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Chemical contaminants in natural environments and human health implications

Edited by
Aina Olubukola Adeogun, Beatrice Opeolu, Olatunde Farombi, Oju Richard Ibor and Azubuike Chukwuka

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Chemical contaminants in natural environments and human health implications

Topic editors

Aina Olubukola Adeogun — University of Ibadan, Nigeria
Beatrice Opeolu — Cape Peninsula University of Technology, South Africa
Olatunde Farombi — University of Ibadan, Nigeria
Oju Richard Ibor — University of Calabar, Nigeria
Azubuike Chukwuka — National Environmental Standards and regulations Enforcement Agency (NESREA), Nigeria

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EDITED AND REVIEWED BY
Sara Cristina Antunes,
University of Porto, Portugal

*CORRESPONDENCE
Aina Olubukola Adeogun,
ainaadeogun@yahoo.com

†PRESENT ADDRESS
Azubuike Victor Chukwuka,
National Environmental Standards and Regulations Enforcement Agency (NESREA), Abuja, Nigeria

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Editorial: Chemical contaminants in natural environments and human health implications

Aina Olubukola Adeogun[1]*, Azubuike Victor Chukwuka[2†], Beatrice Olutoyin Opeolu[3], Oju Ibor[4] and Ebenezer Olatunde Farombi[5]

[1]Department of Zoology , University of Ibadan, Ibadan, Oyo, Nigeria, [2]National Environmental Standards and Regulations Enforcement Agency (NESREA), Abuja, Nigeria, [3]BEE Solutions and Consultancy Services, Cape Town, South Africa, [4]Department of Zoology and Environmental Biology, University of Calabar, Calabar, Cross River, Nigeria, [5]Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria

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Editorial on the Research Topic
Chemical contaminants in natural environments and human health implications

Natural environments are invaluable resources necessary for ecological and human health and chemical contaminants are increasingly recognized as significant threats to public health and environmental integrity. The growing body of research highlights their harmful effects and other human existential threats such as climate change currently add to the burden of pollution effects on the environment. These underscore the urgent need for effective regulatory frameworks and public health initiatives. Manuscripts that focus on topical \issues of chemical contaminants were selected for inclusion in this special edition of Frontiers in Toxicology.

A comprehensive analysis by Zhang et al. examined Bisphenol A (BPA) exposure among the Chinese population from 2004 to 2019, revealing alarmingly high BPA levels, particularly among males and children. The geographical variations in exposure were linked to waste disposal practices, emphasizing the critical need for improved waste management strategies to mitigate BPA exposure and associated health risks. This study not only highlights the health risks posed by BPA but also provides timely information for policymakers to prioritise sustainable waste management practices.

The threat posed by agricultural pollutants is similarly concerning, as illustrated in the study by Abarikwu et al., which focused on atrazine, a widely used herbicide. Authors demonstrated that atrazine induce d cytotoxicity, oxidative stress, and apoptosis, with detrimental effects on reproductive health, including decreased sperm count. Notably, authors observed that antioxidants like vitamin E could alleviate these toxic effects, suggesting potential strategies for risk mitigation. This finding raises the possibility of integrating antioxidant supplements into agricultural practices to counteract pesticide toxicity, thereby protecting both agricultural workers and consumers.

Expanding on the narrative of environmental pollution, Alarape et al. assessed glyphosate residues in African catfish across various markets in Ibadan, Nigeria, and

reported residue concentrations exceeding acceptable limits. This finding underscores the urgent need for stricter pesticide regulations in aquaculture to safeguard consumer health and aquatic ecosystems. Coupled with this, Akinnusotu et al. identified polycyclic aromatic hydrocarbons (PAHs) in sediment and fish from River Owan, Edo State. Although their study indicated minimal cancer risks associated with these contaminants, the presence of pyrogenic PAHs highlights the need for ongoing monitoring to prevent long-term ecological damage and protect aquatic biodiversity.

In the context of water quality, Mhlongo et al. investigated the occurrence of phenolic compounds in potable and treated waters in the Western Cape of South Africa. Although detected at levels below regulatory thresholds, health risk assessment suggested potential non-carcinogenic effects and slight mutagenicity. This underscores the need for vigilance in water quality management, with recommendations for routine monitoring and stricter enforcement of regulations to ensure safe drinking water for all communities.

The impact of environmental chemical exposures on children's mental health is a pressing concern, as highlighted in the narrative review by James and Oshaughnessy. A comprehensive review of 29 studies revealed significant associations between exposure to heavy metals and endocrine disruptors and adverse mental health outcomes in children. This emphasises the critical need for further research into the cumulative effects of these chemical agents during sensitive developmental stages. Policymakers must prioritize educational campaigns and preventive measures to reduce children's exposure to these harmful substances, ensuring a healthier future generation.

Akangbe et al. investigated endocrine-disrupting chemicals in fish from Lagos and Epe lagoons, providing significant gonadal alterations and hormonal imbalances insights, with some fish exhibiting intersex characteristics. This research illustrates the profound impacts of environmental contaminants on aquatic life and highlights the necessity for targeted management strategies to protect these ecosystems. Collaborative efforts among researchers, regulators, and local communities can foster sustainable practices to mitigate these risks in aquatic biota and ensure human health.

Occupational exposure to chemical contaminants was examined by Barros et al. in a study of wildland firefighters, revealing elevated levels of PAH metabolites and increased blood pressure among participants, with higher risks for smokers. This finding emphasises the urgent need for protective measures in high-risk professions and the development of guidelines to minimise occupational exposure to hazardous chemicals.

Lead exposure remains a critical concern, as demonstrated by He et al., whose study measured blood lead levels in residents of Jiangxi Province. Findings suggest that higher lead levels correlated with adverse haematological and biochemical indices, particularly among older adults. This underscores the widespread health risks associated with lead exposure in vulnerable populations and highlights the necessity for comprehensive screening programs and public health initiatives aimed at reducing lead exposure in communities. In a related study, Zhang et al. explored the link between heavy metal exposure and persistent infections, revealing that exposure to metals such as arsenic, cadmium, and lead increased infection risks primarily through immunosuppression. This finding further illustrates the intricate relationship between environmental contaminants and human health, necessitating a multi-faceted approach to address these interconnected issues.

In this study, Tian et al. examined the relationship between perfluoroalkyl and polyfluoroalkyl substances (PFASs) and glucose metabolism, indicating that higher PFAS exposure correlated with increased fasting plasma glucose levels and decreased insulin levels. This association highlights the potential metabolic consequences of chemical exposure, necessitating further investigation into the long-term health effects of PFAS on human health.

Collectively, these studies present a concerning picture of the pervasive threats posed by chemical contaminants to both human health and environmental integrity and underscore the urgent need for robust regulatory frameworks, public health initiatives, and ongoing research to mitigate exposure risks and protect vulnerable populations.

Priority actions for next steps in environmental and human health protection entails adopting proactive approaches that include:

- Implementing stricter regulations on the use of harmful chemicals in agriculture and industry.
- Promoting public awareness campaigns to educate communities about the dangers of chemical exposure and preventive measures.
- Enhancing monitoring and enforcement of water quality standards to protect public health.
- Encouraging interdisciplinary collaborations among researchers, policymakers, and communities to develop and implement effective solutions.

By prioritising these actions, we can address the pressing challenges posed by chemical contaminants and safeguard human and ecosystems health for sustainable exploitation of their abundant resources.

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EDITED BY
Camilo Dias Seabra Pereira,
Federal University of São Paulo, Brazil

REVIEWED BY
Folarin Owagboriaye,
Olabisi Onabanjo University, Nigeria
Marina Trevizan Guerra,
Federal University of Mato Grosso do Sul, Brazil

*CORRESPONDENCE
Ebenezer O. Farombi,
olatunde_farombi@yahoo.com
Sunny O. Abarikwu,
sunny.abarikwu@uniport.edu.ng

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Atrazine: cytotoxicity, oxidative stress, apoptosis, testicular effects and chemopreventive Interventions

Sunny O. Abarikwu[1]*, Ogechukwu E. Ezim[1], Cynthia N. Ikeji[2] and Ebenezer O. Farombi[2]*

[1]Reproductive Biology and Molecular Toxicology Research Group, Department of Biochemistry, University of Port Harcourt, Choba, Nigeria, [2]Drug Metabolism and Toxicology Research Laboratories, Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria

Atrazine (ATZ) is an environmental pollutant that interferes with several aspects of mammalian cellular processes including germ cell development, immunological, reproductive and neurological functions. At the level of human exposure, ATZ reduces sperm count and contribute to infertility in men. ATZ also induces morphological changes similar to apoptosis and initiates mitochondria-dependent cell death in several experimental models. When *in vitro* experimental models are exposed to ATZ, they are faced with increased levels of reactive oxygen species (ROS), cytotoxicity and decreased growth rate at dosages that may vary with cell types. This results in differing cytotoxic responses that are influenced by the nature of target cells, assay types and concentrations of ATZ. However, oxidative stress could play salient role in the observed cellular and genetic toxicity and apoptosis-like effects which could be abrogated by antioxidant vitamins and flavonoids, including vitamin E, quercetin, kolaviron, myricetin and bioactive extractives with antioxidant effects. This review focuses on the differential responses of cell types to ATZ toxicity, testicular effects of ATZ in both *in vitro* and *in vivo* models and chemopreventive strategies, so as to highlight the current state of the art on the toxicological outcomes of ATZ exposure in several experimental model systems.

KEYWORDS

atrazine, oxidative stress, antioxidant vitamins, flavonoids, cytotoxicity, testes, apoptosis

1 Introduction

Atrazine (ATZ; 2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a triazine herbicide that is used to control the growth of broadleaf and grassy weeds. Atrazine is commonly spread pre-emergence to maize, coffee, sorghum, sugarcane, wheat, in conifer forests, on golf courses, on Christmas tree farms and household lawns. Currently, ATZ and related triazines are one the most extensively utilized agricultural herbicides in the world and the most frequently patronized herbicide in Nigeria (Adesina et al., 2014), and with an annual production of over 36,000 tonnes (Jablonowski et al., 2011; Chang et al., 2022). The extensive use of ATZ has given rise to its detection in the environment, largely in surface and groundwater (Goldman, 1994; Owagboriaye et al., 2022) and in maize plants (Santos-Hernández et al., 2018; Yu et al., 2021). ATZ at a concentration of 0.2 ppb has been detected in 2–3 million people who use groundwater as their main source of drinking water

(Goldman, 1994), as such ATZ has been given a sizeable deal of scientific audit by both governmental and scholarly researchers. ATZ can be found in the environment at concentrations as substantial as 21 ppb in groundwater, 42 ppb in surface waters, 102 ppb in water basins in farm areas, and up to 224 ppb in streams (Kolpin et al., 1998; Owagboriaye et al., 2022; Roh et al., 2023). The U.S. Environmental Protection Agency (EPA) has set the maximal limit of ATZ allowed in drinking water (MCL) at 3 ppb and has designated ATZ as a Restricted Use Pesticide in an attempt to minimize human exposure to ATZ through drinking contaminated groundwater (Costa Silva et al., 2010; Powell et al., 2011). However, in occupational set-up, humans are exposed to ATZ at a thousand-fold higher concentration than seen in residential exposure. Because triazine herbicides are dominantly applied to the maize cropping fields, the European legislation has set the maximum residue level for ATZ in corn as 0.25 mg kg^{-1} (Elvis et al., 2015; Yu et al., 2021). In fact, ATZ is thought to be highly selective for maize, since their roots could efficiently metabolize ATZ to OH-atrazine; and the formation of glutathione conjugates in the aerial parts of the plant (Tasli et al., 1996). Evidence from epidemiological reports have also indicated a plausible connection between exposure to ATZ-containing agrochemicals in workers handling the herbicide and an upsurge of diverse neoplastic diseases (Schroeder et al., 2001; Hopenhayn-Rich et al., 2002; Alavanja et al., 2003). Because of its unacceptable levels on groundwater, ATZ use was restricted in the European Union in 2004 (Zeljezic et al., 2006; Ackerman, 2007). However, ATZ-containing formulations have continued to be produced for export in some European member states (United Kingdom, Spain, Portugal and Ireland). In Africa and other developing countries where there are weak policies on agrochemicals usage and disposal, the contamination of drinking waters might even be higher. As a result of this and the constancy of ATZ in groundwater, segments of the European and African populations will still be brought into contact with this active ingredient. Although the strict ranking of ATZ as an endocrine disrupting compound has been discussed, a lot of sequelae on living organisms have been suggested (Solomon, 2009).

The endocrine disrupting effects of ATZ in birds, reptiles, fish, amphibians and mammals (Bisson and Hontela, 2002; Hayes et al., 2003; Fan et al., 2008; Zaya et al., 2011; Abarikwu et al., 2021); immunotoxic properties (Brodkin et al., 2007; Thompson et al., 2015) and effects on sperm qualities in animals at low exposure levels have been reported (Saalfeld et al., 2018). At higher exposure levels, ATZ causes a number of effects that are similar across several animal species, e.g., developmental delays and abnormalities (Tavera-Mendoza et al., 2002; Nieves-Puigdoller et al., 2007), steroidogenesis and spermatogenesis abnormality, induction of oxidative stress and cytotoxicity and apoptosis (Abarikwu et al., 2011a, 2012a; Victor-Costa et al., 2010; Pogrmic et al., 2009; Pogrmic-Majkic et al., 2010). Considering the relevance of genotoxic and oxidative damage in the harmful effects of several environmental and biological toxic chemicals such as agrochemicals, there has been no systematic study on the cytotoxicity, apoptosis and oxidative stress effects of ATZ in animal and cellular model systems. It is consequently imperative to point-out molecular variables of oxidative stress that detect effects of ATZ in both *in vivo* and *in vitro* experimental model systems. We present a review about ATZ effects, focusing on some target *in vivo* and cell-based *in vitro* systems in order to achieve a better grasp of its cellular mechanism of actions.

1.1 The Cytotoxicity of ATZ. data from *In Vitro* Study

The cytotoxic and DNA damaging effects of ATZ have been tested and confirmed in a variety of cell types; although most of the studies present inconsistent and conflicting results (Pino et al., 1988; Clements et al., 1997; Ribas et al., 1998; Taets et al., 1998). For instance, three cytotoxicity techniques: sister-chromatid exchanges, chromosome aberrations and micronuclei assays suggested that ATZ (5–100 μg/mL; 23.2–462 μM) lack the capacity to induce clastogenic and aneugenic damages in cultured human lymphocytes (Ribas et al., 1998). Other *in vitro* studies demonstrated that cultured human lymphocytes exposed to triazines (ATZ, simazine, and cyanazine) did not induce sister-chromatid exchanges or chromosome aberrations up to the limits of the doses of the chemicals that were severely toxic (Kligerman et al., 2000a). The chemical was also found not to be toxic to sheep peripheral blood phagocytes and lymphocytes even up to 100 mM, whereas in some studies, even at concentrations of ATZ as low as 0.0001 μg/mL (0.0005 μM), DNA damage were demonstrated in human lymphocytes cultures as measured by comet assay and chromosomal aberration analysis (Meisner et al., 1992). Similar results were also found when chromosome aberrations, sister chromatid exchanges and mitotic index, were applied in human peripheral lymphocytes exposed *in vitro* to ATZ at concentrations between 5 and 51 μM. Interestingly, the dose-related increase in the percent of aberrant cells and of sister chromatid exchanges were dependent on ATZ induced cytotoxicity as measured by Trypan blue exclusion assay (Lioi et al., 1998). At the highest concentration of ATZ exposed to the cultured human lymphocytes, glucose 6-phosphate dehydrogenase activity, either remained stable or decreased, when the cytotoxicity effect was increased (Lioi et al., 1998). This observation allowed the speculation that the increased sister-chromatid exchanges and chromosome aberrations after ATZ exposure, accompanied by a change in the cell redox state, confirms that reactive oxygen intermediates are involve in the cytotoxicity effect of ATZ in cultured lymphocytes. Thus, the thought that the genetic toxicity of ATZ and triazine chemicals could alter reactive oxygen intermediates in mammalian cells through a mechanism that involves glucose 6-phosphate dehydrogenase was demonstrated (Lioi et al., 1998). Hence, the initial response of the cells to ATZ would involve depleting of intracellular GSH pools, and subsequently, the inactivation of glucose 6-phosphate dehydrogenase activity/expression. It is therefore rationale to assume that when the exposure of cultured human lymphocytes to ATZ is sustained, the efficiency of the hexose monophosphate shunt to replenish the GSH pools is hampered with, together with it capacity to protect human lymphocytes against oxidant injury (Lioi et al., 1998). Furthermore, comet assay applied on erythrocytes isolated from the tadpoles, *Rana catesbeiana* exposed to ATZ showed DNA damage that appears to be dependent on ATZ dosages (Clements et al., 1997). Other studies have shown that these genotoxic effects are minimal, even if it exists. Roloff et al.

(1992) reported little chromosome damage in human lymphocytes exposed to 0.005 μM (0.001 μg/mL) ATZ concentrations. ATZ did not also induce genotoxic damage by comet and DNA diffusion assay in human lymphocytes at concentrations between 0.047 and 4.7 μg/mL, whereas the commercial preparations of ATZ containing adjuvant mixtures could increase DNA damage in lymphocytes (Zeljezic et al., 2006), suggesting that the commercial formulations contain other substances that represent a genotoxic risk to human lymphocytes. Although the DNA damaging effects for non-cytotoxic doses of ATZ appear controversial (Meisner et al., 1992), higher concentrations would be more consistent with the genotoxicity of ATZ. However, sister chromatid exchanges analysis at metaphase cells are not appropriate techniques to use for this endpoint, since affected cells are delayed in G2-phase and do not proceed to mitosis (Malik et al., 2004). Furthermore, as the cytotoxicity studies for high concentrations of ATZ did not also demonstrate any increase in homologous recombinational events (Malik et al., 2004), a genotoxic mode of action of ATZ cannot be assumed in cultured human lymphocytes. Therefore, the variations in the sister chromatid exchanges analysis after exposures to certain concentrations of ATZ are due to polarities in cell cycle kinetics of cultured lymphocytes, rather than to a true biological disparity in the cytogenetic marker used (Malik et al., 2004). Hence, the general notion for most studies on the genotoxicity of ATZ in human cultured lymphocytes is to report negative results.

The cytoxicity of ATZ has also been demonstrated in gonadal experimental model systems. Clastogenicity was reported in the Chinese Hamster ovary (CHO) cells when flow cytometry was the cytotoxicity technique applied to determine the chromosomal damaging potentials of ATZ at the US EPA maximum contamination level of 3 ppb (0.0139 μM), and the highest contamination level of 18 ppb (0.0835 μM) found in a community drinking water supplies (Taets et al., 1998). Another study using the CHO cells found that ATZ at 20 μg/mL (20,000 ppb) caused a 19% growth decrease after 72 h and at 80 μg/mL (80,000 ppb), the cell viability was inhibited by 55% (Kmetic et al., 2008). The rates of porcine oocyte maturation and quality were reduced after exposure *in vitro* to 200 μM ATZ. When 5-bromo-deoxyuridine assay was applied to test the cell proliferative capacity, the population of proliferating cells was found to decrease. The cytotoxicity of ATZ effect also resulted in the disruption of the spindle morphology, maturation-promoting factor activity; mitochondrial membrane potential and DNA damage response as shown by TUNEL assay (Yuan et al., 2017). High levels of superoxide radicals, increase cathepsin B activity and the decrease in GSH concentration was assumed to be responsible for the impaired developmental competence of porcine oocyte (Yuan et al., 2017). The viability of testicular cells (Leydig and Sertoli-germ cells) that were treated with 232 μM (50 μg/mL) ATZ concentration and subjected to the 2,5-diphenyl-2H-tetrazolium bromide salt (MTT) assay assays were found to be decreased (Abarikwu et al., 2011a; Abarikwu et al., 2012). The neutral red uptake (NRU) assay which allows viable cells to incorporate and bind the supravital dye neutral red in the lysosomes, also showed similar cytotoxicity pattern in Leydig cells but were less sensitive than the MTT assays (Abarikwu et al., 2011a). This concentration of ATZ that was found to be cytotoxic in testicular cells was also required to decrease the viability of the rat pheochromocytoma (PC12) cells (Abarikwu et al., 2011b). In this study on ATZ model of neuronal injury, the four cytotoxicity techniques: MTT, NRU, lactate dehydrogenase leakage and trypan blue assays confirmed the cytotoxic damage of ATZ in the PC12 cells. If the 24 h required for the manifestation of the cytotoxic response (Abarikwu et al., 2011b) is compared to the 48 h for the MTT assay and 72 h for the NRU assay; required for the onset of cell death in testicular cells (Leydig cells) (Abarikwu et al., 2011a), it could suggest that neuronal cell lines could be more vulnerable to the cytotoxicity of ATZ than testicular cells. Additionally, ATZ concentration (300 μM, 65 μg/mL) slightly higher than those reported in the above studies (Abarikwu et al., 2011a; Abarikwu et al., 2011b; Abarikwu et al., 2012) was also observed to decrease the proliferation and cellular expansion of the human neuroblastoma (SH-SY5Y) cells (Abarikwu et al., 2011c). This was also detected in cell viability studies as demonstrated by the MTT and NRU assays and DNA ladder-like formation detected when ethidium bromide stained agarose gel electrophoresis was applied to check the mechanism of the cell death (Abarikwu et al., 2011c).

The concentration-dependent cytotoxicity of ATZ has also been demonstrated across other mammalian cell types and fish cells. Tchounwou et al. (2001) found that ATZ concentration at 100 μg/mL (100, 000 ppb) is non-toxic to the human hepatoma cell line, HepG2. The chemical was also not toxic to rat primary hepatocytes (Sawicki et al., 1998). We had previously reported that ATZ at concentration of 300 μM (65 μg/mL); a concentration lower than that used in Tchounwou study was able to inhibit growth of human neuronal cell line (SH-SY5Y cell line) (Abarikwu et al., 2011c). An ATZ concentration of 625 ppb or higher was needed to cause a decrease in HepG2 cell proliferation compared to control cells (Powell et al., 2011). Interestingly, concentrations of ATZ lower than 625 ppb were not sufficient to inhibit growth of immortalized HepG2 cells (Powell et al., 2011). However, in a previous study, ATZ at a concentration as small as 0.8 ppb was sufficient to decrease the growth of normal human fibroblast cells (Dhanwada et al., 2003). This value is 3.2 times less than the MCL of 3 ppb fixed by the EPA and is frequently detected in drinking water supplies (Gely-Pernot, 2017). Furthermore, the 625 ppb value is 3.5–10 times smaller than the concentration reported in studies where immortalized cells had been used (Sanderson et al., 2000; Laville et al., 2006; Rowe et al., 2007) and 160 times smaller than was reported in HepG2 cells (Tchounwou et al., 2001). The cytotoxic effects of ATZ was also reported by the MTT assay in cultured grass carp (*Ctenopharyngodon idellus* cell line, ZC7901), with IC_{50} value ranging from 11.6 ± 6 0.5 mg/L to 199.0 ± 6 7.8 mg/L (Liu et al., 2006), indicating substantial flexibility in the cytotoxic responses of fish cells to ATZ exposure. Moreover, DNA fragmentation was detected by the TUNEL reaction and agarose gel electrophoresis in dose-and time-dependent fashion. The application of the MTT technique on this experimental model system, confirmed the cytotoxicity of low concentration of ATZ (9.4–47.2 mg/L) in fish cells.

The cytotoxicity of ATZ was also tested at doses ranging from 10–500 μM in human embryonic stem cells (hESC), a differentiation model system that is used to test the toxicity of chemicals at divergent phases of neural differentiation *in vitro*. In this study, hESC were differentiated into neural stem cells before they were terminally differentiated to neurons and glial cells after 21 days. The

application of the cell counting kit-8 (CCK-8) cell viability assay showed that ATZ could inhibit hESC viability and proliferation, and the numbers of colonies were dose-dependently decreased reaching almost 50% at 200 μM dose of ATZ after a long period of exposure (Shan et al., 2021).Other studies demonstrated that exposure to low-dose ATZ ranging from 0.0014–0.14 μM (0.3–30 ppb) for 4 days prior to differentiation and completion of differentiation, can result in long-lasting changes in epigenome and increase risks of synuclein alpha (SNCA)-related Parkinson's disease in a pre-differentiation SH-SY5Y model system (Xie et al., 2021). The cytotoxicity observed in this study was minimal at the tested ATZ concentrations when tested by the MTT assay, suggesting that neuronal differentiation was affected without changes in the viability of cells (Xie et al., 2021). The effects of ATZ (12–300 μM) exposure on N27 rat dopaminergic cells, also confirmed that ATZ could alter the morphology of undifferentiated N27 cells, because after 48 h of exposure, differentiating N27 cells exhibited increased numbers of neurites and longer neurite length (Lin et al., 2013) similar to the altered neurite outgrowth reported at pre-differentiation exposure of SH-SY5Y to ATZ (Xie et al., 2021). It is interesting to also note that the sexual differentiation in amphibians also suffers dramatic changes at doses as low as 0.1 μg/L of ATZ (Hayes et al., 2002). It is therefore reasonable to assume that developmental exposure to low dose ATZ has the potential to cause long-lasting neurological variations (neural toxicity) and reproductive phenotypic changes in animal models. This is interesting to know, and has important environmental and public health concern, because the low dose 3 ppb and high dose 30 ppb ATZ concentrations employed in these studies are the present EPA regulation standard of ATZ in drinking waters, and the minimal dose of ATZ that can elicit gene expression changes without cytotoxicity effects, that are considered safe in drinking water or safe for limited human exposure, respectively (Rohr and McCoy, 2010; Xie et al., 2021).

Similarly, conflicting results on the cytotoxicity of ATZ in intestinal cells have been demonstrated. For instance, ATZ concentrations ranging from 1 to 10 μM (215.7–2157 ppb) increased the proliferation of human intestinal epithelial cells after 72 h, a mechanism associated with cancer development (Green and Reed, 1998).The application of trypan blue exclusion assay for cellular viability and the MTT assay for cell growth study on the normal rat IEC-6 intestinal and human colonic epithelial cell cultures produced a growth-stimulatory effect of ATZ, and the lowest dose (0.5–10 μM) was even more potent than higher doses (50 μM) in stimulating cell growth (Green and Reed, 1998). Because the growth and viability of normal rat intestinal cells and human colonic epithelial cell cultures treated with the DMSO vehicle were not influenced, lead to the suggestion that the growth rate increases in ATZ treated rat intestinal and colonic epithelial cell cultures are not due to the vehicle control (DMSO). However, the human colonic epithelial cell cultures are more sensitive to the effects of ATZ in sustaining cell growth than the rat intestinal cell cultures (Green and Reed, 1998). At concentration of 50 μM ATZ and above, ATZ was found to provoke substantial cytotoxic effects on the human intestinal Caco-2 cells. This brings about the decrease in cell proliferation rate and viability (Olejnik et al., 2010). Moreover, long-term exposure of the intestinal cells to ATZ at non-cytotoxic doses (1–10 μM) inhibited cell maturation and decline the transepithelial electrical resistance (Olejnik et al., 2010). The MTT test and the Trypan blue exclusion assay verified the cytotoxicity of ATZ, as both a dose- and time-dependent event in Caco-2 cells cultures (Olejnik et al., 2010). Thus, unlike the normal rat intestinal and human colonic cells, the human intestinal Caco-2 cells are more sensitive to the cytotoxic effect of ATZ (Olejnik et al., 2010). The reasons for these contrasting results are, apart the cancerous nature of Caco-2 cells, it is thought that ATZ causes loss of growth control in normal intestinal cells leading to its fast proliferation which allows the propagation of the mutated cells and ultimately leads to neoplasia (Olejnik et al., 2010). Furthermore, the growth of colonic epithelial cells may be responsive to the estrogenic activity of ATZ, such that the higher the number of estrogen receptors in normal cells, more than those of transformed cells, the higher the capacity of ATZ to stimulate normal intestinal cell proliferations (Olejnik et al., 2010). Additionally, DNA breaks and alkali labile lesions were found in gastric mucosa cells, when DNA alkaline elution technique was applied as the genotoxicity assay (Pino et al., 1988).

It is interesting to speculate that some aromatase sensitive cell types appear to exhibit consistency when they are tested for ATZ cytotoxicity. Laville et al. (2006) reported that ATZ at 10 μM (2157 ppb) did not alter the viability of the human choriocarcinoma JEG-3 cell line after 24 h as verified by the MTT assay; although this concentration could induce the activity of the aromatase enzyme, a very important molecular target of triazine herbicides, and increase or inhibit the viability of other cell types not expressing aromatase (Olejnik et al., 2010; Green and Reed, 1998). Several herbicides with endocrine disrupting effects like ATZ could also induce or inhibit aromatase activity at concentrations even lower than those unable to alter cell viability, suggesting that cytotoxicity and steroidogenic effects share dissimilar molecular events in aromatase responsive cell types (Laville et al., 2006). Another possibility on the unaltered viability of the JEG-3 cells would be that the high expression of aromatase in JEG-3 cells might be protective against cytotoxic responses by ATZ. This appears rationale to assume because human adrenocortical (H295R) and human breast cancer (MCF-7) cell lines which also express high level of aromatase (Sanderson et al., 2001) did not experience cytotoxicity at the same dose applied in the JEG-3 cells (Sanderson et al., 2000). In the work of Sanderson and colleagues, ATZ at concentrations of 0.3–30 μM (64.7–6,471 ppb) did not alter the growth of the H295R cells, even at the highest ATZ concentration tested, and despite the fact that these concentrations caused a dose-dependent increase in aromatase activity (Sanderson et al., 2000) and drastically inhibit the viability of other cell types (Abarikwu et al., 2011c; Powell et al., 2011). These molecular features could be responsible for dampening ATZ cytotoxicity on these cell types.

ATZ cytotoxicity has also been reported in immune cells. For instance, natural-killer cell-specific activity of peripheral blood lymphocytes declined 24 h after ATZ exposure at concentrations of 3–30 μM (647.1–6,471 ppb), however, cell viability was unchanged even at the highest tested concentration of the herbicide (Rowe et al., 2007). $CD4^+$ T cells were activated by 30 μM ATZ after 4 days of exposure (Thueson et al., 2015). The ATZ exposure decreased the antigen-driven build-up of $CD4^+$ cells, an observation that is at variance with the unaltered numbers of $CD4^+$ T cells that was reported earlier in the work of Filipov et al.

(2005). This discrepancy was explained by the fact that *in vivo* ATZ exposure was not accompanied with lymphocyte activation. This is because in the absence of the triggering of T cell receptors (TCR), ATZ has only minimal effect on $CD4^+$ T cell numbers (Shan et al., 2021). This is clinically relevant because the level of ATZ that produced these effects is lower than their urinary concentrations in high-risk people including farmers (Perry et al., 2001) and is similar or smaller than the levels reported in in vitro studies examining the immunotoxicity of ATZ (Devos et al., 2003; Pinchuk et al., 2007). Interestingly, 30 μM ATZ when applied *in vitro* to the $CD4^+$ T cells was not cytotoxic since the frequency of apoptotic or necrotic cells was not raised. However, ATZ completely inhibited $CD4^+$ T cell proliferation in a manner that was correlated with the inhibition of T cell activation, suggesting that people that are chronically exposed to ATZ occupationally or in their drinking water may have an altered immune status (Thueson et al., 2015). Recently, Galbiati et al. (2021) speculated that ATZ act on dopamine receptors expressed in immune cells during inflammatory responses for host defense, and alter inflammation-related diseases. Therefore, triazines immunotoxicology remains an important area to explore as it would influence our understanding of the connection between the immune and nervous systems in triazines disease model, e.g., inflammation-related diseases (Thompson et al., 2015). Dendritic cell viability studies with trypan blue exclusion assay showed that ATZ, at concentrations of up to 100 μM was not cytotoxic to bone marrow-derived immature dendritic cell lines (JAWSII DC) after 24 h. The application of annexin-V apoptosis assay to detect apoptosis found that at concentrations of ATZ up to 200 μM, the number of JAWSII cells undergoing apoptosis was increased, whereas at the highest concentration (300 μM), it increased modestly, the percentage of annexin-V positive cells. The phenotypic changes, including loss of surface major histocompatibility complex −1 (MHC-I) was observed at 1 μM concentration of ATZ. In these studies, the proportions of mature dendritic cells were decreased, suggesting that ATZ inhibited dendritic cell maturation even at doses that are regarded to as not cytotoxic (Pinchuk et al., 2007). Furthermore, ATZ-induced degranulation of mast cells was determined by measuring the release of granule-associated β-hexosaminidase from RBL-2H3 cells in which ATZ between the range of 10 nM and 1 μM showed induced rapid degranulation in mast cells. Interestingly, ATZ did not cause any cytotoxic effects on the cells, as evaluated by the trypan blue exclusion assay (Mizota and Ueda, 2006). It may appear that the doses of ATZ that interfered with major biological functions do not alter the viability of immune cells.

The cytotoxicity of ATZ has been tested with other triazines in four human breast cell lines: MCF-7, MDA-MB-231 and MCF-10A. One common feature of these cells is their capacity to respond to endocrine toxicity (Rich and Gabriel, 2012). These breast cancer cells were found to be responsive to endocrine-disruptors, but the triazines (atrazine, cyanazine and simazine) failed to dramatically alter cell densities (Rich and Gabriel, 2012). Although there was a sign of plausible increase with ATZ at 10 nM and cyanazine at 10,000 nM in the MCF-7 cells, a tendency for raised cell viability was observed at 1,000 nM with simazine in MCF-7 and MDA-MB-231 cells, and with no changes for the MCF-10A cells with any of the tested triazines. It appears that the existence of the estrogen receptors in these cells enhances the stimulatory effects of these chemicals and inflates cell growth, yet when these receptors are missing, the chemicals may be initiating cell death through apoptotic pathways (Rich and Gabriel, 2012). The viability of MDA-MB-231 cells, an estrogen independent breast cancer cell and the non-cancerous MCF-10A breast cells were not altered much after exposure to ATZ, even though it shows a trend to decrease except for simazine which dramatically increased the cell viability, suggesting that this triazine unlike ATZ responded diversely in the cells and/or that different tumors respond differently to triazine chemicals, a scenario that could influence the likely stimulatory and cytotoxicity actions of these chemicals (Rich and Gabriel, 2012). This variance in the responsiveness of different cells to ATZ toxicity are thought to be due to several points such as the choice of solvent (e.g., dimethyl sulfoxide or ethanol) used to dissolve the ATZ (Powell et al., 2011) and the ATZ estrogenic activity (Olejnik et al., 2010) as previously mentioned above. It is known that cultures of non-transformed cells are more estrogen-sensitive than the tumor cells (Potter, 1995). To affirm this speculation, Manske et al. (2004) reported that when DMSO was used as a solvent for ATZ, a 12.5-fold higher level of ATZ (10 ppb) was required to inhibit growth of normal human fibroblasts. Also, the proliferation of the estrogen-sensitive MCF7-BUS human breast cancer cells was not affected by ATZ concentration of up to 10 μM (Oh et al., 2003). It seems that ATZ can inhibit proliferation, viability and induces DNA damage in different cell types under *in vitro* settings, mostly at levels that do not frequently occur in the environment (Table 1). Furthermore, low-level of ATZ at concentrations that are environmentally material can lead to inhibition in the growth of normal cells raising the possibility that normal cells are more susceptible to the cytotoxicity of ATZ than immortalized cells. From all the data above, and within the context of ATZ model of cellular toxicity, cytotoxicity occurs at ATZ concentrations different from the doses that interferes with other important biologic functions, e.g., enzyme activity and/or expression, and that primary cells are more sensitive than cell lines to ATZ cytotoxic effect, which might also be affected by several factors including cell types (estrogen-responsive) and choice of the solvent used to dissolve the ATZ.

1.2 Apoptosis-inducing effects of ATZ

Atrazine-induced apoptosis has been investigated in several experimental model systems (Table 2). A study developed by Liu et al. (2006) demonstrated that cells that are incubated with ATZ-presented a series of structural changes, including shrinking of the nucleus, flanking of chromatin to the shape of thick granular caps, and generation of apoptotic bodies. Moreover, TUNEL assay and agarose gel electrophoresis were used to detect the fragmented DNA. This was the first proof that ATZ could cause apoptosis *in vitro*. Exposure to agrochemicals, e.g., ATZ is assume to be one of the stressful cellular situations that could trigger some molecular events, e.g., expression of p53 protein. This protein gauges DNA damage and function as a transcription factor controlling genes, which regulate cell growth, apoptosis and DNA repair (Cozmei et al., 2002). p53 expression was found to be increased in peripheral lymphocytes of rats chronically treated with ATZ (Cantemir

TABLE 1 Cytotoxicity effects of different concentrations of atrazine (μM) in different cell types.

Concentrations (μM)	Cell Types	Effects on cell viability	References
4×10^{-3}	Human fibroblasts	Decreased	Dhanwada et al. (2003)
10	Caco-2	No change	Green and Reed (1998)
30	H295R	No change	Sanderson et al. (2000)
30	Peripheral blood lymphocytes	No change	Rowe et al. (2007)
2.9–23.2	HepG2	Decreased	Powell et al. (2011)
50–1,000	Rat hepatocytes	No change	Sawicki et al. (1998)
200–300	Mouse dendritic cells	Decreased	Pinchuk et al. (2007)
232	Rats testicular cells	Decreased	Abarikwu et al. (2011a)
232	PC12	Decreased	Abarikwu et al. (2011b)
250	Caco-2	Decreased	Olejnik et al. (2010)
300	SH-SY5Y	Decreased	Abarikwu et al. (2011c)
93–371	CHO-K1	Decreased	Kmetic et al. (2008)
463	HepG2	No change	Tchounwou et al. (2001)
10–500	hESC	Decreased	Shan et al. (2021)
30	$CD4^+$ T	No change	Thueson et al. (2015)
0.01–10	Splenic lymphocytes	No change	Chen et al. (2015)

TABLE 2 Expressions of apoptosis-related genes in differerent cell types exposed atrazine.

Concentrations (μM)	Cell types	Expressions of genes	Genes	Reference
300	SH-SY5Y	Decreased	p53	Abarikwu et al. (2011c)
		Decreased	p21	
		Decreased	Bax	
		Decreased	Bcl-2	
232	PC12	Increased	p53	Abarikwu et al. (2011b)
		Increased	Bax	
		Increased	Caspase-3	
		Increased	Caspase-9	
		Decreased	Bcl-2	
0.23–2.32	HepG2	Decreased	Cyclin B	Powell et al. (2011)
		Decreased	Cyclin E	
0.01 and 0.1	H22	Decreased	p53	Tian et al. (2018)
		Increased	Cyclin-D1	
		Increased	VEGF	
		Increased	C-myc	
10		Increased	Caspase-3	Ge et al. (2021)
	Lymphocytes	Increased	Caspase-9	
		Decreased	Bcl-2	
1–30	Human placenta	Decreased	p53	Ibrahim et al. (2015)
		Increased	Bcl-2	

et al., 1997). The peripheral mononuclear cells of humans occupationally exposed to ATZ also showed increased expression of p53 (Cozmei et al., 2002). Tian et al. (2018) observed that the proliferation of H22 cells after been treated with 0.01 and 0.1 μM ATZ increased in the absence of changes in the percentage of apoptotic cells. Additionally, the tumor size and mass of ascites were remarkably expanded in an orthotopically implanted hepatocarcinoma tumor C57BL/6 mice model. p53 expression was downregulated, whereas those of cyclin-D1, MMP2, Stat3, VEGF and C-myc was upregulated by ATZ, suggesting that ATZ activated Stat3 signaling and induced the proliferation and seizure of hepatocellular carcinoma cells. The H22 cells that were exposed to ATZ at 0.01 μM displayed an increase in proliferation much more than those exposed to 0.1 μM ATZ when tested by the MTT proliferation assay. The increase in H22 cells proliferation was also accompanied with a decline in the number of cells in the G2 phase and a rise in the populations of cells in the S phase, suggesting that ATZ induces the accumulation of cells in the S phase and moves the H22 cells quickly through the G2 phase (Tian et al. (2018). The numbers of apoptotic cells in human lymphocytes have also been reported to escalate when treated with commercial preparations containing ATZ (Zeljezic et al., 2006). The apoptotic nuclei of lymphocytes going through apoptosis or necrosis were distinct from normal cells in having a foggy or vague outline without exact margins because of the nucleosomal-sized DNA dispersing into the agarose. Necrotic cell nuclei are larger and are poorly delineated. They have a distinct, defined outward margin of the DNA halo and a proportionate homogenous halo appearance. Conversely, cells that are not necrotic or apoptotic but have damaged DNA have well-defined nuclei with a broad halo and defined external boundary.

Shan et al. (2021) treated hESC and neural stem cells to ATZ for up to 24 h and observed that ATZ caused dissimilar toxic susceptibility on these cells. For instance, ATZ stopped the G1 phase of neural stem cells *by* down-regulating the cyclin-dependent kinase 4 and 2 (CDK4 and CDK2), which occluded more cells to traverse the G1/S phase nodes and inhibited the mitosis of neural stem cells. Thus, considering the neurotoxicity of ATZ, it is believed that ATZ not only target the dopaminergic system but also the glutamatergic neurons and astrocytes (Filipov et al., 2007; Shan et al., 2021). In addition, MKI67 (marker of proliferation Ki-67) and PCNA (proliferating cell nuclear antigen) gene expressions along with CCND1 which moves cells from G1 into S phase were decreased, but the percentages of early apoptosis and late apoptotic cells showed no obvious changes at the tested doses (100–500 μM) of ATZ treatment (Shan et al. (2021). The recent findings by Galbiati and colleagues on the connection between the immune and nervous systems in endocrine disruption of ATZ, confirms the relevance of the dopaminergic system in the neurotoxicity of triazines (Galbiati et al., 2021). Manske et al. (2004) did not detect any increase in apoptosis in human fibroblast exposed to low doses of industrial grade ATZ. However, in the study of Greenlee et al. (2004) herbicide preparations containing ATZ suitable for commercial purposes increased the rate of apoptosis in exposed murine embryos.

There are numerous possible course of toxicity that would lead to the suppression of cell growth. One conceivable rationale for the decrease in the cell number could be that ATZ exposure cause DNA damage in cells and ultimately apoptosis. In the research of Olejnik and Colleagues, the detection of the DNA damage was conducted utilizing the alkaline single cell micro electrophoresis assay, a helpful and responsive technique for assessing DNA damage (Ventura et al., 2008). Their results showed that intestinal Caco-2 cells responsiveness to ATZ-induced DNA damage was not remarkably contingent on the growth stage and that Caco-2 cells were not sensitive to the genotoxic effect of ATZ at concentrations of up to 50 μM (Figure 1). To understand the reasons for the diminished cell numbers after exposure of cells to ATZ, propidium iodide staining of DNA accompanied with flow cytometry analysis was used to determine if the ATZ treated cells were adjusted in their successions along the cell cycle (Powell et al., 2011). After exposure to ATZ for 48 h, an aggregation of the S phase cells was observed in the 100, 300 and 500 ppb treated samples (Figure 2) and a decrease in the G1 and the G2/M phases cells in the 300 ppb treated cells for 24 h (Powell et al., 2011). This is the same as the report of Freeman and Rayburn (2006) where CHO cells exposed to ATZ at 200 μM (43,000 ppb) produced a notable aggregation of S phase nuclei. In the study of Powell and colleagues a much smaller concentration of ATZ (100 ppb) was indispensable to initiate an effect. The capacity of ATZ to cause DNA fragmentation and apoptosis in fish cell lines was found to include interference on the mitochondrial membrane potential and production of ROS (Liu et al., 2006). Likewise, the clastogenicity of ATZ in the peripheral lymphocytes of rats was accompanied with escalated expressions of p53 proteins (Cantemir et al., 1997). Experiments developed by our research group also reported that high concentrations of ATZ (300 μM) initiated nuclear changes linked with apoptosis; including fragmentation of nuclei, condensation, DNA laddering (Figure 3), and amplified caspase-3 activity in the human neuroblastoma SH-SY5Y cells. This was associated with alterations in the expressions of caspase-3, caspase-9, p21, p53, Bax and Bcl-2, p21, and and decreased proliferation and growth of cells (Abarikwu et al., 2011c). The diminished expressions of

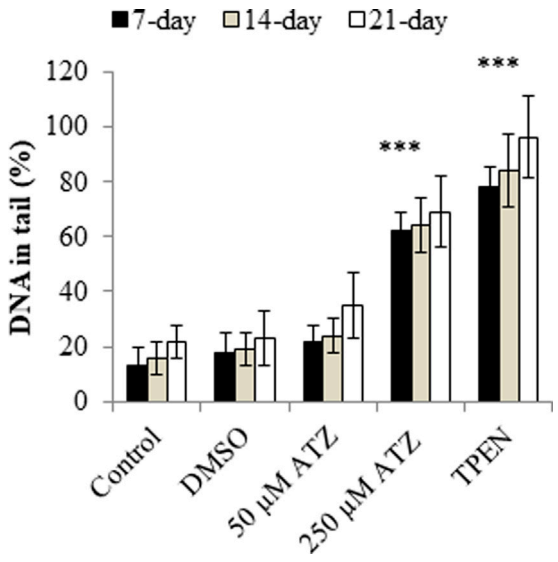

FIGURE 1
DNA damage in 7-, 14-, and 21-day-old Caco-2 cells treated with 50 and 250 μM ATZ for 24 h. DNA damage is expressed as the percentage of DNA in the comet tail. (***$p < 0.001$). Source: Redrawn from data of Olejnik et al. (2010).

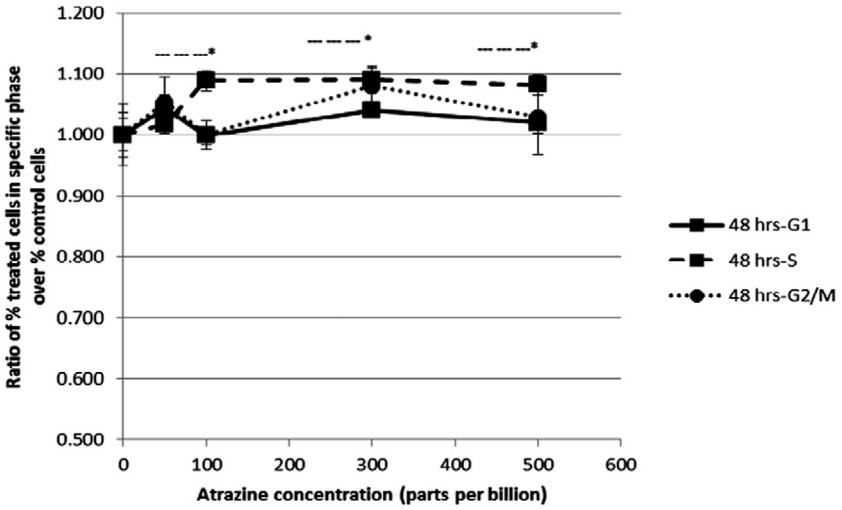

FIGURE 2
Flow cytometric analysis of HepG2 cells after a 48 h exposure to increasing concentrations of atrazine. Cells were harvested and DNA was stained with propidium iodide for flow cytometry analysis. The ratio of the percent of treated cells in a specific phase of the cell cycle (G1, S or G2/M) over the percent of untreated control cells in the same phase was determined. The ratio ±standard error of the mean (SEM) is indicated. Source: Powell et al., 2011.

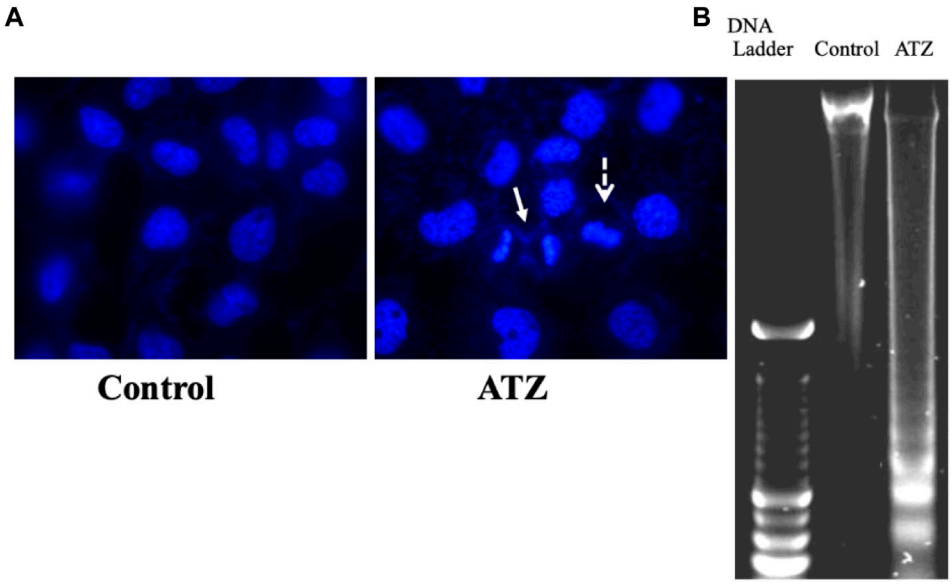

FIGURE 3
Nuclear changes **(A)** and DNA-fragmentation **(B)** in SH-SY5Y cells exposed to 300 μM of atrazine after 48 h culture period. Source: Abarikwu et al., 2011c.

c-fos and *c-Jun* in PC12 cells as observed earlier by us was as a consequence of the observed apoptotic cell death as demonstrated by the MTT assay (Abarikwu et al., 2011b). The alterations in the expressions of apoptosis-related genes implicate the role of apoptosis in the observed cell death. Flow cytometric examination established the implication of ROS in the ATZ-induced apoptosis *in vitro*. We conclude here that the increased ROS levels and alterations in the gene expressions of apoptosis-related genes and proteins in experimental models exposed to ATZ indicates the potential of ATZ to induce apoptosis in mammalian models especially at high concentrations.

1.3 The cytotoxicity of ATZ. data from *In Vivo* study

The DNA-damaging potentials of ATZ in polychromatic erythrocytes of mice were not evident as measured by the bone marrow micronuclei assay, even at large dose exposures that result in bone marrow suppression and/or death (Kligerman et al., 2000b). Other studies reported that, even extremely high concentrations of the triazines, including ATZ, have only marginal DNA-damaging activity *in vivo* in mouse leukocytes, when the isolated leukocytes

were subjected to DNA damage analysis using the alkaline single cell gel electrophoresis assay, one of the most responsive DNA damage protocols available (Tennant et al., 2001). DNA breaks and alkali labile lesions were found in stomach mucosa and kidney, and to a lower degree in the liver, but not in the lungs of rats that were orally administered a single high dose (875 mg/kg b.w) or repeated daily doses (350 mg/kg b.w) of ATZ (Pino et al., 1988) for 5 and 15 days. Interestingly, the DNA damage in the stomach mucosa environment as detected by the DNA alkaline elution technique correlated with the activation of ATZ in the gastic cells (Rajkovic et al., 2011). This observation, couple with the insolubility of ATZ in water and the prolonged contact of ATZ with the stomach mucosa environment, was the reason for the long-lasting DNA-damaging activity of ATZ in gastric cells (Adler, 1980; Rajkovic et al., 2011). High levels of ATZ were reported in the kidneys and liver of mice after single oral exposures to ATZ (5–250 mg/kg b.w) using liquid chromatography/mass spectrometry ((Ross et al., 2009). In rat´s liver, ATZ inhibited the activities of principal enzymes of gluconeogenesis including hexokinase, glucokinase and glycogen synthase resulting in reduced accumulation of glycogen in hepatic tissues, early signs of cytotoxicity and decreased body weight (Gluzczak et al., 2007). This was also detected in the liver of fish (Curic et al., 1999). Atrazine exposure to the Neotropical fish, *Astyanax altiparanae* promoted oxidative stress in their gills, liver and muscles. For example, glutathione *S*-transferase (GST) was decreased and CAT activity was increased in gills. Lipid peroxidation increased in the liver, but endocrine parameters were not altered except for the disruption of the triiodothyronine to thyroxine (T3/T4) ratio, suggesting that in this fish model of ATZ toxicity, oxidative stress variables are better indicators of environmental stress than endocrine disruption. The histological features of the liver included enlarged sinusoid capillaries, vascular congestion and enhanced leukocyte infiltration. The diameter of the hepatocytes and cell size and the hepatocyte nucleus diameter were small. Therefore, authors hypothesized that at low ecologically pertinent and applicable concentrations of ATZ (0.5–10 μg/L), oxidative damage and histological abnormalities in the adult Neotropical fish are better indicators of ATZ toxicity (Destro et al., 2021).

Atrazine was found to increase CAT levels and maintained the expressions of superoxide dismutase (SOD) and GST in the liver of rats. In addition, lipid peroxidation, degeneration of hepatic tissues, activation of heat shock protein-90, escalated expression of connexin mRNA, and genotoxic damage were reported in the liver of the animals. It was concluded that ATZ prompted hepatic oxidative stress that provoked defense mechanisms of the liver, an adaptive strategy essential for maintaining the morpho-physiological integrity of hepatic tissues (Campos-Pereira et al., 2012). The genotoxic potential of ATZ was confirmed by the induced frequency of micronucleated polychromatic erythrocytes that could also be responsible for the elevated lipid peroxidation and cytotoxicity (Campos-Pereira et al., 2012).The role of oxidative stress in ATZ-induced cytotoxicity was further confirmed in the spleen of mice, at doses of ATZ ranging from 100–400 mg/kg b.w after 21 days of daily oral gavage to mice (Gao et al., 2016). The elevated ROS levels and the depletion of GSH concentrations in the serum were all found to occur in a dose-related manner. Additionally, when the splenocytes were removed and tested by comet assay, an increase in DNA comet tail formation was found, confirming the presence of DNA damage. Interestingly, expressions of antioxidant enzymes genes such as heme oxygenase −1 and glutathione peroxidase-1 responded positively in the spleen. It appears that elevated oxidative stress, amongst other factors are implicated in the immunotoxicity effects of ATZ in mammals (Gao et al., 2016). However, the relevance of this hypothesis remains to be clarified, since these concentrations of ATZ are rarely encountered in real-life human scenarios (Lagunas-Basave et al., 2022; Owagboriaye et al., 2022; Li et al., 2023). The cytotoxicity of ATZ at a concentration of 15 μg/L on the erythrocytes of *Lithobates spectabilis* (male frog, native to Mexico) as detected by the micronucleus test, was accompanied with increases in the areas of melanin-containing melanomacrophage centers, and abnormal histological features of the liver along with a rise in the number of membrane with bumpy surfaces, apoptotic, and necrotic erythrocytes (Méndez-Tepepa et al., 2023). Although liver biomarkers that permit for the examination of liver damage were missing in the study, the ATZ effects such as hepatotoxicity and cytotoxicity on the native frog species are relevant indicators of environmental stress especially in countries where ATZ is widely authorized for use in different irrigation systems increasing its potential to contaminate aquatic systems.

There are still no studies on cell cycle proteins to define the mechanisms of apoptosis and necrosis in frog's erythrocytes. *Rana catesbeiana* tadpoles (bull frog) exposed to concentrations of AAtrex Nine-O (ATZ as active ingredient) showed significant increases in DNA damage when compared to the control. The percentage of damaged cells was also increased with increasing dose of the herbicide (Figure 4), but the association between DNA damage and the herbicide dose was modest (r = 0.663). Furthermore, no tadpoles lived after 24 h of exposure to 308 mg/L of the herbicide (Table 3), a concentration that is calculated to approximate one-tenth of the oral LD_{50} (3,080 mg/kg) of AAtrex for mice (Clements et al., 1997). It may be assumed that low concentrations of ATZ threaten the survival of organisms that inhabits small bodies of water draining pesticide runoff. The ability of ATZ to also induce genotoxicity *in vivo* concerning the increase in the prevalence of micronuclei and DNA strand breaks was also demonstrated in the erythrocytes of *Carassius auratus* (Cavas, 2011). Cytosol leakage, pyknosis and karyolysis were the common histological features, findings that are indicative of nuclear damage and cell death. The observed cell death agrees with the biochemical data, illustrated by the elevated malondialdehyde (MDA) concentration, tissue degeneration, and over expression of connexions (Campos-Pereira et al., 2012). It was assumed that the cell death occurred by apoptosis, since pyknosis was noticed without the formation of dense bodies and chromatin marginalization, or it could have happened by necrosis because of the notable cell lysis, cytosolic leakage and karyolysis; however, the levels of alanine aminotransferase did not confirm this mechanism. The third assumption on the mechanism of the cell death is autophagy, which was marked by cellular atrophy, nuclear pyknosis and cytoplasmic vacuolation as a consequence of the macroautophagy (Yin et al., 2008). Because a TUNEL reaction in the liver of the rats models (Campos-Pereira et al., 2012) did not confirm cell death, it was thought that the mechanism of cell death induced by ATZ *in vivo* needs to be verified. The glycogen and lipid content in the liver of tadpoles (*Xenopus laevis*) that were exposed to ATZ was unchanged (Zaya et al., 2011). The liver of the tadpole was smaller and those exposed to higher concentration of

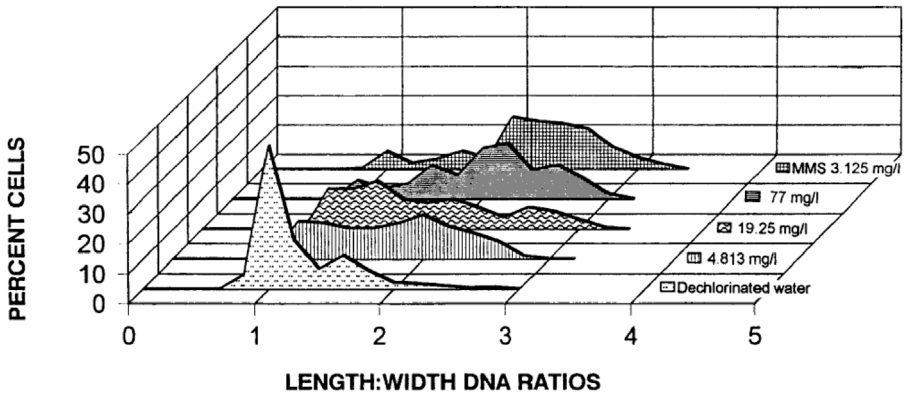

FIGURE 4
Distribution of DNA damage (based on length-width ratios of DNA patterns) observed at the cellular level in *R. catesbeiana* tadpoles (bullfrog) after exposure for a 24-h period to dechlorinated water. Methyl methanesulfonate (MMS) and selected concentrations of the herbicide AAtrex Nine-O; Source: Clements et al., 1997.

TABLE 3 Detection of DNA damage in erythrocytes of *R. catesbeiana* tadpoles exposed to AAtrex Nine-O for 24 h (Source: Clements et al., 1997).

Dose (mg/L)	No. of tadpoles	DNA length: width ratio ± SEM[a]	Range: SD ratio
Negative control	11	1.343 ± 0.022	2.99
4.813	7	1.821 ± 0.095	2.38
19.25	6	1.861 ± 0.138	2.52
77	7	2.131 ± 0.087	2.71
308[c]	4		
Positive control (3.125 mg/L MMS)	9	2.265 ± 0.044	3.45

*The recommended application concentrations ranged from 2.5 to 29.3 g/L.
[a]Ratios based on 25 cells/tadpole.
[b]All comparisons are relative to the negative control.
[c]All animals died within 24 h.
MMS, methyl methanesulphonate.

ATZ (400 μg/L) had larger numbers of activated caspase-3 immuno-positive cells, suggestive of increased rates of apoptosis. The authors believed that the observed changes in body and organs as well as fat body size suggested that ATZ exposure compromised the development of tadpoles (Zaya et al., 2011). Possibly, ATZ decreases the ability of tadpoles to transform and survive the stresses of metamorphosis or diminish their reproductive fitness. The latter is rationale to assume since frogs depends on lipid storage for these molecular events. Histological changes were also found in the liver of ATZ treated zebrafish (*Danio rerio*). ATZ-induced vacuolar degenerations of the liver, biliary hyperplasia and renal tubular necrosis have also been demonstrated in the male Japanese quail, *Coturnix japonica* (Hussain et al., 2011).

To further support the role of apoptosis in ATZ toxicity, low numbers of 3β-hydroxysteroid dehydrogenase positive Leydig cells accompanied with amplified *in situ* cell death fluorescence (Figure 5) and caspase-3 immunoexpression in the testes of mice orally exposed to ATZ (50 mg/kg b.w) for 3 days was demonstrated by us (Abarikwu et al., 2021). The same study also reported elevated immunoexpression for cell cycle gene regulators, including p45, cyclin D2 and E2, suggesting that ATZ bluntly diminish the population of testosterone producing Leydig cells in mice by apoptosis (Abarikwu et al., 2021). It is however possible that metabolites of ATZ are more toxic than ATZ in terms of their endocrine disrupting effects, since diaminochlorotriazine inhibited both serum and testicular testosterone concentrations in mice, unlike ATZ that decreased only testicular testosterone concentrations (Jin et al., 2014).

Another study performed in zebrafish also observed alterations in the expressions of proteins associated with oxidative stress, oncogenesis, lipid metabolism and insulin resistance after ATZ exposures (Yuanxiang et al., 2012). Lipid droplets were found to be accumulated in the liver of rats after 5 months of exposure to ATZ and this was associated with altered mitochondrial morphology in the liver, and additionally in muscle. Since no treatment-associated changes in food or water intake or physical activity were reported throughout the study, it was suggested that the occurrence of insulin resistance as a consequence of ATZ exposure might be related to energy metabolism, and authors suggested that prolonged exposure to ATZ might contribute to the development of insulin resistance

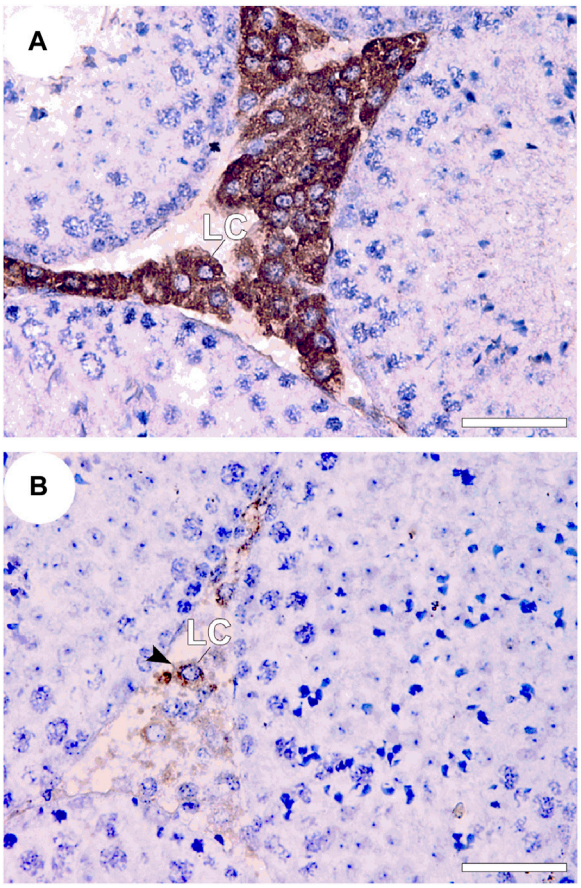

FIGURE 5
3β-HSD immunostaining in the testes of control and treated BalB/c mice 3 consecutive days after treatment with 50 mg ATZ per kg body wt. The immuno-expression of 3β-HSD was intense in the Leydig cells (LCs) of the control **(A)** and was decreased in the testis of atrazine treated mice **(B)**, Note that 3β-HSD is expressed in almost all LCs in controls, but is only expressed in some LCs in atrazine treated mice, whereas other LCs are less immunopositive for 3β-HSD (arrowhead). Scale bar = 50 μm, Mag 400×). Source: Abarikwu et al., 2021.

and obesity, especially where a high fat diet is fashionable (Lim et al., 2009). Evidence of oxidative stress was also reported in the liver of a fresh water fish Channa Punctatus (Bloch), when the animals were exposed to ATZ at concentrations ranging from 4.24 to 10.6 mg/L in a semi static system for 15 days (Nwani et al., 2010). After completion of ATZ exposure, the antioxidant enzymes: superoxide dismutase, catalase and glutathione reductase responded positively to the enhanced lipid peroxidation status of the fish liver in a concentration-dependent fashion (Nwani et al., 2010). From these data, it can be suggested that the liver is one of the important target organ of ATZ toxicity, but the hepatotoxicity in mammalian models appears to be observed only at concentrations of ATZ not likely encountered in the environment, which may not be true for aquatic animals. However, more studies with environmentally applicable doses of ATZ will furnish additional information on the roles of apoptosis and oxidative stress in the hepatotoxicity of ATZ in human, wildlife and mammalian models.

2 Testicular toxicity of atrazine in experimental models

The adverse effect of ATZ on testicular functions has been investigated by few researchers (Table 4). A study developed by Swan et al. (2003) was the first to explore the human reproductive risks that are connected to ATZ exposure. This population-based study demonstrated the link between specific biomarkers of environmental exposures and male reproduction in humans and concluded that the association between pesticides use and diminished semen quality suggested that agrochemicals such as ATZ may have played a part in the lowering of semen quality in fertile men.

In animal studies, it was shown that histopathological features in the testis and epididymis of ATZ treated mice revealed cells that were disorganized and clusters of cells aggregated with spermatocytes (Abarikwu et al., 2021). The electron microscopy of the testicular tissue of rats displayed separately vacuolated cytoplasm, diminished collagen fibre and presence of Sertoli cells with degenerated cytoplasm (Kniewald et al., 2000). Additionally, Leydig cells were of eccentric shape with dissimilar structure and the cisternae of rough endoplasmic reticulum were more noticeable and softly expanded (Kniewald et al., 2000). The Leydig cells cytoplasmic protuberances were minuscule and the intercellular space was sizeable, displaying larger bulk of collagen fibers. Furthermore, the association between Leydig cells and macrophages were also floppier, and macrophages showed scanty lysosomes, patchy nuclei, commonly with profound folds and accentuated nucleoli (Victor-Costa et al., 2010). The altered testis morphology was evidenced by few atrophic seminiferous tubules and their dilation at ATZ doses of 200 mg/kg and 300 mg/kg for 15 and 7 days respectively, and some enlarged tubules appeared that are uneven in shape. Considering these irregular appearances of the tubules, authors did not measure the alterations in the testes. Conversely, the testes of rats treated with ATZ at 200 mg/kg for 40 days were marked by atrophy and substantial reduction in the lumen of the seminiferous tubules. Interestingly, most of the seminiferous tubules in this group of rats were Sertoli cells only. Large multinucleated bodies and numerous apoptotic cells were also common features in the seminiferous tubules (Victor-Costa et al., 2010). Thin layers of cells close to basement membrane with expanded intertubular space in rats exposed to 50 mg/kg body weight ATZ for 60 days were also confirmed by us (Ndufeiya-Kumasi et al., 2022), and when the same dose was applied in BalB/c mice for 3 days, the gonads displayed diminished numbers of germ cells in tubules with large apoptotic cells near the lumen as well as few numbers of Leydig cells (LCs) in the intertubular areas (Abarikwu et al., 2021), supporting the testicular toxicity of ATZ in mammalian animal models. The testicular lesions induced by ATZ were also accompanied with decreased germ cell numbers in amphibians, teleost fish and reptiles (Figure 6), a feature that appears to be congruous across vertebrate classes (Hayes et al., 2011). In an earlier report by Hayes and colleagues (2003), ATZ demasculinizes and feminizes the male gonads of vertebrates by decreasing androgen levels and inducing estrogen synthesis. This feature has been successfully demonstrated in fish, amphibians and reptiles, and may represent the probable mechanisms to explain these effects. It was also found that ATZ decreased testicular testosterone level in male rats and mice

TABLE 4 Expressions of markers of steroidogenesis in the testis and testicular cells of rats and mice after atrazine exposure as reported in literature.

Parameter	Cell types/tissue	Concentrations/doses	Expressions	References
3β-HSD	Testis	>50 mg/kg	Decreased	Victor-Costa et al. (2010)
3β-HSD	Leydig cells	5–50 μg/mL	Increased	Abarikwu et al. (2012)
AR	Sertoli-germ cells	50 μg/mL	Increased	Abarikwu et al. (2012)
ER-alpha	Sertoli-germ cells	50 μg/mL	No change	Abarikwu et al. (2012)
ER-alpha	Leydig cells	10 μg/mL	Increased	Abarikwu et al. (2011a)
SCF	Sertoli-germ cells	50 μg/mL	Decreased	Abarikwu et al. (2012)
CYP11A1	Leydig cells	5–10 μg/mL	Increased	Abarikwu et al. (2011a)
StAR	Leydig cells	5–10 μg/mL	Increased	Abarikwu et al. (2011a)
LHR	Testis	50–200 mg/kg	Decreased	Pogrmic et al. (2009)
17β-HSD	Testis	50–200 mg/kg	Decreased	Pogrmic et al. (2009)
StAR	Testis	50–200 mg/kg	Decreased	Pogrmic et al. (2009)
CYP17A1	Testis	50–200 mg/kg	Decreased	Pogrmic et al. (2009)
SR-B1	Testis	50–200 mg/kg	Decreased	Pogrmic et al. (2009)
17β-HSD	Leydig cells	20 μM	Increased	Pogrmic et al. (2009)
CYP-17A1	Leydig cells	20 μM	Increased	Pogrmic et al. (2009)
SF-1	Leydig cells	20 μM	Increased	Pogrmic et al. (2009)
StAR	Leydig cells	20 μM	Increased	Pogrmic et al. (2009)
3β-HSD	Testis	50 mg/kg	Decreased	Abarikwu et al. (2021)
ARO	Testis	50 mg/kg	Decreased	Abarikwu et al. (2021)
AR	Testis	50 mg/kg	Increased	Abarikwu et al. (2021)
ER-α	Testis	50 mg/kg	Increased	Abarikwu et al. (2021)
p450scc	Testis	100–200 mg/kg	Decreased	Jin et al. (2013)
p450 17α1	Testis	100–200 mg/kg	Decreased	Jin et al. (2013)
17β-HSD	Testis	100–200 mg/kg	Decreased	Jin et al. (2013)
SR-B1	Testis	200 mg/kg b.w	Decreased	Jin et al. (2014)

3 β-HSD, 3β-Hydroxysteroid steroid dehydrogenase; AR, androgen receptor; ER-alpha = estrogen receptor-alpha; SCF, stem cell factor; CYP11A1 = cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc); StAR, steroidogenic acute regulatory protein; CYP17A1 = cytochrome P450 17α-hydroxysteroid dehydrogenase (P450 17α); SF-1, steroidogenic factor-1; ARO, aromatase; scavenger receptor class B type 1 (SR-B1); 17β-HSD, 17β-Hydroxysteroid dehydrogenases.

(Friedmann, 2002; Abarikwu et al., 2021) and this was accompanied with poor semen quality in humans (Swan et al., 2003). This finding agrees with the poor sperm parameters including, membrane integrity, mitochondrial functionality, decrease acrosome integrity of sperms obtained in the mice, *Calomys laucha* after 21 days exposure of the animal to low dosages of ATZ (Saalfeld et al., 2018). In addition to the decrease in the levels of serum and intratesticular testosterone, following ATZ exposure in mice, we also detected notable decline in the levels of pituitary hormones in our recent study (Ikeji et al., 2023). This finding suggests that ATZ may induce dysfunction in hypothalamus orchestrating an adverse reaction in the secretion of gonadotropins and testosterone, thus perturbing the hypothalamic-pituitary–gonadal axis which plays pivotal role in male reproductive functions. We also demonstrated that sperms obtained from the cauda epididymides of rats exposed to ATZ up to 14-days were of poor quality as reflected in the observed low motility, count and high abnormality rate (Figure 7) (Abarikwu et al., 2010) as well as decreased daily sperm production, 7 days post ATZ exposure (Abarikwu et al., 2012). The testis of ATZ treated Japanese quail (*Coturnix japonica*) was diminished in size and the seminiferous tubules revealed reduced numbers of spermatocytes, spermatids with necrotic nuclei and decreased numbers or lack of spermatozoa (Hussain et al., 2011), similar to the observation found in rats for gonadal weight effects ((Abarikwu et al., 2010) and in mice for effect on germ cells (Abarikwu et al., 2021; 2022).

The relevance of the mRNA expressions of steroidogenesis genes: steroidogenic acute regulatory protein (STAR), cytochrome P450 (CYP) 11A1, 3β-hydroxysteroid dehydrogenase (3β-HSD), estrogen receptor-alpha (ER-α) and androgen receptor (AR) in interstitial Leydig cells (ILCs) as a gauge of ATZ induced testicular toxicity in rats ILCs after short-term exposure was

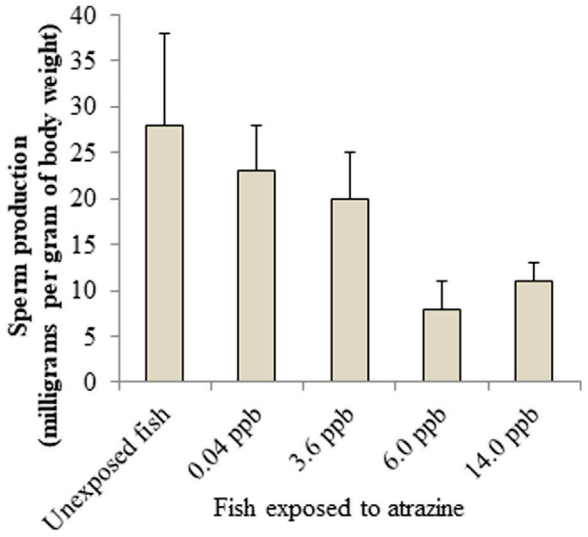

FIGURE 6
Atrazine decreases sperm production in Salmon. Sources: Moore and Waring, 1998; Cox, 2001.

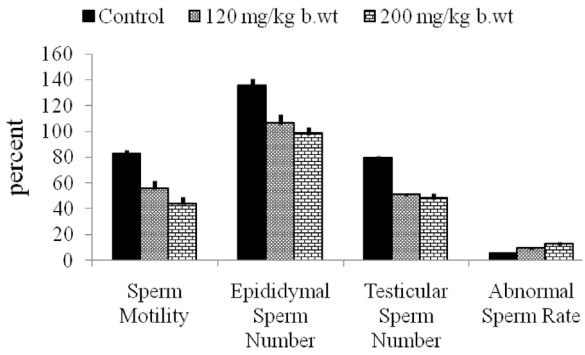

FIGURE 7
Atrazine decreases sperm quality of rats after 7 days of exposure. Adapted from Abarikwu et al., 2010.

reported by us (Abarikwu et al., 2011a). At the level of *in vivo*, immunoexpression of LCs populations from mice sub-acutely exposed to ATZ that were immunopositive for 3β-HSD were dramatically decreased (Figure 5) whereas those of ER-α, aromatase and AR were increased (Abarikwu et al., 2021), pointing to LCs as the primary cell that is sensitive to ATZ exposure in adult mice. The fact that most of these cells were TUNEL positive and were immunopositive for caspase-3 confirmed this assertion (Abarikwu et al., 2021). Furthermore, regarding the capacity for ATZ to induce steroidogenesis genes expressions and activities of CYP17A1 and 17β-HSD, cAMP builds-up, and androgen production were found to be transient resulting to the facilitated androgenesis (Pogrmic-Majkic et al., 2010). Conclusively, Pogrmic-Majkic et al. (2010) demonstrated from their study that *in vivo* exposure to ATZ compromises LCs steroidogenesis by down-regulating steroidogenesis gene expression associated with diminished androgenesis. The capacity of ATZ to change the expression of genes related to spermatogenesis steroidogenesis was also detected in Sertoli-germ cells co-culture and was accompanied with decreased testicular sperm production *in vivo* (Abarikwu et al., 2012). Atrazine at doses higher than 50 mg/kg decreased body weight, elevated adrenal weight and transiently elevated testis weight, accompanied with testis atrophy. The decrease in testosterone was associated with enhanced estradiol concentrations (Victor-Costa et al., 2010) and changes the transcription of the key genes in the testosterone synthetic pathway (Jin et al., 2014). The study of Victor-Costa and colleagues was the first time 3β-HSD protein was observed to decrease in the testes even when it remained unchanged in the adrenal. This is a very interesting finding, because it suggests that 3β-HSD inhibition may represent a probable route through which ATZ impairs androgen production in the testes resulting to the distortion of spermatogenesis in adult mammalian animal models. Our recent findings on the low populations of 3β-HSD$^+$ LCs at the inter-tubular spaces of mice testes treated sub-acutely to ATZ for 3 days confirms that the impaired testicular androgenesis were due to decreased expression of 3β-HSD$^+$ LC (Abarikwu et al., 2021), and supports the hypothesis of 3β-HSD inhibition by ATZ in rodents as opposed to the aromatase induction hypothesis in lower animals (Hayes et al., 2011; Abarikwu et al., 2021), as a plausible mechanism for the decline of testicular androgen production in rodents. Another major concern has been if ATZ enhances estrogen synthesis, perhaps by intensifying aromatase gene expression and activity. To verify this, Tinfo et al. (2011), compared the effect of ATZ ón primary cultures of granulose cells and H295R adrenal cortical carcinoma cell lines, and reported elevated estradiol production and aromatase activity in granulose cell cultures, but not in the H295R cells, that were characterised with escalated estradiol and estrone production only. Apparently, the enhanced progesterone production in both cell types demonstrates a broader effect of ATZ on steroid production (Tinfo et al., 2011).

