SAPIENZA UNIVERSITY OF ROME
Department of Public Health and Infectious Disease

Doctorate Course in Infectious Diseases, Microbiology and Public Health

Cycle XXXVI

EVALUATION OF THE ECOTOXICITY INDUCED BY VETERINARY PHARMACEUTICALS AND ENVIRONMENTAL SAMPLES IN THE AQUATIC VERTEBRATE *DANIO RERIO* THROUGH A ONE HEALTH APPROACH

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2020-2024

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SHORT SUMMARY

The aim of this thesis is to deepen the knowledge on the application of zebrafish, and in particular zebrafish embryos as a model for the detection of the effects induced by mixtures of chemical pollutants using sublethal endpoints. This work was structured on three different phases. To investigate the actual knowledge on the subject and to highlight possible gaps and future development, a scoping review was elaborated with a focus on the use of zebrafish for chemical-induced neurotoxicity identification with sublethal behavioural endpoints.

Then zebrafish embryos were employed for the detection of acute toxicity of known substances (veterinary pharmaceuticals) and environmental samples obtained from the Tiber river after extreme weather events that were cause of fish-kills in Rome. In both cases the Fish Embryo Toxicity test based on mortality was applied, but was also supported by sublethal endpoints. In the activity on the river samples however, the aim was to investigate the possible causes of the fish-kill and to test the use of sublethal endpoints for the detection of the effects of complex mixtures such as those that can be found in a heavily impacted river. In the experimental activity on the known substances a new assay aimed at the detection of neurotoxicity, the CAT, was applied, as well as others sublethal endpoints.

In conclusion, as highlighted in our review the model Zebrafish is knowing growing popularity in the evaluation of the effects of chemical pollutants in mixtures, especially through the use of sublethal endpoints, such as behavioural alterations. Sublethal effects are also a valid tool, especially in early warning systems. Finally, the CAT is a simple and quick method in the assessment of neurotoxicity that could be a valuable addition in the regulatory guidelines for identify the risks linked to neurotoxicity in the ecosystem.

Keywords: neurotoxicity; Zebrafish; environmental pollution; Effect Based Methods

1. Introduction

The term “One Health” (OH) refers to an olistic approach that involves several fields and disciplines recognizing the close relationship between human, animal and environmental health. The concept itself is not new, and evolves from the concept of “One Medicine”, that recognizes the close connection between human and veterinary medicine. Although there is no univocal definition for this concept, throughout the years many notable attempts have been made to give it a clear connotation: it was defined by the One Health Commission and the US Centers for Disease Control and Prevention as “A collaborative, multisectoral, and transdisciplinary approach—working at the local, regional, national, and global levels—with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment", while the the One Health Global Network define that "One Health recognizes that the health of humans, animals and ecosystems are interconnected. It involves applying a coordinated, collaborative, multidisciplinary and cross-sectoral approach to address potential or existing risks that originate at the animal-human-ecosystems interface". The OH concept focuses not only on the relationship between human, animal and environmental health (Figure 1), but also recognizes the importance of a complex and multidisciplinary approach where several sectors work togheter to achieve a common result in a world where several factors, like the increasing human population, an higher livestock demand, a rising urbanization and increasingly deteriorated ecosystems produce increasingly complex scenarios. The term OH was firstly used during the SARS outbreak in 2003 and from that moment it gained rising popularity, at first as an integrated approach in zoonoses management and prevention, and later as as an approach more broadly focused on several other areas, still relevant to human health such as food safety, antimicrobial resistance, climate changes, wildlife conservation, ecosystem health and biodiversity (1).

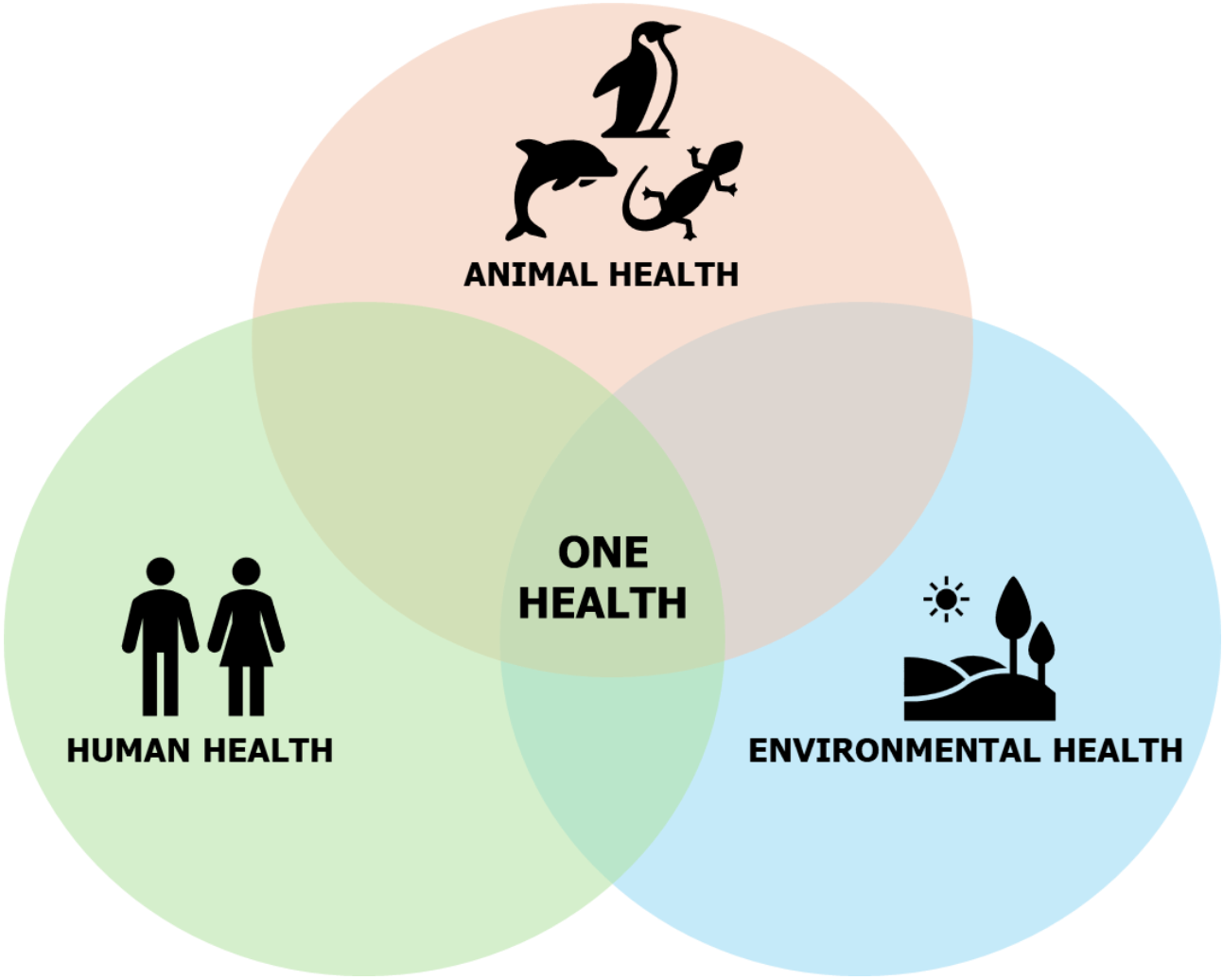

Figure 1: the concept of One Health involves the close relationship between human, animal and environmental health.

Ecosystems relevance to human health is well-known, since they provide services defined in the Millennium Ecosystem Assessment as "the benefits people obtain from ecosystems" (2). The ecosystem services, categorized by the MA in four groups (provisioning, regulating, cultural and supporting) are involved in many aspects of the human life, like basic necessities, diseases prevention and care, mental health, heat and pollution mitigation (3-6). In this context biodiversity, an essential component in a healthy ecosystem, is linked to these services on many levels, and it can affect the way in which such services are delivered (7). Biodiversity seems to prevent the expansion of some diseases, many of which can be transmitted to humans. According to the "dilution effect" hypothesis, to a significant number of vertebrate species in the ecosystem corresponds a lower risk of disease transmission to humans, since the higher number of possible hosts lower the overall prevalence (8). Consequently, the conservation of biodiversity could be essential to prevent the expansion of such diseases (9,10).

Also, many studies observed how the exposure to various natural environments is necessary to develop an adequate immune response to allergens and it is therefore possible that the loss of microbial species may lead to immune dysfunctions in some cases (11,12). Moreover, there is growing evidence that contact with multiple natural environments would have a positive influence on human health (13). Biodiversity decline could have consequences on the growing prevalence of allergies, asthma and other inflammatory diseases, especially in the urban population, where a correlation has been observed between atopy and biodiversity (intended as environmental biodiversity and as macrobiota biodiversity) (11). All the components of biodiversity can play a role in generating ecosystem services, but the value of each element may vary and be difficult to describe. The processes that take place inside an ecosystem depend on the number of species and their characteristics; the ability of the ecosystem to react to external stimuli, and the importance of biodiversity in this kind of response is hugely variable and related to the found species present (14). Ultimately, biodiversity is essential to sustain the processes and the functions of the ecosystems that provide the services on which human health depends, and ecosystems subject to stress provide less services, with negative consequences on human well-being (1,7,14-17).

Partly due to human interference, the planet biodiversity is undergoing a substantial decline at an alarming rate (18). Human activities like agriculture (19, 20), and the introduction of exotic species, can be harmful to biodiversity, with consequences often more serious on terrestrial ecosystems compared to the aquatic ones (21). Over the last centuries the disappearance of a growing number of species has been observed, even though it is difficult to quantify this phenomenon due to the fact that species not yet considered extinct can still be extinct from their original environment (22). Even though the extinction rate tends to be different among vertebrate groups, a significant increase has been observed in the last 200 years; moreover, some authors suggested that the number of vertebrate species disappeared in the last 100 years would have taken about 800 to 10000 years to went extinct considering a background rate of mammal extinctions of 10,000 species per 100 years (23). According to the International Union for the Conservation of Nature (IUCN) around 27% of the over 100000 species reviewed results to be at risk of extinction. The number of species on the planet is therefore rapidly decreasing, and even genetic diversity has declined sharply, especially between cultivated species (2). In many countries the portion of the territory adapted to agricultural use is constantly increasing (Reaping the Benefits, Royal Society, London, 2009) and, similarly, there has been an increase of deforestation in the tropical region of the planet, leading to the loss of a huge amount of forests all over the planet (24). The biodiversity on the planet is increasingly more

isolated between urban and cultivated areas (25). In this process some species seem to be less favoured than others, as they have a larger size, a slower reproductive cycle, and a less efficient reproductive strategy (14).

As a result, more than 60% of the ecosystem services of the planet have been eroded in the last half century, and barely the 11% of protected ecosystems are today in a favourable state (26).

One of the biggest obstacles and causes of concern to both human and environmental health is the presence and release in the environment of chemical pollutants. The production and use of chemical products is growing all over the world, with global sales estimated in 3347 billion euros in 2018. As a consequence, every year a huge amount of chemical waste is released into the environment as a result of human activities such as farming, mining, transport, energy production and many others (27). In addiction to all pre-existing chemicals, every year thousands of new substances are synthetised all over the world, and many of this compounds may have some kind of toxic effect alone or when in mixture with other chemicals. This process, started with the Industrial Revolution, has became more and more widespread and severe over the last decades to the point that chemical pollutants have been found even in the most remote regions of the planet and in the highest layers of the atmosphere (28), and toxic chemicals are commonly found in homes, foodstuff and workplaces, to the point that has been estimated that more than nine million people die each year due to some form of chemical contamination (29).

1.1 Water Framework Directive

For this reason, since the year 2000 in the European Union became effective the legal act for water protection known as the European Water Framework Directive (WFD) or Directive 2000/60/EC, that aims at achieve the “good ecological and chemical status” of the waterbodies within the Union by 2027. The Directive foresees the drafting of new laws and regulations and requires their implementation within member states.

The definition of the good status is based on four assessments:

- chemical status of ground water: good status is achieved when the concentrations of chemical pollutants considered relevant by the European legislation do not exceed the regulatory limits imposed by the Directive and there are no negative consequences on the ecosystems and the surface waterbodies that rely on the groundwater basin

- quantitative status of ground water: a good status is achieved when, considering the annual rate of abstraction, there are no negative consequences on the ecosystems and the surface waterbodies that rely on the groundwater basin
- ecological status of surface waters: it refers to the health of the ecosystems, defined through the status of phytobenthos, phytoplankton, macrophytes, invertebrate fauna and fish
- chemical status of surface waters: is defined as the compliance to the Environmetal Quality Standards (EQSs) of a list of priority chemical pollutants identified in the Directive as relevant for both human and environmental health.

The directive applies to transitional, coastal, inland and groundwaters, and implies the maintenance of the already clean waters and the restoration of polluted waters. The good ecological and chemical status has to be achieved through an integrated approach shared by all member states based on agreed upon meauseres such as River Basin Management Plans (RBMPs) and Programmes of Measures (PoMs). In order to achieve and maintain a good water quality in all waterbodies, a Common Implementation Strategy (CIS), applied to all member states, has been developed with the aim of having a common set of rules, guidelines and management plans among stakeholders. In Italy the Directive has been integrated within the Legslative Decree n 172/2015.

