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Reviewing the use of zebrafish for the detection of neurotoxicity induced by chemical mixtures through the analysis of behaviour

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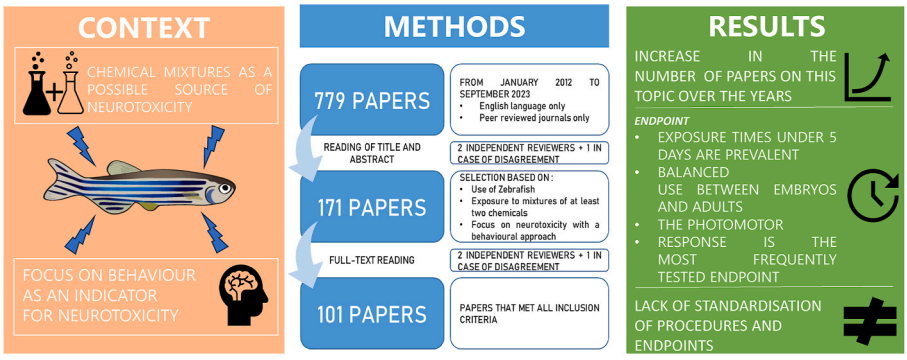
HIGHLIGHTS

- The use of zebrafish for the detection of neurotoxicity increased over the last decade.
- Among the assays, those based on light/dark stimulation are the most commonly used.
- The use of embryos, although common, is not prevalent over that of juveniles/adults.

GRAPHICAL ABSTRACT



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ABSTRACT

The knowledge and assessment of mixtures of chemical pollutants in the aquatic environment is a complex issue that is often challenging to address. In this review, we focused on the use of zebrafish (*Danio rerio*), a vertebrate widely used in biomedical research, as a model for detecting the effects of chemical mixtures with a focus on behaviour. Our aim was to summarize the current status of the ecotoxicological research in this sector. Specifically, we limited our research to the period between January 2012 and September 2023, including only those works aimed at detecting neurotoxicity through behavioural endpoints, utilizing zebrafish at one or more developmental stages, from egg to adult. Additionally, we gathered the findings for every group of chemicals involved and summarised data from all the works we included. At the end of the screening process 101 papers were considered eligible for inclusion. Results show a growing interest in zebrafish at all life stages for this kind of research in the last decade. Also, a wide variety of different assays, involving different senses, was used in the works we surveyed, with exposures ranging from acute to chronic. In conclusion, the results of this study show the versatility of zebrafish as a model for the detection of mixture toxicity although, for what concerns behavioural analysis, the lack of standardisation of methods and endpoints might still be limiting.

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1. Introduction

Thousands of chemical substances released in the ecosystems are defined as "emerging" since they are not yet regulated and there is limited data on risk assessment. They include, *inter alia*, pesticides, pharmaceuticals and nanomaterials which can cause effects on human health and the environment (Geissen et al., 2015). These chemicals can also form mixtures with potential additive or synergistic interactions, amplifying their individual toxicity (Liess et al., 2020) with effects not always predictable through chemical analysis alone (Brack et al., 2019). Consequently, there is a need to improve current knowledge and methodologies and develop holistic approaches for the ecological risk assessment of chemicals under realistic conditions (ECETOC, 2012). New Approach Methodologies (NAMs) are needed to understand the different effects that are caused by emerging chemicals (genotoxicity, neurotoxicity, embryotoxicity, cardiotoxicity, etc.) and mixtures in the ecosystems. Among these effects neurotoxicity is considered an emerging issue due its impact on ecological and human health (Legradi et al., 2018). In 2020 the European Commission published the Chemical Strategy for Sustainability (CSS) (European Commission, 2020), aimed at banning the most harmful chemicals in consumer products (allowing their use only when strictly necessary), and at considering the potential cocktail effect of chemicals when performing risk assessment. The risk posed by chemical substances in the European Union (EU), except for specific pieces of legislation, is mainly evaluated by considering single chemicals, or in some cases mixtures intentionally formulated for specific uses. To adequately address the combined effect of chemical mixtures, legal requirements need to be put in place to ensure that risks posed by mixtures are effectively considered across chemicals-related policy areas.

The widely used approaches (Bopp et al., 2015) that estimate and predict the effects of a chemical mixture, are the dose or Concentration Addition (CA), for substances with the same Mode of Action (MoA), and the Independent Action (IA) or response addition models for substances with a dissimilar MoA. These models are based on known properties of the single components. The interactions are deviations from expected combined effect. The combined effect of two or more substances could be, in some cases, either greater (synergistic) or less severe (antagonistic) than that predicted based on dose addition or response addition.

To date there are still uncertainties in understanding the real effects caused by mixtures in the environment, due to the presence of almost infinite combinations of chemicals (including transformation products and metabolites), exposure durations and concentrations. Several authors concluded that synergistic interactions may rarely occur in the environment, where the concentrations of the single chemicals are usually low (Cedergreen, 2014).

Several studies have been investigated in a systematic review by Martin et al. (2021b) that conducted a quantitative evaluation of 10 years of experimental studies on mixtures to investigate the frequency and reliability of evaluations of mixtures synergistic or antagonistic effects. The authors quantified the extent of the deviations from expected additivity and identified several studies that described such deviations, but they were small in most cases. The application of the precautionary principle has also led to the use of an additional safety factor (Bopp et al., 2019) in the risk assessment procedures for single chemicals to account for possible interaction effects.

Different approaches have been used to better understand the synergistic or antagonistic effects of chemicals mixtures (Bopp et al., 2019). Toxicokinetic/Toxicodynamic (TK/TD) modelling are useful approaches to evaluate the potential for interactions, and in particular, synergistic effects. The application of modelling approaches based on these concepts, such as the General Unified Threshold model of Survival (GUTS) framework applied by Bart et al. (2022), have been extended to mixture toxicity assessment. Other methods that can support mixture assessments include, *inter alia*, the Threshold of Toxicological Concern (TTC), Quantitative Structure-Activity-Relationships (QSAR) models Omics, Read-Across, IATA (Integrated Approaches to testing and assessment). Adverse Outcome Pathway (AOP) is also an important tool in such assessment.

Whole mixture approaches using Effect Based Methods (EBMs) can also help to fill the gaps since they measure directly the combined net effect of the total mixture (Bopp et al., 2019). EBMs include *in vitro* and *in vivo* bioassays suitable for the evaluation of the short-term and long-term effects of chemical pollutants (Brack et al., 2019). Several recent works highlighted the importance of EBMs as a complement to chemical analysis (König et al., 2017; Könemann et al., 2018) and the recent European directive proposal concerning the priority substances in the aquatic environments (European Commission, 2022) has foreseen the inclusion of EBMs for estrogenicity for the first time.

In the 2019 work by Brack et al. a battery of EBMs was proposed, in the context of the EU Project Solutions, involving also different organisms, including algae (with the 72 h inhibition of population growth), crustaceans (with the 48 h daphnia immobilization) and fish (with the 96 h fish embryo acute toxicity, that usually employs zebrafish (*Danio rerio*)).

Zebrafish are freshwater teleosts that, in the last decades, gained growing popularity in several research fields. This organism owes its success to several advantages, such as the low maintenance costs and the small size; they are also easy to keep in captivity and to breed (reviewed by Dai et al., 2014). There is a variety of different strains available on the market (reviewed by Teame et al., 2019), and the zebrafish genome is also completely sequenced (Howe et al., 2013). Moreover, zebrafish has orthologues of more than 71% of human genes (Vilella et al., 2009), and 84% of human disease-related genes have a zebrafish counterpart (Howe et al., 2013). The use of embryos further expands the range of possibilities: the early stages present in fact several advantages over the adults, since they are easier to manipulate, have a short time of generation and are transparent (reviewed by Basnet et al., 2019).

For all these reasons, zebrafish has been successfully employed over the last decades for various applications, as in cancer research, drug toxicity assessment (Fazry et al., 2018), developmental toxicity (Caballero and Candiracci, 2018) and drug discovery (Cassar et al., 2020). Also, in recent years there has been an increase in the number of neuroactive substances detected in the ecosystem, probably because up to 30% of all chemicals used commercially have been estimated to have neurotoxic potential (reviewed by Legradi et al., 2018). Even in this field, zebrafish is considered a powerful and useful tool for assessment (d'Amora and Giordani, 2018; Lin et al., 2023), and is increasingly used as a model organism to understand the mechanisms and neurotoxic effects of these substances, often present in the environment at low concentrations. There is also evidence of numerous similarities at a functional level between mammals and zebrafish in several brain areas (Lau et al., 2011; Randlett et al., 2015), and for this reason the use of zebrafish is becoming more common over time (reviewed by Noyes et al., 2018), even in assays employing embryos and early larvae which show similar characteristics with mammals in some behaviours (reviewed by Ahmad et al., 2012) and follow the Directive No. 2010/63/EU on the protection of animals used for scientific purposes. Among the endpoints proposed there is locomotor activity (Selderslaghs et al., 2013), lateral tail movements (Lacchetti et al., 2022) and the inhibition of the enzyme Acetylcholinesterase (AChE) (Koenig et al., 2016). The aim of this work is to provide a comprehensive view of the literature produced between 2012 and 2023 focused on the use of zebrafish as a model to detect the neurobehavioural effects of chemical mixtures.

2. Materials and methods

For this work, we performed extensive research on Embase with the aim of summarising and discussing the current state of knowledge on the topic of neurotoxicity induced by mixtures of environmental chemicals. We searched for papers aimed at detecting neurotoxicity by the use of

zebrafish (*Danio rerio*) as a model organism through a behavioural approach. We proceeded through several steps, first defining search fields and the most suitable keywords, then a timeframe, and finally performing a screening process on the resulting articles.

2.1. Search items

To perform comprehensive research, we selected four search fields to be merged later in a search string: organism, exposure approach (mixtures), endpoint and timeframe. For each field we selected many broadly used synonyms to include the largest possible number of articles. We then collected all the keywords in the search fields obtaining a single search string. Every synonym was separated from the next by the Boolean operator "OR", to indicate that all the keywords within a field can be interchangeably used in the searching process. Between the searching fields the Boolean operator "AND" was used instead, indicating that at least one term from each field had to be present. The complete search string is included in the supplementary material.

2.2. Screening process

For this stage we chose a screening method involving two reviewers operating independently on every work in the list with the task of defining the correspondence of each paper to our requirements. In case of disagreement, a third senior analyst was involved to make a final decision (Levac et al., 2010).

The screening process was based on inclusion requirements that were defined prior to the launch of the search string. We defined a timeframe ranging from January 2012 to the moment when the string was launched (late September 2023). We considered only papers in English and from peer-reviewed journals. Narrative and systematic reviews that did not present original work were excluded. All papers had to include experimental activity on zebrafish, and all developmental stages were acceptable for inclusion. Also, we did not exclude those papers focused also on other organisms, such as other fish species or crustaceans. All papers had to include the use of behavioural endpoints as an indicator for neurotoxicity, and we included works that tested only for behaviour, and works that included other endpoints as well. Lastly, we considered only papers that exposed zebrafish to chemicals in combination. We did not specify the duration or the route of exposure, but all papers had to include the exposure to at least two chemicals at the same time (mixtures). Lastly, we did not select papers based on specific chemical classes or types, and also environmental samples or other complex preformed mixtures were considered acceptable, at the condition that the chemical composition was known.

A first screening phase was performed by reading title and abstract of every work to verify compliance with our inclusion requirements. All those papers that did not respond to our search criteria were therefore excluded. For all the papers that passed the first screening phase the procedure was then repeated by reading the whole text for a more comprehensive evaluation. All the papers that were found eligible for such further assessment were included in the study (Fig. 1).

3. Results

Research on Embase was performed in September 2023 and resulted in the extraction of 779 papers. The first screening process reduced the total number to 171, while the reading of full texts further reduced the number to 101 (Fig. 1).