To understand the importance of the antioxidant protective network in the testis and epididymis of mammalian animal models, we found that the antioxidant defense set-up in the gonads, e.g., testis and epididymis was decreased, a scenario that is synonymous to the manifestation of oxidative stress (Abarikwu et al., 2010). Our research group reported that glutathione (GSH) concentration and GST activities were increased in rats treated with ATZ at 200 mg/kg b.w., whereas lipid peroxidation levels were unaffected in the testis 7-days post-exposure. When the treatment regimen was elongated to 16 days, GSH concentration remained unaffected whereas malondialdehyde concentration was escalated both inthe testis and epididymis. This correlated to the decrease in GST and superoxide dismutase (SOD) enzymatic activities. Catalase (CAT) activities were unaltered in the testis and then diminished in the epididymis. During the 7-day exposure regimen, the variables of sperm quality including epididymal and testicular sperm numbers and sperm morphology were altered even though there was no evident of oxidative stress (Abarikwu et al., 2010). In addition, the histology of the testis and epididymis were normal. Since the spermato-toxicity occurred earlier than when oxidative stress was manifested, authors inferred that oxidative stress in the testis and epididymis of rats after ATZ exposure might not be responsible for the impaired sperm quality. Doses of ATZ and metabolites of ATZ similar to doses reported in the above studies have also shown to

induce oxidative stress in different experimental model systems (Jin et al., 2014), supporting the oxidative stress hypothesis in the toxicity of ATZ. These effects seem to occur at doses higher than environmental levels and unlikely to occur at lower doses ((Saalfeld et al., 2018), and thus, ATZ effect on the variables of oxidative stress in the testes of mammalian animal models may not exceed a level of concern for humans (Saalfeld et al., 2018). However, in studies where ATZ at much lower doses (10 mg/kg b.w) was found incapable of altering lipid peroxidation status in sperm isolates, the sperm quality markers such as progressive sperm motility were lowered even at doses lower than environmental levels, justifying the claims that sperms in epididymis are responsive to ATZ toxicity than oxidative stress variables in the testis (Abarikwu et al., 2010). To support this assertion, Jestadi et al. (2014) did not observe any testicular toxic effects in rats treated with low doses of ATZ. However, the ′′no observed effect′′ dose of ATZ (0.5 mg/kg b.w) set by the Australian government was demonstrated to alter the sperm quality profiles of weaning mice at 12 weeks of age (Cook et al., 2018). Thus, the low dose effects of ATZ on the metabolic and reproductive features of mammalian male animal models are influence by the age at which the exposure to ATZ occurs.

The capacity of ATZ to alter electron transport and oxidative stress in the mitochondria of *Drosophila melanogaster* has also been reported (Thornton et al., 2010). In a panel of research designed to determine the possible effects of ATZ on male amphibians *in vivo*, gonadal aromatase activity in ATZ-treated animals was found to be comparable to those of the control animals (Coady et al., 2004; Hecker et al., 2004). The explanation for the contrary results was attributed to one of two factors: 1) ATZ does not affect aromatase activity in the examined amphibian species, or 2) that because of the inadequate enzyme activities in the gonads, it was not technically workable to analyse precise variations in aromatase with the applied test model. In another study with *Xenopus laevis*, aromatase enzyme activity and gene expression were comparable to the control, and the tested ATZ concentration was found not to interfere with steroidogenesis because of the unaffected aromatase action (Hecker et al., 2005). Thus, there remain discrepancies on the hormonal effects of ATZ in experimental models, and only at high concentration was ATZ found to alter plasma testosterone homeostasis. Future studies with environmentally relevant doses of ATZ targeting more on a general mode of toxic action as pertaining to their effects on spermatogenesis and steroidogenesis are therefore necessary.

3 Chemopreventive intervention with selected antioxidants

Several pharmacological agents including melatonin, vitamins, quercetin, kolaviron, selenium, tannic acid have been used to reduce ATZ-induced toxicity regarding oxidative damage and apoptosis. These molecules substantially decreased the *in vivo* and *in vitro* toxic actions of ATZ, presumably, because of their antioxidative properties. Melatonin, a principal secretory product from the pineal gland, has been demonstrated to have an effective antioxidant and free radical scavenger properties (Buyukokuroglu et al., 2008). The *in vivo* administration of ATZ in rats inhibited glucose-6-phosphate dehydrogenase activity, ATPases (e.g., Na^+/K^+-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase), and diminished protein, total lipids, cholesterol, and phospholipid concentrations in erythrocyte membrane. Scanning electron microscopic examination showed structural modifications in the erythrocytes of ATZ treated rats. However, melatonin supplementation adjusted the ATZ-induced lipid peroxidation level and the variations in total lipids, ATPases activities as well as GSH concentration and antioxidant enzymes, confirming the antioxidant protective actions of melatonin against ATZ-induced oxidative impairment in rat′s erythrocytes (Bhatti and Sidhu, 2011). Additionally, melatonin upregulated the expressions of E2F-1 and PUMA and diminished Bax expression in an ATZ model of p53 independent mitochondrial apoptosis (Sharma et al., 2014). The endoplasmic reticulum (ER) stress as a result of the enlarged expression of ATF-6α, spliced XBP-1, CREB-2 and GADD153 was also attenuated by melatonin. This molecular event that was also accompanied with the expression LC3B-II and p62 and diminished BECN-1 signals were also attenuated by melatonin. The cytoprotective role of melatonin in an ATZ model of p53 independent mitochondria-mediated apoptosis, autophagy and ER stress was thus established by the Sharma and colleagues (2014). Tannic acid, a glucosyl chemical in gallnuts, has been demonstrated to antagonize ATZ (3 ppb)-induced Grass carp hepatocytes cytotoxicity (Gao et al., 2022). The application of both flow cytometry and dual acridine orange/ethidium bromide fluorescent staining demonstrated a higher ratio of apoptosis and necrosis in the hepatocytes. Additionally, the oxidative stress-related indicators, including ROS and MDA levels that were elevated, and the downregulated anti-oxidative system found after ATZ exposure, were alleviated by tannic acid. The mechanism for the cytotoxicity of ATZ in fish hepatocytes as proposed by Gao and colleagues involves the binding of tumor necrosis factor α (TNF-α) to TNF receptor 1 (TNFR 1) resulting in the upregulated expressions of the markers of apoptosis and necrosis: TRADD, FADD, Caspase-3, p53, RIP1, RIP3 and MLKL, whereas tannic acid abrogated the-induced apoptosis, necrosis and immunotoxicity through a ROS-responsive TNF-α/TNFR 1 pathway (Gao et al., 2022). In an ATZ model of rat hepatic injury, L-carnitine at 100 mg/kg b.w demonstrated a strong potent antioxidant, anti-inflammatory and antiapoptotic properties that had ameliorative effect against ATZ-induced hepatotoxicity (Rashad et al., 2023). In this study, 400 mg ATZ/kg b.w administered daily for 2 weeks induces adverse functional alterations and morphological changes in the rats that were accompanied with increased activities of liver enzymes and oxidative stress, modified expression of apoptotic and antiapoptotic genes, degenerative changes in the liver, and robust immunoreactivity to glial fibrillary acidic protein (GFAP), supporting previous findings on the oxidato-inflammatory and apoptotic mechanisms of ATZ in mammalian models (Campos-Pereira et al., 2012). In a similar study, vitamin E protected against ATZ-prompted lipid peroxidation in the liver and reversed the GSH level and the activity of glucose-6-phosophate dehydrogenase that was found to decline after ATZ treatment. It was deduced that ATZ promoted oxidative stress through lipid peroxidation, and that vitamin E supplementation attenuated these effects, demonstrating it as a possible antioxidant protective molecule against oxidative stress initiated by ATZ (Singh et al., 2011). Administration of selenium to rats by oral gavage did not counteract ATZ-induced impairments of sperm quality and had no beneficial protective effects against the alterations in the biochemical variables caused by ATZ the testis and epididymis. However, selenium

strongly protected against the biochemical changes initiated by ATZ in the liver. Thus, selenium was found to be valuable in reversing hepatic injury but not the reprotoxicity of ATZ (Adesiyan et al., 2011). We attributed this observed pattern of selenium protective effect in ATZ model of hepatotoxic and testicular injury in rats to the disparate redistribution of selenium in the target tissues (Adesiyan et al., 2011). One limitation of this study was that it did not measure the selenium contents both in hepatic and gonadal tissues. In a recently published article, the protective effects of zinc oxide nanoparticles and vitamin C against ATZ-induced hepatotoxicity was explored in rats. After exposing the animals to a single repeated oral dose of ATZ (300 mg/kg b.w) for 21 days, ATZ was also found to elevate liver oxidative stress by prompting the formation of MDA at higher level and decreasing GSH concentration. These biochemical alterations were found to induce inflammation associated with apoptosis through up-regulating Bax, nuclear factor-kappa B, TNF-α and minimising Bcl-2 gene expressions in the hepatic tissues. Light microscopy of liver sections was typified with vacuolated and degenerated hepatocytes surrounding dilated and congested blood sinusoids and central veins (Mohammed et al., 2023). Additionally, the serum samples obtained from the animals were characterised with higher level of aspartate aminotransferase and alanine aminotransferase enzyme activity, and diminished albumin and globulins concentrations confirming liver toxicity. Interestingly, zinc oxide nanoparticles (10 mg/kg b.w) or vitamin C (200 mg/kg b.w) pre-treatment through the oral route for 30 days could dampen the oxidative stress, inflammation, and apoptosis orchestrated by ATZ, suggesting that zinc oxide nanoparticles and vitamin C supplementations can effectively protect the liver from ATZ-hepatotoxicity (Mohammed et al., 2023). Because, the dose 300 mg/kg b.w of ATZ is in the same range of doses as in other *in vivo* studies of ATZ model of experimental hepatotoxicity (Santa Maria et al., 1987; Campos-Pereira et al., 2012; Rashad et al., 2023) or higher than those used in other ATZ mammalian models of experimental toxicology (Liu et al., 2014; Liu et al., 2021; Khozimy et al., 2022), the hepatic injury observed in most of these studies above is of limited clinical relevance. Therefore, it is only at experimental doses, and not at relevant environmental concentrations has ATZ-induced hepatotoxicity been established for mammalian animals. However, one study (Jestadi et al., 2014), have demonstrated hepatotoxicity associated with elevated serum aspartate aminotransferase and alanine aminotransferase activity in animals exposed to ATZ at doses of 300 μg/kg b.w, which is more than 1,000 times lower than was used in the above studies (Campos-Pereira et al., 2012; Mohammed et al., 2023; Rashad et al., 2023). Thus, the proposal that ATZ effects, in mammalian animals may be influenced by differences in methodologies, sources and purity of chemical, solvents, and statistical analyses (Kligerman et al., 2000b), are important concepts to consider in studies focusing on ATZ-induced liver injury in animal models. But, it is also interesting to appreciate that experimental model that is focused on the antioxidant actions of the chemical interventional agent and high doses of the chemical toxicants are important for determining a mechanistic effect of the chemical toxicant (Powell et al., 2011).

Kolaviron, a bioactive bioflavonoid extracted from bitter kola was also found to protect cultured ILCs from ATZ-induced toxicity. Our study with ILCs demonstrated that kolaviron improved the LCs viability and diminished MDA and ROS concentrations. Additional investigations showed a decrease in glutathione peroxidase (GSH-Px), glutathione reductase (GR), GST and escalation of superoxide dismutase 1 (SOD-1) and superoxide dismutase 2 (SOD-2) as measured by mRNA expression. Furthermore, the changes in the mRNA transcript copy numbers of steroidogenesis genes: StAR, CYP11A1, and 3β-HSD prompted by ATZ treatment were reversed to control values, confirming that kolaviron protected ILCs against the toxicity of ATZ. This was speculated to be throughthe suppression of ROS and MDA levels and accompanied by the normalization of mRNA expression of the tested antioxidant and steroidogenesis genes (Abarikwu et al., 2012). This is very interesting considering that a principal defensive strategy against oxidative stress in tissues and cells is the stimulation of the mRNA expression of antioxidant genes. Similarly, our study with quercetin, a flavonol with potent antioxidant properties, showed that quercetin blunted the diminished cell viability and germ cell depletion, and stopped lactate dehydrogenase release caused by ATZ, implying that testicular cell toxicity promoted by ATZ can be arrested by quercetin (Abarikwu et al., 2012) just like kolaviron. Experimental models and designs that combined both kolaviron and quercetin would be important at determining which of them will show better cytoprotective effects in ATZ model of tissue injury related to male reproduction. Because the expressions of cyclooxygenase-2, GATA-4, NF-κB, stem cell factor and androgen receptor in Sertoli-germ cells treated with ATZ were strongly recovered to levels comparable to the control by quercetin, suggested that quercetin might have diminished the capacity of ATZ to initiate spermatogenesis disturbance. The shedding of germ cells from Sertoli cell after ATZ exposure to cultures of Sertoli-germ cells which were inhibited by quercetin could be explained by the fact that quercetin preserved the associations between Sertoli and germ cells, thereby facilitating spermatogenesis (Abarikwu et al., 2012). Quercetin is therefore an important cytoprotective agent against impaired spermatogenesis by environmental factors, such as the triazines.

In a recent study, we confirmed *in vivo* that quercetin at lower doses could improve the histological features of the rat′s testes after treated with ATZ. The tubules with single layers of germ cells including the stem cell spermatogonia next to the basement membrane and enlarged interstitial space after ATZ exposure were seen to have been re-populated with germ cells at different maturity stages in the epithelium over a time course of 60 days after treatment with both curcumin and quercetin (Ndufeiya-Kumasi et al., 2022). Although, curcumin was better than quercetin in protecting the testis from ATZ gonadal toxicity, their combined -treatment improved quercetin protective effects against ATZ-induced testicular injury. It seems plausible because curcumin has a preferable ability compare to quercetin to pass through the blood-testes barrier and on co-administration could promote the passing of quercetin through tissue barriers so as to enhance their protective effect against ATZ-induced injury to the gonads (Sharma et al., 2018; Abdelhamid et al., 2020; Ndufeiya-Kumasi et al., 2022). Additionally, plant extractives like fluted pumpkin seeds that have substantial bioactive substances such as polyphenols that have numerous biological and pharmacological effects on the testes was found to be protective against testicular injury-induced by ATZ in rats by recovery of the sperm quality, biometric data and re-balancing of the MDA and GSH testicular status to the level seen in the control values as well as the activity of testicular lactate dehydrogenase and γ-glutamyl transpeptidase, important enzymes involved in testicular germ cell energetics and epididymal oxidative stress control respectively (Abarikwu et al., 2022). Aside their effect on

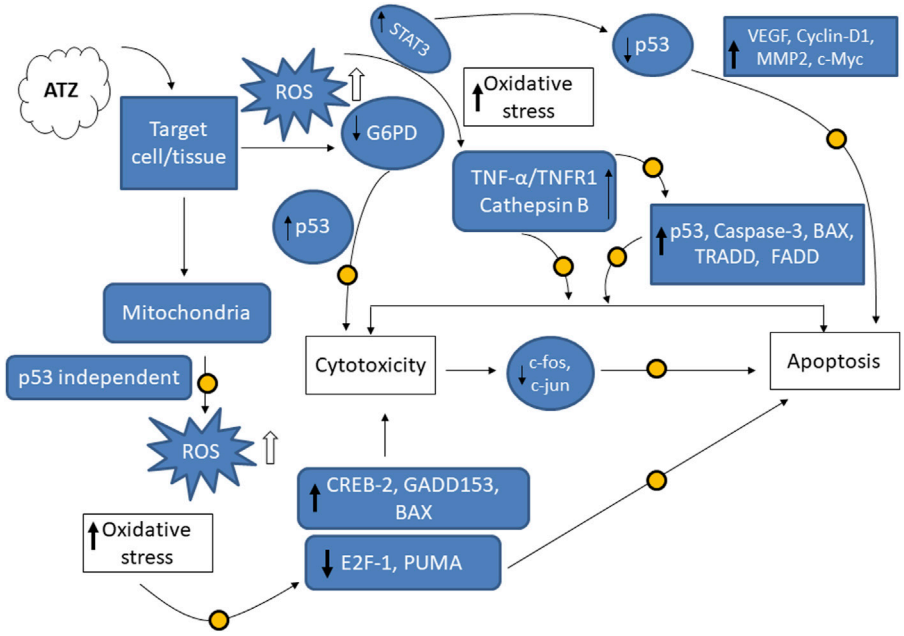

FIGURE 8
Probable graphical representations of the molecular mechanisms of ATZ-induced redox imbalance, cytotoxicity and apoptosis, as well as possible sites of actions of chemopreventives. The probable interventional target sites for the chemopreventives are illustrated as shaded circles ( ).

male reproduction, kolaviron was found to attenuate enhanced ROS generation, cell death and diminished the human neuroblastoma cell line (SHY-SY5Y) proliferation treated with ATZ. Molecular features that characterize nuclear changes due to apoptosis such as DNA laddering, nuclear fragmentation and condensation and escalated caspase-3 activity prompted by ATZ treatment were strongly blunted by kolaviron, including the variations in p53, Bax, Bcl-2, p21, caspase-3 and caspase-9 expressions (Abarikwu et al., 2011c). Similar observations were made with the rat pheochromocytoma (PC12) cell line (Abarikwu et al., 2011b) and confirmed the medicinal prospects of kolaviron in neuronal model of ATZ-induced apoptotic cell death. Myricetin, a flavonoid abundantly found in red wines and grapes, displayed protective effect on testicular injury caused by ATZ, increased levels of the pituitary hormones and recovered normal testosterone production and circulation in both serum and interstitial fluid (Ikeji et al., 2023). Also, myricetin attenuated ATZ-induced diminution in antioxidant enzyme activities and markers of oxidative stress, including the histological aberrations in the epididymis, hypothalamus and testes of mice.

4 Molecular insights on the role of apoptosis and oxidative stress in the cytotoxicity of ATZ

The probable mechanisms contributing to ATZ–induced cytotoxicity, apoptosis and oxidative stress have been summarized in Figure 8. The binding of ATZ to target cells mitochondrial redox systems may cause mitochondrial dysfunction (Galbiati et al., 2021) and activate ROS- induced oxidative stress systems, followed by cytotoxicity, DNA damage and apoptosis. The initial response to ATZ exposure would involve the inactivation of glucose 6 phosphate dehydrogenase activity/expression, and subsequently, the depletion of intracellular GSH pools (Lioi et al., 1998). If the exposure to ATZ is sustained, the resulting redox-imbalance activates p53 expressions directly, leading to cytotoxic responses in lymphocytes (Lioi et al., 1998), or through a ROS-responsive TNF-α/TNFR 1 pathway, and together with increased cathepsin activity could explain the increased caspase-3 mediated apoptosis (Gao et al., 2022). The cytotoxic response that accompanied decreased c-Fos and c-Jun expressions may result in increased apoptosis (Abarikwu et al., 2011b). Oxidative stress may also then activate, as a defense mechanism, the STAT3 transcription factor (Ng et al., 2014), which in turn downregulates p53 transcription (Tian et al., 2018). The latter, together with increased ROS generation and expressions of VEGF, cyclins (cyclin-D1), MMP2 and C-myc, explain increased cell apoptosis (Tian et al., 2018). The p53-independent mitochondria cytotoxicity pathway activates a ROS responsive upregulation of BAX (Bcl-2 Associated X-protein) expression which together with GADD153 and CREB-2 dampen the expressions of E2F-1 and PUMA to drive apoptosis and cytotoxic damage (Nakano and Vousden, 2001; Sharma et al., 2014). However, the experimental settings, such as the concentration, duration and the age at which exposure to ATZ occurs, may influence differently the above-described mechanisms, possibly explaining the contrasting effects of ATZ in different mammalian model systems (Kligerman et al., 2000b; Galbiati et al., 2021).

5 Conclusion

Although ATZ may induce cytotoxicity, oxidative stress, apoptosis and genotoxicity in *in vivo* and *in vitro* experimental

models, the majority of the doses of ATZ required to induce these changes in most of the studies reported in literature are unlikely to be found in the environment, thus the environmental levels of ATZ may not exceed a level of concern for humans and wildlife. However, the contributions of oxidative stress and apoptosis in the cytotoxicity of ATZ have been demonstrated in many experimental model systems. But, it is pertinent to consider that, as humans and wildlife may be continuously exposed to minuscule concentrations of ATZ, more studies with environmentally relevant doses of ATZ will come up with further ideas and assumptions on the mechanism of testicular-, neuronal- and hepato-toxicity caused by ATZ. Furthermore, the actions of ATZ on redox status of cells/animals are important interventional target sites at the mechanistic level, since the induced oxidative stress, cytotoxicity, apoptosis and genotoxicity observed in ATZ-exposed experimental models were blunted by antioxidants: such as vitamins (vitamin E and C and antioxidant bioactive flavonoids such as quercetin, myricetin, curcumin and kolaviron. Finally, 3β-HSD inhibition represents a plausible alternative mechanism by which ATZ affects gonadal androgen secretion and production resulting to changes in spermatogenesis in adult mammalian animal models, which might be different from the aromatase hypothesis that have been established for lower animals.

Author contributions

SA and EF designed the study and wrote the manuscript; OE and CI contributed in the prepration of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Azubuike Chukwuka,
National Environmental Standards and Regulations Enforcement Agency (NESREA), Nigeria

REVIEWED BY
Nosakhare Osazee Erhunmwunse,
University of Benin, Nigeria
Pramita Sharma,
University of Burdwan, India
Emmanuel Omogbemi,
University of Ibadan, Nigeria

*CORRESPONDENCE
Selim Adewale Alarape,
link2sas@yahoo.co.uk

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Assessment of glyphosate and its metabolites' residue concentrations in cultured African Catfish offered for sale in selected markets in Ibadan, Oyo State, Nigeria

Selim Adewale Alarape[1]*, Adekemi Florence Fagbohun[2], Oladeni Adegoke Ipadeola[1], Anthony Ayodeji Adeigbo[2], Ridwan Olamilekan Adesola[3] and Olanike Kudirat Adeyemo[1]

[1]Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria, [2]Federal College of Animal Health and Production Technology, Ibadan, Nigeria, [3]Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria

Introduction: Glyphosate is a non-targeted organophosphate insecticide whose solubility and mobility in hydrophilic solvents enable its rapid leaching into the soil and subsequent contamination of ground and surface water and possible build-up in the aquatic food chain. Based on the public health importance of glyphosate in fish through consumption, it is crucial to determine the current residue concentration in culture *Clarias gariepinus* species. The aim of the present study is to evaluate glyphosate's residue concentrations and its metabolites in cultured African Catfish offered for sale in selected markets in Ibadan.

Methods: A total of twenty-five (25) adult *Clarias gariepinus* (300 $\pm$ 50 g) were sourced from five (5) selected active fish markets (Ojoo, Iwo road, Eleyele, Challenge, and Apata) within the Ibadan metropolis. The collected fish tissue samples (liver, kidney, and spleen) were prepared for glyphosate residue concentration analysis using Liquid Chromatography (LC).

Results: The results showed that glyphosate residues were recorded in all the seventy-five (75) fish tissue samples obtained from the selected fish markets in the Ibadan metropolis and all residue concentrations were above both the recommended Acceptable Daily Intake (ADI) of 1.0 mg/kg (1×10^{-3} mg/L) and Maximum Residue Limits (MRL) of 0.01 mg/kg (1×10^{-5} mg/L). Isopropylamine has the highest residue concentration followed by N-Phosphonomethyl and Aminomethylphosphonic Acid (AMPA), while N-Acetyl Glyphosate has the least residue concentration across the sampled markets.

Discussion: The presence of residues of glyphosate and its metabolites in ready-to-eat fish calls for holistic, systematic, and effective risk management strategies towards monitoring pesticide/herbicide usage in aquaculture production and ensuring the provision of wholesome fish and fish products for the consumers.

KEYWORDS

glyphosate, *Clarias gariepinus*, liquid chromatography, residue, metabolites, herbicides

Introduction

Fish farming is a popular industry in Nigeria with increased demand for nutritious animal protein. Catfish farming is practiced by around 80% of fish farmers in Nigeria, and it is the most popular variety of fish employed in aquaculture. As a result, the usage of agrochemicals for fish growth and sustainability has increased. On farms, various agrochemicals such as Formalin, Copper Sulphate, Malachite green, and Potassium Permanganate among others have been abused and/or misused (Adeyemo et al., 2011; Alarape et al., 2013). The adverse side effects of glyphosate and aminomethyl phosphonic acid, or metabolites, on soil, water quality, plant, animal, and human health have been studied extensively in recent years due to glyphosate's extensive use and accumulation in the environment and food products (Battaglin et al., 2014; Séralini et al., 2014). In 2015, the World Health Organization (WHO) categorized the glyphosate herbicide as possibly human carcinogenic based on their most recent findings on its potential chronic negative effects (EFSA, 2015; Guyton et al., 2015; IARC, 2015).

According to the Rodriguez-Gil et al. (2017) report, a typical and maximum application of glyphosate and runoff rates from the soil resulted in estimated concentrations of 0.21–0.99 mg POEA L−1 surface water, which were responsible for the estimated impairment of 21%–43% of a wide range of aquatic species. Sandrini et al. (2013) found that pure glyphosate suppressed acetylcholinesterase activity in brown mussels (*Perna perna*) and several fish species at low concentrations (1–676 mg L−1) (Menéndez-Helman et al., 2012; Sandrini et al., 2013). These findings are consistent with the effects of glyphosate on terrestrial animals. In zebrafish embryos exposed to Roundup® at higher concentrations (50 mg L−1), developmental (forebrain, midbrain, and eye) damage was seen. Glyphosate was reported to have damaged the zebrafish primary motoneurons at a low concentration of 0.01 mg L−1 resulting in erratic movements at a young age (Roy et al., 2016; Zhang et al., 2017a). The metabolism in many tissues was impacted by the prolonged exposure of goldfish (*Carassius auratus*) to glyphosate (34 mg L−1), which resulted in an excess of oxidative stress and severe kidney damage (Li et al., 2017). The interactions between fish and their diseases are also impacted by glyphosate, in addition to the direct effects on aquatic creatures mentioned above.

Herbicides made of glyphosate may pollute the soil in and surrounding treated regions. Because of its delayed breakdown by soil microbes due to its adsorption to clay and organic matter, which causes its accumulation in soils over time, glyphosate was initially not thought to be an issue for both ground and surface water (Sihtmäe et al., 2013; Banks et al., 2014; Sviridov et al., 2015; Travaglia et al., 2015; Cassigneul et al., 2016; Okada et al., 2016; Sidoli et al., 2016). By multiple earlier observations, glyphosate and metabolites, its breakdown product, may linger for more than a year in soils with a high clay concentration, but swiftly wash out of sandy soils (Bergström et al., 2011; Okada et al., 2016; Sidoli et al., 2016). Despite its attachment to clay and organic matter, glyphosate and metabolites are degraded in part by soil pH (Zhang et al., 2015b), and after a significant amount of rain, some of the chemicals dissolve in groundwater (Maqueda et al., 2017). The soil particles carrying glyphosate and metabolites are also transported into surface water by rain and erosive processes, where they can either persist in the particulate phase or dissolve (Maqueda et al., 2017).

While contaminated particles might settle and mix with the bottom sediment, dissolved glyphosate and metabolites in surface water can sorb down to the bottom (Maqueda et al., 2017). Due to its widespread acceptance, glyphosate and its metabolites are currently present in large quantities in a range of natural waterways and sediments. Glyphosate biodegrades much more slowly in sediment than in water (Grandcoin et al., 2017; Maqueda et al., 2017; Poiger et al., 2017). In the United States, where glyphosate-resistant crops are produced on genetically engineered plants, glyphosate and metabolites are commonly found in soil, surface water, and groundwater (Battaglin et al., 2014). Glyphosate levels in the river and stream water have been found to range from 2 to 430 g L−1 (Coupe et al., 2011; Mahler et al., 2017). Additionally, it has been found in the spring snowmelt, air, and rain during the planting and growing season (Chang et al., 2011). When glyphosate is used, it eventually winds up in seawater, where it is extremely persistent (Mercurio et al., 2014).

While growing genetically modified crops is prohibited in Europe, unlike the United States, glyphosate has been found in several water sources (although at lower concentrations than in the United States) with very low concentrations (0.1–2.5 g L−1) surface water samples in Germany (Skark et al., 1998), northeastern Spain (Sanchis et al., 2012), Hungary (Mörtl et al., 2013), France (Villeneuve et al., 2011), and Switzerland (Poiger et al., 2017). In addition to runoff from agricultural land, Grandcoin et al. (2017) and Hanke et al. (2010) suggest that urban runoff is another source of glyphosate for streams and rivers. According to Rosenbom et al. (2010), numerous Northern European nations have outlawed the use of glyphosate on paved surfaces because it increases runoff from impermeable and linked paved surfaces. Yet glyphosate and metabolites were discovered in samples of sewage and stormwater runoffs, outputs from wastewater treatment plants, and even packaged bottled water (Birch et al., 2011; Grandcoin et al., 2017). According to reports, glyphosate and metabolites are typically found in drinking water, however at very low amounts that are below the recommended daily intake (ADI) that was established in 1997 (WHO, 2005).

Although, Bastos et al. (2020) reprted that there were no published works that have evaluated the toxicological effects of glyphosate in aquatic mammals and birds which demonstrated lack of knowledge on the risk of exposure of aquatic animal in the environments contaminated by glyphosate. But in contrary, Shehata et al. (2014), treported that glyphosate residues could be found in the liver, spleen, lung, intestine, heart, muscles, kidney, and animal feed. This is because glyphosate is applied at higher rates and more frequently than before. Eighty-nine percent (89%) of the corn crop and 94% of the soybean crop in the United States were herbicide-tolerant varieties, and glyphosate was likely sprayed on most of them. Additionally, a considerable portion of the feed used for animals comes from genetically modified crops. According to research, 57% of maize and 85% of soybeans are used annually in livestock diets worldwide (McNaughton et al., 2011). In none of the aforementioned research was it discovered that feeding animals glyphosate-sprayed crops during cultivation decreased their output. In the findings of Muhammad et al. (2021), they concluded that a relatively high concentration of technical grade glyphosate is needed

to induce significant changes in fish, however, it was stated that the bodyweight index is the most sensitive toxicity parameter in that at 25 mg/L of glyphosate, there was reduction in the fish body weight. Negative correlations between the glyphosate concentration and toxicity parameters such as specific growth rate (SGR), hepato-somatic index (HIS), and gonado-somatic index (GSI) were observed (Muhammad et al., 2021). Ruminants are major consumers of genetically modified (GE) crops, and as bacterial protein and end products of microbial fermentation make up a sizable amount of their metabolizable nutrients, they serve as models for research on the impact of pesticide residues on gut microbes. Similar to this, although microbial proteins are not consumed, bacterial fermentation activity in the hindguts of non-ruminant animals is significant for some nutrients. Recently, glyphosate exposure toxicity has been reported to cause several changes such as haematologic and biochemical processes in tissues (Modesto and Martinez, 2010), genotoxicity (Guilherme et al., 2012; de Brito Rodrigues et al., 2019), histopathological damage and immunotoxicity (Antunes et al., 2017; Ma et al., 2019), or cardiotoxicity (Gaur and Bhargava, 2019) in fish.

This present study was designed to evaluate the residue concentrations of glyphosate and its metabolites in cultured African Catfish offered for sale in selected markets in Ibadan.

TABLE 1 Comparison of glyphosate and its metabolites' residue concentrations in organs and muscles of cultured African Catfish offered for sales at different fish markets in Ibadan.

Markets	Organs	*p*-value (≤ 0.05)
Ojoo	Liver	0.0006
	Kidney	
	Muscle	
Eleyele	Liver	0.01
	Kidney	
	Muscle	
Apata	Liver	0.01
	Kidney	
	Muscle	
Challenge	Liver	0.01
	Kidney	
	Muscle	
Iwo Road	Liver	0.007
	Kidney	
	Muscle	

Materials and methods

Sample collection

Five adults *Clarias gariepinus* samples (300 ± 50 g) each were sourced from five selected active cultured fish markets (Ojoo, Iwo road, Eleyele, Challenge, and Apata) within the Ibadan metropolis. A total number of twenty-five adult *Clarias gariepinus* were transported to the Fish and Wildlife Unit Laboratory, Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Ibadan.

Each fish was pithed before sacrifice. Organs (muscle, liver, and kidney) were then harvested through dissection of the fish and separately put inside the sterile sample nylons, sealed, and kept under the ice. The collected samples were then taken to the International Institute of Tropical Agriculture (I.I.T.A) for glyphosate residue concentration analysis.

Solid-phase extraction (SPE) and quantification

The SPE was carried out based on the procedures described by Delmonico et al. (2014). The digestion tubes were washed with water, later rinsed with distilled water, and finally rinsed using 0.5% HCl in ultra-pure water (purest scientific water) and allowed to dry. The fish tissue samples were homogenized and stored in Pyrex tubes in a viscous form. 0.5 mL of homogenized samples were poured into each of the digestion tubes using a precision pipette. About 20 mL of the ammomethyl-phosphonic-nitric acids mixture in the ratio 5:2: 5 was then dispensed into each of the digestion tubes containing the samples. The mix is then digested for two and half hours on a digestion block at 25°C and covered with a condenser.

At the end of the digestion process, the digests were brought out and allowed to cool to room temperature (25°C). Each cooled digest was made up to 25 mL volumes with ultra-pure water, covered with paraffin paper, swirled to mix properly, tightly covered, and shaken on a mechanical shaker for 10 min. They were then centrifuged for 5 min at 5,000 revolutions per minute (rpm). The resultant supernatants were analyzed using Reverse Phase LC (Agilent Technologies, Santa Clara, CA, United States) with C18, 5 µm 120 Â, 4.6 × 250 mm column as stationary phase and methanol/H_2O (90:10) as mobile phase at a flow rate of 1 mL/min for 30 min.

Method validation

The SPE-LC method's specificity, linearity, sensitivity, accuracy, precision, and resistance to matrix effects were all validated.

Data analysis

Data obtained were calculated by the interphase software with LC. The results were expressed in frequency, percentages, and variance (descriptive statistics) on SPSS Statistics Version 26.

Ethical consideration

The University of Ibadan Animal Care and Use in Research Committee accepted the research protocol, which was given the number UI-ACUREC/019-0220/6.

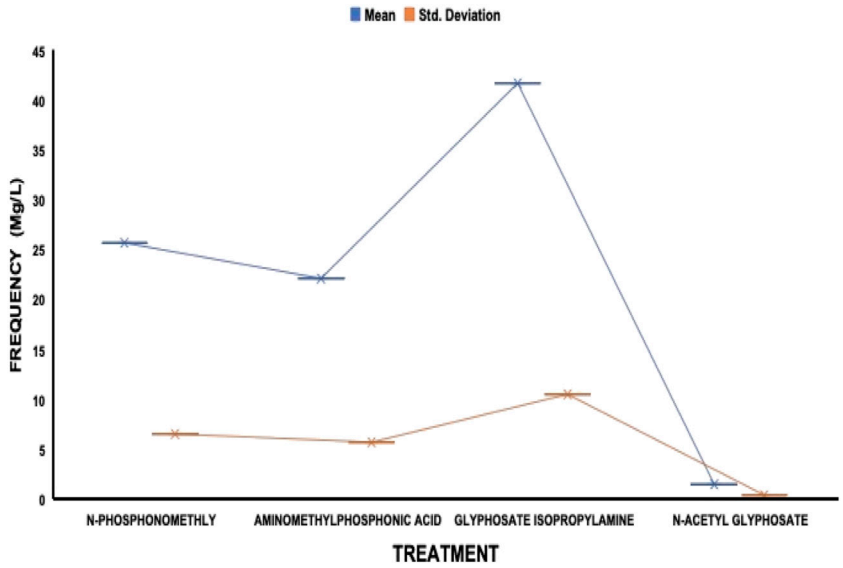

FIGURE 1
Frequency of glyphosate and its metabolites' residue concentrations present in the Catfish organs and muscles offered for sale at Ojoo fish market. The error bars of box plots represent mean and standard deviation.

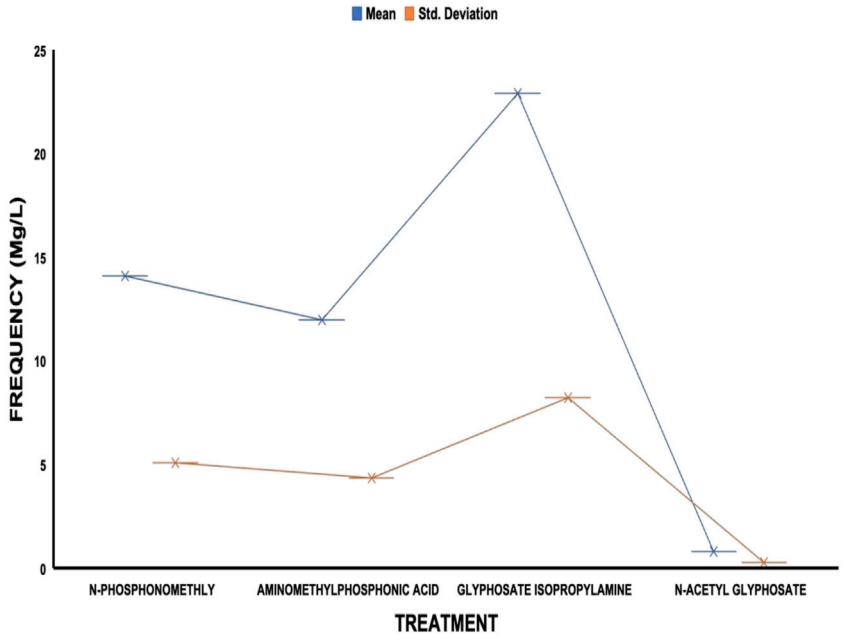

FIGURE 2
Frequency of glyphosate and its metabolites' residue concentrations present in the Catfish organs and muscles offered for sale at Iwo road fish market. The error bars of box plots represent mean and standard deviation.

Results

The residue concentrations of glyphosate its metabolites as detected in fish organs and muscles from different fish markets are shown in Supplementary Figures S1–S15. Isopropylamine has the highest residue concentration followed by N-Phosphonomethyl and Aminomethylphosphonic Acid (AMPA), while N-Acetyl Glyphosate has the least residue concentration in fish organs and muscles obtained from Ojoo fish market (Supplementary Figures S1–S3).

From the ANOVA result obtained in Table 1, there is significance difference (p-value less than 0.05) among the fish organs and muscles across the glyphosate and its metabolites.

The average residue concentrations of glyphosate and its intermediates as detected in the fish organs and muscles samples

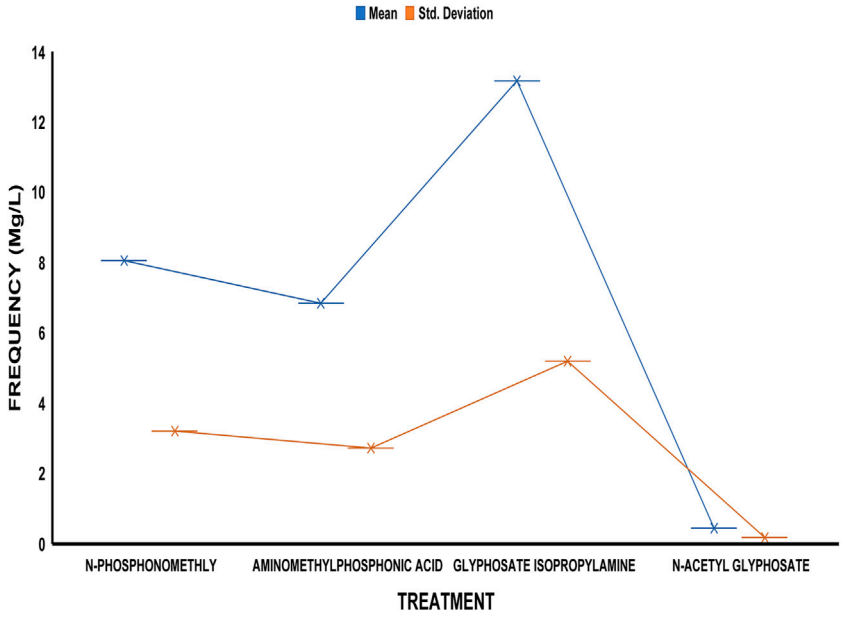

FIGURE 3
Frequency of glyphosate and its metabolites' residue concentrations present in the Catfish organs and muscles offered for sale at Eleyele fish market. The error bars of box plots represent mean and standard deviation.

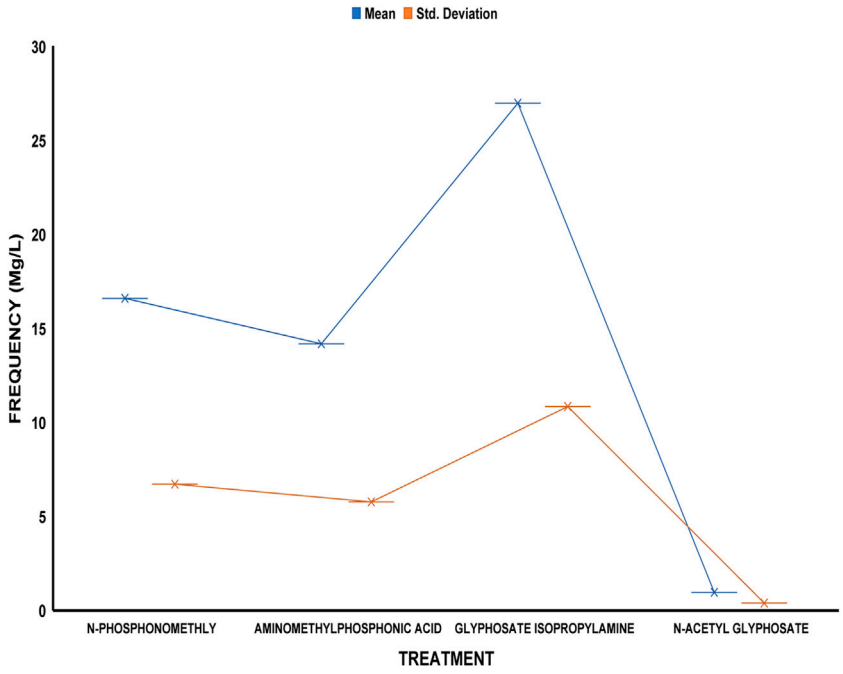

FIGURE 4
Frequency of glyphosate and its metabolites' residue concentrations present in the Catfish organs and muscles offered for sale at Apata fish market. The error bars of box plots represent mean and standard deviation.

(liver, kidney, and muscle) were shown in Figures 1–5. Isopropylamine has the highest statistically significant residue concentration (p-value ≤0.05) followed by N-Phosphonomethyl and Aminomethylphosphonic Acid (AMPA), while N-Acetyl Glyphosate has the least residue concentration in fish organs and muscles obtained across all the fish market (Figures 1–5). Muscle had the highest residue concentration of both glyphosate and its metabolites across all the fish markets. Glyphosate and its

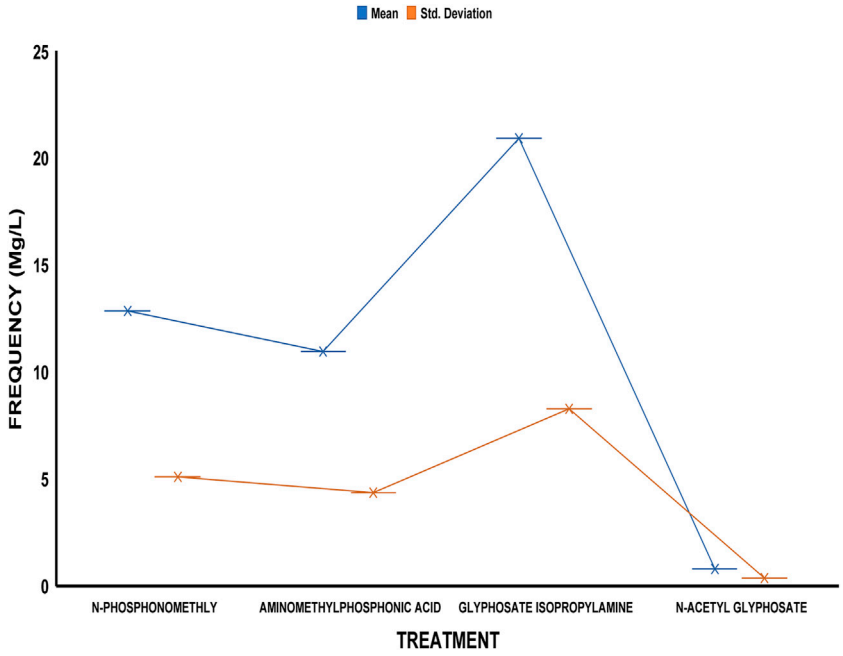

FIGURE 5
Frequency of glyphosate and its metabolites' residue concentrations present in the Catfish organs and muscles offered for sale at Challenge fish market. The error bars of box plots represent mean and standard deviation.

metabolites residue concentration detected in Kidney were lower than those detected in muscle and higher than the detected concentrations in the liver samples across all the fish markets (Figures 1–5).

Isopropylamine has the highest residue concentration followed by N-Phosphonomethyl and Aminomethylphosphonic Acid (AMPA), while N-Acetyl Glyphosate has the least residue concentration in fish tissues obtained across all the fish market (Figures 1–5).

Discussion

Large quantities of glyphosate-based herbicides are applied to crops two to three times per season to get rid of weeds that grow back after herbicides application. Glyphosate residues can remain stable in foods for years, irrespective of the method of food preservation. The health of both humans and animals may be impacted by glyphosate, according to several earlier research. In this study, glyphosate residues were recorded in all the fish samples obtained from the selected fish markets in the Ibadan metropolis which is in agreement with the reports of Kruger et al. (2013). The detection of glyphosate residues in the fish organs and muscles in this study confirmed the use of several glyphosate-based herbicides (Force-Up, Vinash, Para force, and Round-Up) on the fish farms (fish as a non-target organism) in Nigeria (Alarape et al., 2021), use of banned pesticides like pyrethroids, organophosphates, carbamates, and organochlorine in agricultural products in Bangladesh (Jallow et al., 2017) and Ghana (Essumang et al., 2009). Although glyphosate-based herbicides were not used directly on fish, environmental contamination may be a possible source of the herbicide residues found in fish in the research location where such herbicides are used on farms close to fish farms. In addition to demonstrating that glyphosate and its metabolites were present in the fish sold for human consumption, it also demonstrated a definite pattern regarding the degree of chemical contamination in the fish farms' water supplies. This is supported by a 2009 investigation by Essumang et al. (2009) that found variable concentrations of organochlorine (OC) and organophosphorus (OP) pesticides in the lagoons of Etsii, Fosu, Korle, and Chemu in Ghana.

Glyphosate and several of its metabolites (AMPA, N-acetyl glyphosate, and N-acetyl AMPA) have an acceptable daily intake (ADI) for people of 1.0 mg/kg (1×10^{-3} mg/L) (FAO, 2004; FAO, 2011). The findings of this investigation showed that the residue of glyphosate and its metabolites in the fish samples exceeded recognized safety thresholds. The environmentally relevant concentration of glyphosate is about 0.4 mg/L (Glusczak et al., 2007). Despite this, studies based on actual water runoffs from fields freshly applied with glyphosate formulation have demonstrated glyphosate concentrations as high as 5.2 mg/L (Edwards et al., 1980), whilst simulated studies using sand as a matrix showed water runoffs containing as high as 17 mg/L glyphosate (Fu, 2020). According to Vicini et al. (2021), the permitted concentration in drinking water in Europe is less than 0.1 mg/L, but the maximum residue limit (MRL) as suggested by WHO and FAO [Codex, Alimentarius Commission (2009)] was 0.01 mg/kg (1×10^{-5} mg/L) (EPA, 2015). In this study, all fish samples obtained from the fish markets in Ibadan had glyphosate and its metabolites residue concentration above the recommended MRL. Glyphosate Isopropylamine had the highest residue concentration in all the fish samples analyzed while N-Acetyl Glyphosate had the least residue concentration (Figures 1–5). The highest glyphosate and its metabolites' residue concentration

was recorded throughout the fish muscle samples, followed by the fish kidney while the liver had the least residue concentrations when compared with both muscle and kidney (Supplementary Figures S1–S15).

The findings of Akan et al. (2019) are in contrast to the high glyphosate residue concentrations reported in this study, where the concentrations of herbicide residues recorded were below both the WHO and FAO maximum residue limit (MRL) of 0.01 mg/kg (1×10^{-5} mg/L) and the acceptable daily intake value (ADI) of 0.006 mg/kg (6×10^{-6} mg/L), which is considered safe for consumption as of the time of their present research work. The difference in the findings may be due to differences in vegetation, weather, and rates of usage of herbicides in the Northern part of Nigeria. When animals are fed crops cultivated with glyphosate or indirectly exposed to glyphosate, it is not anticipated that animal products, except kidney and liver due to their physiological functions, will contain appreciable residues of glyphosate. This is because glyphosate has a high water solubility (10.5 g/L), a low octanol-water partition constant (log POW = 3.2), and is quickly excreted through the kidney (Bus, 2015). This assertion is in contrast to the findings in this study because glyphosate residues were detected in the muscles of the fish sample with the highest concentrations.

Conclusion

The increased use of herbicides in agricultural sectors is a result of the world's rising food demand, but their residues in agricultural products, particularly fish, are raising serious health issues for consumers and public health officials because they are linked to food safety. The presence of a high residue concentration of glyphosate and its metabolites in the muscles of collected fish samples calls for direct and targeted regulations on its use in food animal-based farms.

Despite the advantages of using glyphosate-based herbicides, the results of this study and many others show that these chemicals can have negative effects on the aquatic environment, human health, and even the global food-chain cycle (Abdulkareem et al., 2014). Through the ingestion of feed and contact with the usage of polluted water, glyphosate residue may come into contact with humans and animals. Singh et al. (2020) claim that the extensive use of glyphosate has caused ecosystems to become contaminated, which is negatively affecting microorganisms, plants, animals, and humans. Therefore, this study has established the presence of glyphosate-based herbicide and its metabolites in cultured *Clarias gariepinus* (African Catfish) offered for sale in fish markets within the Ibadan metropolis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Animal Care and Use Research Ethics Committee, University of Ibadan. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SAA, OKA, and AFF: conceptualization. AAA, ROA, and OAI: data collection. SAA and ROA: formal analysis. SAA, OKA, and AFF: supervision. SAA, AFF, OAI, AAA, and ROA: manuscript preparation and revision. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer EO declared a shared affiliation with the authors SAA, OAI, ROA, and OKA to the handling editor at the time of review.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2023.1250137/full#supplementary-material

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EDITED BY
Azubuike Chukwuka,
National Environmental Standards and regulations Enforcement Agency (NESREA), Nigeria

REVIEWED BY
Hailong Zhou,
Hainan University, China
Balram Ambade,
National Institute of Technology, India

*CORRESPONDENCE
Akinyinka Akinnusotu,
akinnusotuakinyinka@gmail.com,
akinyinka_akinnusotu@rugipo.edu.ng,
aakinnus@ttu.edu

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Source, distribution, and risk assessment of polycyclic aromatic hydrocarbons in sediment and fish samples from River Owan, Edo State, Nigeria

Akinyinka Akinnusotu[1,2,3]*, Justina E. Ukpebor[2] and Felix E. Okieimen[2]

[1]Department of Science Laboratory Technology, Rufus Giwa Polytechnic, Owo, Nigeria, [2]Department of Chemistry, University of Benin, Benin City, Nigeria, [3]Department of Environmental Toxicology, The Institute of Environmental and Human Health (TIEHH), Texas Tech University, Lubbock, TX, United States

Polycyclic aromatic hydrocarbons (PAHs) are persistent environmental contaminants that present several environmental risks including human health. The 16 priority PAHs including its 1-methylnaphthalene, and 2-methylnaphthalene were determined in sediment and fish samples (*Clarias anguillaris and Oreochromis niloticus*) of River Owan, Edo State, Nigeria using gas chromatography (GC) equipped with flame ionization detector (FID) and other standard laboratory protocols. The isomeric ratio was used for source diagnosis, sediment quality guidelines, and risk models of incremental lifetime cancer were used for risk assessment. 1-methylnaphthalene and 2-methylnaphthalene were most predominant in all sediment samples analysed. The ∑LMW PAHs ranged between 0.093—0.250 µg/kg; ∑HMW PAHs were 0.107—0.579 µg/kg. The sediment samples range for ∑PAHs was 0.280–0.810 µg/kg with concentration order of increase: SE5>SE4>SE3>SE6>SE1>SE2>SE7 for the seven sampling locations. The ∑PAHs for *Oreochromis niloticus* was 0.190 µg/kg, which is higher than the value of *Clarias anguillaris* 0.080 µg/kg, and these values were greatly lesser when compared to the European Commission limit of 12.00 µg/kg. The diagnostic ratio indicates that the sources are more pyrogenic than petrogenic, revealing combustion from grass, wood, and bush burning. Sediment quality assessment showed that the ∑PAHs were lower than the regulatory values of sediment quality guidelines (SQG) assessment suggesting no ecotoxicological effects on the benthic organisms in this area at present. The Incremental Life Cancer Risk results were in the range of 9.15×10^{-12}—1.46×10^{-6} for children, and 7.78×10^{-12}—1.76×10^{-6} for adults considering the three routes of exposure. The incremental life cancer risk assessment showed a negligible risk.

KEYWORDS

Eco-toxicology, Risk-Assessment, Owan, PAHs (polycyclic aromatic hydrocarbons), Diagnostic ratio, Sediment

1 Introduction

Organic compounds known as polycyclic aromatic hydrocarbons (PAHs) are made up of at least two fused benzene rings arranged in various ways which are produced as a result of carbon-based materials' incomplete combustion. As a result of their detrimental implications on the health of man, and the environment, which include their carcinogenicity, mutagenicity, teratogenicity, etc., sixteen (16) members of PAHs are documented to be priority contaminants according to the United States Environmental Protection Agency (Barakat et al., 2011; Li et al., 2015; Cui et al., 2016; Adekunle et al., 2018; Kosek and Ruman, 2021; Shariatifar et al., 2021; Ambade et al., 2023; He et al., 2023). The majority of PAHs are generated when organic molecules undergo heat decomposition known as pyrolysis, and then recombination called pyrosynthesis to produce them, e.g., acenaphthylene, anthracene, benz[a]anthracene, acenaphthene, etc. There exist two categories of PAHs which are established by considering the number of benzene rings present in them: low molecular and high molecular weights with the acronym LMW and HMW (Banger et al., 2010; Li et al., 2015; Lawal, 2017; Ambade et al., 2022a; Roy et al., 2022; He et al., 2023).

Due to their high lipophilicity, PAHs are extremely soluble in organic solvents that have fused benzene rings. Each ring structure and isomer of PAHs has a unique UV spectrum due to the compounds' specific UV absorbance spectrum. The majority of PAHs are fluorescent as well, and when activated, they release certain light wavelengths (Yu et al., 2014; Kuppusamy et al., 2016; Lawal, 2017; Gao et al., 2018). Environmental matrices like soil, mixtures of air, water, biota, sediment, and household products like cosmetics (Wang et al., 2010; Xiao et al., 2012; Adekunle et al., 2018; Wang et al., 2019; Ambade et al., 2021a; Ambade et al., 2021b; Ambade et al., 2023), food produce, and its products (Wang et al., 2017; Sadowska-Rociek et al., 2021; Sharma et al., 2021) are common media with PAHs concentrations that have been established, making them ubiquitous chemicals.

Several activities and processes released PAHs into the surroundings including industrial and domestic activities such as wastewater discharges, oil spillage, asphalt particles, vehicular emissions, and exhausts, washing from oil tanks, leakages from marine vessels, gas flaring, disposal of used petroleum products into the river where adequate environmental management policies are not available, including the burning of fossil fuels, forest fire, etc. (Rogowska et al., 2015; Paloluoğlu et al., 2016; Wang et al., 2017; Sharma et al., 2021; Ambade et al., 2022b). Their routes of exposure are inhalation, ingestion, and dermal absorption; these are the three major ways by which PAHs get into humans. Research has shown that the environment is the primary receiver, and sediment is the major sink and reservoir of PAHs and other environmental pollutants due to the ease of their absorption into organic matter (Srogi, 2007; Feng et al., 2009; Wang et al., 2013; Lang et al., 2015; Olayinka et al., 2019; Ambade et al., 2023). The major ways of PAHs transportation, distribution, and dispersal in the environment include atmospheric transport, erosion, wind, storms, and so on. The buildup of PAHs in sediment and biota causes food chains to become contaminated, which poses a threat to the health of man and animals including biota such as fish (Cui et al., 2023).

According to reports, fish are the most susceptible aquatic organisms for detecting contaminants and pollution in an aquatic environment; hence, they have been used for environmental monitoring of contaminants in rivers (Cui et al., 2016; Olayinka et al., 2019; Pang et al., 2021; Ciucure et al., 2023). Arising from their capacity to bio-accumulate in biota, persist in the environment, having mutagenic and carcinogenic potencies in man, the US EPA has banned some PAHs, hence the interest of all stakeholders especially the researchers for many years (US EPA, 1993; US EPA, 2001; Orecchio and Papuzza, 2009; Lawal, 2017; Gao et al., 2018; Baran et al., 2021; Cui et al., 2023). Several researchers have analyzed, characterized concentrations, and carried out health implications of POPs in which PAHs a member of that group in the past decades across the globe (Lau et al., 2010; Yu et al., 2014; Li et al., 2015; Wang et al., 2017; Gao et al., 2018; Ekere et al., 2019; Usese et al., 2021; Cui et al., 2023).

Considering the dangers PAHs represent to the environment, wildlife, and the health of man, scientific research on them has attracted considerable attention in many developed nations. However, there are significant data gaps regarding PAHs sources and levels in developing nations (Ogunfowokan et al., 2003; Nieuwoudt et al., 2011; Okedeyi et al., 2013; Mirza et al., 2014; Zheng et al., 2020; Sharma et al., 2021; Usese and Egbuta, 2021; Ambade et al., 2023). Several samples including water, soil, sediment, fish, and other aquatic organisms from rivers, oil spillage sites, industrial locations, polluted soil, agricultural land use areas, etc. have been published (Ogunfowokan et al., 2003; Jiao et al., 2013; Asagbra et al., 2015; Edokpayi et al., 2016; Adeniji et al., 2019; Zheng et al., 2020; Sharma et al., 2021; Bajt, 2022; Ambade et al., 2023), there are no reports of studies on PAHs from River Owan and its environments except a preliminary study executed by our group (Akinnusotu et al., 2020). There are rivers in Edo State, Nigeria in which River Owan is one of them with several economic significance. These include various agricultural practices (extensive and intensive farming), irrigation of farms, fishing from the river, lumbering, transportation of goods and services to several camps around the river, marketing of the various farm produce around this area, and so on (Omoregbee and Edeoghon, 2006). Some other human activities around the river include forest and bush burning from farms which are common practices, dumpsites located very close to the river, speed boat engine repair workshops constructed around the river, major markets for the community located close to the river as a meeting point, most drainages in the community are channeled towards the river, transportation of logs of wood, etc. It serves as a primary source of water for the use of the community including drinking, and other domestic uses. Hence, this research provides further data on the concentration of PAHs in River Owan, Edo State, Nigeria by determining its concentrations in fish and sediment samples, identifying its possible sources, and evaluating health risk using risk assessment models.

2 Materials and methods

2.1 The area of study

Owan is a community located in Edo State, Nigeria Figures 1A, B with a major River that lies on latitude 06° 45′ 40″ N; and longitude

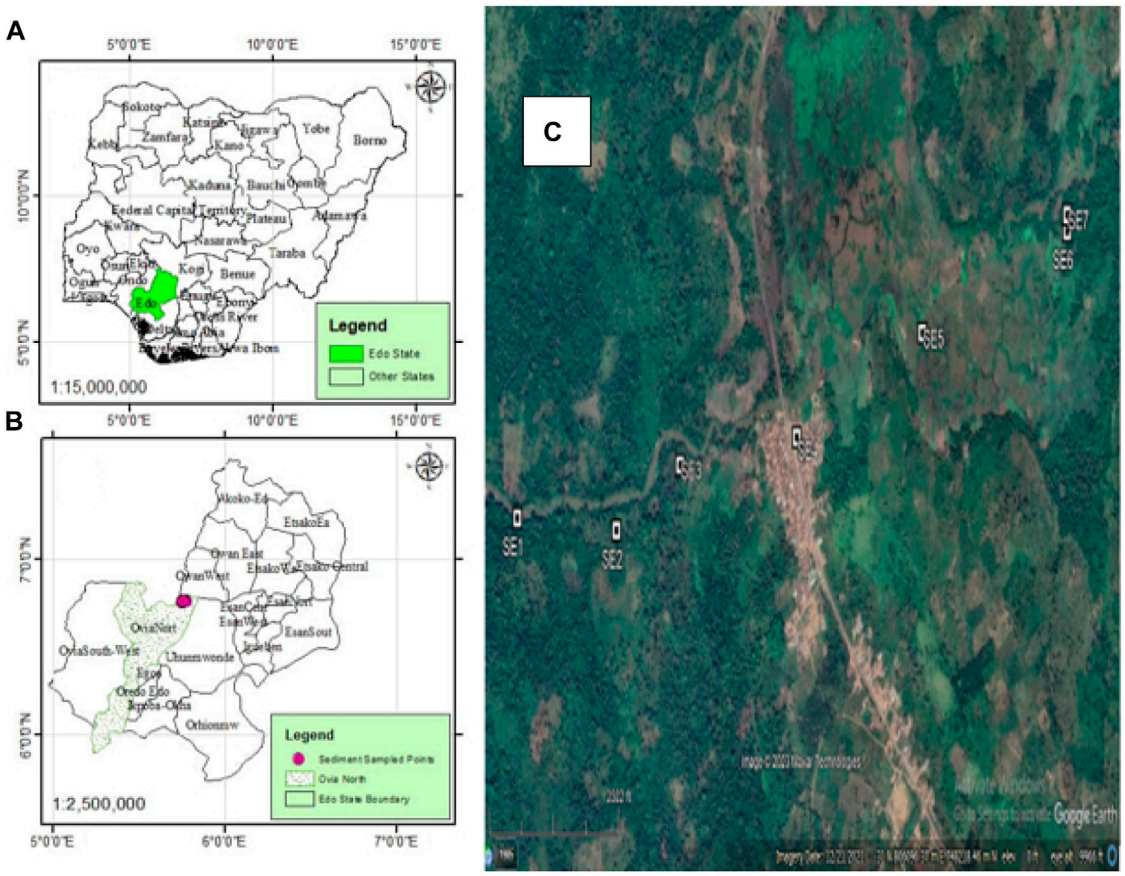

FIGURE 1
Map of the study area and locations of sampling.

005° 46′ 07.4″ E (Ogbeide et al., 2015). The two climatic seasons in Nigeria are the dry season and the wet season. The dry season typically lasts from November through March of the year, whereas the wet season is typically between the months of April and October. Some of the camps and communities around River Owan are Agbenikaka, Odei, Ogbigbi, Okpokhumi, Sabongida, etc. The communities are notable for farming activities that involve both cash and food crops. Some of the crops include cocoa, palm tree, plantain, banana, yam, cassava, etc. The river is used for irrigation of close farm plantations, fishing, and domestic use such as washing, cooking, bathing, and drinking. Other activities in the study area include mechanic workshops for the repairs of various automobiles, the burning of wastes, and major markets located along the river banks (Ogbeide et al., 2015; Akinnusotu et al., 2020). Seven sampling locations were used considering human activities, vegetation, and ecological settings that run across the river.

2.3 Sampling and sampling locations

2.3.1 Sediment

Samples of sediment were taken across the river from seven different sampling locations using a stainless-steel grab sampler, putting the samples into a 1-L glass jar having a cap (cleaned), and transported to the Chemistry Laboratory at the Rufus Giwa Polytechnic, Owo, Nigeria, in an ice-chest at 4°C. Seven different sampling locations were selected where sediment samples were taken across the river. The sampling locations and samples were assigned codes SE1 to SE7 (Figure 1C). Sampling location coordinates were obtained with the aid of a Garmin GPSMAP 76S GPS.

2.3.2 Fish

Ethical clearance was obtained from the ethical committee of Rufus Giwas Polytechnic, Owo, Nigeria before going for the fish sampling in February 2019 during the dry season. Two major types of fish species were selected because they are common (predominant) to River Owan, they are *Clarias anguillaris* popularly known as catfish, and *Oreochromis niloticus* known as tilapia fish which were obtained using nets with the help of fishermen in the community. Three fish of equal size and weight were collected for the two fish species, placed in an ice chest, and frozen at −20°C before dissection. Equal weight of their muscle tissues only was composited for the two fish species - *Clarias anguillaris* and *Oreochromis niloticus* which was used for this study. The fish samples were defrosted, cleaned, and washed thoroughly with distilled water before dissecting them through the tail to the spinal cord, and the muscle tissues were removed for each of the fish. The fish samples were assigned sample codes: F1 for *Clarias anguillaris* and F2 for *Oreochromis niloticus*. The

samples were transferred to the lab in a labeled glass jar, kept at 4°C in a chest cooler, and then stored there. The fish samples were established by an aqua-culturist in the Institutions' Fishery and Aquaculture Department (Rufus Giwa Polytechnic in Owo, Nigeria). *Clarias anguillaris* is known to be a bottom feeder and depends greatly on detritus while *Oreochromis niloticus* is a surface dweller (Adewumi et al., 2014; Wagaw et al., 2021).

2.4 Sediment sample preparation and treatment

A standard reference procedure for the analysis of PAH in sediment was employed (US EPA 8240 method). Samples of the sediment were air-dried in a dust-free laboratory at room temperature, sieved using a 2 mm mesh laboratory sieve, and packed into zip-lock bags. Ten grams (10 g) of the sieved sediment sample was carefully weighed into a vial meant for extraction that had first been washed using chromic acid and dried. A glass rod was used to properly mix 10 g of anhydrous sodium sulphate (Na_2SO_4) which was carefully weighed into the mixture. The sample was mixed with 50 mL (mL) of a 3:1 hexane and dichloromethane mixture (90 mL of n-hexane and 30 mL of dichloromethane made in a standard flask). The material was filtered after being shaken at 500 osc/min for 30 min on a shaker (Chen et al., 2007). The sample was then allowed to concentrate in the extraction bottle at room temperature for at least 24 h until about 2.0 mL of concentrated sample remained. Following this, n-hexane and dichloromethane were fractionated in an activated alumina (neutral)/silical gel column for effective clean-up. This extraction process was repeated twice. Using a rotary evaporator, the fraction was reduced to 1.0 mL volume. The aromatic extract was kept until analysis in dried organic free and chromic acid pre-cleaned glass vials with Teflon rubber closures in a freezer at −4.0°C.

2.5 Fish sample preparation and extraction

The United States Environmental Protection Agency standard for analytical procedures of 1986 was followed (USEPA, 1986). Using a whirling blender, the tissue from the fish sample was pulverized. One hundred (100) mL of acetone and n-hexane was added to a homogenizer cup along with five (5 g) of the fish sample. Samples were combined with 5 g of anhydrous sodium sulphate after being homogenized for 20 min at 100 rpm. A mixture of dichloromethane and n-hexane was used for the extraction using a Soxhlet extraction apparatus for about 5 h and evaporated to dryness the extracted solvent. The resultant extract was reconstituted using 50 mL n-hexane and later evaporated until 1.0 mL.

2.6 Sample analysis

A flame ionization detector (FID) in an Agilent 7890A GC was used for the instrumental analysis. One μL (1.0 μL) of the concentrated sample was injected into the column through a rubber septum using an exmire micro syringe in the splitless mode. Vapour separation occurs as a result of partitions between the liquid and gas phases. The following are the GC settings for analysis:

2.7 Gas chromatography operating conditions

The initial setting for the temperature of the GC oven was 45°C with a 2-min holding time. The temperature was raised to 240°C, while the gradient rate was 15°C/min, and the injector temperature was 280°C. After injecting samples, a gradient increase rate of 10 C/min allowed for the higher maximum working temperature to be reached, which was 300°C. The detector's temperature was 340°C and the carrier gas used was helium. Pressure program (set point) at 14.0psi with an injection volume of 1.00 μL.

2.8 Gas chromatograph calibration

PAH standard comprising the 16 prioritized, 1-methylnaphthalene, and 2-methylnaphthalene PAHs of AccuStandard Inc. was purchased from a reputable commercial vendor in Nigeria and was used for the calibration. The GC temperature was set and allowed to increase gradually to reach optimum. Standards for calibration were set up in the range of 0.10 and 0.50 ppm (five levels) which were utilized for calibration. A standard of 1.00 μL volume was injected and run. The analytes' coefficient of determination (R^2) for the calibration was all in the range of 0.995–0.999. Software for PAH calibration and quantification was employed, having a CLARITY-GC interface.

2.9 Source apportionment using diagnostic ratio

Diagnostic ratio (DR) is one of the techniques available to determine the origin of PAHs in environmental matrices qualitatively using the isomer ratios of the PAH components (Davis et al., 2019). It is applied as a result of the fact that PAH isomers possess analogous chemical characteristic behavior in the natural environment which is quite similar in terms of transformation and degradation. According to Tobiszewski *et al.* (2012) and Santos *et al.* (2017), the isomer ratios remain the same from the time of emission to the time of measurement. In order to recognize pyrogenic sources from petrogenic sources, people typically look at the ratios of Ant/(Ant + Phe) and Fla/(Fla + Pyr). A petroleum source is indicated by a ratio of Ant/(Ant + Phe) 0.1, but the dominance of combustion is indicated by a ratio of Ant/(Ant + Phe) > 0.1. Additionally, Fla/(Fla + Pyr) giving a value greater than 0.5 indicates combustion from biomass or coal sources, while ratio values between 0.4 and 0.5 indicate combustion from petroleum sources. Fla/(Fla + Pyr) of 0.4 ratio value is pointing to petroleum source. Tobiszewski *et al.* (2012) stated that a ratio of the LMW to HMW PAHs <1 shows a pyrogenic source, while >1 indicates a petrogenic source. According to Wang *et al.* (2010); Wang *et al.* (2017); and Chen *et al.* (2012), the ratio of BaA/(BaA + Chr) below 0.2 implies a petroleum source, but values in the

range of 0.2–0.35 suggested petroleum source, especially liquid fossil fuel, vehicle, fuels, and crude oil, while values above 0.35 indicate the combustion of coal, grass, and wood.

2.10 Toxic equivalent factor (TEF), toxic equivalent quantity (TEQ), and lifetime risk assessment

The carcinogenic potential of individual PAHs (Cn) in comparison to that of Benzo[a]pyrene (BaP) is known as the toxic equivalent factor (TEF). As defined by the term "toxic equivalent quantity" (Nisbet and LaGoy, 1992):

$$TEQ = \sum C_n \text{ x } TEF_n$$

In evaluating the lifetime risk assessment, several factors are responsible for the impact of pollutants such as PAHs on human health which include lifestyle, health condition, age, and contact time with the pollutant(s). Incremental lifetime cancer risks (ILCRs) were used to assess the lifetime risk of PAHs. Using Eqs. 1–4, the effects of three main exposure pathways - dermal, ingested, and inhaled were measured. In this study, two age groups - children (0–18 years) and adults (19–70 years) were taken into account. Accordingly, an ILCR of $\leq 10^{-6}$ indicates negligible risk, an ILCR of 10^{-6} to 10^{-4} indicates low risk, an ILCR of 10^{-4} to $\leq 10^{-3}$ indicates moderate risk, an ILCR of 10^{-3} to $\leq 10^{-1}$ indicates high risk, and an ILCR of $\geq 10^{-1}$ indicates extremely high risk (Yahaya et al., 2017; Qu et al., 2019; Xing et al., 2020).

$$BaPeq = \sum \text{Ci x TEF} \quad (1)$$

$$\boldsymbol{ILCRs}(Dermal) = \frac{\text{CS x } \left(\text{CSF (Dermal)} \sqrt[3]{\left(\frac{BW}{70}\right)}\right) \text{ x AF x SA x EF X ABS x ED}}{BW \; x \; AT \; x \; 10^6} \quad (2)$$

$$\boldsymbol{ILCRs}(Ingestion) = \frac{\text{CS x } \left(\text{CSF}(Ingestion) \sqrt[3]{\left(\frac{BW}{70}\right)}\right) \text{ x IR (Ingestion) x EF x ED}}{BW \; x \; AT \; x \; 10^6} \quad (3)$$

$$\boldsymbol{ILCRs}(Inhalation) = \frac{\text{CS x } \left(\text{CSF (Inhalation)} \sqrt[3]{\left(\frac{BW}{70}\right)}\right) \text{ x IR (Inhalation) x EF x ED}}{BW \; x \; AT \; x \; PEF} \quad (4)$$

The sum of converted PAHs for 7 CarPAHs based on toxic equivalents of BaP using the Toxic equivalent factor (TEF) by Nisbet and LaGoy (1992), the carcinogenic slope factor - CSF $(mg/kg/day)^{-1}$, the body weight (BW) of the exposed resident (kg), the average lifespan (AT) in years, the exposure frequency (EF) in days per year, the exposure duration (ED) in years, others include the dermal adherence factor (AF) for sediment ($mg/cm^2/h$), the dermal adsorption factor (ABS), the surface area of dermal exposure (SA) (cm^2), the sediment ingestion rate (IR Ingestion) (mg/day), the inhalation rate (IR Inhalation) (m^3/day), and the particle emission factor (PEF) (m^3/kg). According to Peng *et al.* (2011), the CSF ingestion was taken as 7.30 $(mg\ kg^{-1}\ day^{-1})^{-1}$, CSF for dermal as 25 $(mg\ kg^{-1}\ day^{-1})^{-1}$, and CSF for inhalation to be 3.85 $(mg\ kg^{-1}\ day^{-1})^{-1}$ of BaP based on the cancer-causing ability.

2.11 Statistical analysis and quality assurance

Excel and Minitab packages were employed for the descriptive data analysis, and generating the distribution charts. The GC was air-flushed before starting up to clean the column and get ready for fresh analysis. Quality control (QC) and blank samples were included within each batch of samples that were to be analyzed. Target compounds were not detected in the blank samples. High purity and analytical grade chemicals and reagents were used of >98.8% purity (including sodium chloride and anhydrous sodium sulphate). All solvents used (acetone (99.8%), dichloromethane (99.5%), n-hexane (99.8%), and acetonitrile) were GC grade and of high purity. The correlation coefficient (r^2) from the calibration curves was 0.995 indicating perfect linearity. Recovery studies of PAH surrogates ranged from 81.2% to 95.6%, and the spiked samples were between 79.7% and 91.5%. The signal-to-noise ratio of the examined blanks was multiplied by 3 and 10 to determine the limits of detection and quantitation (LOD and LOQ).

