$$F = 2^{*}\frac{Precision^{*}Recall}{Precision + Recall} \tag{3}$$

Choosing the right number of scientific publications for the training of the model was an important decision to make. Usually, the amount of data required to build a good DL model depends on the complexity of the problem (in our case extracting words and excerpts of information from unstructured scientific publications) and the quality of the training data. Regarding DL, the hypothesis is that the more quality data used to train a model, the higher is the performance (Mitsa, 2019). The impact of the number of publications used for training the IE model on the quality of the results was investigated. We created 5 folders containing 10, 20, 30, 40, and 50 publications randomly selected from the 50 papers that had been selected previously and performed a 5-fold Cross-validation in every folder.

Another form of evaluation was to select new publications (beyond the 50 that were used before) and compare the system's performance on IE with a manual extraction process (the conventional way where a human extracts information from text documents). We selected a set of 10 new scientific publications related to *Legionella* and incorporated them in the IE module. The same publications were processed by a human expert for manual extraction of the IK and the results were compared to assess the usefulness of the proposed approach on extracting information from *Legionella* scientific publications.

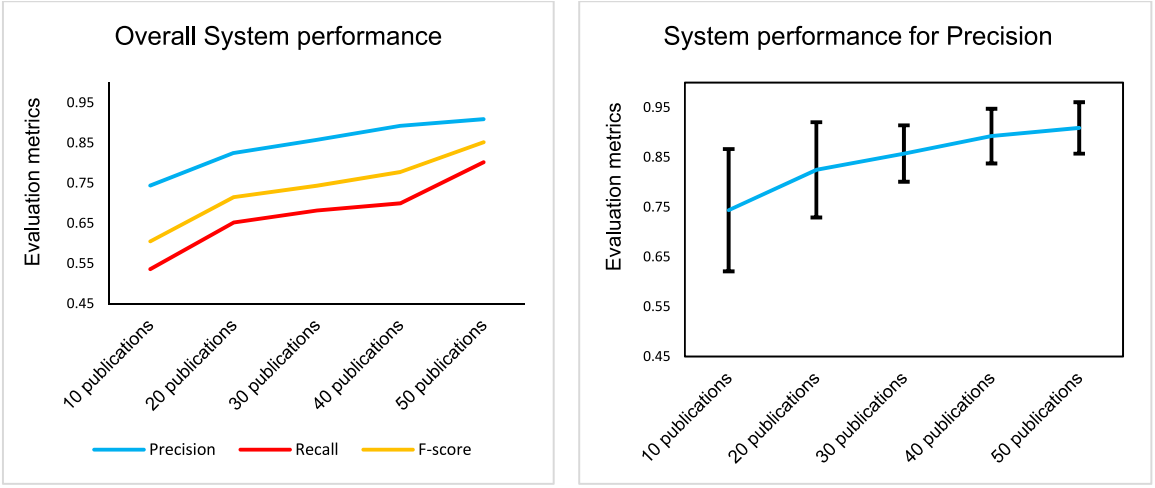

Fig. 3. a) System performance under different number of publications. b) System performance and standard deviation for precision under different number of publications.

3. Results

3.1. Influence of the number of publications on evaluation metrics

Fig. 3a shows that by increasing the number of publications, all metrics improved and the standard deviation of cross-validation regarding precision in Fig. 3b decreased overall (the standard deviation for recall and F-score can be found in the supplementary material). That means that by increasing the number of training data (publications) the model generalizes and thus, there is a smaller variation in its performance. These 2 interpretations go in line with the original hypothesis and since the standard deviation of precision remained constant for 3 consecutive increments of publications, we decided that 50 publications were an adequate and feasible starting point for the creation of the model. All further results were generated using 50 publications to train the model.

3.2. Evaluation of the supervised and rule-based extraction

For the supervised technique with custom-trained NER, the information on "Clinical manifestations", "Environmental habitat", "Route of transmission" and "Source of exposure" was extracted from the 50 publications. After performing a 5-fold cross validation to test the model, Table 3 shows the results of the 1st folder in a confusion matrix. The confusion matrix compares the actual with the predicted IK labels, indicating that the custom-trained NER technique was able to correctly predict the labels in the majority of the tokens. The only label that seemed to have mislabeled many features was the label "O" (which contains all the irrelevant words in a document). That "confusion" was expected to a certain extent since there was an imbalance between the label "O" and the rest of the IK (15897 tokens assigned to label "O" versus 2404 assigned to the rest of the IK) in the testing data. Considering that the desired information was generally organized in a complex and sparse manner within the text, it was expected to see false negatives. The label "O" affected and captured some of the words that should have been assigned to other labels. Another set of IK mislabeling their tokens were the "Source of exposure" and "Environmental habitat". This "confusion" was also expected since in many scientific publications the meaning of these two IK was often mixed and misinterpreted (i.e. "*The source of exposure of Legionella was 2 cooling towers*", " *Legionella* can grow and survive in cooling towers"). We see in this example that cooling towers can be labeled both as "Source of exposure" and "Environmental habitat" and therefore it was difficult for the system to always make correct predictions.

