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RESEARCH ARTICLE

Assessment of receptor-mediated activity (AhR and ERα), mutagenicity, and teratogenicity of metal shredder wastes in Wallonia, Belgium

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Abstract

In this study, hazardous wastes including fluff, dust, and scrubbing sludge were sampled in 2019 from two metal shredding facilities located in Wallonia, Belgium. To assess the extent of the contamination, a global approach combining chemical and biological techniques was used, to better reflect the risks to health and the environment. The samples investigated induced significant in vitro aryl hydrocarbon receptor (AhR) agonistic bioactivities and estrogenic receptor (ERα) (ant)agonistic bioactivities in the respective CALUX (chemical activated luciferase gene expression) bioassays. The mutagenicity of the samples was investigated with the bacterial reverse gene mutation test using the *Salmonella typhimurium* TA98 and TA100 strains. Except for the sludge sample (site 3), all samples induced a mutagenic response in the TA98 strain (± S9 metabolic fraction) whereas in the TA100 strain (+ S9 metabolic fraction), only the sludge sample (site 2) showed a clear mutagenic effect. The in vivo toxicity/teratogenicity of the shredder wastes was further evaluated with zebrafish embryos. Except for the dust sample (site 2), all samples were found to be teratogenic as they returned teratogenic indexes (TIs) > 1. The high levels of contamination, the mutagenicity, and the teratogenicity of these shredder wastes raise significant concerns about their potential negative impacts on both human health and environment.

Keywords Shredder wastes · Persistent organic pollutants (POPs) · CALUX bioassay · Bacterial reverse gene mutation test · Zebrafish embryos · Effect-based method (EBM)

Abbreviations

AhR	Aryl hydrocarbon receptor
AwAC	Walloon Air and Climate Agency
BaP	Benzo[a]pyrene
BEQ	BioEquivalent Quantity
CALUX	Chemically activated luciferase gene expression
DMSO	Dimethyl sulfoxide
DLC	Dioxin-like compound
E2	17β-Estradiol
EBM	Effect-based method
EC50	Half-maximal effective concentration
ELV	End-of-life vehicle
ER	Estrogenic receptor
HIS +	Histidine positive
hpf	Hours post fertilization
IC50	Half-maximal inhibitory concentration
LC50	Concentration resulting in death of 50% of the zebrafish larvae
PAH	Polycyclic aromatic hydrocarbon

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PCB	Polychlorinated biphenyl
PCDD/F	Dibenzo-p-dioxins/furan
POP	Persistent organic pollutant
RLU	Relative light unit
TA100	*Salmonella typhimurium* TA100
TA98	*Salmonella typhimurium* TA98
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TEF	Toxic equivalency factor
TEQ	Toxic equivalent quantity
TI	Teratogenic index
WEEE	Electronic and electrical equipment
WHO-TEQ	The World Health Organization-toxic equivalent quantity

Introduction

Anthropogenic activities have altered and continue to alter the environment by constantly reshaping its physical and ecological elements. Nationwide, solid wastes generated and disposed improperly leads to pollution and increased degradation of environmental resources (Ferronato and Torretta 2019). Every year, in Europe, between 8 and 9 million tons of end-of-life vehicles (ELVs) waste is generated, of which 72,588 tons is processed in Belgium via 20 shredding facilities (Eurostat 2021). These facilities handle waste streams from numerous sources such as ELVs, wastes from electronic and electrical equipment (WEEE), industrial residues from metal working, metal furniture, and railway equipment. In shredding plants, the hazardous fluids and parts are initially removed from metal containing waste, which is further shredded into valuable metal scrap (Dubois et al. 2015). During the shredding processes, mechanical friction releases heat, favoring the volatilization of hazardous chemicals such as polychlorinated dibenzo-p-dioxins/furans (PCDD/Fs) and polychlorinated biphenyls (PCBs). These chemical classes, also known as POPs (persistent organic pollutants), include toxic aromatic compounds that are subject to the "Stockholm Convention on Persistent Organic Pollutants" (Lallas 2001). In addition, incomplete combustion of organic materials (e.g., fossil fuel) during industrial activities can lead to the generation of polycyclic aromatic hydrocarbons (PAHs), which are known mutagenic and carcinogenic environmental pollutants (Kim et al. 2013; da Silva Junior et al. 2021).

Both the native hazardous chemicals that ELV waste contains and the hazardous chemicals that are unintentionally produced during waste treatment pose serious environmental threats. Soils, as well as air and water, are primary sinks for these man-made contaminants, which vary in composition and concentration. The organic pollutants accumulate in soils because of their physicochemical properties: high structural stability, extreme hydrophobicity, low mobility, and high organic carbon–water partition coefficient (KOC) (Mahfouz et al. 2020). They also persist in time, can volatilize, and are subject to long-range atmospheric transport (Scheringer 2009; Vorkamp and Rigét 2014). The greatest concern is that most of these chemicals are extremely toxic, can enter the food chain, and contaminate wildlife or humans through bioaccumulation and biomagnification processes (Wu et al. 2008; El-Shahawi et al. 2010; Sun et al. 2021).

Exposure to PCDD/Fs, PCBs, and PAHs results in a diversity of risks for both wildlife and human health. The most documented effects are observed in animal studies and include developmental toxicity. In humans and other vertebrates, they have been proven to be risk factors for cancer, immune deficiency, reproductive and developmental abnormalities, nervous system pathology, endocrine disruption with regard to diabetes and thyroid disorders, decreased pulmonary functions and bronchitis, liver damage, and other health-related negative impacts (White and Birnbaum 2009; Dai et al. 2020; Mallah et al. 2022). Numerous studies have demonstrated that most of the toxic and biological actions of dioxins and the dioxin-like chemicals, which are known POPs and by-products of industrial processes, are mediated via the aryl hydrocarbon receptor (AhR), a cytosolic receptor protein present in most vertebrate tissues. They can bind and activate the AhR, affect the expression of target genes, and disrupt its endogenous function. However, the complete mechanisms of action are still not well understood. In addition, AhR interacts with other signaling pathways such as that of the estrogen receptor (ER) (Van den Berg et al. 2006; Lindén et al. 2010; Vogel et al. 2020).

Analytical tools that can detect and quantify PCDD/Fs, PCBs, and PAHs in different environmental matrices are fundamental for pollution monitoring and risk assessment. Currently, the gold standard method used is the HRGC/HRMS (high-resolution gas chromatography/high-resolution mass spectrometry), which offers detailed quantification of several congeners (Santos and Galceran 2003; Kanan and Samara 2018). However, this analysis method does not take into consideration the biological effect of all active chemicals in a sample, including the unknown compounds and their cocktails effect (antagonistic and/or synergistic effects of the various compounds between them). For this purpose, effect-based methods (EBMs) using in vitro and in vivo bioassays represent a promising alternative as these methods provide readouts in processes and pathways taking place in living organisms (Escher et al. 2018). To get a comprehensive understanding of the hazard posed by samples from potentially polluted areas to the environment and living organisms, it is thus recommended to first carry out in vitro and in vivo EBMs and then, at a second stage, perform a detailed chemical analysis to identify pollutants in samples that raised concerns in the EBMs (Behnisch et al. 2024).

In this study, the AhR and ERα-CALUX (aryl hydrocarbon receptor / estrogenic receptor – chemically activated

luciferase gene expression) methods were used to assess the receptor-mediated activity of the organic pollutants, present in six hazardous material samples from two shredding facilities in Wallonia, Belgium, and one reference soil sample. Additionally, the potential mutagenicity of the hazardous material samples was investigated via the bacterial reverse gene mutation test ("Ames test"). The Ames test is used to identify chemicals inducing gene mutations, permanent changes in the genetic material that have been associated with serious adverse health effects. Gene mutations in somatic cells may lead to cancer whereas gene mutations in germ cells have been linked to fertility problems and heritable diseases (Mortelmans and Zeiger 2000). Moreover, zebrafish (*Danio rerio*) embryos were used to assess the potential toxicity/teratogenicity induced by the samples. Zebrafish are vertebrates that are particularly suitable for studying the effects of various toxic substances thanks to their semi-transparency during embryonic development and larval stages, which allows for real-time observation of organ formation and physiological processes. In addition, its significant genetic and physiological similarities with humans have contributed to the attractiveness of this animal model for high throughput prediction of human toxicity and teratogenicity (Selderslaghs et al. 2009; Burbank et al. 2023). The combination of these techniques can provide early warning information to support, together with chemical information, the assessment of the risks that compounds present in shredder wastes may pose to the environment, wildlife, and human health.