3 Results and discussion

3.1 PAHs concentration and distribution in the sediment samples

Table 1 shows the concentrations of the various components of PAHs: the low molecular weight (LMW), high molecular weight (HMW), 1-methyl naphthalene, and 1-methyl naphthalene. All the LMW PAHs were detected in all the sediment samples except for acenaphthylene. The concentrations of naphthalene, fluorene, anthracene, phenanthrene, and acenaphthene were in the range: of 0.032—0.112 μg/kg, 0.031—0.016 μg/kg, 0.003—0.019 μg/kg, 0.025—0.059 μg/kg and 0.001—0.029 μg/kg respectively while their mean values were 0.072 μg/kg, 0.020 μg/kg, 0.010 μg/kg, 0.040 μg/kg and 0.014 μg/kg. Figure 2 gives the distribution of the low molecular weight PAHs across the seven sampling locations. The ∑LMW PAHs were in the range of 0.093—0.250 μg/kg. The highest concentration was from sediment sampling location 6 (SE6) (0.250 μg/kg) while the least was from sampling location 7 (SE7) (0.093 μg/kg). A publication by dos-Santos *et al.* (2018) reported total PAHs in sediment samples from the Estuary of Amazon River of Amapa of Brazil to be in the range of 22.20—158.90 ng/g. The values of naphthalene were in the range of 26.40–6.70 ng/g, acenaphthene 0.60–0.20 ng/g, anthracene was 1.00-ND, pyrene, and benzo[a]pyrene was 6.30-ND, and 8.80–0.60 ng/g. Almost all the sixteen PAHs analysed were detected from the study area. The total PAHs PAHes obtained were far greater than those of this study. Research conducted by Olayinka *et al.* (2019) on polycyclic aromatic hydrocarbons in sediment and biota (fish, crab, and shrimp) around Atlas Cove Nigeria revealed the presence of naphthalene with a mean concentration of 0.66 ± 0.01—1.17 ± 0.01 mg/kg,

TABLE 1 Concentration of ∑PAHs in various sediment and fish samples from rivers across the globe.

River	City/Country	∑PAHs in sediment (μg/kg dw)	∑PAHs in fish (μg/kg dw)	References
Around Atlas Cove	Nigeria	2.15–36.46	11.89–71.06	Olayinka *et al.* (2019)
Warri River	Nigeria	4587.7 (mean)	1098.5 (mean)	Asagbra *et al.* (2015)
Jhelum Riverine system	Pakistan	14.54–437.43	-	Raiz *et al.* (2019)
River Benue	Nigeria	55 ± 3–382 ± 9	-	Arowojolu *et al.* (2021)
Mediterranean sea	Egypt	13.5—22,600	-	Barakat *et al.* (2011)
Gulf of Suez	Egypt	1667.02–2671.27	621–4207 wet weight	Younis *et al.* (2018)
Amazon River	Brazil	22.2–158.9	-	Dos Santos *et al.* (2018)
Brisbane River	Australia	148–3079	-	Duodu *et al.* (2017)
Naples harbor	Southern Italy	9–31774	-	Sprovieri *et al.* (2007)
Jakarta bay	Indonesia	257–1511	-	Koike *et al.* (2012)
Langkawi Island	Malaysia	868–1637	-	Nasher *et al.* (2013)
Wei River	China	60.50—10,240.1	-	Pang *et al.* (2021)
Weihe River	China	362–15,667	-	Chen *et al.* (2015)
White Nile	South Sudan	566 to 674	566 to 674 wet weight	Abayi *et al.* (2021)
Guan River	China	43–169	-	He *et al.* (2014)
Mediterranean sea	Italy	40–679	-	Montuori and Triassi (2012)
Hooghly River	India	48–1831	-	Khuman *et al.* (2018)
Paraguacu River	Brazil	443.7–636.1	-	Santos *et al.* (2017)
Estero de Urias Estuary	Mexico	27–418	-	Jaward *et al.* (2012)
Delaware River	USA	3749–22,324	-	Kim *et al.* (2018)
Edremit Bay (Aegea Sea)	Turkey	0.65–175	-	Darilmax *et al.* (2019)
River Owan	Nigeria	0.28–0.81	ND-0.19	This study

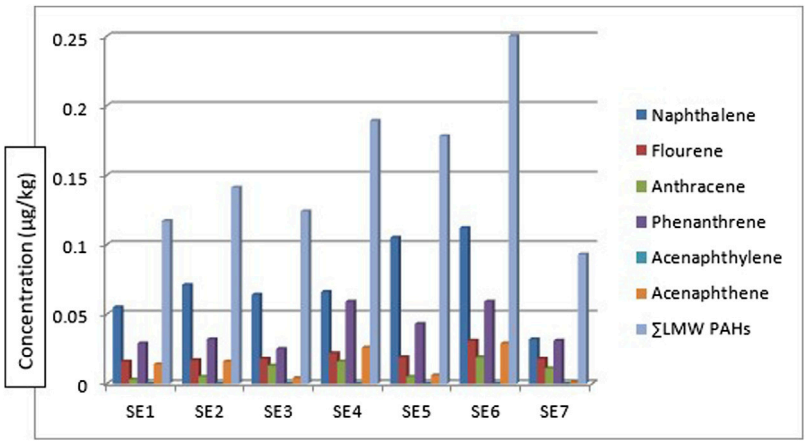

FIGURE 2
Concentration, comparison, and ∑ LMWPAHs in the sediment samples.

acenaphthylene: BDL—1.42 mg/kg while fluorene was only detected in one out of the 5 sampling locations with a concentration of 1.05 ± 0.053 mg/kg. The concentration of LMW PAHs in the sediment samples from Olayinka *et al.* (2019) was higher than the concentration obtained from this study. Gosai *et al.* (2018) reported mean ∑LMW PAHs higher

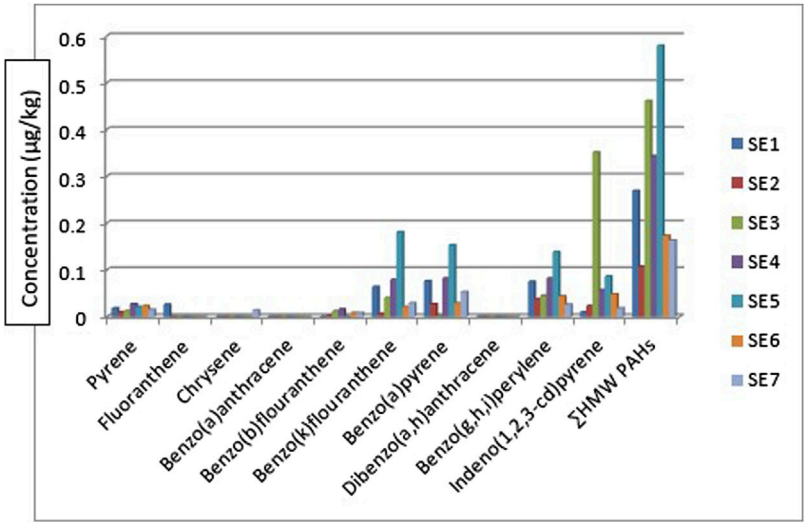

FIGURE 3
Concentration, comparison, and of $\sum$HMWPAH$_s$ in the sediment samples.

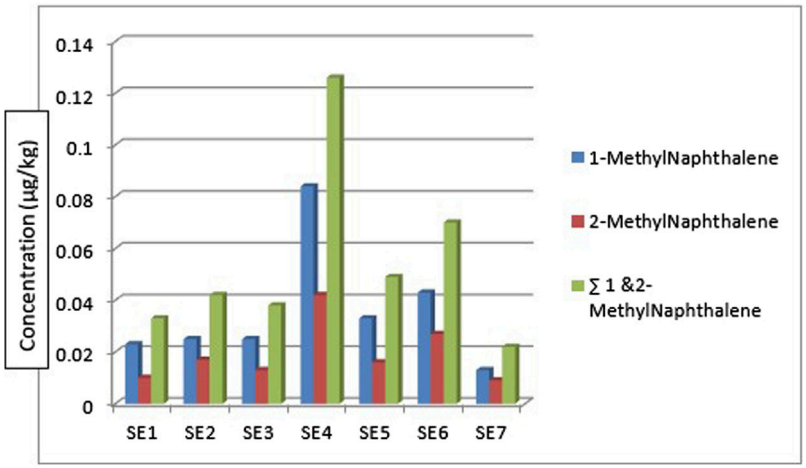

FIGURE 4
Concentration and $\sum$1 and 2-Methyl naphthalene PAH$_s$ in the sediment samples.

than this study (1,437.499–65.36 and 3,024—179.36 ng/g) of sediment from two sampling locations along the Gujarat coastline.

The HMW PAHs such as pyrene, benzo[b]fluoranthene, benzo [k]fluoranthene, benzo[a]pyrene, benzo[g,h, i]perylene, and indeno [1,2,3-cd]pyrene were detected in all the sediment sampling locations in the range: 0.010—0.027 µg/kg, BDL - 0.016 µg/kg, 0.007—0.181 µg/kg, 0.001—0.153 µg/kg, 0.026—0.138 µg/kg and 0.010—0.351 µg/kg respectively. Chrysene was only detected in sediment samples from sampling location 7 with a concentration of 0.014 µg/kg and fluoranthene was detected in sampling location 1 with a concentration of 0.026 µg/kg. Benz[a]anthracene and dibenzo[a,h]anthracene were below the detection limit (BDL) in all the sampling locations. The mean range concentrations of the sediment samples BDL - 0.085 µg/kg. Figure 3 gives the distribution of the high molecular weight PAHs in the sediment samples across the seven different sampling locations. The ∑HMW PAHs range was 0.107—0.579 µg/kg across the sampling locations. The highest concentration was from sampling location 5 (SE5) while the least was from sampling location 2 (SE2). High molecular weight PAHs determined by Olayinka *et al.* (2019) in sediments around Atlas Cove, Nigeria were below the detection limit of the equipment (0.001 mg/kg) with only one site having 4.97 ± 2.45, 3.03 ± 1.44, 2.08 ± 1.67, 4.10 ± 1.95 and 3.38 ± 1.89 mg/kg concentrations for chrysene, benzo[k]fluoranthene, benzo[b]fluoranthene, benzo[a] pyene and benzo[g,h, i]perylene respectively. In the same study (Olayinka et al., 2019), pyrene, fluoranthene, and benzo[a] anthracene are in the range of BDL—5.43 ± 0.18, 0.23 ± 0.02—4.73 ± 0.10 and BDL—2.48 ± 0.45 mg/kg.

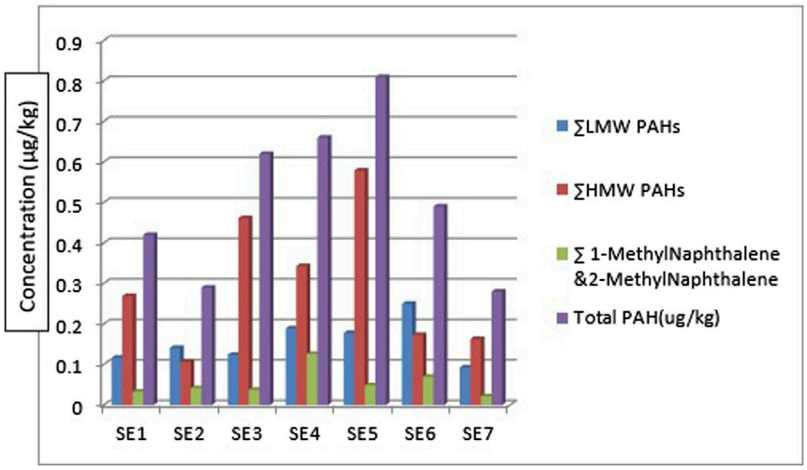

FIGURE 5
∑LMW, ∑HMW, and ∑1 and 2- Methyl naphthalene PAH_s in the sediment samples.

Figure 4 shows the concentration and distribution of 1-methyl naphthalene and 2-methyl naphthalene in the sediment samples across the seven different sampling locations. 1-methyl naphthalene and 2-methyl naphthalene were detected in the sediment samples from all the sampling stations. 1-methyl naphthalene is in the range: 0.013—0.084 µg/kg and 2-methyl naphthalene 0.009—0.042 µg/kg. The highest concentration of 1-methylnaphthalene and 2-methylnaphthalene was from the sediment sample from location 4 (SE4) at 0.126 µg/kg while the least was from sampling location 7 (SE7) at 0.022 µg/kg. The concentration of 1-methylnaphthalene and 2-methyl naphthalene obtained from sediment samples by Olayinka et al., 2019 around Atlas Cove, Nigeria was in the range of 0.19 ± 0.02—0.41 ± 0.02 mg/kg and 0.12 ± 0.00—0.27 ± 0.01 mg/kg for 1-methyl naphthalene and 2-methyl naphthalene respectively.

Considering the ∑PAHs, the highest concentration is from the sediment sample from sampling location 5 (SE5) 0.810 µg/kg. The major contribution to this value is from the HMW components (∑HMW PAH) sampling location 5 (SE5) 0.579 µg/kg. The least ∑PAH components are from sediment samples from sampling location 7 (SE7) 0.280 µg/kg. The range of ∑PAHs in the sediment samples is 0.280–0.810 µg/kg (Concentration order is ∑PAHs SE5>SE4>SE3>SE6>SE1>SE2>SE7). Figure 5 shows the ∑LMW, ∑HMW, ∑1-methylnaphthalene, and 2-methylnaphthalene polycyclic aromatic hydrocarbons across all the sampling locations. These results are lower than what Olayinka *et al.* (2019) obtained from sediment samples around Atlas Cove, Nigeria which range between 2.15—36.46 mg/kg. Assessment of ∑16PAHs in the sediment of Wei River by Pang *et al.* (2021) was in the range of 60.50—10,241.10 ng/g while Raiz et al., 2019 reported 14.54—437.43 ng/g for PAHs sediment samples along Jhelum riverine system of lesser Himalayan region of Pakistan while a report published by Dos Santos *et al.* (2018) of Amazon River in Brazil reported a total PAHs concentration of 22.2—158.9 µg/kg of sediment samples from the river. Asagbra *et al.* (2015) determine the concentration of PAHs in sediment samples from the Warri River at Ubeji, Niger Delta, Nigeria with a mean concentration of 4587.70 ng/g. Barakat *et al.* (2011) also published the ∑PAH concentration of the Mediterranean Sea of Egypt to be in the range of 13.5—22,600 µg/kg. The total concentration of PAHs of sediment from the Delaware River in the USA river was reported by Kim *et al.* (2018) to be in the range of 3749–22,324 µg/kg. All these ∑PAH concentrations were higher than the results obtained from river Owan, Edo State, Nigeria (this study). Table 2 shows some other results obtained by other researchers across the globe. The presence of PAHs in the environment has been a contribution from a series of human activities and natural occurrences ranging from domestic to industrial sources. These include burning of fossil fuel, combustion of wood, vehicular emissions, incinerators, flaring gases from industries, deposition wildfires, leakages from pipelines, gasoline leakage from engine boats, etc. (Davis et al., 2019; D'Agostino et al., 2020). Some of the factors that could affect the concentrations of PAHs in the environment include the type of human activities in the study area (anthropogenic), natural occurrence (naturogenic), seasonal variations, sediment transport processes, physicochemical properties, etc. (D'Agostino et al., 2020; Ambade et al., 2023).

3.2 PAHs concentration and distribution in fish samples

Table 3 shows the concentration of PAHs in the fish samples. Catfish (*Clarias anguillaris)* (F1) and tilapia fish (*Oreochromis niloticus)* (F2) were the two types of fish analysed with varying concentrations of PAH components. Among the LMW PAHs, acenaphthylene was below the detection limit in the two fish samples. Acenaphthene was BDL in *Clarias anguillaris* (F1) but with a concentration of 0.0106 µg/kg in *Oreochromis niloticus* (F2). The concentrations of naphthalene, fluorene, anthracene, and phenanthrene for *Clarias anguillaris* (F1) were in the range of 0.0035–0.0254 µg/kg while for *Oreochromis niloticus* (F2) 0.0066–0.0598 µg/kg. The concentration of LMW PAHs is higher in (F2) 0.1148 µg/kg while F1 is 0.0474 µg/kg. Figure 6 is the chart

TABLE 2 PAHs in sediment samples.

PAH components/Sampling locations	SE1	SE2	SE3	SE4	SE5	SE6	SE7	SD	TEFs	ERL	ERM
Naphthalene (NAT)	0.055	0.071	0.064	0.066	0.105	0.112	0.032	0.003	0.001	160	2100
Fluorene (FLR)	0.016	0.017	0.018	0.022	0.019	0.031	0.018	0.001	0.001	19	500
Anthracene (ANT)	0.003	0.005	0.013	0.016	0.005	0.019	0.011	0.001	0.01	85	1100
Phenanthrene (PHT)	0.029	0.032	0.025	0.059	0.043	0.059	0.031	0.001	0.001	240	1500
Acenaphthylene (ACL)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	-	0.001	44	640
Acenaphthene (ACN)	0.014	0.016	0.004	0.026	0.006	0.029	0.001	0.001	0.001	16	500
∑LMW PAHs	**0.117**	**0.141**	**0.124**	**0.189**	**0.178**	**0.25**	**0.093**				
HMW PAHs											
Pyrene (PYR)	0.018	0.010	0.012	0.027	0.021	0.023	0.015	0.001	0.001	600	5100
Fluoranthene (FLT)	0.026	BDL	BDL	BDL	BDL	BDL	BDL	0.001	0.001	665	2600
Chrysene (CHR)	BDL	BDL	BDL	BDL	BDL	BDL	0.014	-	0.01	380	2800
Benz(a)anthracene (B[a]A)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	-	0.1	260	1600
Benzo(b)fluoranthene (B[b]F)	BDL	0.002	0.012	0.016	BDL	0.008	0.008	0.01	0.1	320	1880
Benzo(k)fluoranthene (B[k]F)	0.064	0.007	0.040	0.079	0.181	0.021	0.029	0.006	0.1	280	1620
Benzo(a)pyrene (B[a]P)	0.076	0.027	0.001	0.082	0.153	0.029	0.053	0.005	1	430	2800
Dibenzo(a,h)anthracene (D[ah]A)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	-	1	63	260
Benzo(g,h,i)perylene (B[ghi]P)	0.075	0.038	0.045	0.082	0.138	0.044	0.026	0.004	0.01	85	330
Indeno(1,2,3-cd)pyrene (IP)	0.010	0.023	0.351	0.057	0.086	0.048	0.018	0.012	0.1	240	950
∑HMW PAHs	**0.269**	**0.107**	**0.461**	**0.343**	**0.579**	**0.173**	**0.163**				
1-methylNaphthalene	0.023	0.025	0.025	0.084	0.033	0.043	0.013	0.010	-	85	800
2-methylNaphthalene	0.010	0.017	0.013	0.042	0.016	0.027	0.009	0.010	-	70	670
∑ 1-methylNaphthalene and2-methylNaphthalene	**0.033**	**0.042**	**0.038**	**0.126**	**0.049**	**0.07**	**0.022**			**3442**	**24290**
Total ∑PAH (ug/kg)	**0.42**	**0.29**	**0.62**	**0.66**	**0.81**	**0.49**	**0.28**				
∑TEQ	**0.084608**	**0.031226**	**0.042003**	**0.09838**	**0.181,324**	**0.037584**	**0.061307**				

HMW, High molecular weight; LMW, Low molecular weight, SE1 to SE7—Sampling locations, SD, Standard deviation; TEF, Toxic equivalent factor; ERL, Effect range low; ERM, Effect range mean (Source: Nisbet and LaGoy, 1992; Adeniji et al., 2019).

showing the distribution of the LMW polycyclic aromatic hydrocarbons in the fish species. A study carried out by Usese *et al.* (2021) on the assessment of PAHs in Nile tilapia from Agboki Creek, Southwestern Nigeria obtained a concentration of 60.51—69.85 μg/kg which is higher than that of this study.

Figure 7 shows the distribution of the HMW polycyclic aromatic hydrocarbons in the fish species. The HMW PAHs such as chrysene, fluoranthene, benzo[a]anthracene, and dibenzo[a,h]anthracene were below the detection limit in the fish samples. The concentration of pyrene, benzo[b]fluoranthene, benzo[a]pyrene, benzo[g,h,i]perylene, and benzo[k]fluoranthene are of these concentrations for *Clarias anguillaris* (F1): 0.0057 μg/kg, 0.0013 μg/kg, 0.0058 μg/kg, BDL μg/kg, and 0.0033 μg/kg while that of *Oreochromis niloticus* (F2): 0.0120 μg/kg, 0.0031 μg/kg, 0.0139 μg/kg, 0.0010 μg/kg and 0.0093 μg/kg respectively. Indeno [1,2,3-cd]pyrene was BDL in *Oreochromis niloticus* but detected in *Clarias anguillarias* (F1) with a concentration of 0.0062 μg/kg. *Oreochromis niloticus* (F2) had the higher concentration of the HMW PAHs with a value of 0.0393 μg/kg while that of *Clarias anguillaris* (F1) was 0.0225 μg/kg. Benzo[a]pyrene was detected by Usese *et al.* (2021) in Nile Tilapia from Agboyi Creek, Southwestern Nigeria with a concentration of 0.08 ± 0.17 μg/kg. This value is higher when compared to this study. The high value recorded might be due to the level of pollution of the river which is in one of the industrialized cities of Nigeria (Lagos).

Figure 8 shows the distribution of the 1-methylnaphthalene and 2-methyl naphthalene in the two fish species. 1-methyl naphthalene and 2-methylnaphthalene are detected in the two fish species with a concentration of 0.0112 μg/kg and 0.0247 μg/kg of 1-methylnaphthalene in *Clarias anguillaris* (F1) and *Oreochromis niloticus* (F2) while that of 2-methylnaphthalene are 0.0038 μg/kg and 0.0106 μg/kg for the two fish species (F1 and F2). The ∑1-methylnaphthalene and 2-methylnaphthalene of *Oreochromis niloticus* are higher (0.0353 μg/kg) than *Clarias anguillaris*

TABLE 3 PAHs in fish samples.

PAHs components	*Clarias anguillaris* (F1)	*Oreochromis niloticus* (F2)
LMW PAHs	(Ug/kg)	(Ug/kg)
Naphthalene	0.0254	0.0598
Fluorene	0.0068	0.0147
Anthracene	0.0035	0.0066
Phenanthrene	0.0117	0.0231
Acenaphthylene	BDL	BDL
Acenaphthene	BDL	0.0106
∑ LMW PAHs	**0.0474**	**0.1148**
HMW PAHs		
Pyrene	0.0057	0.0120
Chrysene	BDL	BDL
Fluoranthene	BDL	BDL
Benzo(a)anthracene	BDL	BDL
Benzo(b)fluoranthene	0.0013	0.0031
Benzo(a)pyrene	0.0058	0.0139
Dibenzo(a,h)anthracene	BDL	BDL
Benzo(g,h,i)perylene	BDL	0.0010
Indeno(1,2,3-cd)pyrene	0.0062	BDL
Benzo(k)fluoranthene	0.0033	0.0093
∑ HMW PAHs	**0.0225**	**0.0393**
1-methylnaphthalene	0.0112	0.0247
2-methylnaphthalene	0.0038	0.0106
∑1and2-methylnaphthalene	**0.015**	**0.0353**
Total ∑PAHs (ug/kg)	**0.08**	**0.19**
∑LMW/∑HMW PAHs	**2.107**	**3.921**
EC recommendation	**12.0 ug/kg**	

EC, european commission, Source, EC (2015).

(0.0150 μg/kg). The ∑PAHs (LMW, HMW, 1-methylnaphthalene, and 2-methylnaphthalene as shown in Figure 9 are higher in *Oreochromis niloticus* (F2): 0.190 μg/kg than *Clarias anguillaris* (F1): 0.080 μg/kg (∑PAHs F2>F1). Figure 9 shows the distribution of ∑LMW, ∑HMW and ∑1-methylnaphthalene, and 2-methylnaphthalene in the fish samples. Asagbra *et al.* (2015) determined the concentration of PAHs in fish tissue from the Warri River at Ubeji, Niger Delta of Nigeria with a mean concentration of 1098.50 ng/g. The total concentration of 16 PAHs in fish samples reported by Younis *et al.* (2018) ranged from 621 to 4207 ng/g wet weight, with the species *Saurida undosquamis* having the highest concentration and *Stephanolepis diaspros* having the lowest from the Gulf of Suez, Egypt. Ekere *et al.* (2019) reported total ∑PAHs concentration of 2.626 and 2.061 μg/kg for catfish and tilapia fish from Rivers Niger and Benue confluence Lokoja, Nigeria. The ratio of LMW/HMW PAHs from this study is lower than what was reported by Ekere *et al.* (2019) of fish from Rivers Niger and Benue confluence, Lokoja, Nigeria. The concentration of PAHs in the fish samples analysed is lower than 12.0 μg/kg recommended regulatory limit by European Commission (2015).

3.3 Diagnostic ratio of PAHs in the sediment and possible sources

The result of the diagnostic ratio of the sediment samples from River Owan, Edo State is shown in Table 4 with their sources (petrogenic or pyrogenic). The percentage of petrogenic to pyrogenic in the ratio of Ant/(Ant + Phe) was 43% in the

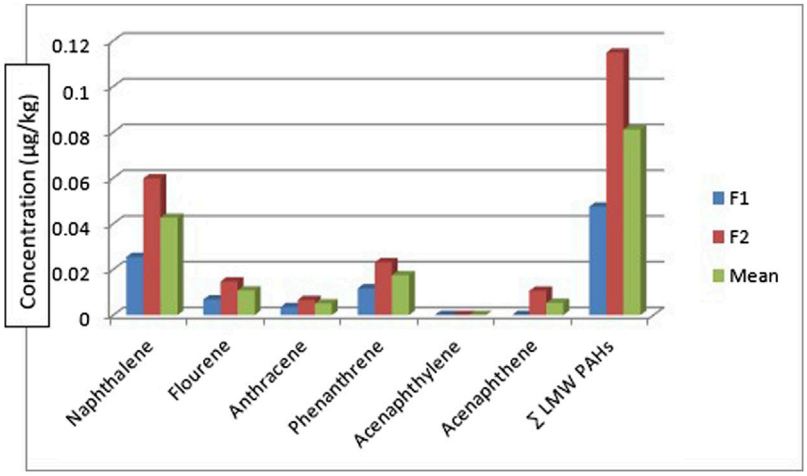

FIGURE 6
Concertration, mean, and ∑LMW PAH_s in the fish samples.

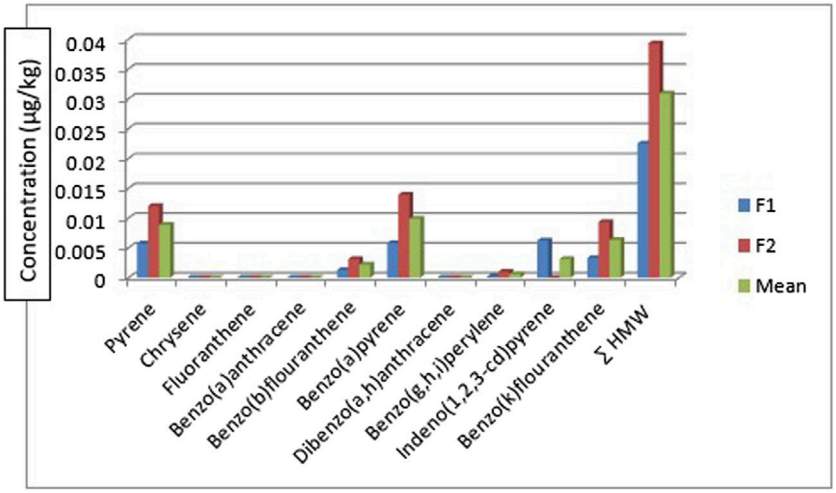

FIGURE 7
Concerntration, mean, and ∑HMW PAH_s in the fish samples.

sediment. The ratio of Flt/(Flt + Pyr) in sediment was >0.1 meaning they are all from pyrogenic sources, showing 100%. The percentages of LMW/HMW PAHs of petrogenic to pyrogenic sources are in the range of 29%–71% in the sediment samples. Generally, petrogenic PAHs are characterized by the predominance of the two to three ring (LMW) PAHs, while pyrogenic PAHs are characterized by a high proportion of above 4-ring (HMW) PAHs (Wang et al.,2013). Furthermore, the ratio of the LMW/HMW <1 except perylene suggests pollution of pyrolytic origin according to Yan *et al.* (2009); and Tavakoly Sany *et al.* (2014). Microbial degradation also accounted for the resistance of HMW PAHs leading to a low LMW/HMW ratio according to Tavakoly *et al.* (2014). Chandru *et al.* (2008) also observed that a low ratio of the LMW/HMW could be caused by high volatility and solubility of LMW. The ratio of Flt/(Flt + Pyr) can also be used as an indicator of PAH origin. Flt/(Flt + Pyr) ratio <0.4 is attributed to a petrogenic source, while a ratio >0.5 suggests a pyrogenic source arising from wood and coal combustion (Yan et al.,2009; Hiller et al.,2011; Tavakoly Sany et al.,2014; Abayi et al.,2021). In this study, the ratio of Flt/(Flt + Pyr) > 0.5, indicates a pyrogenic source. A study by Zhao *et al.* (2017) used diagnostic ratio for PAHs sources in the sediment samples from a river and lake (Qinhuai River and Xuanwu Lake), results obtained revealed that sources were primarily from the burning of biomass and coal during the spring, fall, and winter. Other sites that were sampled revealed mixed sources of pyrogenic and petroleum sources such as this study. Furthermore, Gosai *et al.* (2018) reported PAHs sources in sediment samples from the Gujarat coastline using various isomeric ratios which revealed a mixed origin of petrogenic and pyrogenic sources.

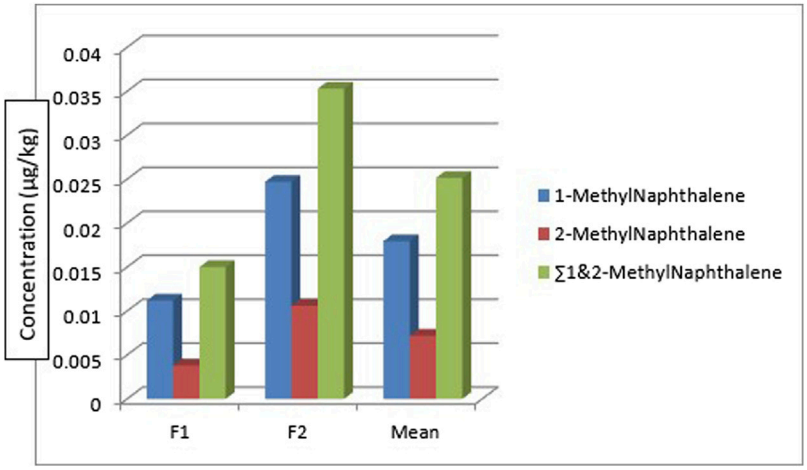

FIGURE 8
Concerntration, mean, and ∑1 and 2 Methyl naphthalene in the fish samples.

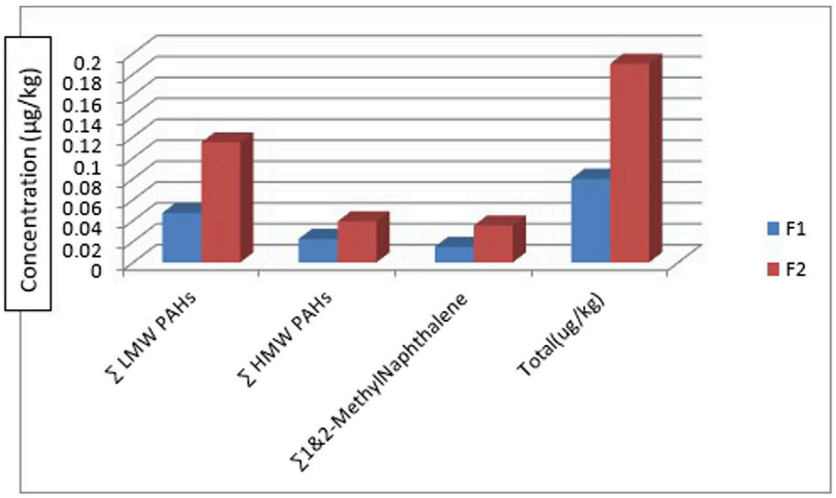

FIGURE 9
Summation and total concerntration of all the PAH_s in the fish samples.

TABLE 4 Diagnostic PAHS in sediment.

Locations	Ant/(Ant + Phe)	Flt/(Flt + Pyr)	LMW/HMW
SE1	0.1 Petrogenic	0.6 Pyrogenic	0.4 Pyrogenic
SE2	0.1 Petrogenic	0.8 Pyrogenic	1.3 Petrogenic
SE3	0.3 Pyrogenic	0.7 Pyrogenic	0.3 Pyrogenic
SE4	0.2 Pyrogenic	0.7 Pyrogenic	0.6 Pyrogenic
SE5	0.1 Petrogenic	0.7 Pyrogenic	0.3 Pyrogenic
SE6	0.2 Pyrogenic	0.7 Pyrogenic	1.5 Petrogenic
SE7	0.3 Pyrogenic	0.7 Pyrogenic	0.6 Pyrogenic

Ant–Anthracene, Phe—Phenanthrene, Flt–Fluoranthrene, Pyr–Pyrene, LMW, Low molecular weight, and HMW, High molecular weight.

TABLE 5 Incremental life cancer risk (ILCR) assessment.

Age groups/Routes	SE1	SE2	SE3	SE4	SE5	SE6	SE7	Mean	Risk implication
ILCR (Children) Dermal	7.28E-07	2.69E-07	9.53E-08	8.06E-07	1.46E-06	2.95E-07	4.94E-07	5.93E-07	Negligible
ILCR (Adult) Dermal	2.55E-07	5.74E-07	2.04E-07	1.72E-06	3.13E-06	6.30E-07	1.06E-06	1.08E-06	Negligible
ILCR (Children) Ingestion	1.65E-06	6.10E-07	2.16E-07	1.83E-06	3.33E-06	6.69E-07	1.12E-06	1.35E-06	Negligible
ILCR (Adult) Ingestion	8.76E-07	3.23E-07	1.15E-07	9.70E-07	1.76E-06	3.55E-07	5.94E-07	7.14E-07	Negligible
ILCR (Children) Inhalation	6.99E-11	2.58E-11	9.15E-12	7.74E-11	1.41E-10	2.83E-11	4.74E-11	5.69E-11	Negligible
ILCR (Adult) Inhalation	5.94E-11	2.19E-11	7.78E-12	6.58E-11	1.20E-10	2.41E-11	4.03E-11	4.84E-11	Negligible

3.4 Sediment quality guidelines (SQG)

Sediment quality guidelines (SQG) are one of the benchmarks employed to evaluate the level of pollution in an aquatic environment (Long and MacDonald, 1998; Darilmax et al., 2019). Comparing the concentrations of the PAHs in the sediment samples with SQG values, the total concentrations (0.280–0.810 μg/kg) were very low when compared with the regulatory values of effect range low and effect range mean (ERL and ERM) for all the sampling stations (Table 1). This shows that no toxic effect occurrence is possible in this area at present. Also, looking at the concentrations of PAHs around the globe from rivers of other sediments across the globe (Table 2), the concentrations from this study are low. A study by Darilmax *et al.* (2019) reported a concentration range of 0.65—175 ng/g of surficial sediments of Edremit Bay (Aegean Sea). The least or minimum value (0.65 ng/g) from Darilmax's team is in the range of concentrations recorded in this study (0.28—0.81 μg/kg). Ambade *et al.*, 2022a also reported values that were low with fewer ecological hazards of sediment samples from the Estuary of the Subarnarekha India. It is important to note that the presence of PAHs in the ecosystem has negative biological implications on various biological species and causes a change in the proper functioning of these organisms from long-term exposure (Akhbarizadeh et al., 2016). Some of the potential human health implications of the presence of PAHs include the capability of causing cancer, mutagenicity, teratogenic effects, damage to the central nervous system (CNS), and so on. Hence, constant monitoring is needed to assess the environmental risk to wildlife and human health. Proper environmental management including a waste disposal plan using the best available practices (bap) should be employed. In addition, indiscriminate disposal of sewage and refuse into the river should be outlawed (Davis et al., 2019; D'Agostino et al., 2020; Ambade et al., 2022b; Ambade et al., 2023).

3.5 ILCR assessment

Looking at the result from the ILCRs evaluated, children and adults are the age groups considered with three routes of exposure (dermal, ingestion, and inhalation). The results of the ILCR are presented in Table 5. The results are in the following range for children's dermal, ingestion, and inhalation 9.53×10^{-8} to 1.46×10^{-6}, $2.16 \times 10^{-7}$to 3.33×10^{-6}, and 9.15×10^{-12} to 1.41×10^{-10} respectively for all the sampling sites. For the adult, the results were in the range of 2.04×10^{-7} to 3.13×10^{-6}, 1.15×10^{-7} to 1.76×10^{-6}, and 7.78×10^{-12} to 1.20×10^{-10} respectively for dermal, ingestion and inhalation. These range values for the three routes of exposure, and the two age groups, ILCR implications show negligible risks. A study reported by Bhutto *et al.* (2021) gave ILCRs for children: 9.51×10^{-2} -2.03×10^{-5} with an average of 4.76×10^{-2} and 4.01×10^{-2}—8.57×10^{-6} with an average of 2.01×10^{-2} for adult indicating a moderate cancer risk to children and threat to adult. Incremental lifetime cancer risks of two locations (ASSBRY and NAV) published by Gosai *et al.* (2018) ranged from 4.11×10^{-6} - 2.11×10^{-5} to 9.08×10^{-6}–4.50×10^{-3} implying cancer risk at a higher level at NAV when compared to ASSBRY.

4 Conclusion

This study showed that LMW PAHs are more predominant than HMW PAHs for the sediment samples from River Owan. Meanwhile, their concentrations vary from one sampling location to the other. The levels of ∑PAHs in the fish samples are in varying concentrations, LMW PAHs were higher than the HMW PAHs in the fish samples. The concentration of PAHs in *Clarias anguillaris* (catfish) is higher than that of *Oreochromis niloticus* (tilapia fish). The concentration of ∑PAHs in the fish samples analysed is far lesser than the European Commission regulation limit. The result of the diagnostic ratio of the sediment samples shows that the sources are more pyrogenic than petrogenic sources. Sediment quality assessment revealed that the total concentrations of PAHs are lower than the regulatory values of SQG (ERL and ERM) for all the sampling stations. This shows that no toxic effect occurrence in this area at present. ILCR assessment evaluated showed negligible risk. To prevent any potential ecotoxicological problems in the near future, constant monitoring of PAH levels in the study area will be required. In addition, assessment of PAHs during the raining season and increasing the frequency of sampling is of interest for further study.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

Conceptualization AA and FO; Methodology AA and JU; Formal analysis and investigation AA; Writing–original draft

preparation AA; Writing–review and editing AA, FO, and JU; Supervision FO. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Aina Olubukola Adeogun,
University of Ibadan, Nigeria

REVIEWED BY
Francheska Merced-Nieves,
Icahn School of Medicine at Mount Sinai, United States
Adesola Ogunniyi,
University of Ibadan, Nigeria

*CORRESPONDENCE
Ashley A. James,
james.ashley@epa.gov

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Environmental chemical exposures and mental health outcomes in children: a narrative review of recent literature

Ashley A. James[1,2]* and Katherine L. OShaughnessy[3]

[1]United States Environmental Protection Agency, Office of Children's Health Protection, Regulatory Support and Science Policy Division, Washington, DC, United States, [2]Oak Ridge Institute for Science Education, Oak Ridge, TN, United States, [3]United States Environmental Protection Agency, Public Health Integrated Toxicology Division, Center for Public Health and Environmental Assessment, Research Triangle Park, NC, United States

Background: Mental health is an important factor for children's overall wellbeing. National health statistics show that millions of children are diagnosed with mental health disorders every year, and evidence from studies on chemical pollutants like lead and bisphenols indicate that environmental exposures are linked to mental health illnesses in youth. However, the relationship between children's mental health and the environment is not well understood. This paper aims to review recent literature on prenatal and/or childhood environmental chemical exposures and mental health problems related to mood, anxiety, and behavior. This work also identifies areas of insufficient data and proposes suggestions to fill the data gaps.

Methods: A narrative review was performed by searching Google Scholar and PubMed for literature published in the last 6 years (2017–2022), using search terms related to children, mental health, and environmental chemical exposure. Additional relevant studies were identified by screening the references in these papers.

Results: A total of 29 studies are included in this review and results are summarized by chemical category: heavy metals, endocrine-disrupting chemicals, and pesticides. The majority of studies reported positive and significant associations between chemical exposures and child mental health outcomes including internalizing and externalizing behaviors.

Conclusion: This review demonstrates that there is a growing body of literature that suggests developmental exposure to some environmental chemicals increases a child's risk of mood, anxiety, and behavior problems. Future research should expand on these findings to understand cumulative impacts, chemical mixtures, neurotoxic mechanisms, sex differences, and windows of vulnerability.

KEYWORDS

children's mental health, behavioral health, environmental justice, environmental exposure, toxicity, heavy metals, endocrine disrupting chemicals, pesticides

1 Introduction

Children's mental health is a critical factor of their overall health and wellbeing. Mental health disorders, including mood, anxiety, and behavioral disorders, impact multiple aspects of children's lives including the development of social skills, academic performance, and physical health. The diagnosis of childhood mental health disorders is also one of the strongest predictors of adult mental health disorders (Fryers and Brugha, 2013). Over the past 2 decades, the prevalence of adverse mental health outcomes among U.S. children has increased (Park-Lee, 2019; Tkacz and Brady, 2021). Based on 2016–2019 surveillance data for children ages 3–17 years living in the United States, 8.9% of children had received a behavior problems diagnosis, 4.4% of children had received a depression diagnosis, and 9.4% of children had received an anxiety diagnosis (Bitsko et al., 2022). Given the inherent gaps in surveillance data, including that many children mental health disorders are undiagnosed or misdiagnosed (Bitsko et al., 2022), these numbers may be underestimates.

The etiology of psychopathology is complex and includes genetic, social, and environmental factors, including exposures to chemicals in the environment. In recent years, the environmental determinants of mental health outcomes have gained increasing attention in multiple scientific disciplines including toxicology, epidemiology, psychology, and psychiatry. For example, there is a growing body of research that indicates air pollution may negatively impact children's mental health (Brokamp et al., 2019; Roberts et al., 2019; Szyszkowicz et al., 2020; Reuben et al., 2021). Data on anthropogenic climate change and its negative impacts on youth mental health outcomes is also growing (Burke et al., 2018; Van Nieuwenhuizen et al., 2021; Clemens et al., 2022), including the consideration of "climate anxiety," or anxiety related to the climate crisis and environmental disasters (Wu et al., 2020; Hickman et al., 2021). Further, the emerging discipline of clinical ecopsychology, which seeks to "systematically examine the direct and indirect mental health impacts of climate change, pollution, environmental degradation, and/or destruction of the air, water, and ecosystems," has shown that environmental crisis and pollution act as stressors and disrupt multiple pathways that can increase a person's vulnerability to adverse mental health (Thoma et al., 2021). Despite this growing interest, several data gaps related to environmental exposures and child mental health outcomes remain.

Scientists, physicians, and decision-makers need to better understand how environmental pollutants may impact children's mental health. There are several plausible mechanisms to link environmental chemical exposures to the induction and/or exacerbation of mental health disorders. Chemical pollutants, such as heavy metals and endocrine disruptors, may alter neurotransmitter systems, damage the blood-brain barrier, modify brain gene expression leading to increased vulnerability to mental health disorders (i.e., depression), dysregulate the hypothalamus-pituitary-adrenal axis, reduce neuronal plasticity, and induce oxidative stress and neuroinflammation (Van Den Bosch and Meyer-Lindenberg, 2019; Thoma et al., 2021). The latter three are also consequences of chronic stress, which can have a synergistic effect on mental health outcomes (Van Den Bosch and Meyer-Lindenberg, 2019; Thoma et al., 2021).

Neurodevelopment is a protracted process that occurs from early embryogenesis and continues until at least 21 years of age (Stiles and Jernigan, 2010). Therefore, children are vulnerable to environmental exposures for years, and any perturbation of normal neurodevelopment could hold lifelong consequences (Stiles and Jernigan, 2010). Furthermore, children have limited ability to handle stress due to immature coping mechanisms, and thus have increased vulnerability to life stressors that can impact brain structure and function (Thoma et al., 2021). For example, racial and economic disparities in child mental health outcomes exist. A recent study found that non-Hispanic Black children had higher rates of mental-health related emergency visits than white children (Abrams et al., 2022). Several studies have also demonstrated that children from low-income households have a higher prevalence of mental health disorders than children from middle- and high-income households (Roberts et al., 2007; Melchior et al., 2010; Najman et al., 2010; Acri et al., 2017; Cree et al., 2018). These studies suggest that children living in environmental justice communities may be more at-risk to mental health conditions, given their burden of both chemical and non-chemical stressors. This review aims to assess the landscape of recent literature on prenatal and/or childhood environmental xenobiotic exposures and consequent symptoms related to mood, anxiety, and behavior disorders.

2 Materials and methods

This review focuses on symptoms and behaviors related to mood, anxiety, and behavior disorders such as depression, generalized anxiety disorder, and conduct disorder, respectively. Studies that exclusively focused on neurodevelopmental disorders (i.e., attention deficit hyperactivity disorder and autism spectrum disorder), cognition, intelligence quotient, and/or learning disorders were excluded. Experimental animal and adult population studies were also excluded, unless related to a childhood exposure. This review considered exposures from conception until age 21 years, in accordance with the U.S. Environmental Protection Agency's Policy on Children's Health (U.S. EPA, 2021).

To include the most recent research, only original research studies from the last 6 years were included, from January 2017 to December 2022. Google Scholar and PubMed were searched using search terms related to children (i.e., "child" or "infant" or "prenatal" or "adolescent"), mental health (i.e., "mental health" or "stress" or "anxiety" or "behavior" or "mood"), and environmental chemical exposure (i.e., "pollution" or "chemical" or "toxins"). Reference lists were also screened for additional relevant studies. Papers were selected based on title and then abstracts were screened. Full texts were then checked according to the inclusion/exclusion criteria, resulting in 29 final papers selected for review. Given that there is limited data on this emerging research area, we chose a narrative review, which is useful for exploring under researched topics (Sukhera, 2022). The approach was not intended to follow systematic review procedures.

3 Results

3.1 Literature overview

A total of 29 studies were included in this review (Table 1). These results are summarized below by chemical category, with some

TABLE 1 Summary of the literature reviewed.

Citation	Chemical class	Chemical name	Assessment tool	Results
Zeng et al. (2021)	Metals	Lead	The Strengths and Difficulties Questionnaire (SDQ)	Blood Pb level was negatively correlated with NPY, but positively correlated with behavioral symptom scores; while serum NPY levels were negatively associated with behavioral symptom scores
Joo et al. (2018)	Metals	Lead	Child Behavior Checklist (CBCL)	Prenatal Pb associated with increased total behavior scores in preschool males. Early childhood Pb associated with increased total behavior scores in preschool females
Horton et al. (2018)	Metals	Lead, zinc, manganese, mixture	The Behavior Assessment System for Children 2nd Edition (BASC-2)	Infant Pb exposure (measured in deciduous teeth) associated with increased anxiety symptoms in mid childhood
Reuben et al. (2019)	Metals	Lead	Big Five Personality Inventory	Mid childhood blood Pb levels associated with increased internalizing symptoms and general psychopathology in adults
Rokoff et al. (2022)	Metals, Pesticides	Organochlorines, lead, manganese	The Behavior Assessment System for Children 2nd Edition (BASC-2) and Conners' Rating Scale (CRS)	Prenatal Pb associated with increased 15-year-old anxiety symptoms and psychosomatic symptoms in males. Prenatal Mn associated with increased internalizing symptoms in girls
Rodrigues et al. (2018)	Metals	Manganese	Child Behavior Checklist (CBCL)	Toenail Mn associated with increased total behavioral and externalizing behavior scores in 7–12-year-olds, with a stronger effect for externalizing behavior in boys
Rahman et al. (2017)	Metals	Manganese	The Strengths and Difficulties Questionnaire (SDQ)	Prenatal Mn exposure from drinking water increased the risk of conduct problems in 10-year old children, with a stronger effect in boys
de Water et al. (2018)	Metals	Manganese	functional magnetic resonance imaging (fMRI)	Maternal blood Mn were associated with reduced functional connectivity of brain areas involved in emotional processing and regulation in 6- to 7-year-old children
Kim et al. (2018)	Metals, EDCs	Phthalates, heavy metals, and persistent organic pollutants	Child Behavior Checklist (CBCL)	Prenatal blood Hg associated with and higher internalizing and externalizing scores in Korean toddlers aged 1–2 years. Prenatal MEP associated with increased internalizing scores
Maitre et al. (2021)	Metals	Copper	Child Behavior Checklist (CBCL)	Cu exposure, measured in blood, associated with higher internalizing symptoms in 6- to-11-year-old children
Jedynak et al. (2021)	Metals, EDCs	BPA, phthalates, copper	The Strengths and Difficulties Questionnaire (SDQ)	Prenatal blood Cu associated with decreased externalizing behavior, prenatal urinary MBzP associated with increased externalizing behavior, and prenatal urinary BPA associated with increased externalizing behaviors in children ages 3 to 7
Singer et al. (2017)	EDCs	Phthalates	Infant Behavior Questionnaire (IBQ) Toddler Behavior Assessment Questionnaire (TBAQ)	Weak positive associations with prenatal exposure to multiple phthalates and temperament in infants
England-Mason et al. (2020)	EDCs	Phthalates	Child Behavior Checklist (CBCL)	Prenatal exposure to high molecular weight phthalates indirectly associated with internalizing and externalizing problems through mean diffusivity (MD) of different parts of the brain
Chen et al. (2019)	EDCs	Phthalates	Child Behavior Checklist (CBCL)	Prenatal DEHP exposure associated with increased behavior scores in all categories except somatic complaints in 8-, 10-, and 14-year-olds
Huang et al. (2019)	EDCs	Phthalates	Child Behavior Checklist (CBCL)	Prenatal DEHP exposure associated with CBCL higher delinquent behavior and externalizing problem scores in 8-to 14-year-olds

(Continued on following page)

TABLE 1 (*Continued*) Summary of the literature reviewed.

Citation	Chemical class	Chemical name	Assessment tool	Results
Colicino et al. (2021)	EDCs	Phthalates	The Behavior Assessment System for Children 2nd Edition (BASC-2)	Prenatal urinary DEHT exposure with increased overall BASC-2 behavioral problems and depression scores in 4- to 6- year-old boys
Philippat et al. (2017)	EDCs	Phthalates, phenols	The Strengths and Difficulties Questionnaire (SDQ)	Prenatal urinary MnBP and MBzP associated with increased internalizing behavior in 3-year-old boys. Prenatal urinary BPA associated with increased internalizing behavior in 3-year-old boys and increased externalizing behavior in 5-year-old boys
Vuong et al. (2021)	EDCs	PFAS	The Behavior Assessment System for Children 2nd Edition (BASC-2)	Prenatal serum PFOS, PFHxS, and PFNA associated with worse externalizing behavior, and PFHxS was associated with worse internalizing behavior at ages 5 and 7 years
Ghassabian et al. (2018)	EDCs	PFAS, phenols	The Strengths and Difficulties Questionnaire (SDQ)	Newborn dried blood spot BPA exposure associated with decreased prosocial behavior difficulties (2nd and 4th quartile only) and newborn PFOS exposure associated with increased conduct and emotional problems at age 7
Luo et al. (2020)	EDCs	PFAS	The Strengths and Difficulties Questionnaire (SDQ)	Prenatal plasma PFNA associated with increased externalizing behaviors at ages 7 and 11 years
Strawn et al. (2022)	EDCs	PBDE	Screen for Child Anxiety Related Emotional Disorders (SCARED)	Prenatal PBDE exposure associated with increased anxiety symptoms in 12-year-olds
Braun et al. (2017)	EDCs	PBDE, BPA	The Behavior Assessment System for Children 2nd Edition (BASC-2)	Prenatal serum BDE-47 exposure associated with increased externalizing behaviors in 2- to 8-year-olds. Prenatal urinary BPA associated with increased externalizing behavior in girls only
Vuong et al. (2017)	EDCs	PBDE	The Behavior Assessment System for Children 2nd Edition (BASC-2)	Concurrent PBDE serum exposure in 8-year-olds associated with increased externalizing symptoms
Shoaff et al. (2019)	EDCs	Phthalates, phenols, parabens	The Behavior Assessment System for Children 2nd Edition (BASC-2)	Sum of 11 antiandrogenic phthalate metabolites collected from adolescent spot urine associated with increased maladaptive behaviors. Null results for exposure to phenols
Stacy et al. (2017)	EDCs	Phenols	The Behavior Assessment System for Children 2nd Edition (BASC-2)	Prenatal urinary BPA increased externalizing behavior in girls (at age 8) and 8-year BPA increased externalizing behavior in boys
Zhang et al. (2022)	EDCs	Phenols	Center for Epidemiologic Studies Depression Scale for Children (CES-DC)	Serum Bisphenol AF exposure associated with increased depressive symptoms in adolescents (7th grade), with males significantly more vulnerable
Rosenquist et al. (2017)	EDCs, pesticides	DDT	The Strengths and Difficulties Questionnaire (SDQ)	Pre- and postnatal serum p,p′-DDE associated with increased conduct problems in 5- and 7-year-olds
Suarez-Lopez et al. (2021)	Pesticides	Organophosphates	Multidimensional Anxiety Scale for Children 2nd Edition (MASC-2) or the Children's Depression Inventory 2nd Edition (CDI-2)	Decrease in AChE activity, from exposure to organophosphate pesticides, associated with increase depression symptoms of 11–17-year-old adolescents
Furlong et al. (2017)	Pesticides	Pyrethroids	The Behavior Assessment System for Children 2nd Edition (BASC-2)	Prenatal urinary 3-PBA and cis-DCCA associated with increased internalizing and externalizing behaviors respectively

papers addressing the effects of more than one chemical class. The distribution of papers by chemical class are as follows: 11 papers on heavy metals, 20 on endocrine disrupting chemicals, and 4 on pesticides. Most of the studies (n = 22) used a prospective cohort design, with 20 studies being longitudinal birth cohorts. The majority of studies (n = 20) assessed prenatal exposure and measured outcomes during early childhood, from 0 to 5 years (n = 13), and mid childhood, from 6 to 10 years (n = 14). Each study utilized various covariates like maternal age and education, and this information can be found in the original publications.

TABLE 2 Summary of the behavioral assessments used to quantify mental health in children.

Name	Abbreviation	Scales	Assessor	Count (n)
The Strengths and Difficulties Questionnaire	SDQ	Emotional Symptoms, Conduct Problems, Hyperactivity/Inattention, Peer Relationship Problems, Prosocial Behavior, Total Difficulties Score	Parent, Teacher, or Self-Report for Adolescents	7
The Behavior Assessment System for Children 2nd Edition	BASC-2	Internalizing Problems (Anxiety, Depression, Somatization), Externalizing Problems (Hyperactivity, Aggression, Conduct Disorder), School Problems (Attention Problems, Learning Problems), and Adaptive Skills (Adaptability, Social Skills, Leadership, Study Skills)	Parent, Teacher, or Self-Report for Adolescents	9
Child Behavior Checklist/Korean Child Behavior Checklist	CBCL/KCBCL	Syndrome Scales: Withdrawn/Depressed, Somatic Complaints, Anxious/Depressed, Social Problems, Thought Problems, Attention Problems, Delinquent Behavior, Aggressive Behavior, Internalizing Score, Externalizing Score, Total Problems Score. Diagnostic and Statistical Manual of Mental Disorders Scales: Anxiety, Oppositional Defiant Disorder, Conduct Problems, Somatic Problems, Affective Problems, Attention Deficit Disorder	Parent, Teacher, or Self-Report for Adolescents	7
Big Five Personality Inventory	BFI	Extraversion, Agreeableness, Openness, Conscientiousness, and Neuroticism	Self-Report	1
Conners' Rating Scale	CRS	Oppositional, Cognitive, Problems/ Inattention, Hyperactivity, Anxious-Shy, Perfectionism, and Social Problems, ADHD Index Score, a DSM-IV: Inattention Score, and a DSM-IV: Hyperactivity' Score	Parent, Teacher, or Self-Report for Adolescents	1
Multidimensional Anxiety Scale for Children 2nd Edition	MASC-2	Separation Anxiety/Phobias, Social Anxiety, Obsessions & Compulsions, Physical Symptoms, Harm Avoidance, GAD Index and Inconsistency Index	Parent or Self-Report	1
Children's Depression Inventory 2nd Edition	CDI-2	Scales: Emotional Problems, Functional Problems. Subscales: Negative Mood/Physical Symptoms, Negative Self-Esteem, Interpersonal Problems, Ineffectiveness	Self-Report	1
The Behavior Rating Inventory of Executive Functioning	BRIEF	Clinical Scales: Inhibit, Shift, Emotional Control, Initiate, Working Memory, Plan/ Organize, Organization of Materials, Monitor. Validity scales: Inconsistency and Negativity. Global Executive Composite	Parent or Teacher	1
Screen for Child Anxiety Related Emotional Disorders	SCARED	Generalized Anxiety, Separation Anxiety, Social Anxiety, Panic or Somatic Symptoms, School Avoidance	Parent or Self-Report	1
Infant Behavior Questionnaire	IBQ	Approach, Vocal Reactivity, High Intensity Pleasure, Smiling and Laughter, Activity Level, Perceptual Sensitivity, Sadness, Distress to Limitations, Fear, Falling Reactivity/Rate of Recovery from Distress, Low Intensity Pleasure, Cuddliness, Duration of Orienting, Soothaability	Parent	1
Toddler Behavior Assessment Questionnaire	TBAQ	Activity level, Anger, Social Fear, Interest, Pleasure	Parent	1
Center for Epidemiologic Studies Depression Scale for Children	CES-DC	One scale with 20 items designed to specifically measure for depression	Self-Report	1

Eleven of the studies were conducted in North America, 7 in Asia, 5 in Europe, 5 in Latin America, and 1 in Oceania (New Zealand). Internalizing behaviors (i.e., anxiety, depression, somatization, withdrawal) and externalizing behaviors (i.e., aggression, impulsivity, conduct disorder) were the most frequently assessed endpoints. The most common measures used to assess these behaviors were the Behavior Assessment System for Children 2nd Edition (BASC-2) (n = 9), the Strengths and Difficulties

Questionnaire (SDQ) (n = 7) and the Child Behavior Checklist (CBCL) (n = 7); information regarding these behavioral metrics can be found in Table 2. All findings summarized in this section are statistically significant, unless stated otherwise.

3.2 Heavy metals

Heavy metals are naturally occurring chemical elements that have a vast range of industrial uses but can be extremely toxic to several organ systems, including the central nervous system. Heavy metal applications include agriculture (e.g., fertilizer), technology (e.g., electronics), home goods (e.g., cookware) and manufacturing (e.g., automobile), which result in multiple possible sources of exposures. Documented exposure routes in humans include inhalation, ingestion, and dermal absorption (Al-Osman et al., 2019).

3.2.1 Lead

Lead (Pb) is one of the most well-established neurodevelopmental toxicants with no known safe level of exposure (U.S. EPA, 2013). Sources of Pb exposure for children include toys, dust, residential lead paint, drinking water, and soil (Njati and Maguta, 2019; Wilson et al., 2022). Pb accumulates in bone, likely due to its similar atomic properties to calcium. During pregnancy, Pb harbored in bone can be released into the bloodstream due to normal maternal bone remodeling, which results in elevated maternal Pb concentrations and consequently increased fetal exposure (reviewed in Goyer, 1996). Hypothesized mechanisms for Pb induced neurotoxicity include oxidative stress and neuronal cell death (Nemsadze et al., 2009; Ramírez Ortega et al., 2021). A study of Chinese pre-school students also found that electronic waste Pb exposure may decrease serum Neuropeptide Y, and that Neuropeptide Y may mediate a positive association between blood Pb level and behavioral deficits. However, this study was cross-sectional and thus could not establish causality (Zeng et al., 2021).

In the U.S., a birth cohort study found a positive association between prenatal Pb exposure (via umbilical cord blood) and BASC-2 anxiety score in 15-year-old adolescents, indicating increased anxiety symptoms (Rokoff et al., 2022). The study found a positive association between prenatal Pb and BASC-2 psychosomatic scores, with a stronger effect in males (Rokoff et al., 2022). A Korean birth cohort study found sex differences as well, with higher prenatal blood Pb levels increasing total CBCL behavior scores more in 5-year-old males, and higher childhood blood Pb levels (measured at ages 2,3, and 5) increasing total CBCL behavior scores more in 5-year-old females (Joo et al., 2018). When analyzing deciduous teeth to estimate developmental Pb exposure, a birth cohort study in Mexico associated increased dentine Pb at 12 months old with increased BASC-2 anxiety symptoms at 8–11 years (Horton et al., 2018).

Only one cohort study, conducted in New Zealand, reported results on early life Pb exposure and adult mental health (up to age 38 years) (Reuben et al., 2019). The study found that higher childhood Pb exposure, as determined by Pb blood concentrations measured at 11 years of age, was associated with increased internalizing symptoms and increased general psychopathology. However, this is a single study conducted in the 1970s, so these results may not be generalizable and should be replicated in other populations. In total, Pb is a recognized neurotoxicant. In addition to established effects like reduced intelligent quotient scores (IQ) (Lanphear et al., 2005; McFarland et al., 2022), these recent epidemiological studies also suggest that increased Pb exposure are correlated to negative mental health consequences in children.

3.2.2 Manganese

Manganese (Mn) is essential for brain function, but neurotoxic in excess. Children can be exposed to Mn prenatally (i.e., maternal blood), and via air pollution, food, and drinking water (Krachler et al., 1999; Rahman et al., 2017; Rodrigues et al., 2018). Inhaled Mn can cross the blood brain barrier, accumulate in the brain, and alter synaptic mechanisms (Davis, 1999; Aschner and Dorman, 2006) Mn has also been shown to impair astrocyte function, disrupt myelination, and disrupt dopamine neurotransmission (Normandin and Hazell, 2002). Using functional magnetic resonance imaging (fMRI), a birth cohort study in Mexico found that higher levels of Mn in maternal blood were associated with reduced functional connectivity of brain areas involved in emotional processing and regulation in 6- to 7-year-old children (de Water et al., 2018).

A cross-sectional study in Brazil assessed behavior in 7- to 12-year-old children residing near a ferro-manganese alloy plant, which resulted in increased exposure to airborne Mn (Rodrigues et al., 2018). The team used toenail clippings to assess long-term (7–12 month) Mn exposure and found significant associations between increased Mn exposure and increased total SDQ behavioral and externalizing behavior scores; this indicates that high Mn correlated to worsening behavioral symptoms. In Bangladesh, Rahman et al. (2017) found increased prenatal Mn exposure from drinking water increased the risk of conduct problems in 10-year-old children, as measure by the SDQ. Both studies saw more pronounced effects for externalizing behavior symptoms in boys (Rahman et al., 2017; Rodrigues et al., 2018). Whereas in the U.S., Rokoff et al. (2022) observed a positive association between prenatal Mn exposure, measured in umbilical cord blood, and internalizing symptoms in girls, at ages 8 years (measured by the Conners' Rating Scale) and 15 years (measured by BASC-2), but saw null results in boys.

Like Pb, Mn is a well-recognized neurotoxicant. A large portion of the Mn literature focuses on adult neurotoxicity, as Mn excess can cause a Parkinson-like syndrome, including memory and motor deficits (Kwakye et al., 2015). The few studies presented here suggests that Mn excess may also contribute to mental health and behavioral problems in children. Further studies examining these neurological effects during development are warranted.

3.2.3 Copper

Copper (Cu) is an essential mineral for brain development but neurotoxic at high concentrations. Children can be exposed to Cu during gestation, and from sources such as drinking water (e.g., Cu pipes), food cooked on uncoated Cu cookware, Cu rich food, and smoke from burning Cu sulfate (Amorós et al., 2019; Royer and Sharman, 2023). Cu exposure may increase the release of proinflammatory cytokines and damage the structure of brain

mitochondria, resulting in neurotoxicity (Kitazawa et al., 2016; Borchard et al., 2018). A cross-sectional European study found increased Cu in blood samples, was associated with higher CBCL internalizing symptoms in 6- to-11-year-old children (Maitre et al., 2021). Another European study found prenatal blood Cu levels to be negatively associated with SDQ externalizing scores in children ages 3–7 years old, suggesting decreased risk (Jedynak et al., 2021). The latter study noted their results should be interpreted with caution because there aren't enough studies on Cu exposure and externalizing problems in children.

3.2.4 Mercury

Elemental, organic, and inorganic forms of mercury (Hg) are neurotoxic to children and have been linked to neurodevelopmental and neurocognitive disorders (reviewed in Al-Osman et al., 2019). Hg bioaccumulates in the food chain, especially seafood, which is a main source of exposure for children and pregnant people (Bose-O'Reilly et al., 2010). One study explored the effects of Hg on behavioral health and found an association between increased maternal blood Hg levels and higher CBCL internalizing and externalizing scores in Korean toddlers aged 1–2 years (Kim et al., 2018). While this suggests that Hg may be implicated in abnormal internalizing and externalizing behaviors, there is a paucity of data. Other studies are needed to ascertain reproducibility across cohorts.

3.3 Endocrine-disrupting chemicals

Endocrine-disrupting chemicals (EDCs) are xenobiotics that mimic or inhibit hormones, and can lead to abnormal hormone signaling in tissues (Shoaff et al., 2019). EDCs are widely used in a myriad of products including, but not limited to, plastic containers, food packaging, personal care products, medical supplies, and building materials.

3.3.1 Phthalates

High molecular weight phthalates are primarily used as plasticizers to increase flexibility and durability of plastics, while low molecular weight phthalates are used in cosmetics and pharmaceuticals (Chen et al., 2019). Phthalates easily leach into the environment and routes of exposure to children include inhalation, ingestion, and dermal absorption (Braun et al., 2013). Phthalates can also cross the placenta, resulting in prenatal exposure (Qian et al., 2020). Possible neurotoxic mechanisms for phthalates include altering steroid hormone concentrations, lipid metabolism, and disrupting neurotransmitters involved in the release of dopamine (Huang et al., 2019). In Canada, England-Mason et al. (2020) used MRI to examine brain white matter in pre-school children, and also conducted behavioral assessments. The authors found by neuroimaging and statistical modeling that increased prenatal exposure to high molecular weight phthalates was associated with changes in white matter microstructure in children (England-Mason et al., 2020). Specifically, increased mean diffusivity in brain regions that control affective function (i.e., mood) and perceptual processing. Further, the authors found indirect associations with increasing phthalate exposure during pregnancy and increased CBCL internalizing and externalizing scores. Together, these data suggest that the behavioral observations could be mediated by pthalate-induced structural changes in brain white matter (England-Mason et al., 2020).

In addition to the aforementioned study, several observational studies have also identified that increased phthalate exposure is correlated to behavioral outcomes. A U.S. birth cohort study found borderline statistical significance between increased prenatal exposure to multiple phthalates and temperament at 12- and 24-months (Singer et al., 2017). A Korean birth cohort study found a positive association between prenatal monoethyl phthalate (MEP) and CBCL internalizing scores in toddlers (Kim et al., 2018). In Taiwan, a birth cohort study assessed prenatal exposure to di (2-ethylhexyl) phthalate (DEHP) in 8-, 10-, and 14-year-old children (Chen et al., 2019). The authors found significant positive associations between prenatal urinary DEHP concentrations and increased CBCL scores in all of the test's categories, except for somatic complaints. This indicates that children with higher phthalate exposure had behavioral problems in the following categories: withdrawn, anxious/depressed, social problems, thought problems, attention problems, delinquent behavior, aggressive behavior, internalizing problems, and externalizing problems. Children with higher DEHP exposure also had consistently higher overall CBCL scores (Chen et al., 2019). The authors noted that the median exposure quantified in their study was 4.54 μg/kg bw/day. This, in addition to previous epidemiological studies where DEHP exposure was associated with worsening behavioral symptoms, is well below the reference levels currently recommended by the EU and U.S., which is 50 and 20 μg/kg bw/day, respectively (Chen et al., 2019).

Another Taiwanese birth cohort study found associations between increasing maternal urinary DEHP metabolites and higher CBCL delinquent behavior and externalizing scores and increasing maternal urinary mono-2-ethylhexyl phthalate (MEHP) and higher CBCL internalizing and externalizing scores in children aged 8–14 years (Huang et al., 2019). The researchers also found a positive association between urinary monobenzyl phthalate (MBzP) at ages 2–8 years and CBCL scores for social problems at the age of 8–14 years (Huang et al., 2019). Colicino et al. (2021), studied di-2-ethylhexyl terephthalate (DEHT), a replacement for DEHP marketed as a less toxic alternative. In this Mexican birth cohort study, the authors linked higher maternal urinary DEHT levels with increased overall BASC-2 behavioral and depression scores in 4- to 6-year-old boys.

A French birth cohort study also reported that increased maternal urinary mono-n-butyl phthalate (MnBP) and MBzP during pregnancy were associated with increased SDQ internalizing behaviors in 3-year-old boys, however the incidence rate ratios were close to the null (Philippat et al., 2017). Jedynak et al. (2021) found prenatal urinary MnBP was associated with increased SDQ externalizing problems in 3-to7-year-old European children. In a cross-sectional design, Shoaff et al. (2019) collected spot urine from 15-year-old adolescents living near a Superfund site in the United States and assessed behavior using the BASC-2. The researchers observed a positive association between a sum of 11 antiandrogenic phthalate metabolites and increased maladaptive behaviors, such as externalizing behavior and developmental social disorders (Shoaff et al., 2019). Together, these data suggest that pthalate exposure during pregnancy is

associated with a wide range of increased behavior problems in children, with ages ranging from infancy to adolescence.

In all, multiple studies conducted worldwide suggest that developmental phthalate exposure is correlated to behavioral deficits in children. Systematic reviews also suggest that increased developmental exposure to some phthalates is correlated with cognitive and psychomotor impairments, in addition to the behavioral issues reviewed here (Ejaredar et al., 2015; Zhang et al., 2019). While the etiology of these observations is unknown, phthalates are a diverse family of compounds with many metabolites. Additional research should be conducted to test the reproducibility of the epidemiological evidence cited herein.

3.3.2 Polybrominated diphenyl ethers

Polybrominated diphenyl ethers (PBDEs) are flame retardants present in a variety consumer goods and materials, such as furniture, electronics, and car seats (Costa and Giordano, 2007). Children can inhale or ingest PBDEs from contaminated dust, as well as ingest these compounds from food (i.e., fatty fish) and breastmilk (Domingo, 2012; Malliari and Kalantzi, 2017). Data suggests that PBDE's could negatively impact neurobehavior via several mechanisms including disrupting thyroid hormone action, inducing oxidative stress, altering brain protein expression, increasing neuronal apoptosis, altering cholinergic system responses, and disturbing neurotransmitter function (Costa and Giordano, 2007; Vuong et al., 2018; Strawn et al., 2022). Early life exposure to PBDEs, which can accumulate in tissues and cross the placenta, has been shown to negatively impact cognition and behavior in children (Costa and Giordano, 2007; Lam et al., 2017; Gibson et al., 2018; Strawn et al., 2022).

Three papers utilized data obtained from the Health Outcomes and Measures of the Environment (HOME) study, an ongoing U.S.-based birth cohort that assesses the health impact of early childhood exposures to environmental toxicants. Strawn et al. (2022) examined prenatal serum PBDE concentrations and found significant associations with increased anxiety symptoms in 12-year-olds, measured by the Screen for Child Anxiety Related Emotional Disorders. The strongest effects were observed for panic and separation anxiety (Strawn et al., 2022). Braun et al. (2017) found that higher prenatal serum BDE-47 exposure was associated with persistent increases in externalizing behaviors from ages 2 to 8, as quantified by BASC-2 scores. Vuong et al. (2017) assessed serum PBDE concentrations in 8-year-old children and saw a positive association between multiple PBDE congeners (BDE-28, BDE-47, BDE-153, ∑PBDEs) and externalizing symptoms. While these three papers provide great preliminary data, more research from different cohorts can help elucidate the relationship between BPDEs and adverse mental health symptoms.

3.3.3 Bisphenols

A few studies investigated bisphenols, such as bisphenol A (BPA), an estrogenic polymer that is used to produce plastics (Ben-Jonathan and Steinmetz, 1998). In addition to prenatal exposure, children can be exposed to bisphenols from canned and packaged food, plastic baby and beverage bottles, and breast milk (Lakind and Naiman, 2011). Research suggests that BPA may disrupt the brain's stress system (hypothalamic pituitary adrenal axis) and thus can increase symptoms of stress-related outcomes, such as anxiety and depression (Wiersielis et al., 2020). Several studies from years earlier than 2017 have linked prenatal and childhood BPA exposure to adverse behavior, anxiety, and depression (reviewed in Wiersielis et al., 2020).

In a China-based case-control study, researchers found that serum bisphenol AF exposure, a common BPA replacement, was associated with increased depressive symptoms in 7th grade students, with males being significantly more vulnerable than females (Zhang et al., 2022). A U.S. birth cohort observed a positive association between increased prenatal urinary BPA concentrations and greater BASC-2 externalizing scores in girls ages 2 through 8 years, but not boys (Braun et al., 2017). Using the HOME study cohort, Stacy et al. (2017) collected urinary BPA from pregnant people and children from the 2nd trimester through 8 years of age. The authors found that higher prenatal BPA concentrations were correlated with increased BASC-2 externalizing behavior in girls at 8 years of age. This same effect was not observed in boys. In contrast, higher BPA urinary concentrations was significantly associated with increasing externalizing behavior in boys, when both BPA and behavior were measured at 8-years of age. These studies suggest that BPA may elicit both temporal and sex-specific effects, similar to previous reports (Braun et al., 2009; Perera et al., 2016).

In the U.S., Shoaff et al. (2019) explored the cross-sectional relationship between 7 phenols (including bisphenol A, F, and S) in spot urine samples and adverse behavior in 15-year-old adolescents but produced null results. Another U.S. study observed an inverse association, indicating improved scores, between newborn BPA concentrations measured from dried blood spots and difficulties in SDQ prosocial behavior at 7 years old; however, this association was not significant when BPA concentrations were categorized in quartiles, making interpretation of these data difficult (Ghassabian et al., 2018). Jedynak et al. (2021) determined that increasing prenatal urinary BPA levels were associated with increased SDQ externalizing behaviors in European children ages 3–7 years. Assessing behavior in males with the SDQ in France, Philippat et al. (2017) found prenatal urinary BPA exposure was associated with increased internalizing behavior at 3 years old and increased externalizing behavior at 5 years old, as well as a positive association between triclosan and externalizing behavior at 3 years old.

Collectively, the bisphenol literature reviewed shows that abnormalities in externalizing and internalizing behaviors in children can be correlated to increased bisphenol exposure during pregnancy or childhood. However, the results of epidemiological studies correlate to factors such as the sex of the child and when bisphenols were measured. Additional studies are needed to confirm these observations, ideally with cohorts large enough in scale to permit stratification by sex and with bisphenol exposure measured sequentially at multiple times during development.

3.3.4 Per- and polyfluoroalkyl substances

Per- and polyfluoroalkyl substances (PFAS) are a chemical class with several thousand different congeners, which are widely used in various consumer products from cookware to clothing (Glüge et al., 2020). Many PFAS are persistent in the environment and in humans and have been categorized as persistent organic pollutants (Baker and Knappe, 2022; Starnes et al., 2022). PFAS can be detected in the

placenta, amniotic fluid, maternal and neonatal blood, and breastmilk, demonstrating that pregnant people and children have some body burden of PFAS (Vuong et al., 2021). These organohalogens can elicit endocrine disruption in animal models and may be neurotoxic via disruption of the thyroid system (Mariussen, 2012). PFAS may also induce oxidative stress in the brain, and alter dopaminergic signaling pathways (Mariussen, 2012; Salgado et al., 2016).

A U.S. prospective birth cohort study found that prenatal serum perfluorooctane sulfonic acid (PFOS), perfluorohexane sulfonic acid (PFHxS), and perfluorononanoic acid (PFNA) were associated with increased odds of externalizing behaviors, and PFHxS was associated with increased internalizing problems at ages 5 and 7 years (Vuong et al., 2021). Another U.S. prospective birth cohort study found that higher PFOS concentrations in dried blood spot from newborns was associated with increased odds of conduct and emotional problems at 7 years of age (Ghassabian et al., 2018). In a Danish prospective birth cohort, authors observed a positive relationship between prenatal plasma perfluorononanoic acid (PFNA) concentrations and externalizing behaviors at ages 7 and 11 years, as detected by the SDQ (Luo et al., 2020). Overall, the epidemiological studies reviewed here suggests that a developmental PFAS exposure may correlate to later abnormalities in both internalizing and externalizing behaviors. However, there are relatively few studies that examine the mental health consequences of PFAS exposure in children and adolescents. Given that ubiquity of PFAS exposure and their biological persistence, this relationship should be explored in future studies.