1.2 Good chemical status

The assessment of the ecological and chemical status is therefore necessary to define the condition of a waterbody and chemical contamination is taken into account in the evaluation of both parameters. The achievement of the good chemical status requires the compliance with the EQSs defined within the Directive. The EQSs are limit concentrations set with the aim of protecting the most sensitive aquatic organisms as well as human health that should not be exceeded under any circumstance. The EQSs are defined as annual average (AA) and maximum allowable concentration (MAC): the AA takes into account a one-year timeframe to ensure the long term quality of the waterbodies. The MAC is defined as the maximum concentration that should never be exceeded, even for a short term period. The good chemical status is defined as the ratio between the concentration of a chemical compound found in the environment and its EQS. A value below one implies good chemical status.

EQSs are defined for water, sediment and biota, following the procedure described in the TGD CIS guidance 27 and are currently applied to a list of 45 priority substances harmonized within the EU

Member States. The list includes substances of wide concern such as pesticides, PCBs and heavy metals. Moreover, the Directive allows single states to eleborate an additional list of “river basin specific pollutants” based on specific needs. In this case the EQSs are defined nationally. This way pollutants of interest can be managed locally using the Directive legal framework. The list of priority substances can be furtherly expanded every six years with the addition of new compounds of concern, usually incorporated from the Watch List introduced by the Directive 2013/39/EU as a new tool to gain informations in chemical risk assessment on chemical substances in surface waters. The list, includes potentially harmful chemical substances on which data are lacking. Such substances are monitored for a four-year period to establish the potential risk for human and environmental health. To mantain reasonable costs only a limited number of substances is included in the list, and every four years an update ensures that all the screened substances are promptly removed while new ones are added.

In 2020, as a new long-term policy aimed at speeding up the transition toward safer chemicals, the Chemicals Strategy for Sustainability was presented by the European Commision as a follow-up to the policies already implemented within the Union. This document adresses some of the weaknesses of the current framework, with the aim of improving the EU approach in terms of management of chemical products. The policy aims to implement the “generic approach to risk management” within the union, banning from consumer products all carcinogenic substances, and improving Environmental Risk Assessment procedures to better counter the issue of chemicals in the environment. Also, the new strategy places emphasys on the issue posed by chemical mixtures in the environment.

The thousands of chemical substances currently released in the aquatic ecosystems can in fact form mixtures with potential additive or synergistic interactions, that might amplify their individual toxicity (30) with complex effects that might not be predictable through chemical analysis (31). Recently, in the European Commission proposal for the directive on priority substances in the aquatic environments (32), Effect Based Methods (EBMs) were included for the first time. The very definition of the Environmental Quality Standard is also modified, including trigger values based on the use of EBMs and aimed at protecting both the aquatic life and human health. This preliminary proposal should also be broadened by taking into account other effects in the aquatic environment such as genotoxicity and neurotoxicity. EBMs include *in vitro* and *in vivo* bioassays suitable for the evaluation of short-term and long-term effects of the present and future priority relevant

substances, even in mixtures (31). A 2019 work from Brack et al. addresses EBMs as an useful tool for the detection of chemical mixture toxicity, and were recently highlighted as a relevant complement to chemical analysis (32).

1.3 Pharmaceuticals in the environment

Pharmaceutical products are commonly part of the everyday life, and are an essential component of our health. This large class of chemicals comprehends thousands of different products that can be classified according to their chemical structure (usually within subgroups of pharmaceuticals), their activity or their Mode of Action (33).

It is now commonly aknowledged that the release of pharmaceuticals in the environment can be a serious source of pollution, causing serious risks for both environmental and human health. Also, it has been estimated that the consumption of pharmaceutical products is destined to increase in the near future due to several different factors, such as the aging trend that is observed in the population of many western countries. The presence of pharmaceutical residues in the aquatic environment and in drinking water in particular has been cause of concern for several decades now, and for this reason risk assessment and risk management activities are becoming more common in the last years.

All the components of a pharmaceutical product (Active Pharmaceutical Ingredient, excipients and additives) might be able to enter the environment through different routes, such as Sewage Treatment Plants (STPs) and landfill effluents, household waste and manufacturing plants. Some compounds in particular tend to be active at very low concentrations, and for this reason they are considered of particular interest. Among this group anticancer treatment (34-36), endocrineactive pharmaceuticals (37-39) and antibiotics can be found. Antibiotics in particular may also cause antibiotic resistance in the environment. In both humans and animals pharmaceutical products usually undergo structural modifications, and in the end only a part of the total amount of product released in the environment is expelled as the parent compound, while the rest is exctreted as metabolites, that can have an effect on the environent themeselves. When released in the environment, both parent compounds and metabolites can be further modified by several different biotic and abiotic processes, such as bacteria (40) and light, or even processes aimed at the treatment of pollluted waters, such as hydrolysis and photolysis (41).

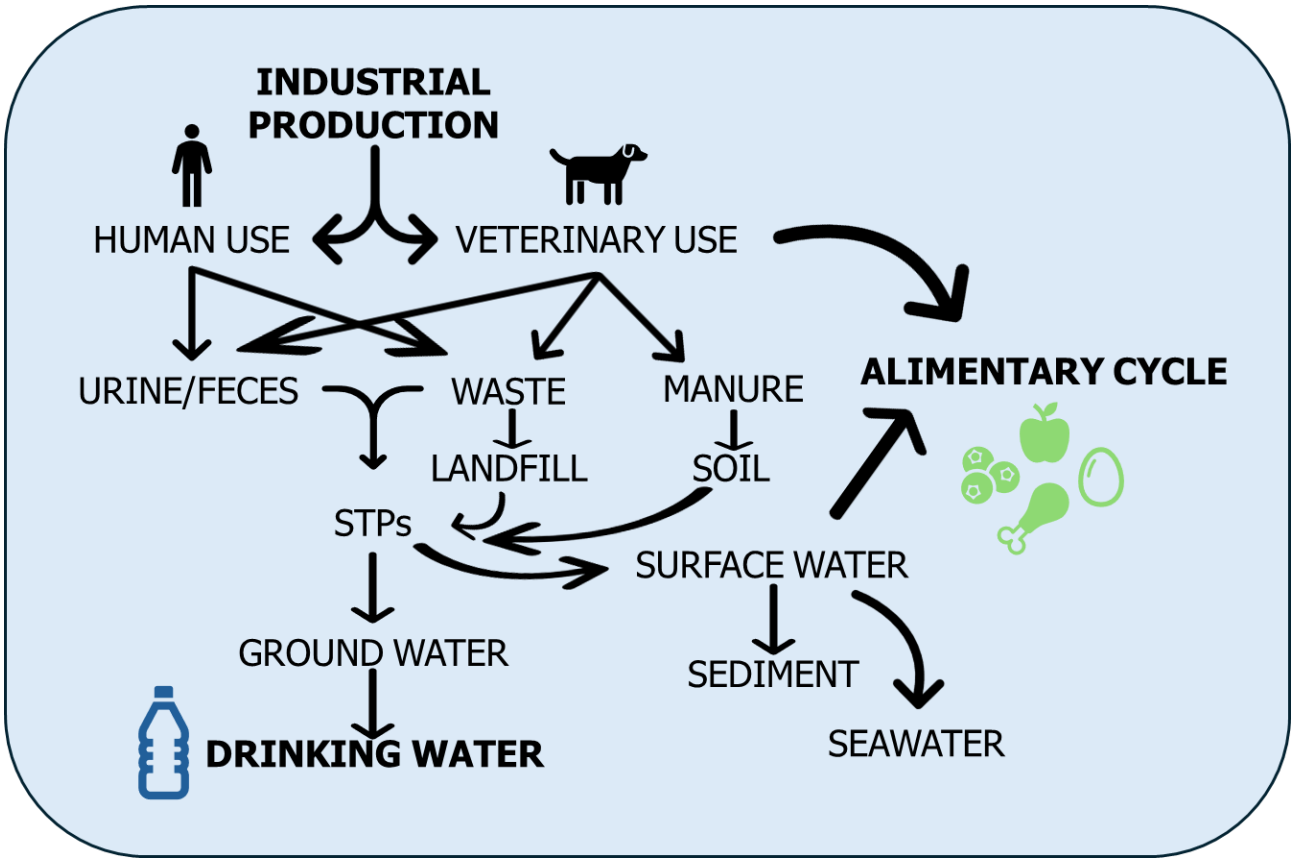

Figure 2: pharmaceuticals can enter the environment through numerous routes.

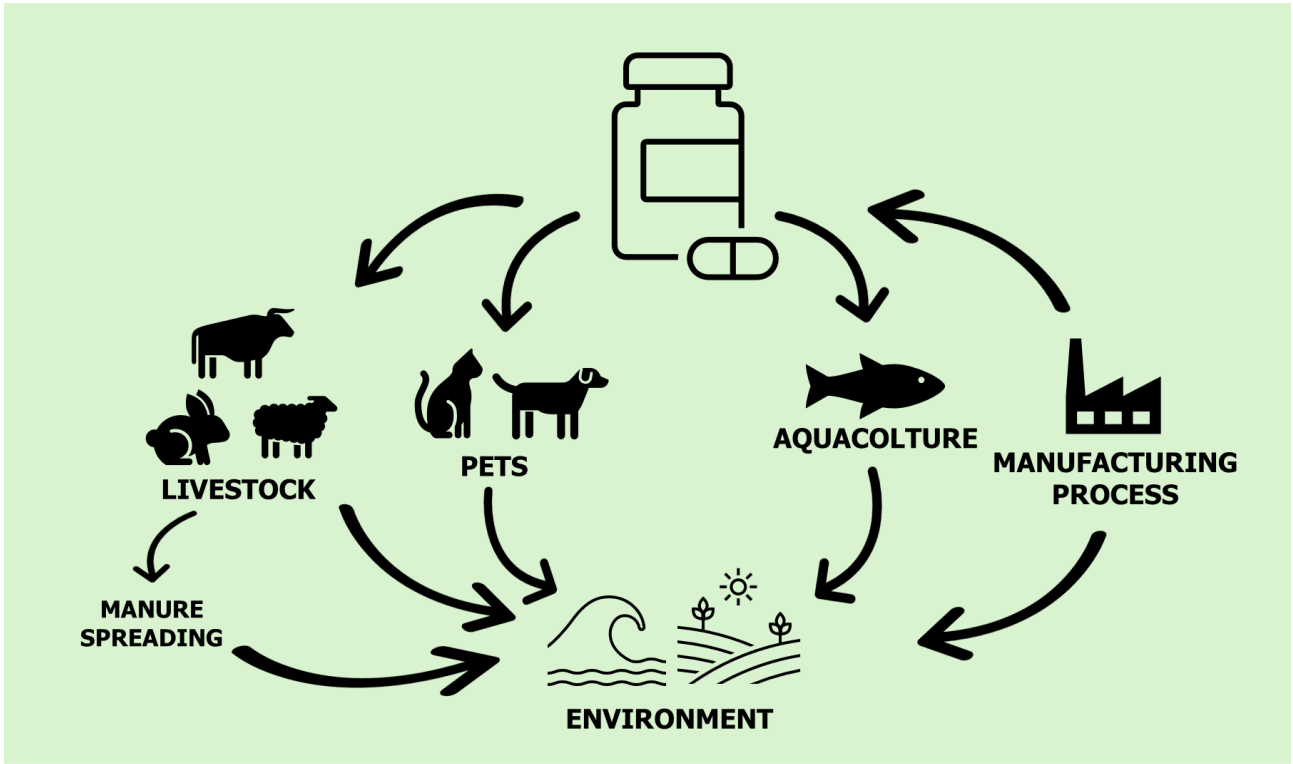

Figure 3: The different applications for veterinary pharmaceuticals that cause their release into the environment.

1.4 Effect Based Methods

Effect Based Methods (EBMs) can be used for the detection of chemical toxicity in the environment through the use of single cells, tissues or whole organisms. The effect can be measured in many different ways, such as an alteration of in the expression of a protein or a gene, an alteration in the integrity of the membrane of a cell or, on a higher level, as an aggregated effect (lethality, reproduction, growth, behavior).

EBMs can be categorized as:

- ***in vitro*** and ***in vivo*** **bioassays**, which measure the toxicity of the environmental sample under controlled laboratory conditions

- **biomarkers,** used to measure the effects of pollution directly on the field

In particular, bioassays are currently applied as part of the Environmental Risk Assessment of several potentially azhardous products, such as pesticides, veterinary pharmaceuticals and biocides. EBMs are also often included in environmental monitoring programs, and they have been used for long to assess effluents (WEA, Whole Effluent Assessments) containing complex mixtures. EBMs are applied in sediment monitoring programmes and in the assessment of the risk posed by hazardous waste. In Italy EBMs are also applied in the evaluations that, in the process that preceed the construction of a potentially impactful activity (such as an incineration plant, an industrial facility or a port), are aimed at estimating the consequence on human and environmental health. EBMs will allow to evaluate the effect in the environment of multiple chemicals on different organisms.

In vivo bioassays are based on the exposure of a whole organism or a population to a sample or a known substance in order to determine short- and long-term toxicity (acute and chronic assays respectively). These assays allow to test the effects of numerous substances with different characteristics, but a chemical should preferebly be tested on several organisms, since different species can show different sensibility towards the same compound. Also, both acute and chronic assays should be performed since the toxicity of a pollutant or a mixture might not be easily detectable in the short-term (a chronic assay covers at least the 50% of the lifespan of the organism). Many of these tests are already regulated at the international level, like those that are defined by the OECD guidelines. These tests are usually performed in laboratory conditions and can take into

account several different endpoints, such as mortality, immobilization, effects on reproduction, effects on growth, molecular/biochemical responses and behavioural changes, and they allow to highlight the Mode of Action (MoA) of the tested substances.