3.1. PFAS and PFAS-containing mixtures

Per- and Polyfluorinated Substances (PFASs) are chemicals with lipo- and hydrophobic properties, that accumulate in the environmental media and biota. Exposure to these pollutants has been linked to several pathologies in humans, and to developmental and behavioural disorders in model animals such as mice and fish (Khezri et al., 2017; Domingo and Nadal, 2019; Menger et al., 2020; Rericha et al., 2021). As shown in Table 1, several studies that emerged from our query focused on this class of chemicals. In the study from Rericha et al. (2021) a screening of the effects of 58 individual PFAS and two mixtures was conducted on dechorionated embryos, one mixture comprising 11 Perfluoroalkyl Carboxylic Acids (PFCAs, 0.27 μM), the other 46 PFASs (0.023 μM). Twenty-one compounds induced an abnormal response, including the PFAS mixture (hyperactive behaviour), while the mixture of 11 PFCAs showed no toxicity. Similar hyperactivity was also observed in the study

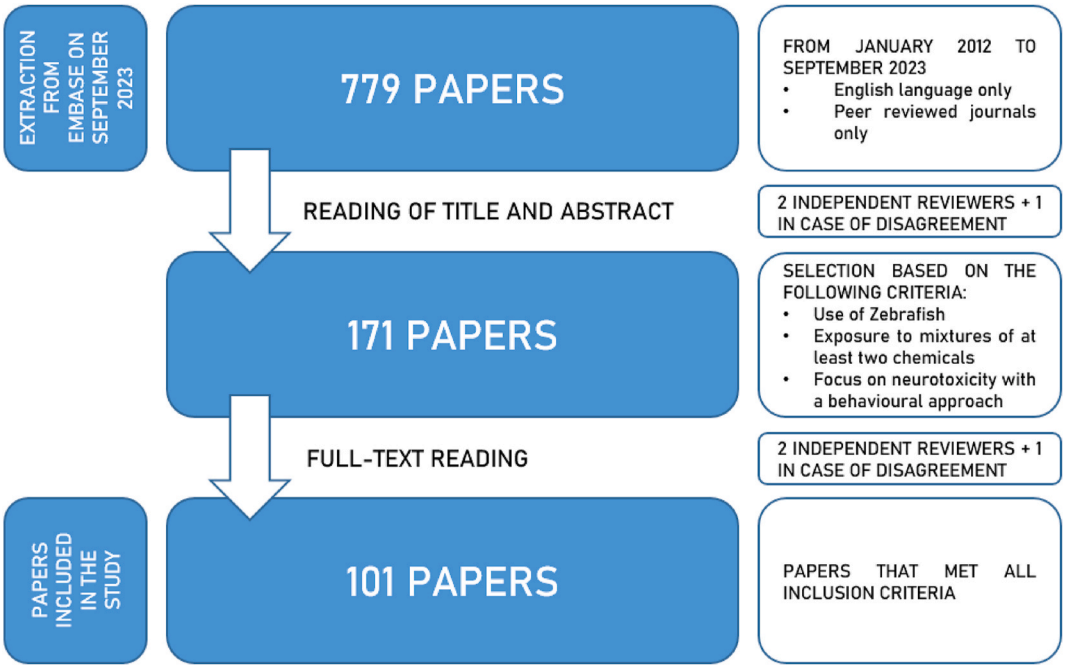

Fig. 1. Screening and inclusion process. The process started with a search on Embase followed by two screening phases operated by independently operating reviewers (for color reproduction on the Web)

Table 1
Summary of the main parameters of the papers focused on PFAS and PFAS-containing mixtures.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae	48 h	POPs mixture	10x, 20x, 100x, 200x the mean human blood concentration	Swimming activity (total distance moved, average swimming speed and total time spent active)	↑Swimming speed	Khezri et al. (2017)
Embryo/ larvae/ adult (*trans*-gen. study)	90 h	POPs mixture	10x, 70x the mean human blood concentration	Novel Tank Test (F0) (distance moved, mean speed and number of crossings between zones), light/dark transition test andthigmotaxis assay (F1)	↑Swimming speed (F1 re-exposure) ↓Swimming speed in adults	Christou et al. (2021)
Embryo/ larvae	90 h	POPs mixture	10x, 70x the mean human blood concentration	Light/dark transition test and thigmotaxis test (total distance travelled, time spent active, mean swimming speed and total distance moved)	↑Swimming speed	Christou et al. (2020a)
Embryo/ larvae	144 h	PFAS mixture	0.0001, 0.001, 0.01, 0.1, 1, 10, 30 mg L-1	Swimming activity (144 hpf, as total swimming distance, burst swimming activity and startle response)	↑Startle response	Menger et al. (2020)
Embryo/ larvae	120 h	PFAS mixture	0.27 μM	Embryonic photomotor response (24 hpf), larval photomotor response and larval startle response assays (120 hpf)	↑Larval photomotor response (lower concentration)	Rericha et al. (2021)
Embryo/ larvae/ adult (*trans*-gen. study)	5 d	PFOS + PFOA	3.5 ng L-1, 35 ng L-1, 350 ng L-1 PFOA +12 ng L-1, 120 ng L-1, 1200 ng L-1 PFOS (low-, medium-, high-dose)	Total distance moved in light/dark condition	↑Swimming distance in F0 ↓Swimming behavior in F1 ↑Activity (very low concentration) ↓Activity (ultra-low concentration) in F2	Haimbaugh et al. (2022)
Embryo/ larvae	96 h	POPs mixture	75 × , 125 × , 250 × the mean human blood concentration	Photomotor response, startle response	↑Swimming speed ↑Activity ↑Swimming distance ↑Startle response	Guerrero-Limón et al., 2023

[a] Exposure time expressed in minutes (min), hours (h) days (d) or months (m).

by Menger et al. (2020), except for the swimming distance. Since the effects of mixtures were less severe than those produced by the single compounds, the study suggests that compounds in mixtures might have reduced their individual effects. In a series of studies by Christou et al. and Khezri et al. (2017), zebrafish embryos and adults were exposed to mixtures that included PFAS, Polychlorinated biphenyls (PCBs) and Brominated diphenyl ethers (BDEs) (Christou et al., 2020a, 2021). Concentrations were 10–70 times higher than those found in the blood of the Scandinavian population. In the 2020 study, the behavioural changes were mainly attributed to the presence of Perfluorooctane sulfonic acid (PFOS), which is, with Perfluorooctanoic acid (PFOA), the most investigated compound in the PFAS class, due to its high toxicity (Domingo and Nadal, 2019; Christou et al., 2020a). In the 2021 study, the effects from the exposure were compared to those related to the single exposure to PFOS. Zebrafish were exposed as larvae (6–96 h post-fertilization, hpf) and their behaviour was tested after 7 months. Behavioural data on their offspring was also collected, with or without re-exposure. Results show lower swimming speed in the F0 adults while an higher speed was observed in the re-exposed F1 larvae. The third study (Khezri et al., 2017) considered the swimming behaviour in zebrafish larvae, observing an increase in swimming speed in larvae exposed between 48 and 96 hpf. Similarly, a study from Guerrero-Limón et al. (2023) tested a mixture of 29 compounds as found in the blood of the Scandinavian population. The behavioural testing was performed at concentrations for each chemical 125 times higher than those found in blood. The effects from the single-component exposure of the mixture (Perfluoroalkyl acids, Br, Cl) were significantly weaker than those of the whole 125x mixture, suggesting potential synergistic or additive effects resulting from other chemicals within the mix. Finally, a 2022 study (Haimbaugh et al., 2022) tested the multigenerational effects of PFOS and PFOA on three generations of zebrafish (F0, F1, F2).

3.2. Flame retardants/PCB mixtures

Polybrominated diphenyl ethers (PBDEs), hydroxylated BDEs and PCBs have been widely produced in complex mixtures, due to their hydrophobicity and non-flammability, as flame retardants for several products including furniture, paper and PVC coatings. Between the 1980s and 2000s, PCB and PBDE production has been progressively banned but they still persist and accumulate in the environment and their high concentrations in exposed fish and mammals they still pose a substantial health and ecotoxicological risk. In the study from Macaulay et al. (2017), 10 d old zebrafish were exposed for 14 d to a mixture of DE-71 (30 μg L-1), a commercial PentaPBDE mixture, tribromophenol (TBP, 4,9 μg L-1) and 6-OH-BDE-47 (0,5 μg L-1, based on average human serum levels). A behavioural assay was conducted with the Novel Tank Test and the tap startle test, and the exposure caused no significant effects on swimming ability and tap response (Table 2). In the studies by Gonzalez et al. (2016) and Lovato et al. (2016), zebrafish embryos were exposed to environmentally relevant concentrations of Aroclor1254, a PCB mixture, for 24 h and their behaviour was observed at 7 dpf. In both cases an anxiety-like response was elicited, suggesting that the correlation between human cognitive disorders and PCB exposure can be found in zebrafish as well. In Péan et al. (2013), adult zebrafish were exposed for eight months to environmental-based concentrations of PCBs-spiked dry food. Behavioural tests were conducted on both F0 adults and their offspring, highlighting higher mobility (F0) and higher activity and less time spent at the bottom of the tank (F1). In a following study (Alfonso et al., 2019), the same research group exposed zebrafish larvae from their first meal to 22 PCB congeners mixed with 7 PBDE congeners for six months, highlighting hyperactivity in the F1 larvae and hypoactivity in the F2, F3 and F4 larvae. Abnormal effects were also observed in adults as anxiety–like behaviour in the F2. A recent follow-up study assessed, after a long-term exposure to other

Table 2
Summary of the main parameters of the papers focused on Flame retardants/PCB mixtures.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Juvenile/adult (F0) larvae/juvenile (F1) (*trans*-gen. study)	8 m	PCBs mixture	∑CB = 515 ng g− 1 dry weight (dw) and ∑CB = 2302 ng g− 1 dw	F0: T-maze, swimming activity (distance travelled) F1: light/dark transition test (5 dpf larvae) and swimming activity (distance travelled, time spent and crossings in and between tank sections in two months old juveniles)	↑Mobility in T-maze in F0 ↑Activity In F1 larvae ↓Frequency of occupation bottom section of the tank in F1 juveniles	Péan et al. (2013)
Larvae/adult (F0) Larvae (F1, F2, F3, F4) (trans—gen. study)	6 m	PCBs + PBDE mixture	1991 ng/g PCB +411.1 ng/g PBDE	F1, F2, F3: Novel Tank test (adults), F1, F2, F3, F4: larval photomotor response (total distance travelled at 5 dpf)	↓Time spent in top area (Novel tank diving test) in F2 ↑Locomotor activity (F1) ↓Locomotor activity (F2, F3, F4)	Alfonso et al. (2019)
Embryo/larvae	24 h	PCBs mixture (Aroclor (A) 1254)	2, 5, 10 ppm	Free swimming with or without visual stimuli	↑Thigmotaxis ↓Swimming speed	Gonzalez et al. (2016)
Embryo/larvae	24 h	PCBs mixture (Aroclor (A) 1254)	2, 5, 10 ppm	Free swimming with or without visual stimuli	↓Avoidance behaviour	Lovato et al. (2016)
Juvenile	14 d	PBDE + OH-BDE mixture	30 μg L-1, 600 μg L-1 DE-71 + 4.9 μg/L, 99.9 μg L-1 TBP, 0.5 μg L-1, 6.0 μg L-1 6-OH-BDE-47 (low- and high-dose)	Novel Tank Test andtap startle test	↓Startle response	Macaulay et al. (2017)
Embryo/larvae	120 h	Tetrabromobisphenol A and Silicon dioxide nanoparticles (n-SiO2)	50, 100, 200 μg L-1 TBBPA + 25 mg L-1 n-SiO2	Spontaneous movement (24 hpf) and locomotor activity in light/dark condition (120 hpf)	↓Swimming speed	Zhu et al. (2022a)
Larvae/Adult (F0) Juvenile (F1, F2) without re-exposure	210 d (F0)	PCBs + PBDE mixture	∑PCBs = 1932.3 ± 90.4 ng/g wet weight (ww) + ∑PBDEs = 479.8 ± 50.8 ng/g ww	Novel tank test, shoal preference test and Z-maze test	↑Boldness in F1 (compared to other generations) ↑Anxiety syndrome in F2 at 120 d	Alfonso et al. (2023)

[a] Exposure time expressed in minutes (min), hours (h) days (d) or months (m).

ecologically relevant behavioural traits, the effects on F0 fish and their unexposed offspring, showing higher boldness in the F1 compared to the other generations and higher anxiety in the F2 compared to the control (Alfonso et al., 2023). Finally, a 2022 work from Zhu and colleagues (Zhu et al., 2022a) investigated the effects of tetrabromobisphenol A and Silicon dioxide nanoparticles (n-SiO2) on zebrafish embryos up to 120 hpf, observing lower swimming speed in the co-exposed group compared to the group exposed to n-SiO2 alone.