3.3.5 Pesticides

Pesticides prevent, repel, and kill pests, and are integral to agriculture. Sources of pesticide exposure to children and pregnant people include contaminated food, water, indoor/outdoor air, household dust, and treated lawns (Roberts et al., 2012). Children who live in households close to agricultural sites, have household members working in agriculture, or who work in agriculture themselves, likely have higher exposure to various pesticides compared to the general population (Fenske et al., 2000; Lu et al., 2000).

Organochlorine pesticides, such as dichlorodiphenyltrichloroethane (DDT), were used heavily worldwide in the 1940s through 1960s. While they have been phased out of use in the U.S., organochlorines are still utilized in South America, Africa, and Asia for vector (i.e., mosquito) control. Nonetheless, these chemicals are persistent in the environment, and bioaccumulate in human tissue due to their lipophilic properties. They also likely cross the placenta, suggesting fetal exposure (Rosenquist et al., 2017). It is hypothesized that organochlorines dysregulate dopamine-mediated functions in animals, which is crucial to the limbic system, or the part of the brain that controls behavior and emotion (Rokoff et al., 2022). In Greenland and the Ukraine, Rosenquist et al. (2017) saw a positive association between a doubling of pre- and postnatal serum exposure to 1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethylene (*p,p′*-DDE), a metabolite of DDT, and odds of conduct problems in 5- and 7-year-olds. Rokoff et al. (2022) also looked at pre- and post-natal *p,p′*-DDE exposure and the effect on anxiety in 8- and 15-year-olds and found no association.

Organophosphate pesticides are applied widely in agriculture and work by inhibiting acetylcholinesterase (AChE) activity in insects. However, organophosphates also inhibit AChE activity in other species, and the cholinergic system plays a key role in mood regulation (Suarez-Lopez et al., 2021). A prospective cohort study in an Ecuadorian agricultural community saw an increase in adolescent (11–17 years) depression symptoms as AChE activity decreased, a relationship that was strongest at older ages and among female participants (Suarez-Lopez et al., 2021). The researchers did not detect any associations between AChE inhibition and anxiety symptoms (Suarez-Lopez et al., 2021).

Pyrethroid pesticides are widely utilized in homes, gardens, and agriculture; they are also used on pets and clothing to prevent ticks, and as vector control (Richardson et al., 2019). Pyrethroids can cross the blood-brain barrier and may alter various neurotransmitter systems and cause oxidative stress (Nasuti et al., 2007; Richardson et al., 2019). A longitudinal birth cohort study in the United States assessed prenatal exposure to pyrethroids and child behavior at ages at 4, 6, and 7–9 years (Furlong et al., 2017). Even with low detection frequencies, researchers found a positive association between detectable urinary levels of 3-phenoyxbenzoic acid (3-PBA) and cis-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis-DCCA) with increased BASC-2 internalizing and externalizing behaviors respectively. However, researchers advised interpreting the results with caution because exposure was categorized only as "detect" or "non-detect," and not quantified. In all, the potential neurotoxicity of pesticides in humans is a known concern. However, there are few studies that specifically investigate if pesticide exposure is related to mental health concerns in children.

4 Discussion

The purpose of this review was to identify and summarize recent literature on environmental xenobiotic exposures and child mental health outcomes, specifically symptoms related to mood, anxiety, and behavioral disorders. The 29 studies included reveal that there is a growing body of literature demonstrating a potential relationship between increased exposure to pollutants like heavy metals, endocrine disrupting chemicals, and pesticides, with increased adverse mental health outcomes in children.

None of the studies in this review assessed the potential impact of environmental injustice. Low income and/or children of color living in communities disproportionately exposed to environmental pollution, often referred as "environmental justice," or "overburdened" communities, may have increased vulnerability to the impact of chemical exposures on mental health outcomes. This may be due to increased chemical exposure(s), and/or an increased susceptibility to such environmental exposures due to additional psychological and physical stressors. For example, children in environmental justice communities may be exposed to higher levels of traumatic experiences, such as racial discrimination and environmental disasters (Jones Harden and Slopen, 2022). Furthermore, these communities may have less access to protective factors, such as greenspaces, healthy foods, quality healthcare and other neighborhood amenities (Reuben et al., 2022). It's essential to understand the degree to which children in environmental justice communities are at increased risk and to what extent chemical exposures contribute to adverse physical health and mental health outcomes.

Potential opportunities to fill data gaps include conducting community based participatory research (CBPR) studies in communities where children and pregnant people have disproportionate exposure to chemically polluted sites (i.e., waste and wastewater facilities, industrial farming, metal working facilities, manufacturers, and oil and gas refineries), and/or xenobiotic exposure from sources like contaminated seafood, lead paint, unsafe water, and certain personal care products.

Along these lines, more research is needed to understand the cumulative impact of prenatal and childhood exposure to chemical and non-chemical stressors (i.e., psychosocial stress) on mental health outcomes, as well as the potential impact of protective factors, For example, Maitre et al. (2021) used an exposome approach to assess chemical and non-chemical environmental stressors and found longer sleep duration, higher family social capital, and a healthy diet during childhood to be protective against behavioral symptoms. Mental health outcomes could also be included in policy and regulatory cumulative impact/risk frameworks, especially given their potential economic burden (Torio et al., 2015; Trautmann et al., 2016; Tkacz and Brady, 2021).

Furthermore, a chemical exposure rarely occurs in isolation, and instead, complex mixtures more accurately represent a human exposure scenario. Two studies included in this review explored chemical mixtures. Horton et al. (2018) found a metal mixture of manganese, zinc and lead was associated with increased anxiety symptoms, with the mixture driven by Mn at 0–8 months and Pb at 8–12 months. When looking at prenatal exposure to a mixture of organochlorines and metals in 8- and 15-year-olds, Rokoff et al. (2022) saw null results. In addition, there have been other recent studies exploring the relationship between chemical mixtures and mental health related outcomes in children (Cowell et al., 2021; de Water et al., 2022). More research to understand chemical interactions in humans is critical, especially during pregnancy and development (Kim et al., 2018).

Hypothesized neurotoxic mechanisms that can translate to adverse mental health outcomes are discussed for each chemical throughout this review. However, only a few studies explored potential mechanisms. There is a need for mechanistic studies to confirm these hypotheses and preliminary results, including additional fMRI and MRI studies showing which regions of the brain may be affected by environmental exposures, as well as hypothesis-driven animal studies. It is difficult for prospective cohort studies to show causation between a chemical exposure and a health effect. As such, if the observations identified in these human studies could be repeated and further explored in an animal model, it would add further weight of evidence linking an exposure to a mental health outcome. In addition, 8 studies in this review saw statistically significant effects, but only in one sex. Future work is also needed to understand sex differences in neurological development, to help explain sex-specific outcomes.

Most studies included in this review are cohorts comprised of mother-child pairs; this is crucial, as it is well established that fetal and early postnatal development is a period of vulnerability to environmental exposures (Bellinger, 2013). However, some of the data reviewed suggest that chemical exposures during later childhood is also linked to mental health outcomes. Therefore, additional longitudinal birth cohort studies would allow for multiple exposure assessments during pregnancy, infancy, and childhood, providing a unique opportunity to explore windows of vulnerability to chemical exposures. Many mental health disorders are not diagnosed until adolescence (Solmi et al., 2022), thus evaluating children in epidemiological studies during this developmental period, instead of infancy and early childhood, may reveal stronger associations between a chemical exposure and neurological health.

Many of the papers in this review controlled for population variables like sociodemographic factors, birth conditions, and family history of mental illness. Future areas of study could explore potential interactions between these risk factors and chemical exposures of interest. And finally, existing national surveys could be expanded to help fill data gaps. For example, the National Survey of Children's Health currently includes a mental health questionnaire but lacks any environmental exposure data. While an exposure would not be quantified due to the lack of biological sampling, researchers could infer exposure risk based on a participant's geographical location if this information was made available. In all, recruiting large cohorts of mother-child dyads for extended observational studies, and leveraging existing tools for environmental health research, could further this important area of study.

5 Conclusion

Understanding the impact of environmental chemicals on children's mental health is critical to promotion of health, wellbeing, and the prevention of disease (Thoma et al., 2021; Reuben et al., 2022). Due to the complex nature of both environmental xenobiotic exposures and mental health outcomes, it is difficult to establish causality with epidemiology studies alone. Unfortunately, the existing data are too limited to formally determine how individual chemicals may influence the development of specific mental health outcomes, though research in this area are steadily growing. With increased studies, this could permit data synthesis through tools like systematic review and metanalysis, and lead to sound scientific conclusions. Not only could this better inform policy and regulations to minimize any potential adverse effects of environmental chemicals, but it could also lead to informed risk management decisions for individuals, communities, and medical professionals. Given the burden of mental health disorders on children's health, wellbeing, and overall life trajectory, it is essential to identify and take action to address the environmental risks that may increase the development of these disorders.

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Author contributions

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Conflict of interest

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Iseult Lynch,
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Izharul Haq,
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University of Ibadan, Nigeria
Azubuike Chukwuka,
National Environmental Standards and regulations Enforcement Agency (NESREA), Nigeria

*CORRESPONDENCE
Michael Ovbare Akharame,
michael.akharame@uniben.edu

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Phenolic compounds occurrence and human health risk assessment in potable and treated waters in Western Cape, South Africa

Nkosiyenzile Londiwe Mhlongo[1], Michael Ovbare Akharame[2]*, Omoniyi Pereao[1], Izanne Susan Human[1] and Beatrice Olutoyin Opeolu[3]

[1]Department of Environmental and Occupational Studies, Cape Peninsula University of Technology, Cape Town, South Africa, [2]Department of Environmental Management and Toxicology, University of Benin, Benin-City, Nigeria, [3]Environmental Chemistry and Toxicology Research Group, Cape Peninsula University of Technology, Cape Town, South Africa

Phenolic pollutants from industrial and agricultural activities pose a major threat to the world's potable water supply. The persistent micro-pollutants often find their way into drinking water sources with possible adverse human health implications. In this study, bottled water, tap water, and wastewater treatment plant (WWTP) effluent samples from the Boland region of the Western Cape, South Africa were assessed to determine 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP) levels using HPLC/DAD instrumentation. The selected area is known for its vast agricultural ventures and wineries. Evaluation of the human health risk (cancer risk) for the pollutants was conducted using the hazard quotient (HQ). The Ames mutagenicity test was also conducted using the *Salmonella typhimurium* T98 and T100 strains and the S9 activation enzyme. Trace levels of the phenolics were detected in the samples with a range of 9.32×10^{-7}—1.15×10^{-4} mg/L obtained for 4-CP, and 8.80×10^{-7}—1.72×10^{-4} mg/L recorded for 2,4-DCP. Both compounds had levels below the limit of 0.01 mg/L prescribed by South African legislation. The assessed HQ for the phenolic concentrations indicates a low level of potential ecological risk and none of the samples had a cancer risk value that exceeded the regulatory limit. The possibility of the analyzed samples causing cancer is unlikely, but non-carcinogenic adverse effects were found. Strong mutagenicity was observed for the T98 strains with a potential ability to cause mutation toward the insertion or deletion of a nucleotide. The T100 bacterial strain showed very slight mutagenicity potential, however, it is unlikely to cause any mutation. The levels of phenolics in the potable water samples may pose a significant threat to human health. Hence, screening persistent organic chemicals in potable water sources and evaluating their potential human health effects is pertinent to prevent associated health challenges.

KEYWORDS

potable water, wastewater, 4-chlorophenol, 2,4-dichlorophenol, mutagenicity, risk assessment, human health risk

1 Introduction

Securing a safe, economical, accessible, and reliable water supply is currently being threatened by the anthropogenic deposition of organic chemicals and micro-pollutants in the different environmental matrices (Akharame et al., 2022). The deposition of these organic compounds in surface waters and underground aquifers poses challenges to the availability of potable water resources. Industries and agricultural ventures are the major culprits responsible for the organic chemicals and micro-pollutants proliferation in the environment. Many industrial facilities and municipalities operate wastewater treatment plants (WWTPs) as a regulatory measure to mitigate the presence of various contaminants in their effluent discharges (Akharame and Ogbebor, 2023). However, the presence of some chemicals and micro-pollutants in the influent streams poses a serious challenge to the treatment capabilities of WWTPs.

The presence of organic chemicals and micro-pollutants is a serious barrier faced by treatment plants, as pollutants reduce the adequacy of treated wastewater to be discharged into water bodies or used as a source of drinking water. Literature reports suggest that the traditional wastewater treatment solutions available in South Africa are largely insufficient to treat wastewater before discharge or reuse (Afolabi et al., 2018; Edokpayi et al., 2018; Olabode et al., 2020; Pereao et al., 2021). Hence, many municipalities and provinces in South Africa have taken the initiative to upgrade some WWTPs to new membrane bioreactor systems which may provide an improved capability to treat the influent (Zhang et al., 2021). Despite the laudable drive to enhance the effluent treatment processes and capabilities in the country, there is the possibility that the persistent organic chemicals can still find their way into drinking water sources which may pose a threat to human health. Phenols and their derivatives are examples of persistent organic chemicals currently regarded as priority pollutants due to their increasing presence in water sources and their potential to cause adverse health effects (Bakthavatsalam, 2019). Ingestion of water with high levels of phenolics can potentially cause unintentional muscle contractions (muscle tremors), walking abnormalities, and death (ATSDR, 2008); more so, they possess endocrine-disrupting characteristics (Alshabib and Onaizi, 2019; Bakthavatsalam, 2019). Levels of phenolics have been reported in environmental media such as the atmosphere, sediments, plants, animals, and human bodies (Lv et al., 2019; Li et al., 2020; Liu and Mabury, 2020; Mykhailenko et al., 2020; Wang et al., 2020; Sun et al., 2022). Particularly, phenolics have been detected in drinking water, rainwater, groundwater, surface waters, urban runoff, as well as industrial effluents (ATSDR, 2008). Phenolic compound deposition in the environmental matrices is aggravated by the production and application of numerous pesticides and the generation of municipal and industrial effluents (Raza et al., 2019; Krithiga et al., 2022; Okoro et al., 2022).

Consequently, this study is focused on two phenolic compounds - 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP). The 4-CP contaminant has been reported to possess carcinogenic and mutagenic properties (Haddadi and Shavandi, 2013; Hidalgo et al., 2013; Lim et al., 2013), and the European Union Directive classifies it as a dangerous substance (Orejuela and Silva, 2002). It is largely present in many agro-chemicals used in commercial farming and is a by-product formed from winery operations which are the main economic activities in the research area. For 2,4-DCP, is listed as part of the eleven phenols deemed as critical contaminants by the United States Environmental Protection Agency (Mohd, 2022). The 2,4-DCP compound is used in copious quantities in the manufacture of certain herbicides and preservatives, such as 2,4-dichlorophenoxyacetic acid and pentachlorophenol (Estevinho et al., 2007; Moszczyński and Białek, 2012; Kuśmierek et al., 2016), and it is also a by-product formed during the chlorination process utilized for water disinfection. The ubiquitous occurrences of chlorinated phenolic compounds in the various environmental matrices elicited the motivation for the investigation. Additionally, our research team has carried out several investigations targeting phenolic compounds due to their endocrine-disrupting potentials. This includes investigating their degradation route during ozonation processes (Oputu et al., 2019; Akharame et al., 2020), and proffering remediation measures using heterogeneous catalytic approaches (Oputu et al., 2015a; Oputu et al., 2015b; Akharame et al., 2022). Phenolic compounds' environmental contamination pathways include the manufacturing of preservatives, pulp and paper, pesticides and dyes, wines, and other phenol-based compounds (Xu et al., 2017; Raza et al., 2019; Yahaya et al., 2019). The compounds pose threatening human health issues due to their quantum of industrial and agricultural usage which has given rise to accumulations in the environment. The levels of 4-CP and 2,4-DCP were assessed in the bottled water, tap water, and wastewater treatment plant (WWTP) effluent samples from the Boland region of the Western Cape, South Africa. A correlation with the human health hazard quotient was created to assess the overall carcinogenic health effect of the water samples. The Ames mutagenicity test was conducted to determine whether the brands of bottled water, tap water, and WWTP effluent could potentially cause a mutagen. Screening of persistent organic chemicals in potable water sources and evaluating their potential human health effects is pertinent to prevent associated health challenges.

2 Materials and methods

2.1 Chemical and standards

The phenolic compounds 4-CP (99%), 2,4-DCP (99%), HPLC grade acetonitrile (>98%), Supelco C18-E cartridges (500 mg/12 mL of adsorbent) were obtained from Sigma Aldrich (South Africa). Milli-Q water-18 Ω (Milli-Q Academic, France) was utilized for all the analytical work.

2.2 Sampling and extraction procedure of phenols

The study was conducted in Cape Town and the surrounding Boland region of South Africa. The selected area is known for its agricultural processes and wineries. The water samples were collected in the autumn and winter months following standard procedures (APHA, 1998). The bottled water (four brands) was purchased from grocery stores, the tap water samples were collected from a University campus, and the influent and effluent samples were taken from a WWTP (a membrane bioreactor system) located

in the region. The water samples were collected using sterile 500 mL amber bottles. The samples were kept in an ice chest for preservation en route to the laboratory. The samples from the WWTP were filtered by using a 0.22 µm polyethersulphone membrane syringe filter and then refrigerated at 4°C.

The extraction procedure for the phenolics was done following the method previously described by (Olujimi et al., 2011). The Sulpeco C18-E cartridges were conditioned with 8.5 mL *n*-hexane: acetone (50:50 v/v), 8.5 mL methanol, and 15 mL Milli-Q water, respectively. Adjustment of the water samples' pH to 2.5 was done using hydrochloric acid before filtering through the conditioned cartridges. This was followed by channeling 5 mL of Milli-Q water through the cartridges and holding it under a vacuum for 30 min to dry (−70 kPa). The analyte elution from the cartridges was achieved by using 3.5 mL of methanol, and 3.5 mL of n-hexane: acetone (50:50 v/v) in a glass flask, respectively. Thereafter, the drying process with a gentle stream of nitrogen was effected, and aliquots from the solution were analyzed with HPLC instrumentation. The concentrations of the assayed analytes were determined using external calibration standards.

2.3 HPLC instrumentation and chromatographic conditions

The separation and identification of the compounds was done using the HPLC instrumentation (Waters Corporations, United States). The instrument setup consists of a terminal solvent delivery system (Waters 1525 binary HPLC pump), an autosampler (Waters 2707 auto-sampler), photodiode array detector (Waters 2487 dual λ absorbance detector), using the Breeze software™ as the analytical software. An Ace 5 C18 column (150 × 4.6 mm i. d) was utilized to achieve the separation of the compounds, with the elution done using optimized binary gradients (SI Table 1). Milli-Q water ("A") and acetonitrile ("B") were the mobile phases operated at a flow rate of 1 mL/min for the chromatographic separations, while the detection of the compounds was at 280 nm. Pre-conditioning of the chromatographic system was effected by a continuous flow of the solvents for 30 min to obtain a stable baseline signal. Thereafter, the assays were done by the injection of 20 mL of the analytes and standards at an operating temperature of 25°C. The retention time values and the UV-spectral of the target analytes were used for the compound identification.

2.4 Human health risk assessment

2.4.1 Cancer risk assessment for 4-CP and 2,4-DCP exposure

The United States Environmental Protection Agency (USEPA) health risk assessment equations used for estimating exposure to phenols were adopted for this study (Rand and Mabury, 2017; Okoro et al., 2022). The cancer risk assessment was evaluated with the average daily dose (ADD) and hazard quotient (HQ) using Eqs. 1–3. The values used for the exposure calculation are presented in Supplementary Table S2.

TABLE 1 Composition of S9 mix.

Constituent	Volume (mL)
S9A: $MgCl_2$ + KCl solution	0.96
S9B: Glucose-6-phosphate	0.22
S9C: NADP	1.94
S9D: Phosphate buffer	23.96
S9E: Sterile water	20.32
S9F: S9 fraction (hydrate with 2.1 mL of sterile H_2O)	0.60
Total	**48.00**

$$ADD = (IR^{*}C^{*}EF^{*}ED)/(BW^{*}AT) \quad (1)$$

Where IR = Ingestion Rate, C= Concentration, EF = Exposure Frequency, ED = Exposure Duration, BW = Body Weight, AT = Averaging Time (Life Expectancy)

$$Hazard\,Quotient\ (HQ) = (ADD)/(RfD) \quad (2)$$

Where RfD is the Reference Dose (IRIS-USEPA)

$$Cancer\ risk = SF^{*}ADD \quad (3)$$

where SF is the slope factor.

2.4.2 Mutagenicity testing (Ames test)

The mutagenicity assay (Ames test) was carried out using 'Muta-ChromoPlate™ Bacterial Strain Kit with S9 Activation TM Version 2.1' for 6 days. The testing followed the 96-well microplates protocol of the *Salmonella typhimurium* test developed for investigating mutagenic constituents in soluble extracts from different environmental matrices (air, water, and land or sediment), food components, chemicals, and cosmetics (Ames et al., 1975). The following reagents in their required measures were utilized: Davis-Mingoli salts 22 mL (A), D-glucose 10 mL (B), bromocresol purple 7 mL (C), D-biotin 4 mL (D), L-histidine 200 mL (WP2 strains substitute L-tryptophan, 100 µL) (E), sterile distilled water, 120 mL (F), growth medium 5 mL (G), and ampicillin 100 mL (V). The composition of the S9 mix is shown in Table 1. In preparing the S9 mix, the constituents S9A to S9E were added in the reverse order before the addition of the supernatant of a liver homogenate (S9F); the addition sequence is to ensure that the S9 fraction is added to a buffered solution (Maron and Ames, 1983). The prepared S9 mix was immediately kept on ice to prevent loss of activity.

2.4.2.1 Lyophilized test strains and standard mutagens

The lyophilized test strains and standard mutagen assays were composed of T98, T100, NaN_3, 110 mL—for use with TA 100, 2-nitro fluorene (2-NF, 110 µL)—for use with TA 98, and 2-amino anthracene (2-AA, 110 µL)—for use with S9 activation kits.

2.4.2.2 Hydration of dried bacteria and incubation

Prior hydration of the dried bacteria and its incubation was initiated a night before the assay. The procedure commences with transferring 10 mL of reagent ampicillin (V) into the growth media (G) and followed by mixing with the lyophilized bacteria (T98 and T100). Thereafter, the growth media was transferred aseptically into

TABLE 2 Experimental setup of the Muta-ChromoPlate™ Assay with S9 activation for T98 and T100 bacterial strains.

Treatment plate (1–12)	Standard	Sample	Water	Reaction mix	S9 mix	Bacteria (5 µL)
Blank (sterility check)	-	15.5	0	2.5	2.0	-
Background	-	-	15.5	2.5	2.0	+
Positive control	0.1	-	15.5	2.5	2.0	+
WWTP effluent I (with S9)	-	15.5	0	2.5	2.0	+
WWTP effluent I	-	15.5	0	2.5	0.0	+
WWTP effluent II	-	3.0	12.5	2.5	2.0	+
Tap water I (with S9)	-	15.5	0	2.5	2.0	+
Tap water I	-	15.5	0	2.5	0.0	+
Tap water II	-	3.0	12.5	2.5	2.0	+
4-CP						
Bottled water A-I (with S9)	-	15.5	0	2.5	2.0	+
Bottled water A-I	-	15.5	0	2.5	0.0	+
Bottled water A-II	-	15.5	0	2.5	0.0	+
2,4-DCP						
Bottled water B-I (with S9)	-	3.0	12.5	2.5	2.0	+
Bottled water B-I	-	15.5	0	2.5	2.0	+
Bottled water B-II	-	3.0	12.5	2.5	2.0	+

Bold values indicate the sectioning of the tables.

vials containing bacteria and mixed. Incubation of the mixed lyophilized bacteria was done for 18 h at 37°C. Visualization to confirm the bacteria growth was done before proceeding with the assay. The ampicillin included in the mutagenicity assay serves as a marker for the presence of plasmid; the plasmid tends to confer ampicillin resistance which is a convenient marker (Maron and Ames, 1983; Tejs, 2008). The TA98 and TA100 strains were tested on the same plate as a control for ampicillin activity.

2.4.2.3 Aqueous sample dilutions

The samples to be tested were filtered through a 0.22 µm membrane filter for sterilization purposes. Subsequently, the sample mix setup of the Muta-ChromoPlate™ assay with S9 activation for T98 and T100 bacterial strains was prepared in 50 mL sterile tubes as shown in Table 2.

2.4.2.4 Preparation of treatments (with and without S9 activation enzyme) and Muta-ChromoPlateTM assay

The reaction mixture (2.5 mL) was transferred aseptically into all the holding vials containing the test samples, followed by the addition of 15.5 mL of the sterile filtered samples or dilutions to be analyzed. The S9-activation enzyme experiments proceeded with the addition of 2.0 mL of S9 mix to each vial requiring S9 activation only. This was followed by the addition of the reaction mixture (2.5 mL) and 15.5 mL of the samples as shown in Table 2. All the assay vials containing materials to be assessed had 5 mL of bacterial test strain broth culture (*S. typhimurium* T98 and TA100) added to ensure that the bacteria were completely suspended. The resulting mixtures in all the vials were poured into a sterile boat and 200 mL of the mixture was dispensed into a 96-well sterile microplate using a multi-channel pipette. Labeling of the plates for facile identification and separation of the bacterial strains was carried out. Incubation in aseptic-sealed plastic bags was done for 3–6 days at 37°C. The addition of the S9-activation enzyme is predicated on the fact that carcinogens are not directly carcinogenic, they become active after metabolism. The S9 mix (consisting of a 9000 supernatant fraction of liver homogenate from rats and other components) strongly induces several xenobiotic metabolizing enzymes (Hengstler and Oesch, 2001). Essentially, the S9 mix is added to the reaction mixture to provide a favorable condition for the activation of the metabolism of the carcinogen.

2.5 Interpretation of results

The statistical significance difference was determined for each treatment plate using the method described by Mortelmans and Zeiger (2000). The 96-well microplate method described by Gilbert (1980) was used for scoring mutagenicity.

3 Results

3.1 Phenol levels in WWTP effluent and potable water samples

The HPLC chromatograms, calibration data, and curves for 4-CP and 2,4-DCP are presented in Supplementary Figures S1, S2A,

TABLE 3 Levels of 4-CP and 2,4-DCP in potable water and WWTP effluent samples in mg/L (mean ± SD, n = 3).

Sample	4-CP					2,4-DCP				
	Sample 1	Sample 2	Sample 3	Mean	SD	Sample 1	Sample 2	Sample 3	Mean	SD
WWTP influent	1.04×10^{-4}	1.15×10^{-4}	6.43×10^{-5}	9.43×10^{-5}	2.66×10^{-5}	9.81×10^{-5}	1.72×10^{-4}	6.39×10^{-5}	1.11×10^{-5}	5.51×10^{-5}
WWTP effluent	5.61×10^{-5}	1.27×10^{-5}	4.04×10^{-6}	2.43×10^{-5}	2.79×10^{-5}	ND	ND	5.40×10^{-6}	1.80×10^{-6}	3.12×10^{-6}
Bottled water brand A	ND	9.32×10^{-7}	5.81×10^{-6}	2.25×10^{-6}	3.12×10^{-6}	ND	3.68×10^{-6}	1.31×10^{-5}	5.60×10^{-6}	6.77×10^{-6}
Bottled water brand B	ND	6.95×10^{-6}	3.42×10^{-6}	3.46×10^{-6}	3.48×10^{-6}	5.56×10^{-6}	1.37×10^{-5}	3.68×10^{-6}	7.66×10^{-6}	5.35×10^{-6}
Bottled water brand C	ND	ND	9.78×10^{-6}	3.26×10^{-6}	5.64×10^{-6}	8.80×10^{-7}	ND	6.85×10^{-6}	2.58×10^{-6}	3.73×10^{-6}
Bottled water brand D	1.97×10^{-6}	8.90×10^{-7}	6.74×10^{-6}	3.20×10^{-6}	3.11×10^{-6}	1.47×10^{-5}	8.37×10^{-6}	6.28×10^{-6}	9.77×10^{-6}	4.36×10^{-6}
Tap water	9.96×10^{-6}	1.90×10^{-5}	ND	9.65×10^{-6}	9.50×10^{-6}	6.23×10^{-6}	1.90×10^{-5}	5.97×10^{-6}	9.27×10^{-6}	5.49×10^{-6}

ND, not detected.

TABLE 4 Cancer risk assessment using mean concentrations of 4-CP and 2,4-DCP of samples.

Sample	ADD	RfD	HQ	SF	CR	Comment
4-CP						
WWTP influent	6.15×10^{-7}	3.00×10^{-1}	2.05×10^{-6}	1.10×10^{-2}	6.76×10^{-9}	Non-carcinogenic adverse effect
WWTP effluent	1.58×10^{-7}	3.00×10^{-1}	5.28×10^{-7}	1.10×10^{-2}	1.74×10^{-9}	Non-carcinogenic adverse effect
BW A	2.35×10^{-5}	3.00×10^{-1}	7.82×10^{-5}	1.10×10^{-2}	2.58×10^{-7}	Non-carcinogenic adverse effect
BW B	3.61×10^{-5}	3.00×10^{-1}	1.20×10^{-4}	1.10×10^{-2}	3.97×10^{-7}	Non-carcinogenic adverse effect
BW C	3.40×10^{-5}	3.00×10^{-1}	1.13×10^{-4}	1.10×10^{-2}	3.74×10^{-7}	Non-carcinogenic adverse effect
BW D	3.34×10^{-5}	3.00×10^{-1}	1.11×10^{-4}	1.10×10^{-2}	3.67×10^{-7}	Non-carcinogenic adverse effect
Tap water	1.01×10^{-4}	3.00×10^{-1}	3.35×10^{-4}	1.10×10^{-2}	1.11×10^{-6}	Non-carcinogenic adverse effect
2,4-DCP						
WWTP influent	7.24×10^{-8}	3.00×10^{-1}	2.41×10^{-7}	1.10×10^{-2}	7.96×10^{-10}	Non-carcinogenic adverse effect
WWTP effluent	1.17×10^{-8}	3.00×10^{-1}	3.91×10^{-8}	1.10×10^{-2}	1.29×10^{-10}	Non-carcinogenic adverse effect
BW A	5.84×10^{-5}	3.00×10^{-1}	1.95×10^{-4}	1.10×10^{-2}	6.42×10^{-7}	Non-carcinogenic adverse effect
BW B	7.9×10^{-5}	3.00×10^{-1}	2.66×10^{-4}	1.10×10^{-2}	8.79×10^{-7}	Non-carcinogenic adverse effect
BW C	2.69×10^{-5}	3.00×10^{-1}	8.97×10^{-5}	1.10×10^{-2}	2.96×10^{-7}	Non-carcinogenic adverse effect
BW D	1.02×10^{4}	3.00×10^{-1}	3.40×10^{-4}	1.10×10^{-2}	1.12×10^{-6}	Non-carcinogenic adverse effect
Tap water	$9{,}67 \times 10^{-5}$	3.00×10^{-1}	3.22×10^{-4}	1.10×10^{-2}	1.06×10^{-6}	Non-carcinogenic adverse effect

HQ > 1 indicates a carcinogenic adverse effect; HQ < 1 connotes non-carcinogenic adverse effect; BW: bottled water; ADD: average daily dose; RfD: reference dose; HQ; hazard quotient; SF: Slope Factor and CR: cancer risk. Bold values indicate the sectioning of the tables.

S2B, and Supplementary Table S3, respectively. The calibration curve R^2 values for both compounds were >0.999 which indicates the suitability of the method used for the analysis. The retention time for 4-CP and 2,4-DCP were 11.7 min and 14.1 min, respectively. The phenolic levels recorded in the tap water, bottled water, and WWTP effluent are presented in Table 3. Both 4-CP and 2,4-DCP levels were detected in the WWTP effluent, however, the concentrations were below the stipulated guideline of 0.01 mg/L set by the South African Department of Water Affairs and Forestry (DWAF, 1996). The levels obtained ranged from 4.04×10^{-6} mg/L - 5.61×10^{-5} mg/L for 4-CP, and 8.80×10^{-7} - 5.40×10^{-6} mg/L for 2,4-DCP.

TABLE 5 Test scores of samples' mutagenicity using the T98 strain.

#	Plate	Concentration	Bacteria	Day 4	Day 5	Day 6
1	Blank –ws9 (tap water)	100%	-	0	0	0
2	Background wS9	-	+	0	0	0
3	Positive control	-	+	95	96	96
4	WWTP effluent I	100%	+	91	91	91
5	WWTP effluent-I ws9	100%	+	95	95	95
6	WWTP effluent-II	19%	+	96	96	96
7	Tap water I	100%	+	95	95	95
8	Tap water-I ws9	100%	+	95	95	95
9	Tap water –II	19%	+	95	95	95
10	Bottled water 'A'-I	100%	+	96	96	96
11	Bottled water 'A'- I wS9-	100%	+	96	96	96
12	Bottled water 'A'-II	19%	+	96	96	96
13	Bottled water 'B'-I	100%	+	96	96	96
14	Bottled water 'B'-I wS9	100%	+	96	96	96
15	Bottled water 'B'-II	19%	+	96	96	96

3.2 Carcinogenic risk assessment

The carcinogenic risk assessment result for the water samples is presented in Table 4. The risk assessment table shows the calculated values for ADD, HQ, RfD, SF, and CR. The ADD, representing the quantity of a substance consumed per day during the duration of exposure is a crucial criterion for health risk assessment. The cancer risk assessment was conducted using an assumed exposure period of 10 years, a life expectancy of 70 years, and a body weight of 70 kg. The mean levels of 4-CP and 2,4-DCP obtained from the tap water, bottled water, and effluent samples were used for the cancer risk estimation. Values obtained from the computation of the HQ are used to interpret the cancer risk assessment. A HQ > 1 indicates a carcinogenic adverse effect, whereas HQ < 1 connotes non-carcinogenic adverse effects. The HQ value for all samples was <1; consequently, all the samples are categorized as a non-carcinogenic risk for lifetime exposure.

3.3 Mutagenicity test

The Ames test is a biological test that tests the mutagenic ability of chemical compounds (Zeiger, 2019). It makes use of bacteria to test the potential of chemicals that could have the potential to cause mutations in the DNA of the test organism. Some mutagens may undergo metabolic conversion to a reactive metabolite with the ability to interact with DNA; hence, mutagenicity assay for compounds with or without metabolism is essential for a robust assessment as mammals exhibit extensive *in vivo* metabolic capabilities (EBPI, 2019). The direct or direct mutagens can be detected if S9 is added to the assay mixture, which forms the basis for the addition of the S9 mix. The wells that changed to yellow coloration were considered positive, whereas those that retained the purple color were termed negative. The results were taken at 24 h intervals as presented in Tables 5 and Table 6.

The T98 bacterial strain results are considered valid when three criteria are met. These criteria include ensuring that the blank wells are sterile; the mean score for the background control (negative control) is ≥0 and ≤30 revertant wells per 96-well section on day 6; and the mean score for the standard mutagen (positive control) is ≥50 revertant wells per 95-well section on day 6.

4 Discussion

4.1 Phenol occurrence in WWTP effluent and potable water samples

Processing chemicals, raw materials, or intermediate products in the agrochemical industry and wood preservation are sources of phenolic compounds in the environment (Yahaya et al., 2019; Ramos et al., 2021). Chlorophenols are produced in pulp bleaching processes, as metabolites of agricultural pesticides, and as by-products of chlorination during water/wastewater treatment operations (Yahaya et al., 2019). The WWTP is situated in the Boland region of the Western Cape Province in South Africa-a vast area that is known for its agricultural process as numerous commercial farms abound. These farms mostly grow grapevines used to produce different types of wines and their farming operation requires the use of several pesticides. The presence of the low levels of 2,4-DCP may be from the agrochemical usage from these farms as the WWTP solely uses the membrane bioreactor in its treatment process. The membrane reactor system does not require chlorine usage for effluent treatment. Rather, it uses a combination of biological methods for suspended growth (generally activated sludge) and membrane filtration operations; hence, no

TABLE 6 Test scores of samples' mutagenicity using the T100 strain.

#	Plate	Concentration	Bacteria	Day 4	Day 5	Day 6
1	Blank –wS9 (tap water)	100%	-	0	0	0
2	Background wS9	-	+	19	21	23
3	Positive Control	-		54	64	68
4	WWTP effluent I	100%	+	30	35	38
5	WWTP effluent-I ws9	100%	+	26	29	30
6	WWTP effluent-II	19%	+	25	32	35
7	Tap water I	100%	+	29	33	39
8	Tap water-I ws9	100%	+	19	21	24
9	Tap water –II	19%	+	21	33	35
10	Bottled water 'A'-I	100%	+	16	19	24
11	Bottled water 'A'- I wS9-	100%	+	30	37	39
12	Bottled water 'A'-II	19%	+	13	20	23
13	Bottled water 'B'-I	100%	+	25	30	32
14	Bottled water 'B'-I wS9	100%	+	19	23	26
15	Bottled water 'B'-II	19%	+	15	17	23

chlorination by-product is generated. Essentially, low-pressure microfiltration or ultrafiltration is utilized to carry out critical solid-liquid separation functions (AMTA, 2016; Nqombolo et al., 2018). Therefore, the levels of 2,4-DCP in the effluent samples are expected to be low due to this process of treatment used because the 2,4-DCP compound in drinking and wastewater occurs mostly as a by-product of water treated by chlorination (Park and Kim, 2018). For 4-CP, the wine production (wineries) in the surrounding areas of the WWTP could be the major contributor to the trace levels detected in effluent samples (Girish and Murty, 2012). Winery effluent which majorly comes from washing operations during grapes harvesting, pressing, and fermentation contains low levels of phenolic compounds (Cassano et al., 2015).

Four brands of bottled water denoted as brands "A, B, C, and D" were assessed for their phenolic content. The maximum concentrations of 4-CP and 2,4-DCP recorded for all the bottled water samples were 9.78×10^{-6} mg/L and 1.47×10^{-5} mg/L, respectively. The guideline for phenol in bottled water as stipulated by the United States Food and Drug Administration (US FDA) is 0.001 mg/L (ATSDR, 2008); the levels obtained for both compounds were below the FDA regulatory limits. A previous investigation on the USEPA 11 priority phenols in three brands of bottled water in Cape Town, South Africa recorded an average concentration of 5.13×10^{-3} mg/L. In the tap water samples, the concentration of 4-CP ranged from 9.96×10^{-6} - 1.90×10^{-5} mg/L, while 2,4-DCP was from 5.97×10^{-6} - 1.90×10^{-5} mg/L. The United States Environmental Protection Agency (USEPA) maximum permissible limit for phenol in potable water is ≤0.3 mg/L, stipulated for the protection of human health from potential adverse effects of phenol exposure via drinking water and/or contaminated animals and plants (Zeatoun et al., 2004). Also, the European Community stipulates a concentration of 0.1 µg/L as the maximum permissible limit for phenol in drinking water (Al-Janabi, 2011). The agencies set benchmarked concentrations for contaminants as maximum permissible limits to prevent adverse human health effects. The measured levels of both phenolics in the tap water were below the set limits which connotes potability. The likely sources of the compounds in the tap water could be from the chlorination process employed for water disinfection. More so, the raw water sources utilized by most water works come from dams which are filled up during the winter season by run-offs which can be contaminated by agricultural chemicals. Aizawa et al. (2015) recorded concentrations in the ranges of 0.01—0.20 mg/L for phenols in tap water; the study did not differentiate between the individual phenolic compounds.

4.2 Non-carcinogenic and carcinogenic risk assessment

The phenol concentrations in the water samples utilized for the cancer risk assessment were all below the stipulated guidelines set by national and international bodies. The guideline set for phenols in WWTP effluent by the Department of Water Affairs and Forestry (DWAF) in South Africa is 0.01 mg/L (DWAF, 1996). The US FDA guideline stipulates a maximum permissible limit of 0.001 mg/L in bottled water, with a lifetime exposure of 2 mg/L not expected to cause an adverse health effect (ATSDR, 2008). The European Union limit is 0.5 µg/L for total phenols and 0.1 µg/L for individual compounds (Fattahi et al., 2007; Mahugo Santana et al., 2009). Contaminants with concentrations exceeding the maximum permissible limits stipulated by environmental protection bodies pose adverse health implications. The trace levels of 4-CP (9.32×10^{-7}—1.15×10^{-4} mg/L) and 2,4-DCP (8.80×10^{-7}—1.72×10^{-4} mg/L) recorded in the water samples translate to the non-carcinogenic risk recorded. None of the samples analyzed had a cancer risk (CR)

value that exceeded any of the regulatory limits. Typically, HQ values > 1 portend potential harmful ecological effects, whereas values < 1 suggest a low possibility of ecological risk (Megahed et al., 2015). The HQ values recorded in this study were all <1, indicating a low level of potential ecological risk. More so, the recorded ADD values for the phenolics were below 10^{-4}, which indicates a low possibility of cancer risk (Yahaya et al., 2019). Cancer risk values < 10^{-6} indicate no likelihood of cancer (Ramos et al., 2021); none of the samples analyzed had a cancer risk value that exceeded the regulatory limit. Therefore, the possibility of the analyzed samples causing cancer is unlikely. Despite the low possibility of cancer risk observed, cognizance of the assessment being done with data for a healthy adult should be noted. This implies that the observed values may pose a cancer risk to children and other adults with compromised or debilitating health conditions.

4.3 Mutagenicity assessment

The assay results show that the background control had no (0) positive well, while the bottled water "A" and "B", tap water, and the WWTP effluent of the undiluted concentrations (100%) recorded 96, 96, 95, and 95 positive revertant colonies on the test plates on day 6, respectively. The positive wells indicate that the levels of 4-CP and 2,4-DCP in the water samples are mutagenic and could act as a carcinogen. Using the scoring tables in a 96-well microplate, there was a clear significance in the Fluctuation Test which indicates the strong mutagenicity of the water samples. The assay results for all the undiluted samples (i.e. 96, 96, 95, and 95) are much higher than the control; hence, there is < 0.001 chance that 0 and 95 or 96 are the same results. The treatment plates produced a significant difference in the reverse mutation rate when compared with the control, indicating that the samples assayed possess mutagenicity on the T98 strain. Statistically, increases in the frequency of revertant colonies compared to the concurrent control with $p < 0.05$ indicate significant variation (Wisher, 2017). Fluctuation test is used to assess the mutagenicity of a particular chemical or treatment over a control; it takes cognizance of the number of positive wells in the treatment to that of the control (Gilbert, 1980).

The mutagenicity results showed that the background control had 23 positive wells at day 6, whereas the bottled water "A" and "B", tap water, and effluent samples recorded 39, 26, 24, and 30 positive wells, respectively. Comparing the number of positive wells in the samples with that of the backgrounds shows significant variation in the Fluctuation Test - an indication of the samples displaying weak mutagenicity. There is < 0.05 chance that the results of bottled water "B" (26), tap water (24), and effluent (30) are the same result as the background control (23), while the bottled water "A" (39) had a <0.001 chance; suggesting a slight chance of mutagenicity for the T100 strain. Mortelmans and Zeiger (2000) suggested that a substance can be categorized as a mutagen when one or more strains induce a reproducible, dose-related increase in the number of reverting colonies; however, a substance is regarded as a poor mutagen when the number of reverting colonies does not double that of the background number of colonies. Essentially, only a sample with twice the number of reverse mutations compared to the background mutation rate is considered to be mutagenic. A positive bacterial reverse mutation assay result suggests that a substance can potentially cause point mutations in the genome of either *S. typhimurium* by base substitution or frameshift (OECD, 1997). Conversely, negative results suggest that the test substance under the defined conditions is not mutagenic in the tested organisms.

5 Conclusion

The quantification and human health risk assessment of 4-CP and 2,4-DCP compounds in potable and treated waters in the Boland region of the Western Cape of South Africa was carried out in this study. The levels of 4-CP and 2,4-DCP measured in the samples were below the stipulated limits recommended by national and international bodies. The potential risks of the phenolic levels to humans were investigated using the cancer risk and mutagenicity assays. The assessed HQ for the phenolic concentrations indicates a low level of potential ecological risk. There was no cancer risk associated with the samples but signs of non-carcinogenic adverse effects were found. The mutagenicity assay of 4-CP and 2,4-DCP using the T98 strains recorded strong mutagenicity as there was a significant variation ($p < 0.05$) in the frequency of revertant colonies compared to the concurrent control. The T98 results indicate the potential ability of phenolic concentrations in the water samples to cause mutation toward the insertion or deletion of a nucleotide. However, the T100 bacterial strain showed very slight mutagenicity potential, with no real threat or possibility of causing mutation involving the replacement or substitution of a nucleotide base with another in the DNA or RNA.

This investigation is an introductory study into the health risk assessment of selected phenolic compounds in potable water and treated effluent samples. The study has provided some insight into the possible human health risks associated with the occurrence of the 4-CP and 2,4-DCP water samples. The health risk studies were carried out at population and organism levels; hence, cellular and molecular-level studies may provide greater clarity on the associated health risks of the phenolic compounds at the observed concentrations.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

NM: Data curation, Formal Analysis, Investigation, Writing–original draft, Methodology. MA: Formal Analysis, Investigation, Writing–review and editing, Methodology. OP: Formal Analysis, Investigation, Writing–review and editing, Methodology. IH: Supervision, Visualization, Writing–review and editing. BO: Conceptualization, Resources, Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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EDITED BY
Aina Olubukola Adeogun,
University of Ibadan, Nigeria

REVIEWED BY
Liangpo Liu,
Shanxi Medical University, China
Charis Liapi,
National and Kapodistrian University of Athens, Greece
Mirco Masi,
Italian Institute of Technology (IIT), Italy

*CORRESPONDENCE
Xiaoya Ji
✉ jxya1992@163.com
Lin Lu
✉ lulin@qdu.edu.cn

†These authors have contributed equally to this work and share first authorship

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Distribution and potential risk factors of bisphenol a in serum and urine among Chinese from 2004 to 2019

Wenjing Zhang†, Yanting Li†, Tao Wang, Xinglin Zhang, Jianzhong Zhang, Xiaoya Ji* and Lin Lu*

Department of Occupational and Environmental Health, School of Public Health, Qingdao University, Qingdao, China

Background: Bisphenol A (BPA) is an oil-derived, large-market volume chemical with endocrine disrupting properties and reproductive toxicity. Moreover, BPA is frequently used in food contact materials, has been extensively researched recently, and widespread exposure in the general population has been reported worldwide. However, national information on BPA levels in general Chinese people is lacking.

Methods: This study collected and analyzed 145 (104 in urine and 41 in serum) research articles published between 2004 and 2021 to reflect the BPA internal exposure levels in Chinese populations. The Monte Carlo simulation method is employed to analyze and estimate the data in order to rectify the deviation caused by a skewed distribution.

Results: Data on BPA concentrations in urine and serum were collected from 2006 to 2019 and 2004 to 2019, respectively. Urinary BPA concentrations did not vary significantly until 2017, with the highest concentration occurring from 2018 to 2019 (2.90 ng/mL). The serum BPA concentration decreased to the nadir of 1.07 ng/mL in 2011 and gradually increased to 2.54 ng/mL. Nationally, 18 provinces were studied, with Guangdong (3.50 ng/mL), Zhejiang (2.57 ng/mL), and Fujian (2.15 ng/mL) having the highest urine BPA levels. Serum BPA was investigated in 15 provinces; Jiangsu (9.14 ng/mL) and Shandong (5.80 ng/mL) were relatively high. The results also indicated that males' urine and serum BPA levels were higher than females, while the BPA levels in children were also higher than in adults ($p < 0.001$). Furthermore, the volume of garbage disposal ($r = 0.39$, $p < 0.05$), household sewage ($r = 0.34$, $p < 0.05$), and waste incineration content ($r = 0.35$, $p < 0.05$) exhibited a strong positive connection with urine BPA levels in Chinese individuals.

Conclusion: Despite using a data consolidation approach, our study found that the Chinese population was exposed to significant amounts of BPA, and males having a higher level than females. Besides, the levels of BPA exposure are influenced by the volume of garbage disposal, household sewage, and waste incineration content.

KEYWORDS

urine bisphenol A, serum bisphenol A, Chinese population health, Monte Carlo simulation, risk factors, environmental pollutants, endocrine disruptor

1 Introduction

Bisphenol A (BPA) is an artificial chemical compound with high production volume, predominantly used in manufacturing polycarbonate (PC) plastics, polysulfones, and epoxy resins (1, 2). Several studies suggest that BPA is widely applied in producing varying consumer products, such as thermal paper, can coatings (3, 4), and BPA can leach from food and beverage containers, dental sealants, and other composites (5). Due to its widespread application, BPA is detected in water (freshwater, seawater, sewage, drinking water), soil or sediment, atmosphere, food, garbage (4, 6), and in humans. Furthermore, since 2007, Asia has become a significant BPA production and consumption region. At the end of 2018, BPA consumption was 1.43 million tons in China, making it the largest producer of BPA globally (7).

Humans are exposed to BPA through various pathways, including food or drinking water (8), skin contact, and breathing (9), in which dietary intake is the main pathway. Exposure to airborne BPA cannot be ignored, as shown by Ribeiro et al. (10). The study conducted by Rudel et al. revealed the presence of BPA in 86% of house dust samples, with concentrations ranging from 0.2 to 17.6 μg/g (11). In the urban outdoor environment, this compound has been detected in air samples at an average level of 0.51 ng/m^3 with mild seasonal variations observed (12). According to a Chinese study on the causes of urinary BPA exposure in young adults, dietary consumption, indoor dust, paper goods, and personal care items contributed 72.5, 0.74, 0.98, and 0.22% of the overall exposure dose, respectively (13). Several epidemiological research have suggested that fatty foods, bracelets, and socks may be sources of BPA exposure for children (14–16). Moreover, in a human study embedded as part of the Europe project EuroMix ("European Test and Risk Assessment Techniques for Mixtures") it was determined that diet and thermal paper (TP) were the factors most responsible for BPA exposure (17). Occupational exposure, in addition, is an important mode of exposure to BPA. The study conducted by He et al. showed that workers in epoxy resin and BPA manufacturing factories are occupationally exposed to BPA at high levels (18).

Upon oral treatment, pharmacokinetic investigations have shown that BPA is swiftly and effectively absorbed in the gastrointestinal tract. It is first metabolized by the gut wall and liver, where its primary metabolite BPA-glucuronide is produced. BPA-glucuronide is rapidly filtered from the blood by the kidneys and excreted in urine (19). Consequently, BPA has been detected in body fluids, including saliva, urine, serum, plasma, placental tissue, umbilical cord serum, placenta and breast milk (20, 21). Though the half-life of BPA in human urine and blood is 6 h and 5.3 h, respectively (22, 23), urine and serum are widely used biological samples for biomonitoring (19).

Due to its endocrine disrupting properties and reproductive toxicity, BPA shows substantial damage to tissues and organs of the body, including those of the cardiovascular, reproductive, immune (24, 25), respiratory, digestive, and neuroendocrine systems (26–30). Previous epidemiological and toxicological studies demonstrated that exposure to BPA can cause endocrine disruption in humans (31), development of obesity (32), diabetes (33), cardiovascular disease (34, 35), etc. Sandra studies indicate that there were no significant differences in BPA exposure levels among the general population based on sex, geographic region, or analysis technique (36). For the moment, developed countries such as the United States and Canada have carried out long-term and systematic biomonitoring programs for urine BPA in their populations. China has carried out biological monitoring of BPA exposure levels in populations in localized areas, such as Shanghai (37, 38), Jiangsu (39), and Guangdong (39, 40) provinces, so there is a lack of exposure level data for the entire Chinese population.

There is currently no long-term detection and spatial variation trend of BPA exposure in the Chinese population. The study used Monte Carlo simulation to estimate urine and serum BPA levels in the Chinese population, which our group had previously established and validated. We used Monte Carlo simulation to examine BPA's regional and temporal distribution in urine and serum from 2004 to 2019. Additionally, relevant potential risk factors influencing urine and serum BPA levels were investigated.

2 Methods

2.1 Aim

The purpose of this study was to analyze the level of BPA exposure in the Chinese population from 2004 to 2019. And analyze the relationship between demographic factors such as age, gender, and region and the level of BPA exposure. In addition, the relationship between BPA exposure levels in the human body and BPA exposure in the environment were evaluated.

2.2 Design

We searched for literature on BPA exposure levels in the Chinese population from May 2011 onwards. Monte Carlo simulation was used to integrate and analyze the data.

2.3 Sample

PubMed, China National Knowledge Infrastructure (CNKI), Weipu (VIP), and Wanfang Data was selected as the academic publication source in this study.

2.4 Literature search

Four databases, such as PubMed, CNKI, VIP, and Wanfang Data, were searched from inception to May 1, 2021. We identified the following keywords: (a) "bisphenol A" and "urine" and "human" (b) "bisphenol A" and "serum" and "human" was decided as the keyword to search the Chinese database. Literature in English searches for anthropological studies using "bisphenol A" and "human" and "China" or "bisphenol A" and "Chinese" as keywords. The duplicate articles were excluded from the four databases.

Inclusion criteria were as follows: (1) All the subjects were Chinese population; (2) The study population were not patients with certain BPA-related diseases, such as obesity, asthma, thyroid disorders, neurobehavioral disturbances, changes in reproductive function, abnormal mammary gland development, and cognitive dysfunctions;

(3) Subjects were not with high BPA exposure history (described as living or working in areas of high BPA concentration); (4) The test samples were urine and serum, and strict quality control was used in the detection procedure. Data were filtered using the following exclusion criteria: (1) Research performing animal tests; (2) Studies including case reports, conference or poster abstracts, reviews, letters, or articles without containing original data; (3) Studies on substitutes for BPA.

A total of 145 articles published from 2004 to 2021 (including 104 on urine BPA and 41 on serum BPA) were collected, including 78 pieces of Chinese literature (50 on urine BPA and 28 on serum BPA) and 67 parts of English literature (54 on urine BPA and 13 on serum BPA) (see Figure 1). The sampling time ranged from 2004 to 2019. These articles included 64,893 subjects with sample sizes ranging from 10 to 3,426 each (see Supplementary Tables S1, S2).

2.5 Data extraction

Eleven elements were then extracted from each paper and entered into an Excel spreadsheet: article title, publication year, sampling time and method, first author, geographical area, sample size, age, gender, the limit of detection (LOD), and the BPA concentration (see Figure 1). We divided the available urine BPA and serum BPA data into five periods (2006–2008, 2009–2011, 2012–2014,2015–2017, 2018–2019) based on sampling time.

The National Health and Nutrition Examination Survey (NHANES) 2003–2016 provided the American urine BPA data (41). The Fifth Report on Human Biomonitoring of Environmental Chemicals in Canada 2007–2017 is the source of Canadian urine BPA (42). The required data on garbage disposal, domestic sewage, and waste incineration were found in the China Statistical Yearbooks (2006–2019) (43).

2.6 Data processing

2.6.1 Unit conversion of urine and serum BPA level

In this study, uniform unit was ng/mL. Other units were converted to ng/mL, e.g., 1 μg/L = 1 ng/mL; 1 ng/mL = 100 ng/dL; 1 ng/mL = 1,000 ng/L.

2.6.2 The calculation of arithmetic means

For most studies, the median and interrogative range values were presented, and to a lesser extent, geometric mean or arithmetic means. The appropriate Monte Carlo simulation formula was determined by consideration for whether the data fit a normal distribution or log-normal distribution or not. We convert the median to arithmetic mean (https://smcgrath.shinyapps.io/estmeansd/).

2.7 Calculation of standard deviation

The standard deviation of urine and serum BPA level not available in articles was estimated by Equation (44) as follows (45):

$$SD = \sqrt{N}\left(U_{CI} - L_{CI}\right) \div 3.92$$

Where N is the sample size; U_{CI} and L_{CI} are the upper and lower 95% confidence intervals, respectively.

2.8 Monte Carlo simulation

We used excel software to accomplish Monte Carlo simulation analysis on the data. The workflow of our approach was composed of

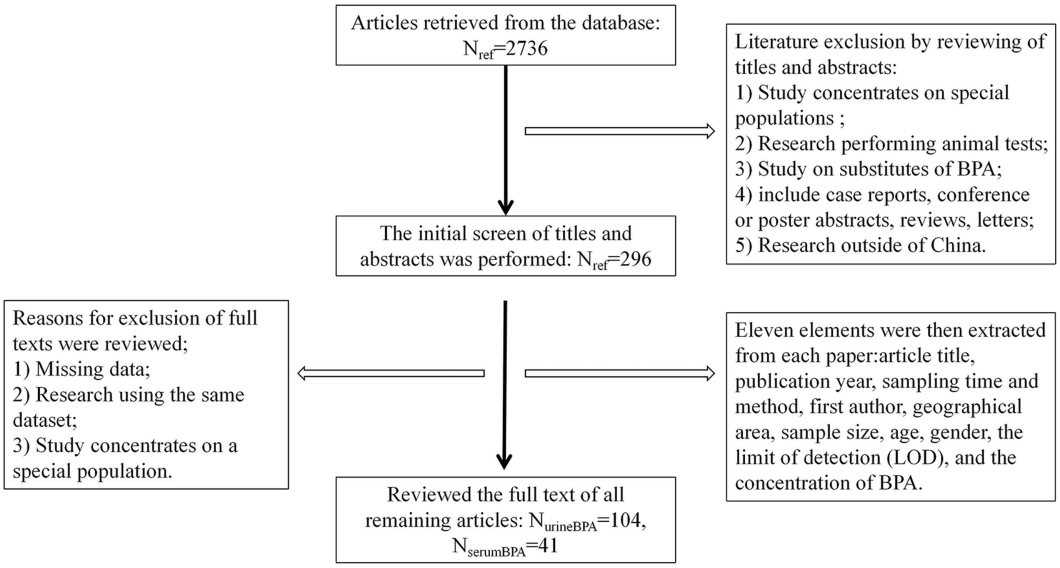

FIGURE 1
Steps taken to identify articles that met our inclusion–exclusion criteria. Urine andserum BPA data were collected from Weipu (VIP) databases, PubMed, China National Knowledge Infrastructure (CNKI), and Wanfang. One hundred forty-five papers reported the urine and serum BPA levels from 2004 to 2019 (sampling time).

three steps: (1) composing a mathematical model for probability simulation; (2) abstracting the simulated random numbers; and (3) arranging statistics and getting the solution to the problem (44).

For the original data of normal distribution and lognormal distribution, we adopted different calculation methods. Formulae used were as follows:

$$= MAX\left(0.5, \text{NORM.INV}\left(\text{RAND}(), \text{AM}, \text{SD}\right)\right)$$

$$= MAX\left(0.5, \text{LOGNORM.INV}\left(\text{RAND}(), \text{Ln}\left(\text{GM}\right), \text{Ln}\left(\text{GSD}\right)\right)\right)$$

Where NORM.INV and LOGNORM.INV denotes interval points that return a given probability normal distribution or lognormal distribution, respectively. RAND returns evenly distributed random numbers greater than or equal to 0 and less than 1. The MAX function ensured that the urine and serum BPA levels were greater than 1/2 LOD. Values were calculated in Excel (Microsoft). Simulations were computed by 100 times to ensure their exactitude (see Supplementary Figure S1).

2.9 Statistical analyses

Statistical analysis was conducted using SPSS version 26 (IBM. Armonk, NY, United States). Figures were prepared using GraphPad Prism 8.3.0 and ArcGIS 10.6. The Mann–Whitney U test was utilized to compare differences between two independent groups. Correlations were evaluated by Pearson's correlation coefficient (r). p-values < 0.05 were considered statistically significant. Urine and serum BPA results were transformed using natural logarithms for data analysis.

3 Results

3.1 Trends of urine BPA and serum BPA concentration in general Chinese population from 2004 to 2019

The Chinese population's urine and serum BPA concentration was calculated from the new database, which was generated by Monte Carlo simulation. Figure 2 depicts the urine and serum BPA concentrations in different time period (2006–2008, 2009–2011, 2012–2014, 2015–2017, 2018–2019 for urine BPA and 2004–2007, 2008–2011, 2012–2015, 2016–2019 for serum BPA). The geometric mean of serum BPA concentration decreased from 1.78 ng/mL in 2004–2007 to 1.07 ng/mL in 2008–2011, while the geometric mean of serum BPA concentration increased from 1.66 ng/mL in 2012–2015 to 2.54 ng/mL in 2016–2019 (see Supplementary Table S3).

3.2 Geographical variation of urine and serum BPA concentration

Figure 3 depicts the urine and serum BPA concentrations in different provinces across two time periods (2008–2011, 2006–2019 for urine BPA and 2006–2011, 2012–2019 for serum BPA). Urine BPA concentrations (2008–2011) and serum BPA concentrations (2006–2011) were detected mostly in coastal locations (see Figures 3A,C), with the area expanding inland from 2012 to 2019 (see Figures 3B,D). Guangdong (3.5 ng/mL), Zhejiang (2.57 ng/mL), and Fujian (2.15 ng/mL) had greater urine BPA levels than the other provinces, whereas Inner Mongolia (0.8 ng/mL) had a comparatively low level (see Supplementary Table S4). The levels of BPA in serum differed substantially between provinces. Jiangxi had the lowest serum BPA content (0.8 ng/mL), and Jiangsu had the highest (9.2 ng/mL) (see Supplementary Table S5).

3.3 Gender difference

Males had higher urine and serum BPA concentrations than females (see Figure 4). Urine BPA concentrations in males and females in China were 2.12 ng/mL and 1.77 ng/mL, respectively, while serum BPA values were 3.03 ng/mL and 1.07 ng/mL (see Figures 4A,C, Supplementary Table S6). The Mann–Whitney U test showed that the BPA concentration of males was significantly higher than that of females (all $p < 0.05$). Additionally, slowly increase in urine and serum BPA concentration in both males and females was noted before 2014, with decrease gradually from 2015 to 2019 (see Figures 4B,D).

3.4 Age difference

Taking ages into consideration, urine and serum BPA concentrations in China among different age ranges were 1.80 ng/mL and 2.88 ng/mL in the group of 0–18 years, 1.50 ng/mL and 1.36 ng/mL in the group of 19 and above years old (see Figures 5A,C). The Mann–Whitney U test showed that the BPA concentration of children was significantly higher than that of adults (all $p < 0.05$). Besides, urine BPA concentration was higher in school-age and young adults compared with people of other ages, with the geometric mean of 2.12 ng/mL and 1.85 ng/mL, respectively (see Figure 5B, Supplementary Table S7). Serum BPA concentration was highest in groups of 0–6 years old, with a geometric mean of 6.44 ng/mL (see Figure 5D, Supplementary Table S7).

3.5 Comparison with U.S. and Canada

In the United States and Canada, urine BPA levels showed an apparent downward trend, while urine BPA concentrations of the Chinese exhibited a significant fluctuation (see Figure 6). Before 2011, the concentration of BPA in urine in China was lower than that in the United States. However, from 2011 to 2019, the concentration of urine BPA in China was higher than that in the United States and Canada (see Figure 6).

3.6 The association between urine BPA concentration in Chinese and the external environment

Environmental factors can influence humans BPA inhalation, ingestion, and skin absorption. As shown in Figure 7A, possible risk

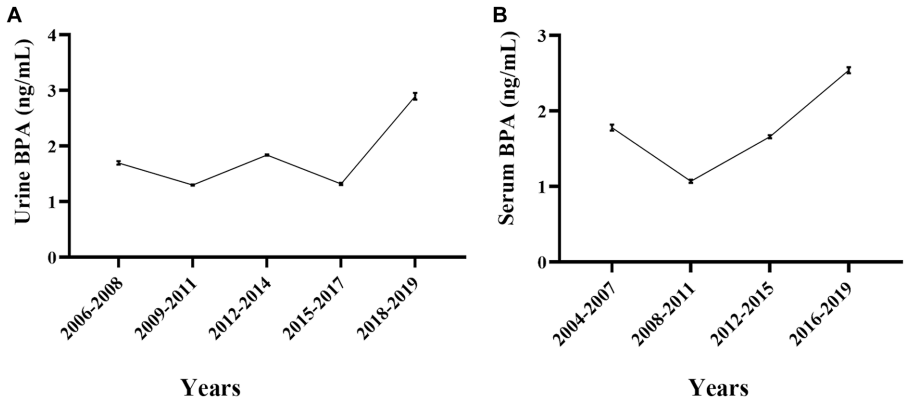

FIGURE 2
The temporal trend of urine ad serum BPA levels in general Chinese population from 2004 to 2019. **(A)** Overview of the general Chinese population's urine BPA levels in different periods. **(B)** Overview of the general Chinese population's serum BPA levels in different periods.

factors such as garbage disposal, residential sewage, and waste incineration content were connected to urine BPA concentration in Chinese provinces (see Supplementary Table S8). Guangdong had the highest urine BPA level, as well as the biggest volume of rubbish disposal and a high level of residential sewage and waste incineration. Domestic sewage levels are also higher in Heilongjiang and Zhejiang provinces. Furthermore, urine BPA levels revealed a strong positive connection with garbage disposal volume ($r = 0.39$, $p = 0.003$) (see Figure 7B), as did household sewage and waste incineration content ($r = 0.34$, $p = 0.01$; $r = 0.35$, $p = 0.009$) (see Figures 7C,D).

4 Discussion

Though BPA-related health problems have been attracting increasing attention (46), large-scale population studies with BPA exposure levels are still lacking in China. Several studies have shown that the average Chinese person had a high BPA exposure level (47). Furthermore, BPA has been widely investigated for its toxicity and shown adverse effects even at low-dose in animals and humans (48), making it a public health problem in China.

Urine and serum BPA level have acted as validated biomarkers to assess BPA exposure in humans. It mainly reflects short-term BPA exposure because of BPA's relatively short-life in urine and serum. He et al. reported BPA concentration in urine and serum of 952 ordinary residents (49). In September 2010, Gao's team conducted a national urine BPA level survey (13). However, most studies have been cross-sectional, and few have evaluated changes in human urine and serum BPA level over time. Based on the nationwide data investigation, our results suggested that the Chinese population's urine BPA had a smooth fluctuation then increased to a high level, while serum BPA gradually increased after reaching its lowest concentration in 2011. Like the United States and Canada (50), China has begun to restrict the use of BPA. However, BPA has been banned in the use of baby bottles, but BPA was still permitted to be used in the production of food packaging materials, containers, and coatings. Before 2011, BPA was in considerable demand, but the imports of BPA were on the decline (51). It has been shown that BPA imports were 8.8% lower in 2008 than in 2007 (52). Additionally, demand for BPA is growing in industries such as home appliances, electronics, urban construction, and automobiles (53).

Instead of focusing on the whole population in China, these present studies paid more attention to localized areas, such as Shanghai, Jiangsu, and Guangdong. This study collected urine and serum BPA data from different provinces. Through comparative analysis, we found that the Chinese population's BPA concentration of biological samples was predominantly distributed in the coastal areas (2004–2011). However, since 2012, the monitoring of BPA has expanded to inland areas. An important reason is that the abundance of seafood products may contain BPA in coastal areas. On the one hand, BPA manufacturers are mainly distributed in Jiangsu, Shanghai, and Zhejiang, among which a chemical company in Jiangsu is the one of the major thing BPA manufacturer in China (54). On the other hand, BPA is extensively used in the production of plastics and microplastics detected in 80–100% of seafood in southeastern China, leading to high levels of BPA in marine fish (55, 56). Additionally, in the atmosphere, BPA concentration is still at a high level in southeast China (57).

In general, the difference in gender and age has influenced the distribution of BPA. This study *shows that* urine and serum BPA concentrations were higher in males than females, *which* was in agreement with the previous studies (49). The elevated level of BPA in male urine may be associated with the glucuronide-conjugated coupling of BPA in urine (58). Moreover, a study in Korea found that gender differences in serum BPA concentrations may involve androgen-related BPA metabolism (59). In the 2015–16 period serum levels were higher in women and in the 2016–18 period females exhibited higher urine levels compared to other periods. On the one hand, the sample size in the urine BPA study conducted between 2016 and 2018 on women ($n = 2{,}204$) was significantly larger compared to men ($n = 439$). The size of the serum Bisphenol A exposure sample was smaller in 2015–2016. On the other hand, factors that cannot be assessed, such as a person's employment, lifestyle choices, or even biological factors, were significant in this regard. BPA levels were two times higher among female employees, according to research by González et al. (0.68 g/L in men and 1.20 g/L in women), although this variation did not achieve statistical significance ($p > 0.05$), which may be connected to the fact that the study's gender-specific workplaces were different (60).

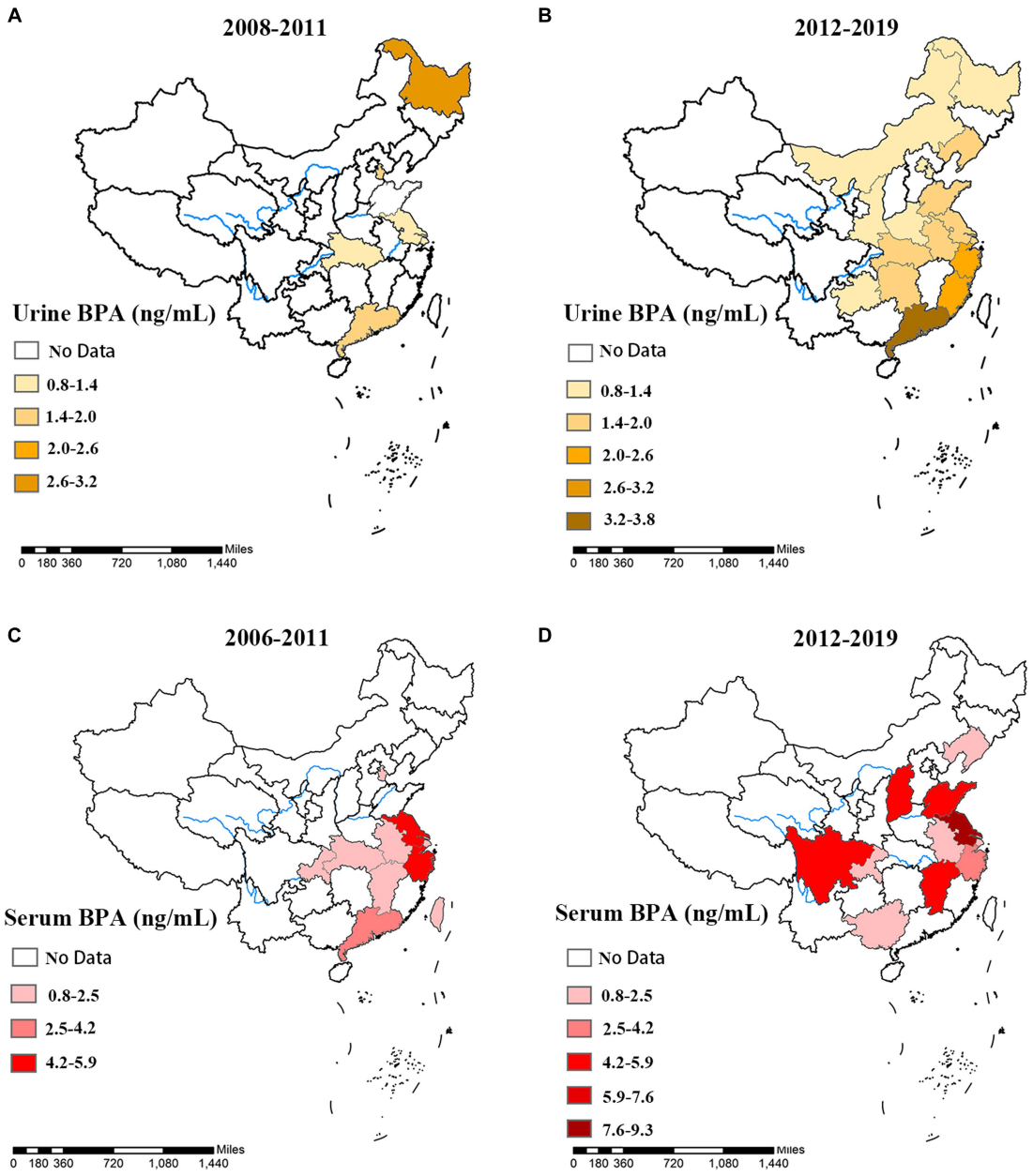

FIGURE 3
The spatial and temporal distribution of Urine and serum BPA concentration of different provinces in China. Urine BPA concentration in different provinces, **(A)** 2008–2011, and **(B)** 2012–2019 were exhibited. Serum BPA concentration in different provinces, **(C)** 2006–2011, and **(D)** 2012–2019 were exhibited.

Otherwise, we also found that urine and serum BPA concentrations were higher in children than that in adults. High concentrations of BPA in children may be associated with their high food consumption (such as fried food and snacks), relevant product usage (such as plastic toys), air inhalation rates in relation to their body weight and different toxic dynamic of their absorption, distribution, metabolism of BPA (61–63). Sugar and confectionery consumption were positively correlated with serum BPA levels in a Spanish sample (64). Additionally, Larsson et al. reported greater BPA levels in kids who consumed chocolate frequently and hypothesized that this could be due to a higher frequency of food consumption tainted by food packaging materials (65). There is BPA levels discrepancy between urine and serum values in the age groups 0–6, and 7–18 years. Children under the age of six are more probable to be exposed to plastic toys, and school-age children between the ages of seven and 18 spend more time in school as a result of dietary and airborne BPA exposure. These differences in lifestyle may be the primary cause. Additionally, differences in one's own health, the economy, and other underlying factors may have an impact. Therefore, public health interventional measures should be tailored to the needs of different populations.

From 2011 to 2019, the concentration of urine BPA in China was greater than in the United States and Canada, according to the

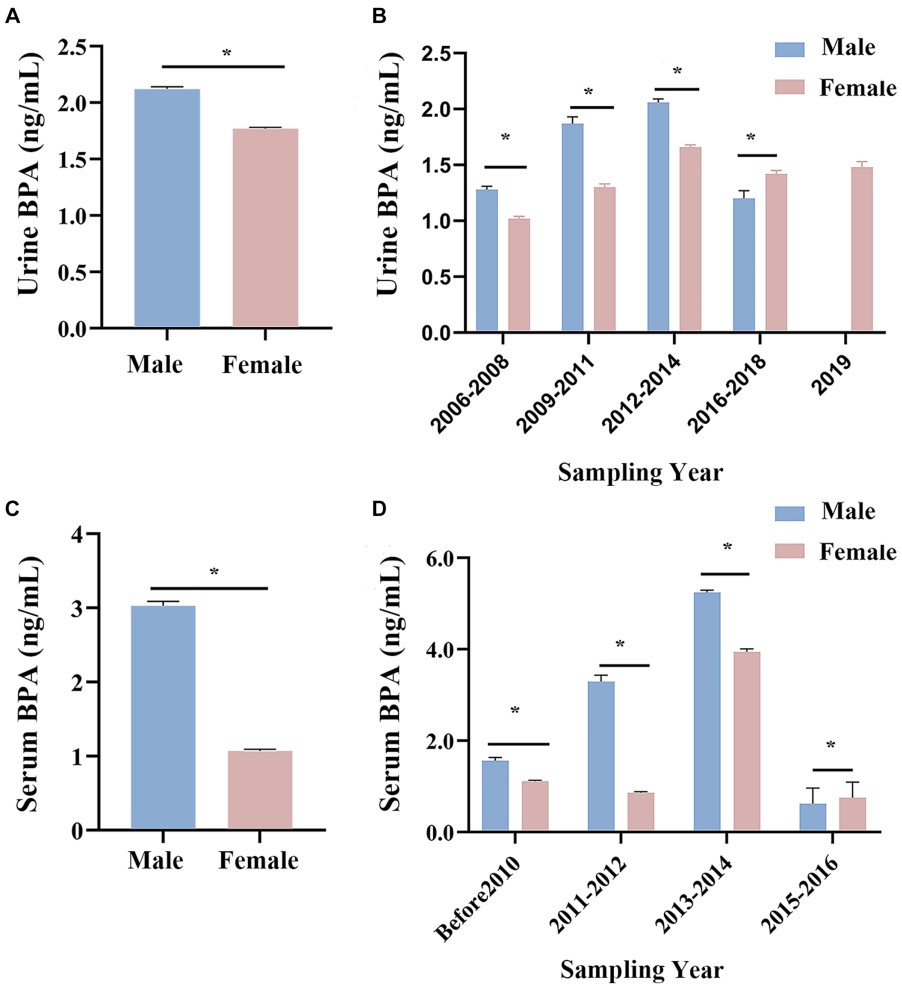

FIGURE 4
Changes in Chinese urine and serum BPA concentration by gender. **(A)** An overview of Chinese urine BPA concentration in different gender. **(B)** Histogram of Chinese urine BPA concentration of male and female in different time. **(C)** An overview of Chinese serum BPA concentration in different gender. **(D)** Histogram of Chinese serum BPA concentration of male and female in different time. $*p < 0.05$.

current study. Based on a great number of animal trials and epidemiological studies in Europe and the United States established the daily intake of BPA at 50 g/kg bw/day, but China has no equivalent legislation (66, 67). Furthermore, Wu et al. examined BPA exposure concentrations in natural surface water (freshwater, estuaries, and beaches) in 55 nations. They discovered that BPA exposure concentrations in China were greater than in the United States and Canada (68). The widespread use of BPA in China is the primary reason BPA exposure in the human body is higher than in the United States and Canada, highlighting the need for lowering BPA requirements and use. We collected data from May 2003 and May 2006 as part of the German Environmental Survey (GerES IV). A total of 599 youngsters were chosen, and the BPA level in their urine was 2.66 ng/mL (69). Between 2014 and 2017, the urine levels of BPA in German children were 1.905 ng/mL (70), which was still higher than in Chinese children.