This term refers to the changes that may occur after the exposure to a chemical compound or a mixture at various level of organization, from cellular level up to anatomycal level. The understanding of the MoA can lead to the identification of the target (e.g. biological receptor) of a substance and extrapolation to anticipated effects or biological responses. By taking into account the MoA of a chemical informations on the toxicity of the substance can be collected, and this also applies to mixtures of pollutants. Despite the lacking of harmonization between different networks, to date various classification schemes have been developed to categorize chemical toxicity based on the MoA evaluation, and this tool will be useful to improve chemical risk assessment procedures in the near future (42).

1.5 Zebrafish

Among the organisms commonly used for *in vivo* bioassays there is growing interest for the Zebrafish (*Danio rerio*), a freshwater teleosts that gained popularity in several research fields in the last decades (43).

This fish is native to South Asia, and is also among the most commonly sold aquarium fish. Their ideal habitat is found in shallow ponds, canals and streams, with still or slow-flowing waters. They feed on a wide range of organisms such as insects, worms and zooplankton. This species tends to live in social groups composed of a varying number of individuals, depending on the environment and mainly on the water flow of their habitat. In still or slower waters they are usually found in smaller groups of 7–10 individuals, while in fast-flowing rivers, they can be found in larger groups of up to 300 fish. They tend to prefer warm (24–35°C) and slow-flowing waters, slightly alkalin (pH 7–8), with high transparency and good plant coverage (4, 5). Zebrafish are asynchronous, batch spawners. Their reproduction depends on food availability and is positively correlated with increased rainfall. Also, olfactory signals play an essential role in zebrafish reproduction: the release in the environment of glucuronic steroids, released by males, induces ovulation in females. After ovulation, females release other hormones, which in turn induce the release of gametes by males

Adults measure about 3-6 cm in length (Figure 4). Their body is elongated, the head is compressed dorsally and the mouth turned slightly upwards. Their color is usually silver, with five lateral blue stripes starting behind the head and reaching the tail.

Figure 4: Zebrafish adults

The average zebrafish lifespan is of three and a half years, and they show the period of maximum fertitility between three months and one year of age. Adult males are distinguishable from females by the reddish tint along the silver longitudinal lines on their body. Females tend to be larger in size with a rounded belly when they are ready for eggs deposition, while male Zebrafish are often more slender with a golden color on their belly. The life-cycle of *Danio rerio* completes within three months.

Fertile females spawns between 50 to 200 eggs daily. Fertilization is planktonic and eggs are completely transparent and telolecitic. The segmentation starts 40 minutes after fertilization when the first cleavage occurs.

Seven broad periods of embryogenesis could be identified:

1- zygote
2- cleavage
3- blastula
4- gastrula
5- segmentation
6- pharyngula
7- hatching

After the first cleavage the cells, or blastomeres, undergo a series of meroblastic divisions of the cytoplasm at about 15-minute intervals. During the divisions, blastomeres remain interconnected by cytoplasmic bridges. This may take between 40 minutes and 2 hours (stage of 64 cells), after which eggs enter the blastula period (128 cell stage). Following the gastrula period within the primary germ layers and the embryonic axis production, occurs the segmentation period (10 – 24 h) characterized by the development of somites, the sketch of the primary organs and the elongation of the embryos with tail buds. In the next stages (pharyngula period, 24 h - 48 h) the embryos mature typical features of vertebrates, with evident bilateral symmetry, a well-developed notochord and complete somites that extend parallel to the post-anal tail.

Hatching period, 48 h – 72 h, is the last developmental stage, characterized by a steadily growth of the embryos. Morphogenesis of the primary organs is rather complete, and the yolk shrinks proportionally to the growth of the head. At the end of this period, the mouth of the embryos is wide open and protrudes anteriorly, the pectoral fins continue to expand and, as the gill filament develop, circulation in the pharyngeal arch region become more complex and the primordium of the operculum extends posteriorly to cover the branchial arch. By day 3 the hatched larva has completed most of its morphogenesis, and it continues to grow rapidly. Prominent changes during the next day include the inflation of the swim bladder and the continued anterior-dorsal protrusion of the mouth. The gut tube drops more ventrally and the yolk extension nearly empties. Whereas during the hatching period the embryo is usually at rest, the early larva gradually begins to swim about actively and moves its jaws, opercular flaps, pectoral fins, and eyes. These developments produce swift escape responses and herald respiration, the seeking of prey, and feeding.

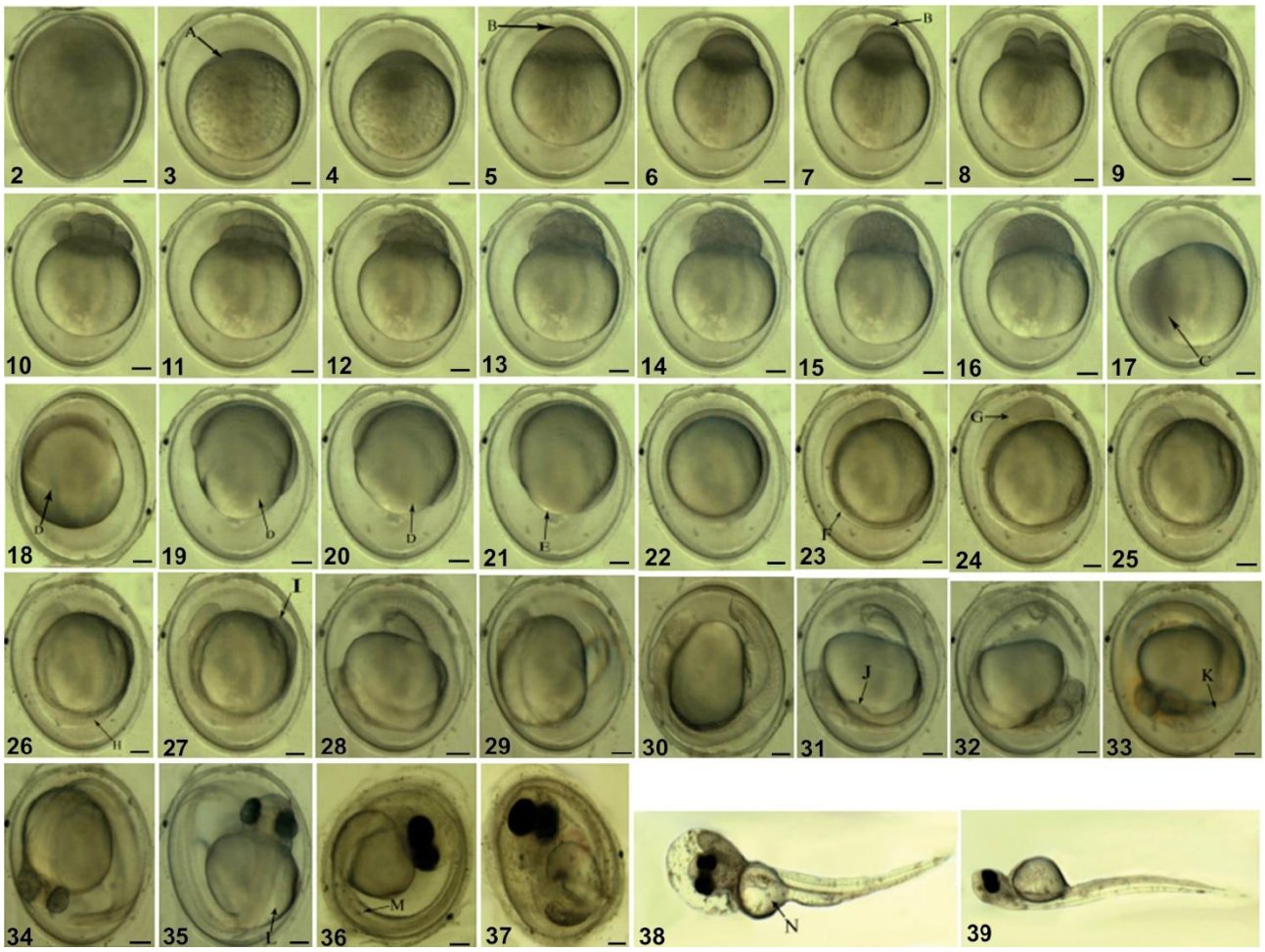

Figure 5: Zebrafish development from egg to larvae

Its success in the biomedical field depends on several advantages respect to other organisms, such as the low maintenance costs and the small size; it can also easily be kept in captivity and is easy to breed (44). Moreover the zebrafish genome is completely sequenced (43) and has orthologues of more than 71% of human genes (45), and 84% of human disease-related genes have a zebrafish counterpart (46). For all these reasons, the zebrafish has been successfully employed for various applications, such as cancer research, drug toxicity assessment (47), and developmental toxicity (48).

A recent report from the Joint Research Center (JRC) summarize the MoAs of the chemicals included in the Priority Substances list of the WFD, as well as other chemicals of interest, outlining the link between EBMs and MoAs whenever possible. The report identified several MoAs such as stress proteins, endocrine disruption, photosynthesis inhibition, oxidative stress, activation of metabolising/detoxifying pathways, genotoxicity and histopathology. Among MoAs, neurotoxicity is considered one of the most commonly occuring in the environment, and it has been estimated that almost a third of all commercially currently used chemicals have neurotoxic potential (49).

In this field zebrafish is considered an useful tool for assessment (50), and there is evidence of numerous similarities at a functional level between mammals and zebrafish in several brain areas (51), and for this reason the use of zebrafish is becoming more common over time (52) even with assays employing zebrafish embryos. In recent years a growing use of zebrafish embryos in place of adults is taking place: the use of embryos further expands in fact the the potential of this model. The early stages have several advantages over the adults, since they are easier to manipulate, have a short time of generation and are transparent (53). Also, they show similar characteristics to mammals in some behaviours (54) and follow the Directive No. 2010/63/EU on the protection of animals used for scientific purposes. Among the endpoints proposed for embryos neurotoxicity there is the evaluation of the lateral tail movements that occur around 24 hours post-fertilization (55).

The aim of this work is to evaluate the potential application of zebrafish embryos in the assessment of the acute toxicity and neurotoxicity induced by chemical pollutants. With this aim, we started by elaborating a scoping review, currently in publication on Chemosphere, focused on the use of zebrafish for the detection on neurotoxicity induced by chemical mixtures. We chose to focus on this endpoint as it is one of the most commonly occurring Mode of Action in the environment. Then we evaluated sublethal endpoints applications in an environmental context, where the zebrafish embryos were also exposed to water samples taken from the Tiber river after weather events that caused a high mortality in the river's fish population (56). Finally, we also tested the effects of commonly used veterinary pharmaceuticals on zebrafish embryos. The future perspectives of this work include the evaluation of the joint toxicity of known chemicals in binary or more complex mixtures, to better understand possible additive, antagonistic or synergistic interaction. The experimental work on known substances involved the β-lactam antibiotic Ceftriaxone (CTX) and the antibiotic Amoxicillin, a beta-lactam aminopenicillin, two widely used veterinary pharmaceuticals that were tested on zebrafish embryos up to 96 hours post-fertilization to investigate acute toxicity and neurotoxicity.

2. Application studies

2.1 Review

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 that aimed to detect neurotoxicity with 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.

2.1.1 Search items

To perform comprehensive research, we selected four search fields to merge 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.

2.1.2 Screening process

For this stage we chose a screening method involving two reviewers operating independently on every work on 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. 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 that 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 the exposure, but all papers had to include the exposure to at least two chemicals at the same time (mixtures). Lastly, we did not selected papers based on specific chemical classes or types, and also

environmental samples or other complex preformed mixtures were considered acceptable, to the condition for the chemical composition to be 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 this further assessment were included in the study (Figure 6).