3.3. PAH mixtures

Polycyclic aromatic hydrocarbons (PAHs) are a class of chemicals with carcinogenic and mutagenic activity, found in complex mixtures from environmental crude oils or from the combustion of organic matter. A few studies focus on the neurotoxic effects of PAH mixtures on zebrafish, although single and combined compound exposure have been associated with cognitive disorders in humans and in other animal models (Ozaki et al., 2012; Kim et al., 2013; Geier et al., 2018; Vuong et al., 2020; Mallah et al., 2022).

As summarised in Table 3, Geier et al. (2018) exposed zebrafish larvae to 10 PAHs, alone or in combination. Results from the study show significant effects, both after direct exposure (lower rate of habituation) and on the long-term behaviour of adults (differences in learning compared to the control), validating the representative mixture approach. However, as the high exposure concentrations are not comparable to those found in the environment, such results rather provide insight into the toxicity potential of the mixtures. In the study from Vignet et al. (2014), fish were exposed through diet to three PAH mixtures, with different ratios and concentrations of PAHs found in the environment depending on their nature (heavy PAHs or alkylated PAHs). The fraction with an intermediate presence of heavy and alkylated PAHs showed the most significant effects, possibly suggesting an

Table 3
Summary of the main parameters of the papers focused on PAH mixtures.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Larvae/juvenile (F1, F2)	3 m	3 PAH mixtures	0.3X, 1X, and 3X, 1X = Σ[16 EPA PAH] at 5 μg g−1 dw (1 pyrolytic fraction, 2 petrogenic fractions)	Locomotor activity in light/dark conditions (F1, 4–7 dpf), 72 h swimming activity (F1, 5 dpf) and Novel Tank Test (F1, 2 months old)	↑Swimming distance ↑Time spent in upper tank area	Vignet et al. (2015)
Juvenile/adult	6 m	3 PAH mixtures	0.3X, 1X, and 3X, 1X = Σ[16 EPA PAH] at 5 μg g−1 dw (1 pyrolytic fraction, 2 petrogenic fractions)	24 h swimming activity assay (6 months old) and photomotor response assay (2 and 6 months old, distance travelled and the time spent without movement), maze test and the Novel Tank Test	↑Activity (stronger response in juveniles)	Vignet et al. (2014)
Embryo/larvae/adult	114 h	PAHs mixture	0.0625, 0.125, and 0.25% of SuperMix10 (embryo, larvae), 0.1% SM10 (adult)	Embryonic photomotor assay (24 hpf), larval photomotor response (120 hpf), and startle stimulus and active avoidance conditioning test (6 months of age)	Behaviour abnormalities (larvae) ↓Habituation, ↓ avoidance (adults)	Geier et al. (2018)

[a] Exposure time expressed in minutes (min), hours (h) days (d) or months (m).

additive effect of the two types of compounds. In a following study (Vignet et al., 2015) the behaviour of F1 unexposed larvae (4–7 and 5 dpf) and juveniles (2 months of age) from the exposed F0 was analysed. In all cases, offspring presented an abnormal behaviour, suggesting maternal transfer to the egg, although PAH metabolites did not accumulate in the F1 generation.

3.4. Mixtures with other compounds

Finally, three works tested compounds from the previous classes mixed with chemicals of different nature (Table 4). In the first paper (Chen et al., 2019) the authors exposed 6 hpf embryos to two widespread environmental pollutants, alkyl phenanthrene (3-MP) and Dechlorane Plus (DP), singly or in combination, until 120 hpf. Both compounds showed neurotoxic activity on developing zebrafish, with additive or synergistic interaction in mixture. In a 2021 study (Holloway et al.) tocofersolan, a synthetic analogue of vitamin E, was used as a possible remedy for benzo[*a*]pyrene (BaP) induced toxicity, with results that suggest the ability of Tocofersolan to prevent or reverse the detrimental effects caused by BaP. In 2022 Hu et al. co-exposed zebrafish embryos to Perfluorobutanesulfonate (PFBS) and probiotics (*Lactobacillus rhamnosus*). The aim of the study was to deepen the knowledge on the antagonistic action of probiotics towards PFBS toxicity. The results of this study suggest an ameliorative ability of probiotics against the toxicity induced by PFBS.

3.5. TiO_2 NP as vectors for mixtures of pollutants

Nanoparticles of titanium dioxide (nano-TiO_2) are widely used in the industrial manufacturing of products and in processes such as the photodegradation of organic solvents in wastewater and enter the environment through domestic and industrial sewage wastewater discharge. Due to their persistent nature, they are likely to exert their action in sites contaminated by different kinds of pollutants. In addition to the sublethal toxicity associated with their environmental concentrations (0.01–16 μg L-1) (Wang et al., 2014; Li et al., 2018; Lei et al., 2020) their extremely low size and high surface area facilitate the absorption and entry in the aquatic organisms of contaminants such as metals and organic compounds, possibly aggravating the risks associated to the single exposure of the compound (Miao et al., 2015; Li et al., 2018; Lei et al., 2020).

A recent study by Sun et al. (2023) observed the effects of nano-TiO2 in combination with common organic UV filter benzophenone-3 (BP3), as they are usually found together, e.g. in cosmetics (Table 5), highlighting increased spontaneous movement at 24 hpf, and decreased touch response at 30 hpf.

In Chen et al. (2021) Tetrabromobisphenol A (TBBPA) (2 μM) and nano-TiO2 (0.1 mg L-1) together elicited slight but significant behavioural changes in zebrafish larvae compared to the single exposures (less time spent in the light, increased number of attacks and time spent at the mirror and less social contact). Similarly, in Wang et al. (2014) 7 d co-exposure of nano-TiO2 with Decabromodiphenyl ether (BDE-209) at comparable concentrations significantly depressed locomotor activity of larvae, and the uptake and metabolism of BDE-209. A mixture of bisphenol A (BPA) and nano-TiO2 induced negative effects on the locomotor behaviour (reduced swimming speed) on directly exposed larvae in Fu et al. (2020), while in Guo et al. (2019) the effects were evaluated in the F1 after a chronic parental exposure, observing lethargic behaviour.

Interestingly, absorption of pesticides such as cypermethrin (CYP) (Li et al., 2018), pentachlorophenol (PCP) (Lei et al., 2020) and difenoconazole (DIF) (Zhu et al., 2023) was enhanced after exposure to nano-TiO2, and both CYP (10 μM) and DIF (0.5 mg L-1) co-exposed with nano-TiO2 reduced the swimming speed in light/dark conditions of zebrafish larvae at 120 hpf, while the exposure of PCP + nano-TiO2 led to similar effects on thyroid hormone levels and on neurobehavior of the fish as for PCP exposed alone (Lin et al., 2022). Miao et al. (2015) evaluated the combined toxicity in zebrafish larvae of lead (Pb) and n-TiO2 in light/dark conditions and again nano-TiO2 significantly enhanced Pb bioaccumulation and uptake in zebrafish larvae. Lastly, TiO2 particles were also tested with Cadmium on embryos for 168 h in a recent work (Mamboungou et al., 2022), without evidencing substantial effects. As discussed in Lei et al., Li et al. and Miao et al., the observed neurotoxic effects might be attributable to the endocrine-disrupting nature of the chemicals, as the thyroid system was shown to play a significant role in the development and regulation of the nervous system. Overall, these results indicate zebrafish neurotoxicity as a highly sensible endpoint for the evaluation of nano-TiO2-induced increased bioaccumulation and toxicity of organic and metal contaminants.

3.6. Nano- and microplastics as vectors for mixtures of pollutants

Nano- (NPs, <100 nm) and microplastic (MPs, <5 mm) are ubiquitous contaminants able to bioaccumulate in aquatic organisms thanks to their small size (Zhang et al., 2019; Feng et al., 2019). In water and sediments, it is possible to find NPs and MPs that originate from polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) that can act as vectors of pollutants into the organisms, facilitating their accumulation. As summarised in Table 6, using 17α-ethynylestradiol (EE2) as positive control Chen et al. (2017a) observed a significant accumulation of NPs in the exposed fish and a decrease in swimming activity in zebrafish larvae exposed to both MPs and NPs, compared to single EE2 exposure, although NPs-EE2 displayed stronger effects. In Chackal et al. (2022) PS nanoplastic particles accumulated in zebrafish larvae and, when co-exposed with flame retardant 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), significant effects on the behaviour (short, long and total movement) at 7 dpf were observed. Chen et al. (2017a), and Liu et al. (2022), respectively performed absorption and accumulation tests on zebrafish exposed to BPA + NPs and Avobenzone (Butyl Methoxydibenzoylmethane, AVO) + NPs. In both

Table 4
Summary of the main parameters of the papers focused on mixtures containing POPs mixed with different chemicals.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae	96 h	PFBS and probiotics	10 mg L-1 PFBS +106 CFU/ mL L. *rhamnosus*	Black or white background preference, phototaxis and swimming speed	Protective effects of probiotics on PFBS exposure	Hu et al. (2022)
Embryo/ larvae	115 h	BaP and tocofersolan	0.3 μM, 1 μM, 3 μM tocofersolan + 5 μM BaP	Swimming activity in light/dark condition (6 dpf, total distance moved)	Protective effects of tocofersolan on BaP exposure	Holloway et al. (2021)
Embryo/ larvae	114 h	3-MP and DP	5, 20 μg L-1 3-MP + 60 μg L-1 DP	Spontaneous movement (24 hpf), touch response (72 hpf) and swimming activity (120 hpf)	↓Spontaneous movement frequency in co-exposure group (24 h) ↓Swimming distance (synergistic effect) at 72 and 120 hpf	Chen et al. (2019)

[a] Exposure time expressed in minutes (min), hours (h) days (d) or months (m).

Table 5
Summary of the main parameters of the papers focused on mixtures containing nano-TiO_2-.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae/ juvenile	16 h, 40 h, 112h, 13 d	nano-TiO_2 + TBBPA	2 μM TBBPA +0.1 mg L-1 n-TiO_2	Spontaneous movement (24 hpf), touch response (48 hpf), light/dark locomotor response (120 hpf)	↑Spontaneous movement ↓Time spent in the light ↓Touch response ↓Swimming speed ↓Shoaling ↓Social contact ↑Mirror attack	Chen et al. (2021)
Embryo/ larvae	6 d	nano-TiO_2 + BPA	1, 4, 20 μg L-1 BPA + 100 μg L-1 n-TiO_2	Locomotor activity (6 dpf, distance travelled and frequency and total duration of movement)	↓Swimming speed	Fu et al. (2020)
Embryo/ larvae	6 d	nano-TiO_2 + PCP	0, 3, 10, 30 μg L-1 PCP + 0.1 mg L-1 n-TiO_2	Locomotor activity in light/dark conditions (6 dpf)	↓Swimming speed	Lei et al. (2020)
Embryo/ larvae	120 h	nano-TiO_2 + CYP	0, 0.4, 2, 10 μg L-1 CYP + 1 mg L-1 TiO_2	Distance travelled and total duration of movement both in continuous light and dark to light photoperiod	↓Swimming speed	Li et al. (2018)
Adult/ embryo/ larvae *Trans*-gen. study	4 m	nano-TiO_2 + BPA	0, 2, 20 μg L-1 BPA +100 μg L-1 n-TiO_2	F1: swimming speed; frequency and total duration of movement, distance travelled in light to dark transition (10 dpf)	↓Swimming speed (F1 10 dpf larvae)	Guo et al. (2019)
Embryo/ larvae	7 d	nano-TiO_2 + BDE-209	0.38 mg L-1 BDE-209 + 0.1, 0.5, 1.0 mg L-1 nano-TiO_2	Frequency and duration of movement, total distance travelled in dark to light transition (7 dpf)	↓Swimming speed	Wang et al. (2014)
Embryo/ larvae	6 d	nano-TiO_2 + PB	0, 5, 10, 20, 30 μg L-1 Pb + 0.1 mg L-1 n-TiO_2	Swimming activity in larvae in light/dark conditions	↓Swimming speed	Miao et al. (2015)
Embryo/ larvae	168 h	nano-TiO_2 + Cd	10 μg L-1 Cd + 0.1, 1.0, 10 μg L-1 TiO_2	Spontaneous movement (24 hpf)	No effects	Mamboungou et al. (2022)
Embryo/ larvae	120 h	nano-TiO_2 + DIF	0, 0.1, 0.5 mg L-1 DIF + 100 μg/n-TiO_2	Locomotor behaviour in light/dark conditions (6 dpf)	↓Swimming speed	Zhu et al. (2023)
Embryo/ larvae	24 h, 30 h, 48 h	nano-TiO_2 + BP3	10 μg L-1 BP3 + 100 μg L-1 n-TiO_2	Spontaneous movement (24 hpf), touch response (30, 48 hpf)	↑Spontaneous movement ↓Touch response (at 30 hpf)	Sun et al. (2023)