Previous research has demonstrated that BPA has become ubiquitous in the environment as a result of its widespread manufacture, ingestion, and application (71). In addition, BPA environmental sources can be classified as pre-consumer sources for BPA synthesis, BPA-containing items, and post-consumer sources (6). Post-consumer sources, such as garbage disposal, incineration, and sewage discharge, are significant exposure pathways for the general population. BPA levels in landfills in the United States and Japan have been deemed high (72). This study found a link between garbage cleansing and incineration, sewage outflow, and human BPA exposure. As a result, more specific BPA control strategies should be maintained as a public health priority.

Nevertheless, the main limitation of the study is the lack of original work such as experimental or epidemiological investigations. The data of this study were mainly derived from the published literature, which itself resulted from outcome bias. Some provinces have less study data (such as Hongkong, Liaoning) thus the data from a few provinces may not be representative enough of the whole nation. As a result, a population-based epidemiologic survey should be carried out in China to determine the level of BPA exposure there as well as to look into any potential influencing

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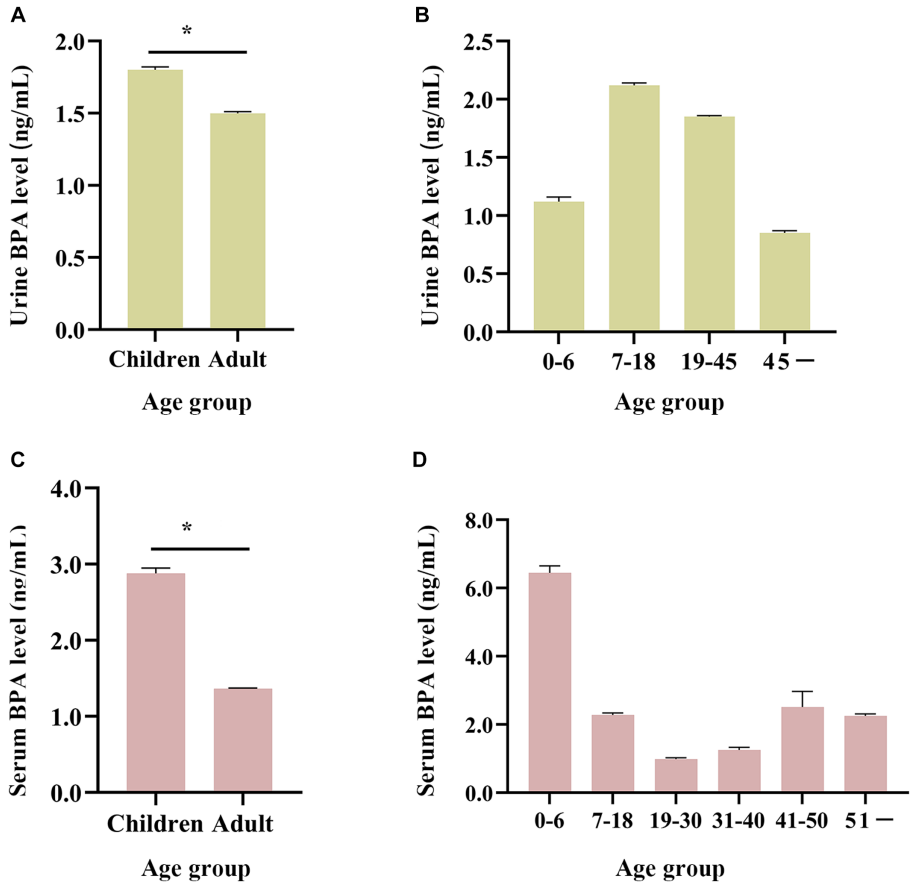

FIGURE 5
Age distribution of BPA levels in the general Chinese population (from 2004 to 2019). **(A,B)** Age changing trend of urine BPA concentration. **(C,D)** Age changing trend of serum BPA concentration. Children are 0–18 years old and adults are ≥19 years old. *$p < 0.05$.

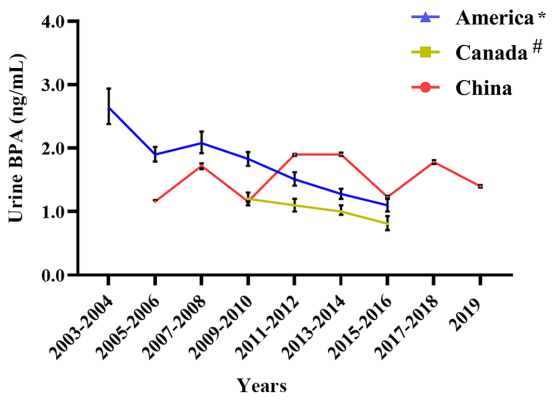

FIGURE 6
Urine BPA concentration in China compared with foreign countries. * Denotes that the source of American urine BPA data is NHANES 2003–2016; # Means that the data of urine BPA of Canadian are from The Fifth Report on Human Biomonitoring of Environmental Chemicals in Canada 2009–2016.

factors, such as lifestyle choices, residential location, health status, age, and gender. We may have omissions in the literature search screening process, resulting in biased results. For example, the study of neonatal urinary BPA exposure by Wang et al. (73). Therefore, we further screened the literature and combined it with epidemiologic studies in the next study. In addition, detection bias may exist in this study. For example, it is detected primarily by ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) (32%) and high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/

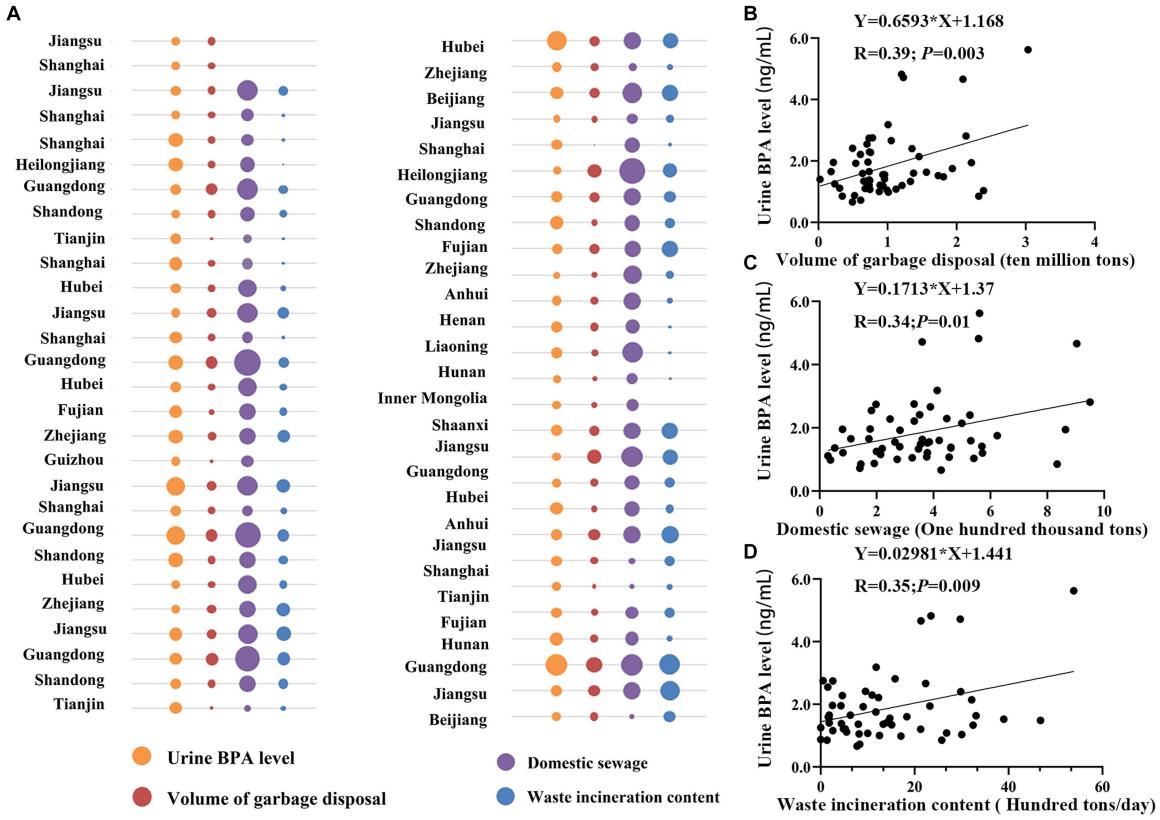

FIGURE 7
The correlation between urine BPA levels and potential risk factors. **(A)** Bubble chart; **(B)** volume of garbage disposal; **(C)** domestic sewage; **(D)** waste incineration content.

MS) (47%), whereas serum BPA is detected by linked immunosorbent assay (ELISA) (32%) and HPLC–MS/MS (46%). The LOD values of all those methods reported from 2004 to 2019 for BPA in human urine samples varied greatly (0.001–1.0 ng/mL). LC-MS/MS is the most sensitive and extensively used method for BPA detection in human urine (74). In addition, it was shown that there was A strong correlation between HPLC/FLD and LC/MS/MS for the determination of BPA levels (75). Even so, the detection bias hardly affected the results due to the high detection consistency and strict quality control.

5 Conclusion

Data mining and analysis based on Monte Carlo simulation showed wide fluctuation in the urine of BPA in the Chinese population; serum BPA showed an apparent decrease during 2004–2011, and a noticeable increase during 2012–2019. In addition, the exposure level of urine BPA in the Chinese population was significantly higher than in the United States and Canada. In space, the detection of BPA was mainly in coastal areas, but the scope was extended to the inland. Males' urine and serum BPA levels were higher than females and children higher than adults. In addition, household garbage cleaning and sewage discharge may affect human BPA levels. In conclusion, this study recommends strengthening the biological detection of BPA and comprehensive management of garbage and sewage discharge to mitigate BPA exposure.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

WZ and YL: data collection and curation, manuscript writing, and language services. TW: methodology and validation. XZ and JZ: validation. XJ and LL: validation, writing—review and editing, supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2024.1196248/full#supplementary-material

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EDITED BY
Camilo Dias Seabra Pereira,
Federal University of São Paulo, Brazil

REVIEWED BY
Wen-Jun Shi,
South China Normal University, China
Katherine Katie O'Shaughnessy,
Center for Public Health and Environmental Assessment, United States

*CORRESPONDENCE
Aina O. Adeogun,
ainaadeogun@yahoo.com

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Gonad pathology, sex hormone modulation and vitellogenin expression in *Chrysichthys nigrodigitatus* from Lagos and Epe lagoons within the southern-lagoon system, Nigeria

Olusola A. Akangbe[1], Azubuike V. Chukwuka[2], Maurice E. Imiuwa[3] and Aina O. Adeogun[1]*

[1]Department of Zoology, University of Ibadan, Ibadan, Oyo, Nigeria, [2]National Environmental Standards and Regulations Enforcement Agency (NESREA), Wupa, Nigeria, [3]Department of Animal and Environmental Biology, University of Benin, Benin, Nigeria

Introduction: Estrogenic chemicals in aquatic environments impact fish reproductive health, with vitellogenin protein levels serving as a crucial biomarker for xenoestrogen exposure. Limited knowledge exists on estrogenic effects in tropical environments, prompting an investigation into the influence of environmental estrogens on *Chrysichthys nigrodigitatus* in Lagos and Epe lagoons.

Methods: A total of 195 fish samples underwent analysis for vitellogenin protein, sex hormones (testosterone and 17 β-estradiol), and gonad pathology in effluent-receiving areas of the specified lagoons.

Results: Gonadal alterations were observed in male and female fish, including empty seminiferous tubules and distorted ovaries. Intersex occurred in 3.81% of Lagos and 3.33% of Epe. Testosterone levels were generally higher in females and males from both lagoons, while E2 levels were higher in females from both lagoons, with Lagos showing higher levels than Epe. Vtg levels were higher in males than females in Lagos samples but showed no significant difference in Epe samples.

Discussion: Contaminant analysis revealed similar trends in metals (Hg, As, Cr) and phthalates (DEHP, DBP, DEP) in both sexes in the Epe population. Multivariate depictions from the PCA showed sex-specific patterns of metal uptake (Cd) in male fishes at the Lagos Lagoon. The positive association between higher pH loadings and metal and DBP levels in sediment at the Lagos lagoon suggests the influence of higher alkalinity in lower bioavailability of contaminants.

Conclusion: Endocrine disrupting effects were observed in male and female *Chrysichthys nigrodigitatus* in Lagos and Epe lagoons populations, with notable differences in hormone and contaminant concentrations between the two

lagoon systems. Identification of specific contaminants and their spatial and temporal trends can inform targeted management and remediation efforts to protect and restore these valuable aquatic ecosystems.

KEYWORDS

environmental estrogens, *Chrysichthys nigrodigitatus*, vitellogenin protein, gonadal alterations, intersex, endocrine disruption

Introduction

The pollution of aquatic ecosystems and implications for local and regional fisheries are issues of important societal and scientific concerns because this has direct bearing on the sustainability of ecosystem services (Häder et al., 2020). Some of the most significant and far-reaching deleterious effects have been attributed to the introduction of chemicals with endocrine disrupting effects into waterways through anthropogenic activities and the resultant exposure of fauna populations in affected aquatic systems (Adeogun et al., 2016b). Adverse impacts have been noted at minimal concentrations of endocrine-disrupting chemicals (EDCs) in the environment, underscoring the potential risks associated with exposure (Walf et al., 2011; Windsor et al., 2018). While reports of endocrine-disrupting effects in fish have demonstrated marked hormonal imbalance by mimicking endogenous estrogen (Rehberger et al., 2020), effects in gonochoristic fish (separate sexes) has also emerged as a concern (Muller et al., 2020). The spectrum of EDC-related effects includes the disrupted process of sexual differentiation and altered gonadal development resulting in temporary or permanent damages to reproductive systems (Naderi et al., 2014; Luzio et al., 2016) including the development/incidence of intersex individuals within exposed populations (Barnhoorn et al., 2010). Other markers of endocrine disruption reported within fish species include modulated production of sex hormones and vitellogenin (Adeogun et al., 2016b; Ibor et al., 2016).

Several studies have indicated a correlation between hormone dynamics, stages of gonadal development, and fish maturation events (Maitra et al., 2013; Díaz and Piferrer, 2017; Amenyogbe et al., 2020). EDCs including metals and phthalates entering aquatic habitats from diffuse sources interfere with hormonal systems and impact the reproductive health of local populations (Gonsioroski et al., 2020). The expression of liver-synthesized vitellogenin (a female-specific precursor protein) in male and juvenile fish is considered an important physiological indicator of endocrine disruptive effects (Hara et al., 2016). As such, the expression of this protein is considered a classical indicator of endocrine disruption in captive and wild fishes and has been linked with the presence of EDCs in several studies (Adeogun et al., 2016a; Ibor et al., 2016). Several reports have indicated that intersex, inhibited gonadal development and aberrations, alteration of sex steroid hormone and vitellogenin levels are related to exposure to endocrine-disrupting chemicals (Adeogun et al., 2019; Delbes et al., 2022).

Tropical lagoons including those off the Gulf of Guinea like Lagos and Epe lagoons are characterized by a wide biologically diverse and highly productive brackish stretch of lagoon systems surrounding the island of Lagos and impacted by unabated pollution incidence which has negative implications for survival of local fisheries (Olakolu and Chukwuka, 2014; Adeogun et al., 2015a). The presence of EDCs originating from various industrial and domestic activities in the southern-lagoon system of Nigeria has been reported (Adeyi, 2020; Jerome et al., 2020). The unabated and indiscriminate discharge of untreated effluents from these adjacent land-based sources into the Lagos Lagoon has also been documented (Adeogun et al., 2015b; Jerome et al., 2017a). The Silver catfish, *Chrysichthys nigrodigitatus* is one of the most landed fisheries from the southern-lagoon system of Nigeria (Abidemi-Iromini et al., 2019) and a species of interest following its habitat range within the Lagos and Epe lagoons and benthopelagic preference that ensures significant risks of contaminant uptake via considerable contact with sediment repositories (Olarinmoye et al., 2011; Kanu and Idowu, 2017). However, knowledge gaps on pollution risks for this catfish based on gross gonadal examination and implications for other similar lagoon species is considerably large. This study seeks to provide a comparative report of the relationship between the presence of EDCs and markers of endocrine disruptions in *Chrysischthys nigrodigitatus* populations exposed to phthalates and metals in Lagos and Epe lagoons. While seeking to uncover potential endocrine disparities and deepen our understanding of environmental influences on the reproductive health of *Chrysichthys nigrodigitatus* in the Southern-Lagoon system, we specifically hypothesize that *Chrysichthys nigrodigitatus* in Lagos and Epe Lagoons may exhibit sex-specific differences in hormone modulation, driven by environmental factors.

Materials and methods

Site description

Lagos lagoon is the largest of four lagoon systems in the Gulf of Guinea (Webb, 1958) and stretches for about 250 km from Cotonou in the Republic of Benin to the western edge of the Niger Delta. The lagoon includes the forest belt and receives several important large rivers such as Yewa, Ogun, Ona, and Osun rivers, draining more than 103,626 km^2 of the country and empties into the Atlantic Ocean (Figure 1A). The Lagos opening is the largest and forms an extensive harbor, which serve as the major outlet of fresh water from the lagoon system during the rainy season. The central body of the lagoon is located between longitude 3° 23′0° and 3° 40′0 E and latitude 6° 22′0° and 6° 38′0 N. The brackish region is a significant area of concern for the transportation of pollutants from both the hinterland and the immediate shores of the lagoon (Ajao and Fagade (1990). Due to the fact that the lagoon watershed encompasses both

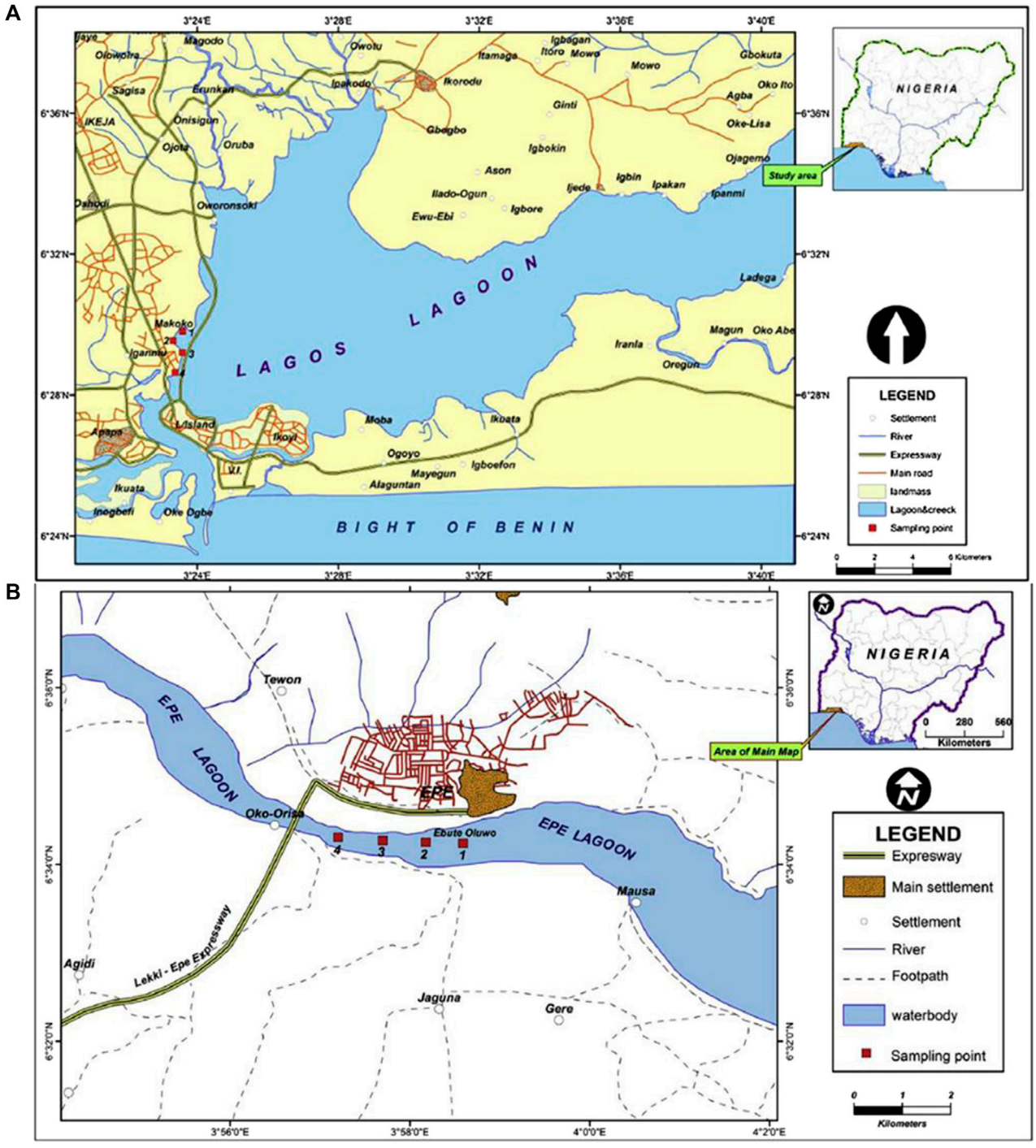

FIGURE 1
Map showing sampling sites. **(A)** Lagos lagoon. **(B)** Epe lagoon.

residential and industrial areas, it is frequently used as a dumping site for untreated anthropogenic effluent. (Jerome et al., 2017b).

The surface area of the Epe lagoon spans approximately 247 km^2, with a maximum depth of 6.4 m and shallow regions accounting for most of the lagoon and measures less than 3.0 m deep. It is situated between latitudes 6° 25′ and 6° 37′N, and longitudes 4° 00′ and 4° 15′E. The lagoon receives River Osun that drains a number of cities and agricultural lands (Figure 1B). The study area is bordered on the west by a number of cultivated lands and receives wood wastes from local wood processing industries located at the bank of the lagoon. The lagoon is used for transportation of timber logs (possible source of wood particles and leachates) from the villages to the city of Lagos and is the second largest contributor to the viable commercial artisanal fisheries of the southern lagoon complex. The lagoon houses a major jetty at Epe, where different forms of

anthropogenic wastes within and around the jetty are indiscriminately deposited (Edokpayi et al., 2010).

Sample collection

Fish and sediment samples were collected from the Makoko site of Lagos Lagoon where anthropogenic activities such as fishing and transportation are common as well as domestic waste discharge from residencies on this axis of the Lagoon. A long stretch of large sawmills and wood processing industries that dump sawdust into the lagoon at this site. Similarly, fish was collected at the Oluwo landing jetty of Epe Lagoon where several commercial activities and anthropogenic wastes can be found. A total of 195 samples were collected comprising 105 samples from Lagos Lagoon and 90 samples from Epe Lagoon with the aid of artisanal fishermen using a combination of gill and cast nets (mesh size 50–55 mm). This was done between 6–11 a.m. once a month from Nov. 2020 to May. 2021. Sediment samples were also collected in duplicates on-site using a van Veen grab at points close to where fish were sampled, wrapped with foil paper and kept at −20°C before extraction for contaminant analysis (Frias et al., 2018).

Blood samples were quickly collected on site from the caudal vein of *C. nigrodigitatus* using a sterile 2 mL syringe and transferred into a 5 mL Heparin vacutainer (Denslow et al., 1999). Serum was separated from the blood by placing the vacutainer in a standing position and the supernatant was placed in 1.5 mL cryogenic polypropylene Eppendorf tubes, preserved in ice, and transferred to the laboratory pending further analysis. Fish samples were tagged, preserved on ice, and transferred to the laboratory for further analysis.

Biometric analyses

Morphometric measurements

Morphometric parameters measured with an Ohaus digital meter (Mettler Instruments) and an Absolute Digital Caliper (Tresna Instruments) were standard length (SL), total length (TL), and Wet weight (W). Condition index was also calculated using the Fulton's formula.

Condition Factor (k) = 100 W/L^3 where W is wet weight (g) and L is total length (cm).

Gonad evaluation

Gonads were harvested, observed macroscopically, and classed into stages of development. As a gonochoristic species with distinct male and female individuals, sex identification relied on external and internal morphological characteristics (Rodriguez et al., 1997; Edem et al., 2021). Genetic analyses were omitted from the sex determination process. Acknowledging the potential influence of endocrine-disrupting chemicals (EDCs) on sex ratios and gonad morphology in species with genetic sex determination (Scholz and Klüver, 2009), this was recognized as a study limitation.

For histological examination, excised gonads were placed in Bouin's fluid for 72 h to enable tissue hardening (Culling, 1974). Gonads were then transferred to 10% phosphate-buffered formalin for preservation, dehydrated in a graded series of ethanol dilutions, and embedded in paraffin wax (Barnhoorn et al., 2010). Section of 5 µm was cut and stained with hematoxylin and eosin (H&E) and examined (Liu et al., 2018). Over the 7-month study duration, six individuals each (male and female per site) were sampled each month per site, and three sections were obtained for each sampled individual.

The gonadosomatic index (GSI), was calculated according to the equation: gonad weight/(body weight − gonad weight) × 100.

Plasma sex hormones and vitellogenin quantification

Sex hormones (17β-estradiol and testosterone) were measured in fish serum using ELISA kits from Randox Laboratories. For estradiol quantification, 15 µL of plasma samples and standard solution were added to a pre-coated microliter plate, incubated for 60 min, and washed before adding TMB. The color reaction was stopped, and the intensity of the color was measured at 450 nm using a Robonik 11–2,000 ELISA plate reader after 15 min. For testosterone quantification, 25 µL plasma sample were pipetted into pre-coated microliter plate, and HRP substrate was added 4 times before incubating at 37°C for 60 min. After washing the plate, TMB was added and further incubated for another 60 min at the same temperature. The color intensity (OD) was measured for 20 min after the addition of 1N HCl, at 450 nm using a Robonik 11–2000 ELISA plate reader.

In this study, a highly sensitive Fish Vitellogenin ELISA Kit (Bioassay Technology Laboratory) was procured to measure the serum levels of VTG in fish blood. The kit had a sensitivity of 0.55 µg/mL, allowing for precise measurements of VTG levels and the standards used in the study were prepared following the manufacturer's protocol. To ensure the reliability and validity of the VTG measurements obtained in this study, a standard curve was generated using the standards, which fell within the recommended range advised by the manufacturer (Supplementary Material SI).

Quantification of chemicals in fish tissue and sediment

The levels of five heavy metals/metalloids: Chromium, Cadmium, Lead, Arsenic, and Mercury, in sediment and fish muscle samples were quantified using Atomic Absorption Spectrophotometer (AAS). Three phthalic esters (DEHP, DEP, DBP) that were previously identified and quantified in sediment were included in chemical analysis (Adeogun et al., 2015b).

For the analysis of fish muscle samples, 2 g of flesh (wet weight) was weighed into an open beaker, and 10 mL of 1:1 Nitric acid – Hydrogen peroxide was added. The beaker was covered with a watch glass and left for 1 h. Subsequently, the beaker was placed in a water bath and heated gradually to 160°C, and the content boiled for about 2 h (Aderinola, et al., 2012). The digested sample was allowed to cool and made up to 25 mL with de-ionized water for AAS analysis.

Sediment samples were air-dried, pulverized and sieved with a 2 mm sieve. To this, 9 mL of Nitric acid (65%) and 3 mL of HCL (37%) in a ratio of 3:1 was added to 5 g of sediment sample, and the mixture was digested for 1 h at 160°C (Uddin et al. (2016) After cooling, the solution was filtered into a volumetric flask, and deionized water was added to make the total volume up to

TABLE 1 Distribution and abundance of *Chrysichthys nigrodigitatus* from Lagos and Epe lagoons.

	Male	Female	Total
Lagos lagoon	60	45	105
Epe lagoon	45	45	90
Total	105	90	

100 mL. The resulting sample solution was then transferred to sample bottles for analysis of metals (Hg, Cd, Cr, Pb, and As) using an Atomic Absorption Spectrophotometer (AAS). The recovery rates for the analyzed metals were within acceptable ranges: Mercury (Hg) demonstrated a recovery rate between 95% and 105%, Cadmium (Cd) ranged from 90% to 110%, Chromium (Cr) showed a recovery rate between 95% and 105%, Lead (Pb) fell within the range of 90%–110%, and Arsenic (As) exhibited a recovery rate between 95% and 105%. These recovery rates indicate a high level of accuracy in the analytical procedures employed for metal analysis in the sediment samples.

Phthalate sample preparation, extraction and analysis

Muscle samples weighing 10 g were collected and homogenized into a paste-like consistency using a glass mortar and pestle. The resulting mixture was then dried with anhydrous sodium sulfate, following the USEPA (2012) protocol. For the water samples, 200 mL was collected and spiked with butyl benzoate, and 6 g of sodium chloride was added to prevent persistent emulsion. Three portions of 25 mL dichloromethane (DCM) were used for extraction. To remove free fatty acid (FFA) interferences, further extraction with sodium carbonate was carried out. The organic extracts were then dried with anhydrous sodium sulfate, as described by Ogunfowokan et al. (2006). To extract sediment samples, the Soxhlet extractor was used. Approximately 5 g of sample was added to the extraction chamber, and 120 mL of DCM was added to a round-bottom flask. The mixture was heated for six to 8 hours or cycles for complete extraction, and the extracts were stored in a fume hood before clean-up to prevent loss of volatile extractable compounds Peterson and Freeman (1982).

Statistical analysis

Data were subjected to descriptive statistics, Students' t-test, one-way ANOVA. Statistical significance was considered at 0.05 levels of significance. In addition, Principal Component Analysis (PCA) was used to visualize the relationship between metals (fish muscle and sediment) and phthalates (DEHP, DBP, and DEP) in sediment (Adeogun et al., 2015b) for both Lagos and Epe Lagoon (SM II). Prior to the analysis, data transformation procedures, i.e., standardization were applied to ensure that all variables have the same weight in the analysis. The PCA was conducted using Statistica® version 12 software. The output of the analysis provided a visual representation of the relationship between metals, physicochemical properties and phthalate contaminants in the lagoons. All other analysis was achieved using SPSS®, and GraphPad Prism® 5.

Results

Biometric assessment

A total of 195 samples of *C. nigrodigitatus* were encountered in this study with Lagos Lagoon accounting for 53.84%, of the total population consisting of 60 males and 45 females while Epe Lagoon accounted for 46.15%, consisting of 45 males and 45 females (Table 1). Males from Lagos Lagoon were significantly longer and heavier than females, while for Epe Lagoon, males were heavier than females. The GSI was significantly higher in females than males across both lagoons (Table 2). Condition factor (CF) for males and females at two locations, Lagos and Epe revealed higher values in males than females at both locations. Additionally, condition factor at Epe was higher for both males and females compared to Lagos Lagoon.

Gonad pathology and intersex

Gross morphological examination of *C. nigrodigitatus* gonads showed the occurrence of gonad alteration in samples from both Lagos and Epe lagoons (Figure 2). A male fish gonad with one testis was observed in samples from Lagos Lagoon while an Intersex female showing a pair of ovaries alongside a testis was observed in Epe Lagoon samples. Pathological examination of gonads showed the occurrence of oocytes alongside testicular tissues in both female (Figure 3) and male gonads (Figure 4) of *C. nigrodigitatus* from Lagos and Epe lagoons respectively. Intersex was observed in 3.81% of Lagos (males: 50%; females: 50%) and 3.33% Epe (males: 33.3%; females 66.7%) lagoons fish population respectively. Other gonadal alterations ranged from empty seminiferous tubules to dead spermatids in males and distorted ovaries in females in these two lagoon ecosystems (Figure 4).

Serum hormone and vtg protein levels

Serum hormone levels showed sex-related variations and an indication of hormonal disruption in *C. nigrodigitatus* from Lagos and Epe lagoons with females having a higher testosterone level than males in both lagoons (Figure 5). Testosterone (ngL^{-1}) levels in female fish (2.20 ± 0.36) were slightly higher than males (2.16 ± 0.31) in *C. nigrodigitatus* from Lagos Lagoon. In Epe Lagoon, however, the serum testosterone levels were significantly higher in females (3.00 ± 0.71) than male (1.94 ± 0.48) fish (Figure 5A). For 17β-estradiol (E2: ngL^{-1}), females showed higher levels in both lagoons (Lagos: female: 9.24 ± 0.82; male: 2.02 ± 0.57; Epe: female: 7.07 ± 2.26; male: 4.60 ± 1.89) with Lagos Lagoon *C. nigrodigitatus* having a higher E2 level than fish from Epe Lagoon (Figure 5B). Vitellogenin protein (ngL^{-1}) levels also showed sex-related variations in *C. nigrodigitatus* from Lagos and Epe lagoons with the male having a higher Vtg level than females in both lagoons. This is also an indication of hormonal disruption given that vitellogenesis is a female-specific process (Figure 5C). Vitellogenin levels in males (0.28 ± 0.08) were significantly higher than in females (0.23 ± 0.02) in *C. nigrodigitatus* from Lagos Lagoon while in Epe Lagoon the Vtg levels were also higher in males (0.31 ± 0.03) than in female (0.28 ± 0.0.5) fish (Figure 5C).

Physicochemical properties

The mean and standard deviation of various water quality parameters across the two sites, Lagos and Epe Lagoons, are

TABLE 2 Total length (TL), body weight (BW) and gonad somatic index (GSI) of *Chrysichthys nigrodigitatus* in Lagos and Epe lagoons.

Location	Sex	TL (cm)	BW (g)	GSI	Condition factor
Lagos Lagoon	Male	28.28 ± 1.97^{a} (13.30–134.00)	160.44 ± 16.00^{a} (21.00–603.00)	0.21 ± 0.12^{a} (0.06–0.90)	0.79 ± 0.25 (0.72–1.47)
	Female	25.03 ± 0.91^{a} (15.8–41.8)	146.88 ± 20.12^{a} (31.00–715.70)	0.47 ± 0.37^{a} (0.10–1.20)	0.77 ± 0.06 (0.66–0.82)
Epe Lagoon	Male	28.12 ± 0.55^{a} (21.50–39.60)	207.02 ± 11.22^{b} (90.00–464.80)	0.11 ± 0.02^{a} (0.01–0.15)	0.90 ± 0.35 (0.75–1.64)
	Female	35.12 ± 6.74^{a} (15.8–41.80)	222.32 ± 12.31^{b} (130.00–580.00)	0.52 ± 2.75^{b} (1.02–9.30)	0.86 ± 0.09 (0.75–0.98)

Note: Values in the table are represented as mean ± standard deviation. Values within parentheses represent the range of the data. Values with different superscripts (a,b) within the same row are significantly different ($p < 0.05$).

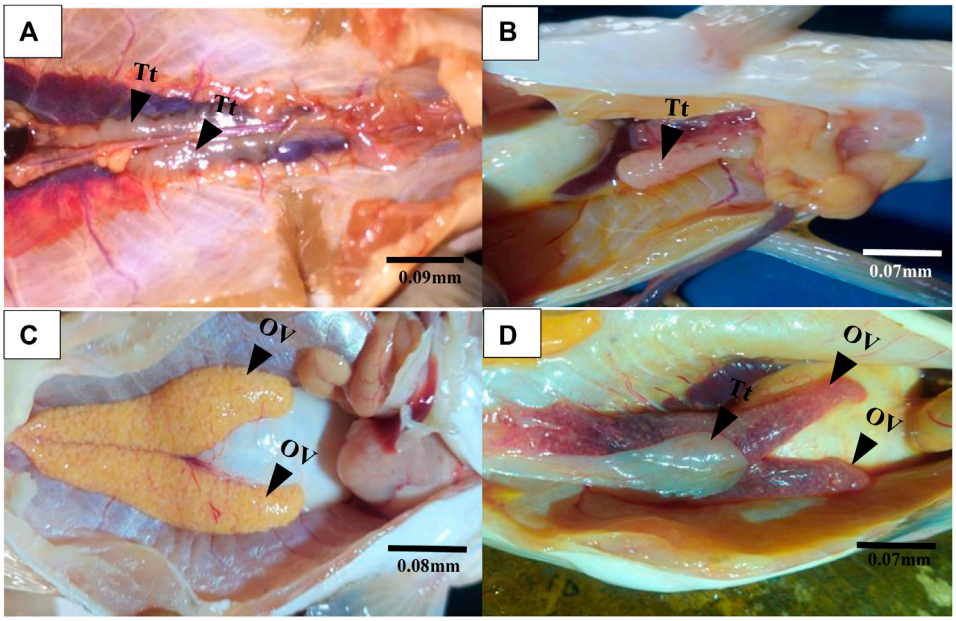

FIGURE 2
Gross morphological examination of gonads from Lagos and Epe lagoons. **(A)** Normal male *C. nigrodigitatus* from Lagos Lagoon with a pair of testis (Tt). **(B)** male *C. nigrodigitatus* from Lagos Lagoon with one testis (Tt). **(C)** Normal female *C. nigrodigitatus* from Epe Lagoon with a pair of ovaries (OV). **(D)** Intersex female *C. nigrodigitatus* from Epe Lagoon showing a pair of ovaries (OV) alongside a testis (Tt).

presented in Supplementary Material SII. In Lagos Lagoon, the pH value was 7.35 ± 0.01, indicating slightly alkaline water. The mean dissolved oxygen (DO) value was 10.33 ± 0.22, indicating well-oxygenated water. The conductivity value was 37.73 ± 0.1, relatively low, while total dissolved solids (TDS) was 32.83 ± 1.80, indicating low mineralization levels in the water. In contrast, Epe Lagoon had a lower pH value of 7.1 ± 0.17, indicating a wider variation in pH. The DO mean value was 3.97 ± 0.1, indicating relatively low levels of oxygen in the water. Conductivity values were much higher than that of Lagos Lagoon, with a value of 1,060.33 ± 1.80, indicating more mineralization in the water. The mean value of TDS was 549 ± 2.29, which was also higher than that of Lagos Lagoon (Supplementary Material SII).

Metals in sediment and muscle of *Chrysichthys nigrodigitatus* from Lagos and Epe lagoons

Table 3 shows the level of five toxicological relevant metals (Cr, Cd, Pb, As, and Hg: mg/g) measured in sediment from Lagos and Epe lagoons. Chromium levels were higher in Lagos Lagoon (0.21 ± 0.00) than in Epe Lagoon (0.19 ± 0.00). The levels of Cadmium were higher in Epe Lagoon (0.02 ± 0.00) than in Lagos Lagoon (0.01 ± 0.00). Lead levels were higher in Lagos Lagoon (0.42 ± 0.00) than in Epe Lagoon (0.39 ± 0.00). Arsenic levels were higher in Epe Lagoon (0.21 ± 0.02) than in Lagos Lagoon (0.14 ± 0.01). Mercury levels were also higher in Epe Lagoon (0.08 ± 0.01) than in Lagos Lagoon (0.04 ± 0.01).

Tissue concentrations of five toxicological-relevant metals (Cr, Cd, Pb, As, and Hg) and arsenic measured in male and female *Chrysichthys nigrodigitatus* from Lagos and Epe lagoons were generally higher in females compared to males for both Lagos and Epe lagoons (Table 4). Metal concentrations were also above the limits specified for food by Food and Agricultural Organization and World Health Organization (Joint FAO/WHO, 2011). In Lagos Lagoon females had higher Chromium levels (0.10 ± 0.12 μg/g) than males (0.02 ± 0.00 μg/g). Cadmium levels were higher in females (0.01 ± 0.00 μg/g) than in males (0.00 ± 0.00 μg/g). Lead levels were

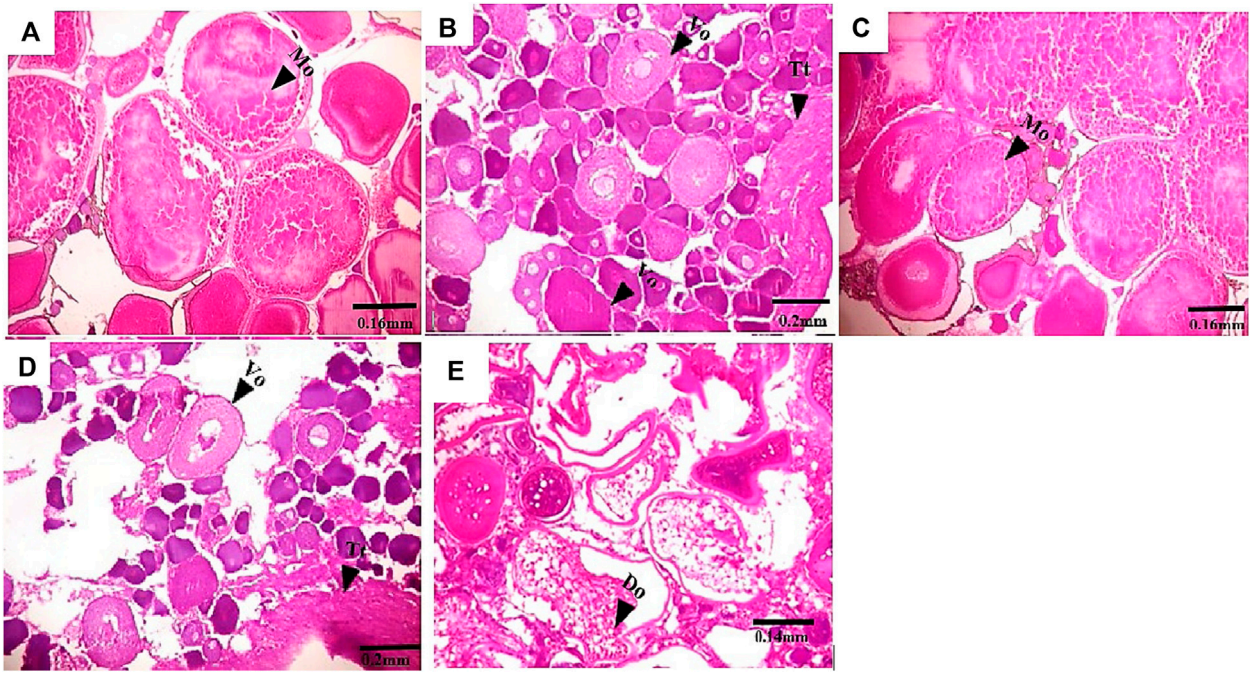

FIGURE 3
Transverse section of *C. nigrodigitatus* ovaries from Lagos and Epe lagoons. **(A)** Normal ovary from Lagos Lagoon with mature ovary (Mo). **(B)** Intersex female ovary from Lagos Lagoon showing vitellogenic oocyte (Vo) present alongside testicular tissues (Tt). **(C)** Normal ovary from Epe Lagoon showing mature oocyte (Mo). **(D)** Intersex ovary from Epe Lagoon showing Vitellogenic oocyte (Vo) present alongside testicular tissues (Tt). **(E)** Ovary from Epe Lagoon showing distorted ovaries (Do).

higher in males (0.35 ± 0.00 μg/g) than in females (0.17 ± 0.00 μg/g). Arsenic levels were higher in females (0.02 ± 0.00 μg/g) than in males (0.01 ± 0.01 μg/g). Mercury levels were also higher in females (0.02 ± 0.01 μg/g) than in males (0.01 ± 0.00 μg/g). In Epe Lagoon Chromium levels were higher in females (0.23 ± 0.00 μg/g) than in males (0.18 ± 0.00 μg/g). Cadmium levels were higher in females (0.01 ± 0.00 μg/g) than in males (0.00 ± 0.00 μg/g). Lead levels were also higher in females (0.44 ± 0.00 μg/g) than males (0.35 ± 0.00 μg/g) and females had higher Arsenic levels (0.03 ± 0.00 μg/g) than males (0.02 ± 0.00 μg/g). Mercury levels (0.02 ± 0.00 μg/g) were similar in both male and female fish from Epe Lagoon.

Multivariate analysis

The PCA analysis performed in this study generated five principal components (PCs) that captured most of the variance in the dataset. Among these PCs, PC1 accounted for 89.75% of the total variation in relationship between contaminant trends in fish and sediment of Lagos and Epe lagoon environments. Analysis of the variable loadings in PC1 revealed strong positive associations with two sediment phthalate levels (DEP and DEHP), metals (Hg male, As male, As female, Cd female, Cr male, Cr female, Pb female) in fish muscle and physicochemical parameters (Conductivity and TDS) in Epe lagoon while one phthalate (DBP) and metals in sediment (Cr, Pb, Hg) and muscle metal levels (Cd male) showed strong positive associations with Lagos lagoon environment. The positive values of these loadings suggest that higher concentrations of these compounds are associated with Epe lagoon (Figure 6) (SM 4). This implies that the use of phthalate-containing products may be more prevalent in Epe lagoon, leading to higher levels of phthalate contamination in the area.

While the Lagos lagoon was associated with high loadings of pH, conductivity and TDS were associated with high phthalate ester concentrations in Epe lagoon. The high loading of DPB sediment as the only phthalate with marked trends at Lagos lagoon (Makoko), while DEHP and DEP in sediments associated with Epe lagoon is notable. Overall, PC1 primarily captured variability in the concentration of phthalate esters and pH across the different samples (Figure 6). Furthermore, Lagos lagoon shows sex-specific trends in metal uptake where associations with high positive loadings for Cd in male *Chrysichthys* fish was observed. Epe lagoon on the other hand did not show sex-specific trends as this environment was associated with high loadings of both metals and phthalates in both sexes.

Discussion

Gross gonadal anomalies

Gross gonadal examination of *C. nigrodigitatus* from Lagos Lagoon revealed occurrences of gonadal anomalies including of intersex, enlarged gonad, and gonad with only one testis. Epe population on the other hand showed a singular occurrence of a

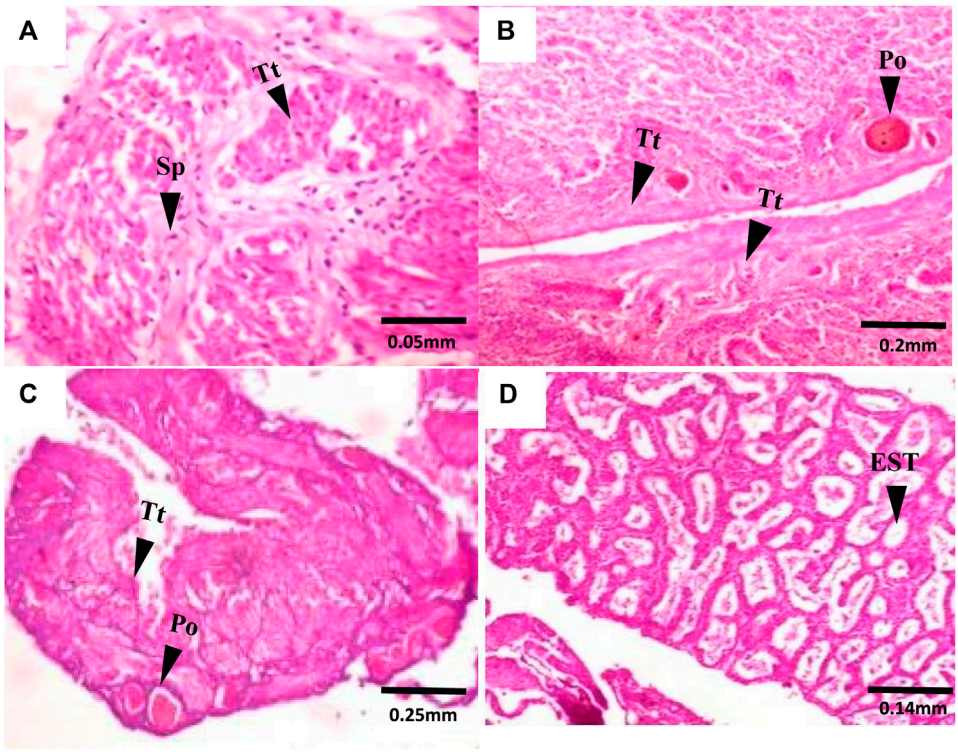

FIGURE 4
Transverse section of C. nigrodigitatus Testes from Lagos and Epe lagoons: **(A)** Normal testis from Lagos Lagoon showing testicular tissue (Tt) and Spermatid (Sp). **(B)** Intersex testis from Lagos Lagoon showing Primary oocyte (Po) present alongside testicular tissues (Tt). **(C)** Intersex testis from Epe Lagoon showing Primary oocyte (Po) present alongside testicular tissues (Tt). **(D)** Testis from Epe Lagoon showing empty Seminiferous tubule (EST).

female fish gonad consisting of a pair of ovaries alongside a testis. Similar phenomena have been documented in gonad of fish exposed to estrogenic wastewater effluents indicating exposure to xenobiotics (Woodling et al., 2006; Vajda et al., 2008). However, such co-occurrence of gonads in non-hermaphroditic species are suggestive of intersex due to endocrine disruptive effects (Adeogun et al., 2019).

Furthermore, lesions in gonadal tissue including empty seminiferous tubules, degenerating spermatids and distorted ovaries observed in this study may be linked to exposure of fish to estrogenic and testicular growth inhibition and degeneration agents (Kunz et al., 2006; Rey Vázquez et al., 2009). Empty seminiferous tubules may eventually affect the ability of male fish to produce spermatocytes which ultimately disrupts spermatogenesis. Fish with abnormal seminiferous tubules has been reported to not contain spermatocytes at any reproductive stage (Ünal et al., 2007). Furthermore, distorted ovaries and other ovarian abnormalities have been attributed to reproductive failure in fish (Nash et al., 2004; Abdel-Moneim et al., 2015). Fewer spermatogenic cysts in testis and much fewer mature follicles in ovaries of fish exposed to UV filter 3-benzylidene camphor have been demonstrated to have a significant effect on gonadal development, fertility and reproduction of fish with potential consequences at the population level (Kunz et al., 2006). Exposure of African catfish, *Clarias gariepinus* to EDCs in the wild has resulted in vacuolization of the seminiferous tubules, empty and disintegrated seminiferous tubules, degeneration of germ cells, and hypertrophy of Sertoli cells (Bhaisare et al., 2022).

Although the marginally higher intersex occurrence in Lagos Lagoon *C. nigrodigitatus* (3.81%) compared to Epe Lagoon (3.33%) confirms reports of endocrine disruption events among Lagos and Epe Lagoons fish populations (Adeogun et al., 2015b), it also suggest that the magnitude of effects due to pollution are similar for both lagoons. Intersex has also been reported in similar studies in which fish were exposed to EDCs from their natural habitat. Harris et al. (2011), reported the occurrence of intersex in Adult roach (*Rutilus rutilus*) from wild populations living in effluent-contaminated rivers in the United Kingdom. Adeogun et al. (2016b) reported 33% and 34% intersex and alterations in reproductive development of tilapia species from two municipal domestic water supply Lakes (Eleyele and Awba) in Southwestern Nigeria. Furthermore, studies on development in *Sarotherodon melanotheron* fish from Lagos lagoon showed a 27.4% prevalence of intersex, with severe evidence feminization of male fish (Adeogun et al., 2019).

Intersex patterns observed in this study is an indication that females had a higher occurrence when compared with males suggesting that females may be more susceptible to EDCs than males. This is consistent with the reports on benthic and pelagic fish from the Owan River in south-south Nigeria which recorded a higher incidence of intersex and gonadal anomalies in female fish (42%) compared to males (12%) (Chukwuka et al., 2019).

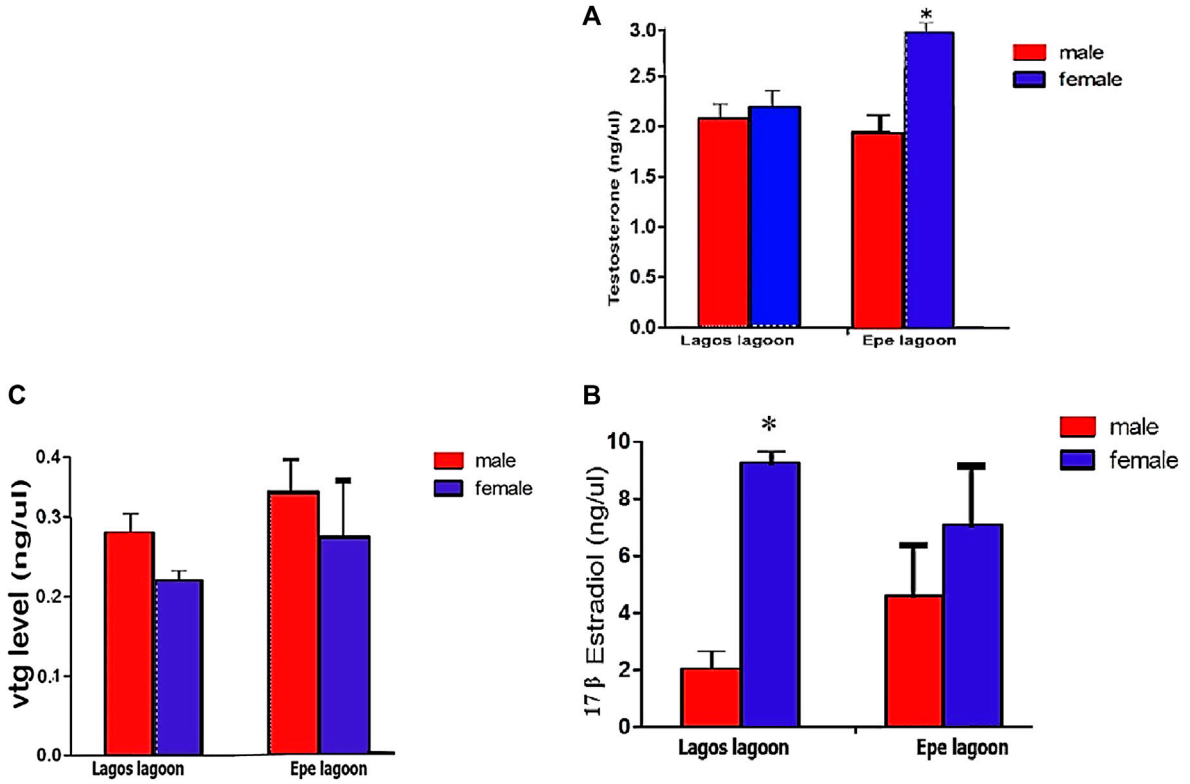

FIGURE 5
Concentrations of **(A)** Testosterone (ng/μL) **(B)** 17-βestradiol level (ng/μL) and **(C)** Plasma Vitellogenin levels in *Chrysichthys nigrodigitatus* from Lagos and Epe lagoons analyzed using Students's t-test. Error bars represent standard error of mean (SEM). The level of significance was set at $p < 0.05$. * significant difference between sexes.

TABLE 3 Concentration of heavy metals in sediment from Lagos and Epe lagoons.

Location	Cr (μg/g)	Cd (μg/g)	Pb (μg/g)	As (μg/g)	Hg (μg/g)
Lagos	0.21 ± 0.00	0.01 ± 0.00	0.42 ± 0.00	0.14 ± 0.01	0.04 ± 0.01
Epe	0.19 ± 0.00	0.02 ± 0.00	0.39 ± 0.00	0.21 ± 0.02	N/A
WHO, 1993	N/A	0.6	N/A	N/A	0.08 ± 0.01

Note: Different letters denote significant difference ($p < 0.05$) across different standards (μg/g). N/A = not available.

TABLE 4 Concentration of heavy metals in the muscle of *Chrysichthys nigrodigitatus* from Lagos and Epe lagoons.

	Chromium (Cr)	Cadmium (Cd)	Lead (Pb)	Arsenic (As)	Mercury (Hg)
Lagos M	0.02 ± 0.00^a	0.01 ± 0.00^a	0.35 ± 0.00^a	0.01 ± 0.01^a	0.01 ± 0.00^a
Lagos F	0.10 ± 0.12^a	0.00 ± 0.00^a	0.17 ± 0.00^b	0.02 ± 0.01^a	0.02 ± 0.01^a
Epe M	0.18 ± 0.00^a	0.00 ± 0.00^a	0.35 ± 0.00^a	0.02 ± 0.01^a	0.02 ± 0.00^a
Epe F	0.23 ± 0.00^a	0.01 ± 0.00^a	0.44 ± 0.00^a	0.03 ± 0.00^a	0.02 ± 0.00^a
Joint FAO/WHO 2011	0.005	0.03	0.5	0.02	0.005

Different letters denote significant differences ($p < 0.05$) across lagoons. M = male; F = Female. Joint FAO/WHO, standards (μg/g).

Biochemical evidence of endocrine disruption

The significantly higher 17β-estradiol level recorded in male fish from Epe Lagoon compared to male fish from Lagos lagoon indicates higher likelihood for feminization of male fish. On the other hand, the lower average levels of 17β-estradiol in Epe female fish compared to Lagos lagoon fish indicates masculinization of females. This possibility is corroborated by the higher male vtg levels compared to female and significantly lower testosterone levels in

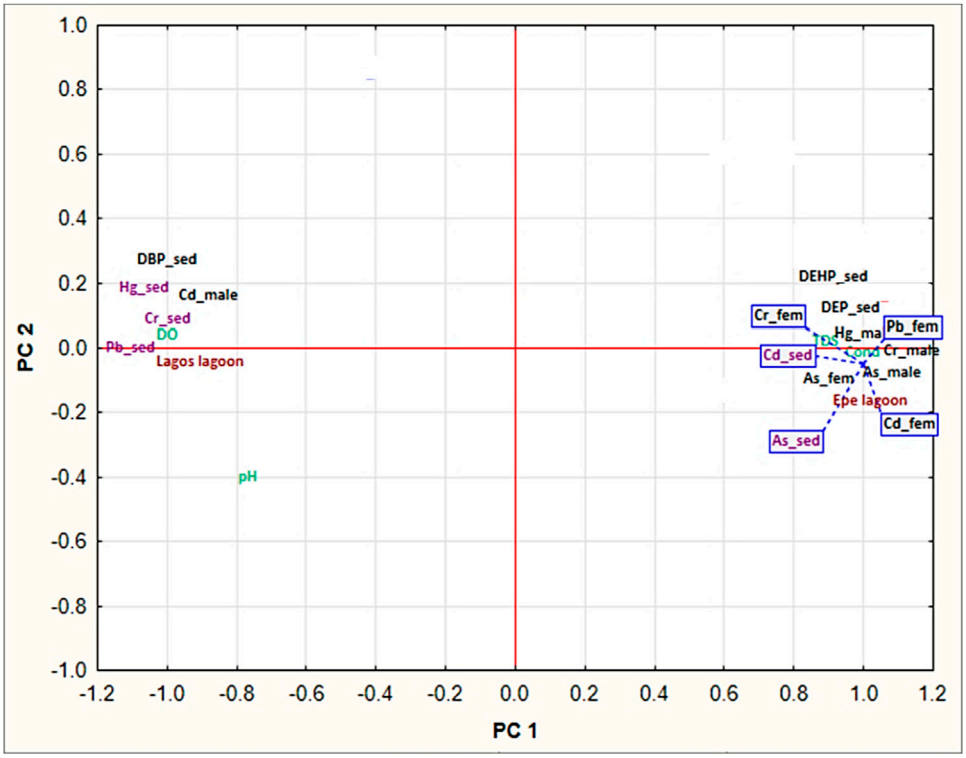

FIGURE 6
Principal component analysis (PCA) of metal and phthalate contaminants in fish muscle and sediment.

males compared to females. The relationship between elevated testosterone levels in female fish and development of intersex condition have been linked with xenoestrogenic effects (Jobling et al., 2003). Feminization in male fish has been also been associated with the presence of estrogenic substances in water (Kidd et al., 2007). Xenoestrogens exert its effect on sex differentiation by altering the expression level of steroidal receptors and steroidogenic enzymes.in addition to influencing steroidogenesis and steroid receptor expression. They also act directly on sex-determining genes and thus influence sex differentiation in gonochoristic species. Furthermore, female fish encountered in this study generally showed higher testosterone levels than 17β-estradiol with potential negative consequences on the quality of oocytes produced. Bugel et al. (2011) correlated decreased 17β-estradiol levels with inhibition of oocyte development and decreased sensitivity of the Vtg pathway.

While the testosterone and vtg levels in male and female fish from the Lagos lagoon showed abnormalities with no significant difference between them, the levels of 17β-estradiol were significantly higher in females than males, indicating that the mechanisms of endocrine disruption in fish populations in the Lagos and Epe lagoons may differ. The changes in 17β-estradiol levels are typically associated with endocrine disruption through estrogenic pathways (E Haut, 2005). However, the observed patterns in Lagos lagoon suggest the possibility of a different steroidogenic pathway, likely influenced by a distinct type of xenoestrogenic exposure. As xenoestrogens exhibit varying structural complexity and produce numerous metabolites or biodegradation products in the environment, they are capable of displaying a range of mechanisms of action (Kerdivel et al., 2013).

Multivariate relationships

The implications of the findings from the PCA analysis in this study are significant for ecological effects. The higher concentration of phthalate esters and metals in sediment at Epe lagoon may imply greater bioavailability, thus portending risks of reproductive failure, population declines and disruptions in aquatic food web. pH had a relatively high loading associated with Lagos lagoon in PC1 also has important ecological implications on the bioavailability and toxicity of metals and other contaminants in the aquatic environment. The higher loading for pH implies greater alkalinity at the Lagos lagoon site, which may explain why the Lagos lagoon site was predominantly associated with metals in sediments and DBP in sediment. Lower pH values can increase the solubility and toxicity of metals, while higher pH values can decrease their bioavailability and toxicity (Peng et al., 2009). In particular, pH dictates metal speciation, influencing their reactivity and toxicity. In acidic conditions, protons compete for ligand binding, yielding more toxic free metal ions, while alkaline conditions promote less reactive metal-ligand complexes or precipitates, reducing toxicity (Namieśnik and Rabajczyk, 2010). Furthermore, the association of high phthalate ester concentrations with conductivity and TDS suggests that these physicochemical parameters may play a role in the transport and fate of phthalate esters in the aquatic environment at the Epe site (Zheng et al., 2014). The elevated ion content, indicated by increased conductivity, has significant implications for the fate of phthalate esters by potentially enhancing their mobility and dispersion in the aquatic environment through the formation of ion-pair complexes, thereby affecting their solubility and facilitating movement through water (Dueñas-Moreno et al., 2022; Zhu et al., 2022). This heightened mobility and dispersion of phthalate esters in the aquatic environment,

facilitated by increased ion content and ion-pair complex formation, may elevate the risk of organism uptake, influencing their exposure to these contaminants (Sardiña et al., 2019).

Since phthalates are known to be endocrine disruptors that can affect the reproductive and developmental processes of aquatic organisms, leading to population declines and ecosystem instability, species at Epe may be at greater risks of reproductive toxicity that the Lagos lagoon site. On the other hand, the high loadings of metals in sediment samples from Lagos lagoon, particularly Hg_sed, Pb_sed, Cr_sed, and DPB_sed, suggest that the contamination of Makoko area of the Lagos lagoon for these metals is a site-specific feature. This further implies that exposures of resident fish fauna to this phthalate and metal species could be chronic, since sediment repository can ensure sustained exposures over time. These metals could eventually accumulate in the tissues of local fish populations, leading to toxicity and biomagnification in higher trophic levels.

To further understand the ecotoxicological implications of the findings, it is worth noting that the sex-specific trends observed in metal uptake in Lagos lagoon may be attributed to the ecological features or habitat terrain of the site at Makoko. The high positive loadings for Cd in male Chrysichthys fish suggest that male fish in Lagos lagoon may exhibit sex-specific ecological behavior, which can result in sex-specific contaminant uptake and toxicity (Jerome et al., 2017b). This is an important finding that underscores the need to consider sex-specific responses in ecotoxicological studies. By contrast, Epe lagoon fish did not show any sex-specific trends in metal uptake but was associated with high loadings of both metals and phthalates in both sexes. Overall, the PCA analysis highlights the complex relationships between environmental variables and contaminants in the Lagos and Epe lagoon systems, providing insights into the sources and pathways of contamination in these aquatic ecosystems. The association of conductivity and TDS with high phthalate ester concentrations in Epe lagoon suggests that these physicochemical parameters may be influencing the transport and fate of phthalates in the environment. High conductivity and TDS can increase the solubility and mobility of contaminants, leading to increased exposure and potential harm to aquatic organisms.

Conclusion

In this study we have demonstrated that pollution of Lagos and Epe lagoons may have negative effects on *Chrysichthys nigrodigitatus*, with phthalates and heavy metals identified as significant pollutants. The discharge of industrial and anthropogenic effluent containing these contaminants results in hormonal imbalances in male and female fish, as shown by disrupted steroid hormone levels and Vtg detection in males. Both male and female fish also experience gonad alterations. Therefore, this study provides insights into the sources and pathways of contamination in these tropical aquatic ecosystems, which may explain the site-specific occurrences of gonadal alterations in male and female fish. Targeted management and remediation efforts can be informed by identifying specific contaminants and their temporal and spatial trends.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because the fish was commercially available fish that was harvested by local fishermen for food in these locations and is not an endangered species.

Author contributions

OA: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing–original draft. AC: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing–review and editing, Writing–original draft. MI: Project administration, Writing–review and editing, Resources. AA: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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EDITED BY
Azubuike Chukwuka,
National Environmental Standards and Regulations Enforcement Agency (NESREA), Nigeria

REVIEWED BY
Pramita Sharma,
University of Burdwan, India
Rabindranath Majumder,
Tamralipta Mahavidyalaya, India

*CORRESPONDENCE
Simone Morais
✉ sbm@isep.ipp.pt

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Baseline data and associations between urinary biomarkers of polycyclic aromatic hydrocarbons, blood pressure, hemogram, and lifestyle among wildland firefighters

Bela Barros[1], Ana Margarida Paiva[1], Marta Oliveira[1], Sara Alves[2], Filipa Esteves[3,4,5], Adília Fernandes[2], Josiana Vaz[6,7], Klara Slezakova[8], Solange Costa[3,5], João Paulo Teixeira[3,5] and Simone Morais[1]*

[1]REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Porto, Portugal, [2]Instituto Politécnico de Bragança, UICISA: E, Unidade de Investigação em Ciências da Saúde: Enfermagem, Instituto Politécnico de Bragança Campus de Santa Apolónia, Bragança, Portugal, [3]Environmental Health Department, National Institute of Health Dr. Ricardo Jorge, Porto, Portugal, [4]Department of Public Health and Forensic Sciences, and Medical School, Faculty of Medicine, University of Porto, Porto, Portugal, [5]EPIUnit – Instituto de Saúde Pública da Universidade do Porto, Porto, Portugal, [6]CIMO, Instituto Politécnico de Bragança, Bragança, Centro de Investigação de Montanha Campus Santa Apolónia, Bragança, Portugal, [7]SusTEC, Instituto Politécnico de Bragança, Bragança, Sustec – Associate Laboratory for Sustainability and Technology in Inland Regions – Campus Santa Apolónia, Bragança, Portugal, [8]LEPABE-ALiCE, Departamento de Engenharia Química, Faculdade de Engenharia, Rua Dr. Roberto Frias, Porto, Portugal

Introduction: Available literature has found an association between firefighting and pathologic pathways leading to cardiorespiratory diseases, which have been linked with exposure to polycyclic aromatic hydrocarbons (PAHs). PAHs are highlighted as priority pollutants by the European Human Biomonitoring Initiative in occupational and non-occupational contexts.

Methods: This cross-sectional study is the first to simultaneously characterize six creatinine-adjusted PAHs metabolites (OHPAHs) in urine, blood pressure, cardiac frequency, and hemogram parameters among wildland firefighters without occupational exposure to fire emissions (> 7 days), while exploring several variables retrieved via questionnaires.

Results: Overall, baseline levels for total OHPAHs levels were 2 to 23-times superior to the general population, whereas individual metabolites remained below the general population median range (except for 1-hydroxynaphthalene+1-hydroxyacenaphtene). Exposure to gaseous pollutants and/or particulate matter during work-shift was associated with a 3.5-fold increase in total OHPAHs levels. Firefighters who smoke presented 3-times higher total concentration of OHPAHs than non-smokers ($p < 0.001$); non-smoker females presented 2-fold lower total OHPAHs ($p = 0.049$) than males. 1-hydroxypyrene was below the recommended occupational biological exposure value (2.5 μg/L), and the metabolite of carcinogenic PAH (benzo(a)pyrene) was not detected. Blood pressure was above 120/80 mmHg in 71% of subjects. Firefighters from the permanent intervention team presented significantly increased systolic pressure than those who performed other functions ($p = 0.034$). Tobacco consumption was significantly associated with higher basophils ($p = 0.01–0.02$) and hematocrit ($p = 0.03$). No association between OHPAHs and blood pressure was found.

OHPAHs concentrations were positively correlated with monocyte, basophils, large immune cells, atypical lymphocytes, and mean corpuscular volume, which were stronger among smokers. Nevertheless, inverse associations were observed between fluorene and pyrene metabolites with neutrophils and eosinophils, respectively, in non-smokers. Hemogram was negatively affected by overworking and lower physical activity.

Conclusion: This study suggests possible associations between urinary PAHs metabolites and health parameters in firefighters, that should be further assessed in larger groups.

KEYWORDS

firefighters health, biomonitoring, biomarkers of exposure, smoking, hydroxylated polycyclic aromatic hydrocarbons, biomarkers of effect

1 Introduction

The International Agency for Research on Cancer (IARC) has classified the occupational exposure as a firefighter as carcinogenic to humans (Group 1) (1, 2). Hazards include heat, noise, and exposure to fire emissions composed of a vast list of harmful pollutants (i.e., particulate matter (PM), polycyclic aromatic hydrocarbons (PAHs), and other volatile organic compounds (e.g., benzene), heavy metals, phthalates, perfluoroalkyl acids, organophosphorus insecticides, dioxins, flame retardants, etc.) (3, 4). Fire hazards can vary by country due to different types of burnt vegetation and materials, climate, forest area, construction materials, urbanization, and protective and preventive measures, which influence firefighters' exposure. Research has been emphasizing the importance of PAHs exposure due to their ubiquity, inherent toxicity, and because they are among the most abundant pollutants formed during wildfires, being of particulate relevance for wildland firefighters exposome (5, 6). IARC has classified some PAHs as known, probably, and possibly carcinogenic to humans (7). Also, the U.S. Environmental Protection Agency (USEPA) has included 16 PAHs in the list of priority pollutants (8). Exposure to PAHs has been associated with respiratory (9, 10) and cardiovascular problems (11–13). Available epidemiological studies have identified oxidative stress, systemic inflammation, hypertension, and atherosclerosis as the main pathological pathways involved in cardiorespiratory toxicological effects of PAHs (9, 13, 14). Apart from exposure during fire combat, PAHs have been identified in the air of the fire stations, off-gassing from stored personal protective equipment (PPE), dirty tools, and vehicles (15–17). Jackobsen et al. (18) suggested that a higher number of diesel-fueled vehicles at Norwegian fire stations, regular live fire training, and synthetic firefighting foams have contributed to increased carcinogenic exposure among firefighters. Moreover, lifestyle habits such as diet (e.g., grilled, barbecued, and smoked meat), tobacco consumption, second-hand smoking, cooking, and traffic pollution also contribute to the total PAHs exposure burden (19–21). Once absorbed by the human body via inhalation, ingestion, or/and dermal contact, PAHs are distributed, metabolized, and mainly excreted through urine and feces (22). Biomonitoring is a useful tool to assess occupational exposure to pollutants, and urinary hydroxylated PAH metabolites (OHPAHs) are the most important biomarkers in the context of wildland firefighting (23). Moreover, the Initiative "Human Biomonitoring for Europe (HBM4EU)" has acknowledged PAHs as priority substances that need to be characterized in occupational and non-occupational biomonitoring studies of the European population (24). Therefore, to support the development of legislation and health-based guidance values for human biomonitoring, it is of utmost importance to characterize PAHs biomarkers. Besides hazardous exposure, firefighters are also under physical and mental stressors such as strenuous exercise under elevated temperatures, long work-shifts, anxiety, and sleep disturbance, which can further increase their susceptibility to disease development (25).

Associations between OHPAHs and blood cell alterations, e.g., sister chromatid exchange, have been reported for North American wildland firefighters [United States of America (USA) (26)]. DNA damage was reported in Portuguese wildland firefighters (27) and brain alterations in the South Korean firefighter's cohort (28). Moreover, hypertension (South Korea), inflammation [United Kingdom (UK) and USA], and cardiovascular disease (CVD) development (Canada, Denmark, USA, and South Korea) have been linked with firefighting activity (29–36). Besides being important biomarkers of disease diagnosis, hematological parameters can also be used for occupational health assessment (37–39). There is currently no information about the simultaneous evaluation of these three parameters, i.e., urinary OHPAHs, blood pressure, and hematologic status in firefighters, or their possible correlations. Furthermore, since male subjects are predominant in wildland firefighters, health effects in women have been poorly characterized.

Portugal is among the European countries most affected by wildfires, with over 10,000 forest fires registered in 2022 (40, 41). Current research on Portuguese firefighters is mainly related to exposure during fire combat activities (17, 42, 43). Additionally, PM-bound PAHs have been found at fire stations at higher levels than outdoors due to inadequate layout, building materials, internal ventilation profile, parking of firefighting vehicles in closed garages with direct access to the main buildings, and storage of contaminated PPE and tools without proper cleaning procedures or air-extraction systems, which all contribute to occupational exposure at the headquarters (16, 44, 45).

Thus, this study characterizes the baseline levels (i.e., with no recent participation in firefighting activities) of OHPAHs in Portuguese wildland firefighters and provides a general assessment of their current health status (including blood pressure, cardiac

frequency, and hematologic parameters) and lifestyle choices that can influence their performance and health risks. Statistical associations between the studied (bio)markers and these characteristics were explored while subgrouping by smoking status and exploring gender differences.

2 Materials and methods

2.1 Study population

This cross-sectional study characterized firefighters from the Northern region of Portugal, one of the most affected by large and intense wildfires (additional information in Supplementary material S1.1). Firefighters from all the cities and towns of this region (i.e., Alfândega da Fé, Bragança, Carrazeda de Ansiães, Freixo de Espada à Cinta, Izeda, Macedo de Cavaleiros, Miranda do Douro, Mirandela, Mogadouro, Torre de Dona Chama, Vila Flor, Vimioso, and Vinhais) were enrolled in the study (Figure 1), which was carried out in accordance with the Declaration of Helsinki (approved protocol – Report Nr. 92/CEUP/2020 – by the Ethics Committee of the University of Porto). The subjects signed an informed consent and completed a structured questionnaire [adapted from World Health Organization (46)] self-reporting biometric characteristics (age, gender, weight, height), lifestyle (smoking, alcohol, diet within the last week, and physical activity), clinical information (medication, and existence of diagnosed diseases/health symptoms), firefighter information (years of service, career categories, signed off work leaves due to fire suppression, job tasks, mean work hours, etc.), and environmental/occupational exposure. Firefighters who acknowledged being diagnosed with neoplasia, respiratory or cardiovascular disease and having participated in firefighting activities within the last week were excluded. Given the importance of smoking on the urinary OHPAH levels, individuals were further organized into two groups: S (current smokers), and NS (non-smokers). Since there was a low participation of female firefighters, no further subdivision was made.

2.2 Sampling

Biological sample collection was performed during the pre-fire season (July 2021 and June 2022), i.e., during a period when firefighters had not been involved in fire combat activities for at least 7 days. Each firefighter collected a spot urine sample into a sterilized polycarbonate container. A health professional collected the blood samples following WHO guidelines (47). Transportation of collected samples was operated as requested by WHO specifications (48). Transportation and manipulation of blood samples were done on the same day of collection within 3–4 h after venipuncture, whereas urine samples were stored at −20°C until analysis.

The blood pressure measurement was executed following the regulations published by the Portuguese National Health Service (49) at the fire station in a calm and welcoming environment to ensure the correct performance of this practice. The participants were asked to be seated and relaxed for at least 5 min, to have previously emptied their bladder and refrained from smoking or ingesting stimulant drinks such as coffee in the last hour. Eating before the blood pressure measurement was not controlled.