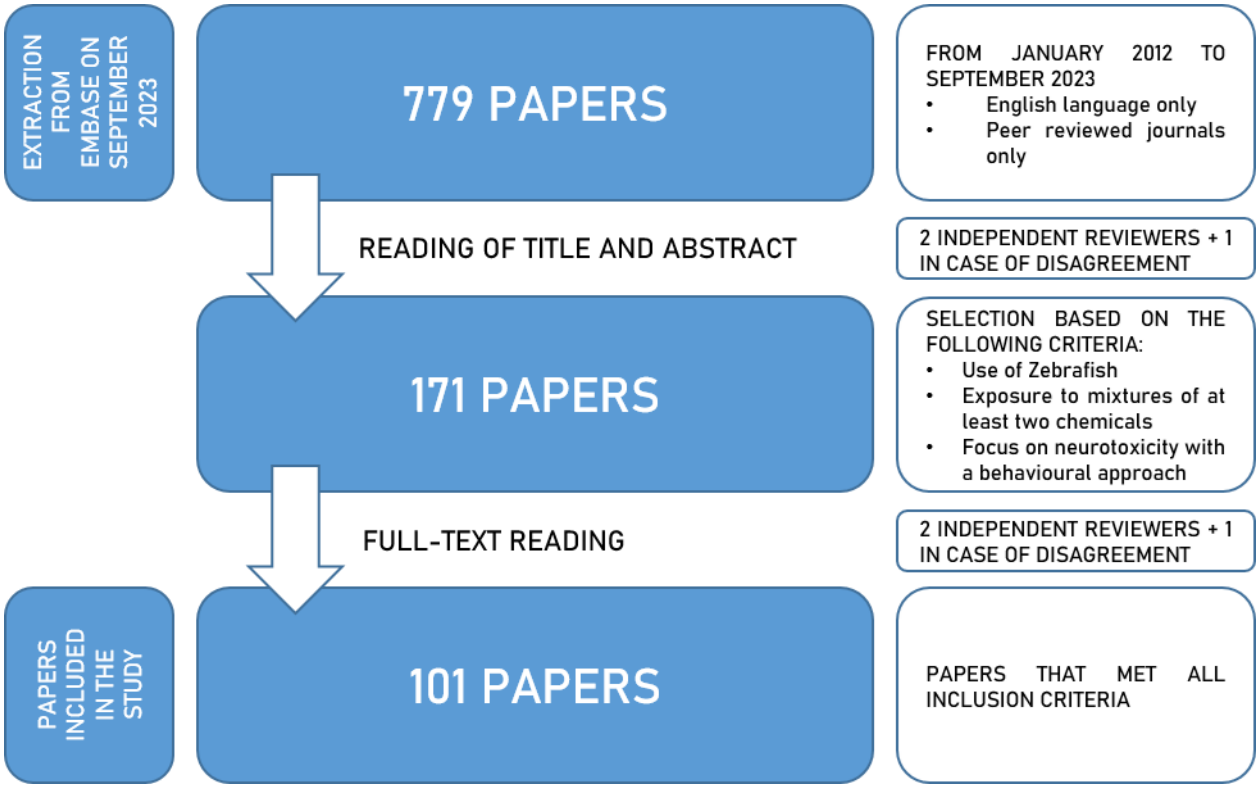

Figure 6: screening and inclusion process. The process started with a search on Embase followed by two screening phases operated by independently operating reviewers

2.2 Experimental activity

Two different short-term assays were carried out in the experimental phase of this thesis. The first method follows the guidelines of the OECD 236 on describing the Fish Embryo Toxicity (FET) test. This method was selected in order to verify the acute toxicity of the single substances at different concentrations and was also applied on the environmental samples. The other method, the Coiling Toxicity Test (CAT), was aimed at the detection of neurotoxicity alone and was based on a recently-

developed protocol described in a 2022 work from Lacchetti and colleagues (55). The eggs used for testing were collected from AB wild-type zebrafish.

2.3.1 OECD 236

For this assay, newly fertilised eggs are collected and then examined under a stereomicroscope. This allows to evaluate the developmental stage and to discard unfertilised and damaged eggs. When the eggs reach the 16/32 cells developmental stage they are placed inside the wells of 24-wells plates and exposed to the test solutions. In every well, a single egg and 2 ml of solution are placed for a total exposure of 96 hours. This assay also requires the set up of both a positive and a negative control. The negative control consists of a separated plate where eggs are exposed to the standard test water alone. Moreover, four wells inside every plate containing the test solutions are also filled with only standard test water as internal control. The positive control also requires an entire plate where single eggs are exposed to a 4mg/L solution of 3,4 Dichloanilyne. For the test to be considered valid, a mortality of at least 30% should be observed in the positive control at the end of the 96 hours exposure, while a survival rate of at least 90% should be observed in the negative control. Also, in order to guarantee throughout the test the ± 20% of the nominal chemical concentration required by the guideline, in this study a semi-static renewal apporach was applied, with the complete renewal of the test solutions every 24 hours.

For the duration of the test the plates have been kept in the dark at a stable temperature of 26±1°C. Every 24 hours, the embryos were examined to highlight the effects of the ongoing exposure. In accordance with the OECD guidelines, we considered the acute toxicity as lehality through the assessment of four endpoints: coagulation of the embryo (at 24 hpf), lack of somite formation (from 24 to 96 hpf), non-detachment of the tail (from 24 to 96 hpf) and lack of heartbeat (from 24 to 96 hpf). Also, sub-lethality endpoints such as deformation, late hatching and edemas have been considered. Each test was performed in three replicates for a total of 60 embryos per sample.

2.3.2 Coiling Toxicity Test

The method used in this thesis for the evaluation of neurotoxicity as an alteration of the coiling movement is based on the method described in a recently published work from Lacchetti et al., (55). Briefly, the first phase of this test requires the same procedures applied in the FET test for egg

collection and examination. Also, the exposure starts at the same developmental stage and the eggs are exposed in the same type 24-wells plates. In order to facilitate the video aquisition required for the evaluation of the neurotoxicity, however, three eggs are posed into every well and sorrounded by a plastic ring made with inert material (PTFE), aimed at keeping the eggs in the centre of the well. The ring has a internal diameter of 4 mm, an external diameter of 8 mm and a thickness of about 1.5 mm. For each concentation 18 embryos are exposed, and kept at 26±1°C in the dark.

For the negative control standard freshwater is used, while two different positive controls are required, one for hypoactivity (a 1% ethanol solution) and one for hyperactivity (a 4% ethanol solution). The observations are made at 23 hpf, when the coiling movement reach the maximum frequency, with tha aid of a digital camera. 1 minute-long videos are aquired for each group of three embryos. The plate is left under the microscope for a 5-minutes period before starting the video acquisition. Every time the plate is moved to a new well, a 30 second resting period is observed before starting the video aquisition.

Videos are then analysed with the DanioScope® software provided by Noldus that is based on changes in the grayscale values of the image. The software is able to detect the embryos and analyse their movements individually as independent subjects and at the same time. DanioScope® can evaluate three main parameters related to the CAT: Burst activity, Mean burst duration and Burstcount/minute.

2.4 Tiber River

2.4.1 Study Area and Sampling Site

The Tiber river rises from “Monte Fumaiolo,” in the Apennine Tosco-Emiliano, and has a water flow with a yearly average of 230 m^3/s. The river is 405 km long, it runs through four regions (Emilia-Romagna, Umbria,Tuscany, and Latium), and its last stretch flows through the city of Rome and to the Tyrrhenian Sea. The Tiber river has a catchment area that exceeds 10,000 km^2, and its water is used mainly for irrigation, recreational fishing, and hydroelectric production although future uses as drinking water sources are under evaluation. The river is affected by numerous anthropogenic activities that may deteriorate its water quality: agricultural, urban (including sewage treatment plants), and industrial (mainly small enterprises). Two water sampling campaigns were carried out in May 2020 and August 2021 at the same sites of the river, shortly after two extreme weather

events that took place in the city of Rome. Such events followed prolonged dry periods (Fig. 1) and resulted in floods and in the enlargement of the river. The site where the water sampling collection has been performed is located under Ponte Duca d'Aosta (Fig. 7) in the city of Rome. It is important to remark that the sampling site is included in the stretch where the fish-kills were observed. This site is also located downstream of the inlet of the Aniene river (the second river of the city). Ten liters of water samples (TB20-year 2020 and TB21-year 2021) were collected between 0 and 30 cm in depth in inert containers and were then filtered at 0.45 µm and stored for 1 month at 4°C in dark conditions at the Laboratory of Ecosystems and Health unit of the Italian Institute of Health (ISS). In the same stretch ARPA Lazio (Lazio Environmental Protection Agency) has detected physico-chemical parameters (ARPA Lazio, 2021).

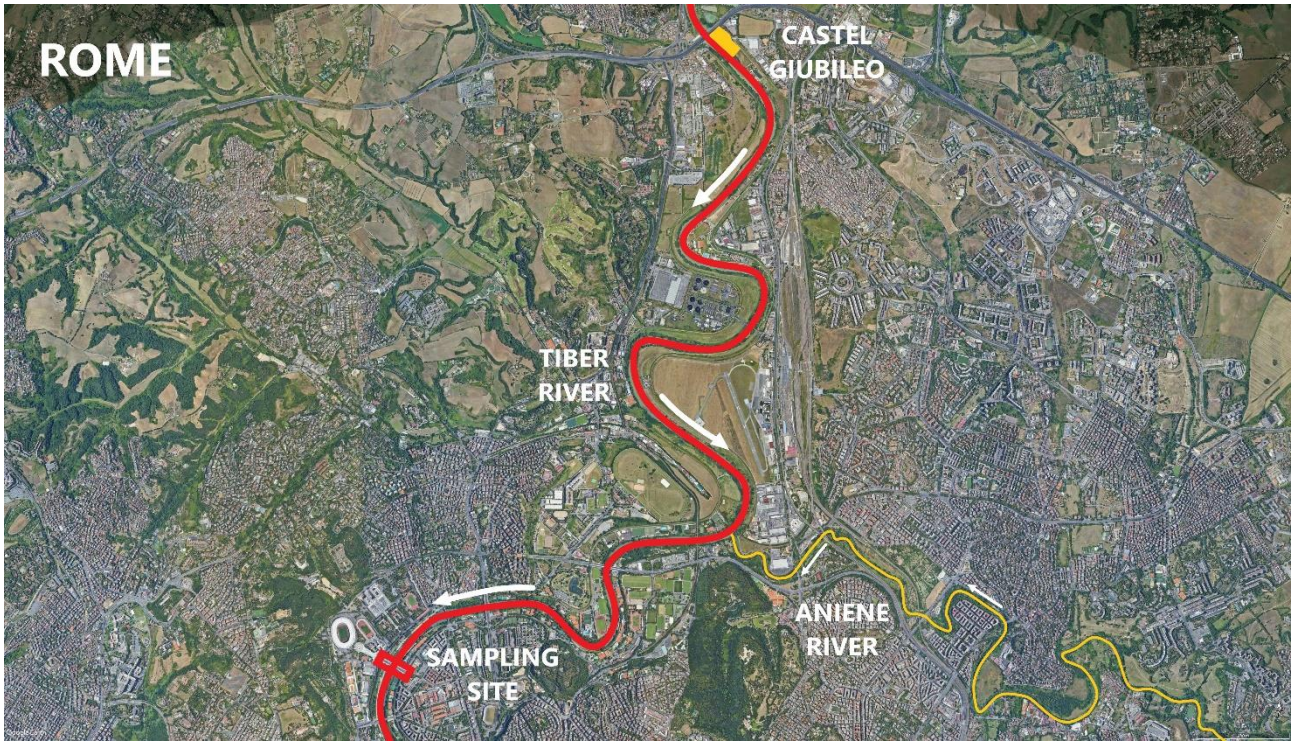

Fig. 7 Water sampling area, ponte Duca D'Aosta. Coordinates: (41° 55' 52.53" N and 12° 27' 38.16" E)

3. Results

3.1 Literature review results

The 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. Based on the differences between the papers we have collected, the works were divided into different categories based on the chemicals tested.

3.1.1 PFAS and PFAS-containing mixtures

Per- and Polyfluorinated Substances (PFAS) are chemicals with lipo- and hydrophobic properties, that accumulate in the environmental media and biota and their exposure has been linked to several human pathologies and developmental and behavioural disorders in model animals such as mice and fish (57). 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. (58) 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 of 46 PFAS (0.023 µM). 21 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 by Menger et al. (57), except for the swimming distance. Since the effects of the mixture were less severe than the single exposures, the study suggests that the compounds might have reduced their individual effect. In a series of studies by Khezri et al., (59), zebrafish embryos and adults were exposed to mixtures that included PFAS, Polychlorinated biphenyls (PCBs) and Brominated diphenyl ethers (BDEs) (60, 61). The concentrations ranged from 10 to 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 (61). In the 2021 study, the effects derived from the exposure were compared to the single exposure to PFOS. Zebrafish were exposed as larvae (6 – 96 hours post-fertilization, hpf) and their behaviour was tested after 7 months. Behavioural data coming from their offspring was also collected, with or without re-exposure. The results show lower swimming speed in the F0 adults while an higher speed was observed in the re-exposed F1 larvae. The third study (59) 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. (62) 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 on part of the compounds present in the mixture (perfluoroalkyl acids, Br, Cl) were significantly weaker than those of the whole POP 125x mixture, suggesting potential synergistic or additive effects resulting from other chemicals within the mix. Finally, a 2022 study (63) tested the multigenerational effects of PFOS and PFOA on three generations of zebrafish (F0, F1, F2).