[a] Exposure time expressed in minutes (min), hours (h) days (d) or months (m).

cases, NPs particles carried the organic compounds and induced various effects on the fish nervous system, including a decrease in AChE enzymatic activity and locomotion. A 165 d co-exposure of BPAF, a BPA structural analogue, and 100 nm NPs to adult zebrafish, led to a significant decrease in locomotor and social behaviour (Wang et al., 2023). The same decrease was also observed in their offspring at 6 dpf. In a study by Varshney et al. (2023), the neurotoxic effects of one of the breakdown products of dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyltrichloroethilene (DDE), were worsened by PS-NPs in zebrafish larvae with a decrease in locomotor activity. Santos et al. (2022a) tested NPs with the herbicide phenmedipham (PHE). The mixture of 0.015 mg L-1 NPls +20 mg L-1 PHE highlighted hyperactive behavior. Zhang et al. (2022c), exposed zebrafish embryos and larvae to MPs with or without melatonin, with the aim of verifying melatonin's protective capabilities against neurotoxicological damage. This ability was highlighted on several different damages, included behaviour between 144 and 168 hpf. Three recent studies exposed zebrafish at different life-stage points to a mixture of MPs and metals found in the environment. Zhu et al. (2022b) carried out a fish embryotoxicity test with MPs and methylmercury (MeHg), confirming hypoactivity after 96 hpf, aggravated in the combined exposure. In Santos et al. (2021), after 14 d of exposure to Cu and MPs, the toxicity of Cu was not modulated by the presence of MPs, although a stronger AChE inhibition was observed in the co-exposed groups. Santos et al. (2020), exposed zebrafish embryos from 2 to 96 hpf to MPs and copper. Several behavioural endpoints were considered, and a noticeable decrease in social behaviour was observed in the mixture groups. Also, an increase of the distance moved in the dark was highlighted, with a significant difference compared to the control group and to the Cu-treated group, suggesting a modulation of the Cu toxicity from the MPs. A second study (Santos et al., 2022b) focused on the effects of MPs and Cu on zebrafish adults, after a 30 d exposure. In this case the exposure to Cu or MPs alone often resulted in overall hypoactivity, while the mixtures induced a state of hyperactivity in different endpoints, such as the mean speed and the time spent in the centre of the tank. In a 4-month study (Cormier et al., 2021), adult fish were exposed through diet to different types of MPs spiked with PFOS, benzo[*a*]pyrene (BaP), and BP3. Only MPs + PFOS seemed to accumulate in the fish tissue, although a decrease in body weight was observed from all the exposure conditions. This study presents a generally realistic environmental scenario, both for the timing and conditions of exposure. Co-exposure of various concentrations of MPs and the antidepressant amitriptyline (AMI) led to a general activity increase of zebrafish larvae after 7 d (Zhang et al., 2023). Finally, Hanslik et al. (2022) exposed embryos to a mixture of MPs previously exposed for 21 d to the water of a polluted site. A targeted analysis revealed the presence of 94 compounds, and sorption on the MPs was observed only for a few pollutants, while the contaminants seemed to accumulate more on the natural particles (sediments and particulate suspended matter). In a similar study, Mancia et al. (2023) fed for 15 d adult zebrafish with food supplemented with 0.4 mg L-1 of MPs sorbed after incubation in the sea, showing a state of hypoactivity. These studies show that MPs and NPs can in some instances act as carriers for organic pollutants. However, when neurotoxicity is considered, it might be difficult at present to evaluate any further effect on living organisms compared to the exposures to single chemicals given the contrasting results that are often put together.

3.7. Pharmaceutical mixtures

Pharmaceutical products are released into the environment as a

Table 6
Summary of the main parameters of the papers focused on micro- and nano-plastics mixtures.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae	7 d	MPs + MeHg	1 mg L-1 MPs +1 μg L-1 MeHg	Locomotor activity in light/dark transition	↓Swimming distance	Zhu et al. (2022b)
Embryo/ larvae	120 h	NPs + PHE	0.015 mg L-1, 1.5 mg L-1 NPls + 2 mg L-1, 20 mg L-1 PHE	Locomotor activity (120 hpf, type of movement, total time and distance, larvae path angle)	↑Swimming distance	Santos et al. (2022a)
Embryo/ larvae/ juvenile	14 d	MPs + Cu	60 μg L-1, 125 μg L-1 Cu + 2 mg L-1 MPs	Locomotor activity, anxiety, avoidance and shoaling behaviour	↓ Swimming behaviour (total distance travelled, absolute turn angle, percentage of time active and mean speed) ↓Avoidance behaviour	Santos et al. (2021)
Adult (F0) Embryo/ larvae (F1)	120 d	MPs + PFOS, MPs + BaP, MPs + BP3	Spiked PE and PVC with 70.22, 159.54 ng/g PFOS; 16.87, 11.50 ng/g BaP; 106, 107 ng/g BP3	Larval photomotor response (F1, total distance travelled)	No effects on adult behaviour, ↑Distance travelled after MP + BP3 exposure (F1)	Cormier et al. (2021)
Embryo/ larvae	94 h	MPs + Cu	15, 60, 125 μg L-1 Cu + 2 mg L-1 MPs	Mean speed, mean distance to the centre zone of the well, total distance moved, mean absolute turn angle and the percentage of time active were assessed in the light; swimming behaviour in light/dark condition; avoidance response with or without stimulus and social behaviour	↓Social cohesion ↑Swimming distance in dark	Santos et al. (2020)
Embryo/ larvae	7 d	NPs + BDE-47	2.5 ppm, 25 ppm PS + 10 ppt BDE-47	Total movement count and duration, speed and distance covered in light/ dark condition	Overall reduction of short movement counts and duration	Chackal et al. (2022)
Embryo/ larvae	140 h	Polystyrene MPs + Melatonin	25 mg L-1 PS + 1 μM melatonin	Swimming activity (144 hpf and 168 hpf)	Swimming impairment rescued by melatonin	Zhang et al. (2022c)
Adult	120 h	MPs + EE2 NPs + EE2	1 mg L-1 MPs + 2, 20 μg L-1 EE2; 1 mg L-1 NPs+ 2, 20 μg L-1 EE2	Swimming activity	↓Swimming distance	Chen et al. (2017a)
Embryo/ larvae	96 h	MPs + environmental samples	MPs mixture exposed to river water	Swimming activity in light/dark conditions	No effects	Hanslik et al. (2022)
Embryo/ larvae	72 h	NPs + AVO	10 μg L-1 AVO + 10 μg L-1 NPs	Swimming activity	↓Swimming speed	Liu et al. (2022)
Adult	30 d	MPs + Cu	2 mg L-1 MPs + 25 μg L-1 Cu	Locomotor activity (swim in the absence of external stimuli as mean distance from the centre, mean speed, total distance moved, mean absolute turn angle and percentage of time spent active or inactive; swimming behaviour in light/dark conditions)	↑Swimming speed ↑Time spent in the centre of the tank	Santos et al. (2022b)
Embryo/ larvae	96 h	NPs + DDE	50 mg L-1 NPs + DDE 100 μg L-1	Swimming activity	↓Swimming speed ↓Swimming distance	Varshney et al. (2023)
Adult (F0) Embryo/ larvae (F1) *Trans*-gen. study	45 d	NPs + BPAF	1 mg L-1 NPs and 200 μg L-1 BPAF	Swimming activity and social behaviour	↓Swimming speed ↓Swimming distance (F0, F1)	Wang et al. (2023)
Adult	7 d	MPs + AMI	0.44 mg L-1 MPs + 2.5 μg L-1 AMI	Startle behaviour	↑Swimming speed ↓Freezing	Zhang et al. (2023)
Adult	15 d	MPs + sorbed marine contaminants	food supplemented with 0.4 mg L-1 PE-MPs incubated in seawater	Exploratory and social behaviour	↓Activity	Mancia et al. (2023)

[a] Exposure time expressed in minutes (min), hours (h) days (d) or months (m).

result of human activities predominantly through wastewaters, and via this way they enter in a variety of waterbodies such as lakes, rivers, creeks and seas and may even reach drinking water sources (Hejna et al., 2022). Their presence in surface waters has been reported in many regions of the world such as China (Liu and Wong, 2013), the United States (Kostich et al., 2014) and the EU (Loos et al., 2013). Specific types of compounds (e.g. psychoactive drugs, hormones, antibiotics) are more likely to be found together as they are often co-prescribed and co-produced (Rodrigues et al., 2020; Tan et al., 2022). Other sources of chemical discharge are wastewater treatment plants (WWTPs) and sewage treatment plants (STPs). As shown in Table 7, Brunelle et al. (2022), sampled 10 treated WWTPs effluents detecting 94 chemical residues, including eight targeted pharmaceuticals, six of them being psychoactive drugs: buprionon, desvenlafaxine (DVS), venlafaxine (VNX), carbamazepine (CBZ), citalopram (CPM) and primidone. Zebrafish larvae were exposed to the samples and hyperactivity was observed in some cases, but, as pointed out by the authors such effect is unlikely to be caused by neuroactive pharmaceuticals alone, but rather than the co-presence of other chemical contaminants and synergistic effects. Atzei et al. (2021), exposed zebrafish embryos to single and combined CBZ, fluoxetine (FLX) and VNX. All exposures induced an inhibitory effect on locomotor activity. Combining compounds with dissimilar and similar modes of action resulted in dose addition, excluding synergistic interactions. In the case study conducted by García-Cambero et al. (2012), exposure to surface water samples from the Tagus River, decreased mobility of zebrafish larvae exposed to the samples. A previous study revealed, through targeted analysis, the presence of CBZ, VNX, CPM, caffeine and erythromycin in the water