In Belgium, previous studies examining soil and atmospheric depositions near shredding facilities found significant contaminations of PCDD/Fs, PCBs, and PAHs (François et al. 2004; Dufour et al. 2021; Doan et al. 2022). This current study builds on two previous research studies of Dufour et al. (2021) and Doan et al. (2022) conducted in the same two metal shredding sites (2 and 3). Dufour et al. (2021) investigated 17 PCDD/Fs and 18 PCBs in fallout dust collected in the vicinity of these shredding sites in Wallonia and reported median deposition levels of 1.9 pg TEQ/m^2 day for ΣPCDD/Fs, 4.4 pg TEQ/m^2 day for Σdioxin-like PCBs, and 246.5 ng/m^2 day for $5 \times \Sigma 6$ DIN-PCBs. Doan et al. (2022) conducted the initial study of POPs in shredder wastes, and provided POPs chemical profiles of occurrence of 47.7–627.2 WHO pg TEQ/g for PCDD/Fs/dl-PCBs and 450–6914 pg TEQ/g for PAHs. Moreover, the toxic potencies of POPs were identified with several bioassays, and the shredder waste samples induced significant dioxin-like activities, high estrogenic activities, and low androgenic activities. While the initial study provided significant insight into the POPs in these shredder wastes, it also highlighted the need to further investigate their toxic potential, considering the threat that these shredding sites pose to the health of the workers of these facilities and the surrounding area.

In this sequel study, we aim to extend the previous work of Doan et al. (2022) by investigating the AhR and ER bioactivities and the mutagenicity and teratogenicity of hazardous material samples collected from two shredder plants in Wallonia. More specifically, the study included (i) the assessment of the AhR- and ERα-CALUX bioactivities for shredder waste samples, (ii) the relationship between the bio-equivalent quantity estimated with CALUX and the toxicological equivalent quantity estimated through chemical analyses, (iii) the investigation of the potential mutagenicity of shredder waste samples using the Ames test, and (iv) the assessment of toxicity and teratogenicity in zebrafish embryos.

It should be highlighted that the three methods implemented here differ from those used by Doan et al. (2022), and provide crucial additional information on the toxicity of organic contaminants and their potential risks to health and the environment. For the AhR-CALUX bioassay, third-generation cell lines (Hepa1c1c7), which are characterized by a significantly higher luciferase induction magnitude and a significantly lower minimum detection limit than previously existing CALUX-type cell lines, were used (He et al. 2011). Extraction was conducted according to US EPA (2014) method 4435, and the sample extract was cleaned to obtain a more accurate representation of AhR-mediated PCDD/F and dl-PCB activities, without other interfering substances such as PAHs (Baston and Denison 2011). Regarding PAH AhR-mediated activity, a tailored procedure was applied to ensure a more precise detection and quantification of activities in crude extract (Boonen et al. 2020). For the ER-CALUX bioassay, the same cell line as Doan et al. (2022), i.e., human breast carcinoma VM7Luc4E2 (OECD 1995), was used, but ER-mediated activity was studied in a purified/cleaned sample extract on OASIS cartridges and activities (anta vs agonist) were estimated according to a generic response addition model developed for complex chemical mixtures of xenoestrogens (Elskens et al. 2023). It should also be noted that the chemical analyses conducted in the shredder waste samples of both the initial and the sequel study were achieved by the same laboratory (SGS Belgium NV) under DIN EN ISO/IEC 17025:2005 accreditation, but in two independent measurements for each study respectively. This study provides valuable information to improve our understanding and assessment of the possible risks associated with organic soil contamination, which can be a major input pathway to the ecosystems.

Materials and methods

Chemicals and standards

Toluene (for PCDD/Fs and PCBs, minimum 96%), n-hexane (for PCDD/Fs and PCBs, minimum 96%), acetone (Pesti-S

grade, minimum 99.9%), and ethyl acetate (LC–MS grade) were purchased from Biosolve (The Netherlands). Methanol (99.9%, gradient grade), silver nitrate (5% wt.% on silica gel), silica gel 60 for column chromatography, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Germany). Sulfuric acid (95–97%, ASC reagent) and celite 545 (0.02–0.1 mm) were purchased from Merck (Germany). Anhydrous sodium sulfate (Na_2SO_4) was from Boom (The Netherlands) and XCARB™ was from XDS (Durham, USA). HLB cartridges (6 cc, 200 mg, glass) were purchased from Waters. The standard solution of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, 99% purity) was purchased from Campro Scientific (The Netherlands). The standard powder of benzo[a]pyrene (BaP, > 96%) and 17β-estradiol (E2, minimum 98%) were purchased from Sigma-Aldrich (Germany).

All media and other compounds for CALUX cell culturing were supplied by Life Science Technology (UK): minimum essential medium α (MEMα), Dulbecco's modification of Eagle's medium (DMEM without phenol red), sodium pyruvate (100 mM, sterile-filtered), penicillin–streptomycin, fetal bovine serum (FBS), FBS charcoal-stripped, PBS (Ambion), L-glutamine (200 mM), and trypsin with or without phenol red. Luciferin and lysis reagent were obtained from Promega (The Netherlands). All glassware was of borosilicate grade and baked at 450 °C in a muffle furnace for at least 4 h prior to use. The recombinant, estrogen-responsive BG1Luc4E2 ovarian carcinoma and the aryl hydrocarbon-responsive H1L7.5c1 mouse hepatoma cell lines were previously described (Rogers and Denison 2000; Denison et al. 2002).

Salmonella typhimurium strains TA98 and TA100, minimal glucose agar plates, Aroclor 1254–induced rat S9, and NADPH regenerating system (RegensysTM A and RegensysTM B) were all purchased from Trinova Biochem (Germany). The positive control chemicals for the Ames test (i.e., 4-nitroquinoline 1-oxide (4NQO), sodium azide, and 2 amino anthracene) were obtained from Merck Life Science BV (Belgium). The DMSO pro-analysis from VWR (Belgium) was used for the dilution of the samples.

Reagents for Danieau's medium for the maintenance of zebrafish embryos and NaCl were purchased from Fischer Chemical (UK); KCl, $Ca(NO_3)_2$, and $MgSO_4$ from Chemlab (Belgium); and HEPES from Sigma-Aldrich (Germany). DMSO (99.9% spectroscopy grade) used for extracting compounds from the samples was purchased from Acros Organics (Belgium).

Sampling and sample processing

This study is part of a regional survey on toxic organic pollutants emitted by metal shredding facilities in the Walloon Region of Belgium conducted by the Walloon Air and Climate Agency (AwAC). Three different types of samples were collected from two shredding plants by AwAC in 2019–2020: fluff samples (2F, 3F) as the light fraction separated from the shredded bulk, dust samples (2D, 3D) from the ground surface, and sludge samples (2S, 3S) from gas scrubbing. A control (CT) soil sample was taken in the Walloon countryside, Havelange (50.385751, 5.153126), in July 2020. All the samples were uniformly collected from each shredding site using a clean scoop into a glass jar, and then dried, ground, and homogenized by SGS Belgium NV, Antwerp, Belgium. Both site 2 and site 3 belong to the same company, which recycles ELVs, e-waste, tires, and shredder residues.

Sample extraction and clean-up for the biological analyses

Sample extraction for AhR-CALUX bioassay and Ames test

Targeted organic compounds were extracted from the hazardous material samples in order to analyze them with both CALUX bioassay and the Ames test. Approximately 2 g of each sample was extracted using a manual extraction method involving ultrasonication and Vortexing (Baston and Denison 2011). Celite columns (25 mL) were prepared by using a plug of glass whole, 1.9 cc of celite, and 4.3 cc sodium sulfate (Na_2SO_4). They were rinsed with n-hexane 3×10 mL. On the other side, 1 to 2 g of samples was transferred into a 40-mL glass vial (baked at 450 °C, 8 h), followed by the addition of 10 mL of methanol/toluene mixture (20/80) and sonication for 5 min with intermittent vortexing. Once the sample particles settled, the supernatant was transferred to the celite column. The same procedure was repeated two times, whereas, the last time, the vials were centrifuged for 3 min to ensure solvent recovery. Finally, the celite column was flushed with 10 mL toluene and the collected elute was evaporated until near dryness using a vacuum centrifuge (CentriVap Cold Trap—Labconco). Hexane (7 mL) was used to redissolve and concentrate the extracted analytes (vortex, sonicate). This extract is further referred to as "crude extract," since all PCDD/Fs, PCBs, and PAHs were collected. The crude extracts were used to investigate the total AhR activity (including PAHs) and genotoxicity using the AhR-CALUX bioassay and the Ames test, respectively.