Table 1: summary of the main parameters of the papers focused on PFAS and PFAS-containing mixtures

LIFE STAGE	EXPOSURE TIME*	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/larvae	48 h	POPs mixture	10x, 20x, 100x, 200x the mean human blood concentration	Swimming behaviour (total distance moved, average swimming speed and total time spent active)	↑Swimming speed	59
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 and the thigmotaxis assay (F1)	↑Swimming speed (F1 re-exposure) ↓Swimming speed in adults	60
Embryo/larvae	90 h	POPs mixture	10x, 70x the mean human blood concentration	Light/dark transition test and thigmotaxis test (cumulative distance travelled, time spent	↑Swimming speed	61

				active, mean swimming speed and total distance moved)		
Embryo/larvae	144 h	PFAS mixture	0.0001, 0.001, 0.01, 0.1, 1, 10, 30 mg/L	Swimming activity (144 hpf, as total swimming distance, burst swimming activity and startle response)	↑Startle response	57
Embryo/larvae	120h	PFAS mixture	0,27 µM	Embryonic photomotor response assay (24 hpf), larval photomotor response and larval startle response assays (120 hpf)	↑Larval photomotor response (lower concentration)	58
Embryo/larvae/ adult (trans-gen. study)	5 d	PFOS + PFOA	3.5 ng/L, 35 ng/L, 350 ng/L PFOA + 12 ng/L, 120 ng/L, 1200 ng/L 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	63

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	62

*Exposure time expressed in minutes (min), hours (h) days (d) or months (m)

3.1.2 Flame retardants/PCB mixtures

Polybrominated diphenyl ethers (PBDEs), hydroxylated BDEs and PCBs were produced in complex mixtures and used, due to their hydrophobicity and non-flammability, as flame retardants for several products including furniture, paper and PVC coatings. Between the 1980s and 2000s, PCBs and PBDEs production was progressively banned but they still persist and accumulate in the environment and their high levels in exposed fish and mammals pose a substantial health and ecotoxicological risk. In the study from Macaulay et al. (64), 10 days old zebrafish were exposed for 14 days to a mixture of DE-71 (30 µg/L), a commercial PentaPBDE mixture, tribromophenol (TBP, 4,9 µg/L) and 6-OH-BDE-47 (0,5 µg/L, 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. (65) and Lovato et al. (66), zebrafish embryos were exposed to environmentally relevant concentrations of Aroclor1254, a PCB mixture, for 24 hours 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. (67), 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 (68), 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 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 (69). Finally, a 2022 work from Zhu and colleagues (70) 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.

Table 2: summary of the main parameters of the papers focused on Flame retardants/PCB mixtures

LIFE STAGE	EXPOSURE TIME*	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Juvenile/ adult (F0) larvae / juvenile (F1) (trans-gen. study)	8 m	PCB 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	67
Larvae/ Adult (F0) Larvae (F1, F2, F3, F4) (trans— gen. study)	6 m	PCB + 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)	69

Embryo/ larvae	24 h	PCB mixture (Aroclor (A) 1254)	2, 5, 10 ppm	Free swimming with or without visual stimuli	↑Thigmotaxis ↓Swimming speed	65
Embryo/ larvae	24 h	PCB mixture (Aroclor (A) 1254)	2, 5, 10 ppm	Free swimming with or without visual stimuli	↓Avoidance behaviour	66
Juvenile	14 d	PBDE + OH-BDE mixture	30 µg/L, 600 µg/L DE-71 + 4.9 µg /L , 99.9 µg/L TBP, 0.5 µg/L , 6.0 µg/L 6-OH-BDE-47 (low- and high-dose)	Novel Tank Test and the tap startle test	↓Startle response	64
Embryo/ larvae	120 h	Tetrabromobisphenol A and Silicon dioxide nanoparticles (n-SiO2)	50, 100, 200 µg/L TBBPA + 25 mg/L n-SiO2	Spontaneous movement (24 hpf) and locomotor activity in light/dark condition (120 hpf)	↓Swimming speed	70
Larvae/ Adult (F0) Juvenile (F1, F2) without re-exposure	210 d (F0)	PCB + 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 the Z-maze test	↑Boldness in F1 (compared to other generations) ↑Anxiety syndrome in F2 at 120 d	69

*Exposure time expressed in minutes (min), hours (h) days (d) or months (m)

3.1.3 PAHs mixtures

Polycyclic aromatic hydrocarbons (PAHs) are a class of hydrophobic chemicals with carcinogenic and mutagenic activity, found in complex mixtures in environmental crude oils or as products of organic matter combustion. Few studies focus on the neurotoxic effects of PAHs mixtures on zebrafish,

although single and combined compound exposure have been associated with cognitive disorders in humans and in other animal models (71).

As summarised in Table 3, Geier et al. (72) exposed zebrafish larvae to 10 PAHs, alone or in combination. The 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. (73), 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 had the most significant effects, possibly suggesting an additive effect of the two types of compounds. In a following study (74) the behaviour of F1 unexposed larvae (4-7 and 5 days dpf) and juveniles (2 months of age) from the exposed F0 was analysed. In all cases, the offspring presented an abnormal behaviour, suggesting maternal transfer to the egg, although PAH metabolites did not accumulate in the F1 generation.

Table 3: summary of the main parameters of the papers focused on PAHs mixtures

LIFE STAGE	EXPOSURE TIME*	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Larvae/ juvenile (F1, F2)	3 m	3 PAHs 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	74
Juvenile/adult	6 m	3 PAHs mixtures	0.3X, 1X, and 3X, 1X = Σ[16 EPA PAH] at 5 µg g−1 dw (1 pyrolytic fraction, 2 petrogenic fractions)	24-hours swimming activity assay (6 months old) and photomotor response assay (2 and 6 months	↑Activity (stronger response in juveniles)	73

				old, distance travelled and the time spent without movement), maze test and the Novel Tank Test		
Embryo/ larvae/ adult	114h	PAHs mixture	0.0625, 0.125, and 0.25% of SuperMix10 (embryo, larvae), 0.1% SM10 (adult)	Embryonic photomotor assay (24hpf), larval photomotor response (120 hpf), and startle stimulus and active avoidance conditioning test (6 months of age)	Behaviour abnormalities (larvae) ↓Habituation, ↓ avoidance (adults)	72

*Exposure time expressed in minutes (min), hours (h) days (d) or months (m)

3.1.4 Mixtures with other compounds

Finally, three works tested compounds from the previous classes with chemicals of different nature (Table 4). In the first paper, Chen and colleagues in 2019 (75) 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 (76) 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., (77) co-exposed zebrafish embryos to Perfluorobutanesulfonate (PFBS) and probiotics (*Lactobacillus rhamnosus*). The aim of the study was to deepen the knowledge about the antagonistic action of probiotics towards PFBS toxicity. The results of the study suggest an ameliorative ability of probiotics against the toxicity induced by PFBS.

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

LIFE STAGE	EXPOSURE TIME*	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/larvae	96 h	PFBS and probiotics	10 mg/L PFBS + 106 CFU/mL *L. rhamnosus*	Black or white background preference, phototaxis and swimming speed	Protective effects of probiotics on PFBS exposure	76
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	76
Embryo/larvae	114 h	3-MP and DP	5, 20 µg/L 3-MP + 60 µg/L DP	Spontaneous movements (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 & 120 h↑	75

*Exposure time expressed in minutes (min), hours (h) days (d) or months (m)

3.1.5 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 properties in the sites contaminated by different kinds of pollutants. In addition to the sub-lethal toxicity associated with their environmental concentrations (0.01-16 µg/L) (84) their extremely low size and high surface area facilitate the absorption and entry in aquatic organisms of contaminants such as metals and organic compounds, possibly aggravating the risks associated to the single exposure of the compound (78).

A recent study by Sun et al. (79) 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. (80) Tetrabromobisphenol A (TBBPA) (2 µM) and nano-TiO2 (0.1 mg/L) 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. (81) 7 days co-exposure of nano-TiO2 with Decabromodiphenyl ether (BDE-209) at comparable concentrations significantly depressed locomotor activity of the 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. (82), while in Guo et al. (83) the effects were evaluated in the F1 after a chronic parental exposure, observing lethargic behaviour.

Interestingly, absorption of pesticides such as cypermethrin (CYP) (84), pentachlorophenol (PCP) (78) and difenoconazole (DIF) (85) was enhanced after exposure to nano-TiO2, and both CYP (10 µM) and DIF (0.5 mg/L) 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 lead to similar effects on thyroid hormone levels and on neurobehavior of the fish as for PCP exposed alone. Miao et al. (86) evaluated the combined toxicity in zebrafish larvae of lead 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 (87), without evidencing substantial effects. As discussed in (78), 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, the results indicate zebrafish neurotoxicity to be a highly sensible endpoint for the evaluation of nano-TiO2-induced increased bioaccumulation and toxicity of organic and metal contaminants.

Table 5: summary of the main parameters of the papers focused on mixtures containing nano-TiO_2-

LIFE STAGE	EXPOSURE TIME*	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 n-TiO_2	Spontaneous movement (24hpf), touch	↑Spontaneous movement	80

				response (48 hpf), light/dark locomotor response (120 hpf)	↓Time spent in the light ↓Touch response ↓Swimming speed ↓Shoaling ↓Social contact ↑Mirror attack	
Embryo/larvae	6 d	nano-TiO_2 + BPA	1, 4, 20 µg/L BPA + 100 µg/L n-TiO_2	Locomotor behaviour (6 dpf, distance travelled and frequency and total duration of movement)	↓Swimming speed	82
Embryo/larvae	6 d	nano-TiO_2 + PCP	0, 3, 10, 30 µg/L PCP + 0.1 mg/L n-TiO_2	Locomotor behaviour in light/dark conditions (6 dpf)	↓Swimming speed	78
Embryo/larvae	120 h	nano-TiO_2 + CYP	0, 0.4, 2, 10 µg/L CYP + 1 mg/L TiO_2	Distance travelled and total duration of movements both in continuous light and dark to light photoperiod	↓Swimming speed	84
Adult/ embryo/larvae Trans-gen. study	4 m	nano-TiO_2 + BPA	0, 2, 20 µg/L BPA + 100 µg/L n-TiO_2	F1: swimming speed; frequency and total duration of movemen	↓Swimming speed (F1 10 dpf larvae)	83

				ts, distance travelled in light to dark transition (10 dpf)		
Embryo/larvae	7 d	nano-TiO_2 + BDE-209	0.38 mg/L BDE-209 + 0.1, 0.5, 1.0 mg/L nano-TiO_2	Frequency and duration of movement, total distance travelled in dark to light transition (7 dpf)	↓Swimming speed	81
Embryo/larvae	6 d	nano-TiO_2 + PB	0, 5, 10, 20, 30 µg/L Pb + 0.1 mg/L n-TiO_2	Swimming activity in larvae in light/dark conditions	↓Swimming speed	86
Embryo/larvae	168 h	nano-TiO_2 + Cd	10 µg/L Cd + 0.1, 1.0, 10 µg/L TiO_2	Spontaneous tail movements (24 hpf)	No effects	87
Embryo/larvae	120 h	nano-TiO_2 + DIF	0, 0.1, 0.5 mg/L DIF + 100 µg/ n-TiO_2	Locomotor behaviour in light/dark conditions (6 dpf)	↓Swimming speed	85
Embryo/larvae	24 h, 30 h, 48 h	nano-TiO_2 + BP3	10 µg/L BP3 + 100 µg/L n-TiO_2	Spontaneous tail movements (24 hpf), touch response (30, 48 hpf)	↑Spontaneous movement ↓Touch response (at 30 h)	79

*Exposure time expressed in minutes (min), hours (h) days (d) or months (m)

3.1.6 Nano- and microplastics as vectors for mixtures of pollutants

Nano- (NPs, < 100 nm) and microplastic (MPs, < 5 mm) particles are ubiquitous contaminants, able, thanks to their small size, to bioaccumulate in aquatic organisms. In water and sediment, 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, Chen et al. (88), using 17α-ethynylestradiol (EE2) as positive control, 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 the NPs-EE2 displayed stronger effects. In (89) 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., (88), and Liu et al., (90), respectively performed absorption and accumulation tests on zebrafish exposed to BPA + NPs and Avobenzone (Butyl Methoxydibenzoylmethane, AVO) + NPs. In both 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-day co-exposure of a new substitution of BPA, BPAF and 100 nm NPs to adult zebrafish, lead to a significant decrease in locomotor and social behaviour (91). The same decrease was also observed in their offspring at 6 dpf. In a study by Varshney et al., (92), 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 locomotion activity. Santos and colleagues (93) tested NPs with the herbicide phenmedipham (PHE). In particular, the mixture of 0.015 mg/L NPls + 20 mg/L PHE highlighted hyperactive behavior. Zhang et al., (94), 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., (95) 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., (96), after 14 days 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., (97), 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 (98) focused on the effects of MPs and Cu on zebrafish adults, after a 30-day 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 (99), 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) lead to a general activity increase of zebrafish larvae after 7 days (100). Finally, Hanslik et al., (101), exposed embryos to a mixture of MPs previously exposed for 21 days 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., (102) fed for 15 days adult zebrafish with food supplemented with 0.4 mg/L 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 taken into account, 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.