Table 7
Summary of the main parameters of the papers focused on pharmaceutical mixtures.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae	120 h	93 in total including bupropion, CBZ, ciprofloxacin, CPM, desvenlafaxine, iopamidol, primidone, VNX	N/A	Total distance travelled in light/ dark condition	↑Swimming distance	Brunelle et al. (2022)
Embryo/ larvae	120 h	Binary mixtures of CBZ + FLX + VNX	Relative potency factor-based mixtures	Light/dark transition test	↓Swimming activity	Atzei et al. (2021)
Embryo/ larvae	80 h	FLX + VNX	3.2 ng L-1 FLX +2000 ng L-1 VFN	Sensorymotor reflexes (light touches)	No effect	Rodrigues et al. (2020)
Embryo/ larvae	104 h	FLX + NFLX + CBZ	2.9 and 289.2 nM equimolar concentrations	Visual motor response test (total distance moved and maximum velocity in light and dark periods)	↓Swimming distance	Zindler et al. (2020)
Adult	45 d	CBZ + Cu	1, 10, 100 μg L-1 CBZ + 0.5, 5, 10 μg L-1 Cu	AChE activity	No effect	Jia et al. (2020)
Embryo	48 h	Ibuprofen + DCF + caffeine + CBZ + clarithromycin + sulfamethoxazole + bezafibrate + triclosan	Low-, medium-, high-exposures of 8 pharmaceuticals	Angular velocity and swimming speed in light/dark conditions	↓Swimming speed in the darkness	Zhou et al. (2019)
Embryo/ larvae	6 d	Water river samples containing previously detected CBZ, VNX, CPM, caffeine, erythromycin, benzoylecgonine, norBE, ephedrine	N/A	Spontaneous movement (24 hpf), locomotion assay (6 dpf, mean distance travelled)	↑ Spontaneous movement frequency ↓Swimming speed ↓Swimming distance (6 dpf)	García-Cambero et al. (2012)
Larvae	24 h	Deprenyl + PCPA, FLX + PCPA	2.5 mM PCPA + 5 μM deprenyl, 2.5 mM PCPA + 0.5 μM FLX	Basal locomotor activity, visual-motor response and vibrational startle response in light/dark conditions	Recovery of behavioral changes induced by Deprenyl/ FLX after combined exposure	Faria et al. (2021)
Embryo/ larvae	120 h	Metformin + ranitidine + bisoprolol + sotalol	Binary mixtures combinations and quaternary mixture at 0.1, 1.0, 10, 100 μg L-1 each	Total swimming distance and total swimming time in light/ dark conditions	No effect	Godoy et al. (2019)
Embryo/ larvae	144 h	EE2 + GES	41, 423 ng L-1 EE2 + 49, 514, 4873 ng L-1 GES in binary combinations	Total distance travelled and mean speed in the dark	↓Swimming distance ↓Swimming speed	Tan et al. (2022)
Embryo/ larvae	144 h	River samples and representative mixture of E2 + EE2 + DCF	160, 1700 ng L-1 DCF + 5, 11 ng L-1 E2 + 7, 115 ng L-1 EE2 (low- and high-dose exposures)	Total distance travelled and swimming speed in light condition (6 dpf)	↓ Touch response ↓ Swimming distance and speed (only after river sample exposure)	García-Cambero et al. (2021)
Adult	14 d	EE2 + CPM	0.9, 1 ng L-1 EE2 + 0.1, 0.4 μg L-1 CPM	Swimming activity (scototaxis, shoaling and Novel Tank test)	Recovery of anxiogenic effect of EE2 by CTP, ↑Latency in novel tank test	Porseryd et al. (2017)
Embryo/ larvae	114 h	DKAs (tetracyclines + fluoroquinones)	0, 4.69, 9.38, 18.75 mg L-1	Swimming activity and swimming speed in light/dark condition	↑Basal swimming rate (lower mixture concentrations ↓Basal swimming rate (higher mixture concentrations	Zhang et al. (2014)
Embryo/ larvae/ adult	120 d	DKAs (tetracyclines + fluoroquinones)	6.25, 12.5 and 25 mg L-1	Bottom dwelling test (time spent in the upper part of the tank, distance travelled, mean speed and number of line crossings), conditioned place preference and shoaling behaviour	↑Swimming distance ↑Time spent in the upper portion of the tank (low mixture concentration) ↓Time spent in the upper portion of the tank (higher mixture concentration) ↓Social cohesion	Wang et al. (2016)
Embryo/ larvae	90 h	DKAs (tetracyclines + fluoroquinones)	0, 4.69, 9.38, 18.75, 37.5 mg L-1 DKAs	Swimming speed and light/dark stimulation response (120 hpf)	↑Spontaneous movement	Yin et al. (2014)
Juvenile/ adult	130 d, 21 d	SSRIs (Fluoxetine, Paroxetine, Sertraline)	10, 100, 200 μg L-1	Novel tank test (90 and 120 dpf)	↓Swimming distance ↓Time spent at the bottom of tank (at 120 dpf)	Venkatachalam et al. (2023)

[a] Exposure time expressed in minutes (min), hours (h), days (d) or months (m).

downstream of STP effluent discharge into the Tagus River (Valcárcel et al., 2011), in addition to the psychoactive drugs benzoylecgnina, norBE and ephedrine (<41 ng L-1), detected in the samples used by García-Cambero et al. The effect observed in the study might be a result of the presence of such psychoactive chemicals continuously introduced in the environment, although their interactions were not explored. Zindler et al. (2020) evaluated the neuroactive potential of selective serotonin reuptake inhibitors (SSRIs) FLX, norfluoxetine (NFLX) and CBZ in single and combined equimolar exposure, with results suggesting an additive interaction between FLX and NFLX. CBZ was considered for another pharmaceutical mixture in Zhou et al. (2019), along with six other substances, including caffeine, which was shown to have neuroactive effects on zebrafish (de Farias et al., 2021; Steenbergen et al., 2011; Tran et al., 2017). Only the highest mixture concentration elicited hypoactivity in larvae. In Jia et al. (2020), significant inhibition of the enzyme acetylcholinesterase (AChE) was observed in larvae exposed to CBZ (1, 10, 100 μg L-1) and Cu (0.5, 5 and 10 μg L-1), but the combined exposure did not exacerbate the decrease in enzymatic activity.

Zebrafish embryos were exposed to FLX (3.2 ng L-1) and VNX (2 μg L-1) in Rodrigues et al. (2020). No significant differences between the control group and the exposed group were found, although a general activity decrease was observed. In a study conducted on 8 dpf zebrafish larvae FLX was combined with 2 other serotonin system modulators, deprenyl and 4-Chloro-DL-phenylalanine (PCPA), with respectively inhibiting and inducing activity. Complete recovery of the reduced activity induced by deprenyl and FLX was achieved after PCPA exposure, suggesting an interaction between pharmaceuticals with different MoAs (Faria et al., 2021). In a recent study by Venkatachalam et al. (2023), three SSRIs (FLX, paroxetine, sertraline) were tested alone and in combination in a short-term and long-term exposure experiment. The study showed an inhibitory effect on the swimming activity and on the exploratory behaviour. In the two already discussed river case studies, other types of pharmaceuticals that affect zebrafish behaviour were found in the river samples, e.g. antibiotics. Yin et al. (2014) and Wang et al. (2016) exposed zebrafish larvae to a mixture of beta-diketone antibiotics (DKAs) frequently detected in polluted internal water systems in high concentrations. In Wang et al. (2016), after three months of exposure, adult fish were screened for behavioural changes. Interestingly, in Yin et al. opposite effects to Wang et al. were detected: lower concentrations induced an increase in swimming activity, that was on the other hand inhibited at the two highest concentrations. Similar results were obtained in a study by Zhang et al. (2014), that observed the combined effect of tetracyclines and fluoroquinones antibiotics. Nevertheless, in all the three studies, the synergic or additive properties of the mixture were not explored. As more classes of antibiotics are commonly detected in the environment (Valcárcel et al., 2011; Zhou et al., 2019; Kovalakova et al., 2020; Lei et al., 2020), their potential interactions should be further analysed since they have, among other endpoints, significant effects on zebrafish behaviour. Another type of ubiquitous organic pollutants detected in effluents from agricultural and domestic STPs are steroid hormones such as progestins and estrogens (Ojoghoro et al., 2021). García-Cambero et al. (2021) detected estradiol (E2), ethinylestradiol (EE2) and diclofenac (DCF), an anti-inflammatory pharmaceutical, in surface water and sediment samples from the Manzares River (Spain). A significant change in the behaviour of zebrafish larvae was observed, but this did not seem to correlate to the presence of the three pharmaceuticals, as exposure to an experimental mixture containing representative concentrations of these chemicals did not cause any relevant effects. EE2 was also tested in combination with CPM (Porseryd et al., 2017) and gestodene (GES) (Tan et al., 2022). The co-exposure with GES, a synthetic progestin, caused a significant decrease in the swimming activity of zebrafish larvae. Moreover, AChE activity increased compared to the single exposures, suggesting that the enhanced enzymatic activity reduced the levels of neurotransmitters and consequently locomotor activity (Tan et al., 2022). EE2, according to Porseryd et al. (2017), potentially interacts with psychoactive substances as well, such as citalopram, as their combined exposure affected the locomotor behaviour of zebrafish compared to the single exposures. In a 2019 study (Godoy et al.) embryos were exposed to a mixture of metformin, bisoprolol, ranitidine and sotalol: in this case no relevant effects were observed at any tested concentrations. In general, among the presented studies, only a few showed significant additive or synergistic effects.

3.8. Pesticide mixtures

Pesticides encompass a wide array of products, including fungicides, herbicides and insecticides (Alavanja, 2009). They are used worldwide in a variety of fields, including food production, gardening, medicine and road maintenance (Schleiffer and Speiser, 2022), and according to a 2017 report from the United Nations (United Nations, 2017) around 200,000 people die each year in the world because of pesticides acute poisoning. Several works we found tested the insecticide chlorpyrifos in conjunction with other products (Table 8); this is not surprising given its widespread use (Tzatzarakis et al., 2020) and its known neurotoxic action as acetylcholinesterase inhibitor (Kopjar et al., 2018). In a 2013 work, Pérez and colleagues tested the widely used herbicides chlorpyrifos, atrazine and terbuthylazine (Wacksman et al., 2006) exposing zebrafish embryos to the single substances and two mixtures (chlorpyrifos + atrazine and chlorpyrifos + terbuthylazine). Results show synergistic interaction between the compounds on behaviour and AChE. In a 2021 paper, Fan and colleagues investigated the individual and combined toxicity of carbendazim and chlorpyrifos on zebrafish embryos until 96 hpf. The binary mixture showed an increase in tail movement as well as synergistic effects on several endpoints. The same compounds were also tested in a 2022 paper (Zhang et al., 2022a) and in a second work from the same author (Zhang et al., 2022b) chlorpyrifos was tested with the insecticide cyfluthrin on zebrafish eggs. Chlorpyrifos was also tested in conjunction with Deltamethrin, a widespread pyrethroid, on zebrafish embryos through a 142-h acute exposure (Hu et al., 2021), suggesting a possible additive interaction. Deltamethrin was tested in combination with *Citrus aurantifolia*, an essential oil used for biological control (Cadena et al., 2023). Their combination resulted in a reduction in forage frequency. A 2014 work (Yang et al.) investigated the toxicity of a type I pyrethroid (Permethrin) and of a type II pyrethroid (Cypermethrin), both widely used pesticides (Oros and Werner, 2005) by exposing zebrafish eggs to environmentally relevant concentrations. The authors suggested additive or synergistic interactions. A 2019 work (da Costa Chaulet et al.) investigated the effects of glyphosate, the most widely used herbicide in the world (Ivantsova et al., 2022) and fipronil, a broad-spectrum insecticide (Wilde et al., 2001) and their mixtures on adult zebrafish. Exposures induced an anxiolytic-like response, with higher intensity in the mixture. The effects of glyphosate and its metabolite aminomethylphosphonic acid (AMPA) were investigated in a 2022 work (Ivantsova et al.) using zebrafish embryos. While the exposure to glyphosate alone induced hyperactivity, in line with other recent works (Zhang et al., 2017), neither the AMPA nor the mixtures were cause of behavioural alterations or changes in AChE expression. In a 2020 paper Rozmánková and colleagues exposed zebrafish embryos to S-metolachlor, a widely used pesticides, and its two metabolites, metolachlor oxanilic acid (MOA) and metolachlor ethanesulfonic acid (MESA). No effects were observed at 5 dpf, however, S-metolachlor and the mixtures induced a notable reduction in the coiling frequency.