2.3 Urinalysis of OHPAHs

Six urinary biomarkers of exposure to PAHs [1-hydroxynaphthalene (1-OHNaph), 1-hydroxyacenaphtene (1-OHAce), 2-hydroxyfluorene (2-OHFlu), 1-hydroxyphenanthrene

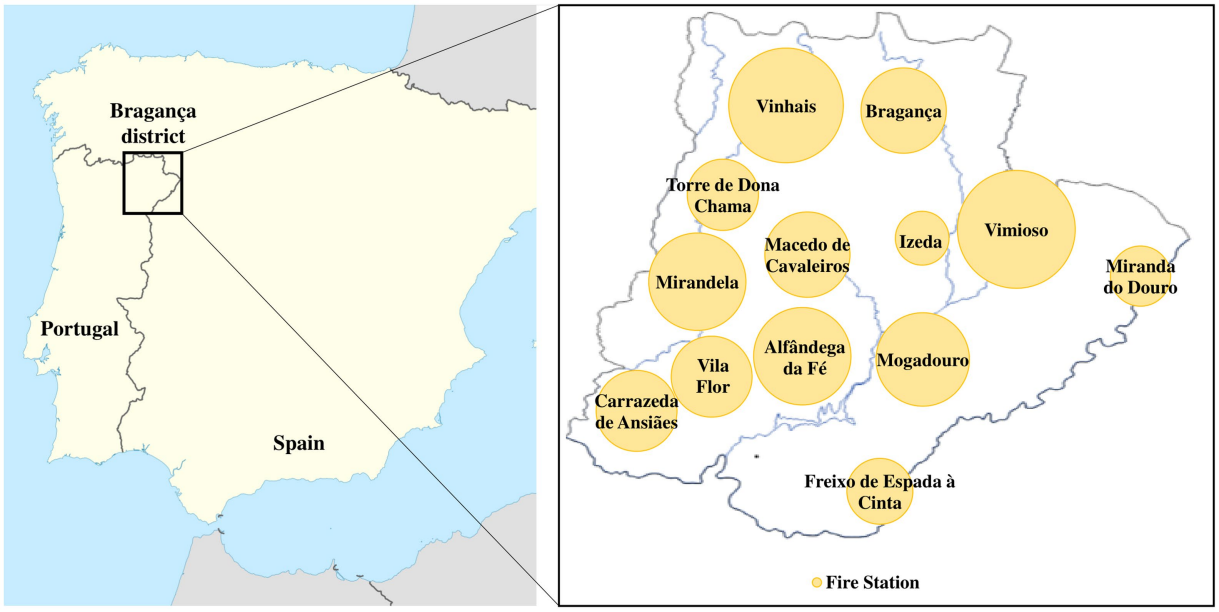

FIGURE 1
Bubble chart displaying the geographic distribution of firefighters for each enrolled fire station from the Bragança district (Northern of Portugal).

(1-OHPhe), 1-hydroxypyrene (1-OHPyr), and 3-hydroxybenzo(a) pyrene (3-OHBaP)] were determined. Their extraction and quantification were performed by solid-phase extraction and high-performance liquid chromatography with fluorescence detector based on previous studies (27). The limits of detection (LODs) varied from 0.018 (1-OHPyr) to 9.59 µg/L urine (1-OHNaph+1-OHAce) whereas the respective limits of quantification (LOQs) varied from 0.06 (1-OHPyr) to 31.96 (1-OHNaph+1-OHAce) µg/L urine (50). Daily blanks and standards were analyzed to check for inter- and intra-day instrument performance. Intra-day precision was assessed through the calculation of the relative standard deviation (RSD) of triplicate urine samples injections (range: 0.1–9.6%) while inter-day precision (reproducibility) was checked every day distributed over 1 month (RSD varied 5–23%). Methodology validation yielded a recovery of 70.0–117.0% (27). Concentrations of OHPAHs were normalized with creatinine levels (µmol/mol creatinine), determined by the Jaffe colorimetric method (51).

2.4 Hemogram

The determined (analyzer PentraES60, Horiba Medical Diagnostics, Montpellier, France) hematological parameters were as follows: red blood count (RBC; cells $\times 10^6$/µL), hemoglobin (HGB; g/dL), hematocrit (HCT;%), mean corpuscular volume (MCV; fL), mean corpuscular hemoglobin (MCH; pg), mean corpuscular hemoglobin concentration (MCHC; g/dL), red cell distribution width (RDW; %); platelet count (PLT; cells $\times 10^3$/µL) and mean platelet volume (MPV; fL), plateletcrit (PCT; %), platelet distribution width (PDW;%); white blood count (WBC; cells $\times 10^3$/µL), and differentiated cells percentage (%) and count (cells $\times 10^3$/µL): neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), atypical lymphocytes (ALY), and large immature cells (LIC).

2.5 Statistical methods

The statistical software SPSS (IBM statistics 29) was used. Whenever the concentration of a OHPAH was below its LOD, the concentration was replaced by its LOD divided by $\sqrt{2}$ for statistical purposes (52). Since normal distribution was not observed by Kolmogorov–Smirnov test ($p \leq 0.05$) for most (bio) markers, statistical differences were verified by the non-parametric Mann–Whitney U test for independent samples (or independent-samples Kruskal-Wallis Test for more than two categories). However, a normal distribution (Kolmogorov–Smirnov test, $p \geq 0.05$) was observed for body mass index (BMI), heart beats per minute, LYM (percentage and count), MON (count), RBC, HCT, PLT, PCT, and PDW for which independent samples *t* tests were applied. Differences and possible correlations of individual and total concentration of OHPAHs (ΣOHPAHs), blood pressure and hemogram within groups (NS versus S) and across categorical variables retrieved from the self-reported questionnaire data were explored by Spearman's rank correlation test. Statistical significance was defined as $p \leq 0.05$ (two-tailed). For those physiological levels that have different guideline values according to gender, results were shown separately for RBC, HGB, HCT and compared accordingly with their respective reference values available for each gender. Non-parametric tests, i.e., Mann–Whitney U test, were used to avoid sample size effects. The significance of the *p*-value was also set below 0.05, which reduces the likelihood of observing significant differences due to random chance.

3 Results

3.1 Study population

The enrolled 135 firefighters (median of 36.1 years old) were mainly male (86.7%) and presented 1 to 43 years of service (Table 1).

TABLE 1 Characteristics of the Portuguese firefighters.

	Non-smoker (*n* = 76)	Smoker (*n* = 59)	Total (*n* = 135)
Age (years), mean ± SD (median, min.–max.)	38.1 ± 11.3 (38.0, 20–65)	33.4 ± 10.1 (32.0, 19–60)	36.1 ± 11.0 (36.0, 19–65)
BMI (kg/m^2), mean ± SD (median, min.–max.)	27.4 ± 3.8 (26.7, 18.5–38.7)	27.2 ± 4.4 (27.3, 18.9–41.3)	27.3 ± 4.0 (26.7, 18.5–41.3)
Female (%)	13.2	13.6	13.3
Male (%)	86.8	86.4	86.7
Number of smoked cigarettes per day, mean ± SD (median, min.–max.)	n.a.	15.7 ± 8.9 (15.0, 1–50)	n.a.
Number of years as a smoker, mean ± SD (median, min.–max.)	n.a.	15.9 ± 10.4 (13, 2–45)	n.a.
Years of service as a firefighter, mean ± SD (median, min.–max.)	16.0 ± 9.9 (14.3, 2–39)	14.2 ± 10.6 (9.0, 1–43)	15.2 ± 10.2 (12.0, 1–43)
Work demands (yes, %):			
Mental	2.7	5.2	3.8
Physics	13.5	10.3	12.1
Both	83.8	84.5	84.1
Having a health problem during the last month (yes, %):			
Cough (many times)	6.8	5.1	6.1
Phlegm (most days)	5.5	20.3	12.1
Wheezing or chest tightness while breathing	4.1	6.9	5.3

Max., maximum; Min., minimum; n.a., not applicable; SD, standard deviation.

The NS group was composed of 76 subjects (56.3%, 66 males and 10 females), whereas 59 individuals (43.7%, 51 males and 8 females) were included in the S group. Firefighters smoked a mean of 15.7 ± 8.9 cigarettes per day (females tended to smoke less: median 10 cigarettes a day; $p = 0.093$), with a median smoking duration of 15.9 years (Table 1). Among NS, females presented significantly shorter firefighter careers than males, i.e., 8 versus 16.5 years of service ($p = 0.045$). The age range was similar, i.e., 20–65 years old for NS and 19–60 years old for the S group; there were no significant differences in the years of service (NS: 16.0 ± 9.9, S: 14.2 ± 10.6; $p = 0.160$) and mean BMI (NS: 27.4 ± 3.8 kg/m^2 [females had non-significant lower BMI: 24.7 versus 28.6 (males), $p > 0.05$], S: 27.2 ± 4.4 kg/m^2; $p = 0.879$) (Table 1). Moreover, 68.8% of firefighters were overweight (≥25 kg/m^2). Regarding physical activity, 22.6% of firefighters acknowledged not practicing exercise, 30.5% practicing sometimes a year, 38.3% weekly, and 8.6% daily (Supplementary Table S1).

Concerning firefighter hierarchical position and related activities, most individuals were 3rd grade firefighters (51.3%), followed by 2nd grade (18.8%), subchief (12.8%), 1st grade (10.3%), chief (4.3%), and other (2.5%). Most firefighters spend more than 8 h per day at the fire station (84.2%), performing a maximum of six different tasks (i.e., permanent intervention team (35.0%), driver (30.9%), paramedic (29.3%), administrative board, commander, rescue, diver, telephone operator, stock management, or other) (Supplementary Table S1). In this study, during their work-shift, subjects reported to be exposed to air pollution (gaseous pollutants and/or PM – 87.2%) and solvents (28.6%) on a weekly and/or daily basis (Supplementary Table S1). Few subjects lived near (~ 500 m) a factory (3.1%), industrial area (5.7%), or (~ 200 m) a farming area in which pesticides were used (27.3%) (Supplementary Table S1). Moreover, 84% of the subjects identified their activity as being both physically and mentally demanding (Table 1). Only 12.2% of subjects reported having a health problem during the last year, and 2.3% in the last 2 months. Few firefighters reported having cough, phlegm, wheezing, or chest tightness while breathing (5.1–12.1%), feeling more tired than other people of the same age (13.1%), and feeling shortness of breath while climbing a ladder (5.3%) within the last month. Also, 12.4% of the study population reported taking chronic medication (Supplementary Table S1).

3.2 Urinary biomarkers of exposure to PAHs

To decrease inter-individual variation due to urine dilution or differences in hydration status, creatinine adjusted levels of OHPAHs (μmol/mol creatinine) are presented in Figure 2A; unadjusted levels (μg/L) can be found in Supplementary Table S2. No differences were observed in creatinine according to smoking status ($p = 0.175$). All urinary OHPAHs were detected in 90–97% of samples, except for 3-OHBaP – metabolite of the marker of exposure to carcinogenic PAHs (7).

ΣOHPAHs in the S group reached a median value of 23.40 μmol/mol creatinine, significantly higher ($p < 0.001$) than in the NS group (7.87 μmol/mol creatinine) (Figure 2). In this study, 45.8% of subjects smoked more than 20 tobacco cigarettes per day. Moreover, moderate to strong positive significant correlations ($r = 0.366$–0.999; $p < 0.001$–0.004) were obtained between all urinary metabolites and the ΣOHPAHs within the S group (Figure 3A); this was not observed for all OHPAHs in NS, nor when all firefighters were included (Figure 3B), NS females presented 2-fold lower urinary concentrations of ΣOHPAHs ($p = 0.049$) than NS males.

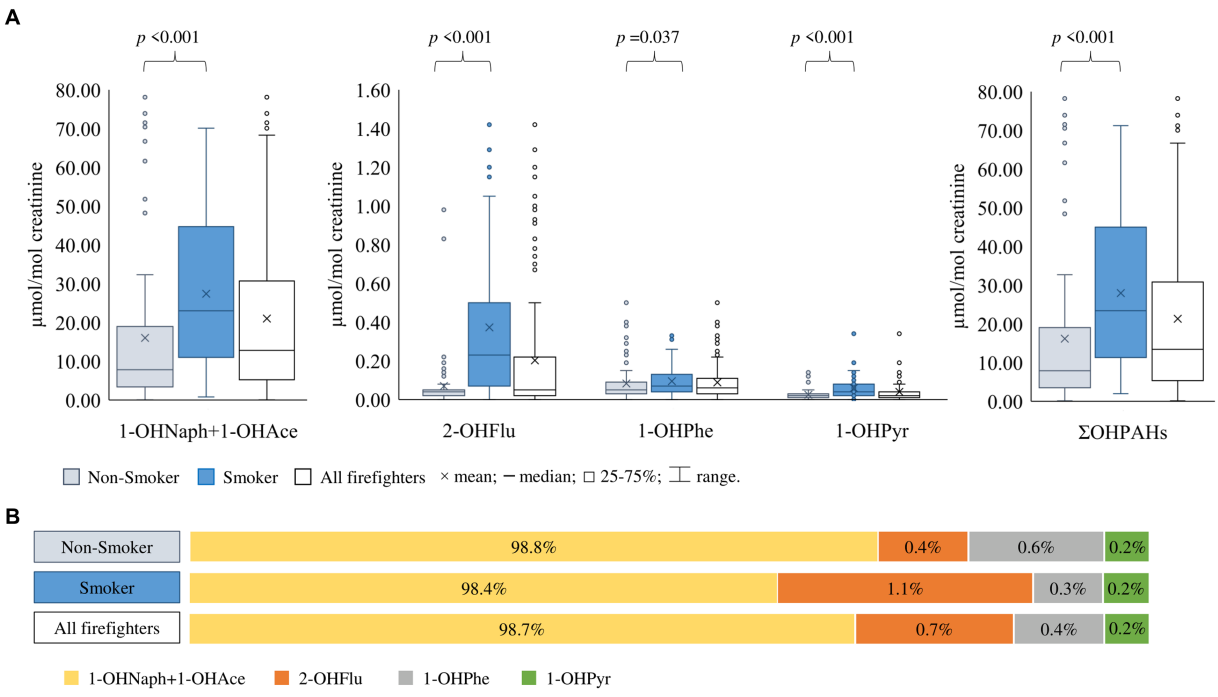

FIGURE 2
Individual and total concentrations of urinary PAH metabolites (∑OHPAHs) detected in firefighters (data expressed as μmol/mol creatinine) **(A)** and distribution (%) of the different OHPAHs **(B)** Statistical significance set at $p < 0.05$ using the Mann-Whitney U test for independent samples.

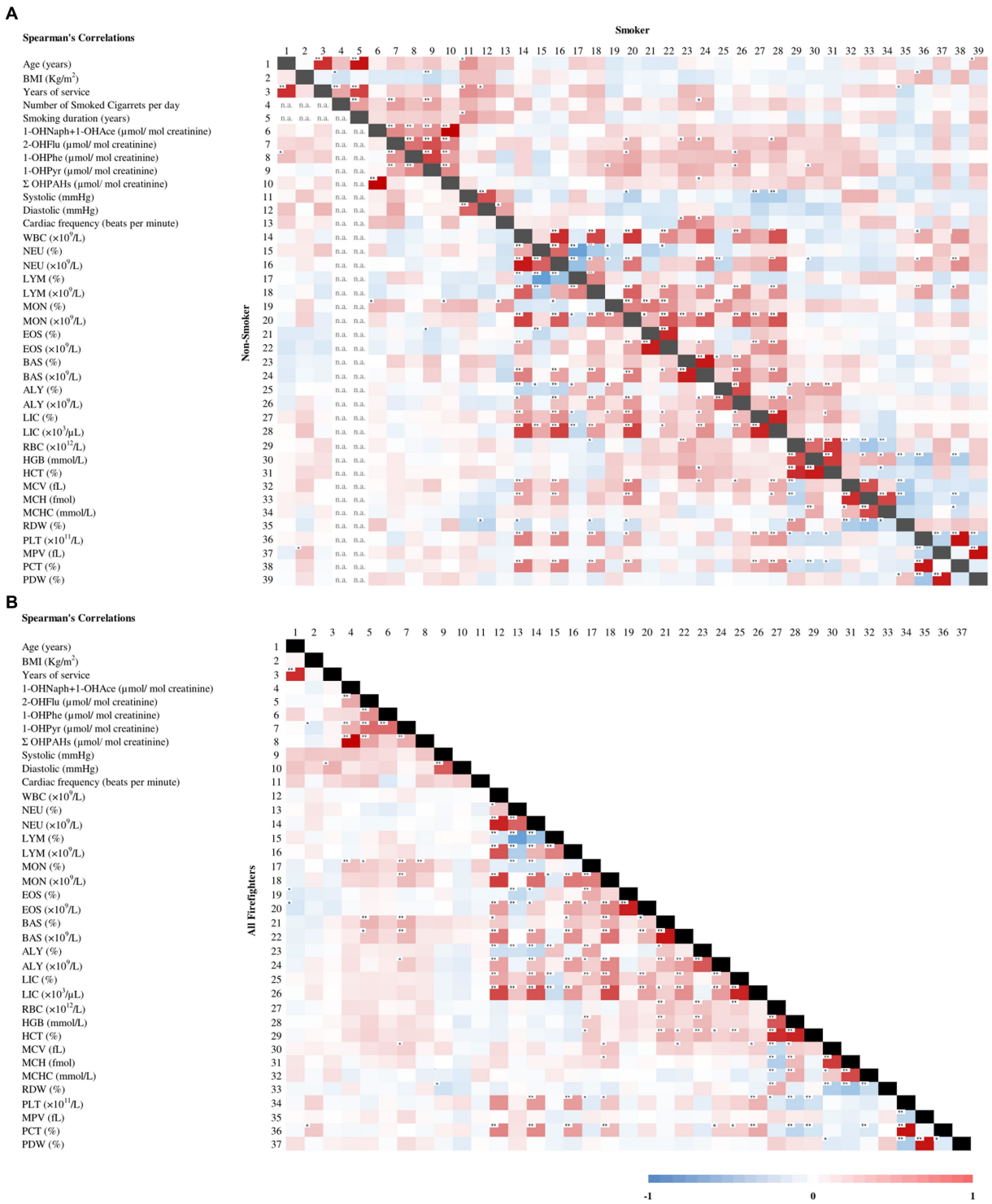

FIGURE 3
Spearman's rank correlation found between population characteristics, blood pressure, cardiac frequency, and the determined urinary and blood (bio) markers in **(A)** non-smoker and smoker individuals; and **(B)** all firefighters. 1-OHNaph+1-OHAce: 1-hydroxynaphthalene+1-hydroxyacenaphtene; 2-OHFlu: 2-hydroxyfluorene; 1-OHPhe: 1-hydroxyphenanthrene; 1-OHPyr: 1-hydrpxypyrene; ALY: Atypical lymphocytes; BAS: Basophils; EOS: Eosinophils; fL: femtoliter; HBG: Hemoglobin; LIC: Large immature cells; LYM: Lymphocytes; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; MON: Monocytes; MPV: Mean platelet volume; n.a.: Not available; NEU: Neutrophils; PCT: Plateletcrit; PLT: Platelet count; RDW: Red blood cell distribution range; WBC: White Blood Cell count. *Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed).

The most abundant OHPAHs (Figure 2B) were 1-OHNaph+1-OHAce (98.7%), followed by 2-OHFlu (0.7%), 1-OHPhe (0.4%), and 1-OHPyr (0.2%). Urinary PAHs' biomarkers were 3-, 6-, and 2-fold higher in the S group than in the NS group for 1-OHNaph+1-OHAce, 2-OHFlu, 1-OHPyr, respectively ($p < 0.001$; Figure 2A). 1-OHPhe median concentration had the lowest difference in the S group

compared to the NS group (40%), but it still reached statistical significance (0.07 versus 0.05 µmol/mol creatinine, $p = 0.037$; Figure 2A). Urinary 2-OHFlu ($r = 0.379$, $p = 0.003$) and 1-OHPyr ($r = 0.359$, $p = 0.006$) were significantly correlated with the number of smoked cigarettes per day (Figure 3A). Regarding gender differences, only 1-OHNaph+1-OHAce metabolite was borderline significantly lower in females in comparison to males (4.16 versus 8.57 µmol/mol creatinine; $p = 0.053$) within the NS group; no significant differences were observed for the other exposure biomarkers. Urinary 1-OHPyr was inversely correlated with BMI in all firefighters ($r = -0.187$; $p = 0.030$), especially in the S group ($r = -0.336$; $p = 0.009$). Interestingly, BMI was also negatively correlated with the number of cigarettes smoked per day ($r = -0.216$; $p = 0.042$).

The ΣOHPAHs was 3.5-fold higher in firefighters who acknowledged exposure to gaseous pollutants and/or PM during their work-shift in comparison to the ones who did not ($p = 0.006$; Supplementary Table S3). No differences in individual or ΣOHPAHs were found between subjects who indicated additional exposure to smoke in the 5–10 km surrounding their workplace and those who did not. However, for subjects who reported additional exposure within 5–10 km, differences in urinary 1-OHPyr concentrations were observed among firefighters from different fire stations ($p = 0.034$; Supplementary Figure S1). Subjects from the Bragança fire station presented 5-times higher median 1-OHPyr levels than those from Carrazeda de Ansiães (Supplementary Figure S1). However, based on the information gathered from the questionnaires, no significant associations were found (data not shown) with any of the variables representing potential sources of exposure (type of diet, heating use, candle lighting, and pesticide use).

3.3 Blood parameters

Blood pressure, cardiac frequency, hemogram parameters, and their respective reference values (considered normal for the Portuguese population) are displayed in Table 2.

Overall, median diastolic and systolic blood pressure were 134 and 85 mmHg, respectively. No differences were observed by gender. Blood pressure in firefighters exceeded the 120 and 80 mmHg guidelines for optimal blood pressure set by the Portuguese Hypertension Society (53). Among all subjects, 28% could be considered hypertensive. However, 71% of firefighters presented values higher than the considered optimal blood pressure; of these, 39% showed values corresponding to hypertension (≥140/90 mmHg). On the other hand, the median heartbeat was 71 beats/min for all firefighters, and only 18% presented a cardiac frequency below the normal range (60–100 heartbeats/min) (58); only 3% exceeded 100 beats/min (Table 2); no differences by gender ($p > 0.05$). No significant differences were found in systolic or diastolic pressure, and cardiac frequency between S and NS firefighters ($p \geq 0.09$; Table 2). Still, there was an almost 2-fold higher frequency of individuals with hypertensive measures (≥140/90 mmHg) in the S than in the NS group (36% versus 19%; Table 2). A significant positive correlation between systolic pressure and duration of smoking (in years) was found ($r = 0.419$; $p = 0.017$; Figure 3A), Additional significant positive correlations were found within the S group (Figure 3A), i.e., (i) age and systolic pressure ($r = 0.416$; $p = 0.016$); and (ii) both systolic and diastolic pressure with years of service ($r = 0.426$, $p = 0.013$; and $r = 0.357$; $p = 0.041$, respectively). Firefighters who were part of the permanent intervention team showed a 5% increase in systolic pressure in comparison to those who were not ($p = 0.036$; Figure 4), no significant differences were found for other variables retrieved from the questionnaires ($p > 0.05$).

Regarding hemogram parameters, the measured WBCs were within the normal range for the Portuguese population (55) for both NS and S groups (Table 2). However, the median percentage for the different leucocyte types was predominantly near (LYM) or slightly above the upper normal limit (MON and BAS), principally for smokers. An inversion was observed for the percentage of NEU since median levels were near the minimum normal percentage (Table 2). Female NS firefighters presented higher number of LYM (+30.6%, $p = 0.005$) and lower MON percentage (−5.3%, $p = 0.034$) at baseline. Women firefighters who smoke presented higher ALY (+28.6%, $p = 0.037$) than men. Even so, the median ALY percentage in the Portuguese firefighters were normal (0.90%; Table 2), and 61% of firefighters presented optimal ALY values. As for LIC, median percentage was below the recommended level (1%), but 12% of firefighters presented LIC above this percentage (LIC maximum of 2.60%). Nevertheless, the median number of LIC [0.05 (0.01–0.20) $\times 10^3/\mu L$] was below the reference values, i.e., $1.0 \times 10^3/\mu L$ (57). No other differences by gender were observed.

The maximum LYM concentration of $5.22 \times 10^9/L$ in the S group was above the set reference limit (Table 2). Moreover, besides smokers presenting a significantly higher BAS than non-smokers (28% higher BAS percentage, $p = 0.01$, and 40% BAS count, $p = 0.02$; Table 2), there was also a significant correlation between BAS count and the number of smoked cigarettes per day ($r = 0.281$; $p = 0.044$; Figure 3A). Although non-significant, there were also higher median leucocyte type counts in the S group in comparison to the NS, i.e., $3.82 \times 10^9/L$ versus $3.65 \times 10^9/L$ for NEU, $2.50 \times 10^9/L$ versus $2.32 \times 10^9/L$ for LYM, and $0.59 \times 10^9/L$ versus $5.40 \times 10^8/L$ for MON, respectively (Table 2). Firefighters who reported having exposure to smoke (within 5–10 km radius from their workplace) presented significantly higher NEU (+5.2%; $p = 0.026$) and lower LYM (−7.2%; $p = 0.036$) percentage than those who did not (Figures 5A,B). These associations were stronger among non-smokers (NEU: +10.1%, $p = 0.011$; LYM: −12.1%, $p = 0.017$; Figures 5A,B). A 16% reduction in LYM count was observed in firefighters who practice physical activity weekly versus sometimes/year ($p = 0.006$; Supplementary Table S3). Moreover, spending more than 10 h a day at the fire station decreased NEU (7%, $p = 0.038$) and increased LYM (11%, $p = 0.022$), when compared to spending 8–9 h (Supplementary Table S3). Lastly, subjects who were drivers had a significantly decreased percentage of MON (7%, $p = 0.032$) and ALY (10% $p = 0.021$) (Supplementary Table S3).

Median levels were within the normal range for RBC, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, MPV, PCT, and PDW (Table 2); no significant differences by gender were observed. Despite being within normal, irrespectively of gender, HCT percentage was significantly higher in the S group than in the NS group (48.10% versus 46.85%, $p = 0.03$) while RBC and HBG were borderline non-significant among these subgroups ($p = 0.05$ and $p = 0.07$, respectively; Table 2). A significantly negative correlation between RDW and years of service ($r = -0.313$, $p = 0.025$; Figure 3A) was found within the S group.

Work environment characteristics such as exposure to smoke, PM, and/or gaseous pollutants during firefighters' work-shifts were associated with a slight decrease in MCHC (1%; $p = 0.011$; Supplementary Table S3). Moreover, reporting solvent exposure during

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TABLE 2 Blood pressure, cardiac frequency, and hemogram characteristics of the studied Portuguese firefighters [data presented as median (minimum-maximum)] and *p* value of statistical tests for distribution differences between non-smoker and smoker firefighters (Independent-samples Mann–Whitney U test, unless indicated otherwise).

	Non-smoker	Smoker	*p* value	Total	Reference range
Blood pressure					(53)
Systolic (mmHg)	129.5 (114–170)	135 (109–209)	0.09	134 (109–209)	120
Diastolic (mmHg)	85 (69–113)	85 (70–128)	0.63	85 (69–128)	80
Cardiac frequency					
Beats per minute	69.5 (49–113)	73 (53–100)	0.61*	71 (49–113)	60–100
Hemogram					† (54);§ (55); ¥ (56); §: (57)
WBC ($\times 10^9$/L)	7.20 (4.40–15.50)	7.20 (4.40–12.70)	0.59	7.20 (4.40–15.50)	$4.5–11.0 \times 10^9$/L †
NEU (%)	55.40 (32.80–70.60)	54.60 (29.70–68.00)	0.42	54.90 (29.70–70.60)	54–62% †
NEU ($\times 10^9$/L)	3.65 (1.97–10.98)	3.82 (1.69–7.82)	0.69	3.78 (1.69–10.98)	$1.5–8.0 \times 10^9$/L §
LYM (%)	33.10 (20.40–54.40)	33.20 (19.30–56.80)	0.51*	33.20 (19.30–56.80)	25–33% †
LYM ($\times 10^9$/L)	2.32 (1.30–4.13)	2.50 (1.33–5.22)	0.21*	2.43 (1.30–5.22)	$0.8–4.0 \times 10^9$/L §
MON (%)	7.45 (4.00–13.90)	8.00 (5.40–11.10)	0.19	7.60 (4.00–13.90)	3–7% †
MON ($\times 10^9$/L)	0.54 (0.32–1.10)	0.59 (0.33–1.00)	0.12*	0.55 (0.32–1.10)	$\leq 1.2 \times 10^9$/L §
EOS (%)	2.20 (0.70–9.70)	2.30 (1.00–7.10)	0.58	2.20 (0.70–9.70)	1–3% †
EOS ($\times 10^9$/L)	0.16 (0.05–0.72)	0.17 (0.07–0.49)	0.52	0.17 (0.05–0.72)	$\leq 0.3 \times 10^9$/L §
BAS (%)	0.70 (0.30–3.10)	0.90 (0.40–10.60)	0.01	0.80 (0.30–10.60)	≤0.75% †
BAS ($\times 10^9$/L)	0.05 (0.01–0.19)	0.07 (0.02–1.24)	0.02	0.05 (0.01–1.24)	$\leq 0.3 \times 10^9$/L §
ALY (%)	0.90 (0.07–3.40)	1.00 (0.50–2.50)	0.11	0.90 (0.07–3.40)	n.a.
ALY ($\times 10^9$/L)	0.07 (0.03–0.80)	0.07 (0.04–0.25)	0.22	0.07 (0.03–0.80)	n.a.
LIC (%)	0.70 (0.30–2.60)	0.80 (0.30–1.40)	0.42	0.80 (0.30–2.60)	n.a.
LIC ($\times 10^3$/μL)	0.05 (0.02–0.20)	0.05 (0.01–0.18)	0.46	0.05 (0.01–0.20)	1.0×10^3/μL §
RBC ($\times 10^{12}$/L)	5.03 (4.07–6.05)	5.20 (4.26–6.37)	0.05*	5.06 (4.07–6.37)	
Male	5.01 (4.07–6.05)	5.16 (4.26–5.98)	0.17*	5.05 (4.07–6.05)	$4.3–5.9 \times 10^{12}$/L †
Female	5.06 (4.14–5.46)	5.39 (4.77–6.37)	0.08*	5.11 (4.14–6.37)	$3.5–5.5 \times 10^{12}$/L †
HGB (mmol/L) [a]	9.74 (7.76–1.92)	9.99 (8.19–12.85)	0.07	9.87 (7.76–12.85)	
Male	9.62 (7.76–11.92)	9.99 (8.19–10.92)	0.09	9.81 (7.76–11.92)	8.38–10.86 mmol/L †
Female	9.90 (7.88–10.74)	10.18 (9.12–12.85)	0.36	9.93 (7.88–12.85)	7.45–9.93 mmol/L †
HCT (%)	46.85 (37.00–54.70)	48.10 (39.00–61.80)	0.03*	47.50 (37.00–61.80)	
Male	46.75 (37.00–54.70)	47.95 (39.00–54.10)	0.09*	47.45 (37.00–54.70)	41–53% †
Female	47.25 (38.10–49.30)	48.70 (44.10–61.80)	0.12*	47.60 (38.10–61.80)	36–46% †
MCV (fL)	93.00 (73.00–103.00)	93.00 (75.00–100.00)	0.70	93.00 (73.00–103.00)	80–100 fL †
MCH (fmol) [b]	1.94 (1.43–2.20)	1.92 (1.43–2.14)	0.61	1.93 (1.43–2.20)	1.55–2.17 fmol †
MCHC (mmol/L) [a]	20.98 (19.05–21.97)	20.98 (18.99–21.53)	0.89	20.98 (18.99–21.97)	19.25–22.36 mmol/L †
RDW (%)	10.90 (9.80–5.60)	10.90 (9.70–13.10)	0.38	10.90 (9.70–15.60)	11.5–14.5% †
PLT ($\times 10^{11}$/L)	2.23 (0.54–3.46)	2.30 (1.13–3.56)	0.30*	2.24 (0.54–3.56)	$1.5–4.0 \times 10^{11}$/L †
MPV (fL)	9.10 (7.70–11.30)	9.10 (7.70–11.60)	0.45	9.10 (7.70–11.60)	6.5–12.4 fL §
PCT (%)	0.20 (0.05–0.31)	0.21 (0.10–0.30)	0.44*	0.21 (0.05–0.31)	0.10–0.50% ¥
PDW (%)	16.15 (11.00–21.30)	16.00 (11.80–22.50)	0.39*	16.00 (11.00–22.50)	10.0–18.0% ¥

* *p* value obtained by *t*-test for Equality of Means (significance < 0.05); ¥, these recommended values are set for Brazilian Portuguese general population; %, percentage; ALY, atypical lymphocytes; BAS, basophils; EOS, eosinophils; fL, femtoliter; HBG, hemoglobin; HCT, hematocrit; LIC, large immature cells; LYM, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MON, monocytes; MPV, mean platelet volume; n.a., not available; NEU, neutrophils; PCT, plateletcrit; PDW, platelet distribution width; PLT, platelet count; RBC, red blood cell count; RDW, red blood cell distribution range; WBC, white blood cell count. *p* value is a result from Independent-Samples Mann–Whitney U Test (significance < 0.05, in bold).

[a]Data were converted from g/dL to mmol/L.

[b]Data were converted from pg to fmol.

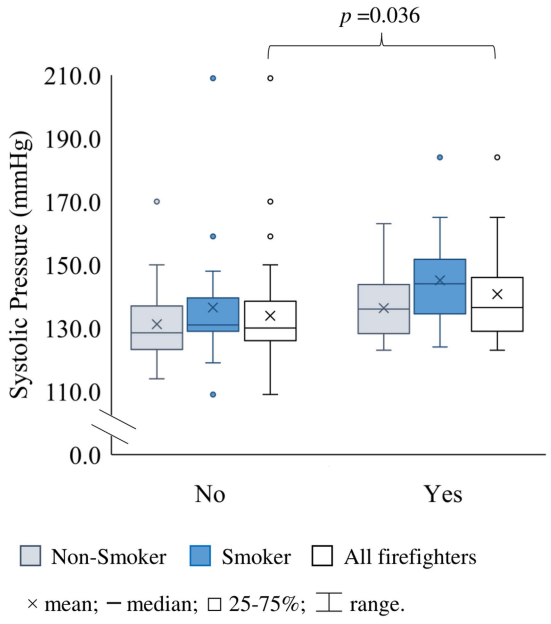

FIGURE 4
Systolic pressure distribution in Portuguese firefighters based on their inclusion in the permanent intervention team (No *versus* Yes) (data expressed as mmHg). Statistical significance set at $p < 0.05$ using the Mann-Whitney U test for independent samples.

the work-shift was associated with a decreased HGB (4%; $p = 0.009$) and increased PCT (10%; $p = 0.027$) (Supplementary Table S3). Being a driver was associated with a 7% decrease in HGB, a 5% reduction in HCT, and a 11% increase in PCT ($p \leq 0.021$; Supplementary Table S3). Significant differences in MCV (fL) and MCH (fmol) were only observed in NS firefighters who reported having exposure to smoke/air pollutants within a 5–10 km radius from work in comparison to those who were not exposed (MCV: +2.2%, $p = 0.013$; MCH: +3.7%, $p = 0.035$; Figures 5C,D). Regarding lifestyle characteristics, firefighters who exercise weekly presented significantly lower PLT count (−13%, $p = 0.005$) and PCT (−12%, $p = 0.006$) than those who exercise only sometimes a year, while the latter presented significantly reduced PDW (−8%, $p = 0.005$) and higher PLT count (+15%, $p = 0.019$) than firefighters who do not exercise (Supplementary Table S3). Additionally, BMI was positively correlated with PLT count in the S group ($r = 0.291$, $p = 0.034$; Figure 3A), MPV in the NS group ($r = 0.245$, $p = 0.044$; Figure 3A), and PCT in all firefighters ($r = 0.213$, $p = 0.009$; Figure 3B).

3.4 Correlations between biomarkers of exposure and health (bio)markers

No associations were found between PAH metabolites (individual or ∑OHPAHs) with blood pressure parameters (Figure 3). Considering hemogram parameters, a positive correlation between 1-OHNaph+1-OHAce and MON was found in non-smokers (Figure 3A) and all firefighters (Figure 3B), and because these urinary metabolites were the main contributors to ΣOHPAHs, the latter was also correlated with MON (Figure 3). For the other PAH metabolites, moderate positive correlations were found for all firefighters: (i) 2-OHFlu with MON and BAS ($0.199 < r < 0.297$; $p \leq 0.029$) and (ii) 1-OHPyr with MON, BAS, ALY, and MCV ($0.188 < r < 0.309$; $p \leq 0.039$) (Figure 3B). These correlations were positive and predominantly significant in the S group ($0.283 < r < 0.384$; $p \leq 0.042$ except for 1-OHNaph+1-OHAce and ΣOHPAHs, $p > 0.05$; Figure 3A). 1-OHPhe was correlated with MON ($r = 0.341$; $p = 0.013$) and ALY ($r = 0.310$; $p = 0.024$) for smokers only (Figure 3A). However, 2-OHFlu, 1-OHPhe, and 1-OHPyr were not correlated with any immunologic parameter in the NS group. On the other hand, an inverse correlation was observed for NEU and EOS and 2-OHFlu and 1-OHPyr, respectively, only in the NS group.

Interestingly, there were negative correlations between systolic pressure and MON and LIC in the S group, and systolic pressure with RDW without subgrouping (Figure 3), whereas diastolic pressure was negatively correlated with RDW in the NS group. Regarding cardiac frequency, the results displayed a positive correlation with MON and BAS in the NS and S groups, respectively (Figure 3A).

4 Discussion

4.1 Study population

This study characterizes firefighters contributing for a baseline profile of these workers without any exposure to fire combat activities. The frequency of smokers in Portuguese firefighters (47.3%) is comparable with the ones reported for USA firefighters in the last decades, i.e., 41.6–51.3% of smokers (59–61). The frequency of overweight firefighters (68.8%) is higher than what was observed for the general Portuguese population (55.9%) and the northern Portuguese population (58.6%) in 2019 (62). Available literature suggests an association between weight gain and working as a firefighter, specifically an increase of 0.5–1.5 kg per year (63, 64). Accordingly, 10 years of service could represent a weight augment of 5 to 15 kg. In fact, more than 60% of our study population had more than 10 years of service, which could help explain the high frequency of overweight individuals. However, these data should be considered with caution since recent studies have highlighted that the percentage of body fat is a better measurement of obesity than BMI (65, 66) mainly because weight can reflect a higher muscle mass rather than body adipocyte mass, thus leading to possible misclassification of obesity. Moreover, the self-reported low physical activity in most of the subjects (Table 1) is concerning because sedentarism has also been linked to higher cardiovascular risk among firefighters (11, 67).

4.2 Urinary biomarkers of exposure to PAHs

This study characterizes firefighters who did not participate in fire combat activities within the last week. This period was selected based on urinary excretion half-lives of 6–35 h (1-OHPyr via inhalation) and 2.3–23.5 h (1-OHNaph, 2-OHFlu, 1-OHPhe, and 1-OHPyr via ingestion), and 13 h (1-OHPyr via dermal contact) (3, 68, 69) yet inhalation and dermal route data is still limited to 1-OHPyr, and information regarding excretion rates based on all routes of exposure and possible chemical interactions in mixed exposures is lacking. The non-detection of 3-OHBaP has been previously observed since it is mainly excreted through feces (70). The median ΣOHPAHs is 2 to

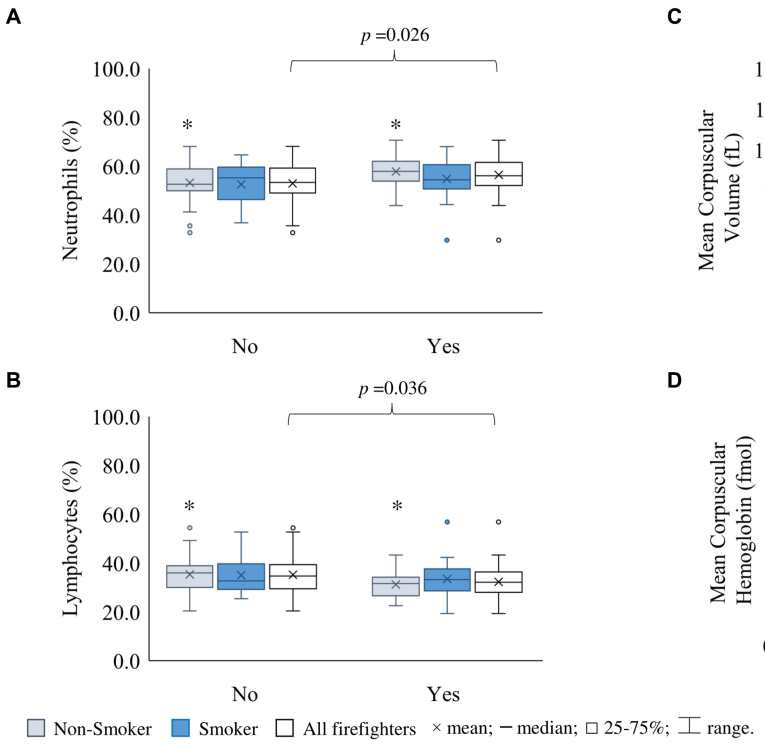

FIGURE 5
Hemogram parameters among firefighters who have acknowledged exposure to smoke/air pollutants in a 5-10 km radius from their workplace (No *versus* Yes). **(A)** Neutrophils (%); **(B)** Lymphocytes (%); **(C)** Mean corpuscular volume (fL); **(D)** Mean corpuscular hemoglobin (fmol). *Significant differences between "No" and "Yes" within the non-smoker group for **(a)** p = 0.011; **(b)** p = 0.017; **(c)** p = 0.013; **(d)** p = 0.035. Statistical significance set at $p < 0.05$ using the Mann-Whitney U test for independent samples.

14-times higher than the range of mean/median concentrations that have been reported for firefighters not exposed to fire emissions [0.95–6.96 µmol/mol creatinine; (69)]. For smokers, there is a known contribution from tobacco consumption to urinary OHPAHs (71–73). The significantly higher levels in smokers (Figure 2) could be related to the number and type of cigarettes smoked per day, since significant correlations between OHPAH and number of smoked cigarettes a day were also found, highlighting a common and prevalent route of exposure to PAHs in the S group. Regardless of smoking status, firefighters presented 5–8 times higher median creatinine adjusted ΣOHPAHs than non-occupationally exposed Italian population (74–77), whereas non-adjusted median values (µg/L) were 11 to 23-times higher than those for the Slovenian and Italian population (74, 78). The identified sources of exposure in these studies were diet, biomass-burning emissions from heating systems during winter, living near busy roads or near a waste-to-energy incinerator (74, 78). On the other hand, in the present study, subjects did not use biomass-burning heating systems during sample collection, nor was the fire station located near incineration centers, suggesting other potential sources of PAHs exposure for this population. As for other countries, Portuguese firefighters displayed ΣOHPAHs concentrations that were 2-fold above those reported by the USA National Health and Nutrition Examination Survey (NHANES: 2009–2016) (79), and 2-times below the ΣOHPAHs reported for the urban Wuhan-Zhuhai cohort in China (80). The latter populations were from heavily polluted cities (81), which helps explaining the high metabolite concentrations found in Chinese.

The sequence of most abundant OHPAHs (Figure 2B) is similar to what has been previously described in Portuguese and USA firefighters (27, 82–85). The significant difference found in individual OHPAHs according to smoking status (Figure 2) reflect the impact of smoking on urinary levels of these compounds. Moreover, the unequally significant distribution of 2-OHFlu concentrations between the NS and S groups (Figure 2B) suggests that tobacco smoke is a main source of fluorene exposure in the characterized Portuguese firefighters. The correlations found between 2-OHFlu and 1-OHPyr with smoked cigarettes per day, support the hypothesis that these two compounds are among the most selective biomarkers of tobacco consumption. These results are in accordance with Helen et al. (72), who also suggested that hydroxyfluorenes and 1-OHPyr may have discriminant power regarding smokers and non-smokers. Since no differences were observed in creatinine levels by smoking status, an influence of PAHs exposure in creatinine levels in association with cigarette smoking was not observed. Nevertheless, ongoing research has conflicting results concerning the influence on estimated glomerular filtration rate in smokers (86), thus further studies should aim to understand the impact of PAHs exposure in glomerular filtration rate and if impacts urinary creatinine clearance. In comparison to the general population, individual 2-OHFlu, 1-OHPhe, and 1-OHPyr (µmol/mol creatinine) in the Portuguese population [2-OHFlu: 0.13–0.16; 1-OHPhe: 0.06–0.08; 1-OHPyr: 0.04–0.06; (87)], the USA NHANES [2-OHFlu: 0.16; 1-OHPhe: 0.07; 1-OHPyr: 0.07; (79)], Italy [2-OHFlu: 0.07–0.11; 1-OHPhe: 0.05–0.10; 1-OHPyr: 0.02–0.04; (74–77)], and Germany [1-OHPyr: median range

0.16–0.38; (88)] were higher than those determined in the characterized Portuguese firefighters (median for all firefighters: 2-OHFlu: 0.05; 1-OHPhe: 0.06; 1-OHPyr: 0.02; Figure 2A).

1-OHPyr, considered the biomarker of exposure to PAHs, has an occupational biological exposure index of 2.5 µg/L proposed by the American Conference of Governmental Industrial Hygienists (89) and the guidance level of 1.0 µmol/mol creatinine for coke oven workers with a pyrene/benzo(a)pyrene ratio equal to 2.5 (90) which, adjusted for the studied firefighters, would be equivalent to approximately 0.59 µmol/mol creatinine. These values were not surpassed in this study (maximum of 0.35 µmol/mol creatinine and 1.63 µg/L; Figure 2A; Supplementary Table S2, respectively), suggesting that most firefighters should not experience any adverse health effects from pyrene/PAHs-related exposure. Moreover, median levels for 1-OHPyr (0.02 µmol/mol creatinine) were below those reported for firefighters from Denmark, German Sweden, and USA, at baseline level before exposure to fire emissions, i.e., 0.03 to 0.72 µmol/mol creatinine (69, 88). The inverse association between 1-OHPyr and BMI (Figure 3) was also observed in other studies due to the potential effects of nicotine on appetite reduction and/or augmented metabolic rate among smokers (91–95).

Multiple sources of PAHs have been identified at fire stations including, vehicle exhaust in unventilated garages, dust, and off-gassing compounds from contaminated PPE and tools (44, 45, 83, 96). A recent UK survey (>10,000 subjects) has revealed that only one third of firefighters cleaned their PPE after use, and both cleaned and contaminated gear were often stored together (97). Even though cleaning and storage practices were not included in this study's questionnaire, they are important to characterize possible sources of (air and surface) PAHs contamination at fire stations. On the other hand, a study conducted in 2014 (during the pre-fire season) has identified traffic emissions, lubricant oil use, and both fuel and biomass combustions as main sources of PAHs at Portuguese fire stations, which promoted lung cancer risks that exceeded 9 to 44-times the WHO-based guideline (98). For chronic PAHs inhalation, minimal risks levels for humans have only been reported for naphthalene [0.0007 ppm; (99)] by the Agency for Toxic Substances and Disease Registry and, based on studies on coke-oven workers, WHO has estimated the unit risk of genotoxic carcinogenicity for benzo(a)pyrene (has an indicator of ambient air PAHs) to be 8.7×10^{-5} per ng/m^3. Available European legislation established 1 ng/m^3 benzo(a)pyrene as the annual mean target value for human health protection (100). A previous study by Oliveira et al. (83) has quantified PM-bound PAHs at Portuguese fire stations from the same district. The reported values ranged from 46.4 to 428 ng/m^3 for total PM-bound PAHs in the personal breathing air zone of Portuguese firefighters, which air concentrations correlated with the sum of urinary PAHs' metabolites ($r = 0.367–0.886$). Occupational limits for total PAHs in the air has been set at 100 and 200 µg/m^3 by the National Institute for Occupational Safety and Health (101) and the North American Occupational Safety and Health Administration, respectively (102), the median levels in the personal air of Portuguese firefighters did not surpass both limits (83). In the present study, the main contributors to ΣOHPAHs were 1-OHNaph+1-OHAce, which parent compounds have been identified as the most abundant in indoor air at Portuguese fire stations (98). Moreover, at Polish fire stations, the highest naphthalene and acenaphthene air concentrations were found in the garage and changing rooms areas (103). Indeed, the combustion of diesel and gasoline, and the evaporation/sublimation of, crude oil, petroleum products, pest repellent, deodorant, and air fresheners have been identified as sources of naphthalene (104). Therefore, even without involvement in firefighting activities over the last week, firefighters' exposures to air pollution and solvents during their work-shift on a weekly and/or daily basis (Supplementary Table S1) could have influenced the levels of urinary OHPAHs concentrations. The observation of 3.5-fold higher ΣOHPAHs in firefighters who acknowledge exposure to PM and/or gaseous pollutants during their work-shift (Supplementary Table S3) agrees with previous studies that reported fire station contamination and firefighters' exposure to PAHs at their headquarters (without fire combat) in Australia, Portugal, and the USA (44, 83, 105). Therefore, suggesting that there must be other significant sources of exposure to PAHs at Portuguese fire stations. There is only a single ground air monitoring station within the Bragança district (Portugal) that quantifies outdoor benzo(a)pyrene, which data were not available during the campaign days (100). No difference in individual or ΣOHPAHs was observed in firefighters with and without additional exposure to smoke in 5–10 km from their workplace. However, Bragança firefighters had 5-times higher median 1-OHPyr levels than those from Carrazeda de Ansiães (Supplementary Figure S1). Despite the Portuguese inland districts such as Bragança having less intense traffic than those on the coastline (106, 107), the city of Bragança has a higher population density and far more trafficked roads (i.e., inside 2 km radius from the fire station there are four national roads and one main itinerary) than Carrazeda de Ansiães. Indeed, some studies have identified an increase in PM_{10} and $PM_{2.5}$ air concentrations in Bragança due to traffic pollution (108, 109).

Other sources of PAHs, e.g., diet (grilled/barbecued food, smoked meat), open burning, candle lighting, and house insecticides use (19, 21, 23) can influence the levels of biomarkers of exposure. Despite Portuguese people having a Mediterranean diet (110), the Northern region is known for increased smoked meat production and consumption. No differences were observed for OHPAHs according to diet choices, heating use, candle lighting, or pesticide use. Although cooking activities were not evaluated in this survey, a study has shown a 9-fold increment in ΣOHPAHs measured in Portuguese workers who performed grilling activities for 4.6 ± 2.2 h in restaurants compared to non-exposed workers (0.2–42.3 versus 0.097–1.66 µmol/mol creatinine), suggesting that it might also be a possible source of PAHs exposure (111).

4.3 Blood parameters

The European Society of Cardiology and the European Society of Hypertension have classified hypertension as a blood pressure measure at the physician's office that is above 140/90 mmHg (53). The frequency of hypertensive firefighters (28%) was lower than the 35–40% prevalence found for the Portuguese population (112), yet, within those with higher blood pressure (> 120/80), 39% presented a pressure ≥ 140/90 mmHg. It is important to notice that having one-time measured blood pressure ≥ 140/90 mmHg is not indicative of a hypertension diagnosis, but suggests an increased risk of hypertension, thus representing an important risk factor for cardiovascular disease development in the studied population. Available literature has described a higher prevalence of hypertension among males (113). No differences were observed by gender in firefighters; this observation could be due to the age of female firefighters (median 39 years old), which could indicate a

higher number of female subjects in their late 30s. At this age, hypertensive disorders related to pregnancy and female hormonal alterations (e.g., pre-menopausal state) that interact with the renin-angiotensin-aldosterone system can be associated with higher blood pressure (113). An augmented proportion of individuals with high blood pressure has also been reported among firefighters worldwide (USA, Canada, France, and South Africa), i.e., frequency of 11–69% (31, 114–116).

Smoking has been recognized as a risk factor for cardiovascular disease (53, 117, 118). However, no differences were observed between NS and S yet, the latter group presented a higher frequency of hypertensive subjects. Also, a significant correlation between blood pressure and years as a smoker was found, which has also been previously observed for non-occupational populations (118, 119). Moreover, moderate significant correlations were found within the S group, i.e., systolic pressure with age, and both systolic and diastolic pressure with years of service (Figure 3A). Therefore, these results are suggestive of an association between age, smoking duration, years of service, and blood pressure, which could cumulatively contribute to an increased risk for hypertension development in smoking firefighters.

The activities of a firefighter are predominantly stressful and can influence their physiological parameters (120, 121). The intervention team replies to any emergency call, e.g., accidents, fires, or disaster occurrences. It is possible that the permanent team members could experience higher stress levels due to the unpredictability of their workday, which could explain the higher systolic pressure found for firefighters from this team (Figure 4), yet more studies are needed to characterize this relationship.

The determined number of white blood cells (Table 2) is comparable with previously published data for UK firefighters and fire service instructors before exposure to fire combat training, i.e., 7.20×10^9/L versus 5.7–7.1 $\times 10^9$/L for WBC, 3.78×10^9/L versus 3.1–4.1 $\times 10^9$/L for NEU, 0.55×10^9/L versus 5.0–6.0 $\times 10^8$/L for MON, 0.17×10^9/L versus 1.0–2.0 $\times 10^8$/L for EOS, and 0.05×10^9/L versus 3.0–8.0 $\times 10^7$/L for BAS, respectively (34, 122). Concerning LYM, the number of cells was slightly higher in this study than in the range found in other UK studies for firefighters and fire service instructors [2.43×10^9/L versus 1.7–2.1 $\times 10^9$/L; (34, 122)]. However, these values remain in the normal range established for LYM count [<5.0 $\times 10^9$/L; (123)]. Interestingly, Watt et al. (122) reported leucocyte counts for control subjects with no heat exposure that were below those found in fire service instructors both pre- and post-exposure, suggesting a more active immune system in workers in comparison to controls, both at the baseline and after fire instruction drills. NS female firefighters had higher number LYM and lower MON percentage than NS male. García-Dabrio et al. (124) also observed higher LYM (CD3+ and CD4+) in Spanish healthy women in comparison to males while Varghese et al. (125) reported higher MON activity/recruitment in males in comparison to females. Smoking tobacco has been reported to be associated with elevated counts of NEU, LYM, MON, and/or BAS (126–130). The significantly higher BAS in S in comparison to NS firefighters support the association between smoking habits and mild basophilia among the studied population. Augmented ALY is an indicator of recent immune activity (usually towards a viral infection); values below 12% are normal, whereas optimal ones are below 1% (131). Since in this study women generally smoked less than men, the significance of having higher ALY among female smokers in comparison to males needs further study. Similarly, an increase in LIC is suggestive of recent development of viral infections and/or inflammation; in normal conditions, there are less than 1% of LIC in the whole blood (132, 133); only 12% of firefighters surpassed this percentage, yet median LIC number was within its recommended level (Table 2).

On the other hand, decreased indoor air quality due to higher air PM concentration has been highlighted as a potential factor for increased systemic inflammation (134). The observation of higher NEU and lower LYM among those who acknowledged having exposure to smoke (5–10 km radius from their workplace) suggests the contribution of an additional source of exposure, especially on the NS group (Figure 5). BAS are also important for allergenic responses, which can be associated with increased air pollution originated from urban traffic (135–137); higher BAS were observed in firefighters from Bragança, Mirandela and Vinhais that acknowledged exposure to smoke (5–10 km radius from their workplace; Supplementary Figure S2). As mentioned previously, within the district and, in comparison to Carrazeda de Ansiães, Bragança is a bigger city with higher road traffic. Similarly, Mirandela has an aeroclub, an industrial zone, and a highway, two national and one municipal roads within 2–4 km of the Mirandela fire station, which contributes to air pollution in the city (138). Regarding Vinhais, there is only a national road, which could mean that there might be other sources of health relevant agents in this fire station in comparison to Carrazeda de Ansiães and thus further studies are needed. Moreover, there seems to be a cumulative effect of smoking habits and other environmental exposures at the fire stations that might be related to the increase of blood BAS in firefighters.

Besides relevant exposure to exogenous agents, endogenous characteristics and lifestyle can contribute to altered immunological parameters (67, 71, 139). Less active firefighters presented lower LYM than those that are more active (Supplementary Table S3). Recurrent exercise has been associated with a decrease in LYM due to the migration of these differentiated cells out of blood during the recovery phase (140). Moreover, more hours of work were associated with lower NEU and higher LYM among firefighters (Supplementary Table S3). The NEU are the first line of defense against pathogens as part of the innate immune response, while LYM will appear later on as part of the adaptative immune response previously triggered by NEU activity (141). Stress conditions can upregulate adaptative immunity while recruiting innate response cell out of the blood stream (142). An altered distribution of leucocyte subsets was observed in students who endured acute academic stress (143). Furthermore, one of the causes of chronic stress is sleep deprivation, which can also alter the body's defense mechanisms (142). Night work-shifts were also associated with increased LYM in health workers (144). Consequently, firefighters who spend more hours at work can present cumulative stress and sleep pattern disruption/sleep deprivation due to night-shifts (145). Therefore, stress/sleep disruption could be a possible trigger for blood cell count alterations, yet to the best of the authors' knowledge, there are no studies that have explored the impact of working hours on hematological parameters in this occupational context. On the other hand, regarding firefighters' function, most drivers were non-smokers (56%) and physically active (69%), which could be one of the reasons why they displayed a lower percentage of MON and ALY. However, future studies need to better characterize the impact of job function on firefighters' health.

Concerning the erythrocyte and platelets in firefighters blood (Table 2), the available literature has reported similar mean values before exposure to fire combat training for South Korean fire service instructors, i.e., 4.9×10^{12}/L for RBC, 9.5–9.6 mmol/L for HGB, 45.2–46.3 ± 1.7% for HCT, 92.9–94.5 fL for MCV, 1.94–1.95 fmol for MCH, and 2.42×10^{11}/L for PLT (146), and in UK firefighters, i.e., 2.09×10^{11}/L for PLT (34).

Tobacco consumption can stimulate the production of red blood cells. Smoking is associated with the inhalation of carbon monoxide (CO). Once hemoglobin adsorbs CO, an irreversible reaction occurs, and erythrocytes can no longer transport oxygen. Thus, the renewal of these cells is stimulated and a consequent rise in their number can be observed in smokers (126) and corroborate the significant higher HCT, borderline non-significant higher RBC and HBG that was found in this study (Table 2). On the other hand, a reduction in the percentage of RDW by itself among smokers with less years of service (Figure 3A) does not have clinical significance. It must be crossed with other hemogram parameters, and depending on the results, the RDW could be used for differential anemia diagnosis (147). Smoking has been associated with higher RDW (147), yet, it is possible that smoking firefighters with a higher number of years of service (moderate/strong intercorrelation of smoking duration with years of service, and number of smoked cigarettes/day; Figure 3A), might produce more macrocytic RBCs, thus their size is more similar, and consequently, the subjects present a lower RDW with the increased number of years of service.

Trace smoke from fossil fuel vehicles exhaust (e.g., in closed garages), PPE storage rooms, and common areas with tobacco smoke may contain CO, which can irreversibly react with hemoglobin, thus a possible reason for the slight decrease in MCHC found for firefighters who acknowledged exposure to PM and/or gaseous pollutants during their work-shift (Supplementary Table S3). Also subjects who self-reported exposure to solvents had lower HGB and higher PCT, which was also observed for firefighters who were drivers (Supplementary Table S3). Similar findings have been observed in Pakistani auto-repair workers exposed to aromatic solvents (148). In fact, at fire stations, the lack of engine exhaust hoods in the garages can contribute to higher air levels of benzene, ethylbenzene, toluene, and xylene (149). Since some of the characterized Portuguese fire stations have a direct connection between the garage and the common areas inside the building, it is possible that the differences in HGB and PLTs are related to exposure to emissions from the heavy motor vehicles that can also cross-contaminate the air inside the headquarters. On the other hand, decreased indoor air quality can have hematopoietic effects (134). The finding of higher MCV and MCH in NS firefighters who self-report exposure to smoke within a 5–10 km radius from work (Figures 5C,D) suggests a possible association between decreased air quality due to smoke at the fire station surroundings and its contribution to larger red blood cells with a higher content of hemoglobin protein in firefighters.

Acute, strenuous exercise provided by a low frequency activity can lead to platelet activation, while regular physical activity can decrease/prevent platelet activation and favorably modulate platelet function (130, 150, 151). This could help to explain the findings of lower PCT and PLT among firefighters who exercise weekly (Supplementary Table S3), corroborating the positive correlations that were found between BMI and PLT (S group), MPV (NS group), and PCT (all firefighters) (Figure 3). A higher BMI has been related with platelet activation, thus, an associated inflammation and increased thrombosis risk (152–154). Therefore, these findings indicate that the characterized firefighters could benefit from including regular exercise in their routine to reduce cardiovascular disease risk. Accordingly, Durand et al. (155) reported that vigorous physical activity can reduce cardiometabolic profile parameters such as blood cholesterol-HDL ratio, triglycerides, glucose, and HDL increments in USA firefighters. Moreover, a recent study performed in male BALB/c mice observed that exercise can positively impact inflammatory cytokines and modulate gene expression related with REDOX imbalance induced by PAHs exposure (156).

4.4 Correlations between biomarkers of exposure and health (bio)markers

The lack of association between individual or ∑OHPAHs and higher blood pressure contrast with recent studies that reported significant correlations for in USA, Chinese, and South Korean petrochemical industry workers, coke oven workers, and chimney sweeps (12, 157). However, in this study, firefighters were not exposed to fire emissions for at least 1 week before sample collection, thus, lower metabolites concentrations were expected. Despite that, it is possible that the workers mentioned have far greater exposures at their workplace than firefighters have at their headquarters. A recent meta-analysis gathered information from available studies on the general population, which showed an overall meta-association between individual OHPAHs and blood pressure. However, after trim and fill analysis, no association was found (157). Also, subgroup analysis revealed an important contribution of USA and Asian ethnicity to the previous positive relationship found between urinary OHPAHs and blood pressure (157). Moreover, the high percentage of smokers and overweight subjects may pose some limitations to the obtained results in firefighters because a higher BMI, lower physical activity, and tobacco smoking are known risk factors for hypertension development.

Recent studies have associated PAHs exposure with inflammatory processes (21, 157–160). The obtained correlations found for 2-OHFlu (with MON and BAS) and 1-OHPyr (MON, BAS, ALY, and MCV) suggest a possible contribution from cigarette smoke exposure. Smoking is a recognized risk factor for cardiovascular disease development, whose mechanisms are mainly related with inflammation through increased leucocyte count and mutagenic alterations (127, 129, 151, 159, 161, 162). The negative correlations of NEU with 2-OHFlu and EOS with 1-OHPyr only in the NS group suggest that (without tobacco consumption as a variable for increased inflammation) there can be a possible smoking-independent negative impact of the two respective PAHs (fluorene and pyrene) on the first line of immune defense (NEU) and allergenic pathways (EOS). A recent study has identified a relationship between years of service as a firefighter and increased leucocyte epigenetic age (162). Since there were significantly older firefighters in the NS than in the S group and a strong correlation between years of service and age, it is plausible that these results (NEU and EOS) can also be age-related in the NS group. On the other hand, being a smoker, along with occupational exposure to air pollutants synergistically increases individual susceptibility to higher cardio-respiratory health effects, this is mainly through overstimulation of oxidative stress and inflammatory processes in the body caused by chronic exposure to toxic compounds, including PAHs (27, 72, 86, 161).

Inflammation has been associated with increased values of blood immune cell counts (163, 164), and a recent review reported that activated macrophages (matured MON) and regulatory T cells can have a protective role against hypertension (165). The inverse correlations between blood pressure parameters and MON, LIC, and RDW values could reflect a latent immune response state in firefighters with increased blood pressure. Thus, suggesting a decreased presence

of MON (migrated and transformed into macrophages), the cells with the biggest size, while other WBCs have similar sizes, therefore culminating in a reduced LIC and RDW. Elevated heart rate itself has been independently associated with augmented immune cell count and inflammation biomarkers (166–168), corroborating the correlations found in this study, i.e., cardiac frequency with MON (NS group) and BAS (S group). Once more, there seems to be a cumulative contribution of smoking status to these associations. Tobacco consumption has been previously associated with hypertension, increased cardiac frequency, and immune cell counts (118, 119, 129, 169). All routes, i.e., respiratory, dermal, and gastrointestinal, can be responsible for cardiovascular effects of PAHs exposure (14). However, inhalation is the major concern among firefighters at fire combat scenarios. Nevertheless, the other routes cannot be overlooked, especially because dermal contact has been identified as a potential source of PAHs in firefighting occupational context and the gastronomic culture of these Portuguese firefighters includes smoked/processed meat, expanding their exposome.

5 Conclusion

This study provided evidence of high exposure to PAHs in Portuguese firefighters without exposure to fire combat activities during the last week, by assessing the urinary levels of six OHPAHs while evaluating health status based on blood pressure, cardiac frequency, and hemogram parameters. Therefore, it contributes to an occupational exposure-based input for PAHs biomonitoring and supports ongoing efforts to track and mitigate PAHs exposure to reduce inherent health risks for the European population. Overall creatinine-corrected ΣOHPAHs levels varied from 1.20×10^{-1} to 78.20 μmol/mol creatinine, which were significantly higher in smokers. Subjects who acknowledged having exposure to PM and/or gaseous pollutants during their work-shift at the fire stations presented significantly higher urinary 1-OHPyr levels, which were still below the occupational recommended level of 2.5 μg/L proposed by ACGIH. This study presented 2 to 10-fold higher ΣOHPAHs compared to the available data concerning firefighters without exposure to fire emissions. The baseline ΣOHPAHs concentrations were 2–23 times higher than in general populations from Europe and the USA, while remaining 2-fold lower than in Chinese. However, all individual metabolite levels, except for 1-OHNaph+1-OHAce, were below those reported for USA, Italian, German, and Portuguese populations. Thus, further investigation of PAHs exposure in firefighters during regular occupational activities at fire stations is warranted. The urinary biomarkers 2-OHFlu and 1-OHPyr have the potential to discriminate tobacco consumption. Smoking firefighters were predominantly hypertensive, which was attributed to smoking duration, age, and years of service of the subjects. Firefighters in the permanent intervention team presented higher systolic pressure than those who had other functions. On the other hand, despite hemogram parameters being within the recommended values, exercising regularly (weekly) and spending less than 8 h per day at the fire station seemed to have a protective role in firefighters' health, whereas smoking was associated with higher BAS and HCT. A non-significant association was found between individual or ΣOHPAHs and blood pressure. On the other hand, blood pressure was inversely correlated with MON, LIC, and RDW. Individual PAH metabolites were positively correlated with differentiated leucocyte counts, but smoking could have been a possible contributor to these associations.

Future studies should aim to (i) enroll a greater number of females, if feasible, to more accurately characterize the exposure and health of women firefighters statistically; (ii) establish cross-contamination sources of PAHs and other pollutants at fire stations; (iii) establish follow-up studies to detect possible alterations in the blood pressure and hemogram of firefighters; (iv) explore the differences in lifestyle between firefighters from other countries; and (v) apply interventive measures to promote a healthier lifestyle among firefighters. Also, the use of biomarkers of relevant toxic mechanisms, such as oxidative stress, DNA damage, lung injury, etc., would be highly valuable to comprehensively characterize this population.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Accredited Ethics Committee of the University of Porto, Portugal, Report Nr. 92/CEUP/2020, under the project BioFirEx project (PCIF/SSO/0017/2018): "A panel of (bio)markers for the surveillance of firefighter's health and safety." The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

BB: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. AP: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. MO: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing, Funding acquisition. SA: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing. FE: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing. AF: Investigation, Methodology, Supervision, Writing – review & editing. JV: Investigation, Methodology, Supervision, Writing – review & editing. KS: Investigation, Methodology, Supervision, Writing – review & editing. SC: Funding acquisition, Project administration, Supervision, Writing – review & editing. JT: Funding acquisition, Project administration, Supervision, Writing – review & editing. SM: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2024.1338435/full#supplementary-material

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EDITED BY
Azubuike Chukwuka,
National Environmental Standards and Regulations Enforcement Agency (NESREA), Nigeria

REVIEWED BY
Pramita Sharma,
University of Burdwan, India
Tope Atere,
Osun State University, Nigeria

*CORRESPONDENCE
Lijian Lei
✉ wwdlijian@sxmu.edu.cn

†These authors have contributed equally to this work

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Association of exposure to multiple perfluoroalkyl and polyfluoroalkyl substances and glucose metabolism in National Health and Nutrition Examination Survey 2017–2018

Qinghua Tian[1,2†], Yutong Yang[1,2†], Qi An[1,2], Yang Li[1,2], Qingyao Wang[1,2], Ping Zhang[1,2], Yue Zhang[1,2], Yingying Zhang[1,2], Lina Mu[3] and Lijian Lei[1,2*]

[1]Department of Epidemiology, School of Public Health, Shanxi Medical University, Taiyuan, China, [2]MOE Key Laboratory of Coal Environmental Pathogenicity and Prevention, Shanxi Medical University, Taiyuan, China, [3]Department of Epidemiology and Environmental Health, School of Public Health and Health Professions, The State University of New York at Buffalo, Buffalo, NY, United States

Objective: To investigate the relationships between perfluoroalkyl and polyfluoroalkyl substances (PFASs) exposure and glucose metabolism indices.

Methods: Data from the National Health and Nutrition Examination Survey (NHANES) 2017–2018 waves were used. A total of 611 participants with information on serum PFASs (perfluorononanoic acid (PFNA); perfluorooctanoic acid (PFOA); perfluoroundecanoic acid (PFUA); perfluorohexane sulfonic acid (PFHxS); perfluorooctane sulfonates acid (PFOS); perfluorodecanoic acid (PFDeA)), glucose metabolism indices (fasting plasma glucose (FPG), homeostasis model assessment for insulin resistance (HOMA-IR) and insulin) as well as selected covariates were included. We used cluster analysis to categorize the participants into three exposure subgroups and compared glucose metabolism index levels between the subgroups. Least absolute shrinkage and selection operator (LASSO), multiple linear regression analysis and Bayesian kernel machine regression (BKMR) were used to assess the effects of single and mixed PFASs exposures and glucose metabolism.

Results: The cluster analysis results revealed overlapping exposure types among people with higher PFASs exposure. As the level of PFAS exposure increased, FPG level showed an upward linear trend ($p < 0.001$), whereas insulin levels demonstrated a downward linear trend ($p = 0.012$). LASSO and multiple linear regression analysis showed that PFNA and FPG had a positive relationship (>50 years-old group: $\beta = 0.059$, $p < 0.001$). PFOA, PFUA, and PFHxS (≤50 years-old group: insulin $\beta = -0.194$, $p < 0.001$, HOMA-IR $\beta = -0.132$, $p = 0.020$) showed negative correlation with HOMA-IR/insulin. PFNA (>50 years-old group: insulin $\beta = 0.191$, $p = 0.018$, HOMA-IR $\beta = 0.220$, $p = 0.013$) showed positive correlation with HOMA-IR/insulin, which was essentially the same as results that obtained for the univariate exposure-response map in the BKMR model. Association of exposure to PFASs on glucose metabolism indices showed positive interactions between PFOS and PFHxS and negative interactions between PFOA and PFNA/PFOS/PFHxS.

Conclusion: Our study provides evidence that positive and negative correlations between PFASs and FPG and HOMA-IR/insulin levels are observed, respectively. Combined effects and interactions between PFASs. Given the higher risk of glucose metabolism associated with elevated levels of PFAS, future studies are needed to explore the potential underlying mechanisms.

KEYWORDS

perfluoroalkyl and polyfluoroalkyl substances, National Health and Nutrition Examination Survey, glucose metabolism, least absolute shrinkage and selection operator, Bayesian kernel machine regression

1 Introduction

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) are a class of synthetic chemical that is widely used in various human production and daily life applications, such as paper, textiles, furniture, and foam fire extinguishers because of their thermal stability, hydrophobicity, and oil repellency (1–3). PFASs have high migration and contaminated ability and can be detected in environmental samples (such as water and soil), sera from various animal tissues, and human bodies (4–6). Additionally, PFASs have a significant bioaccumulation effect and a long half-life in the human body, making their degradation difficult (7). Animal experiments and epidemiological studies have demonstrated that PFASs have genotoxicity, reproductive toxicity, neurotoxicity, and developmental and endocrine-disrupting effects (8, 9).

There is growing evidence that PFASs are associated with a variety of health problems, with glucose metabolism disorder among them (10). Glucose metabolism disorder can cause many diseases, with diabetes being the most common, and has become a major public health issue (11). FPG, Insulin and homeostasis model assessment for insulin resistance (HOMA-IR) are important detection indices of glucose metabolism. FPG level was highly correlated with the presence of diabetic complications (12). Insulin is secreted by pancreatic β-cells, and human blood insulin levels can assess pancreatic β-cell function (13). Insulin resistance refers to the target organs of insulin action, such as liver, muscle and other reduced sensitivity to insulin action, and the normal physiological response of insulin cannot be performed (14). The most widely used assessment of insulin resistance is HOMA-IR (15). Early identification and control of these indices can reduce the harm of glucose metabolism disorder to the body and improve the prognosis.

Abbreviations: PFASs, perfluoroalkyl and polyfluoroalkyl substances; NHANES, National Health and Nutrition Examination Survey; FPG, fasting plasma glucose; HOMA-IR, homeostasis model assessment for insulin resistance; LASSO, least absolute shrinkage and selection operator; BKMR, Bayesian kernel machine regression; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFUA, perfluoroundecanoic acid; PFHxS, perfluorohexane sulfonic acid; PFOS, perfluorooctane sulfonates acid; PFDeA, perfluorodecanoic acid; PPAR, peroxisome proliferator activated receptors; IQR, interquartile range; VIF, variance inflation factor; PIP, posterior inclusion probability; GPR40, human G protein-coupled receptor 40; PPAR-α, peroxisome proliferator-activated receptor alpha; n-PFOA, n-perfluorooctanoic acid; Sb-PFOA, branch perfluorooctanoic acid isomers; n-PFOS, n-perfluorooctane sulfonic acid; Sm-PFOS, perfluoromethylheptane sulfonic acid isomers.

Currently, epidemiological studies on the effects of PFASs on glucose metabolism have yielded conflicting and inconclusive results. The Diabetes Prevention Project analyzed the relationship between serum PFASs concentrations and blood glucose indices and found that perfluorooctane sulfonates acid (PFOS) and perfluorooctanoic acid (PFOA) concentrations were positively associated with the function of HOMA-IR, fasting blood glucose and β cells function (1). The level of serum PFASs 1871 adults was measured in the 2013–2014 National Health and Nutrition Examination Survey (NHANES) in the United States revealed that branched-chain PFOA level was positively correlated with increased FPG (10). However, a study on obese children in Ohio found no statistical significance between PFASs and blood glucose levels (16). Nelson et al. analyzed data from NHANES (2003–04) and found no significant association between the PFASs (PFOA, perfluorononanoic acid (PFNA), PFOS, and perfluorohexane sulfonic acid (PFHxS)) and HOMA-IR (2). Although several studies demonstrated a positive association between serum PFASs levels and glucose metabolism indices, various studies have also determined there to be a non-significant or inverse association. Therefore, further investigation into the relationships between PFASs exposure and glucose metabolism is warranted.

At present, the mechanism of PFASs affecting glucose metabolism is also not clear, and some researchers believe that it may be related to the activation of Peroxisome proliferator activated receptors (PPAR) (17, 18). PPAR belongs to the nuclear hormone receptor superfamily that regulates lipid, hormonal, and glucose metabolism and is considered possibly the major target of PFASs (19). PFASs can activate signaling pathways mediated by all PPAR isoforms (PPARα, PPARβ, PPARγ) (20). Moreover, PPARα may be a preferential target for PFAS above the other PPAR isoform (21). Toxicological studies have found that PFOA can also increase insulin sensitivity and glucose tolerance in mice by affecting the PI3K-AKT signaling pathway in the liver, causing an increase in fasting blood glucose level and a decrease in liver glycogen content in mice (22).