For the extraction of the information on "Incubation period", "Species", and "Symptoms" with rule-based techniques, almost all of the tokens were correctly labeled to their respective IK (Table 4). One IK that mislabeled some tokens, resulting in false negative results, was the "Incubation period". Looking into the testing dataset, this happened because in some publications, although the authors were describing the incubation period, they did not mention specifically the word "incubation" and therefore the regex rules did not apply. Another IK that mislabeled some tokens was the "Symptoms". Out of 521 tokens describing symptoms, 20 of them were not assigned correctly, probably because during the pool parsing technique, the respective pool did not contain those specific symptoms.

Table 4
Confusion matrix of the rule-based techniques.

	Predicted labels				
Actual labels	**Information keywords**	Incubation period	O	Species	Symptoms
	Incubation period	70	20	0	0
	O	0	46226	0	2
	Species	0	1	1011	0
	Symptoms	0	20	0	501

The classification reports in Tables 5 and 6, give an overview of the evaluation metrics of the system for the supervised and rule-based techniques. For the custom-trained NER in Table 5, the overall score of the system has a precision, recall, and F-score of 0.91, 0.80, and 0.85 respectively. While the precision score is high for IE tasks, the recall score of 0.80 leaves room for improvement (Patrick and Li, 2010; Kiritchenko et al., 2010). As explained earlier, the label "O" influenced to a certain extent the recall score of all individual IK (too many False Negatives for all IK), which resulted in a low overall score. The IK with the lowest metrics (both precision and recall) is the "Environmental habitat". This is because sometimes the environmental habitat of *Legionella* can also be presented as its source of exposure and vice versa. For the remaining IK, both precision and recall scores are high numbers.

For the rule-based techniques, as it was expected, the evaluation metrics for all IK are high with an overall precision and recall of 1 and 0.91 respectively.

3.3. Alternative evaluation with new publications

3.3.1. Improving the regex rules

After comparing the IE results with the human extraction, we identified a few setbacks on the proposed rule-based technique. Specifically, during the extraction of IK "Incubation period", the system could not distinguish the semantic difference between the actual incubation period of *Legionella* in patients prior to symptom onset, and the number of days required for the growth of colonies on solid media in a laboratory environment (a scientific publication can include both, i.e. *"L. gormanii and L. wadsworthii isolates resulted in no visible growth after 96 h incubation in BYE broth"*). Although both instances describe incubation period, their semantic is different. Therefore, a new set of rules was added that

Table 5
Classification report of the system's performance for the custom-trained NER.

Classification report	Precision	Recall	F-score	Total number of actual labels
Clinical Manifestation	0.95	0.88	0.91	725
Environmental habitat	0.81	0.73	0.77	286
Route of transmission	0.97	0.81	0.88	114
Source of exposure	0.91	0.79	0.85	1279
Average	0.91	0.80	0.85	–

Table 3
Confusion matrix of the custom-trained NER performance.

	Predicted labels					
Actual labels	**Information keywords**	Clin. Man/on	Env. habitat	O	Route of transmission	Source of Exposure
	Clin. Man/on	637	0	88	0	0
	Env. habitat	3	207	58	0	9
	O	32	31	15517	2	91
	Route of transmission	1	0	20	92	1
	Source of Exposure	2	19	273	1	984

Table 6
Classification report of the system's performance for the rule-based techniques.

Classification report	Precision	Recall	F-score	Total number of actual labels
Incubation period	1	0.78	0.88	90
Species	1	1	1	1012
Symptoms	1	0.96	0.98	521
Average	1	0.91	0.95	–

would exclude all mentions of *Legionella* associated with laboratory results.

3.3.2. Comparing the system with a human extraction

The alternative evaluation of the model (input of 10 new publications into the model and comparison with a human extraction) shows that the model returned results similar to the human extraction and extracted most of the IK from the text document. The classification report in Table 7 supports this argument. Although the sample is small and conclusions cannot be drawn, the evaluation metrics of both precision and recall are high. Table 8 depicts the extraction of information (and comparison) for 2 publications as example. The rest of the comparison tables can be found in the supplementary material.