Sample clean-up for AhR-CALUX bioassay

Dioxin (PCDD/Fs) and PCB fractions were obtained from the crude extracts after a clean-up procedure using a preconditioned multi-layer silica column coupled in series with a carbon column (Sanctorum et al. 2007). The first column (acid silica column, 10 mL) consisted of a plug glass of wool, 0.5 cc sodium sulfate, 1.6 cc silver nitrate 10% on silica gel, 4.3 cc of 33% (w/w) sulfuric silica gel, and 0.5 cc

sodium sulfate. The column was rinsed with 30 mL (6 × 5 mL) of n-hexane prior to use. The second column (the carbon column, 10 mL) was packed with a plug of glass wool, 0.5 cc of sodium sulfate, 1 cc XCARB (packed), and 0.5 cc sodium sulfate. The initial rinsing of the column was conducted with 5 mL acetone, 20 mL toluene, and 10 mL hexane. The crude extract of 5 mL hexane was transferred to a glass vial and 4 mL contracted sulfuric acid was added. The vials were vortexed and left for 10 min to allow reaction. The acid silica column was placed above the carbon column and the hexane phase of the sample extract was loaded on the columns. Hexane was added two more times (2 × 5 mL) to the vials. Afterward, the vials were vortexed, and their content was loaded to the columns. The acid silica column was rinsed with hexane (3 × 5 mL) and removed. Then, the carbon column was rinsed with 8 mL of hexane/acetone (9:1). The PCB fraction was eluted with 15 mL (3 × 5 mL) hexane/toluene/ethyl acetate (8:1:1) and the dioxin fraction was eluted with 20 mL toluene (2 × 10 mL). Both collected elutes in glass vials were further evaporated near dryness using a vacuum centrifuge (CentriVap Cold Trap—Labconco) and redissolved in hexane (5 mL). This extract is further referred to as "cleaned extract," since PCDD/F and PCB fractions are collected separately and further analyzed with the AhR-CALUX.

Sample clean-up for ERα-CALUX bioassay

To investigate the estrogenic potency, a solid-phase extraction (SPE) was performed using OASIS HLB (Vandermarken et al. 2018a). This extraction entails (i) a conditioning step with 3 mL of methyl-t-butyl ether (MTBE), followed by 3 mL methanol (MeOH) and 3 mL of ultra-pure water; (ii) sample loading onto the cartridges; and (iii) the elution of targeted compounds with 6 mL 10% MeOH/MTBE and redissolution in 5 mL hexane through a solvent exchange using purified air (CentriVap Cold Trap—Labconco).

Sample extraction for the zebrafish embryo toxicity/teratogenicity assay

To investigate the in vivo toxicity/teratogenicity of the samples using zebrafish embryos, 500 mg of each sample was suspended in 1 mL of DMSO (99.9% spectroscopy grade). The samples were vortexed for 1 min, ultrasonicated (Ultrasonic cleaner — VWR) for 15 min, shaken at 100 rpm for 1 h (IKA KS4000i Control Shaker — VWR), and finally centrifuged at 15,000 rpm (Eppendorf 5424R — Eppendorf) for 10 min. All steps were carried out at room temperature. The supernatants were collected and kept at − 20 °C until the initiation of the experiments.

Chemical analyses

The samples were analyzed for the content of several groups of organic pollutants (PCDD/Fs, PXDD/Fs, dl- and non-dl-PCBs, PBDEs, PAHs, phthalates, PFASs, PCNs, plasticizers, and SCCPs) by SGS Belgium NV under DIN EN ISO/IEC 17025:2005 accreditation.

Biological analyses

CALUX bioassays

The CALUX assay is a reporter gene mammalian cell bioassay that is used in this study to investigate the AhR- and ER-mediated activity of shredder waste samples. The AhR-CALUX uses the cell line H1L7.5c1 (mouse hepatoma cells, RRID:CVCL_DC24) which responds to chemicals that activate the AhR receptor, a complex protein that binds with halogenated aromatic hydrocarbons and PAHs. The results are expressed in bioequivalents of a standard compound, where benzo[a]pyrene (BaP) was used for PAHs, and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) for PCDD/Fs and PCBs (Baston and Denison 2011; Tomašek et al. 2021). The ERα-CALUX uses the cell line VM7Luc4E2 (human breast carcinoma cells, RRID:CVCL_6571), which responds to chemicals that activate the ER receptor. The results are expressed in 17β estradiol (E2) equivalents (Rogers and Denison 2000; Vandermarken et al. 2016).

Cell treatment and measurements were conducted based on CALUX protocols. Briefly, cells were grown and maintained in Φ100 mm tissue culture plates (TCP) with alpha-medium (α-MEM). TCPs with cells were incubated under specific conditions (37 °C, 5% CO_2, 80% humidity). The VM7Luc4E2 cells, prior to use for measurements, were transferred to an estrogen-free medium (DMEM) and incubated for 48 h. At 80% confluency, cells were harvested and seeded into 96-well plates, with a density of 30,000 cells/well for AhR-CALUX (PAHs, dioxins, and PCBs) and 40,000 cells/well for ERα-CALUX. After the required incubation time (48 h for PAHs, 24 h for dioxins and PCBs, and 24 h for the active substances on the estrogenic receptor), plates were dosed with a certain volume of medium/sample mixture containing 1% DMSO (100 µL/well for PAHs, dioxins, and PCBs and 188 µL/well for the active substances on the estrogenic receptor). First, the standard compound (BaP, 2,3,7,8-TCDD or E2) was dosed in duplicate. Then, the plates were dosed in duplicate with the samples and the mixture sample/standard at 50% response (EC50), to investigate the agonist and the potential antagonist activity respectively. At last, the plates were incubated for 2 h 45 min for PAHs, 24 h for dioxins and PCBs, and 24 h for the active substances on the estrogenic receptor. After the required exposure time,

the medium was removed from the 96-well plate, and cells were rinsed with phosphate-buffered saline (PBS, 100 µL/well) followed by a visual inspection through the microscope. Next, cell lysis reagent (50 µL/well) was added, and the plate was shaken for 10 min (600 rpm). Then, the plate was placed in the luminometer, where luciferin reagent was automatically injected in each well (50 µL), and finally, relative light units (RLUs) were measured.

Ames test

The Ames test was performed in *Salmonella typhimurium* TA100 (detection of base pair substitutions) and *Salmonella typhimurium* TA98 (detection of frameshifts) with and without metabolic activation system (i.e., S9 metabolic fraction). Normally, the Ames test needs to be performed in five different bacterial strains (OECD 2020). However, given the limited amount of extract available, the Ames test was only performed in TA100 and TA98. Previously, it has been shown that testing only in these two strains allows identification of approximately 90% of the mutagens (Mortelmans and Zeiger 2000).

The standard plate incorporate version of the Ames test was performed on the crude extracts as described by Maron and Ames (1983). Briefly, fresh cultures of bacteria were grown up to the late exponential or early stationary phase of growth. Afterward, 0.5 mL sterile phosphate buffer (experiments without metabolic activation) or 0.5 mL S9-mixture (experiments with metabolic activation), 0.1 mL of fresh bacterial culture (containing approximately 108 viable cells), and 0.1 mL of test solutions (extracts) were mixed with 2 mL of an overlay agar containing minimal histidine, and poured over the surface of a minimal glucose agar plate and incubated at 37 °C for 48 h. Following incubation, the number of HIS + revertant colonies (mutants) in each plate was counted. Dilutions of the extracts were made in DMSO and four concentrations from each extract, expressed as milligram crude sample per (minimal glucose agar) plate, were tested, i.e., 2.5, 6.25, 12.5, and 25 mg/plate. A control for spontaneous revertants, a solvent control, and strain-specific positive controls with/without S9 were included in every test. More specifically, 4NQO (2 µg/mL; TA98 without S9), sodium azide (20 µg/mL; TA100 without S9), and 2AA (10 µg/mL; TA98 and TA100 with S9) were used as positive control compounds, whereas DMSO was used as the solvent control. Microscopic (40 ×) examination of the background lawn, densely packed micro-colonies that form a uniform though somewhat granular thin film in the absence of toxicity, was performed to evaluate cytotoxic effects (thinning or complete absence of the background lawn, compared to the solvent control).

Zebrafish embryo toxicity/teratogenicity assay

Adult wildtype (AB strain) zebrafish were reared at 28.5 °C on a 14-/10-h light/dark cycle according to standard zebrafish aquaculture conditions (Westerfield 2007). Food was given to the adult fish three times per day (two times commercial fish diet and one time hatched nauplia of Artemia salina) while minimizing the surplus. Eggs were collected from natural spawning and fostered in Danieau's medium (17 mM NaCl, 2 mM KCl, 1.8 mM Ca(NO3)2, 0.12 mM MgSO4, and 1.5 mM HEPES buffer (pH 7.1–7.3)) in an incubator at 28.5 °C. At blastula stage (approx. 4 h post fertilization, hpf), embryos were selected from a pool of developing embryos and transferred to a 24-well plate containing Danieau's medium and samples (diluted in 1% DMSO) at multiple concentrations. For each condition, 10 embryos were used. The well plates were kept in the incubator at 28 °C until the embryos were 119 hpf. Each embryo was then assessed under a dissection microscope (Leica M80) by an experienced technician for signs of toxicity, i.e., lethality, skeletal deformities, body positioning (i.e. loss of posture), and their ability to swim (i.e., impaired motility) (protocol modified from (Selderslaghs et al. 2009)). All larvae were euthanized before the age of 120 hpf and were hence not considered animals under animal testing regulations (European Commission 2020).