Additionally, most of the previous studies have focused on the biological toxicity of individual PFASs; however, in real-life settings, multiple PFASs often co-exist and interact during exposure, uptake, and metabolism processes, and this interaction can result in complex effects on body glucose metabolism. Currently, the specific effects of combined exposure to multiple PFASs on glucose metabolism remain unknown. Therefore, to provide new evidence on the relationships of PFASs exposure and glucose metabolism, we aimed to examine the relationships between exposure to multiple PFASs and glucose metabolism indices in this study by analyzing the NHANES data from

2017 to 2018, using the Least absolute shrinkage and selection operator (LASSO) and multiple linear regression analysis and Bayesian kernel machine regression (BKMR) models.

2 Materials and methods

2.1 Study population

Data on the study participants were obtained from the NHANES databases. NHANES is a unique 2 years cross-sectional survey of the health and nutrition status of the U.S. population that collects data on demographic, socioeconomic, and health-related issues through interviews, standardized exams, and biometric specimen collection. The health screening was conducted at a mobile Screening Center (MEC) after the participants had already participated in a household interview. The methods and processes used by NHANES for data collection are available on NHANES website[1]. In the current study, we used data from 2017–2018 wave which is the latest test data on PFASs in NHANES.

The total sample size in 2017–2018 was 9,254, of which 1929 were tested for serum PFASs. Considering that type 1 diabetes mellitus accounts for about 90% of total diabetes in children and adolescents and is the most common form of childhood diabetes in most parts of the world (23); at the same time, pregnant women are at risk of gestational diabetes mellitus. 14% of pregnant women worldwide are affected by gestational diabetes mellitus which is a global health problem, affecting a considerable number of pregnant women (24). Therefore, 313 individuals <20 years old, 1,005 individuals who were pregnant, taking anti-hyperglycemic drugs or missed main research indices were excluded. Finally, a total of 611 individuals were included in this study. The National Center for Health Statistics Research Ethics Review Board approved NHANES, and all participants provided written informed consent. The selection process of research participants is summarized in Figure 1.

2.2 Covariates

The demographic database provided information on the gender (male, female), age, race (mexican-American, other Hispanic, non-Hispanic white, non-Hispanic blacks, other races), marital status (married, bereaved spouse, divorce, separation, unmarried, cohabitation), poverty, and education level (less than high school education, high School Degree, university and above). Information on weight and body mass index (BMI) were obtained from obtained from Examination database. Information on smoking, alcohol and leisure-time physical activity were obtained from Questionnaire database. Smoking status was classified as never smoking (fewer than 100 cigarettes or other tobacco products in their life), previously smoking (over 100 cigarettes or other cigarettes in their life but now quit smoking), and currently smoking (100 cigarettes or other cigarettes in their life and still smoking). Drinking status was classified as never drinking (no kind of alcohol in their life), previously smoking (drinking previously, but not in the past 12 months), and now smoking (drinking in the past 12 months). Leisure-time physical activity for each participant was categorized based on the recommended weekly amount of moderate-intensity to vigorous-intensity activity as follows: (1) below, indicating less than 150 min per week; (2) meet, indicating 150 to 300 min per week; (3) exceed, indicating more than 300 min per week.

2.3 Laboratory measurement methods

2.3.1 Blood specimen collection

Each study participants need to meet the 8 to less than 24 h fasting criteria and draw venous blood in a fasting state. The phlebotomist collected study participant's peripheral venous blood into 2 mL gray tubes for FPG and into 15 mL red top tubes for PFASs and insulin. Centrifuge the 2 mL gray tube to yield plasma and transfer at least 0.5 mL plasma from this tube into 2 mL vessels. Centrifuge the red top tubes to yield serum and remove serum into 5 mL sterile cryovials for PFASs and 2 mL vessels for insulin. Store under appropriate frozen (−30°C) conditions until they are tested.

2.3.2 Measurement of serum PFASs concentration

Online solid phase extraction coupled to high-performance liquid chromatography-turbo ion spray ionization-tandem mass spectrometry was used for the quantitative detection of the PFASs. A total of six perfluorinated compounds, including PFOA, PFOS, perfluorodecanoic acid (PFDeA), PFHxS, PFNA, and perfluoroundecanoic acid (PFUA), were analysed in this study. Notably, the PFOA and PFOS used in this manuscript refer to the sum of linear and branched. (The description of measurements of PFASs for NHANES 2017–2018 presents n-perfluorooctanoic acid (n-PFOA), Branch perfluorooctanoic acid isomers (Sb-PFOA), n-perfluorooctane sulfonic acid (n-PFOS), Perfluoromethylheptane sulfonic acid isomers (Sm-PFOS)). The limits of detection (LOD) of the six PFASs were all 0.1 ng/mL. Following NHANES analysis guidelines, PFASs below the LOD were expressed using LOD/$\sqrt{2}$. Details on the analytical methodology can be found on the NHANES website[2].

2.3.3 Measurement of glucose metabolism indices

FPG were determined by hexokinase initiation using the Roche/Hitachi cobas c system (c311). Insulin was measured using the Tosoh AIA system analyzer. Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as follows: HOMA-IR = FPG (mmol/L) × insulin (mIU/L)/22.5.

2.4 Statistical analysis

We calculated weighted means (±standard deviation [SD]) using the NHANES primary sampling unit, strata, and weights of environmental samples for continuous variables and frequencies (proportions) for categorical variables. Means and standard deviations (SDs) for continuous variables with normally distributed distribution, medians and interquartile ranges (IQRs) for continuous variables with non-normally

1 https://www.cdc.gov/nchs/nhanes/

2 https://wwwn.cdc.gov/Nchs/Nhanes/2017-2018/PFAS_J.htm

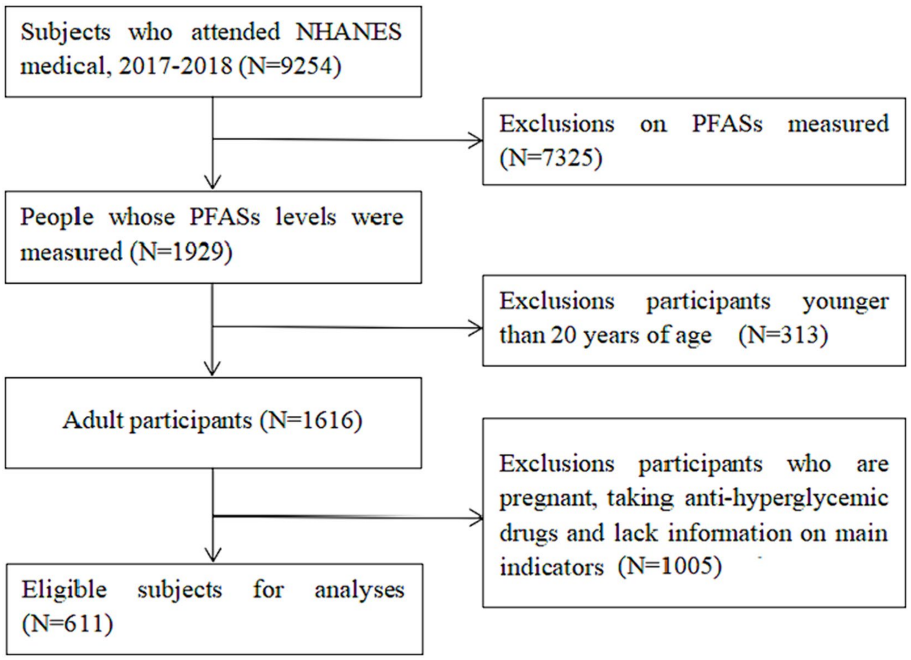

FIGURE 1
NHANES database research participants screening flow chart. (Data screening from top to bottom as indicated by the arrow).

distributed distribution, and proportions for binary or categorical variables were displayed. The distributions of serum PFAS were generally right-skewed, therefore, were ln-transformed was conducted. Spearman correlation analysis were presented using correlation heat maps.

Cluster analysis was performed based on the concentration of PFASs. K-means algorithm is the most used clustering method, which is simple to operate, computationally efficient, so K-means algorithm was used in this study (25). First, the logarithmic transformation of PFASs concentration was performed to achieve an approximate normal distribution. After the data was standardized, the index of sum of squared error provided by Factoextra package in R 4.2.2 was used to determine the optimal number of clusters. The overall population was divided into separate subgroups using the k-means algorithm. The ratio of the average concentration in each subgroup to the average concentration in the total participants of each PFASs was calculated to further assess the exposure level. The Kruskal–Wallis H and Chi-square tests were used to compare differences in baseline information and the levels of glucose metabolism indices between subgroups. Variables showing significant differences ($p < 0.05$) were used as covariates for multiple liner regression analysis to control for potential confounding factors.

Since diabetes is often diagnosed before the age of 50 (26); people under the age of 50 are the most active workforce, the working group affected by diabetes imposes a high economic burden on the country, so the study of glucose metabolism in people under 50 is significant (27); considering that glucose metabolism indices are easily influenced by age and the age range of the study population was large, the study participants were categorized into two groups: ≤50 years old and >50 years old for correlation and regression analyses. Next, The Kruskal–Wallis H test was used to compare differences in PFASs levels between different age groups. Then, the mixed effects of PFASs were analyzed using LASSO and BKMR models.

To explore the association of single PFASs with glucose metabolism indices in the mixture of PFASs exposure, and to avoid the potential collinearity among the variables included in the regression model, the multi-PFASs exposure model was established using LASSO regression for the six PFASs based on adjusting the confounding factors of sex, age, alcohol consumption, race, leisure-time physical activity, BMI, weight and poverty ratio. The coefficient distribution of these six PFASs was used as the penalty parameter for LASSO regression path selection, and the PFASs related to glucose metabolism indices were screened using five cross-validations. Subsequently, the selected elements of PFASs were included in the multiple linear regression model and analyzed the regression coeffcients and 95% confidence intervals (95% CI). The variance inflation factor (VIF) was calculated to evaluate the multicollinearity of PFASs in the model. It is generally believed that if the VIF of an independent variable is >10, there is a multicollinearity problem between the independent variable and other independent variables.

Furthermore, we used the BKMR model as part of the sensitivity analysis and further explored the mixed effects of multiple PFASs exposure on glucose metabolism indices. BKMR utilizes a flexible non-parametric approach to assess dose–response relationships, overcoming the disadvantage that conventional methods may be limited by multicollinearity and model selection error, to more reliably assess the health effects of environmental chemical mixtures (28). This study used the BKMR model to present the cumulative effect of the mixture of the six PFASs, the univariate expose-response relationships between PFASs and glucose metabolism indices and the interactions among individual PFASs. The BKMR analysis included the same covariates as the LASSO regression and calculated the posterior inclusion probability (PIP) to quantify the relative importance of each element to glucose metabolism, with values ranging from 0 to 1. The "BKMR" and "ggplot2" packages of R4.2.2 were used to build the BKMR model and present the results. The data were combined and analyzed using R4.2.2 software, and $p < 0.05$ was presented at the significance level.

3 Results

3.1 General condition of the study participants and distribution of blood PFASs

As shown in Table 1, 611 participants were included in this study comprising 308 males and 303 females, which accounted for 50.4 and 49.6% participants, respectively. Never smokers and individuals with a history of alcohol consumption accounted for 56.3 and 91.3%, respectively, and the median age was 53 years.

Except for PFUA, the blood detection rate of the other five PFASs exceeded 85%. The detection rate and concentration of PFASs in the blood of the study participants are presented in Table 2. Figure 2 illustrates that the correlation coefficients between PFDeA and PFUA, PFNA and PFUA, and PFNA and PFDeA were 0.85, 0.71, and 0.72, respectively. This suggests a strong correlation between some PFASs ($r_s > 0.7$).

TABLE 1 Basic characteristics of study participants, grouped by gender (*N* (%)/M (P25, P75)).

	Total (*N* = 611)	Male (*N* = 308)	Female (*N* = 303)	p
Age (median [IQR])	53.00 [36.00, 64.00]	54.00 [39.00, 65.00]	52.00 [34.00, 64.00]	0.200
Smoking (%)				<0.001
Now	111 (18.2)	65 (21.1)	46 (15.2)	
Previously	156 (25.5)	95 (30.8)	61 (20.1)	
Never	344 (56.3)	148 (48.1)	196 (64.7)	
Drinking (%)				0.012
Now	451 (73.8)	240 (77.9)	211 (69.6)	
Previously	107 (17.5)	51 (16.6)	56 (18.5)	
Never	53 (8.7)	17 (5.5)	36 (11.9)	
Race (%)				0.646
Mexican-American	77 (12.6)	38 (12.3)	39 (12.9)	
Other Hispanic	48 (7.9)	20 (6.5)	28 (9.2)	
Non-Hispanic White	238 (39.0)	125 (40.6)	113 (37.3)	
Non-Hispanic Blacks	143 (23.4)	75 (24.4)	68 (22.4)	
Other races	105 (17.2)	50 (16.2)	55 (18.2)	
Marriage (%)				0.113
Married	306 (50.1)	168 (54.5)	138 (45.5)	
bereaved spouse	41 (6.7)	16 (5.2)	25 (8.3)	
Divorce	81 (13.3)	38 (12.3)	43 (14.2)	
Separation	24 (3.9)	8 (2.6)	16 (5.3)	
Unmarried	104 (17.0)	54 (17.5)	50 (16.5)	
Cohabitation	55 (9.0)	24 (7.8)	31 (10.2)	
Education (%)				0.806
Less than high school education	108 (17.7)	57 (18.5)	51 (16.8)	
High school degree	140 (22.9)	68 (22.1)	72 (23.8)	
University and above	363 (59.4)	183 (59.4)	180 (59.4)	
Leisure-Time Physical Activity				<0.001
Below	365 (59.7)	159 (51.6)	206 (68.0)	
Meet	44 (7.2)	30 (9.7)	14 (4.6)	
Exceed	202 (33.1)	119 (38.6)	83 (27.4)	
BMI(median [IQR])	28.40 [24.50, 33.80]	27.80 [24.98, 31.80]	29.00 [23.95, 35.85]	0.062
Weight(median [IQR])	80.60 [67.00, 95.90]	84.50 [73.35, 98.62]	2.43 [60.10, 91.45]	<0.001
Poverty ratio (median [IQR])	2.20 [1.24, 4.25]	2.43 [1.31, 4.56]	2.03 [1.22, 4.06]	0.165
SBP (median [IQR])	125.00 [113.00, 137.00]	125.50 [116.00, 137.00]	123.00 [109.00, 136.50]	0.009
DBP (median [IQR])	73.00 [65.00, 79.00]	74.50 [67.00, 82.00]	71.00 [64.00, 77.00]	<0.001

Data are presented as the median (IQR) or frequency (percentage).

3.2 Subgroup analysis of the relationship between PFASs and glucose metabolism indices

3.2.1 Cluster analysis based on PFASs exposure

By observing the the cluster heatmap (Figure 3), we found that individuals highly exposed to one type of PFASs were also likely to be exposed to other PFASs simultaneously. As can be seen in the Figure 4, the exposure levels of the six PFASs in subgroup 3 were significantly higher than those in subgroups 2 and 1, while subgroup 2 was significantly higher than subgroup 1. From this, we identified subgroups 1, 2, and 3 as low, medium, and high exposure subgroups, respectively. Figure 5 shows the comparison results of glucose metabolism indices among the three subgroups, with statistically significant differences in FPG among all subgroups and insulin between the high-exposure and low-exposure groups. The trend of FPG and insulin levels showed statistical significance ($p < 0.05$, Table 3), indicating a linear trend between exposure and these two glucose metabolism indices. Combined with the box plots (Figure 5), it was found that FPG levels showed an elevated trend with increasing exposure to PFASs. However, insulin demonstrated a decreasing trend with increasing exposure to PFASs, indicating an association between PFASs exposure and changes in glucose metabolism levels. The weighted basic characteristics of the three subgroups determined based on cluster analysis are presented in Table 4. Statistically significant differences were observed between the groups in terms of gender, age, alcohol consumption, race, and poverty ratio.

TABLE 2 Detection rate and concentration of PFASs (ng/mL) in the population (median [IQR]).

PFASs (median [IQR])	%>LOD	Median [IQR]
PFOA	100.0%	1.47 [0.97, 2.17]
PFOS	99.7%	5.00 [2.90,8.30]
PFDeA	88.5%	0.20 [0.10, 0.30]
PFHxS	99.2%	1.20 [0.70, 2.00]
PFNA	92.5%	0.50 [0.30, 0.80]
PFUA	68.4%	0.10 [0.07, 0.20]

Data are presented as the median (IQR) or frequency (percentage).

3.2.2 Comparison of PFASs levels between the two age groups

As shown in Table 5, the differences in serum levels of six PFASs were statistically significant between the two groups with all $p < 0.001$. Except for PFUA and PFDeA, the remaining four PFASs levels were significantly higher in ≤50 year old group than in >50 year old group.

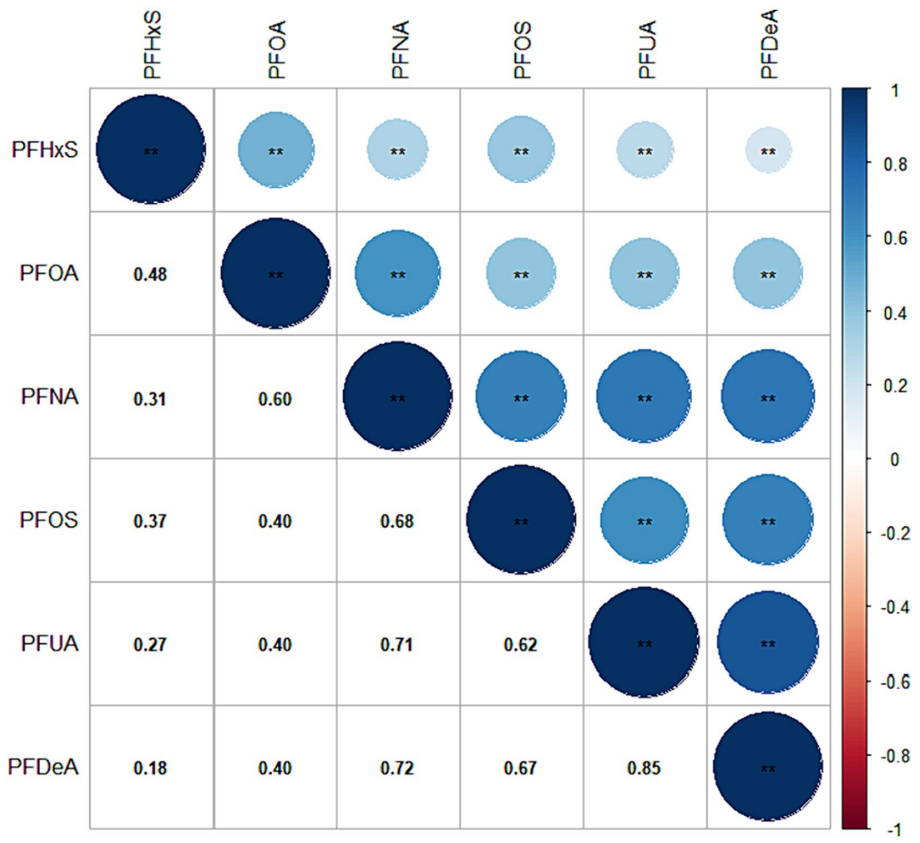

FIGURE 2
Heat map of PFAS correlation in blood. (The values in the box are the correlation coefficient, with values ranging from −1 to 1).

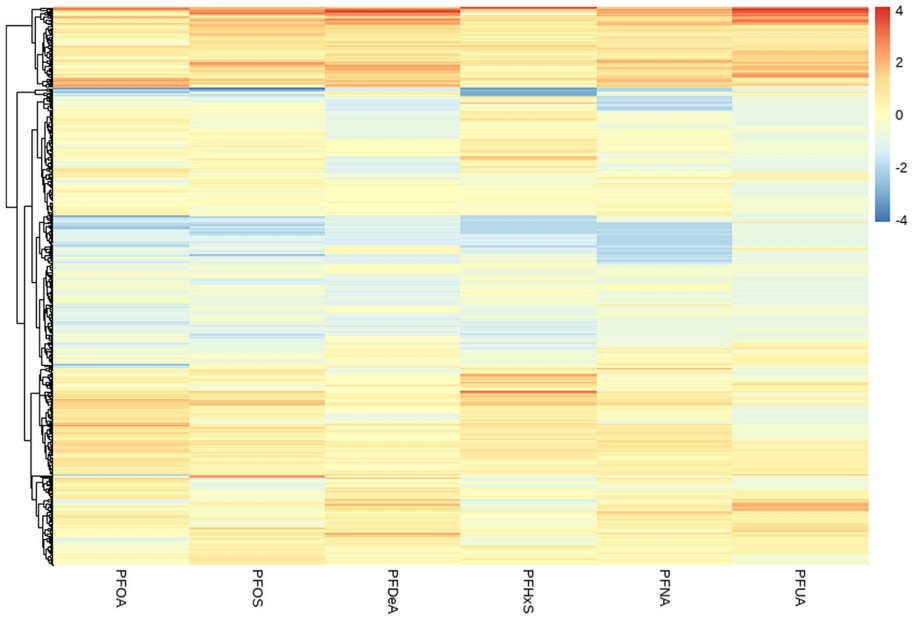

FIGURE 3
Heat map of clustering based on the concentration of PFASs in blood. (The vertical axis represents different PFASs. The horizontal axis represents the sample).

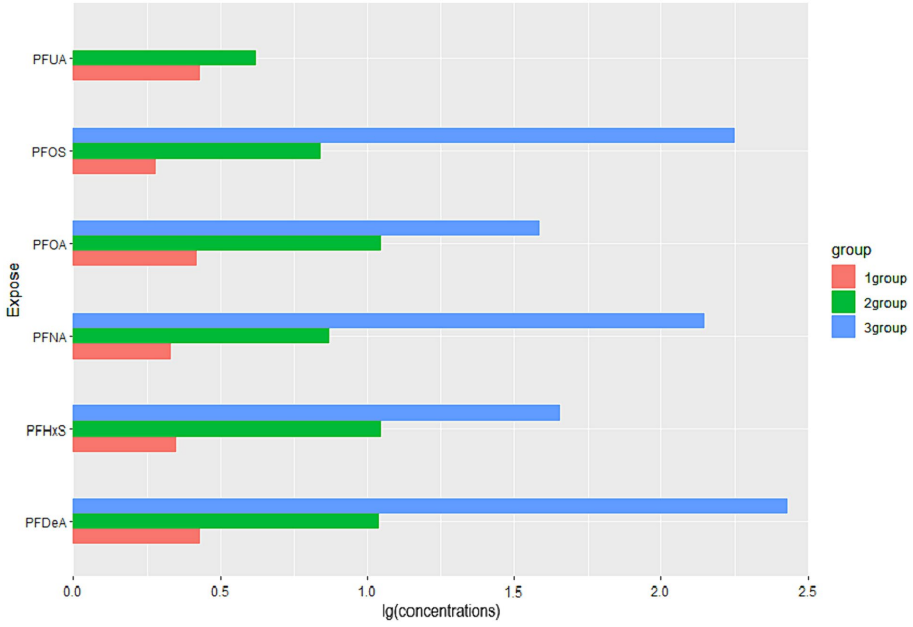

FIGURE 4
Comparison of PFASs concentrations among three exposure subgroups. (Data are expressed as the ratio of the mean concentration of PFASs in subgroups to the population mean; all groups ($n = 6$). The horizontal coordinates indicate the relative levels of the mean concentrations of the subgroup PFASs to the overall mean, and the vertical coordinates are the six PFASs).

3.3 The relationships of single PFASs and glucose metabolism indices

As shown in Table 6, among aged ≤50 years group, PFHxS (Insuliu:$\beta = -0.194$, $p < 0.001$; HOMA-IR: $\beta = -0.132$, $p = 0.020$) was found to be correlated with insulin and HOMA-IR. In the >50 years old population, PFNA exhibited a positive correlation with FPG ($\beta = 0.059$, $p < 0.001$); insulin and HOMA-IR were negatively correlated with PFUA (Insulin: $\beta = -0.133$, $p = 0.037$; HOMA-IR: $\beta = -0.141$, $p = 0.041$), and PFOA (Insulin: $\beta = -0.159$, $p = 0.047$; HOMA-IR: $\beta = -0.163$, $p = 0.042$) but positively associated with PFNA (Insulin: $\beta = 0.191$, $p = 0.018$; HOMA-IR: $\beta = 0.220$, $p = 0.013$). Additionally, the

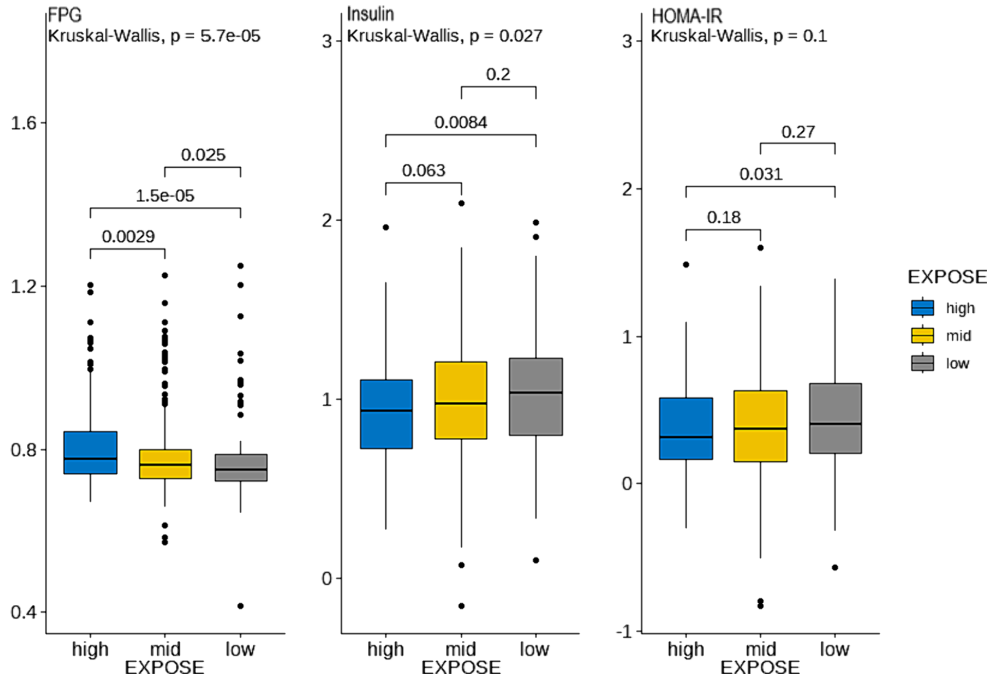

FIGURE 5
Comparison of glucose metabolism indices between the three exposure subgroups. (Blue, yellow, and gray represent the high, medium, and low exposure groups, respectively. (Data are expressed as mean ± SD; all groups (n = 3)). The ordinate represents the logarithmic concentration of glucose metabolism indices, and the horizontal coordinate is the exposure subgroup).

TABLE 3 Trend test of glucose metabolism indices in three exposure subgroups.

	Linear term	Sum of squares	F	p
FPG	Unweight	0.153	17.408	<0.001
	Weighted (E)	0.147	16.682	<0.001
HOMA-IR	Unweight	0.385	3.243	0.072
	Weighted (E)	0.410	3.453	0.064
Insulin	Unweight	0.634	6.125	0.014
	Weighted (E)	0.654	6.320	0.012

Data are presented as equality of variances or equality of variances.

multiple linear regression models indicated that the VIF of all PFASs was less than 10, indicating no multicollinearity among the PFASs variables in the regression process.

3.4 The effects of multiple PFASs on glucose metabolism indices

As shown in Figure 6, in the ≤50 years-old group, the level of FPG showed an increasing trend with the increase of the total level of PFASs mixture. PFOS showed a positive expose-response relationship with FPG. Negative interaction between PFOS and PFHxS may exist. Insulin and HOMA-IR decreased with the increase of the total level of PFASs mixture. Further, PFHxS demonstrated a clear negative linear relationships with these two indices in the expose-response relationship plot, which was consistent with the LASSO regression screening results. As PFNA concentration percentiles changed from low to high, the negative effect of PFOA on insulin/HOMA-IR decreased, indicating the possibility of negative interactions between PFNA and PFOA. The results presented in Table 7 highlight the significant role of PFHxS in the relationships of PFASs on both insulin and HOMA-IR, with the highest PIPs (0.990 and 0.796, respectively).

As shown in Figure 7, in the >50 years-old group, the level of FPG also showed an increasing trend, corresponding to the total PFASs mixture levels. The univariate expose-response diagram showed a linear relationships between the six PFASs and the level of FPG. No interactions were observed between the PFASs. The insulin and HOMA-IR results were similar-both levels demonstrated a downward trend with the increase in the overall level of the PFASs mixture. Furthermore, the univariate expose-response relationships showed that all PFASs had linear relationship with these two indices. PFOA, PFUA and PFHxS showed a negative expose-response relationship with these two indices while PFOS and PFNA demonstrated a positive relationship, which was consistent with the LASSO regression screening results. In the bivariate exposed-response relationship plots of insulin or HOMA-IR, negative interactions were found between PFOA and PFNA/PFOS, and positive interaction was observed between PFOA and PFHxS in the bivariate exposed-response relationship plots of HONA-IR. The results presented in Table 5 highlight the significant role of PFNA in the relationships of PFASs on FPG, with the highest PIPs (0.356).

As shown in Figure 8, all possible interactions between PFASs were summarized, and it was found that PFOA could interact with multiple PFASs, and PFOA played an important role in the combined influence of multiple PFASs on glucose metabolism.

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TABLE 4 Characteristics of the high-, medium-, and low-exposure groups (*N* (%)/M (P25, P75)).

	Low exposure group (*N* = 160)	Medium exposure group (*N* = 320)	High exposure group (*N* = 131)	p
Age (median [IQR])	41.00 [29.75, 56.00]	54.00 [38.00, 65.00]	61.00 [49.00, 73.00]	<0.001
Gender (%)				<0.001
Male	41 (25.6)	193 (60.3)	74 (56.5)	
Female	119 (74.4)	127 (39.7)	57 (43.5)	
Smoking (%)				0.216
Now	30 (18.8)	65 (20.3)	16 (12.2)	
Previously	35 (21.9)	85 (26.6)	36 (27.5)	
Never	95 (59.4)	170 (53.1)	79 (60.3)	
Drinking (%)				0.022
Now	113 (70.6)	242 (75.6)	95 (72.5)	
Previously	23 (14.4)	59 (18.4)	24 (18.3)	
Never	24 (15.0)	19 (6.0)	12 (9.1)	
Race (%)				<0.001
Mexican-American	31 (19.4)	40 (12.5)	6 (4.6)	
Other Hispanic	11 (6.9)	30 (9.4)	7 (5.3)	
Non-Hispanic White	61 (38.1)	139 (43.4)	38 (29.0)	
Non-Hispanic Blacks	35 (21.9)	69 (21.6)	39 (29.8)	
Other races	22 (13.8)	42 (13.1)	41 (31.3)	
Marriage (%)				0.100
Married	69 (43.1)	162 (50.6)	75 (57.3)	
Bereaved spouse	8 (5.0)	21 (6.6)	12 (9.2)	
Divorce	19 (11.9)	46 (14.4)	16 (12.2)	
Separation	9 (5.6)	10 (3.1)	5 (3.8)	
Unmarried	34 (21.2)	53 (16.6)	17 (13.0)	
Cohabitation	21 (13.1)	28 (8.8)	6 (4.6)	
Education (%)				0.584
Less than high school education	25 (15.6)	62 (19.4)	21 (16.0)	
High School Degree	43 (26.9)	68 (21.2)	29 (22.1)	
University and above	92 (57.5)	190 (59.4)	81 (61.8)	
Leisure-Time Physical Activity				0.031
below	94 (58.8)	177 (55.3)	94 (71.8)	
meet	11 (6.9)	26 (8.1)	7 (5.3)	
exceed	55 (34.4)	117 (36.6)	30 (22.9)	
BMI(median [IQR])	30.15 [25.30, 37.88]	28.50 [24.80, 33.50]	26.90 [23.40, 30.80]	<0.001
Weight(median [IQR])	83.35 [66.92, 102.95]	82.10 [68.97, 96.25]	73.20 [36.60, 90.50]	0.001
Poverty ratio (median [IQR])	1.69 [0.98, 2.79]	2.44 [1.35, 4.29]	3.04 [1.46, 5.00]	<0.001
SBP (median [IQR])	120.00 [109.00, 134.25]	125.00 [114.00, 137.00]	126.00 [116.00, 135.00]	0.018
DBP (median [IQR])	71.50 [65.00, 79.00]	73.00 [66.00, 79.00]	73.00 [67.00, 80.00]	0.679

Data are presented as the median (IQR) or frequency (percentage).

4 Discussion

In this study, 611 participants from the 2017–2018 NHANES cohort were selected. Cluster analysis, LASSO regression, and BKMR regression models were used to explore the relationships between the six PFASs and glucose metabolism indices. The results showed that with the increase of PFASs exposure, the FPG level showed an upward trend, while HOMA-IR/insulin demonstrated a downward trend. PFNA and FPG had a positive relationship. PFOA, PFUA, and PFHxS showed negative correlations with HOMA-IR/insulin, whereas PFNA

mainly had positive correlation. Negative interactions were observed between PFOA and PFNA/PFOS, PFOS and PFHxS, while positive interactions were found between PFOA and PFHxS. Notably, PFOA can combine with various PFASs (PFOS/PFNA/PFHxS) to affect glucose metabolism indices.

PFASs exposure was closely associated with the level of FPG. The results of this study revealed that higher exposure to PFASs corresponded to a higher level of FPG, with PFNA having the greatest influence. A cross-sectional studies for adolescents and adults demonstrated that elevated serum PFNA concentration was associated with hyperglycemia (95% CI: 1.39–7.16) (29, 30). A nested case-control study also found that mixed PFASs homologs could affect glucose homeostasis by increasing 1 h glucose levels, with PFNA being the main contributor (31). The reason may be that PFNA is a kind of long-chain PFASs that is difficult to degrade and can lead to higher PPARα activation (32), which also explains the prominent position of PFNA in the relationship between PFASs and FPG.

Insulin is a protein hormone synthesized and secreted by islet β cells, which binds to target cell receptors and activates signaling pathways leading to various metabolic changes, most notably increasing glucose uptake and lowering blood glucose levels (33). Another marker of diabetes is insulin resistance that is measured using HOMA-IR. The results of this study demonstrated that exposure to mixed PFASs was associated with lower insulin and HOMA-IR levels, while PFOA, PFUA, and PFHxS were negatively correlated with both. Another study of the NHANES database also found PFASs mixture exposure were associated with decreased INS and HOMA-IR (34). Studies conducted in Cincinnati found a marginal negative correlation between PFOA levels and insulin/HOMA-IR, and another study in the New York reported a negative correlation between PFHxS and both (35, 36), which were consistent with the present study. In addition, we also found that PFNA was positively associated with both insulin and HOMA-IR levels. Zeeshan et al. analyzed data from the "Isomers of C8 Health Project in China," and also found that PFNA was significant positive associations with insulin and HOMA-IR (37). However, there were some studies with opposite results to the present study. For example, Zeeshan et al. found significant positive correlations between PFOA, PFUA, PFHxS, and both insulin and HOMA-IR (37); some researchers found no significant correlations between PFOA/PFNA/PFHxS and HOMA-IR (2, 38). The analysis of similar and contradictory epidemiological results with this study found that different overall exposure levels in the study population

TABLE 5 Comparison of PFASs levels between the two age groups.

PFASs (median [IQR])	≤50 years old group (*N* = 278)	>50 years old group (*N* = 333)	p
PFOA	1.27 [0.77, 1.77]	1.67 [1.07, 2.47]	<0.001
PFOS	3.40 [2.12, 5.70]	6.30 [3.80, 11.20]	<0.001
PFUA	0.10 [0.07, 0.20]	0.10 [0.07, 0.20]	<0.001
PFNA	0.30 [0.20, 0.60]	0.50 [0.40, 0.90]	<0.001
PFHxS	0.95 [0.50, 1.60]	1.40 [0.90, 2.20]	<0.001
PFDeA	0.20 [0.10, 0.30]	0.20 [0.10, 0.30]	<0.001

Data are presented as the median (IQR).

TABLE 6 Regression coefficients of population glucose metabolism indices and blood PFASs concentrations (95% CI).

Age groups	Glucose metabolism indices	PFASs	β	95% CI	p	VIF[a]
≤50 years old	FPG	PFOS	0.006	(−0.019, 0.033)	0.6149	1.301
	Insulin	PFOA	−0.013	(−0.171, 0.1145)	0.874	2.238
		PFHxS	−0.194	(−0.244, −0.143)	<0.001	2.114
		PFUA	−0.028	(−0.143,0.101)	0.735	1.406
	HOMA-IR	PFOA	−0.027	(−1.934, 0.139)	0.749	2.238
		PFHxS	−0.132	(−0.243, −0.021)	0.020	2.114
		PFUA	−0.021	(−0.149, 0.108)	0.754	1.406
>50 years old	FPG	PFNA	0.059	(0.026, 0.091)	<0.001	1.112
	Insulin	PFOA	−0.159	(−0.219, −0.103)	0.047	2.613
		PFOS	0.053	(−0.080, 0.186)	0.436	3.414
		PFHxS	−0.031	(−0.160, 0.099)	0.645	2.510
		PFNA	0.191	(0.033, 0.349)	0.018	3.763
		PFUA	−0.133	(−0.258, −0.009)	0.037	2.442
	HOMA-IR	PFOA	−0.163	(−0.258, −0.092)	0.042	2.631
		PFOS	0.554	(−0.090, 0.200)	0.455	3.414
		PFHxS	−0.029	(−0.170,0.112)	0.685	2.510
		PFNA	0.220	(0.048, 0.391)	0.013	3.763
		PFUA	−0.141	(−0.277, −0.006)	0.041	2.442

[a]VIF refers to the variance inflation factor, which is generally accepted if the VIF of an independent variable is >10. It indicates that there is a covariance problem between that independent variable and other independent variables.

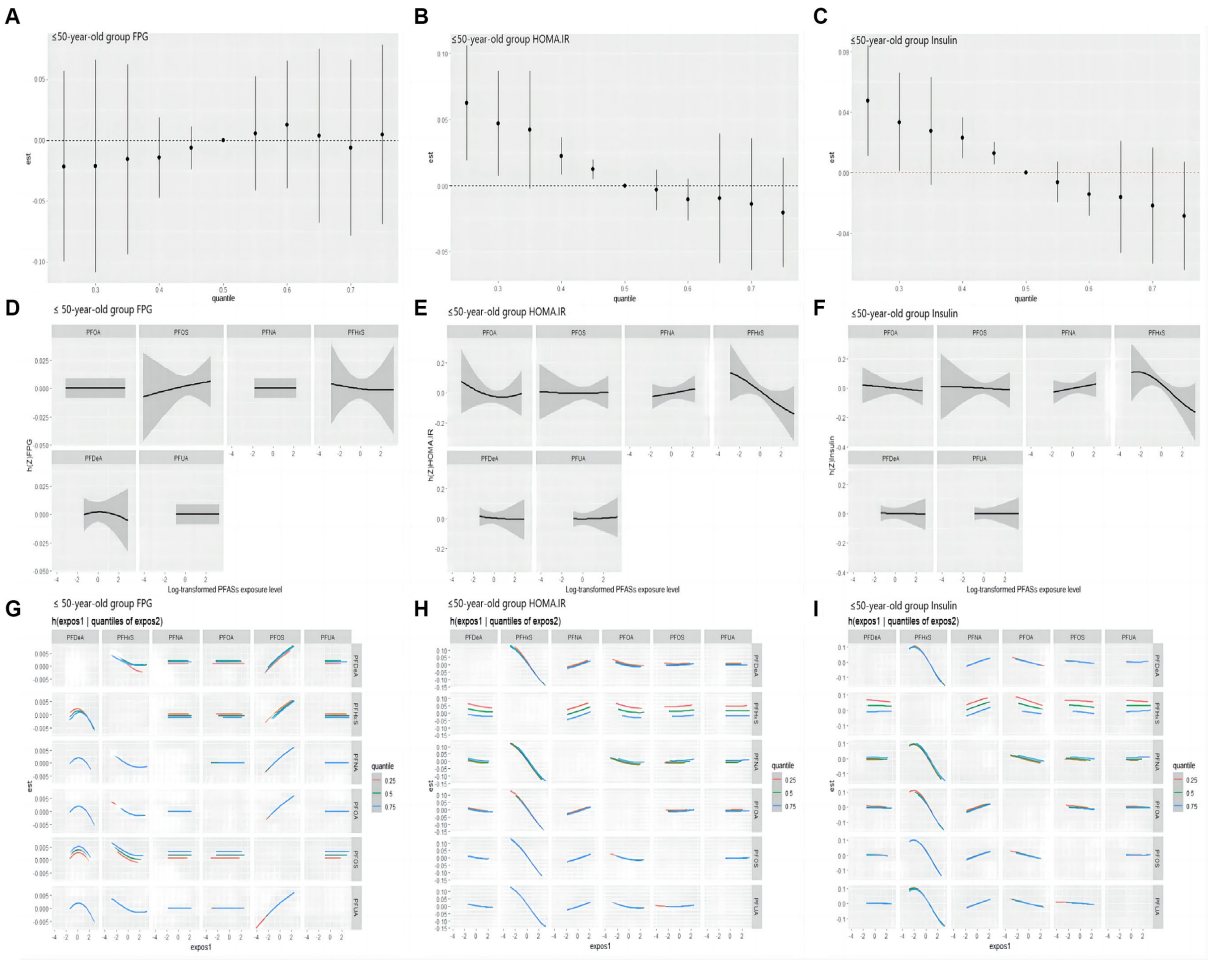

FIGURE 6
BKMR study the correlation of FPG, HOMA-IR and Insulin with PFASs in the ≤50 years-old group. **(A–C)**: overall effect (95%CI) of PFASs. *h*(*Z*) can be interpreted as the correlations of FPG, HOMA-IR and insulin with blood PFASs. **(D–F)**: exposure-response plots of FPG, HOMA-IR and Insulin against each PFAS, with other PFASs held at the median. *h*(*Z*) can be interpreted as the correlations of FPG, HOMA-IR and Insulin with blood PFASs. **(G–I)**: bivariate expose-response relationship. Each cell represented the exposure-response curve for the column PFASs when the row PFASs was fixed at 25th, 50th, or 75th percentile and the remaining PFASs were fixed at their medians.

TABLE 7 A posteriori inclusion probability (PIPs) of the effect of PFASs on glucose metabolism indices.

Variable	≤50 years-old group			>50 years-old group		
	FPG	Insulin	HOMA-IR	FPG	Insulin	HOMA-IR
PFOA	0.016	0.130	0.376	0.012	0.856	0.866
PFOS	0.488	0.096	0.374	0.048	0.576	0.586
PFDeA	0.326	0.076	0.488	0.032	0.231	0.355
PFHxS	0.136	0.990	0.796	0.016	0.688	0.458
PFNA	0.012	0.042	0.312	0.356	0.832	0.832
PFUA	0.026	0.056	0.326	0.028	0.720	0.796

Data are presented as PIP. PIP value reflects the relative importance of PFASs influence on outcome indices of glucose metabolism, and the value range is (0, 1).

may influence the association of PFASs with indicators of glucose metabolism. The results of studies analyzing populations or countries with lower concentrations of serum PFASs were more consistent with this study, concluding that there was a negative or nonsignificant correlation between PFASs exposure and insulin and HOMA-IR. For instance, the median serum concentration of PFOA was 3.8 ng/mL in NHANES (2003–2004) (2), 2.46 ng/mL in the Canadian Health Measures Survey (2007–2009) (38), and 1.47 ng/mL in this study. In studies where there were significant positive correlations, participants had a higher median PFOA

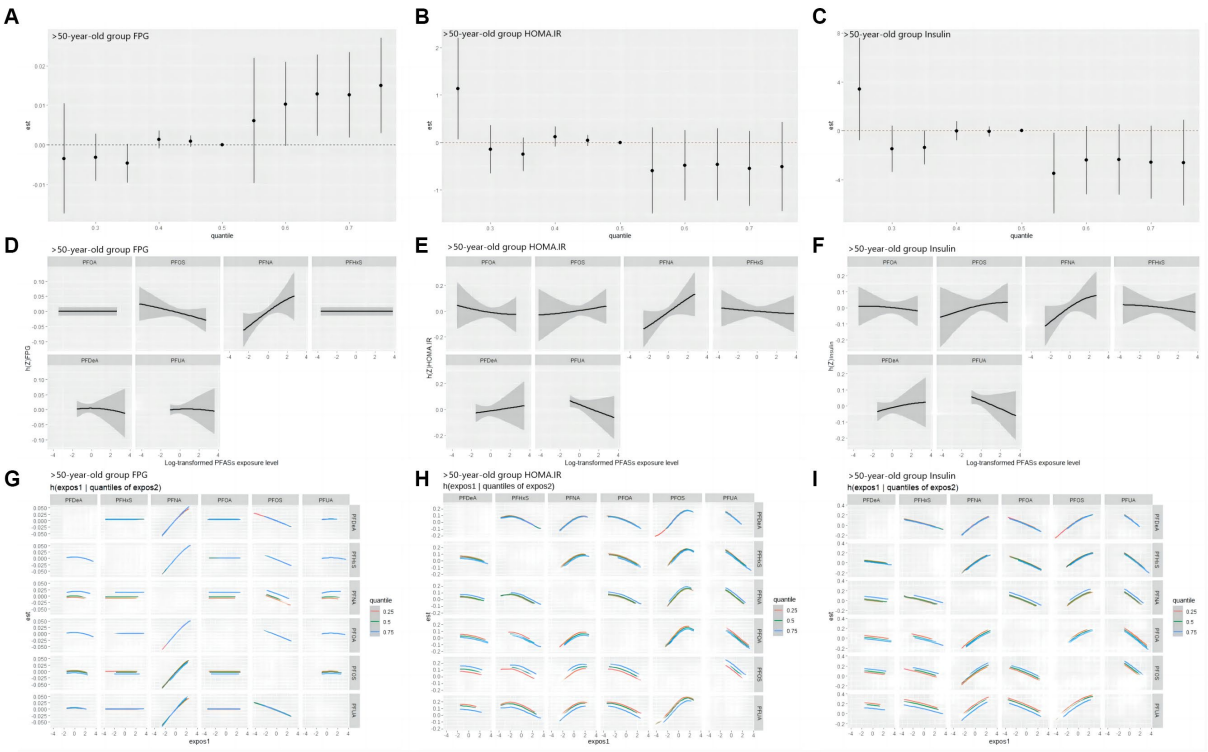

FIGURE 7
BKMR study the correlation of FPG, HOMA-IR and Insulin with PFASs in the >50 years-old group. **(A–C)**: overall effect (95%CI) of PFASs. *h*(*Z*) can be interpreted as the correlations of FPG, HOMA-IR and Insulin with blood PFASs. **(D–F)**: exposure-response plots of FPG, HOMA-IR and Insulin against each PFAS, with other PFASs held at the median. *h*(*Z*) can be interpreted as the correlations of FPG, HOMA-IR and insulin with blood PFASs. **(G–I)**: bivariate expose-response relationship. Each cell represented the exposure-response curve for the column PFASs when the row PFASs was fixed at 25th, 50th, or 75th percentile and the remaining PFASs were fixed at their medians.

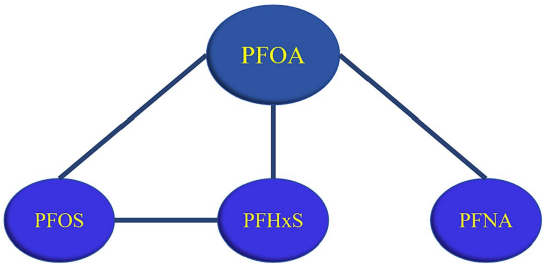

FIGURE 8
Summary of PFASs interactions. (A line between PFASs indicates possible interaction, with a black line indicating negative interaction and a red line indicating a positive interaction).

concentration, such as the European Young Heart Study, which measured a median PFOA concentration of 9.7 ng/mL and 9.0 ng/mL in men and women, respectively (39). The biological mechanisms associated with PFASs and insulin resistance are unclear. Animal studies have shown that in mice exposed to PFAS, PFAS negatively regulates the protein kinase B (PKB) pathway in white adipose tissue, resulting in increased glucose and decreased insulin and insulin resistance (40). PFASs also have affinity to PPAR-γ and exposure to PFASs may also trigger expression of store free fatty acids and regulate the transcription of various insulin-related genes through activation of PPAR-γ and ultimately enhance insulin sensitivity (17, 34, 41). Although the findings from toxicology studies provide valuable insights, population data are lacking, so further research is needed to clarify the underlying mechanisms.

The results of this study showed that PFASs had different interactions in different age groups, for example, in terms of the relationships of PFASs and FPG, a negative interaction was observed between PFOS and PFHxS in ≤50 years old groups, while no interaction was found in >50 years old groups. At the same time, the differences in the serum levels of the six PFASs were all statistically significant between the two age groups in this study. Some researchers found that the combined toxic effects between PFASs may vary with the concentration (42). A study found that the toxic effect of high dose PFASs exposure experiment is not the same as that of low dose PFASs exposure experiment, that is, no effect under high dose exposure does not mean that there is no effect under low dose exposure (43, 44). Therefore, different concentrations may explain the different interaction of PFASs in different age groups. Furthermore, the results of the BKMR model indicate that there is an interaction between PFASs, especially between PFOA and multiple PFASs (PFOS/PFUA/PFHxS). PFASs are a large family containing thousands of compounds, of which PFOA is the most typical and

most widely used (45). Studies show that PFOA is the final metabolite of various PFASs in the environment (46). Activation of PPAR-α is thought to play a key role in the production of toxic effects by PFASs, and PFOA is a potent agonist of PPAR-α (47). The above may be the reason for the interaction of PFOA and multiple PFASs.

Previous studies have primarily focused on the effects of a single PFAS on glucose metabolism, with limited analysis of mixed exposures, and the toxic mechanism of mixed exposure of PFASs is currently unknown. A study found that the six PFASs (PFHxS, PFOA, PFNA, PFDA, PFUA, and PFDeA) can bind to human G protein-coupled receptor 40 (GPR40), and the increase in intracellular calcium level mediated by GPR40 can promote the fusion of insulin-containing vesicles with plasma, leading to insulin secretion, disrupting glucose homeostasis and ultimately aggravating insulin resistance (48, 49). An animal study found tha the PFAS mixture could cause mitochondrial dysfunction and further disrupt glucose and lipid metabolic pathways, ultimately causing metabolic disorders (50). Further studies are needed to clarify the combined mechanism of action of PFASs in the future.

The strengths of this study are as follows: this study first used the method of cluster analysis to automatically categorize the study participants into three groups based on PFASs exposure levels. By comparing the differences in glucose metabolism levels among these exposure groups, the distribution of PFASs among the participants and the influence of PFASs exposure levels on glucose metabolism indices were effectively presented. Further, unlike previous studies on the health effects of exposure to a single PFAS, this study explores the relationship between exposure to multiple PFASs and glucose metabolism indices. This study used LASSO regression to screen PFASs variables and used the BKMR model to evaluate the overall mixed effects and interactions of multiple PFASs. These two approaches complemented each other.

However, some limitations should be recognized. First, we cannot rule out residual and unmeasured confounders (for example, total fat or high fructose dietary intake), or consumption of foods packaged with food contact materials containing PFASs, which could lead to more PFAS exposure. Additionally, the cross-sectional design could not tell the causal relationship between PFAS exposure and glucose metabolism and biological mechanisms linking PFASs exposure to glucose metabolism have yet to be established. Therefore, further experimental studies are required to explore the relevant mechanisms underlying the association of serum PFASs with glucose homeostasis and metabolic indices.

5 Conclusion

In this study, we analyzed 2017–2018 United States NHANES data to assess the relationships between serum concentrations of the six PFASs and glucose metabolism indices. It was found that PFOA, PFOS, PFUA, PFNA, and PFHxS could play a significant role in the relationships of PFASs and glucose metabolism. Moreover, interactions were observed between PFOS and PFHxS, and PFOA and PFOS/PFHxS/PFNA. Our study provides new evidence for the harmful effects of PFASs exposure; however, further longitudinal studies are needed to confirm these findings and clarify the underlying mechanisms.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: https://www.cdc.gov/nchs/nhanes/.

Ethics statement

The studies involving human participants were reviewed and approved by National Center for Health Statistics Research Ethics Review Board. The participants provided their written informed consent to participate in this study.

Author contributions

QT: Writing – original draft, Visualization, Software, Methodology, Formal analysis, Conceptualization. YY: Writing – review & editing, Visualization, Software, Resources, Data curation, Conceptualization. QA: Writing – review & editing, Software, Methodology. YL: Software, Methodology, Writing – review & editing. QW: Writing – review & editing, Validation, Resources. PZ: Writing – review & editing. YuZ: Writing – review & editing. YiZ: Writing – review & editing, Funding acquisition. LM: Project administration, Writing – review & editing, Supervision, Data curation. LL: Project administration, Writing – review & editing, Supervision, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Azubuike Chukwuka,
National Environmental Standards and Regulations Enforcement Agency (NESREA), Nigeria

REVIEWED BY
Guilherme Sgobbi Zagui,
University of Ribeirão Preto, Brazil
Gabriel O. Dida,
Technical University of Kenya, Kenya

*CORRESPONDENCE
Lina Jin
jinln@jlu.edu.cn
Hui Li
leehui@jlu.edu.cn

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Association between heavy metals exposure and persistent infections: the mediating role of immune function

Huiling Zhang, Juan Wang, Kunlun Zhang, Jianyang Shi, Yameng Gao, Jingying Zheng, Jingtong He, Jing Zhang, Yang Song, Ruifei Zhang, Xuening Shi, Lina Jin* and Hui Li*

School of Public Health, Jilin University, Changchun, China

Introduction: Persistent infections caused by certain viruses and parasites have been associated with multiple diseases and substantial mortality. Heavy metals are ubiquitous environmental pollutants with immunosuppressive properties. This study aimed to determine whether heavy metals exposure suppress the immune system, thereby increasing the susceptibility to persistent infections.

Methods: Using data from NHANES 1999–2016, we explored the associations between heavy metals exposure and persistent infections: Cytomegalovirus (CMV), Epstein–Barr Virus (EBV), Hepatitis C Virus (HCV), Herpes Simplex Virus Type−1 (HSV−1), *Toxoplasma gondii* (*T. gondii*), and *Toxocara canis* and *Toxocara cati* (*Toxocara* spp.) by performing logistic regression, weighted quantile sum (WQS) and Bayesian kernel machine regression (BKMR) models. Mediation analysis was used to determine the mediating role of host immune function in these associations.

Results: Logistic regression analysis revealed positive associations between multiple heavy metals and the increased risk of persistent infections. In WQS models, the heavy metals mixture was associated with increased risks of several persistent infections: CMV (OR: 1.58; 95% CI: 1.17, 2.14), HCV (OR: 2.94; 95% CI: 1.68, 5.16), HSV−1 (OR: 1.25; 95% CI: 1.11, 1.42), *T. gondii* (OR: 1.97; 95% CI: 1.41, 2.76), and *Toxocara* spp. (OR: 1.76; 95% CI: 1.16, 2.66). BKMR models further confirmed the combined effects of heavy metals mixture and also identified the individual effect of arsenic, cadmium, and lead. On mediation analysis, the systemic immune inflammation index, which reflects the host's immune status, mediated 12.14% of the association of mixed heavy metals exposure with HSV−1 infection.

Discussion: The findings of this study revealed that heavy metals exposure may increase susceptibility to persistent infections, with the host's immune status potentially mediating this relationship. Reducing exposure to heavy metals may have preventive implications for persistent infections, and further prospective studies are needed to confirm these findings.

KEYWORDS

heavy metals, heavy metal mixtures, pathogens, infectious disease, immune system

Introduction

Heavy metals are a kind of metals and metalloids with density above 4 g/cm^3, including arsenic (As), cadmium (Cd), cobalt (Co), mercury (Hg), lead (Pb), molybdenum (Mo), antimony (Sb) and tungsten (W) (1). Besides existing widely in the natural environment, heavy metals are widely used in manufacturing, agriculture and

homes. Multiple heavy metals are commonly found in daily necessities, such as batteries, paint, cosmetics, and color pigments (2). Humans can be exposed to heavy metals through ingestion, inhalation and dermal absorption (3). In addition, humans can expose to multiple metals simultaneously due to similar exposure routes (2). There may be joint effects between multiple heavy metals on health, such as additive, antagonistic or synergistic effects (4). Due to widespread human exposure and long elimination half–lives, heavy metals have become a concerning class of environmental contaminants.

Existing literature focusing on the impacts of heavy metals on health indicates that exposure to heavy metals could lead to numerous diseases such as cardiovascular disease, nervous system disorders, multiple organ injury and cancers (5, 6). Notably, the potential toxic effect of heavy metals on immune system is receiving increasing attention and being actively researched. Environmental exposure to heavy metals has been noted to disturb immune system and impair its ability against infections (7, 8). Previous epidemiological studies on the impacts of heavy metals exposure on immune system focused on vaccine–induced antibody responses. For instance, prenatal exposure to Cd has been associated with reduced levels of vaccine–induced streptococcal antibodies in the offspring (9, 10). In a cohort study, exposure to Pb was found to be associated with low measles antibody titers in girls, and lower *Haemophilus influenzae* type B titers among children of HIV–positive mothers (11). Exposure to certain heavy metals has also been inversely associated with vaccine–induced diphtheria, pertussis, tetanus, hepatitis B, Japanese encephalitis, polio and measles virus antibodies in children (12). These findings indicate that heavy metals exposure compromises the humoral response, leading to lower vaccine effectiveness.

Currently, numerous pathogens can evade immune clearance to achieve persistent infections, including Cytomegalovirus (CMV), Epstein–Barr Virus (EBV), Hepatitis C Virus (HCV), Herpes Simplex Virus Type–1 (HSV–1), *Toxoplasma gondii* (*T. gondii*), and *Toxocara canis* and *Toxocara cati* (*Toxocara* spp.) (13–16). Recognized as risk factors for all–cause mortality, these infections represent a significant threat to human health, exacerbated by the lack of available vaccines (17). Furthermore, compelling evidence from experimental studies suggests that heavy metals exposure can suppress the innate immunity. Based on these findings, we hypothesize that the immunotoxicity of heavy metals may increase the susceptibility to persistent infections in humans.

In the present study, we included six types of persistent infections: CMV, EBV, HCV, HSV–1, *T. gondii* and *Toxocara* spp. The single and combined effects of multiple heavy metals exposure on these persistent infections were evaluated using logistic regression, weighted quantile regression (WQS), and Bayesian kernel machine regression (BKMR) models. Additionally, this study employed the systemic immune inflammation index (SII) to evaluate the host's immune status and identified its mediating role in the relationships between heavy metals exposure and the risks of persistent infections. Recognized as a stable and comprehensive biomarker, SII provides an accurate reflection of immune responses and inflammatory conditions (18). Its predictive and prognostic utility has been substantiated in numerous studies (19, 20). Our study might provide a new insight into the impacts of heavy metals exposure on persistent infections.

Materials and methods

Study design and population

National Health and Nutrition Examination Survey (NHANES) is a nationally cross–sectional survey conducted by the National Central for Health Statistics. The program aims to estimate the health and nutrition status of residents in the U.S. Participants are interviewed at home and received physical examinations and specimen collection in examination center. National Center for Health Statistics Research Ethics Review Board approved the NHANES survey and all participants provided written informed consents.

In this study, the analyzed data were extracted from public data in the 1999–2016 cycles. We included participants who had measurements of urinary heavy metals and available antibody testing results of CMV, EBV, HCV, HSV–1, *T. gondii* and *Toxocara* spp. Participants with missing data for covariates and mediating variable were excluded. We also excluded pregnant women, as pregnancy could increase susceptibility to infectious diseases (21). Finally, 959, 2,559, 3,725, 4,388, 2,309, and 2,173 participants were left for estimating the association of heavy metals exposure with CMV, EBV, HCV, HSV–1, *T. gondii*, and *Toxocara* spp. infections, respectively. More information about inclusion and exclusion for the study can be found in Supplementary Figure S1.

Measurement of heavy metals

In 1999–2016 survey years, urinary heavy metals were measured by inductively coupled plasma mass spectrometry technology. Eight elements, including As, Cd, Co, Hg, Pb, Mo, Sb, and W were analyzed in the study. When the concentrations of heavy metals were below the lower limit of detection (LLOD), the values were replaced by LLOD divided by the square root of two. In order to correct urinary dilution on the measurement of urinary heavy metals, urinary creatinine was applied to normalize the concentrations of urinary heavy metals which were expressed as μg/g creatinine.

Measurement of persistent infections

Available antibody testing results for CMV (1999–2004), EBV (2003–2010), HCV (2007–2012), HSV–1 (2003–2016), *T. gondii* (2009–2012) and *Toxocara* spp. (2011–2014) were examined. With the exception of antibody testing results for EBV, the testing results for other five pathogens were collected from participants aged 18 years or older. For EBV, available serum testing results from subjects aged 6–19 years were determined for EBV VCA IgG antibody using commercial enzyme immunoassay kits. According to the guideline of NHANES, the IgG antibody testing results for these pathogens were defined as positive and negative. The subjects with equivocal testing result were excluded in this study. Detailed laboratory methods can be found on the NHANES website.[1]

1 https://www.cdc.gov/nchs/nhanes/index.htm

Covariates assessment

All confounders were selected based on prior evidence related to heavy metals exposure and infections. Covariates included gender, age, race/ethnicity, education level, the ratio of family income to poverty (PIR), body mass index (BMI), serum cotinine, smoking status and drinking status. Among them, some covariates were entered into models as continuous variables, including age, PIR, BMI and serum cotinine. The race/ethnicity was divided into non-Hispanic white, non-Hispanic black, Mexican American, other. Education level was categorized into three groups: less than, equal to, and above high school. Smoking status was defined based on whether individuals had smoked at least 100 cigarettes in their lifetime and their current self–reported smoking status. The categories were: never smokers (non-current smoker and smoked less than 100 cigarettes), former smokers (non-current smoker but smoked at least 100 cigarettes) and current smokers (current smoker and smoked at least 100 cigarettes). Based on self–reported daily drinking level, alcohol consumption was defined as never (0 drink for women and men), moderate (≤1 drink for women or ≤2 drinks for men) and heavy (>1 drink for women or >2 drinks for men) (22). Because data on daily alcohol consumption and smoking status are not available for juveniles, the exploration of association between heavy metals exposure and EBV infection was not adjusted for smoking status and alcohol consumption.

Statistical analysis

The quantitative data was tested for the distribution type using the Kolmogorov–Smirnov test. In this study, the continuous variables were presented with medians and interquartile ranges, as the data were not normally distributed. Categorical variables were presented with *n* (%). The Mann–Whitney *U* test and Chi–square test were used to compare differences of participants characteristics by persistent infections status. The concentrations of heavy metals were ln–transformed due to the seriously skewed distribution. The Spearman rank correlation analysis was applied to calculate the correlation coefficients among ln–transformed concentrations of heavy metals.

We performed multivariable logistic regression models with ln–transformed concentration of each heavy metals as continuous variables to estimate the single effects of heavy metal exposure on the risks of persistent infections.

The WQS regression models were used to evaluate the overall effect of multiple heavy metals exposure on persistent infections. The model estimated an empirically weighted index based on the quantiles of chemicals (23). In the present study, WQS index was created based on quartiles of the heavy metals. The average empirical weights of individual heavy metals were calculated to assess the contributing effects of single heavy metal by using bootstrap sampling. The WQS weight index ranges from 0 to 1, with the sum of all weight index totaling 1. We initially constrained the model in the positive direction based on prior evidence, and subsequently constrained the direction to be negative to determine if there were negative relationships.

We further used the BKMR models to assess the potential associations between heavy metals and persistent infections. Under non-parametric Bayesian variable selection framework, BKMR model can estimate the non-linear and non-additive relationships among mixture exposures (24). Posterior inclusion probability (PIP) was calculated to measure the relative importance of individual exposures and the exposure variable with PIP greater than 0.5 was considered significant (25). Here, we evaluated the effects of heavy metals by using the Markov Chain Monte Carlo method for 10,000 iterations. All covariates were adjusted in BKMR models.

To explore the potential mediating effect of SII, we carried out mediation analyses. SII was calculated using the following formula: platelet count × neutrophil count/lymphocyte count (26). The concentrations of heavy metals mixture were calculated by WQS models and analyzed as continuous variables in mediation analysis. The proportion of mediated effect was calculated as follows: (indirect effect/total effect) × 100%. Bootstrapping was used for significance testing and the models adjusted for all covariates.

Finally, two sensitivity analyses were conducted. First, participants with extreme values (>90%, or <10%) of urinary heavy metals were excluded. Second, considering the limitation of the WQS model in limiting the direction of all exposure factors and outcome, we conducted qgcomp model. This model can calculate both the positive and negative weight coefficients for each component within the mixture.

All statistical analyses were conducted using SPSS statistical software (version 24.0; SPSS Inc., Chicago, IL, United States) and R software (version 4.2.1) with "gWQS" (version 3.0.4), "bkmr" (version 0.2.2) and "mediation" (version 4.5.0) packages. *P*–value <0.05 (two–tailed) was considered significant.

Results

Population characteristics

The basic characteristics of the study population were presented in Table 1. The prevalence of CMV, EBV, HCV, HSV–1, *T. gondii*, and *Toxocara* spp. infection was 62.25, 77.49, 2.41, 62.23, 9.59, and 6.26%, respectively. The median ages of the CMV, EBV, HCV, HSV–1, *T. gondii*, and *Toxocara* spp. infection groups were 36, 13, 48.5, 34, 48, and 47 years, respectively. In the EBV infection group, the population had a low level of BMI and serum cotinine. In the other five infection groups, about half of the population was non-Hispanic white, had a high school degree or above and were never smokers. Race, education level and PIR were significantly different between infected and non-infected participants ($p < 0.05$).

Distribution of urinary metals and correlations

The distribution information about urinary heavy metals is exhibited in Supplementary Table S1. The detection frequency of Mo was nearly 100%, which was the highest detection frequency. Mo had the highest and Sb had the lowest concentrations in urinary heavy metals. And the Spearman's rank correlation coefficient illustrated the relatively weak correlations between heavy metals (Supplementary Figure S2).

Associations of heavy metals exposure with CMV infection

The associations between individual heavy metal exposure and persistent infections based on multivariable logistic regression models were presented in Table 2. Each one–fold increase in ln–transformed

TABLE 1 Baseline characteristics of study population.

Characteristics	CMV infection		EBV infection		HCV infection		HSV-1 infection		*T. gondii* infection		*Toxocara* spp. infection	
	Total (*N* = 853)	*P*[c]	Total (*N* = 2,545)	*P*[c]	Total (*N* = 3,696)	*P*[c]	Total (*N* = 4,239)	*P*[c]	Total (*N* = 2,295)	*P*[c]	Total (*N* = 2,155)	*P*[c]
Age[a], years	36.00(28.00, 4.300)	<0.001	13.00(10.00, 16.00)	<0.001	48.50(34.00, 63.00)	0.210	34.00(27.00, 42.00)	<0.001	48.00(33.00, 62.00)	<0.001	47.00(32.00, 62.00)	0.024
PIR[a]	2.38(1.24, 4.00)	<0.001	1.51(0.79, 2.99)	<0.001	2.20(1.15, 4.20)	<0.001	2.15(1.11, 4.06)	<0.001	2.13(1.10, 4.12)	0.009	2.20(1.10, 4.34)	0.004
BMI[a], kg/m^2	27.47(23.61, 32.12)	0.001	21.00(17.85, 24.92)	<0.001	27.84(24.29, 32.27)	0.984	27.40(23.81, 32.28)	<0.001	27.80(24.20, 32.44)	0.026	27.60(24.00, 32.30)	0.111
Serum cotinine[a], ng/mL	0.24(0.04, 109.00)	0.410	0.07(0.02, 0.56)	<0.001	0.05(0.02, 31.58)	<0.001	0.12(0.02, 87.60)	0.271	0.05(0.01, 23.20)	0.928	0.04(0.01, 30.30)	0.089
Gender[b]		<0.001		0.046		0.027		0.006		0.153		<0.001
Male	263(30.83%)		1,328(52.18%)		2025(54.79%)		2,254(53.17%)		1,262(54.99%)		1,175(54.52%)	
Female	590(69.17%)		1,217 (47.82%)		1,671(45.21%)		1985(46.83%)		1,033(45.01%)		980(45.48%)	
Race/ethnicity[b]		<0.001		<0.001		0.004		<0.001		0.075		<0.001
Non-Hispanic White	434(50.88%)		715(28.09%)		1797(48.62%)		1892(44.63%)		1,106(48.19%)		1,011(46.91%)	
Non-Hispanic Black	182(21.34%)		740(29.08%)		746(20.18%)		831(19.60%)		454(19.78%)		419(19.44%)	
Mexican American	174(20.40%)		790(31.04%)		518(14.02%)		769(18.14%)		304(13.25%)		249(11.55%)	
Other	63(7.39%)		300(11.79%)		635(17.18%)		747(17.62%)		431(18.78%)		476(22.09%)	
Education level[b]		<0.001		0.002		<0.001		<0.001		0.001		<0.001
<High School	173(20.28%)		2,318(91.08%)		912(24.68%)		850(20.05%)		513(22.35%)		413(19.16%)	
High School/GED	234(27.43%)		138(5.42%)		835(22.59%)		988(23.31%)		502(21.87%)		459(21.30%)	
>High school	446(52.29%)		89(3.50%)		1949(52.73%)		2,401(56.64%)		1,280(55.77%)		1,283(59.54%)	
Smoking status[b]		0.543		NA		<0.001		0.148		0.101		0.593
Never	436(51.11%)		NA		1794(48.54%)		2,344(55.30%)		1,138(49.59%)		1,120(51.97%)	
Former	138(16.18%)		NA		1,046(28.30%)		699(16.49%)		641(27.93%)		566(26.26%)	
Current	279 (32.71%)		NA		856(23.16%)		1,196(28.21%)		516(22.48%)		469(21.76%)	
Alcohol consumption[b]		0.011		NA		<0.001		<0.001		0.693		0.009
Never	135(15.83%)		NA		761(20.59%)		530(12.50%)		428(18.65%)		401(18.61%)	
Moderate	282(33.06%)		NA		1,397(37.80%)		1,371(32.34%)		865(37.69%)		841(39.03%)	
Heavy	436(51.11%)		NA		1,538(41.61%)		2,338(55.15%)		1,002(43.66%)		913(42.37%)	

BMI, body mass index; CMV, Cytomegalovirus; EBV, Epstein–Barr Virus; GED, general educational development; HCV, Hepatitis C Virus; HSV-1, Herpes Simplex Virus Type-1; NA, not available; PIR, family income to poverty ratio; *T. gondii, Toxoplasma gondii*; *Toxocara* spp., *Toxocara canis* and *Toxocara cati*. [a]Continuous variables were presented as weighted median (P_{25}, P_{75}). [b]Categorical variables were presented as sample number (percentage). [c]*p*-value were derived from Mann–Whitney U test for continuous variables and Chi-square test for categorical variables.

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TABLE 2 Odd ratios and 95% confidence intervals for associations between heavy metals and persistent infections using multivariable logistic models[a].

	CMV infection[b]	EBV infection[c]	HCV infection[b]	HSV-1 infection[b]	*T. gondii* infection[b]	*Toxocara* spp. infection[b]
Arsenic	NA	0.99(0.87, 1.13)	1.04(0.82, 1.30)	**1.10(1.03, 1.18)**	**1.22(1.06, 1.40)**	**1.27(1.06, 1.51)**
Cadmium	**1.43(1.11, 1.84)**	1.00(0.85, 1.17)	**2.41(1.70, 3.41)**	**1.25(1.13, 1.39)**	**1.28(1.03, 1.61)**	1.20(0.93, 1.55)
Cobalt	1.10(0.84, 1.45)	0.92(0.77, 1.11)	**1.38(1.01, 1.89)**	1.01(0.90, 1.14)	1.17(0.95, 1.44)	1.06(0.79, 1.42)
Mercury	0.85(0.72, 1.00)	1.09(0.97, 1.21)	0.95(0.73, 1.23)	**1.10(1.03, 1.18)**	1.17(0.99, 1.37)	0.92(0.76, 1.12)
Molybdenum	0.97(0.76, 1.24)	0.85(0.70, 1.02)	1.07(0.77, 1.50)	0.93(0.84, 1.04)	1.17(0.94, 1.47)	1.17(0.89, 1.55)
Lead	1.10(0.85, 1.43)	**1.45(1.23, 1.71)**	1.36(0.99, 1.87)	**1.25(1.13, 1.39)**	**1.55(1.25, 1.91)**	1.28(0.99, 1.64)
Antimony	0.87(0.69, 1.11)	**1.24(1.04, 1.48)**	1.24(0.91, 1.70)	**1.15(1.04, 1.27)**	1.14(0.92, 1.40)	0.89(0.68, 1.17)
Tungsten	0.87(0.72, 1.06)	1.00(0.89, 1.13)	1.25(0.98, 1.60)	0.96(0.89, 1.03)	0.85(0.71, 1.02)	0.88(0.71, 1.09)

CMV, Cytomegalovirus; EBV, Epstein–Barr Virus; HCV, Hepatitis C Virus; HPV, Human papillomavirus; HSV-1, Herpes Simplex Virus Type-1; NA, not available; *T. gondii*: *Toxoplasma gondii*; *Toxocara* spp., *Toxocara canis* and *Toxocara cati*. [a]Confidence intervals that do not overlap the null value of odd ratio = 1 are shown in blod. [b]Covariates adjusted include gender, age, race, education level, family income to poverty ratio, body mass index, serum cotinine, smoking status and alcohol consumption. [c]Covariates adjusted include gender, age, race, education level, family income to poverty ratio, body mass index and serum cotinine.

TABLE 3 Associations between weighted quantile sum regression index and persistent infections.

Model[a]	Outcomes	OR (95% CI)	*P*-value
Positive			
	Cytomegalovirus infection	1.58(1.17, 2.14)	0.003
	Epstein–Barr Virus infection	1.16(0.94, 1.43)	0.166
	Hepatitis C Virus infection	2.94(1.68, 5.16)	<0.001
	Herpes Simplex Virus Type-1 infection	1.25(1.11, 1.42)	<0.001
	Toxoplasma gondii infection	1.97(1.41, 2.76)	<0.001
	Toxocara spp. infection	1.76(1.16, 2.66)	0.008
Negative			
	Cytomegalovirus infection	1.15(0.87, 1.52)	0.338
	Epstein–Barr Virus infection	1.06(0.84, 1.33)	0.626
	Hepatitis C Virus infection	0.98(0.63, 1.51)	0.919
	Herpes Simplex Virus Type-1 infection	1.06(0.93, 1.21)	0.373
	Toxoplasma gondii infection	0.98(0.78, 1.24)	0.894
	Toxocara spp. infection	0.94(0.69, 1.28)	0.682

OR, odds ratio; CI, confidence interval. [a] "Negative" and "Positive" indicate the index in weighted quantile sum regression models was restricted in the negative and positive directions.

urinary Cd was associated with 43% (OR: 1.43, 95% CI: 1.11, 1.84) higher odds of CMV infection.

The WQS analysis was performed to estimate the effects of exposure to heavy metals mixture on the risks of persistent infections. After adjusting all confounders, exposure to heavy metals mixture was significantly positively associated with CMV infection (OR: 1.58, 95% CI: 1.17, 2.14) (Table 3). The estimated weights of heavy metals are displayed in Figure 1A. Cd had the highest weight, with a weight of 0.58. Whereas Hg (weighted <0.01) weighted lightest in CMV infection. In addition, when the direction in WQS models was constrained to be negative, mixed exposure to heavy metals had no significant negative correlations with CMV infection.

To further identify the combined effect of heavy metals on persistent infections and potential relationships between heavy metals, we treated the concentrations of heavy metals as continuous variables to fit the BKMR models. The PI*p* values of each heavy metal exposure were summarized in Supplementary Table S2. Results showed that Cd (PIP = 0.996) played a major role in CMV infection. The finding of Cd as the main heavy metal contributing to CMV infection in BKMR models was similar to the result produced by WQS models. Figure 2 represented the joint and independent effects of heavy metals mixture on persistent infections. The latent continuous outcomes of CMV infection showed a significant increase when all heavy metals at 65th percentiles or above in comparison with their median values, indicating significant positive associations between heavy metals mixture and CMV infection (Figure 2A). Cd was significantly related to the increased risk of CMV infection when other heavy metals were fixed at the 25th, 50th, and 75th percentiles (Figure 2B). We also found the non-linear association between Cd and CMV infection when other heavy metals were fixed at their median values (Figure 3A).