4. Discussion

4.1. Evaluation of the IE model

The proposed IE model demonstrated very good performance on a set of 7 information keywords and extracted both quantitative and qualitative information regardless of the complexity of the targeted information. After testing it with 50 testing publications (10 publications per 5 folds of cross-validation) from various aspects of research on *Legionella* (scientific reviews and outbreak reports) the system was able to extract meaningful information. For the set of IK, both supervised and rule-based techniques were needed. The results of the evaluation metrics showed that the IE approach can adequately extract the desired information from scientific publications regarding the waterborne pathogen *Legionella*. Overall, the IE system identified and extracted the targeted IK with high precision (0.91) and provides proof of concept for automated extraction of this type of information from scientific publications. The lower recall score (0.80) indicated that the IE model missed some of the information. While the system's performance was not perfect and there is room for improvement, it is comparable with other IE tasks from biomedical sciences. In Kiritchenko et al. (2010), the results of precision and recall were 0.93 and 0.91 respectively whereas in Patrick & Li (2010), their precision had a score of 0.89 and recall 0.82. Finally, although not focused on NER, an IE task from tables in biomedical literature had 0.94 score for both precision and recall (Milosevic et al., 2019).

The alternative evaluation of the IE model confirmed the validity of our approach: when comparing the system's results with the manual extraction in 10 new publications on *Legionella*, the IE system returned similar results for all 7 IK. Although in some cases the IE model extracted irrelevant information for some of the IK, considering the complexity of the desired information, the results of the proposed IE model were of sufficiently high quality.

Table 7
Classification report of the custom-trained NER on the 10 new publications.

Classification report	Precision	Recall	F-score
Clinical Manifestation	0.76	0.91	0.81
Environmental habitat	0.63	0.92	0.71
Route of transmission	0.66	0.89	0.72
Source of exposure	0.68	0.87	0.75
Incubation period	1	0.75	0.83
Species	1	1	1
Symptoms	1	0.72	0.82
Average	0.82	0.87	0.81

4.2. Limitations and recommendations

Although the proposed approach showed promising results, it is accompanied by limitations. The main limitation stems from the very nature of the study's objective. IE tasks have not been implemented for data extraction on waterborne pathogens from scientific publications before. Therefore, there is still no relevant work to allow for a comprehensive comparison with the results of the proposed IE model. Although the proposed approach is based on similar work applied to biomedical data extraction using ML approaches, an established open-access benchmark dataset related to waterborne pathogens data extraction utilizing DL methods is missing. Considering the plethora of methods available in the literature for AI-data extraction using ML and DL methods, it is recommended that other approaches should also be tested.

Considering the proposed approach, the complexity of some of the IK is another limitation which resulted in missing some of the information (lower recall score). It was relatively easy to extract straightforward information, but when the desired information was unstructured, lengthy, or vague, the system sometimes failed to correctly identify its label. For example, for the IK "Clinical manifestation", the system would potentially have to target and extract words such as "Legionnaires' disease", "Pontiac Fever", and "pneumonia". The problem, in this case, is that the targeted fragment of words can be mentioned anywhere in a text document, each time in a different semantic context. Another limitation was the choice of pool parsing technique for the IK "Symptoms". Although the pool of symptoms included a variety of symptoms (more than 40), it was limited only to the symptoms collected manually from the literature. That means that there could be symptoms that the IE model would fail to recognize simply because they were not included in the respective pool. To tackle this limitation, an enrichment of the symptoms pool is recommended by incorporating all symptoms listed in the National Library of Medicine's Unified Medical Language System (UMLS) associated with the waterborne pathogen *Legionella* (Bodenreider, 2004). Finally, although the choice of regex rules showed good results, it also presented some difficulties in the information extraction process. The inability of the IE model to extract the incubation period in sentences where the word "incubation" is not mentioned, indicated the need for a slightly different approach. Instead of first isolating the word "incubation" from the whole text prior to applying the regex rules, it is recommended to first perform a sentence-level classification, extracting the sentences that contain the relevant information, and then apply the regex rules in the sentences that have been classified correctly. Doing that can ensure that all the values of the IK "Incubation period" can be extracted from the text.

4.3. Potential applications of IE tasks

Experts can use the IE model to extract high quality information in substantially less time (compared to the conventional way) for meta-analysis purposes. A meta-analysis can help recognize patterns, enrich the knowledge on *Legionella* (or other pathogens), and/or generate hypotheses. For example, by gathering information from multiple scientific publications (reviews and/or outbreak reports) regarding the incubation period of *Legionella*, it would be possible to create a distribution curve of the incubation time. Other examples are to collect and categorize various transmission pathways, or to identify the most common symptoms based on their frequency in Legionella outbreaks. Finally, by measuring the frequency of reported Legionellosis (the clinical manifestation of Legionella infection) case studies associated with exposure events, it is possible to estimate the likelihood of sources of exposure. All of these meta-analysis examples demonstrate the potential and

Table 8
Comparison between the system's performance and manual extraction of IK from 2 publications (Beauté et al., 2020; Couturier et al., 2020). Red highlighted shade = erroneous results. Red bold font = Missed result (either by the IE model or by the manual extraction).