3.9. Cyanotoxin mixtures

Cyanobacteria are a vast group of photosynthetic microorganisms that can be found in a wide range of habitats throughout the planet (Zakhia et al., 2008; Ionescu et al., 2010), able to produce under certain conditions a large number of secondary metabolites (Dittmann et al., 2015) some of which (i.e. the cyanotoxins) of great concern for both

Table 8
Summary of the main parameters of the papers focused on pesticides.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae/ juvenile	70 d	Commercial pesticides + essential oils	Binary mixtures 9:1 (pesticide and essential oil)	Forage frequency	↓ Forage frequency	Cadena et al. (2023)
Embryo/ larvae	116 h	Chlorpyrifos + cyfluthrin	1.16 mg L-1 chlorpyrifos + 7.06, 14.12 μg L-1 cyfluthrin (low-, middle-dose exposures)	Average velocity, total distance travelled, meander	↓Swimming speed ↓Swimming distance ↑Meander	Zhang et al. (2022b)
Embryo/ larvae	96 h	Carbendazim + chlorpyrifos	Mix1 = 0.59–2.35 mg L-1 (in total, equipotent concentration ratio) Mix2 = 0.38–1.51 mg L-1 (total, based on maximum residue limits)	Spontaneous movement (24 hpf)	↑ Spontaneous movement frequency	Fan et al. (2021)
Embryo/ larvae	116 h	Carbendazim + chlorpyrifos	0.51, 0.57, 0.64 mg L-1 carbendazim + 1.00, 1.26, 1.58, 2.00, 2.52 mg L-1 chlorpyrifos (low-, middle-, high-dose exposures)	Light/dark transition test (total distance travelled, meander, average swim velocity, turn angle, angular velocity)	↓Swimming speed ↓Swimming distance ↓Light/dark acceleration	Zhang et al. (2022a)
Embryo/ larvae	142 h	Chlorpyrifos + Deltamethrin	4.80, 39.06, 78.13 μg L-1 CPF + 0.06, 1.60, 3.19 μg L-1 DM (low-, middle-, high-dose exposures)	Light/dark transition test, swimming speed	↑Frequency of spontaneous tail coiling (24 hpf) ↓Swimming speed ↓Habituation	Hu et al. (2021)
Embryo/ larvae	7 d	GLY + AMPA	1 μM GLY + AMPA	Total distance travelled (locomotor activity assay) and light/dark preference	No effect	Ivantsova et al. (2022)
Embryo/ larvae	120 h	S-Metolachlor + metolachlor oxanilic acid and metolachlor ethanesulfonic acid	1 μg L-1 of each substance in the mixture	Spontaneous movement (23 hpf), total swimming distance in light/dark condition (120 hpf)	↓ Spontaneous movement frequency (23 hpf)	Rozmánková et al. (2020)
Adult	96 h	Glyphosate-based herbicide + Fipronil-based insecticied	1, 3, 5 mg L-1 GBH + 0.009, 0.018, 0.027 mg L-1 FBI (low-, middle-, high-dose exposures)	Novel Tank test	↑Time spent in the top of the tank	da Costa Chaulet et al. (2019)
Embryo/ larvae	141 h	Permethrin + cypermethrin	100, 200, 300 μg L-1 PM + 10, 20, 30 μg L-1 CP	Involuntary body movement (spasms)	↑Spasms	Yang et al. (2014)
Embryo/ larvae	96 h	Atrazine, terbuthylazine + chlorpyrifos	7 binary mixtures from 5 concentrations of each pesticide (0.5–40 mg L-1	Swimming activity	Altered swimming behaviour	Pérez et al. (2013)

[a] Exposure time expressed in minutes (min), hours (h), days (d) or months (m).

human and animal health (Moreira et al., 2014).

As shown in Table 9, a 2022 paper (Martin et al., 2022) assessed on zebrafish embryos the toxicity of a widely studied cyanotoxin, β-methylamino-L-alanine (BMAA), and of other two less known cyanotoxic compounds, 2,4-diaminobutyric acid (2,4-DAB) and *N*-(2-aminoethyl) glycine (AEG), alone or in 7 different binary or ternary mixtures. BMAA and AEG enhanced startle sensitivity, and showed additive interactions. Binary mixtures also caused a reduction in average speed and in the total distance travelled.

The same authors also tested BMAA and microcystin leucine and arginine (MCLR) on zebrafish larvae (Martin et al., 2021a). A dose-related increase in acoustic startle sensitivity was found with both compounds, furtherly enhanced by the combined exposure, and the authors hypothesized a synergic interaction between the two compounds. Lastly, Roegner et al., in 2019 tested a wide variety of cyanobacterial metabolite standards and bloom components from the Rio de La Plata waters on 4 hpf dechorionated embryos.

3.10. Metal mixtures

Heavy metals are naturally occurring elements whose concentration in the environment is often increased by human activities such as mining, agricultural activities and industrial processes (Shallari et al., 1998; He et al., 2005). These elements tend to accumulate in the environment and in living organisms, and exposure can result in significant toxicity in both humans and animals (Patel et al., 2021). Because of this, over the last decades many metals have been cause of health concern at international an level (Tchounwou et al., 2012). Exposure of living organisms

Table 9
Summary of the main parameters of the considered papers focused on cyanotoxins.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae	6 d	BMAA +2,4DAB + AEG	7 mixtures with different ratios of the cyanotoxins (0.167, 0.3, 0.5, 0.667, 1 μM)	Acoustic startle response and spontaneous locomotion (6 dpf, average speed and total distance travelled)	↓Spontaneous movement ↓Swimming speed ↓Swimming distance	Martin et al. (2022)
Embryo/ larvae	6 d	BMAA + MCLR	100 μM BMAA + 1 μM MCLR	Swimming activity (startle response and locomotor activity)	↓Swimming speed	Martin et al. (2021a)
Embryo/ larvae	114 h	16 cyanotoxins and 8 bioactive cyanopeptides	Environmental samples with different concentrations of cyanobacteria metabolites	Photomotor response in light/dark transition and total movement in light/dark transition (5 dpf)	↑Activity in photomotor response assay after river extract exposure	Roegner et al. (2019)

[a] Exposure time expressed in minutes (min), hours (h), days (d) or months (m).

to mixtures of metals is likely to occur in the environment, and for this reason a more comprehensive understanding of their effects is needed (Patel et al., 2021). Eight works that met our inclusion criteria tested metals in combination with other metals or different chemicals (Table 10). In a 2018 work (Heffern et al.) zebrafish were exposed to common metals to investigate their activity as olfactory toxicants. Zinc, arsenic and chromium were tested singularly on zebrafish embryos to investigate possible alterations in the response behaviour to two odorants, taurocholic acid and L-Cysteine. Binary mixtures of cadmium (Cd) and zinc (Zn) were also tested.

In the study from Xia et al. (2023), lead (Pb) and manganese (Mn) showed combined effects on 7 dpf zebrafish larvae, while in 2021, Patel et al. exposed for 21 d zebrafish adults to Cd chloride and mercury (Hg) chloride alone or in combination, for an environmentally realistic exposure. The exposure to the mixture and to Cd alone induced a significantly longer permanence in the dark side of the tank, and the Novel Tank Test showed a similar preference for the lower part of the tank in all treated groups, showing an anxiety-like behaviour without synergistic effects.

Zhang and colleagues in 2016 tested the joint toxicity of Cd and sodium dodecyl benzene sulfonate (SDBS) on both zebrafish adults and *Daphnia magna*. The test on zebrafish was performed with a flow-through system and a three-dimensional recording system with several combinations of concentrations. The mixture showed synergistic effect on the vertical position but an antagonistic effect on the swimming speed.

In another 2016 study (Zhu et al.) the effects of the exposure of zebrafish larvae to decabromodiphenyl ether (BDE-209, a polybrominated diphenyl ether widely used as flame retardant) and environmentally relevant concentrations of Pb, either alone or in combination were investigated. In a 2017 paper, Chen and colleagues (Chen et al., 2017b) performed a transgenerational study involving the same compounds, starting with chronic parental exposure: adult individuals were exposed to environmentally relevant concentrations of Pb, BDE-209 and their mixtures for a 3-months period. A recent work (Lanzarin et al., 2022) tested Cu, Zn, and Mn in combination with the herbicide glyphosate on zebrafish embryos. After a 96-h exposure the exposed larvae were tested at 120 hpf. Finally, the effects of cypermethrin (5 mg L-1) together with Pb (50 μg L-1) were assessed over 96 h on zebrafish larvae (Jijie et al., 2023).

3.11. Environmental samples-complex mixtures

Several works that emerged from our query focused on the use of zebrafish for detection of environmental pollution in freshwater (Table 11). This is not surprising, given the growing release of neurotoxic substances all over the world (García-Cambero et al., 2012).

A 2017 work by Michiels and colleagues exposed zebrafish embryos to river water samples and to a reconstituted mixture of eight metals found in the river samples at the same concentrations. The aim of the work was to compare the results obtained from the two different mixtures to better understand which effects could be attributed to the metal mixture alone. A 2021 work (Pompermaier et al.) used adult zebrafish to assess the effect of an acute exposure to environmental samples taken from an area subjected to pollution from old vineyards. Given the widespread release from this kind of agricultural activity of copper (Cu) from phytosanitary products, the fish was also exposed to several Cu concentrations for comparison. The study highlighted anxiety-like behaviour and stress.

Several works were also focused on environmental samples subjected to a purification process through wastewater treatment plants. The effluents from these structures are often source of chemical pollution (Petrie et al., 2015), with several known ecotoxicological effects (Babić et al., 2017; Stelzer et al., 2018).

In a 2020 work (Ribeiro et al., 2020) zebrafish eggs were exposed to water samples from wastewater treatment plants effluents, a known possible source of environmental toxicity (Babić et al., 2017). A 2021 paper (Rothe et al., 2021) was aimed at assessing the effects derived

Table 10
Summary of the main parameters of the papers focused on metals.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Larvae	24 h	Cd + Zn	76 μg L-1 Zn + [0, 4, 16, 36, 85, 326 μg L-1 Cd] 36 μg L-1 Cd + [0, 22, 47, 76, 122, 262 μg L-1 Zn	Olfactory response	↓Olfactory response (high concentration mixture)	Heffern et al. (2018)
Adult	21 d	Cd chloride + Hg chloride	1 mg L-1 Cd + 30 μg L-1 Hg	Light/dark preference (7 dpf) and Novel Tank test (21 dpf)	↓Exploratory behaviour ↑Time spent in lower zone of the tank	Patel et al. (2021)
Adult	130 min	Cd + SDBS	2, 4, 6, 12 mg L-1 Cd + 2.5, 5, 10, 20, 40 mg L-1 SDBS	Swimming speed and vertical position	↓Vertical position ↓Time spent on surface	Zhang et al. (2016)
Embryo/ larvae	142 h	Pb + BDE-209	5, 10, 20 μg L-1 Pb + 50, 100, 200 μg L-1 BDE-209 (low-, middle-, high-dose co-exposure)	Larval swimming behaviour in response to light/dark transition (distance travelled, the frequency of movements, and the total duration of movements)	↓Swimming speed	Zhu et al. (2016)
Adult/ embryo/ larvae *Trans*-gen. study	90 d	Pb + BDE-209	0, 1, 10, 100 μg L-1 BDE-209 + 10 μg L-1 Pb	F1: Locomotor activity (5 dpf, average speed under light/dark conditions)	↓Swimming speed	Chen et al., 2017b
Embryo/ larvae	96 h	Cu, Zn and Mn complexed with glyphosate-based herbicide	1 μg a.i./mL GBH +100 μg L-1 Cu/Mn/Zn	Speed, distance travelled and distance from the centre of the well, thigmotaxis and behaviour in response to visual stimuli in different light conditions	↓Swimming distance in darkness	Lanzarin et al. (2022)
Embryo/ larvae	96 h	Pb + Ciprofloxacin	50 μg L-1 Pb + 5 mg L-1 Ciprofloxacin	Swimming activity	↓Swimming activity in top part of the tank ↑Freezing duration	Jijie et al. (2023)
Embryo/ larvae	7 d	Pb + Mn	50 μg L-1 Pb + 300 μg L-1 Mn	Light/dark photoperiod stimulation test	↓Activity count ↓Swimming distance	Xia et al. (2023)

[a] Exposure time expressed in minutes (min), hours (h), days (d) or months (m).

Table 11
Summary of the main parameters of the papers focused on environmental samples.