Data treatment

CALUX bioassays

When the CALUX response variable is normalized between 0 and 100, agonist dose–response curves for standard and sample can be fitted using a three-parameter parallel-line logistic regression model (Gottschalk and Dunn 2005):

$$y = 100 \cdot \frac{\left(\frac{s_0 \cdot x}{c_0}\right)^n + \left(\frac{s_1 \cdot x}{c_1}\right)^n}{1 + \left(\frac{s_0 \cdot x}{c_0}\right)^n + \left(\frac{s_1 \cdot x}{c_1}\right)^n} \quad (1)$$

where s_0 is 1 if the observation comes from the standard, and 0 if not, and where s_1 is 1 if the observation is from the sample of interest, and 0 if not; x corresponds to the explanatory variable and y to the response variable; n is the slope parameter; c_0 and c_1 are the abscissa of the mid-height point which ordinate is 50 for both response curves. This is a constrained model because the observations corresponding to the standard curve influence the optimization of the sample curve. From Eq. (1), we can see that this model generates two parallel curves, the only difference between which is the positioning of the curve, the offset being given by $(c_1 - c_0)$. If c_1 is greater than c_0, the curve corresponding to the sample

is shifted to the right of the standard curve and vice versa. When both agonist and antagonist activities are observed, as in the ER-CALUX assay, Eq. (1) can be rewritten as follows:

$$y = 100 \cdot \frac{\left(\frac{s_0 \cdot x}{c_0}\right)^n + a_1 \cdot \left(\frac{s_1 \cdot x}{c_1}\right)^n}{1 + \left(\frac{s_0 \cdot x}{c_0}\right)^n + \left(\frac{s_1 \cdot x}{c_1}\right)^n + \left(\frac{s_2 \cdot x}{c_2}\right)^n} \quad (2)$$

where $s_0 = 1$; $s_1 = s_2 = 0$ if the observation comes from the standard alone, where $s_1 = 1$; $s_0 = s_2 = 0$ if the observation comes from the sample for the detection of agonist activity and $s_0 = c_0/x$, $s_1 = 0$, and $s_2 = 1$ if the observation comes from the sample for the detection of antagonist activity on E2.

Examples of dose-responses curves are shown in Supplementary Fig. 1 a, b, c, d. Best-fit parameters for the dose–response curves were obtained by minimizing the least squares residuals using a Levenberg–Marquardt algorithm (Elskens et al. 2023). The accuracy goodness of fit was assessed by comparing the predicted curves with the observed values using root mean square error (RMSE). Overall, the fitting quality expressed by the RSQ values (% of explained variance) ranged from 97 to 99% (Supplementary Table S2). Sample bioactivity below the 10% threshold values was designated as < LoQ. Quality control samples were systematically run in triplicate on 96-well plates. They consisted of the standard at the semi-maximal effective concentration (EC_{50}). The recovery rates of these quality controls were between 89 and 120%, which was considered satisfactory. In addition, the characteristic parameters of dose–response curves for standard curves, such as EC_{50} and Hill slope (Supplementary Table S2), were checked in control charts. The precision under repeatability conditions (rsd%) for these parameters in the different assays was always < 15%.

The results of the CALUX bioassay are expressed as bioanalytical equivalent quantity (BEQ) of the standard compound (TCDD/BaP/E2) and expressed as:

$$\mathrm{BEQ} = \frac{\mathrm{EC}_{50}^{\mathrm{Standard}}}{\mathrm{EC}_{50}^{\mathrm{Sample}}} \quad (3)$$

For the antagonistic assays (ERα-CALUX), the half-maximal inhibitory concentration (IC_{50}) was calculated for the sample by determining the sample concentration needed to inhibit half of the maximum biological response of E2. It corresponds to c_2 in Eq. (2). The higher the IC_{50} value, the less potent is the sample.

Ames test

The Ames test results were reported as number of histidine positive (His +) revertant colonies in *Salmonella typhimurium* strain per plate. The concentration of the test chemical was expressed as milligrams/plate. The results were further expressed as a fold-increase in the number of (His +) revertants over the solvent control value. An extract was considered mutagenic in vitro in case the amount of histidine positive (His +) revertants had doubled compared to the solvent control. The effect also needed to be concentration-dependent.

Zebrafish embryo toxicity/teratogenicity assay

Based on the toxicity parameters observed, a score for the overall effect was generated for each of the larvae. A zebrafish larva was considered either normal (all scores = 0), malformed (score = 1 for one or more toxicity parameters observed), or dead (score = 1). For each concentration, the percentages of larvae that were normal, malformed, or dead after exposure to the different samples were analyzed by non-linear regression (curve fit) (Prism 10) and depicted in graphs in two separate curves, one showing malformation and the other lethality for the different samples. The teratogenic index (TI) was calculated for each of the samples by dividing the lethal concentration 50 (LC50), i.e., the concentration where 50% of larvae are dead, by the effective concentration 50 (EC50), i.e., the concentration where 50% of larvae are malformed. Previous studies where known teratogenic compounds in human were analyzed in a similar manner to this study, determined compounds with TI > 1(Selderslaghs et al. 2009) or TI > 2 (Burbank et al. 2023), respectively, to be teratogenic.

Statistical analysis

Apart from zebrafish toxicity/teratogenicity assay, which were analyzed with non-linear regression (curve fit) (Prism 10), all analysis were performed using R statistical software version 4.3.2 (2023–10-31) with the following packages: Hmisc, Factoextra, FactoMineR, ggplots 2, corrplot, and minpack.lm.

Results

Distribution of chemical compounds in shredder wastes

Table S1 shows the concentrations of several groups of hazardous chemical compounds detected in the hazardous waste samples: dioxin-like compounds, estimated total polychlorinated biphenyls, plasticizers, flame retardants, and dispersants in samples of fluff (F), dust (D), and sludge (S) taken from two shredding plants (sites 2 and 3). The data presented in Table S1 is obtained via chemical analysis as previously reported by Doan et al. (2022). However, these results were obtained as part of independent tests/

measurements. Therefore, the values are comparable to those of Doan et al. (2022), but with slight differences due to experimental uncertainties. In general, the level of sample contamination from these shredding plants is several orders of magnitude higher than the AwAC toxicological quality criteria for atmospheric deposition (Table S1). Concentrations for PFAS, PBBs, TCEP, and hexachlorobutadiene were below the limits of quantification and PCNs were subject to interference.

A principal component analysis shows that pollutants are grouped into three main categories: a group including phthalates and substances with estrogenic activity (DBP, DEHP, BPA, and 4-NP), a group including PCBs and SCCPs, and finally a group including BAP and the Σ16 EPA priority PAHs (Doan et al. 2022). It should also be noted that the brominated flame retardant (TBBPA) is the only measured hazardous chemical that meets the quality criteria approved by AwAC. The sludge at both sites is on average more contaminated by PAHs than dust and fluff, which are on the other hand more contaminated by other pollutants, particularly at site 3. At site 2, dust appears to be relatively enriched in PAHs but less contaminated by the remaining pollutants, while fluff is essentially contaminated by PCBs, BPA, and 4-NP (Fig. 1A). The significant Spearman's rank-order correlation between the different pollutant groups is summarized in Fig. 1B. There is a positive monotonic relationship ($r \geq 0.7$, $p \leq 0.1\%$) between HBCD, DIN-PCBs, PCB126, BPA, and DEHP.