	Healthcare-Associated Legionnaires Disease, Europe, 2008–2017. (Beauté et al., 2020)		*Transmission of Legionnaires Disease through Toilet Flushing* (Couturier et al., 2020)	
	System	Manual extraction	System	Manual extraction
Clinical manifestation	"ld" **"community acquired ld** "pneumonia" "legionnaires disease" **"knoxville"**	"Legionnaires disease" "pneumonia""ld"	"legionnaires disease" "pneumonia" "renal failure "bilateral pneumonia"	"legionnaires disease" "pneumonia" **"respiratory** and renal failure "bilateral pneumonia"
Environmental habitat	"human made water systems" "aquatic environments"	"aquatic environments" "human-made water systems"	**"hot** water" **"hospital hematology" "respiratory"**	"water"
Route of transmission	"inhalation" "aspiration"	"inhalation" "aspiration"	-	**"inhales" "person to person"**
Source of exposure	"potable water" "pools" **"nursing homes" "peaking"** "humidifiers" "decorative fountains" **"school" "activities"** **"composts"** **"demographic variables" "medical epidemiologist"** "potting soil" "medical devices"	"potable water" **"bathing" "steam-heated towels"** "humidifiers" "decorative fountains" "medical devices" "birthing pools"	"windshield washer fluid" "fountains" "dental unit" "cooling towers faucets" **"hospital" "aerosols" "contaminated** toilet" "air filtration" "shower" **"filters"**	"showers" "cooling towers" "faucets" "fountains" "windshield washer fluid" "dental unit" "waterlines" **"flushing toilets"** "air filtration systems" **"sink"** "toilet **water"**
Species	"L wadsworthii""anisa""L longbeachae" "L feeleii" "sainthelensi""micdadei" "pneumophila" "L bozemanii" "L dumoffii" "cincinnatiensis" "macechernii"	"L wadsworthii""anisa""L longbeachae" "L feeleii" "sainthelensi""micdadei" "pneumophila" "L bozemanii" "L dumoffii" "cincinnatiensis" "macechernii"	"L pneumophila"	"Legionella pneumophila"
Symptoms	-	-	"dyspnea" "fever"	"dyspnea" "fever" **"shivering"**
Incubation	"20 days"	**"2-10 days"** "20 days"	-	-

importance of using AI and specifically IE tasks to automatically extract high-quality information from scientific publications.

4.4. Future research

Future research should focus on improving the overall performance of the proposed approach. A hybrid system (a combination of the proposed DL method with another discriminative classifier such as CRF or SVM) could potentially improve the system's overall performance as previous research has shown (Lê, T., & Burtsev, 2019; Patrick and Li, 2010). For example, assigning the NER task to the custom-trained NER developed here and then coupling it with another classifier to classify relationships between entities could potentially further unravel the complexity of some of the IK. Another approach would be to consider using another DL approach, namely the Bidirectional Encoder Representations from Transformers (BERT). Based on the so-called Transformer neural network, this technique has gained attention and has become a ubiquitous baseline in NLP tasks, since it examines the context of words in both directions within a sentence (Kalyan et al., 2021).

4.4.1. Extrapolate the process to other pathogens and/or fields

Although this paper is focused on the waterborne pathogen *Legionella*, the IK are generic for waterborne pathogens. The good results with *Legionella* indicate that the IE model could also be successful for other waterborne pathogens, although many of those are not uniquely waterborne, but also spread via other matrices (food) or via person-to-person contacts, adding more complexity. The ability of DL methods (coupled with rule-based techniques) to unravel the complexity of information found in scientific publications enables experts to create more custom-train NER models using sufficient and representative training data from other waterborne pathogens publications. The proposed approach also enables scientists from different scientific domains to explore the power of using AI to extract complex, qualitative, or quantitative information from scientific publications. For example, the use of IE could be tested for the ability to extract functions such as inactivation rates (at different temperatures), disinfection kinetics, or log removal values of pathogens from various treatment processes found in scientific case studies.

5. Conclusions

This paper aimed to evaluate the feasibility and performance of a newly developed IE model to extract both qualitative and quantitative information from scientific publications about the waterborne pathogen *Legionella*. For the IE model, we adopted a combination of supervised (custom-trained NER model) and rule-based (regex pattern-matching, and pool parsing) techniques. The evaluation metrics showed a satisfactory performance for extraction of both qualitative and quantitative information: the custom-trained NER model had an overall F-score of 0.85, and the rule-based techniques had an F-score of 0.95. The IE model returned similar results with the manual extraction indicating that the extracted information is of high quality, and it can be further used by experts who seek to extract meaningful information from scientific publications using AI.

Overall, this study indicates that IE can provide an efficient and adequate approach for extracting qualitative and quantitative information on waterborne pathogen characteristics from the complex body of environmental microbiology literature. Scientists and experts can therefore begin to harness the power of Artificial Intelligence and Deep Learning techniques in this science field.

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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.2022.114018.

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