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae	144 h	Wastewater treatment plants effluents samples	N/A	Spontaneous movement (24 hpf)	↑ Spontaneous movement frequency	Ribeiro et al. (2020)
Embryo/ larvae	24 h, 120 h	Wastewater treatment plants effluents samples/ river samples	N/A	Spontaneous movement (24 hpf), swimming behaviour in light/dark conditions (120 hpf)	↓Swimming distance	Rothe et., al 2021
Embryo/ larvae	120 h	A mixture of Al, Mn, Fe, Ni, Cu, Zn, As, Cd and water samples	N/A	Swimming activity (5 dpf, speed and distance travelled)	↓Swimming distance	Michiels et al. (2017)
Adult	48 h	Enviromental water and sediment samples, Cu	N/A	Novel Tank Test	↓Time spent in the bottom area of the tank	Pompermaier et al. (2021)
Embryo/ larvae	120 h	Extract from sediment samples	N/A	Total swimming distance,swimming speed and swimming time in light/dark changes	↑Locomotor behaviour	Shuliakevich et al. (2022)
Embryo/ larvae	7 d	Groundwater samples	N/A	Swimming speed	↓Swimming speed	Yang et al. (2022)
Adult	96 h	Hospital effluents	N/A	Novel Tank Test (Time spent in top and bottom, time frozen, mean speed, total distance travelled, distance travelled in the top and bottom)	↓Swimming distance	Rosales-Pérez et al. (2022)
Adult	72 h	River samples	N/A	Swimming activity	↓Swimming activity	Sánchez-López et al., 2023

[a] Exposure time expressed in minutes (min), hours (h), days (d) or months (m).

from the exposure to conventionally treated wastewaters, ozone treated wastewaters compared to untreated river samples in both zebrafish embryos and larvae. Also, aim of this work was to assess the sensibility of several endpoints not covered in the OECD 236 guideline. Behavioural analysis showed to be a suitable endpoint for this kind of investigation, showing greater sensitivity compared to other tested endpoints, such as blood flow and body length. Shuliakevich et al. (2022) exposed zebrafish embryos to the extract solution from sediment samples from a polluted river, and in 2022 Yang and colleagues exposed zebrafish embryos to Sri Lanka's groundwater for 7 d. A work from Rosales-Pérez et al. (2022) assessed the effects on zebrafish adults of the exposure to hospital effluents samples. Finally, Sánchez-López et al. (2023) observed the daily rhythm of swimming activity of adult zebrafish exposed acutely for 3 d to water samples coming from Lerma River and detected a significant reduction of the swimming activity, considering the circadian variation in locomotor activity.

3.12. Other mixtures

In this paragraph we included mixtures not easily placeable in the previous categories (Table 12). Two works that emerged from our query used zebrafish to investigate the effects of cigarette total particulate matter (TPM), coherently with previous works that assessed the suitability of the zebrafish model for this purpose (Ellis et al., 2014). The first study (Massarsky et al., 2015) investigated the effects of cigarette particulate matter on zebrafish embryos through a 94 h exposure, with the aim of highlighting its toxicity and compare it with that exhibited by nicotine. In this case the exposure to the mixture showed effects on behaviour that could not be replicated though the exposure to nicotine alone. In 2018 Massarsky and colleagues set up a *trans*-generational study aimed at verifying if developmental exposure to TPM can cause long-term toxicity in zebrafish adults (F0) and their offspring (F1). In 2019 Gauthier and Vijayan exposed zebrafish embryos to isoproterenol, serotonin and ethanol. Isoproterenol, ethanol or a combination of both were used to assess behavioural changes. A 2019 paper (Samarut et al., 2019) used 5 dpf larvae to investigate the effects derived from a brief exposure to Δ-9-tetrahydrocannabinol (THC), cannabidiol (CBD) and their mixture at different concentrations, while electronic cigarette flavourants, with and without nicotine were tested in a 2020 paper (Gauthier et al., 2020).

In a 2020 paper by Christou et al. (2020b), zebrafish embryos were exposed to solvents dimethyl sulfoxide (DMSO) and methanol in combination with flutamide and perfluorooctanesulfonic acid (PFOS), until 98 hpf.

The effects of leachate from plastic bags on zebrafish reproduction and behaviour was tested in a 2022 work from Lin et al. and a recent paper from Haridevamuthu et al. (2022) tested the ameliorative ability of benzo[*b*]thiophene analogues on the neurotoxicity induced by acrylamide on 3 dpf zebrafish larvae through a 3 d exposure. Finally, a 2022 paper (Jessup et al., 2022) tested the effects of a mixture of siloxanes on newly hatched zebrafish embryos. The effects on behaviour were considered after 7 and 14 d of exposure.

4. Discussion

In general, peer-reviewed studies included in this work show a good overall quality. However, as highlighted in a 2021 paper by Martin et al. (2021b), in the next future a better knowledge and description of the concepts of synergism and additivity is desirable.

Among the works we surveyed, 13% focused on behavioural parameters alone. All the other papers combined behavioural observations with other endpoints, only in some cases directly related to neurotoxicity. In particular, among the most frequently tested parameters, 24% of works tested for oxidative stress, 37% for genetic expression, 37% for lethality and 48% for other sub-lethal parameters such as morphological and developmental alterations. Only 3% of the papers tested other organisms in combination with zebrafish (Daphnia species and Medaka). 16% of works tested for AChE activity, a well-known marker for neurotoxicity (Roda et al., 2020), whose inhibition can be correlated with alterations of behaviour (Serafini et al., 2019). In our case, all papers that tested for AChE activity also observed behavioural alterations, showing high correspondence between these two parameters (87.5%).

Results from our query highlight a growing number of research papers that fit our inclusion criteria (Fig. 2). This confirms the growing popularity that the zebrafish has gained throughout the years (Teame et al., 2019; Collin and Martin, 2017); there is also evidence of the increase of the use of zebrafish for chemical screening (Rennekamp and Peterson, 2015) for several classes of chemicals, such as environmental pollutants (Ankley and Johnson, 2004) and pharmaceuticals (Gibert et al., 2013).

This same growing trend can also be observed by considering the

Table 12
Summary of the main parameters of the papers focused on "other mixtures".

LIFE STAGE	EXPOSURE TIME[a]	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae	30 min	Electronic cigarette flavourants + nicotine	0, 1, 5, 10% dilutions of electronic cigarette liquid with or without nicotine (80 μM)	Embryo photomotor response (32 hpf)	↑Activity	Gauthier et al. (2020)
Embryo/ larvae	94 h	Cigarette TPM	0.4, 1.4 μg mL-1 TPM	Spontaneous movement (24 hpf), swimming activity in light/dark conditions (96 hpf)	↑Spontaneous movement frequency ↑Swimming distance	Massarsky et al. (2015)
Adult/ embryo/ larvae *Trans*-gen. study	90 h	Cigarette TPM	1.4, 2.8, 4.2 μg mL-1 TPM	F0 and F1: swimming distance (144 hpf) F0: shoaling test, novel tank test, predator avoidance and startle tap test (6 months adults)	↑Swimming distance (F0 larvae) ↓Swimming distance (F1 larvae) ↑Swimming distance (F0 adults) in startle tap test, shoaling test ↓Perception to danger (predator avoidance test) ↓Anxiety in males (novel tank dive test) ↑Anxiety in females	Massarsky et al. (2018)
Embryo/ larvae	30 min	Isoproterenol + serotonin (1) Isoproterenol + ethanol (2)	20 μM isoproterenol + 100 μM serotonin, 100 μM isoproterenol + 2% ethanol	Spontaneous activity (32 hpf) Swimming activity in different light conditions (80 hpf, total distance travelled)	(1) Suppression of anxiolytic effect of isoproteronol after serotonin exposure (2) Activity increase after single exposures	Gauthier and Vijayan, 2019
Adult	2 m	Leachate from plastic bags	Room-temperature water treatment with plastic bag, leaching group from heated water treatment	Swimming activity and social behaviour (mirror attack and exploration in different light conditions)	↓Swimming speed (males) ↓Crossings in light/dark assay ↓Mirror attacks	Lin et al. (2022)
Embryo/ larvae	92 h	DMSO/MeOH, flutamide, MB andPFOS	1.0, 3.2, 5.5, 7.8, 10.0 μM flutamide or 0.25, 0.50, 1.00, 2.00, 4.00 μM PFOS + 0.1, 1.0% DMSO 0.10, 0.32, 0.55,0.78, 1.00% DMSO + 0.0005% MB	Swimming speed and total distance travelled in light/dark conditions (98 hpf)	Additive effects on swimming speed (DMSO + flutamide/PFOS)	Christou et al. (2020b)
Larvae	1 h	Pentylenetetrazole,THC, CBD	2.5, 5 mM PTZ + 1.5, 2 μM THC + 1 μM CBD	Total distance travelled at high and low speed	Synergistic effects on distance travelled induced by THC + CBD on pentylenetetrazole	Samarut et al. (2019)
Larvae	72 h	Benzo[*b*]thiophene analogues + acrylamide	0.75 mM acrylamide + 80 μM BP, BN, EP, EN	Swimming distance and swimming pattern	Rescue of reduced swimming activity caused by acrylamide after combined exposure	Haridevamuthu et al. (2022)
Embryo/ larvae/ juvenile	14 d	Water samples including siloxanes	D4 + D5 in water samples	Startle response and locomotor activity (angular velocity, total distance moved, frequency of immobility and turn angle)	Age-related differences (7 vs 14 dpf) on: frequency of visits to centre of tank, swimming velocity, total distance travelled and turn angle ↓Activity (14 dpf)	Jessup et al. (2022)
Adult	30 d	SARS-CoV-2-derived peptides + mix of emerging pollutants	222.6 ng/SARS-CoV-2-derived peptides + different concentrations of 14 pollutants	Open field test (locomotor activity at 29 d, social aggregation at 30 d)	No effect	Freitas et al. (2023)
Adult	120 d	Sucralose + acesulfame	50, 75, 125 μg L-1 Sucralose + 50, 75, 125 μg L-1 Acesulfame	Novel Tank Test, Dark/Light test	↓Swimming distance ↑Freezing time ↑Time spent in dark area	Colín-García et al., 2023

[a] Exposure time expressed in minutes (min), hours (h), days (d) or months (m).

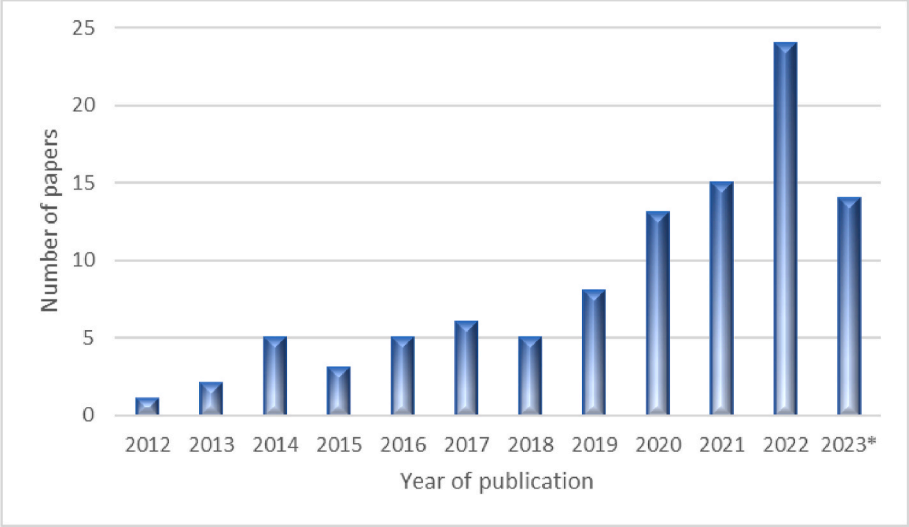

Fig. 2. Year of publication of the papers included in this study after the screening process. Research included papers published between January 2012 and September 2023. (only papers included in this study are showed) (for color reproduction on the Web). *: until September 2023

many different systems that can be used in zebrafish to assess toxicity, such as the auditory system, the circulatory system, the digestive system and the nervous system itself (Cassar et al., 2020). At the same time, the number of research papers that focused their attention on the effects of chemical mixtures in the environment has also grown steadily, showing an increasing concern for the issue at a global level (Fig. 2).

In the first 5 d after the egg's fertilization the zebrafish show the complete development of a vertebrate embryo, and this age marks the transition to a self-sustainable organism. This particular phase in the embryo's development does also have practical consequences on the study planning, since to this day the use of zebrafish embryos not older than 5 dpf is not acknowledged as an *in vivo* study under the current European legislation (Bertelli et al., 2017), and can be an interesting alternative to the classic animal testing (Cassar et al., 2020). This is particularly relevant in the view of the recent "3R" strategy (Replacement, Reduction, Refinement) (MacArthur and Clark, 2018) on animal studies, since the use of zebrafish embryos could allow to reduce the use, or even replace some *in vivo* studies, and provide a refined model that makes possible a quick and easy observation of the internal effects on the organism (Cassar et al., 2020).