The AhR-CALUX bioactivities

The AhR-CALUX results for the crude sample extracts are expressed as μg BaP-BEQ g^{-1} dry weight (dw) and presented in Table 1. All samples exhibit positive AhR receptor agonistic responses. Antagonism was also studied by mixing a standard concentration of BaP equivalent to 50% of the cellular receptor response with the sample extract. However,

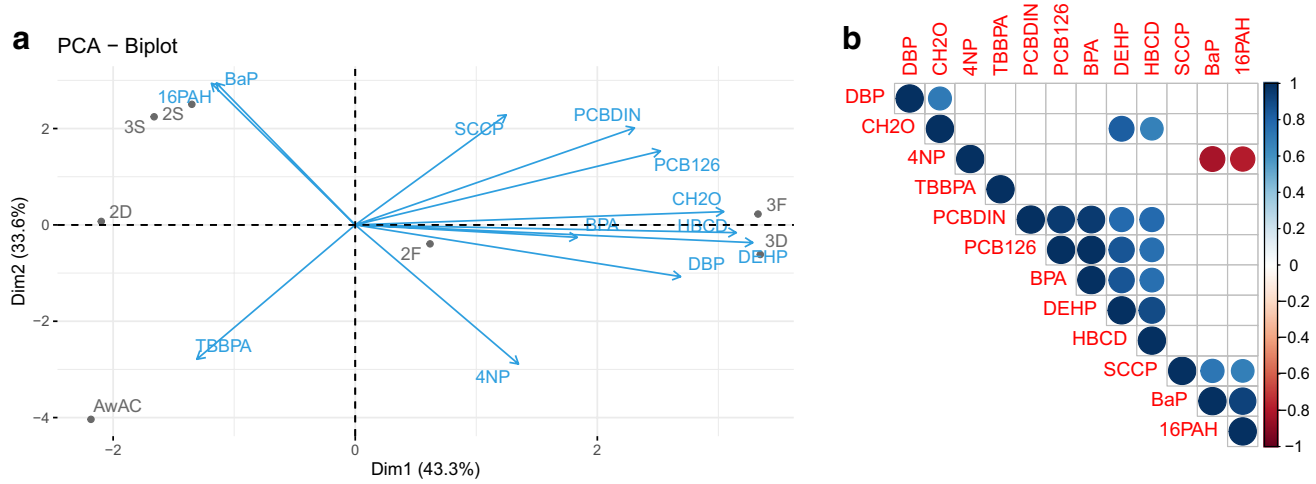

Fig. 1 PCA (**a**) and correlation analyses (**b**) of chemical compounds in shedder waste. The name and concentration of each compound are given in Table S1

Table 1 AhR bioactivities in bio-equivalent (BEQ) and the toxic equivalent quantity (TEQ) in shredder waste at sites 2 and 3

Crude extract	2F	2S	2D	3F	3S	3D	CT
AhR ligands (μg BaP-BEQ g^{-1} dw)	47 ± 9	80 ± 15	36 ± 9	100 ± 27	182 ± 31	37 ± 10	3 ± 1
16 PAHs (μg EPA TEQ g^{-1} dw)	1.2	7.8	3.7	2.2	8.8	2.2	NA
Cleaned extract							
PCDD/Fs (pg TCDD-BEQ g^{-1} dw)	572 ± 101	157 ± 19	57 ± 8	128 ± 23	33 ± 4	119 ± 23	4.4 ± 0.2
dl-PCBs (pg TCDD- BEQ g^{-1} dw)	288 ± 38	85 ± 10	26 ± 2	54 ± 9	19 ± 1	39 ± 7	0.9 ± 0.2
DLCs* (pg TCDD- BEQ g^{-1} dw)	860 ± 108	242 ± 21	83 ± 8	182 ± 25	52 ± 4	158 ± 24	5.3 ± 0.3
DLCs*[a] (WHO 2005 pg TEQ g^{-1} dw)	89	53	73	79	49	68	NA
PCB126 (WHO 2005 pg TEQ g^{-1} dw)	50	29	20	46	27	38	NA

NA, not available

*DLCs = PCDD/Fs + dl-PCBs

[a]Calculated based on toxicity equivalent factor (TEF) from Van den Berg et al. (2006), as in Doan et al. (2022)

no antagonism could be detected. The highest BEQs were detected at sites 2 (sludge) and 3 (sludge and fluff). For the sludge samples, this is probably linked to contamination by PAHs (see also Fig. 1A). It is noted that samples from both sites show significantly higher contamination levels than those detected in the control soil sample (CT). The AhR-CALUX results for the cleaned extracts corresponding to PCDD/F and dl-PCB fractions are expressed as pg TCDD-BEQ g^{-1} dw and presented in Table 1. All samples are positive, indicating the presence of PCDD/Fs and dl-PCB active ligands. Antagonism was also studied by mixing a standard concentration of TCDD equivalent to 50% cellular receptor response with the sample extract, but no antagonism could be detected. The highest levels of bioactivity for both fractions are observed at site 2 in fluff and sludge samples. The overall bioactivity levels of the dl-PCB fraction are lower than that of PCDD/Fs. However, all samples show bioactivities that are several orders of magnitude higher than the control sample (CT).

These results were cross-checked with those calculated for the dioxin toxicity equivalence (pg TEQ g^{-1} dw) based on congener concentrations for the dioxin/furan group (17 congeners) and the dioxin-like PCB group (12 congeners). Also included in this analysis is the TEQ calculated from PCB 126 concentrations, the congener exhibiting the highest WHO-TEF among dl-PCBs. Finally, the BaP-BEQ values evaluated from crude extracts were cross-referenced with those of the TEQs derived from the concentrations of 16 EPA priority PAHs (Doan et al. 2022), and their corresponding TEF values (Chen et al. 2014). The results are summarized in Table 1. The PCA analysis clearly indicates a consistent association (covariance) between BEQ/TEQ pairs, even though BEQs are higher than TEQs (Fig. 2A). Measuring total bioactivity in these complex mixtures of dioxin- and polycyclic aromatic hydrocarbon-type compounds using an effect-based method such as CALUX has the advantage of covering both known pollutants (e.g., chemicals on the restricted dioxin-like compounds (DLCs) and PAH list) and yet unknown pollutants. This leaves room for the presence of other ligands active on AhR in both crude and cleaned extracts. Thus, it can be estimated that (i) the TEQ from PCB 126 alone represents between 17 and 100% of the BEQ of the congeners in the dl-PCB fraction, (ii) the TEQ from DLCs represents 10 to 89% of the BEQ of the PCDD/F + dl-PCB fractions, and (iii) the TEQ estimated from 16 EPA PAHs represents between 1 and 10% of the BAP-BEQ estimated from the crude extract. There are several groups of unmeasured chemicals in this study that could explain these differences, firstly polybrominated dibenzo-p-dioxins (PBDDs), dibenzofurans (PBDFs), and some dioxin-like brominated biphenyls (PBBs) as well as halogenated aromatic hydrocarbons, particularly PCNs. The statistically significant ($p \leq 0.1\%$) Spearman's rank-order (anti)-correlations between the different congener groups are summarized in Fig. 2B.

The ERα-CALUX bioactivities

The ERα-CALUX results for active substances on the ERα receptor show positive agonist and antagonist responses for all samples from the metal shredding sites. Agonist responses are expressed in ng E2-BEQ g^{-1} dry weight and presented in Table 2. In Fig. 3A, these activities seem essentially driven by the sum of the estrogenically active substance response SUM = [DBP, DEHP, BPA, 4-NP]. Statistically significant Spearman's rank correlations are summarized in Fig. 3B. There are positive monotonic relationships ($r \geq 0.7$–0.8, $p \leq 0.1\%$) between E2-BEQs, phthalates, 4-nonyl phenol, and SUM.

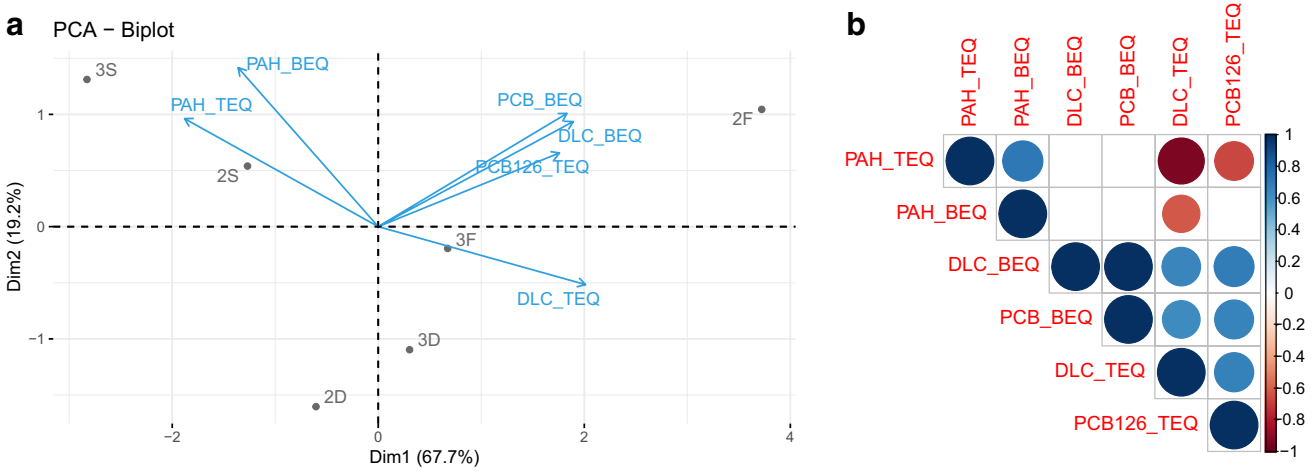

Fig. 2 PCA (**a**) and correlation analyses (**b**) in toxicological equivalent (TEQ) and bio-equivalent (BEQ) for the pollutant groups DLCs = PCDD/Fs + dl-PCBs, dl-PCBs, and PAHs. The TEQ and BEQ values are shown in Table 1