Associations of heavy metals exposure with EBV infection

In multivariable logistic regression model, Pb (OR: 1.45, 95% CI: 1.23, 1.71) and Sb (OR: 1.24, 95% CI: 1.04, 1.48) were positively associated with the risk of EBV infection (Table 2).

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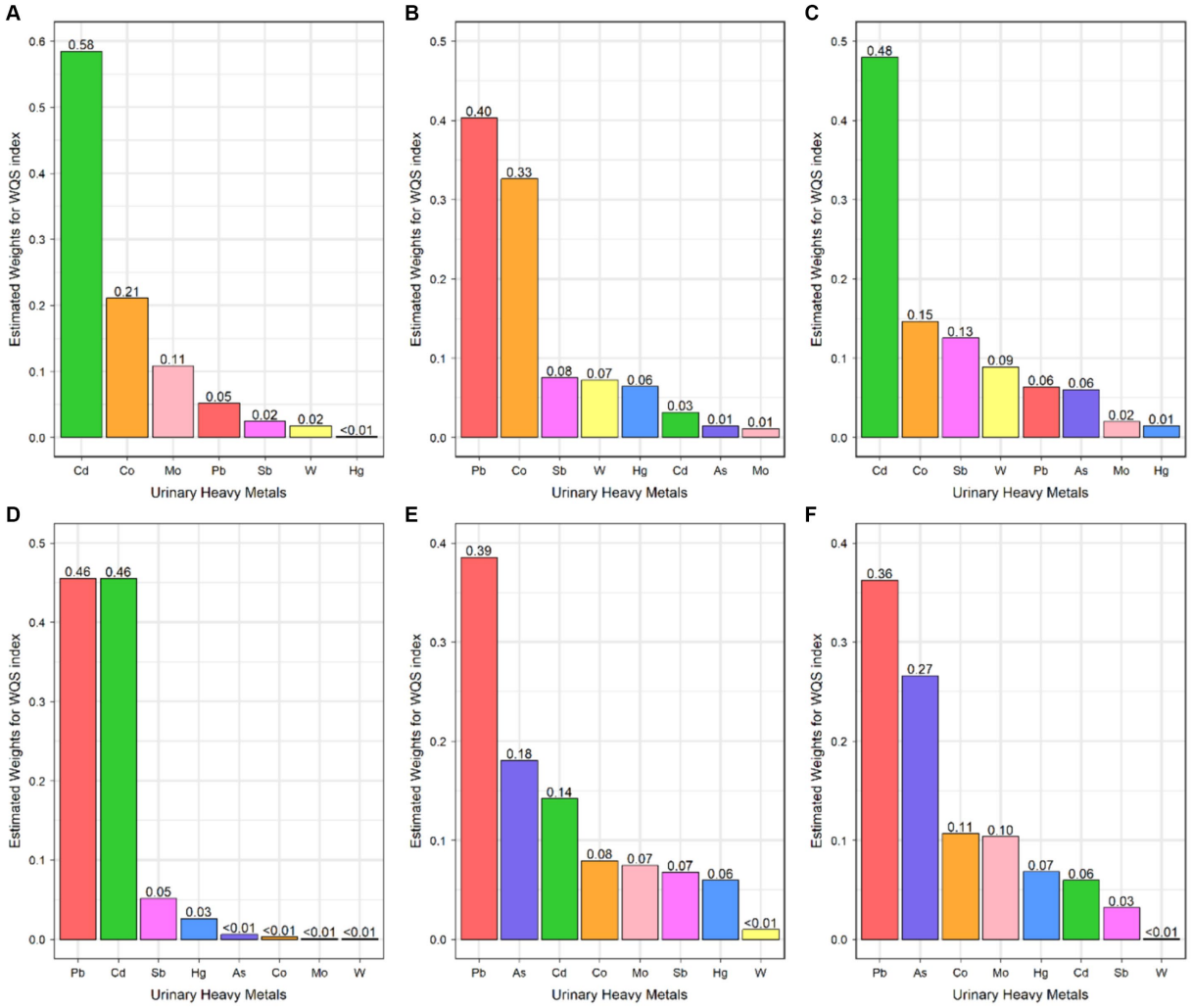

FIGURE 1
Weighted quantile sum regression index weights for Cytomegalovirus infection **(A)**, Epstein–Barr Virus infection **(B)**, Hepatitis C Virus infection **(C)**, Herpes Simplex Virus Type-1 infection **(D)**, *Toxoplasma gondii* infection **(E)**, and *Toxocara* spp. infection **(F)**.

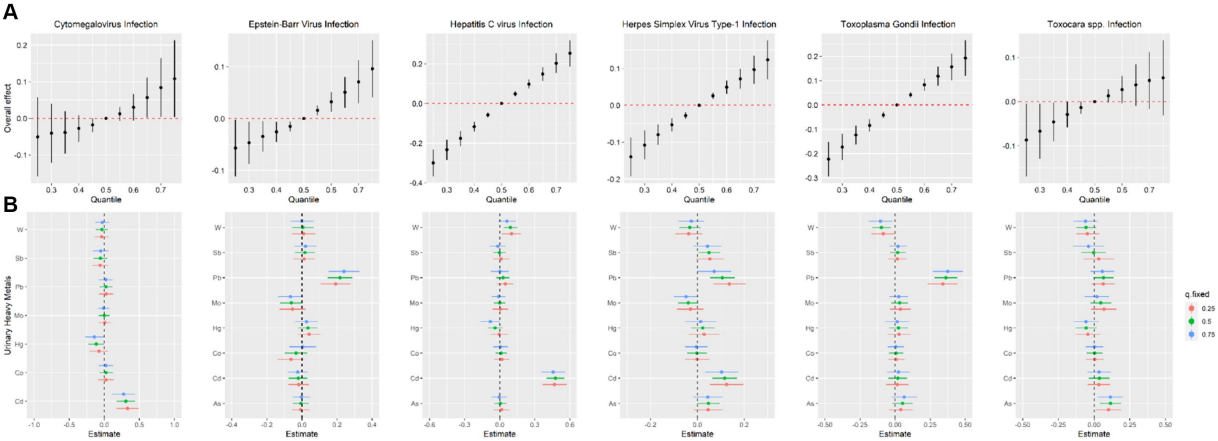

FIGURE 2
Estimated joint effects **(A)** and independent effects **(B)** of urinary heavy metal mixtures on Cytomegalovirus infection, Epstein–Barr Virus infection, Hepatitis C Virus infection, Herpes Simplex Virus Type-1 infection, *Toxoplasma gondii* infection and *Toxocara* spp. infection by Bayesian kernel machine regression models.

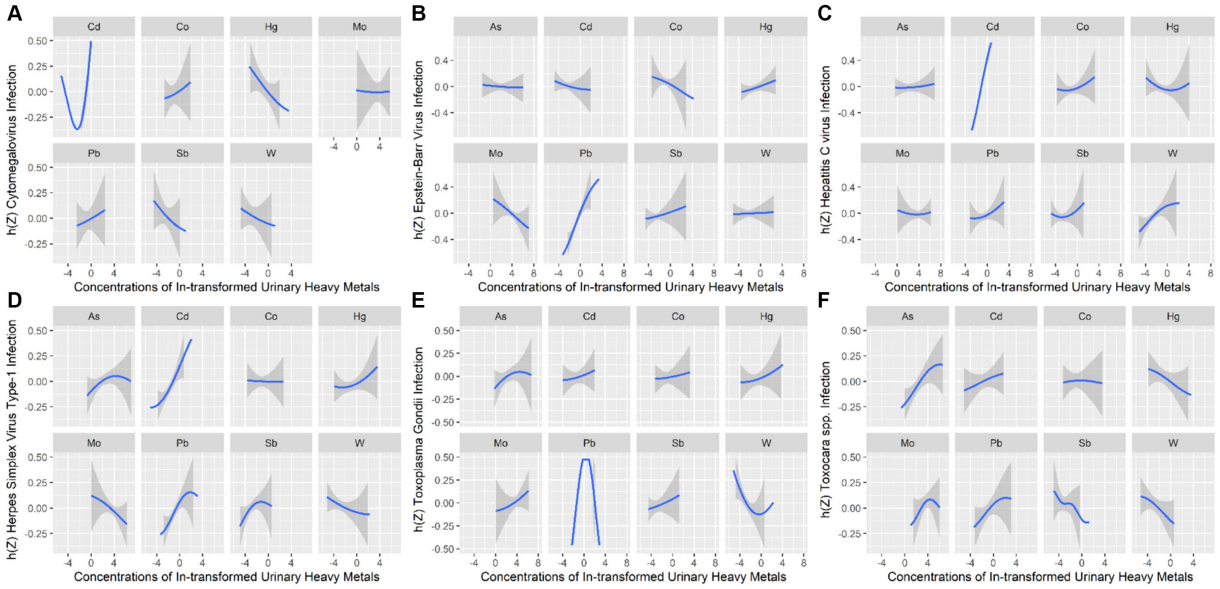

FIGURE 3
Univariate exposure–response relationships between heavy metals exposure and Cytomegalovirus infection **(A)**, Epstein–Barr Virus infection **(B)**, Hepatitis C Virus infection **(C)**, Herpes Simplex Virus Type-1 infection **(D)**, *Toxoplasma gondii* infection **(E)**, and *Toxocara* spp. infection **(F)**. h(Z) can be interpreted as the relationship between heavy metals and persistent infections.

As for the combined effect of heavy metals, the significant association between heavy metals mixture and EBV infection in the WQS model was not found (Table 3; Figure 1B).

In BKMR model, Pb appeared to drive the overall effect (PIP = 1.000) (Supplementary Table S2). The joint effect on the risk of EBV infection increased as the cumulative level with heavy metal mixture exposure increased (Figure 2A). Pb displayed a significantly independent effect on the risk of EBV infection, and the association was non-linear (Figures 2B, 3B). Besides, the bivariate exposure–response functions reflecting the interactions between each pair of heavy metals on persistent infections were determined. When Pb was fixed at 10th, 50th, and 90th percentiles, the slope of Co was inconsistent, indicating that there might be interactions between Pb and Co (Supplementary Figure S3B).

Associations of heavy metals exposure with HCV infection

After adjusting for covariates, the multivariate–adjusted ORs (95% CIs) of HCV risk were 2.41 (1.70, 3.41) for Cd and 1.38 (1.01, 1.89) for Co (Table 2).

In WQS model, a quartile increase in the WQS index of heavy metals mixture was statistically significantly associated with HCV infection (OR: 2.94, 95% CI: 1.68, 5.16) (Table 3). Cd (weighted 0.48) was the most heavily weighted heavy metal responsible for the positive association with HCV infection (Figure 1C).

The PIPs calculated by BKMR model showed that Cd (PIP = 1.000) and W (PIP = 0.555) were found to be relatively important (>0.5) for association between heavy metals mixture and HCV infection (Supplementary Table S2). The overall association between heavy metals mixture and HCV infection is shown in Figure 2A, from which we found a significant and positive association when heavy metals mixture concentration was at 55th percentile and above, compared to their median concentration. When the levels of all heavy metals were below their respective median values, significant negative associations of mixed heavy metals exposure with HCV infection were found, with decreasing negative correlations with increasing exposure levels of heavy metals mixture. Figure 2B displayed the significant and positive effect of Cd on HCV infection, which was consistent with the finding from WQS model. In addition, we found that multiple heavy metals (Co, Hg, Pb, Sb, and W) had non-linear correlations with the risk of HCV infection, with increasing trends in the high concentration (Figure 3C).

Associations of heavy metals exposure with HSV–1 infection

Multiple heavy metals were associated with the increased odds of HSV–1 infection. The positive associations of As, Cd, Hg, Pb, and Sb with the elevated risks of HSV–1 infection were found and the corresponding ORs and 95% CIs were 1.10 (1.03, 1.18), 1.25 (1.13, 1.39), 1.10 (1.03, 1.18), 1.25 (1.13, 1.39), and 1.15 (1.04, 1.27), respectively (Table 2).

The WQS index of heavy metals mixture was significantly associated with increased odds of HSV–1 infection (OR: 1.25, 95% CI: 1.11, 1.42) (Table 3). Besides, Pb (weighted 0.46) and Cd (weighted 0.46) were the top two weighted heavy metals contributing to the overall effect on HSV–1 infections (Figure 1D).

The BKMR analyses further identified the potential association between heavy metals and HSV–1 infection. In the eight urinary heavy metals, the highest PIP was found for Cd (PIP = 0.990), followed by Pb (PIP = 0.975), Sb (PIP = 0.523), and Mo (PIP = 0.513) (Supplementary Table S2). The concentration of heavy metals mixture at or above the 55th percentile was positively associated with the risk of HSV–1 infection (Figure 2A). Besides, when other heavy metals

were fixed at 25th, 50th, and 75th percentile, Cd displayed significant independent effect on the risk of HSV–1 infection (Figure 2B). The trends in exposure–response functions were displayed in Figure 3D. When other heavy metals were fixed at their median values, Cd, Hg, and Pb exhibited non-linear positive relationships with HSV–1 infections. We further investigated the interactions between the heavy metals. The results suggested the existence of potential interactions among As, Cd, Pb, Hg, and Sb (Supplementary Figure S3D).

To determine if SII, reflecting the host immune status, is a mediator on the associations of heavy metals exposure with persistent infections, we calculated the mixed exposure concentration of eight heavy metals based on WQS models and performed mediation analyses (Figures 4A–F). The significant mediation effect of SII in the association between heavy metals mixture and HSV–1 infections was found. The total effect of heavy metals mixture on HSV–1 infection was 0.0585 and a direct effect of 0.0514. The mediating effect of SII was 0.0071, indicating that SII mediated 12.14% of the association between exposure to heavy metals mixture and HSV–1 infection (Figure 4D).

Associations of heavy metals exposure with *T. gondii* infection

As for the independent effect of heavy metals, there were significant associations of As, Cd, and Pb with increased odds of *T. gondii* infection, with the adjusted OR (95% CI) of 1.22(1.06, 1.40), 1.28(1.03, 1.61), and 1.55(1.25, 1.91), respectively (Table 2).

Combined exposure to heavy metals was significantly associated with the increased odds of *T. gondii* infection (OR: 1.97; 95% CI: 1.41, 2.76) (Table 3). The highest weighted heavy metal in *T. gondii* infection model was Pb (weighted 0.39). As and Cd were highly weighted in *T. gondii* infection (weighted 0.18 and 0.14, respectively) (Figure 1E).

Similar results were found in BKMR model which identified Pb (PIP = 1.000) as the most important contributor for the positive association with *T. gondii* infection (Supplementary Table S2). Figure 2A showed a significant positive correlation between mixed heavy metals exposure and *T. gondii* infection, suggesting that the risk of *T. gondii* infection will increase significantly when multiple heavy metals are increased. Pb was significantly associated with increased risk of *T. gondii* infection when the concentrations of other heavy metals were fixed at their 25th, 50th, and 75th percentiles (Figure 2B). In univariate exposure–response functions, Co and Sb had approximately linear positive relationships with *T. gondii* infection, and As and Hg exhibited non-linear relationships when each of heavy metals was held at its median value (Figure 3E). Besides, the parallel lines presented in Supplementary Figure S3E indicated no evidence of interaction among these heavy metals.

Associations of heavy metals exposure with *Toxocara* spp. infection

In logistic regression model, As was found to be significantly associated with *Toxocara* spp. infection (OR: 1.27, 95% CI: 1.06, 1.51) (Table 2).

In the WQS positive model, we found that mixed exposure to eight heavy metals was positively associated with *Toxocara* spp. infection (OR: 1.76, 95% CI: 1.16, 2.66) (Table 3). Pb was the predominant heavy metal responsible for the positive association with *Toxocara* spp. infection (weighted 0.36), followed by As (weighted 0.27) (Figure 1F).

The PIPs derived from the BKMR model showed that all PIP values of eight heavy metals were higher than 0.5, and As (PIP = 0.855), Mo (PIP = 0.692), Pb (PIP = 0.687) had the highest rankings within association between the concentrations of eight heavy metals and *Toxocara* spp. infection (Supplementary Table S2). Although no statistically significant overall associations between heavy metals mixture and *Toxocara* spp. infection were found, there was an increasing trend (Figure 2A). As was significantly related to the risk of *Toxocara* spp. infection when other heavy metals were fixed at 25th, 50th, and 75th percentiles (Figure 2B). When other heavy metals were at the median level, As, Pb, and Cd

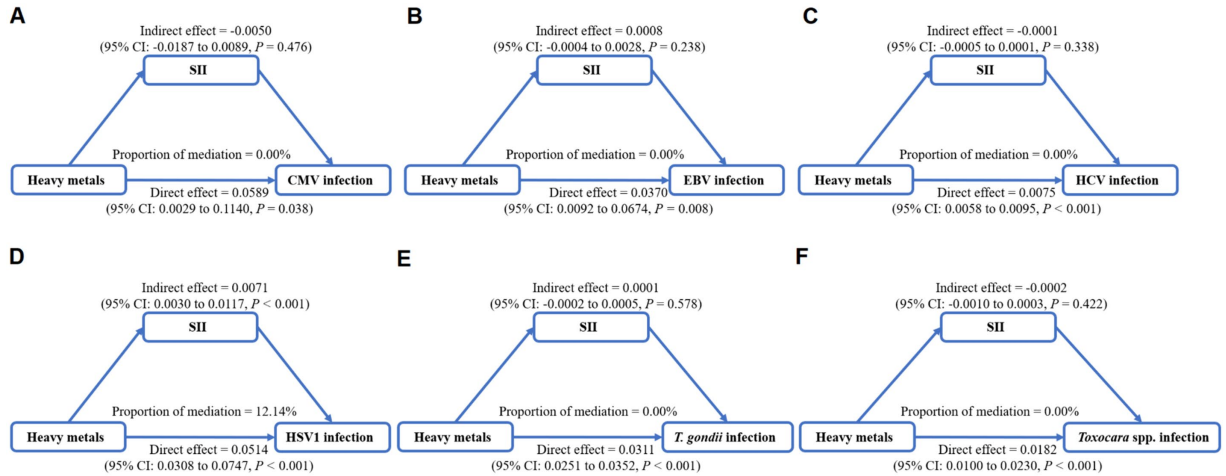

FIGURE 4
Mediation analysis of systemic immune inflammation index (SII) on the interactions between heavy metals mixture and Cytomegalovirus infection **(A)**, Epstein–Barr Virus infection **(B)**, Hepatitis C Virus infection **(C)**, Herpes Simplex Virus Type-1 infection **(D)**, *Toxoplasma gondii* infection **(E)**, and *Toxocara* spp. infection **(F)**.

exhibited positive non-linear association with *Toxocara* spp. infection (Figure 3F).

Sensitivity analyses

Two sensitivity analyses were conducted to confirm the robustness of our results. The positive associations between heavy metals exposure and persistent infections remained when excluding participants with extreme values of heavy metals (Supplementary Table S3). Besides, qgcomp analysis showed the significant associations of heavy metals mixture exposure with HCV (OR: 2.21; 95% CI: 1.38, 3.56), HSV–1 (OR: 1.29; 95% CI: 1.12, 1.47) and *T. gondii* (OR: 2.03; 95% CI: 1.49, 2.76) infections (Supplementary Table S4 and Supplementary Figure S4).

Discussion

In this cross–sectional study, we investigated the associations of heavy metals exposure with several persistent infections and the mediating effect of SII which reflected the host immune function in these associations. Using logistic regression, multiple heavy metals (As, Cd, Co, Hg, Pb, and Sb) were identified to be positively associated with the risk of persistent infections. The results of WQS and BKMR analyses consistently showed the association between heavy metals mixture exposure and the increased risk of persistent infections, and emphasized As, Pb, and Cd as the heaviest contributors for persistent infections. In mediation analyses, SII was found to contribute 12.14% in the relationship of heavy metals mixture exposure with HSV–1 infection.

Our findings were supported by previous studies, which suggested associations between higher exposure levels of Pb and Cd and increased risks of *Helicobacter pylori*, herpes simplex virus type 2, hepatitis B virus, *T. gondii* and human immunodeficiency virus infections (27–30). In a prospective study of 214 mother–infant pairs, the higher maternal As exposure was found to relate to infant infections and there was an increasing trend in the number of infant infections with higher maternal As concentrations (31).

The biological mechanisms underlying the association between heavy metals exposure and persistent infections remain inconclusive. A possible situation is that heavy metals exposure affects the risk of infections by altering the immune function. The outcome and severity of infections are highly dependent on innate and adaptive immunity (32). The weakened immune function has been confirmed to be a risk factor for infections. Immunocompromised individuals have been shown to have a higher risk for persistent infections and developing multiple diseases (33, 34). Heavy metals are ubiquitous environmental pollutants with immunotoxicity. Epidemiological investigations found that exposure to heavy metals can suppress the antibody–mediated immunity (35). Data from animal studies suggested that heavy metals exposure also suppresses non-specific immune responses. Exposure to heavy metals can disturb immune function by affecting the production of immune cells, inducing alterations of inflammatory markers, and altering the levels of cytokines (36, 37). These immune substances play an important role in killing virus–infected cells and engulfing parasites (38, 39), and as such, impaired immune function can increase susceptibility to infections. Prior studies have demonstrated that the immunotoxicity of heavy metals could impair the ability to defend infectious agents, subsequently augmenting the host's susceptibility to infections. Findings by Cox et al. (40) found that exposure to Cd can significantly disrupt the immune function of macrophages, key immune cells that play a pivotal role in the body's defense against pathogens. This disruption can result in an increased susceptibility to infections, particularly among individuals suffering from chronic obstructive pulmonary disease. Exposure to Pb can exert toxic effects on the immune system, potentially leading to an increased susceptibility to various infections, including Influenza virus and hepatitis B virus (41, 42). A prospective cohort study revealed that prenatal exposure to As in drinking water was associated with an increased risk of developing acute respiratory infection in children (43). Similarly, an experimental study utilizing a mouse model for both prenatal and postnatal periods has discovered that early life exposure to As could aggravate inflammatory responses, thereby elevating the risk of developing respiratory infections (44). Several *in vitro* and experimental studies have also reported similar findings regarding the effects of nickel and Hg (35, 45). Recent studies have revealed a positive correlation between heavy metal exposure and SII, indicating that as the level of heavy metal exposure increases, the body's immune–inflammatory response intensifies (46–48). Prolonged immune–inflammatory responses can lead to immune system dysregulation and affect the normal functioning of the immune system. Based on a nationally representative sample, we discovered the intermediary role of SII in the interaction of heavy metals exposure with the increased risk of HSV–1 infection, suggesting that the alteration of host immune function might be responsible for the increased risk of persistent infections. The result provided epidemiological evidence for our hypothesis.

In this study, we employed three different statistical methods to explore the association between heavy metals exposure and persistent infections. Logistic regression model is a common analysis approach to assess the effect of single exposure factor on health. Our results of logistic regression analysis showed that typical heavy metals, such as As, Pb, Hg, and Cd, were positively related to several persistent infections. However, relying solely on logistic models might ignore the interactions between heavy metals, contributing to misleading results. WQS and BKMR are two analysis models recently developed to estimate the effects of mixture. By using WQS models, we found that exposure to higher levels of heavy metals mixture could increase the risks of CMV, HCV, HSV–1, *T. gondii* and *Toxocara* spp. infections. Due to the restriction of correlations in one direction, WQS model is unable to evaluate the joint effect of exposure factors in diverse effect directions (49). BKMR is a more comprehensive analysis method for identifying the joint effects of mixture, single exposure effects, non-linear associations, and potential interactions. Besides further confirming the findings in the WQS model, BKMR analysis also found the major contributing effect of As for *Toxocara* spp. infection, Cd for CMV, HCV and HSV–1 infections, and Pb for EBV and *Toxocara* spp. infections. With this approach, we observed the non-linear associations between multiple heavy

metals and persistent infections, and potential interactions between heavy metals. These three models estimated the effect of heavy metals on persistent infections from different aspects, verifying the comprehensiveness and reliability of this study.

Several limitations within our study should be mentioned. Firstly, the findings are derived from cross–sectional survey data, which imposes constraints on establishing temporality and causality. Persistent infections are diseases contributed by a variety of factors and developed over a long time. Multiple heavy metals, with bioaccumulation potential, exhibit long half–lives within the human body (50, 51). It remains unclear whether exposure to heavy metals precedes initial infections. Further research employing experimental and prospective design studies are needed to provide more evidence. Secondly, for infections such as CMV (52), EBV (53), HCV (54), HSV–1 (55), *T. gondii* (56), and *Toxocara* spp. (57), IgG antibodies are typically produced within the initial months following infection and generally persist for lifetime of the individual. This timeline may result in the exclusion of very recently acquired infections from our analysis. Thirdly, the accurate assessment of heavy metal exposure is impeded by the body's metabolism of these substances and the practice of substituting values below LLOD with a fixed value, which may introduce measurement bias. Lastly, our study may not have adequately controlled for all residual confounders that could influence the outcomes, including dietary habits, occupational exposures, and behavioral factors.

Conclusion

In summary, our results provided evidences for the associations between heavy metals exposure and persistent infections. The typical heavy metals, such as As, Cd, Pb, and Hg, were the main contributors for the increased risk of persistent infections. Furthermore, our study highlighted the mediating role of host immune function in the causal pathway linking mixed heavy metals exposure to an increased risk of HSV–1 infection. Reducing the exposure level of heavy metals could potentially decrease an individual's susceptibility to infectious diseases and long–term impacts of persistent infections. This study suggested new perspectives for the prevention of persistent infections and provided clues for further studies in this area.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cdc.gov/nchs/nhanes/index.htm.

Ethics statement

The studies involving humans were approved by National Center for Health Statistics Research Ethics Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

HZ: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing – original draft, Visualization, Writing – review & editing. JW: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. KZ: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. JS: Data curation, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft. YG: Data curation, Methodology, Software, Writing – original draft. JZhe: Investigation, Supervision, Validation, Writing – original draft. JH: Investigation, Supervision, Validation, Writing – original draft. JZha: Investigation, Supervision, Writing – original draft. YS: Investigation, Supervision, Writing – original draft. RZ: Supervision, Writing – original draft. XS: Supervision, Writing – original draft. LJ: Investigation, Methodology, Supervision, Writing – review & editing. HL: Conceptualization, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2024.1367644/full#supplementary-material

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EDITED BY
Azubuike Chukwuka,
National Environmental Standards and Regulations Enforcement Agency (NESREA), Nigeria

REVIEWED BY
Khaled Salama,
Imam Abdulrahman Bin Faisal University, Saudi Arabia
Birhanu Sewunet,
Wollo University, Ethiopia

*CORRESPONDENCE
Junjie Fu
✉ 1326621040@qq.com

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Internal blood lead exposure levels in permanent residents of Jiangxi Province and its effects on routine hematological and biochemical indices

Wenxin He[1,2], Junjie Fu[1]*, Ruiyi Fu[3], Xiaoguang Song[1], Siyue Huang[4], Yujue Wang[5], Keke Lu[6] and Hao Wu[1]

[1]Jiangxi Provincial Center for Disease Control and Prevention, Nanchang, Jiangxi, China, [2]School of Public Health, Nanchang University, Nanchang, Jiangxi, China, [3]Faculty of Business and Economics, The University of Melbourne, Parkville, VIC, Australia, [4]Dermatology Hospital of Jiangxi Province, Nanchang, Jiangxi, China, [5]Nanchang Health Promotion Center, Nanchang, Jiangxi, China, [6]Jiangxi Provincial Patriotic Hygiene and Health Promotion Center, Nanchang, Jiangxi, China

Background: Lead exposure levels are closely linked to human health and can cause damage to multiple organ systems, including the blood system and liver. However, due to insufficient evidence, the effects of lead exposure on hematological and biochemical indices have not been fully established.

Objective: This study aims to explore the blood lead levels of permanent residents in Jiangxi Province and analyze the factors affecting blood lead levels and the impact of blood lead levels on hematological and biochemical indices.

Methods: We conducted a cross-sectional study including questionnaires, health examinations, and blood sample examinations on 720 randomly selected permanent residents (3–79 years) in Jiangxi Province in 2018. The blood lead levels were measured using inductively coupled plasma mass spectrometry. Routine hematological and biochemical tests were determined by qualified medical institutions using automated hematology analyzers and biochemistry analyzers.

Results: The geometric mean of blood lead concentration in permanent residents of Jiangxi Province was 20.45 μg/L. Gender, age, annual household income, smoking, and hypertension were the influencing factors for blood lead levels. For each 1 μg/L increase in blood lead, the risks of elevated red blood cell count (from low to high), platelet volume distribution width, alkaline phosphatase (from low to high), and cholesterol increased by 2.4, 1.6, 3.6, and 2.3%, respectively, whereas the risks of elevation of direct bilirubin and total bilirubin both decreased by 1.7%.

Conclusion: The blood lead level in permanent residents of Jiangxi Province is higher than the national average. Higher blood lead levels were found in men than in women; blood lead levels were positively correlated with age but negatively correlated with annual household income; smoking and hypertension are risk factors for elevated blood lead; and blood lead levels affect routine hematological and biochemical markers such as red blood cell count, platelet volume distribution width, direct bilirubin, total bilirubin, alkaline phosphatase, and cholesterol.

KEYWORDS

blood lead levels, hematological indices, biochemical indices, heavy metals, biomonitoring

1 Introduction

Lead, also known as Pb, is a toxic heavy metal element that is widely found in human production and the living environment. Humans are exposed to Pb mainly through their diet, drinking water, air, and industrial production such as heavy metals, fuel, batteries, and gasoline manufacturing (1). In terms of diet, the test results of a total of 1,065 food items in six categories in Jiangxi Province during the period from 2018 to 2021 showed that the exceedance rate of lead was 0.75% (2). In terms of soil and water, some river water bodies in Jiangxi Province have high levels of lead (3–5); the lead content in river sediments is generally higher than the soil background value (3, 6, 7); and the lead content of Jiangxi soils, especially those in mining areas, is serious soil background value (8–12). In terms of air, atmospheric lead emissions in Jiangxi Province have increased in recent years, from 23rd in the country in 2002 (less than 200 tons) to 11th in the country in 2017 (nearly 400 tons), with non-ferrous metal smelting, industrial coal combustion, and iron and steel smelting being the main sources of lead (13). In terms of agriculture and industry, there have been reports indicating that workers in certain industries in Jiangxi Province have seriously exceeded blood lead levels (14, 15), and incidents of lead contamination, such as from improperly treated sewage from storage batteries (16) and lead contamination due to irrigation of swine wastewater (17), have also occurred occasionally. In summary, there is a certain risk of environmental lead exposure in Jiangxi Province.

Blood is both a transport medium and a key toxic target for lead. Once in circulation, blood lead levels reflect the balance of tissue absorption and accumulation. According to the clinical guidelines for blood lead levels of the China Health Council, 400 μg/L is considered harmful to health for adults and 100 μg/L for children. However, none of the blood lead values are considered safe. Due to the high toxicity and slow clearance rate, even small amounts of lead can cause damage to the nervous system, cardiovascular system, reproductive system, hematopoietic system, and liver health (18–20).

Lead blood exposure has been found to have significant effects and alterations on many hematological indicators (21). Most studies consider Pb as a harmful hematological factor that can lead to a decrease in red blood cell count, mean platelet volume, and hemoglobin content. However, some studies have come to the opposite conclusion (22, 23), Chwalba et al. (24) suggested that long-term lead exposure at levels of <50 μg/dL did not affect red blood cell counts and hemoglobin levels, and that long-term lead exposure elevated mean platelet volume compared to short-term exposure, suggesting that the relationship between lead exposure and hematological parameters is unclear and that different levels of lead exposure may have different effects on hematological indices The liver is both an important detoxification organ in the body and the site of initial lead storage and damage. Lead absorbed into the circulation reaches the liver rapidly, where it is metabolized, accumulated, and excreted through the liver, causing pathological and biochemical alterations in these organs and thus indirectly affecting blood biochemical parameters. Some studies have shown that blood lead is positively correlated with total cholesterol (25), and liver enzymes (ALP, ALT, and AST) while negatively correlated with direct bilirubin (26). However, an animal study by Pandi Prabha showed that lead toxicity reduced the levels of the liver marker enzymes alanine aminotransferase and aspartate aminotransferase in fish serum (27).

In conclusion, it is important to pay attention to the lead exposure issue in Jiangxi Province. However, the current research on blood lead and human health indicators focuses on high-risk groups or high levels of lead exposure, and there are not many human studies on the monitoring of lead exposure in the general population and the impact of low-level blood lead exposure on blood and biochemical indicators, and the relevant studies in Jiangxi Province are still blank. The connections among blood lead and hematological and biochemical indices are not conclusive yet. Therefore, we investigated the status of lead blood internal exposure in permanent residents of Jiangxi Province, aiming to understand the blood lead levels and their effects on routine hematological and biochemical indices in permanent residents of the province, to provide research evidence to elaborate the mechanisms of lead effects on routine hematological and biochemical indicators, and to provide a scientific basis for the development of targeted health strategies.

2 Materials and methods

2.1 Sample size estimation

The minimum sample size of the monitoring sites was determined using the formula sample size:

$$n = \left(\frac{z_{\alpha/2} S}{\varepsilon \bar{X}} \right)^2 deff$$

Where $\alpha = 0.05$, the degree of certainty $z_{\alpha/2}$ was 1.96, the relative error ε was taken as 10%, $\bar{X}$ and S was taken as the mean and standard deviation of environmental lead exposure in China, 24.98 and 10.75, respectively (28), and the design effect *deff* was estimated as 2. The minimum sample size n was calculated to be 142. Taking into account the feasibility of the project, national and local financial support, and the balanced distribution of gender and age, it was determined that the monitoring would be carried out in five monitoring sites across the province based on meeting the sampling requirements, with 144 people in each monitoring site for a total sample size of 720 people.

2.2 Study population

The study population was permanent residents aged 3–79 years in the survey area (living in the monitoring area for more than 6 months in

the 12 months prior to the survey). Permanent residents for the purpose of this monitoring were defined as citizens of Chinese nationality who had lived in the monitoring area for more than 6 months in the 12 months prior to the survey, excluding residents in the functional areas of their residences, such as the military, schools, nursing homes, and so on.

2.3 Sampling methods

The method of multi-stage stratified random sampling was adopted, based on the urbanization rate and secondary industry employment rate of 100 counties (districts and cities) in Jiangxi Province, after stratification by clustering method, systematic sampling was used to select five monitoring sites in Duchang County, Jizhou District, Jinxian County, Lushan City and Qingshanhu District. Each monitoring site was divided into two strata according to urban and rural areas, and the urban/rural sampling ratio for the three survey sites was determined on the basis of the urbanization rate. Based on the demographic information obtained from the survey sites, the population was divided into 6 strata according to age: 3–5, 6–11, 12–18, 19–39, 40–59, and 60–79 years old, and within each stratum, it was further divided into 2 strata according to gender, for a total of 12 strata, with 4 randomly selected samples in each stratum, for a total of 720 permanent residents.

2.4 Questionnaires and health checks

Participants were interviewed in person using face-to-face questioning by uniformly trained investigators, and younger children were answered by their parents on their behalf. The survey included basic information such as gender, age, place of origin, transportation travel, diet and home environment, as well as daily behavioral habits such as smoking and drinking. Smoking and drinking status were defined as current cigarette consumption and alcohol consumption within 1 year, and any subject under 10 years of age was considered to be a non-smoker and non-drinker.

The health examination includes height, weight, hematology routine, and blood biochemistry items. Height measurement was performed using a metal column type height meter with an accuracy of 0.1 cm; weight measurement was performed using an electronic weight scale with an accuracy of 0.1 kg The body mass index (BMI) of subjects aged 3 ~ 6 years, 7 ~ 17 years and 18 years and above were categorized as wasting, normal, overweight, and obesity according to the appropriate criteria (29–32). The clinical hematology routine and biochemical tests were determined by qualified medical institutions using automated hematology analyzers and biochemistry analyzers. The routine blood tests refer to WS/T 406–2012 Clinical Hematology Testing Routine Items Analysis Quality Requirements, and the biochemical tests refer to WS/T 403–2012 Clinical Biochemistry Testing Routine Items Analysis Quality Indicators. The test results were uploaded to the information management platform system of the center in Excel format.

2.5 Sample collection and processing

Sample collection and processing were carried out in strict compliance with the Biological Monitoring Quality Assurance Specification (GB/T 16126) (33). Blood samples were collected using 5 mL vacuum blood collection tubes, 3 tubes of fasting venous blood non-anticoagulated whole blood, and 1 tube of anticoagulated whole blood of 4 mL each for children over 12 years old and adults; 1 tube of fasting venous blood anticoagulated whole blood and 2 tubes of non-anticoagulated whole blood of 4 mL each for children 6–12 years old; and 1 tube of 4–5 mL anticoagulated blood for children under 6 years old.

2.6 Sample transport, preservation, and testing

All samples were dispensed within 4 h. Samples for hematological tests and blood biochemical tests could only be stored at 2 ~ 6°C within 24 h of dispensing and were not to be frozen. Samples for blood lead tests were transported to the Chinese Center for Disease Control and Prevention within 24 h of dispensing for detection by inductively coupled plasma mass spectrometry (ICP-MS). The detection limit (LOD) for blood lead was 0.035 μg/L, and all test values were above the detection limit.

2.7 Quality control

Organize unified training for investigators before conducting the survey, standardize the questionnaire survey process and filling methods, and arrange for supervisors to supervise and guide the survey site to ensure that the survey is conducted in strict accordance with the requirements of the unified workbook. The collection of blood samples from survey subjects is undertaken by qualified medical institutions; sampling supplies are uniformly issued; each batch of samples is measured in a standard series; the linear correlation coefficient of the standard curve for lead elements should be ≥0.999; at least 3 sets of field blanks are prepared for each batch of samples; and the sample blanks are not higher than the detection limit. Health checkups were conducted in qualified medical institutions, and the testing instruments all met the requirements of national metrological certification. The data were entered into the computer system and reviewed by Jiangxi CDC and China CDC to ensure the reliability of the data.

2.8 Statistical methods

Statistical analysis using SPSS (version 26.0). The blood lead levels of the study subjects were skewed and approximately obeyed normal distribution after logarithmic transformation, described by geometric mean (G), median and interquartile range [M (P_{25} ~ P_{75})], maximum and minimum values. Combining the questionnaire and health examination data, the Mann–Whitney rank sum test was used for the comparison of two independent samples, the Kruskal-Wallis rank sum test was used for the comparison of multiple independent samples, and Spearman's correlation was used to determine the correlation between the two skewed distribution indicators. Variables for which the rank-sum test or Spearman's correlation was statistically significant were included in the linear regression. The data on routine hematological and biochemical indexes were skewed and classified

into low (< P_{25}), medium (P_{25} ~ P_{75}), and high (> P_{75}) levels according to the range of P_{25} ~ P_{75}. After correcting for potential confounders, logistic regression was used to explore the dose–response relationship between blood lead levels and routine hematological and biochemical parameters. A $p < 0.05$ was considered a statistically significant difference.

3 Results

3.1 Demographic characteristics of the study population

The demographic characteristics of the study population are shown in Table 1. There were 720 study subjects, of which 360 (50.00%) were male and 360 (50.00%) were female; 384 (53.33%) were urban residents and 336 (46.67%) were rural residents; the age of the study subjects (29.29 ± 23.21) years, range 3 ~ 78 years, 3 ~ 5, 6 ~ 11, 12 ~ 18, 19 ~ 39, 40 ~ 59, 60 ~ 79 years old were 121 (16.81%), 119 (16.53%), 119 (16.53%), 120 (16.67%), 121 (16.81%), 120 (16.67%), respectively. 47 (6.53%), 427 (59.30%), 183 (25.42%), and 63 (8.75%) of the study subjects were wasting, normal, overweight, and obesity, respectively. Annual household income was less than 30 thousand yuan, 30 ~ 100 thousand yuan, and more than 100 thousand yuan were 168 (23.33%), 369 (51.25%), and 146 (20.28%). Seventy (9.72%), 49 (6.81%), 167 (23.19%), and 434 (60.28%) of the study population were engaged in primary, secondary, tertiary and other occupations, respectively. Nine (1.25%) of the study population had occupational pollution from metals and metalloids, 31 (4.31%) had occupational pollution from pesticides, 36 (5.00%) had occupational pollution from production dusts, and 37 (5.14%) had other occupational pollution.105 (14.58%), 185 (25.69%), and 44 (6.11%) were smokers, alcohol drinkers, and hypertensive patients, respectively (Table 1).

3.2 Transportation travel, diet, and home environment of the study population

The study subjects traveled to and from work or school mainly on foot, 250 (34.72%) of them, and the travel time to and from work or school was mainly within one hour. Most of the study subjects made breakfast, lunch and dinner at home, 561 (77.92%), 659 (91.53%) and 477 (66.25%) respectively. The median and quartiles of mean daily intake of beverages, boiled water, coffee, freshly squeezed juice, bottled water, raw water, tea, staple food, meat, fish intake, eggs, vegetables, fruits, milk, mushrooms, and other food items of the study population in the past year were, respectively, 0 (0 ~ 20) mL/d, 600 (400 ~ 800) mL/d, 0 (0 ~ 0) mL/d, 0 (0 ~ 0) mL/d, 0.83 (0,57 ~ 14) mL/d, 0 (0 ~ 0) mL/d, 0 (0 ~ 0) mL/d, 346.76 (264.07 ~ 487.99) g/d, 60.14 (27.72 ~ 113.83) g/d, 14.29 (4.12 ~ 35.12) g/d, 30 (14.29 ~ 50) g/d, 155 (80 ~ 300) g/d, 50 (14.29 ~ 120) g/d, 35.71 (0 ~ 200) g/d, 3.57 (1 ~ 14.29) g/d, 8.09 (1.67 ~ 23.33) g/d. Among the study subjects, 539 (74.86%) had tap water as their main type of drinking water, 652 (90.56%) had a frequency of consumption of fried foods <1 time/week, and 673 (93.47%) had a frequency of consumption of barbecued foods <1 time/week (Tables 1, 2).

Among the study subjects, 52 (7.22%) used air purifiers or activated charcoal. Most of the study subjects did not use insecticides, moth-proofing agents, air fresheners, air purifiers, disinfectants, toilet cleaners, and hoods on a regular basis, 451 (62.64), 598 (83.06%), 661 (91.81%), 698 (96.94%), 527 (73.19%), 286 (39.72%), and 326 (45.28%). 352 (48.89%) used mosquito repellent occasionally. Among the study population, 365 (50.69%) did not renovate their dwellings, 442 (61.39%) did not replace their furniture, 349 (48.47%) had simple buildings, 319 (44.31%) had actual usable area of the dwelling less than 100 m2, 473 (65.69%) used closed kitchens, 384 (53.33%) had kitchens with exhaust fan for ventilation, 595 people (82.64%) use gas/LPG/natural gas/biogas as the first domestic fuel for cooking, 518 people (71.94%) cook frequently, 516 people (71.67%) use electricity as the main heating method in winter, 653 people (90.69%) in spring, 686 people (95.28%) in summer, and 627 people (87.08%) in fall, 455 (63.19%) in winter, and the frequency of indoor ventilation was characterized by >5 times/week.

3.3 Blood lead internal exposure levels and univariate analysis

The blood lead concentration of 720 study subjects participating in this survey ranged from 7.27 to 103.73 μg/L, with a geometric mean of 20.45 μg/L, a median of 20.03 μg/L, and P_{25} and P_{75} were 15.12 and 26.54 μg/L, respectively. The results of the rank sum test showed that blood lead levels differed significantly by gender ($p < 0.001$), age group ($p < 0.001$), fat and thinness ($p = 0.003$), annual household income ($p = 0.001$), occupation ($p < 0.001$), occupational pollution ($p < 0.05$), smoking ($p < 0.001$), drinking ($p < 0.001$), hypertension ($p < 0.001$), mode of travel to and from work or school ($p < 0.05$), travel time to and from work or school ($p < 0.05$), frequency of hood use ($p < 0.05$), type of housing ($p < 0.05$), and frequency of cooking ($p < 0.05$). Blood lead levels were positively correlated with tea intake ($p < 0.001$) (Tables 1, 2, 3).

3.4 Multiple linear regression analysis of factors influencing blood lead

Blood lead concentration was approximately normally distributed after log-transformation, and by stepwise linear regression, gender ($\beta = 0.078$, $p < 0.001$), age groups ($\beta = 0.022$, $p < 0.001$), annual household income (>100 thousand yuan) ($\beta = -0.042$, $p < 0.05$), smoking ($\beta = 0.075$, $p < 0.001$), hypertension ($\beta = 0.092$, $p < 0.05$), and frequency of hood use ($\beta = -0.014$, $p < 0.05$) still had significant effects on blood lead concentration (Table 4).

3.5 Effects of blood lead levels on routine hematological and biochemical indices

The data of routine hematological and biochemical indexes of the surveyed subjects were skewed, and their median, P_{25} and P_{75} distributions are shown in Table 5. We classified the data of routine hematological and biochemical indexes into low, medium, and high

TABLE 1 Univariate analysis of blood lead exposure level (μg/L) and demographic characteristics, smoking, alcohol consumption, and hypertension in permanent residents of Jiangxi Province.

Features	Number of cases (composition ratio/%)	G	M ($P_{25\sim P75}$)	Statistical values	p
Blood lead	720 (100.00)	20.45	20.03 (15.12 ~ 26.54)		
Gender					
Male	360 (50.00)	22.84	22.00 (17.21 ~ 29.75)	−7.48[a]	< 0.001
Female	360 (50.00)	18.30	17.20 (13.69 ~ 23.21)		
Age group (years)					
3 ~ 5	121 (16.81)	19.44	19.09 (15.43 ~ 24.38)	117.06[b]	< 0.001
6 ~ 11	119 (16.53)	18.34	18.13 (13.69 ~ 23.25)		
12 ~ 18	119 (16.53)	16.71	16.78 (13.52 ~ 20.80)		
19 ~ 39	120 (16.67)	18.18	17.21 (14.09 ~ 23.74)		
40 ~ 59	121 (16.81)	25.01	24.57 (17.94 ~ 32.3)		
60 ~ 79	120 (16.67)	26.88	25.76 (20.11 ~ 36.53)		
Fat and thinness					
Wasting	49 (6.81)	19.55	20.68 (13.65 ~ 24.6)	13.61[b]	0.003
Normal	420 (58.33)	19.82	19.02 (14.92 ~ 25.02)		
Overweight	188 (26.11)	22.35	21.70 (16.52 ~ 29.65)		
Obesity	63 (8.75)	20.03	18.40 (13.98 ~ 28.87)		
Urban and rural					
Urban	384 (53.33)	20.10	18.97 (14.92 ~ 25.81)	−1.69[a]	0.091
Rural	336 (46.67)	20.86	20.81 (15.69 ~ 26.84)		
Annual household Income (thousand yuan)					
<370	168 (23.33)	22.19	21.79 (16.42 ~ 29.06)	16.80[b]	0.001
30–100	369 (51.25)	20.62	20.08 (15.52 ~ 26.92)		
>100	146 (20.28)	18.63	17.41 (14.34 ~ 23.84)		
Unknown	37 (5.14)	18.77	17.77 (13.65 ~ 24.81)		
Occupation**					
Primary industry	70 (9.72)	24.81	24.05 (17.96 ~ 35.16)	60.44[b]	< 0.001
Secondary sector	49 (6.81)	27.37	26.67 (22.42 ~ 33.89)		
Tertiary	167 (23.19)	21.62	20.62 (15.65 ~ 29.67)		
Other occupations	434 (60.28)	18.77	18.08 (14.61 ~ 23.6)		
Occupational pollution					
No	607 (84.31)	19.99	19.2 (14.96 ~ 25.73)	17.56[b]	0.002
Metals and Metalloids	9 (1.25)	30.14	27.31 (23.06 ~ 40.05)		
Pesticides	31 (4.31)	22.48	21.39 (17.77 ~ 28.58)		
Production Dust	36 (5)	24.07	23.66 (18.40 ~ 29.65)		
Other Pollution	37 (5.14)	21.33	21.82 (14.16 ~ 29.99)		
Smoking					
Yes	105 (14.58)	28.37	28.28 (21.85 ~ 36.57)	−8.46[a]	< 0.001
No	615 (85.42)	19.34	18.79 (14.76 ~ 24.54)		
Drinking					
Yes	185 (25.69)	24.12	23.70 (17.28 ~ 32.27)	−5.93[a]	< 0.001
No	535 (74.31)	19.31	18.64 (14.81 ~ 24.57)		

(Continued)

TABLE 1 (Continued)

Features	Number of cases (composition ratio/%)	G	M ($P_{25 \sim P75}$)	Statistical values	p
Hypertension					
Yes	44 (6.11)	28.08	29.42 (20.19 ~ 36.89)	−24.21[a]	< 0.001
No	676 (93.89)	20.03	19.47 (14.95 ~ 25.47)		

**The primary sector refers to agriculture, forestry, animal husbandry and fisheries. The secondary sector refers to mining, manufacturing, electricity, heat, gas and water production and supply, and construction. The tertiary industry includes: information transmission, software and information technology services, finance, wholesale and retail trade, education, health and social work, transportation, warehousing and postal services, accommodation and catering, real estate, international organizations, leasing and business services, culture, scientific research, social security and social organizations, water conservancy, environment and public facilities management, and repair and recreation. [a]Statistics are Z-values; [b]Statistics are H-values.

levels according to P_{25} and P_{75}, and analyzed the effects of blood lead levels on routine hematological and biochemical indexes of the survey respondents after correcting for gender, age, annual household income, smoking, and hypertension. Logistic regression analysis showed that each unit increase in blood lead resulted in a 2.4% (OR = 1.024, 95%CI: 1.001 ~ 1.048), 1.6% (OR = 1.016, 95%CI: 1.002 ~ 1.031), 3.6% (OR = 1.036, 95%CI: 1.000 ~ 1.072), and 2.3% (OR = 1.023, 95%CI: 1.007 ~ 1.039) increased risk of elevated RBC (from low to high levels), PDW, ALP (from low to high levels), and CHO, respectively, while the risks of elevated DBIL and TBIL decreased by 1.7% (OR = 0.983, 95%CI: 0.969 ~ 0.998) ($p < 0.05$) (Table 6).

4 Discussion

The study found that the geometric mean of blood lead concentration in permanent residents of Jiangxi Province was 20.45 μg/L, which was significantly lower than that of Liaoning Province, higher than that of Jilin Province (34) and the national (35). Compared with other countries, the geometric mean of blood lead in our province is higher than that in the United States (36) and Korea (37). The range of blood lead concentration of permanent residents in Jiangxi Province is 7.27 ~ 103.73 μg/L, which is far below the limit value of lead poisoning proposed by the China Health and Wellness Commission (400 μg/L for adults and 100 μg/L for children) and is still at a low concentration level.

In the study, the blood lead levels of males was higher than that of females, which was consistent with Guizhou Province (38). This difference may be due to differences in men's and women's jobs and lifestyles, as well as physiological processes and hormone levels. Females are mainly engaged in family activities, and their positions require avoiding lead working environment as much as possible, while males have long outdoor activities and more opportunities for lead occupational exposure, so their blood lead levels are relatively higher. This study found that age was positively correlated with blood lead levels ($p < 0.001$), which may be related to the duration of exposure and metabolic levels in different age groups. Lead tends to accumulate in the human body, and as age increases, the exposure time of the human body to lead increases, while the functions of the body decrease and the metabolic rate also slows down, which makes the accumulation of lead in the body more serious and the internal exposure level increase. It was also found that those with an annual household income of more than 100 thousand yuan had lower blood lead levels ($p < 0.05$), probably because higher-income people have better living environments and pay more attention to the quality of foods than lower-income people, and therefore are less likely to consume lead-containing foods and have less exposure to lead in their living environments. The study showed that the blood lead level was lower in residents with frequent use of hoods, which may be due to the fact that lead is contained in grease fumes, and those with frequent use of hoods inhaled less grease fumes and therefore had a lower blood lead level. There was no statistically significant difference in blood lead exposure between urban and rural residents ($p > 0.05$), which may be related to the industrial layout and the proximity of urban and rural residents' living standards, as factories tend to build their plants at the urban–rural border to save costs, and as China's strategy of revitalization of the countryside advances, the living standards of rural residents have significantly improved, and urban and rural residents' living conditions have become more and more convergent, so that urban and rural residents have similar levels of exposure to lead. Human lead comes from the outside environment, and tobacco smoke is an important source of lead exposure for permanent residents. Tobacco plants can capture lead from soil and air and enrich it in tobacco leaves. The results of this study demonstrated that human blood lead concentrations were higher in the smoking group than in the non-smoking group. The study (39) showed that each gram of cigarette contains about 0.54 μg of lead, and 33–60% of lead is transferred to cigarette smoke during the smoking process and enters the body as aerosols through the respiratory tract, thus the lead level in smokers is usually higher. It is suggested that changing the living habits of the population and advocating smoking cessation are feasible measures to reduce lead exposure. There is a lack of evidence regarding the mechanism of the effect of lead and hypertension, but many cross-sectional studies (40–42) have shown that lead exposure levels are positively associated with hypertension, which is consistent with the results of this study.

Currently, most of the studies on lead and routine hematological and biochemical indices focus on high blood lead internal exposure, and there is a lack of studies on the effects of low blood lead internal exposure on routine hematological and biochemical indices. The low blood lead concentration explored in the study makes up for the shortcomings of this type of study. High concentrations of lead can interfere with the redox reactions and energy metabolism of cells by binding to enzymes containing sulfhydryl groups involved in cellular metabolism, resulting in damage to multiple organ systems such as the hematopoietic system and liver, causing changes such as a decrease in RBC and Hb. The study found that low blood lead concentrations were positively correlated with RBC and CHO and negatively

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TABLE 2 Univariate analysis of blood lead exposure level (μg/L) and transportation and diet among permanent residents in Jiangxi Province.

Features	Number of cases (composition ratio/%)	G	*M* ($P_{25\sim P75}$)	Statistical values	*p*
Mode of travel to and from work or school					
No travel	191 (26.53)	22.85	22 (15.99 ~ 31.97)	25.10[b]	0.001
Walking	250 (34.72)	18.78	18.12 (14.76 ~ 22.98)		
Bicycle	33 (4.58)	19.77	19 (16.1 ~ 25.39)		
Electric scooter/ motorcycle	156 (21.67)	20.39	20.35 (14.82 ~ 26.94)		
Car	54 (7.5)	21.2	20.62 (15.83 ~ 26.63)		
Public Transportation	27 (3.75)	18.62	17.67 (14.76 ~ 24.17)		
Subway	3 (0.42)	21.56	20.21 (13.33 ~ 20.21)		
Other	6 (0.83)	28.74	28.26 (19.62 ~ 43.05)		
Travel time to and from work or school					
0	192 (26.67)	22.96	22.4 (16.02 ~ 32.08)	16.12[b]	0.001
≤1 (h/d)	394 (54.72)	19.72	18.98 (14.86 ~ 24.6)		
1 ~ 2 (h/d)	92 (12.78)	19.43	19.34 (15.84 ~ 25.11)		
>2 (h/d)	42 (5.83)	18.91	18.61 (14.41 ~ 25.31)		
Main dining place for breakfast					
Made at home	561 (77.92)	20.45	19.91 (15.18 ~ 26.69)	0.67[b]	0.880
Bought at home	25 (3.47)	21.35	21.8 (15.68 ~ 26.42)		
Restaurant	66 (9.17)	20.71	19.61 (15.38 ~ 25.82)		
School	68 (9.44)	19.90	20.2 (14.66 ~ 25.98)		
Main place for lunch					
Make at home	659 (91.53)	20.52	19.97 (15.24 ~ 26.46)	0.52[b]	0.915
Buy at home	3 (0.42)	19.08	20.51 (14.78 ~ 20.51)		
Restaurants	12 (1.67)	18.89	16.93 (14.81 ~ 26.38)		
Schools	46 (6.39)	19.97	20.94 (13.08 ~ 29.5)		
Main place for dinner					
Make at home	477 (66.25)	20.86	20.21 (15.24 ~ 27.26)	3.40[b]	0.334
Buy at home	6 (0.83)	24.12	23.53 (15.81 ~ 36.05)		
Restaurant	21 (2.92)	19.1	16.29 (14.19 ~ 24.98)		
School	216 (30)	19.61	19.63 (14.92 ~ 24.7)		
Beverage intake (mL/d)			0 (0 ~ 20)	−0.024[c]	0.525
Boiled water intake (mL/d)			600 (400 ~ 800)	0.052[c]	0.161
Coffee intake (mL/d)			0 (0 ~ 0)	−0.016[c]	0.677
Freshly squeezed fruit juice intake (mL/d)			0 (0 ~ 0)	−0.042[c]	0.262
Bottled water intake (mL/d)			0.83 (0 ~ 57.14)	−0.039[c]	0.296
Raw water intake (mL/d)			0 (0 ~ 0)	0.031[c]	0.413
Tea intake (mL/d)			0 (0 ~ 0)	0.163[c]	< 0.001
Staple food intake (g/d)			346.76 (264.07 ~ 487.99)	−0.009[c]	0.807
Total meat intake (g/d)			60.14 (27.72 ~ 113.83)	0.027[c]	0.464
Fish intake (g/d)			14.29 (4.12 ~ 35.12)	−0.006[c]	0.882
Total egg intake (g/d)			30 (14.29 ~ 50)	−0.047[c]	0.204

(Continued)

TABLE 2 (Continued)

Features	Number of cases (composition ratio/%)	G	M ($P_{25\sim P75}$)	Statistical values	p
Vegetable intake (g/d)			155 (80 ~ 300)	−0.019[c]	0.606
Fruit intake (g/d)			50 (14.29 ~ 120)	−0.042[c]	0.259
Milk intake (g/d)			35.71 (0 ~ 200)	−0.070[c]	0.059
Intake of mushrooms (g/d)			3.57 (1 ~ 14.29)	0.029[c]	0.433
Other food intake (g/d)			8.09 (1.67 ~ 23.33)	−0.002[c]	0.958
Drinking water type					
Tap water	539 (74.86)	20.28	19.48 (15.05 ~ 26.46)	2.46[b]	0.482
Bucket/bottle water	37 (5.14)	19.79	20.8 (14.1 ~ 23.91)		
Well water	137 (19.03)	21.28	20.92 (16.13 ~ 27.77)		
Others	7 (0.97)	20.86	18.95 (15.99 ~ 28.45)		
Frequency of consumption of fried food					
< 1 time/week	652 (90.56)	20.54	19.99 (15.22 ~ 26.72)	−0.72[a]	0.470
≥ 1 time/week	68 (9.44)	19.56	20.18 (14.4 ~ 24.47)		
Barbecue food consumption frequency					
< 1 time/week	673 (93.47)	20.58	20.08 (15.2 ~ 26.72)	−1.26[a]	0.206
≥ 1 time/week	47 (6.53)	18.69	18.32 (13.69 ~ 24.29)		

[a]Statistics are Z-values; [b]statistics are H-values; [c]statistics are r_s.

correlated with DBIL and TBIL. This may be related to the toxic excitatory effect (hormesis), which is the stimulating effect of low-concentration blood lead on the organism.

Lead inhibits the activity of heme synthetase, so lead poisoning is often manifested by a decrease in hematocrit, and the body shows signs of anemia. However, the results of this study showed a positive correlation between blood lead exposure and red blood cell count. Although lead blocks the binding of protoporphyrin to iron to form heme and reduces heme synthesis, the low blood lead concentration also leads to a lower degree of hematocrit decline, and the body stimulates the hematopoietic system to produce more erythrocytes through a feedback mechanism, leading to a compensatory increase in the number of erythrocytes to reduce the adverse effects caused by lower hematocrit (43), which results in an increase in the red blood cell count as shown in the assay index. Therefore, the positive correlation between low blood lead exposure and erythrocyte count shown in this study is not contradictory to blood Pb causing anemia. Bilirubin is a metabolite of hemoglobin, and lead may increase bilirubin levels by inducing hemoglobin degradation, while the study found that the risk of elevated DBIL and TBIL levels decreased with increasing blood lead concentration, which is consistent with the study of Ye M et al. (44), probably due to a decrease in bilirubin synthesis caused by the depletion of hemoglobin.

ALP is an enzyme widely distributed in human liver, bones, intestines, kidneys, placenta and other tissues and excreted by liver to bile, which can reflect the function of liver and bile (45). Ali Firoozichahak et al. showed that blood lead levels were positively correlated with ALP levels, which is consistent with my findings (46). On the one hand, lead causes disturbance and disruption of cell membranes (47). Phosphate is known as an intracellular anion and it increases serum ALP levels when cell membranes are damaged or disrupted (48). On the other hand, bones contain large amounts of ALP. Lead replaces bone calcium, leading to structural damage to the bones, thus causing an increase in serum ALP levels (49).

Park et al. (25) found that blood lead levels were positively correlated with total cholesterol levels, which is consistent with the findings of the study, and it may be that elevated blood lead levels induce lipid peroxidation (50), which increases cholesterol through lipid peroxidation. PDW indicates the size distribution of platelets produced by megakaryocytes and is an important marker of platelet activation. Kooshki et al. (51) found a positive correlation between lead exposure and platelet distribution width, which is consistent with the results of the study, suggesting that lead may cause inflammatory responses and altered platelet morphology in the body.

In conclusion, this study found that there is a certain correlation between blood lead concentration and blood routine hematological and biochemical indexes in permanent residents, suggesting that low blood lead concentration may be related to the number of red blood cells, platelet morphology, as well as liver functions. The mechanism of its effect still needs to be studied in depth, and the threshold value of blood lead concentration that has a damaging effect on the hematological system and liver function needs to be further determined. In this study, the effect of low blood lead concentration on routine hematological and biochemical indexes of permanent residents was found, which provides a scientific basis for early identification, prevention, and control of potential health damage from lead and reduction of its health risk. However, our study is a prospective study, which can only provide clues to

TABLE 3 Univariate analysis of blood lead exposure level (μg/L) and home environment in permanent residents of Jiangxi Province.

Features	Number of cases (composition ratio/%)	G	M ($P_{25\sim P75}$)	Statistical values	*p*
Whether to use air purifier or activated carbon					
No	668 (92.78)	20.49	19.99 (15.22 ~ 26.54)	−0.52[a]	0.601
Yes	52 (7.22)	19.88	20.48 (14.02 ~ 26.6)		
Frequency of insecticide use[d]					
No	451 (62.64)	20.26	19.58 (14.83 ~ 26.67)	2.60[b]	0.457
Occasionally	208 (28.89)	20.84	20.34 (15.86 ~ 26.6)		
Sometimes	55 (7.64)	20.15	19.67 (14.83 ~ 24.11)		
Often	6 (0.83)	24.52	25.91 (18.64 ~ 35.35)		
Frequency of mosquito repellent use[d]					
None	152 (21.11)	20.72	19 (15.29 ~ 27.09)	1.80[b]	0.616
Occasionally	352 (48.89)	20.12	19.44 (14.88 ~ 26.1)		
Sometimes	190 (26.39)	20.87	21.02 (15.25 ~ 27.12)		
Often	26 (3.61)	20.31	20.39 (15.59 ~ 23.66)		
Frequency of use of moth-proofing agents[d]					
No	598 (83.06)	20.42	20.15 (15.16 ~ 26.68)	4.37[b]	0.225
Occasionally	73 (10.14)	19.76	18.32 (14.79 ~ 25.07)		
Sometimes	20 (2.78)	19.34	19.41 (14.01 ~ 26.43)		
Often	29 (4.03)	23.71	22.28 (17.06 ~ 33.78)		
Frequency of air freshener use[d]					
None	661 (91.81)	20.45	19.97 (15.1 ~ 26.45)	0.49[b]	0.921
Occasionally	49 (6.81)	19.96	20.34 (14.31 ~ 27.6)		
Sometimes	5 (0.69)	23.77	22.97 (14.28 ~ 44.15)		
Often	5 (0.69)	21.6	20.62 (17.42 ~ 27.63)		
Frequency of use of air purifiers[d]					
None	698 (96.94)	20.47	20.03 (15.16 ~ 26.53)	0.65[b]	0.885
Occasionally	16 (2.22)	19.75	18.37 (13.72 ~ 26.68)		
Sometimes	2 (0.28)	22.86	25.04 (14.83 ~ 25.04)		
Often	4 (0.56)	18.57	17.31 (13.49 ~ 28.38)		
Frequency of toilet cleaner use[d]					
None	286 (39.72)	21.23	20.69 (15.24 ~ 28.43)	5.46[b]	1.141
Occasionally	248 (34.44)	19.74	19.16 (14.95 ~ 24.29)		
Sometimes	119 (16.53)	20.78	19.67 (16.06 ~ 26.99)		
Often	67 (9.31)	19.32	18.14 (14.72 ~ 24.46)		
Frequency of hood use[d]					
None	326 (45.28)	21.28	20.63 (15.72 ~ 27.76)	11.88[b]	0.008
Occasionally	230 (31.94)	20.39	20.07 (16.15 ~ 25.85)		
Sometimes	105 (14.58)	19.43	18.47 (13.95 ~ 24.18)		
Often	59 (8.19)	18.18	16.37 (13.52 ~ 22.97)		
Renovation time					
Not renovated	365 (50.69)	20.29	19.47 (14.81 ~ 26.85)	1.45[b]	0.695
< 2 years	55 (7.64)	19.51	19.67 (14.95 ~ 23.86)		
2 ~ 5 years	106 (14.72)	20.88	20.24 (16.32 ~ 25.45)		
> 5 years	194 (26.94)	20.78	20.35 (16.21 ~ 25.97)		

(Continued)

TABLE 3 (Continued)

Features	Number of cases (composition ratio/%)	G	M (P_{25} ~ P_{75})	Statistical values	*p*
Furniture replacement time					
Not replaced	442 (61.39)	20.56	19.73 (15.16 ~ 27.24)	1.42[b]	0.702
< 2 years	77 (10.69)	19.74	18.29 (14.43 ~ 25.61)		
2 ~ 5 years	100 (13.89)	20	20.14 (16.36 ~ 24.43)		
> 5 years	101 (14.03)	20.95	20.81 (15.52 ~ 26.89)		
Type of housing					
Simple Bungalow	18 (2.5)	23.85	24.52 (19.58 ~ 29.89)	15.90[b]	0.003
Brick Bungalow	27 (3.75)	19.77	20.63 (14.53 ~ 25.48)		
Simple House	349 (48.47)	21.37	20.92 (16.03 ~ 27.76)		
Commercial house	323 (44.86)	19.38	17.98 (14.63 ~ 24.39)		
Villa	3 (0.42)	20.49	20.34 (16.4 ~ 20.34)		
Actual use area of housing (m^2)					
≤100	319 (44.31)	20.06	19.09 (14.82 ~ 25.83)	3.32[b]	0.190
100–200	301 (41.81)	20.43	20.29 (15.15 ~ 26.12)		
>200	100 (13.89)	21.82	21.5 (16.07 ~ 28.13)		
Kitchen Type					
Closed	473 (65.69)	20.55	20.36 (15.26 ~ 26.29)	−0.67[a]	0.505
Open	247 (34.31)	20.25	19.09 (14.96 ~ 27.03)		
Kitchen ventilation					
Range hoods	15 (2.08)	21.46	23.66 (12.39 ~ 27.73)	4.45[b]	0.217
Exhaust fan	384 (53.33)	20.2	19.46 (15.14 ~ 25.72)		
No measures taken	197 (27.36)	19.98	19.67 (14.83 ~ 26.49)		
Natural window ventilation	124 (17.22)	21.92	20.69 (16.5 ~ 28.29)		
First domestic fuel for cooking					
Firewood/charcoal/ wood/animal manure	92 (12.78)	22.33	21.87 (17.02 ~ 28.27)	9.01[b]	0.061
Coal	4 (0.56)	29.25	27.71 (15.91 ~ 63.46)		
Gas/Liquefied Petroleum/Natural Gas/ Biogas	595 (82.64)	20.19	19.48 (14.95 ~ 25.99)		
Solar/Electricity	17 (2.36)	20.36	23.04 (14.11 ~ 26.86)		
None	12 (1.67)	17.36	17.14 (12.92 ~ 20.87)		
Frequency of cooking[d]					
None	53 (7.36)	19.11	19.2 (15.15 ~ 23.37)	8.01[b]	0.046
Occasionally	42 (5.83)	17.48	16.57 (13.43 ~ 22.33)		
Sometimes	107 (14.86)	20.37	19.48 (14.68 ~ 26.89)		
Often	518 (71.94)	20.87	20.2 (15.63 ~ 27.36)		
Main heating method in winter					
No	113 (15.69)	21.68	21.37 (15.23 ~ 27.76)	9.10[b]	0.105
Centralized heating	7 (0.97)	19.54	19.48 (17.42 ~ 23.08)		
Gas	5 (0.69)	18.15	19.79 (11.26 ~ 31.01)		

(Continued)

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TABLE 3 (Continued)

Features	Number of cases (composition ratio/%)	G	M ($_{P25\sim P75}$)	Statistical values	*p*
Coal-fired	5 (0.69)	19.74	18.14 (13.79 ~ 31.05)		
Electric heating	516 (71.67)	19.94	19.05 (14.83 ~ 25.72)		
Other	74 (10.28)	22.65	21.81 (17.16 ~ 28.69)		
Frequency of indoor ventilation in spring					
No window	3 (0.42)	18.65	20.36 (14.53 ~ 20.36)	1.45[b]	0.695
1–3 times/week	28 (3.89)	20.2	21.49 (16.95 ~ 25.28)		
3–5 times/week	36 (5)	18.71	19.39 (13.75 ~ 25.54)		
>5 times/week	653 (90.69)	20.57	19.97 (15.13 ~ 27.05)		
Frequency of indoor ventilation in summer					
Without opening windows	1 (0.14)	18.93	18.93 (18.93 ~ 18.93)	0.60[b]	0.897
1–3 times/week	12 (1.67)	21.48	23.28 (16.62 ~ 26.48)		
3–5 times/week	21 (2.92)	19.81	22.06 (15.77 ~ 24.75)		
>5 times/week	686 (95.28)	20.45	19.91 (15.04 ~ 26.72)		
Frequency of indoor ventilation in fall					
No windows open	4 (0.56)	16.89	15.96 (11 ~ 29.07)	2.46[b]	0.483
1–3 times/week	37 (5.14)	18.32	19.19 (13.96 ~ 23.71)		
3–5 times/week	52 (7.22)	20.26	21.58 (15.88 ~ 26.34)		
>5 times/week	627 (87.08)	20.62	19.84 (15.13 ~ 27.03)		
Frequency of indoor ventilation in winter					
No windows open	32 (4.44)	19.95	20.78 (14.65 ~ 26.45)	2.45[b]	0.484
1–3 times/week	142 (19.72)	20.6	21.16 (14.56 ~ 27.33)		
3–5 times/week	91 (12.64)	21.56	20.8 (16.91 ~ 25.03)		
>5 times/week	455 (63.19)	20.22	19.21 (14.95 ~ 26.2)		

[a]Statistics are *Z*-values; [b]statistics are H-values; [d]"Occasionally" refers to a frequency of use of not more than once a week; "Sometimes" refers to a frequency of use of at least once a week but not more than once a day; "Often" refers to a frequency of use of once a day or more.

TABLE 4 Multiple linear regression results of blood lead levels and related factors in permanent residents of Jiangxi Province.

Variables	*β*	*β* standard error	*t*	*p*
Gender [e]	0.078	0.014	5.79	<0.001
Age group [f]	0.022	0.004	5.31	<0.001
Annual household income (>100 thousand yuan) [g]	−0.042	0.015	−2.74	0.006
Smoking [h]	0.075	0.020	3.64	<0.001
Hypertension [h]	0.092	0.027	3.38	0.001
Frequency of hood use[i]	−0.014	0.007	−2.09	0.037

Variable assignment: [e]0 = female ~ 1 = male; [f]1 = 3 ~ 5 years old ~ 2 = 6 ~ 11 years old ~ 3 = 12 ~ 18 years old ~ 4 = 19 ~ 39 years old ~ 5 = 40 ~ 59 years old ~ 6 = 60 ~ 79 years old; [g]0 = else ~ 1 = > 100 thousand yuan; [h]0 = no ~ 1 = yes; [i]1 = None ~ 2 = Occasionally ~ 3 = Sometimes ~ 4 = Often.

investigate the association between blood lead and routine blood and biochemical indicators, but cannot determine the causal relationship between them. Therefore, we need to conduct further prospective studies to investigate the causal relationship between blood lead and routine blood and biochemical indicators. In addition, since blood lead is an important indicator of recent lead exposure, but lead tends to accumulate in the body over a long period of time, our future studies could further measure lead in urine and bone, and explore the effects of lead exposure in urine and bone on the health of the population.

TABLE 5 Distribution of routine hematological and biochemical indices of permanent residents in Jiangxi Province.

Routine hematological and biochemical indices	M	P_{25}	P_{75}
RBC (10^{12}/L)	4.65	4.25	5.00
Hb (g/L)	140.00	127.25	150.00
MCV (fL)	87.60	82.93	92.40
MCHC (g/L)	341.50	330.00	351.00
PDW (%)	13.35	10.10	15.90
MPV (fL)	9.60	8.70	10.20
AST (IU/L)	24.00	19.35	29.00
ALP (IU/L)	101.00	70.00	191.88
ALT (IU/L)	16.05	12.00	26.00
DBIL (μmol/L)	2.40	1.60	3.70
TBIL (μmol/L)	11.00	7.93	14.50
CHO (mmol/L)	4.33	3.82	5.18

RBC, red blood cell counts; Hb, hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; PDW, platelet volume distribution width; MPV, mean platelet volume; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; DBIL, direct bilirubin; TBIL, total bilirubin; CHO, cholesterol; Ref, reference category.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Institute of Environmental and Health-Related Product Safety, Chinese Center for Disease Control and Prevention. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

WH: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. JF: Conceptualization, Formal analysis, Validation, Writing – review & editing. RF: Writing – review & editing. XS: Project administration, Supervision, Writing – review & editing. SH: Data curation,

TABLE 6 Association of blood lead levels with routine hematological and biochemical indices in permanent residents of Jiangxi Province.

Dependent variable**	*β*	OR (95% CI)	Wald	*p*
RBC (low level) [j]	Ref	Ref	Ref	Ref
RBC (medium level) [j]	0.010	1.01 (0.989 ~ 1.030)	0.871	0.351
RBC (high level) [j]	0.024	1.024 (1.001 ~ 1.048)	4.115	0.043***
Hb (low level) [j]	Ref	Ref	Ref	Ref
Hb (medium level) [j]	0.004	1.004 (0.984 ~ 1.024)	0.128	0.721
Hb (high level) [j]	0.009	1.009 (0.986 ~ 1.033)	0.619	0.431
MCV (low level) [j]	Ref	Ref	Ref	Ref
MCV (medium level) [j]	−0.003	0.997 (0.974 ~ 1.021)	0.062	0.803
MCV (high level) [j]	0.004	1.004 (0.978 ~ 1.031)	0.107	0.743
MCHC [k]	−0.004	0.996 (0.981 ~ 1.01)	0.326	0.568
PDW [k]	0.016	1.016 (1.002 ~ 1.031)	4.816	0.028***
MPV [k]	0.003	1.003 (0.988 ~ 1.018)	0.155	0.694
AST (low level) [j]	Ref	Ref	Ref	Ref
AST (medium level) [j]	0.013	1.013 (0.989 ~ 1.038)	1.198	0.274
AST (high level) [j]	0.024	1.024 (0.998 ~ 1.051)	3.237	0.072
ALP (low level) [j]	Ref	Ref	Ref	Ref
ALP (medium level) [j]	0.014	1.014 (0.995 ~ 1.034)	2.200	0.138
ALP (high level) [j]	0.035	1.036 (1.000 ~ 1.072)	3.916	0.048***
ALT [k]	0.005	1.005 (0.99 ~ 1.02)	0.458	0.499
DBIL [k]	−0.017	0.983 (0.969 ~ 0.998)	5.262	0.022***
TBIL [k]	−0.017	0.983 (0.969 ~ 0.998)	4.901	0.027***
CHO [k]	0.023	1.023 (1.007 ~ 1.039)	8.044	0.005***

[j]Parallel line test $p < 0.05$ using unordered logistic regression with low level as the reference category. [k]Ordered logistic regression. RBC, red blood cell counts; Hb, hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; PDW, platelet volume distribution width; MPV, mean platelet volume; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; DBIL, direct bilirubin; TBIL, total bilirubin; CHO, cholesterol; Ref, reference category. ***$p < 0.05$.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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