The use of embryos comes with several other advantages: external fertilisation and development of the eggs (which reduces maternal behavioural influences) and the transparency of the embryo (which allows live cell imaging) are beneficial aspects when studying multiple developmental processes. Zebrafish embryos kept at 28.5 °C hatch between 48 and 72 hpf, when they become free-swimming larvae with a complex behavioural repertoire (Colwill and Creton, 2011). In relation to chemical mixtures, the study from Martin et al. (2021b) highlighted the possibility of detecting synergistic effects with zebrafish embryos. Moreover, Jakobs et al. (2020) compared measured toxicity with predicted toxicity of nine mixtures in zebrafish embryos to investigate the applicability of the CA and IA models to predict phenotypic effects in a whole organism through a short-term acute assay, and also suggested the importance of using *in vivo* bioassays to confirm the predictivity of models such as the CA.

However, it has to be considered that the use of embryos implies some limitations as well: before hatching, the embryo is confined within the chorion, a relatively impermeable membrane that might act as a barrier to the entry of some compounds (Pelka et al., 2017), although not so frequently (Gustafson et al., 2012). For this reason, in some studies the chorion is forcefully removed before the exposure (Rericha et al., 2021; Roegner et al., 2019). Moreover, the behavioural patterns of the younger stages might be not so sophisticated when compared to the adults (Palmer et al., 2017). This might at least partially explain why, despite the large number of papers focused on short or very short exposures (less than five days, Fig. 3), roughly the 60% of the studies exceed the 5 dpf threshold. Moreover, we did not find a consistent increase in the number of papers relying on the use of embryos within 5 dpf following the year 2013, when the OECD 236 was released (Fig. 4).

Finally, there is a wide variety of different behaviours that the zebrafish exhibit at every developmental stage (Kalueff et al., 2013), therefore it is not surprising that several different assays were developed throughout the years based on different behaviours and senses (Ahmad et al., 2012). This emerges quite clearly also in our review, where several different assays were considered. Among the behavioural endpoints, the most frequently considered is the swimming activity in response to different light conditions (light compared to dark or light/dark switch), followed by the free-swimming activity, that considers the swimming patterns in the absence of external stimuli (Fig. 5). It has to be noted that the choice of the assay to apply can be influenced by the developmental stage of the organism and vice versa: not all behaviours are in fact observed at all stages. The thigmotaxis and the acoustic startle response for example appear around 5 dpf (Schnorr et al., 2012; Best et al., 2008), while the tactile and the visual startle response appear earlier, at 2 and 3 dpf respectively (Colwill and Creton, 2011; Basnet et al., 2019). The coiling emerges at around 17 hpf but only last a few hours (McKeown et al., 2009), while social behaviours, such as shoaling tend to develop and gain complexity in juveniles and adults (Orger and de Polavieja, 2017). Among the assays we surveyed the photomotor response was the most widely used, and this is not surprising given the high sensibility of the zebrafish to light changes and conditions (Orger and de Polavieja, 2017). Very common are also the assays based on different forms of swimming activity, and this might be explained by the possibility to study this behaviour for almost every developmental stage, since it firstly clearly appears at around 4 dpf (Hernandez et al., 2018).

In the environmental context, neurotoxicity and the abnormal behaviours often highlighted in the papers we surveyed may have tangible consequences on fish populations. Behavioural alterations in swimming activity and avoidance may result in less food consumption, with negative consequences on reproduction and growth (Kar and Senthilkumaran, 2024) also leading to health decline and vulnerability to infections (Miranda et al., 2019). The consequent decline in fish populations may affect biodiversity and environmental health, but also potentially human health, through the reduction of ecosystem services and food availability (Barboza et al., 2018).

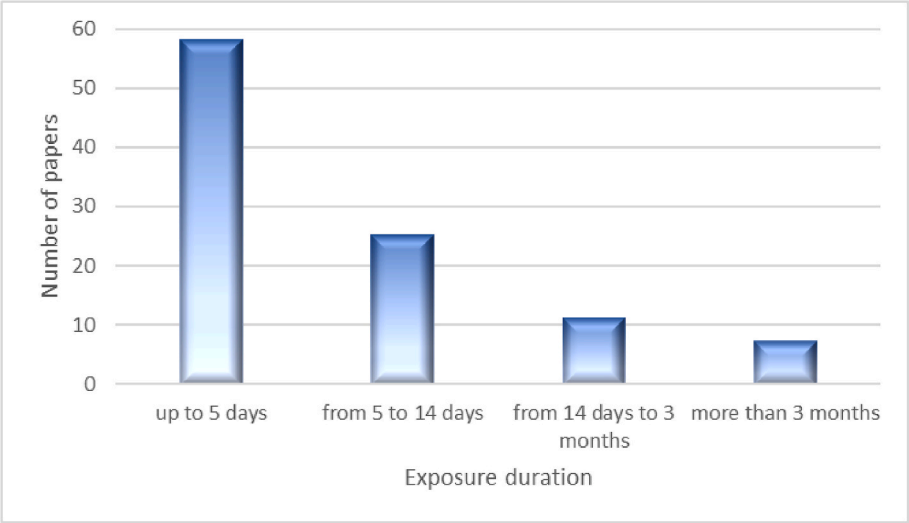

Fig. 3. Categorisation according to exposure duration. The papers selected for this review are categorized according to the duration of the exposure of zebrafish to the chemical mixture. The most prevalent exposure window is "up to 5 d" (58 papers). (for color reproduction on the Web)

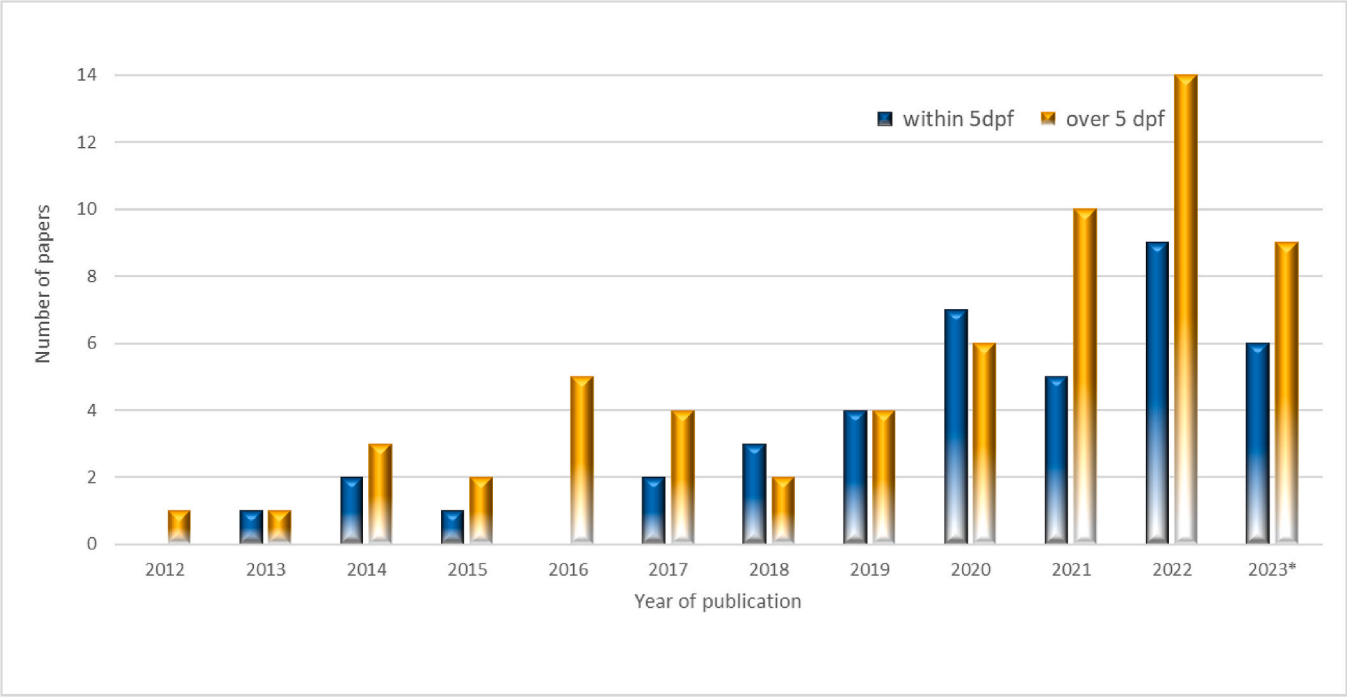

Fig. 4. Categorisation based on year of publication and duration of exposure. The chart shows the number of works in which exposure to chemical mixtures was carried out within (blue bars) or over (orange bars) 5 dpf, between 2012 and September 2023 (for color reproduction on the Web)

5. Conclusions

Results of this review allow to elaborate some considerations that could also be useful for future studies:

- Neuroactive chemicals are commonly present in the ecosystem in simple and complex mixtures as a result of different human activities. Therefore, the application of EBMs based on behavioural analysis is useful to improve the knowledge of the neurotoxic effects of mixtures in the environment.
- The use of additional parameters at a molecular and cellular level as a complement to the behavioural analysis is a key aspect for a better understanding of the effects of neurotoxic mixtures.
- Future mixture studies should also include a clear description and evaluation of the possible synergistic, antagonistic and additive effects detected.
- Given the wide variety of different endpoints and approaches for behavioural analysis, the necessity emerges to develop more standardized and shared methods for neurotoxicity assessment that could improve the replicability and reproducibility.
- In the future, prioritization of relevant neurotoxic mixtures could provide a useful contribution to improve legislation and in the application of safety measures.

In conclusion, zebrafish proves to be a very sensitive model organism even at low concentrations of neurotoxic compounds and, as emerged

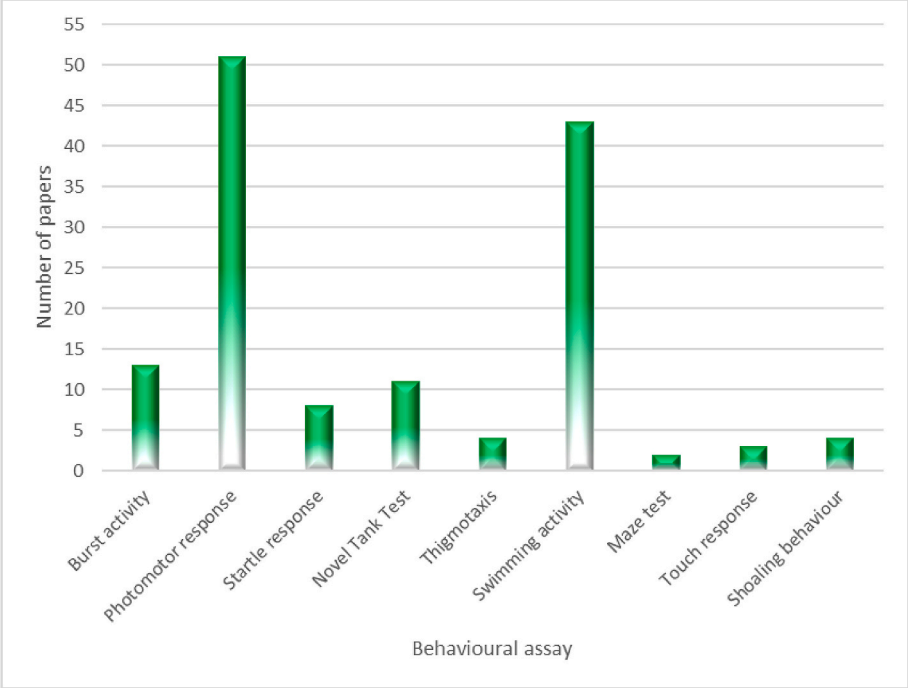

Fig. 5. Categorisation of the considered papers based on the selected behavioural assays/endpoints. Among the assays/endpoints considered, swimming activity without stimuli (swimming activity) and swimming activity under different light conditions (photomotor response) were the most widely applied (for color reproduction on the Web)

from our review, there is evidence of a growing interest on this topic. Finally, the use of zebrafish embryos, as highlighted in several studies, represents an important step for the implementation of the "3R" principle on animal well-being that should be applied whenever possible.

CRediT authorship contribution statement

Kevin di Domenico: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Ines Lacchetti:** Formal analysis, Conceptualization. **Giulia Cafiero:** Writing – original draft, Investigation, Conceptualization. **Aurora Mancini:** Investigation. **Mario Carere:** Writing – review & editing, Methodology. **Laura Mancini:** Supervision.

Declaration of competing interest

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

Data availability

I have shared the link to my data at the attach file step

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142246.

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