Table 2 (Anti)-estrogenic bioactivities in shredder waste at sites 2 and 3

Agonist effect	2F	2S	2D	3F	3S	3D	CT
BEQ (ng E2 g^{-1} dw)	1.2 ± 0.6	1.4 ± 0.8	0.35 ± 0.23	1.9 ± 0.8	0.63 ± 0.1	2.0 ± 1.3	< LoQ
Antagonist effect							
IC_{50} (mg dw mL^{-1} md)	23 ± 6	17 ± 4	48 ± 13	7.1 ± 2.7	8.4 ± 4.5	4.7 ± 0.9	< LoQ

LoQ, limit of quantification

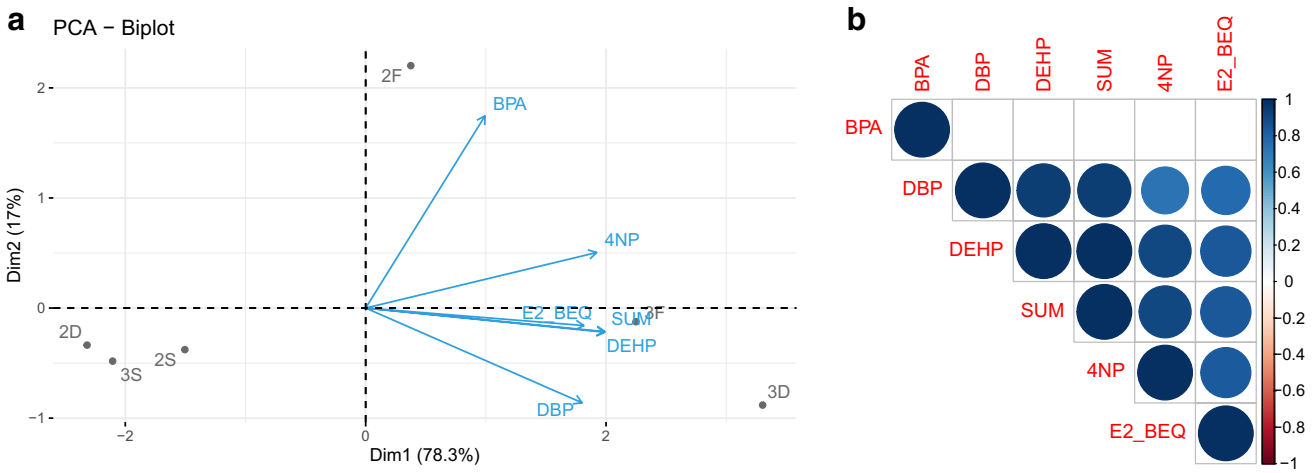

Fig. 3 PCA (**a**) and correlation analyses (**b**) for bio-equivalent (BEQ) and chemical concentration of estrogenic active substances (SUM = [DBP, DEHP, BPA, 4-NP]). The BEQ and concentration values are reported in Table 2 and Table S1, respectively

The antagonist activity has a strong impact on the agonist activity of the samples. Significant cytotoxicity is also observed at the highest extract concentrations leading to cell death. The inhibition curve for the agonist response of the endogenous ligand (E2) tested at its EC50 follows a decreasing trend as a function of the concentration of the extract to which the CALUX cells are exposed. The half-maximal inhibitory concentration (IC50) is calculated for the sample by determining the concentration of extract necessary to inhibit half of the maximum biological response of E2 using parallel fitting techniques as described in Elskens et al. (2023) (see Fig. S1). The higher the IC50 values, the less significant the inhibition effect exerted by the sample. IC50 values are thus inversely related to pollutant concentrations such as SSCPs, CH2O, TBBPA, HBCD, DIN-PCBs, and PCB126 (Fig. 4A). More specifically, there is a negative Spearman's correlation ($r \geq -0.8$, $p \leq 0.1\%$) between IC50 and DIN-PCBs and CH_2O (Fig. 4B). Samples from site 3 appear to have a more pronounced inhibitory effect than those from site 2 but given the value of the standard error on IC50, it is not possible to establish a clear pattern of inhibition between fluff, dust, and sludge. All samples exhibit higher ER agonist and antagonist potencies than the control sample (CT).

The Ames test

The mutagenicity of the shredder waste samples was examined with the Ames test using *Salmonella typhimurium* strain TA100 and strain TA98, and the results are presented in Table 3 and Table 4, respectively. The data show that for the *Salmonella typhimurium* strain TA100, no mutagenic effect was observed in the absence of the S9 metabolic fraction, although there was an increase (less than two-fold) in the number of His + revertants at the highest concentration of sample 2S (site 2, sludge). In the presence of S9 metabolic fraction, sample 2S (site 2, sludge) was clearly mutagenic. The other samples 2F, 2D, 3F, 3D, and 3S showed an increase in the number of (His +) revertant colonies compared to the solvent control (DMSO) in the presence of S9 metabolic fraction. However, this increase was less than doubled compared to the solvent control.

For the *Salmonella typhimurium* strain TA98, mutagenic effects were observed both in absence and in presence of the S9 metabolic fraction for all the samples, with exception of sample 3S (site 3, sludge) and CT (control sample). Sample 2S (site 2, sludge) showed the most pronounced mutagenic effect. Although no clear mutagenicity was found with sample 3S and CT, there appeared to be an increase in

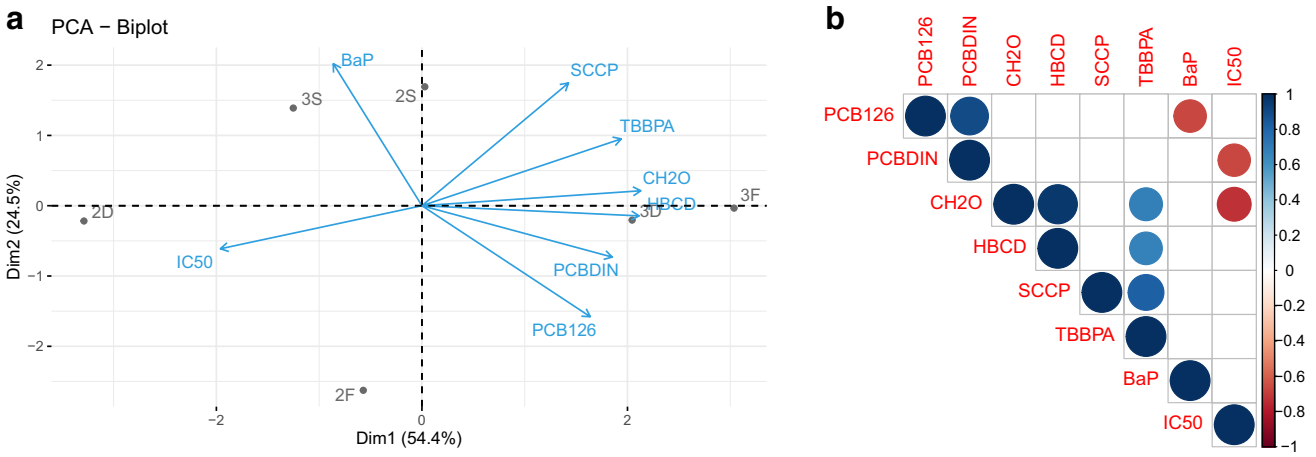

Fig. 4 PCA (**a**) and correlation analyses (**b**) for ER-CALUX IC50 and concentrations of hazardous chemicals. The IC50 and concentration values are reported in Table 2 and Table S1, respectively

Table 3 Ames results for the *Salmonella typhimurium* strain TA100 with/out S9 in shredder waste at sites 2 and 3

Sample	TA100 — without S9						TA100 — with S9					
Concentration (mg/plate)	SC	2.5	6.25	12.5	25	PC*	SC	2.5	6.25	12.5	25	PC**
Concentration (%)	0%	10%	25%	50%	100%	PC*	0%	10%	25%	50%	100%	PC**
2F	89	116	106	110	95	*768*	102	137	134	168	168	*1993*
2D	100	111	116	112	108	*768*	94	104	142	151	182	*3107*
2S	89	101	122	136	162	*768*	102	166	172	176	*212*	*1993*
3F	100	98	111	108	116	*768*	94	145	136	150	159	*3107*
3D	89	82	105	126	124	*768*	102	126	170	155	156	*1993*
3S	89	98	110	113	99	*768*	102	112	112	111	158	*1993*
CT	100	98	94	102	108	*768*	94	98	100	112	97	*3107*
Blank	100	102	90	93	97	*768*	94	119	114	104	84	*3107*