Table 6: summary of the main parameters of the papers focused on micro- and nano-plastics mixtures

LIFE STAGE	EXPOSURE TIME*	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/larvae	7 d	MPs + MeHg	1 mg/L MPs + 1 µg/L MeHg	Locomotor activity in light/dark transition	↓Swimming distance	95
Embryo/larvae	120 h	NPs + PHE	0.015 mg/L, 1.5 mg/L NPls + 2 mg/L, 20 mg/L PHE	Locomotor behaviour (120 hpf, type of moveme	↑Swimming distance	93

				nt, total time and distance, larvae path angle)		
Embryo/larvae/juvenile	14 d	MPs + Cu	60 µg/L, 125 µg/L Cu + 2 mg/L MPs	Locomotor activity, anxiety, avoidance and shoaling behaviour	↓ Swimming behaviour (total distance traveled, absolute turn angle, percentage of time active and mean speed) ↓Avoidance behaviour	96
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)	99
Embryo/larvae	94 h	MPs + Cu	15, 60, 125 µg/L Cu + 2 mg/L MPs	Mean speed, mean distance to the centre zone of the well, total distance moved, mean absolute	↓Social cohesion ↑Swimming distance in dark	97

				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		
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	89
Embryo/larvae	140 h	Polystyrene MPs + Melatonin	25 mg/L PS + 1 µM melatonin	Swimming activity (144 hpf and 168 hpf)	Swimming impairment rescued by melatonin	94
Adult	120 h	MPs + EE2 NPs + EE2	1 mg/L MPs + 2, 20 µg/L EE2	Swimming activity	↓Swimming distance	88

			; 1 mg/L NPs+ 2, 20 µg/L EE2			
Embryo/larvae	96 h	MPs + environmental samples	MPs mixture exposed to river water	Swimming activity in light/dark conditions	No effects	101
Embryo/larvae	72 h	NPs + AVO	10 µg/L AVO + 10 µg/L NPs	Swimming activity	↓Swimming speed	90
Adult	30 d	MPs + Cu	2 mg/L MPs + 25 µg/L Cu	Locomotor behaviour (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	98
Embryo/larvae	96 h	NPs + DDE	50 mg/L NPs + DDE 100 µg/L	Swimming activity	↓Swimming speed	92

					↓Swimming distance	
Adult (F0) Embryo/larvae (F1) Trans-gen. study	45 d	NPs + BPAF	1 mg/L NPs and 200 µg/L BPAF	Swimming activity and social behaviour	↓Swimming speed ↓Swimming distance (F0, F1)	91
Adult	7 d	MPs + AMI	0.44 mg/L MPs + 2.5 µg/L AMI	Startle behaviour test	↑Swimming speed ↓Freezing	100
Adult	15 d	MPs + sorbed marine contaminants	food supplemented with 0.4 mg/L PE-MPs incubated in seawater	Exploratory and social behaviour	↓Activity	102

*Exposure time expressed in minutes (min), hours (h) days (d) or months (m)

3.1.7 Pharmaceuticals products

Pharmaceutical products are released in the environment as a 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 even reach drinking water sources. Their presence in surface waters has been reported in many regions of the world such as China, the USA and the European Union. 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. Other sources of chemical discharge are wastewater treatment plants (WWTPs) and sewage treatment plants (STPs). It is not surprising that the among the many categories of chemicals that were tested in the works examinated in our review the category of pharmaceuticals was the most represented, and is reported in this paragraph. As shown in the table below, Brunelle et al., (103), 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 (CPMand primidone. Zebrafish larvae were exposed to the samples and hyperactivity was observed in some cases, and as pointed out by the authors the effect is unlikely to be caused by neuroactive pharmaceuticals alone, but rather by other chemical contaminants and synergistic effects. Atzei et al., (104), 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., (105), exposure to surface water samples from the Tagus River, decreased mobility of zebrafish larvae exposed to the samples. A previous detection study revealed, through targeted analysis, the presence of CBZ, VNX, CPM, caffeine and erythromycin in the water downstream of STP effluent discharge into the Tagus River (106), in addition to the psychoactive drugs benzoylecgnina, norBE and ephedrine (<41 ng/L), detected in the samples used by García-Cambero et al. The effect observed in the study might be a result of the discardedpresence of such psychoactive chemicals continuously introduced in the environment, although their interactions were not explored. Zindler et al. (107) 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., (108), along with six other substances, including caffeine, which was shown to have neuroactive effects on zebrafish (109). Only the highest mixture concentration elicited hypoactivity in larvae. In Jia et al., (110), significant inhibition of the enzyme acetylcholinesterase (AChE) was observed in larvae exposed to CBZ (1, 10, 100 µg/L) and Cu (0.5, 5 and 10 µg/L), but the combined exposure did not exacerbate the decrease in enzymatic activity.

Zebrafish embryos were exposed to FLX (3.2 ng/L) and VNX (2 µg/L) in Rodrigues et al., (111). No significant differences between control and exposure 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 (112). In a recent study by Venkatachalam et al., (113), 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., (114) and Wang et al., (115) exposed zebrafish larvae to a mixture of beta-diketone antibiotics (DKAs) frequently detected in polluted internal water systems in high concentrations. In (115), after three months of exposure, adult fish were screened for behavioural changes. Interestingly, in (114)., 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., (116), that observed the combined effect of tetracyclines and fluoroquinones antibiotics. Nevertheless, in all three studies, the synergic or additive properties of the mixture were not explored. As more classes of antibiotics are commonly detected in the environment (106; 108), 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. García-Cambero et al., (117) 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 the exposure to an experimental mixture containing representative concentrations did not cause any relevant effects. EE2 was also tested in combination with CPM (118) and gestodene (GES) (119). 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 (119). EE2, according to (118), 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 (120) 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 few showed significant additive or synergistic effects.

Table 7: summary of the main parameters of the papers focused on pharmaceutical mixtures

LIFE STAGE	EXPOSURE TIME*	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/ larvae	5 d	93 in total including bupropion, CBZ, ciprofloxacin, CPM, desvenlafaxine, ipamidol, primidone, VNX	N/A	Total distance swam in light/dark condition	↑Swimming distance	103
Embryo/larvae	5 d	Binary mixtures of CBZ + FLX + VNX	Relative potency factor-based mixtures	Light/dark transition test	↓Swimming activity	104

Embryo/larvae	80 h	FLX + VNX	3.2 ng/L FLX +2000 ng/L VFN	Sensorymotor reflexes (light touches)	No effects	111
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	107
Adult	45 d	CBZ + Cu	1, 10, 100 µg/L CBZ + 0.5, 5, 10 µg/L Cu	AChE activity assay	No effects	110
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 darkness	108
Embryo/larvae	6 d	Water river samples containing previously detected CBZ, VNX, CPM, caffeine, erythromycin, benzoylecgnina, norBE, ephedrine	N/A	Spontaneous movement frequency (24 hpf), locomotion assay (6 dpf, mean distance travelled)	↑ Spontaneous movement frequency ↓Swimming speed ↓Swimming distance (6 d)	105
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	112
Embryo/larvae	120 h	Metformin + ranitidine + bisoprolol + sotalol	Binary mixtures combinations and quaternary mixture at 0.1,	Total swimming distance and total swimming	No effects	120

			1.0, 10, 100 µg/L each	time were evaluated in light/dark conditions		
Embryo/larvae	144 h	EE2 + GES	41, 423 ng/L EE2 + 49, 514, 4873 ng/L GES in binary combinations	Total distance travelled and mean speed in the dark	↓Swimming distance ↓Swimming speed	119
Embryo/larvae	144 h	River samples and representative mixture of E2 + EE2 + DCF	160, 1700 ng/L DCF + 5, 11 ng/L E2 + 7, 115 ng/L 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)	117
Adult	14 d	EE2 + CPM	0.9, 1 ng/L EE2 + 0.1, 0.4 µg/L CPM	Swimming activity (scototaxis, shoaling and Novel Tank test)	Recovery of anxiogenic effect of EE2 by CTP, ↑Latency in novel tank test	118
Embryo/larvae	114 h	DKAs (tetracyclines + fluoroquinones)	0, 4.69, 9.38, 18.75 mg/L	Swimming activity and swimming speed in light/dark condition	↑Basal swimming rate (lower mixture concentrations ↓Basal swimming rate (higher mixture concentrations	116
Embryo/larvae/adult	120 d	DKAs (tetracyclines + fluoroquinones)	6.25, 12.5 and 25 mg/L	Bottom dwelling test (time spent in the upper part of the tank, distance travelled, mean speed and number of line crossings), conditioned place preference	↑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	115

				and shoaling behaviour	concentratio n) ↓Social cohesion	
Embryo/larvae	90 h	DKAs (tetracyclines + fluoroquinones)	0, 4.69, 9.38, 18.75, 37.5 mg/L DKAs	Swimming speed and light/dark stimulation response (120 hpf)	↑Spontaneo us movement	114
Juvenile/adult	130 d, 21 d	SSRIs (Fluoxetine, Paroxetine, Sertraline)	10, 100, 200 µg/L	Novel tank test (90 and 120 dpf)	↓Swimming distance ↓Time spent at the bottom of tank (at 120 dpf)	113

*Exposure time expressed in minutes (min), hours (h), days (d) or months (m)

3.1.8 Pesticide mixtures

Pesticides encompass a wide array of products, including fungicides, herbicides and insecticides . They are used worldwide in a variety of fields, including food production, gardening, medicine and road maintenance, and according to a 2017 report from the United Nations around 200,000 people die each year in the world because of pesticides acute poisoning. Several work we found tested the insecticide chlorpyrifos in conjunction with other products (Table 8); this is not surprising given its widespread use and its known neurotoxic action as acetylcholinesterase inhibitor (121). In a 2013 work (122), Pérez and colleagues tested chlorpyrifos, atrazine and terbuthylazine, widely used herbicides on zebrafish embryos with the exposure to the single substances and two mixtures (chlorpyrifos + atrazine and chlorpyrifos + terbuthylazine). The results show synergistic interaction between the compounds on behaviour and AChE. In a 2021 paper (123), Fan and colleagues investigated the individual and combined toxicity of carbendazim and chlorpyrifos, widely used pesticides, 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 (124) and in a second work from the same author (125) 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 (126), suggesting a possible additive interaction. Deltamethrin was tested in combination with *Citrus aurantifolia*, an essential oil used for biological control (127) and their combination resulted in a reduction in forage frequency. A 2014 work (128) investigated the toxicity of a type I pyrethroid (Permethrin) and of a type II pyrethroid (Cypermethrin), both widely used pesticides by exposing

zebrafish eggs to environmentally relevant concentrations. The authors suggested additive or synergistic interactions. A 2019 work (129) investigated the effects of glyphosate, the most widely used herbicide in the world (130) and fipronil, a broad-spectrum insecticide and their mixtures on adult zebrafish. The exposures induced an anxiolytic-like response, with higher intensity in the mixture. The effects glyphosate and its metabolite aminomethylphosphonic acid (AMPA) were investigated in a 2022 work (130) using zebrafish embryos. While the exposure to glyphosate alone induced hyperactivity, in line with other recent works (131), neither the AMPA nor the mixtures were cause of behavioural alterations or changes in AChE expression. In a 2020 paper Rozmánková (132) 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.

Table 8: summary of the main parameters of the papers focused on pesticides

LIFE STAGE	EXPOSURE TIME*	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	127
Embryo/larvae	116 h	Chlorpyrifos + cyfluthrin	1.16 mg/L chlorpyrifos + 7.06, 14.12 µg/L cyfluthrin (low-, middle-dose exposures)	Average velocity, total distance travelled, and meander	↓Swimming speed ↓Swimming distance ↑Meander	125
Embryo/larvae	96 h	Carbendazim + chlorpyrifos	Mix1 = 0.59 - 2.35 mg/L (in total, equipotent concentration ratio) Mix2 = 0.38 - 1.51 mg/L (total, based on maximum residue limits)	Spontaneous movement frequency (24 hpf)	↑ Spontaneous movement frequency	123

Embryo/larvae	116 h	Carbendazim + chlorpyrifos	0.51, 0.57, 0.64 mg/L carbendazim + 1.00, 1.26, 1.58, 2.00, 2.52 mg/L 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	124
Embryo/larvae	142 h	Chlorpyrifos + Deltamethrin	4.80, 39.06, 78.13 µg/L CPF + 0.06, 1.60, 3.19 µg/L DM (low-, middle-, high-dose exposures)	Light/dark transition test, swimming speed	↑Frequency of spontaneous tail coiling (24 h) ↓Swimming speed ↓Habituation	126
Embryo/larvae	7 d	GLY + AMPA	1 µM GLY +AMPA	Total distance travelled (locomotor activity assay) and light/dark preference	No effects	130
Embryo/larvae	120 h	S-Metolachlor + metolachlor oxanilic acid and metolachlor ethanesulfonic acid	1 µg/L of each substance in the mixture	Spontaneous movement frequency (23 hpf), total swimming distance light/dark condition (120 hpf)	↓ Spontaneous movement frequency (23 h)	132
Adult	96 h	Glyphosate-based herbicide + Fipronil-based insecticied	1, 3, 5 mg/L GBH + 0.009, 0.018, 0.027 mg/L FBI (low-, middle-, high-dose exposures)	Novel Tank test	↑Time spent in the top of the tank	129

Embryo/larvae	141 h	Permethrin + cypermethrin	100, 200, 300 µg/L PM + 10, 20, 30 µg/L CP	Involuntary body movement (spasms)	↑Spasms	128
Embryo/larvae	96 h	Atrazine, terbuthylazine + chlorpyrifos	7 binary mixtures from 5 concentrations of each pesticide (0.5 - 40 mg/L	Swimming activity	Altered swimming behaviour	122

*Exposure time expressed in minutes (min), hours (h), days (d) or months (m)

3.1.9 Cyanotoxin mixtures

Cyanobacteria are a vast group of photosynthetic microorganisms that can be found in a wide range of habitats throughout the planet, able to produce under certain conditions a large number of secondary metabolites some of which (i.e. the cyanotoxins) of great concern for both human and animal health.

As shown in Table 9, a 2022 paper (133) assessed the toxicity of a widely studied cyanotoxin, β-methylamino-L-alanine (BMAA), and of other two lesser 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 on zebrafish embryos. BMAA and AEG enhanced startle sensitivity, and that they have additive interaction. Binary mixtures also caused a reduction in average speed and total distance travelled.

The same authors also tested BMAA and microcystin leucine and arginine (MCLR) on zebrafish larvae (134). A dose-related increase in acoustic startle sensitivity with both compounds, furtherly enhanced by the combined exposure was found, and the authors hypothesized a synergic interaction between the two compounds. Lastly, Roegner et al., in 2019 (135) tested a wide variety of cyanobacterial metabolite standards and bloom components from the Rio de La Plata waters on 4 hpf dechorionated embryos.

Table 9: summary of the main parameters of the papers focused on cyanotoxins

LIFE STAGE	EXPOSURE TIME*	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	133
Embryo/larvae	6 d	BMAA + MCLR	100 µM BMAA + 1 µM MCLR	Swimming activity (startle response and locomotor activity)	↓Swimming speed	134
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	135

*Exposure time expressed in minutes (min), hours (h), days (d) or months (m)

3.1.10 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 . These elements tend to accumulate in the environment and in living organisms, and an exposure can result in significant toxicity in both humans and animals (136), and for this reason over the last decades they have been cause of health concern at international level . Moreover, the exposure of living organisms to mixtures of metals is likely to occur in the environment, and for this reason a more comprehensive understanding of their effects is needed (136). 8 works that met our inclusion criteria tested metals in combination with other metals or different chemicals (Table 10). In a 2018 work (137) exposed zebrafish 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 Lcysteine. Binary mixtures of cadmium (Cd) and zinc (Zn) were also tested.

In the study from Xia et al. (138), lead (Pb) and manganese (Mn) showed combined effects on 7 dpf zebrafish larvae, while in 2021, (136), exposed for 21 days 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 cadmium 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 (139) tested the joint toxicity of cadmium 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 (140) 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 (141) 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 (142) 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) together with Pb (50 µg/L) were assessed over 96 h on zebrafish larvae (143).

Table 10: summary of the main parameters of the papers focused on metals

LIFE STAGE	EXPOSURE TIME*	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Larvae	24 h	Cd+ Zn	76 µg/L Zn + [0, 4, 16, 36, 85, 326 µg/L Cd] 36 µg/L Cd + [0, 22, 47, 76, 122, 262 µg/L Zn	Olfactory response	↓Olfactory response (high concentration mixture)	137

Adult	21 d	Cd chloride + Hg chloride	1 mg/L Cd + 30 µg/L Hg	Light/dark Preference Test (7 dpf) and the Novel Tank test (21 dpf)	↓Exploratory behaviour ↑Time spent in lower zone of the tank	136
Adult	130 min	Cd + SDBS	2, 4, 6, 12 mg/L Cd + 2.5, 5, 10, 20, 40 mg/L SDBS	Swimming speed and vertical position	↓Vertical position ↓Time spent on surface	139
Embryo/ Larvae	142 h	Pb+ BDE-209	5, 10, 20 µg/L Pb + 50, 100, 200 µg/L BDE-209 (low-, middle-, high-dose co-exposure)	Larval swimming behaviour in response to the light/dark transition (distance travelled, the frequency of movements, and the total duration of movements)	↓Swimming speed	140
Adult/ Embryo/ Larvae Trans-gen. study	90 d	Pb + BDE-209	0, 1, 10, 100 µg/L BDE-209 + 10 µg/L Pb	F1: Locomotor activity (5 dpf, average speed under light/dark conditions)	↓Swimming speed	141
Embryo/ Larvae	96 h	Cu, Zn and Mn complexed with glyphosate-based herbicide	1 µg a.i. /mL GBH + 100 µg/L Cu/Mn/Zn	Speed, distance covered and distance from the centre of the well, thigmotaxis and behaviour in response to visual stimuli in different light conditions	↓Swimming distance in darkness	142

Embryo/ Larvae	96 h	Pb + Ciprofloxacin	50 µg/L Pb + 5 mg/L Ciprofloxacin	Swimming activity	↓Swimming activity in top part of the tank ↑Freezing duration	143
Embryo/ Larvae	7 d	Pb + Mn	50 µg/L Pb + 300 µg/L Mn	Light/dark photoperiod stimulation test	↓Activity count ↓Swimming distance	138

*Exposure time expressed in minutes (min), hours (h), days (d) or months (m)

3.1.11 Environmental samples

Several works that emerged from our query revolved around 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.

A 2021 work (144) 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 from phytosanitary products, the fish was also exposed to several copper 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, with several known ecotoxicological effects (145).

In a 2020 work zebrafish eggs were exposed to water samples from wastewater treatment plants effluents, a known possible source of environmental toxicity (145). A 2021 paper (146) was aimed at assessing the effects derived by 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., (147) 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 days (148). A work from Rosales-Pérez et al., (149) assessed the effects

on zebrafish adults of the exposure to hospital effluents samples. Finally, Sánchez-López and colleagues (150) observed the daily rhythm of swimming activity of adult zebrafish exposed acutely for 3 days to water samples coming from Lerma River and detected a significant reduction of the swimming activity, considering the circadian variation in locomotor activity.

Table 11: summary of the main parameters of the papers focused on environmental samples

LIFE STAGE	EXPOSURE TIME*	CHEMICALS	CONCENTRATIONS	ENDPOINT	EFFECT	REFERENCE
Embryo/larvae	144 h	Wastewater treatment plants effluents samples	N/A	Spontaneous movement frequency (24 hpf)	↑ Spontaneous movement frequency	145
Embryo/larvae	24 h, 120 h	Wastewater treatment plants effluents samples/river samples	N/A	Spontaneous movement frequency (24 hpf), swimming behaviour in light/dark conditions (120 hpf)	↓Swimming distance	146
Adult	48 h	Enviromental water and sediment samples, Cu	N/A	Novel Tank Test	↓Time spent in the bottom area of the tank	144
Embryo/larvae	120 h	Extract from sediment samples	N/A	Total swimming distance, the swimming speed and the swimming time were assessed as behavioural endpoints with light/dark changes	↑Locomotor behaviour	147

Embryo/larvae	7 d	Groundwater samples	N/A	Swimming speed of the larvae was assessed	↓Swimming speed	148
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	149
Adult	72 h	River samples	N/A	Swimming activity	↓Swimming activity	150

*Exposure time expressed in minutes (min), hours (h), days (d) or months (m)

3.1.12 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. The first study (151) investigated the effects of cigarette particulate matter on zebrafish embryos through a 94-h exposure, with the aim of highlight its toxicity and compare it to that of 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 (152) 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 & Vijayan (153) exposed zebrafish embryos were to isoproterenol, serotonin and ethanol. Isoproterenol, ethanol or a combination of both were used to assess behavioural changes. A 2019 paper (154) 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 concentration, while electronic cigarette flavourants, with and without nicotine were tested in a 2020 paper (155).

In a 2020 paper by Christou et al. (156), 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. (157) and a recent paper from Haridevamuthu and colleagues (158) tested the ameliorative ability of benzo[b]thiophene analogues on the neurotoxicity induced by acrylamide on 3 dpf zebrafish larvae through a 3-days exposure. Finally, a 2022 paper (159) tested the effects of a mixture of siloxanes on newly hatched zebrafish embryos. The effects on behaviour were considered after 7 and 14 days of exposure.

Table 12: summary of the main parameters of the papers focused on "other mixtures"

LIFE STAGE	EXPOSURE TIME*	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	155
Embryo/larvae	94 h	Cigarette TPM	0.4, 1.4 µg/mL TPM	Spontaneous movement frequency (24 hpf), swimming activity in light/dark conditions (96 hpf)	↑Spontaneous movement frequency ↑Swimming distance	151
Adult/ Embryo/larvae Trans-gen. study	90 h	Cigarette TPM	1.4, 2.8, 4.2 µg/mL TPM	F0 and F1: swimming distance (144 hpf)	↑Swimming distance (F0 larvae) ↓Swimming distance (F1 larvae)	152

				F0: shoaling test, novel tank test, predator avoidance and startle tap test (6 months adults)	↑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	
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	153
Adult	8 weeks	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	157
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,	Swimming speed and total distance travelled in light/dark conditions (98 hpf)	Additive effects on swimming speed (DMSO + flutamide/PFOS)	156

			1.00% DMSO + 0.0005% MB			
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	154
Larvae	3 d	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	158
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 d) on: frequency of visits to centre of tank, swimming velocity, total distance travelled and turn angle ↓Activity (14 d)	159

*Exposure time expressed in minutes (min), hours (h), days (d) or months (m)

3.2 Amoxicillin

For Amoxicillin three concentrations were tested based on data available in literature and on range finding tests aimed at selecting concentrations as relevant as possible in an enviromental scenario. The concentrations tested were 100, 50 and 10 mg/L.

3.2.1 FET test

The 96 hours exposure highlights a mild dose-related mortality, with values up to 20% for the highest concentration (Fig.7).

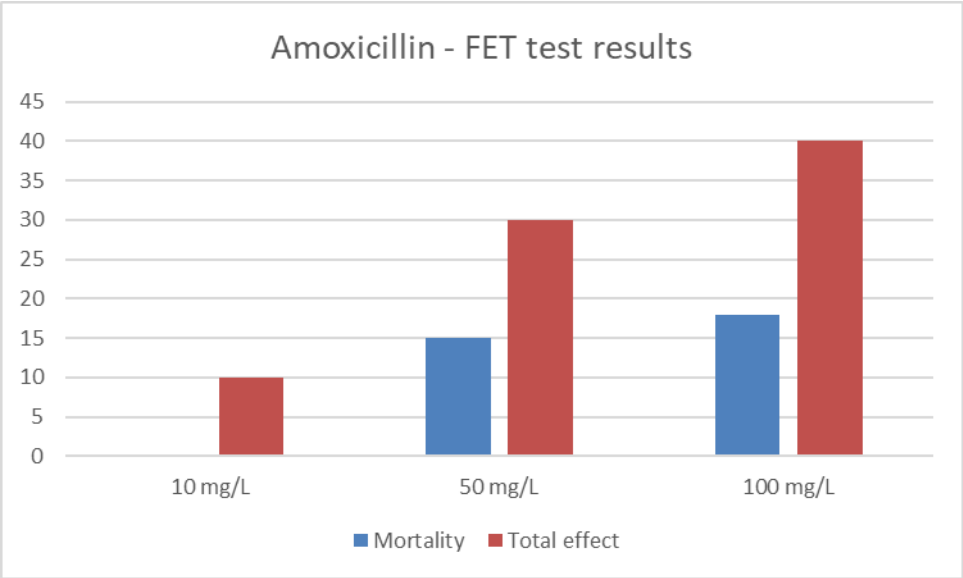

Figure 7: FET test results for Amoxicillin after a 96-hours exposure

The exposure also induced a low percentage of sub-lethal effects. In both cases spine deformation and late hactching were most commonly observed (Table 13, Figure 9, 10 and 11).

Zebrafish (OECD 236)				
Amoxicillin Concentration	% Mortality	% Total Subl. Effect	% Spine deformation	% Late hatching
10 mg/L	0	10	10	0
50 mg/L	15	15	10	5
100 mg/L	18	12	5	5

Table 13: lethal and sublethal effects of Amoxicillin on zebrafish embryos at 96 hpf.

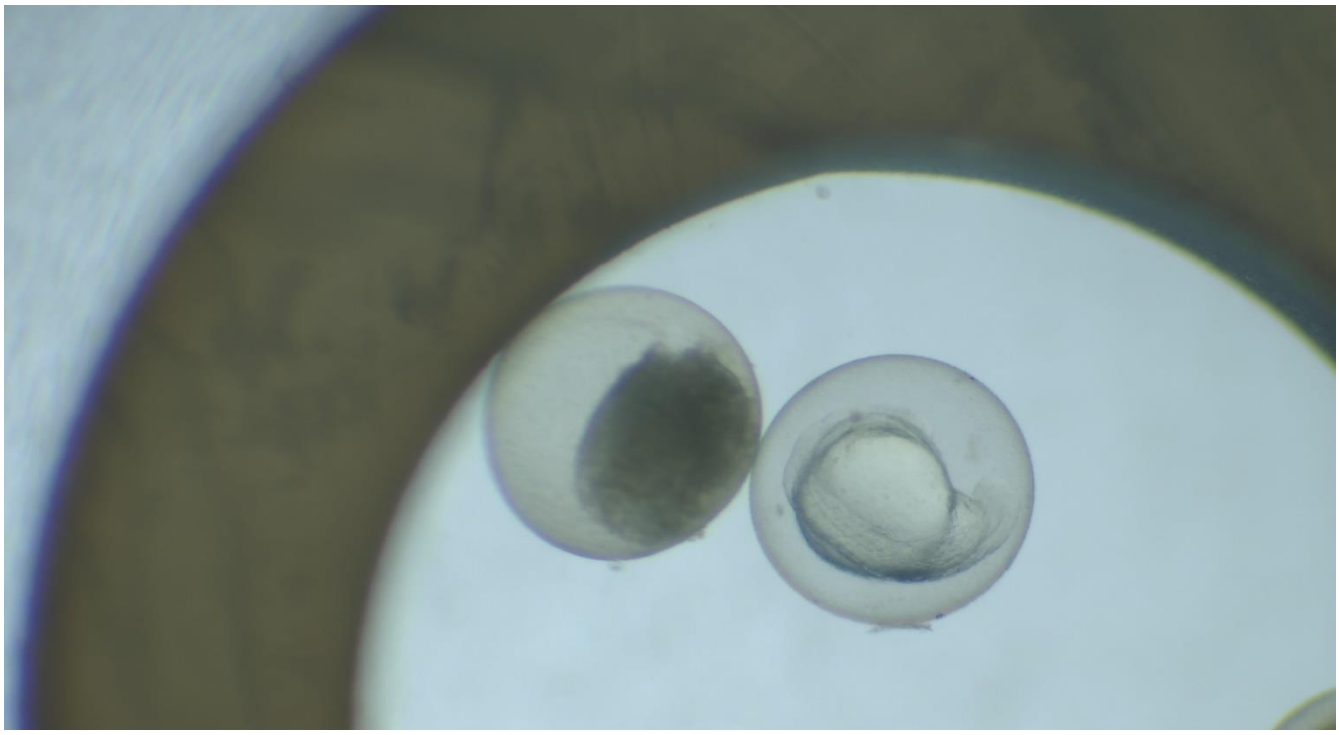

Figure 8: a coagulated embryos and a embryo undergoing irregular development at 24 hpf