PC, Positive Control = *Sodium azide 2 µg/plate – **2-aminoanthracene 1 µg/plate

Values in italic = (His +) revertants ≥ 2 × (His +) revertant of the SC (solvent control)

Table 4 Ames results for the *Salmonella typhimurium* strain TA98 with/out S9 in shredder waste at sites 2 and 3

Sample	TA98 — without S9						TA98 — with S9					
Concentration (mg/plate)	SC	2.5	6.25	12.5	25	PC*	SC	2.5	6.25	12.5	25	PC**
Concentration (%)	0%	10%	25%	50%	100%	PC*	0%	10%	25%	50%	100%	PC**
2F	14	18	20	24	*31*	*328*	18	34	34	*50*	*54*	*462*
2D	13	16	24	*30*	*39*	*342*	15	20	24	*43*	*48*	*634*
2S	14	25	*56*	*72*	*106*	*328*	18	*38*	*64*	*100*	*144*	*462*
3F	13	17	19	24	*26*	*342*	15	24	28	30	*36*	*634*
3D	14	20	16	23	*28*	*328*	18	26	32	35	*38*	*462*
3S	14	12	14	17	11	*328*	18	20	27	25	30	*462*
CT	13	11	23	11	25	*342*	15	22	23	18	25	*634*
Blank	13	16	14	11	18	*342*	15	12	14	18	16	*634*

PC, Positive Control = *Sodium azide 2 µg/plate – **2-aminoanthracene 1 µg/plate

Values in italic = (His +) revertants ≥ 2 × (His +) revertant of the SC (solvent control)

the number of His + revertant colonies at the highest concentration tested for 3S in presence of S9. A blank sample from the extraction and solvent control (DMSO) were also investigated, and they were clearly negative under all tested conditions. For sample sludge from site 2 and potentially for the dust sample from site 2, the observed activity using TA100 also suggests base substitution mutations. For both experiments, the tested sample concentrations induced no cytotoxicity in any of the experiments. However, precipitation of the extracts in the plates was present for 2F (site 2, fluff), 3F (site 3, fluff), and 3D (site 3, dust) at the highest concentration.

The zebrafish embryo toxicity/teratogenicity test

The percentages of malformed and dead zebrafish larvae resulting from exposure to samples from the two shredding sites were plotted as a function of concentration (equivalent (EQ) mg/mL) in Fig. 5 A–G. From the respective curves, LC50 and EC50 were deducted and the teratogenic index, i.e., LC50/EC50 for each of the samples calculated (Table 5.). From metal shredding site 2, two of the samples, i.e., sludge and fluff, were classified as teratogenic. For the sludge sample, LC50 and EC50 calculated from the plotted lethality and malformation curves resulted in a teratogenic index of 1.61. For the fluff sample, LC50 could not be reached with the concentrations analyzed. Even at the highest concentration assessed, i.e., equivalent to 5mg/mL, LC50 was below 10%. By establishing that LC50 > 5 mg/mL, the calculated TI is > 2.15 and therefore this sample is teratogenic. The dust sample caused both minimal malformation and toxicity, i.e., < 10% at the assessed concentrations. A teratogenic index could therefore not be calculated, and the sample was classified as non-teratogenic.

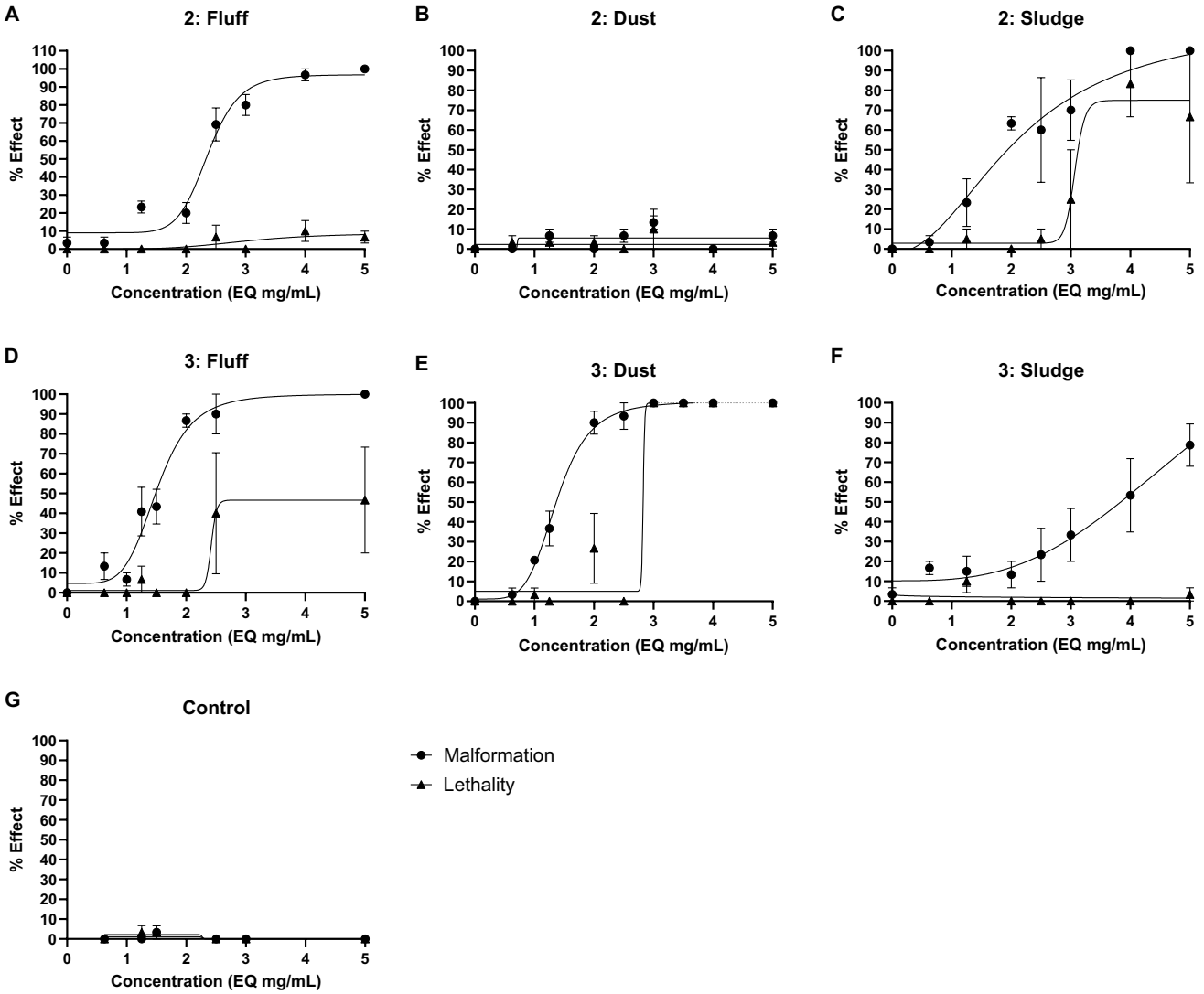

Fig. 5 Concentration–response curve for malformation and lethality for samples from site 2 (**A**–**C**), site 3 (**D**–**F**), or control area (**G**). % Effect (mean ± SEM) is shown versus the concentrations (equivalent (EQ) mg/mL) tested

Table 5 Results of zebrafish embryo toxicity/teratogenicity assay in the shredder waste at sites 2 and 3

Sample	LC50	EC50	TI (LC50/EC50)	Classification
2F	> 5	2.33	> 2.15	T
2D	ND	ND	ND	NT
2S	3.12	1.94	1.61	T
3F	> 5	1.49	> 3.36	T
3D	2.82	1.37	2.06	T
3S	> 5	3.84	> 1.30	T
CT	ND	ND	ND	NT

LC50, concentration lethal for 50% of the embryos; *EC50*, concentration where an effect is demonstrated for 50% of the embryos; *TI*, teratogenic index; *T*, teratogenic; *NT*, non-teratogenic; *ND*, not detected

All samples from metal shredding site 3, i.e., fluff, sludge, and dust, were found to be teratogenic as they returned TIs of > 3.36, 2.06, and > 1.30 respectively. Of note, for the dust and sludge samples, LC50 values could not be reached at the concentrations assessed but the TIs were calculated based on the highest concentration, i.e., LC50 > 5 mg/mL. The sample collected from the control site did not show any malformation or lethality at the concentrations assessed and was hence considered non-teratogenic.

Discussion

The present study applied in vitro CALUX bioassays, the Ames test, and the teratogenicity tests on zebrafish embryos to assess the AhR and ERα bioactivities, as well as the mutagenicity and teratogenicity of the shredder waste samples collected from two metal shredding sites in the Walloon Region of Belgium. Research studies on toxicity in shredding sites are scarce. Data on PCDD/Fs, PCBs, and PAHs in soil or waste matrices are often reported using instrumental techniques such as GC/MS and LC/MS (liquid chromatography–mass spectrometry) and converted in TEQs (toxic equivalent quantity) using established TEF (toxic equivalent factor) for known compounds (e.g., Van den Berg et al. 2006).

Here, however, the differences between the techniques must be considered when comparing results. CALUX bioactivities are expressed as BEQ (bioequivalent quantity) relative to the receptor response of a reference compound (BaP-, 2,3,7,8-TCDD-, and E2-BEQ), assuming that the samples induce an effect by the same mode of action as the reference compounds tested. CALUX bioactivity represents the integrated response of all compounds present in a given sample, including unknown/unidentified compounds and their possible cocktail effect (Escher et al. 2018). Several studies have examined the comparability between BEQ and TEQ values and reported BEQ/TEQ ratios ranging from 1 to 13 times (see for example Du et al. 2011; Croes et al. 2012; Van Langenhove et al. 2012; Zhang et al. 2018; Behnisch et al. 2024). In this study, the ratios obtained were between 10 and 46 for BaP-BEQ/PAH-TEQ and between 1 and 10 for TCDD-BEQ/DLCs WHO-TEQ.

We observed that the AhR-CALUX bioactivities for DLCs in this sequel study were slightly lower than those reported by the previous study of Doan et al. (2022), which is probably linked to differences in the cell lines utilized, and the extraction techniques applied. In this current study, a clean-up was performed on the sample extract to separate the PCDD/F and dl-PCB fractions, to avoid interferences with other AhR ligands such as PAHs. On the other hand, PAH bioactivities were measured with a tailored PAH CALUX method (Boonen et al. 2020). Although the findings of the initial study of Doan et al. (2022) established a foundational understanding of the DLCs AhR-mediated activity in the shredder waste samples, the findings of this subsequent study reinforce the consistency of the initial findings, as the methodology used has been refined and extended. It should be emphasized that the results obtained in this study provide a refined representation of the AhR-mediated activity of PCDD/Fs, dl-PCBs, and PAHs independently of each other. When these results are cross-referenced with chemical analyses, this provides a more dynamic understanding of the toxicity induced by the activation of the aryl hydrocarbon receptor.

Other studies in Belgium report lower BEQ values for PCDD/Fs and dl-PCBs in sludge, soil, and sediments (Van Overmeire et al. 2009; Dumortier et al. 2012; Van Langenhove et al. 2012; Elskens et al. 2013; Vandermarken et al. 2018b). However, research studies in PCDD/Fs and dl-PCBs in contaminated soils in other countries show similar or even higher BEQ magnitude levels (Du et al. 2011; Lin et al. 2014; Liberatori et al. 2021; My et al. 2021). In relation to the AwAC toxicological quality criterion for atmospheric deposition, it can be stated that, although only a limited number of shredder waste samples were examined, the level of contamination in the two metal shredding plants is rather concerning and a more in-depth risk assessment should be considered.

In this study, ERα bioactivations for all shredder waste samples were higher than for the control sample (CT), but cytotoxic and antagonistic effects were detected at the same time. These interactions make the interpretation of the results more complex even if positive correlations were observed between the compounds recognized as estrogenic active substances (phthalates and phenols) and the estimated E2-BEQ values. The apparent inhibition/antagonism observed on cell cultures is correlated with the concentration of DIN-PCB and CH_20. The initial study of Doan et al. (2022) reported significant ER-mediated activities in crude extracts of hexane and acetone. However, in this study,

ER activities were studied in a purified hexane sample cleaned on an OASIS cartridge. Furthermore, Doan et al. (2022) reported moderate antagonism for ER bioactivates in crude hexane extract, and no ER antagonism in crude acetone extract, whereas herein a pronounced antagonism was observed in the purified hexane extract. The use of a generic response addition model to analyze ER responses in complex mixtures of xenoestrogens enabled us to distinguish between (ant)agonistic bioactivities (Elskens et al. 2023).

Mutagenicity of the hazardous material samples was investigated using the Ames test. Except for the sludge sample collected on site 3, all samples induced a concentration-dependent increase in His + revertants in the TA98 strain both in the absence and presence of the S9 metabolic fraction. In the TA100 strain, a clear mutagenic effect was only observed for the sludge sample collected on site 2 in presence of S9 metabolic fraction, suggesting that mostly frameshift mutations were induced. The extract used for the Ames test was crude extract for organic pollutants, and therefore, inorganic contaminants cannot have caused this mutagenic effect. As high contamination levels of PAHs, dioxins, and PCBs are present in almost all the hazardous material sample extracts, and since most of these compounds are mutagenic, these might be responsible for the mutagenic effects detected in the Ames tests (Møller et al. 1985; Donnelly et al. 1986; Man et al. 2013).

The toxicity/teratogenic potential of compounds present in the samples was assessed by zebrafish embryos. All samples apart from the dust sample from site 2 were found to have a TI > 1 and hence classified as teratogenic. The highest TIs were found in the fluff samples with TIs higher than 2.15 and 3.36 for sites 2 and 3, respectively. These samples were also found to have the highest levels of PCBs, DEHP, BPA, and 4-NP. The dust sample from site 2, which was classified as non-teratogenic, was the sample with the lowest amount of these compounds (apart from 4-NP where it was the second lowest). Hence, it is likely that the teratogenicity observed in the zebrafish embryos is caused by exposure to the PCBs, DEHP, BPA, and 4-NP present in these samples. Earlier studies assessing the adverse effect of these compounds in zebrafish embryos observed increased lethality, teratogenicity, and organ toxicity including neurotoxicity and cardiotoxicity. The outcome of this study is thus in accordance with previous findings which validate the usage of zebrafish embryos to assess toxicity/teratogenicity of these compound classes (Ranasinghe et al. 2020).

Several research studies have used these effect-based methods for diagnosis and toxicity monitoring in different environmental matrices (Brack et al. 2019; Dopp et al. 2019; Johann et al. 2020). The current study shows positive responses for the CALUX analyses, the mutagenicity analyses, and the toxicity/teratogenicity analyses. Consequently, combining these techniques can be a suitable approach to screen potential toxic contaminants in environmental samples.

Conclusions and recommendations

The hazardous substances investigated in this study exhibit critical levels of AhR- and ERα-mediated activity, mutagenicity, and teratogenicity. Regular assessment and monitoring of unknown or unknown chemicals with potential adverse effects on the environment, wildlife, and human health should be of crucial importance. These polluted shredding sites are a pathway for organic contaminants to enter ecosystems, harming human health and wildlife and adversely affecting the quality of food, water, and air. Despite the detailed quantification of many congeners offered by sophisticated instrumental chemical analysis, there are also shortcomings such as the challenge of analyzing all substances present in environmental matrices and their potential mixture effects for toxicity diagnosis and monitoring. EBMs such as in vitro and in vivo assays can be used to overcome the difficulties identified above (see Johann et al. 2020; Brack et al. 2019; Dopp et al. 2019). To ensure the protection of environmental quality, we should be able to understand the potential effects caused by all the chemicals present in and around these sites and translate the observed effects into cost-effective management options. The combination of the in vitro CALUX and Ames tests and the in vivo zebrafish embryo toxicity test provides appropriate and additional support for chemical risk assessment.

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Author contribution Matranxhi B: conceptualization, investigation, methodology, formal analysis, data curation, writing — original draft, writing — review and editing. Mertens B: conceptualization, supervision, writing — review and editing. Anthonissen R: investigation, formal analysis, data curation, writing — review and editing. Maes J: investigation, formal analysis, data curation. Ny A: investigation, formal analysis, data curation, supervision, writing — review and editing. de Witte P.: supervision, writing — review and editing. Brouhon JM: project administration, writing — review and editing. De Bast B: writing — review and editing. Elskens M: conceptualization, supervision, formal analysis, data curation, visualization, writing — review and editing.

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Data availability Data will be made available on request.

Declarations

Ethical approval Elskens M. has obtained ethical approval with biosafety permit no. LABO-417843, Risk Level 1, issued on 29/04/2020 and expiring on 29/04/2030 from Brussels Environment in accordance with the decree of the Government of the Brussels-Capital Region dated 8 November 2001.

Consent to participate Not applicable.

Consent for publication All authors have confirmed the article's content and agreed about submission to the journal.

Competing interests The authors declare no competing interests.

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