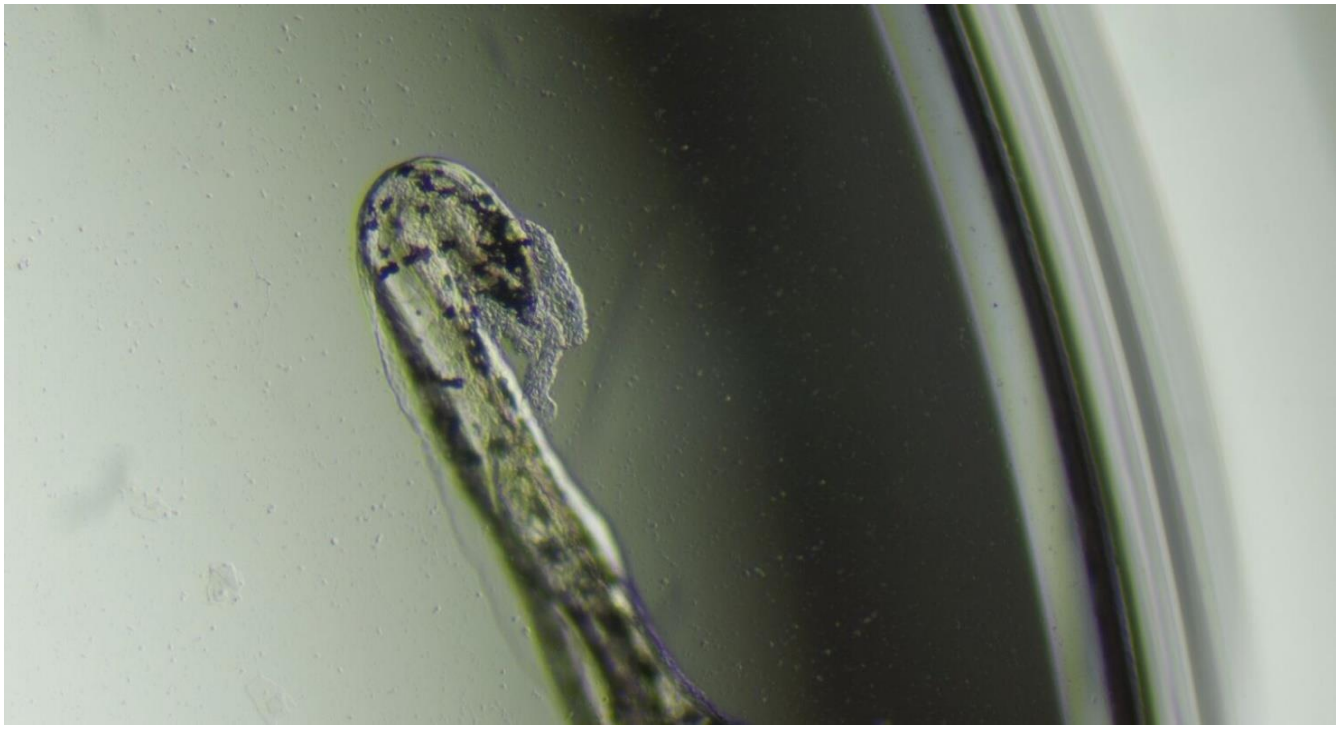

Figure 9, 10 and 11: sublethal effects of Amoxicillin. Spine deformation was the more frequently occurring effect.

3.2.2 CAT test

The CAT test show a dose-dependent hyperactivity. As shown in Fig. 12 and 13, a general decrease in the burst duration is observed in relation to the increase of the chemical concentration, showing faster contractions, while the burst activity and count show an overall increase. The total burst duration also show a dose-dependent increase, while no differences were highlighted on inactivity.

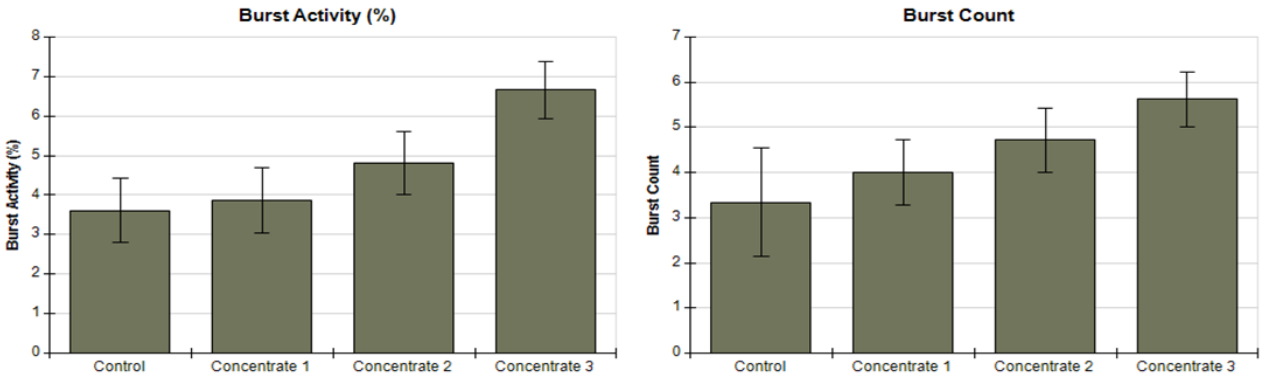

Figure 12: the treated individuals show a clear hyperactivity compared to the control.

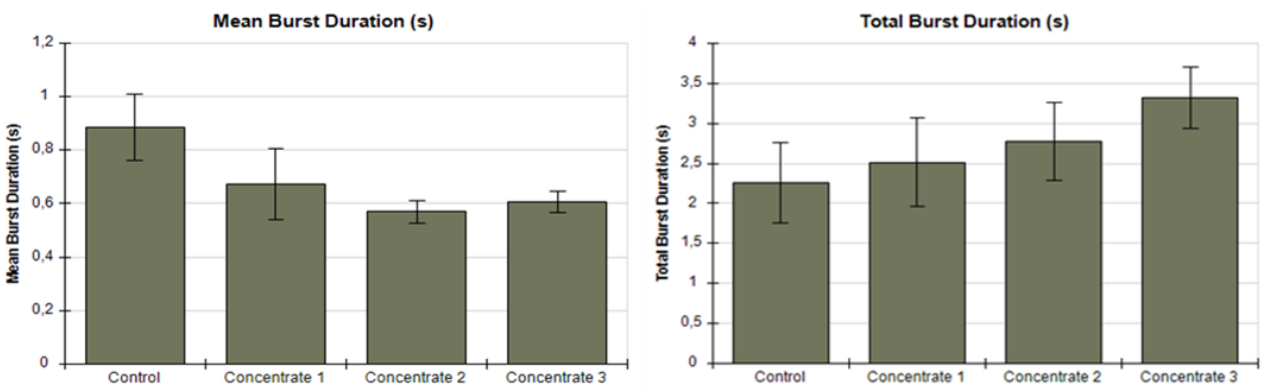

Figure 13: the hyperactivity highlighted in the treated individuals is also clearly visible by taking into account the mean burst duration, that shows a lower value in the exposed embryos, suggesting a faster movement.

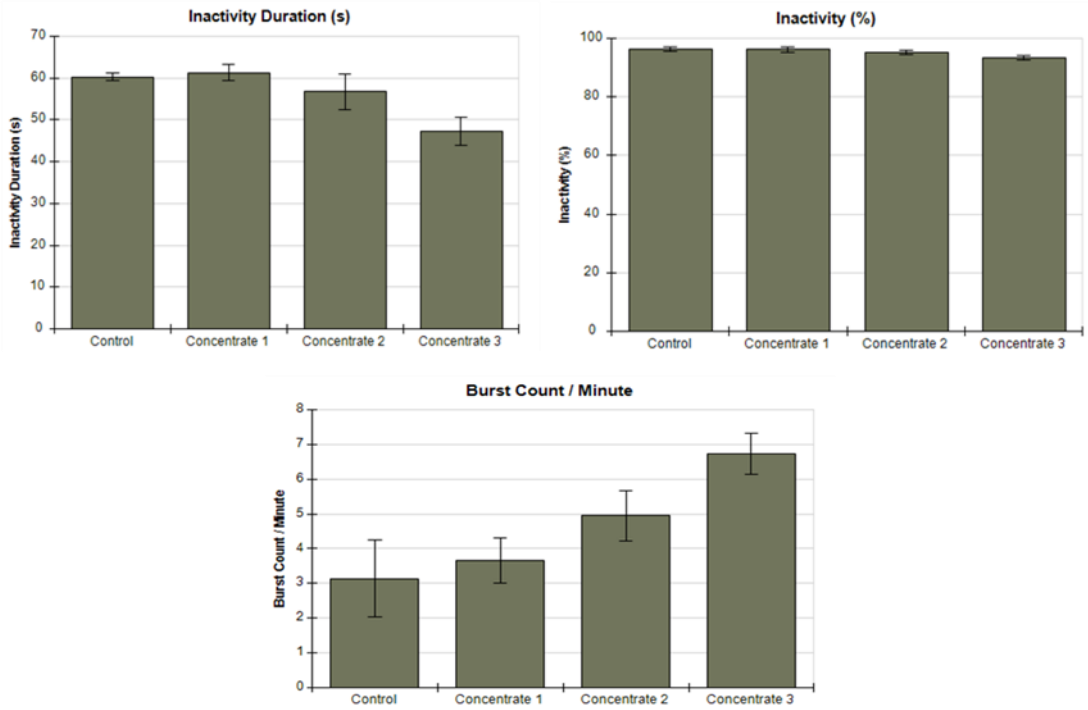

Figure 14: there is no clear difference in inactivity values between treated and untreated individuals.

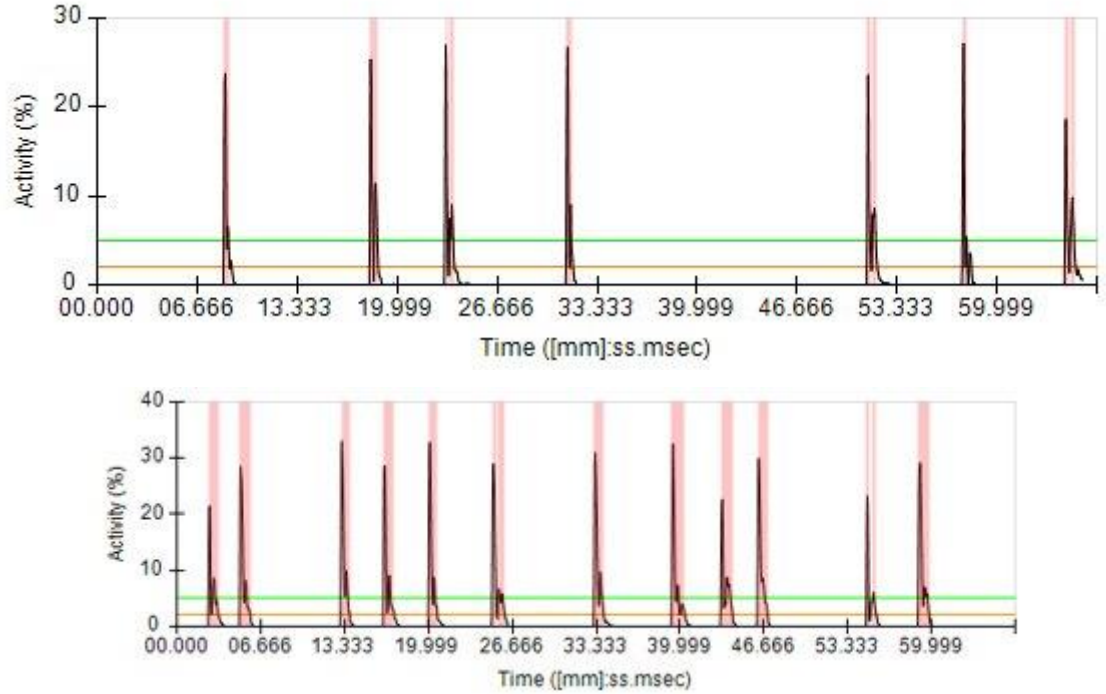

Figure 15: the frequency of movement in the control (image above) compared to the highest Amoxicillin concentration (image below).

3.3 Ceftriaxone

3.3.1 Fish Embryo Toxicity test

As for Amoxicillin, even in this case three concentrations were tested based on data available in literature and on range finding tests. The concentrations tested were 100, 10 and 5 mg/L. The preliminary results with the FET test shows a mild tocixity with a growing trend based on the increase in concentration.

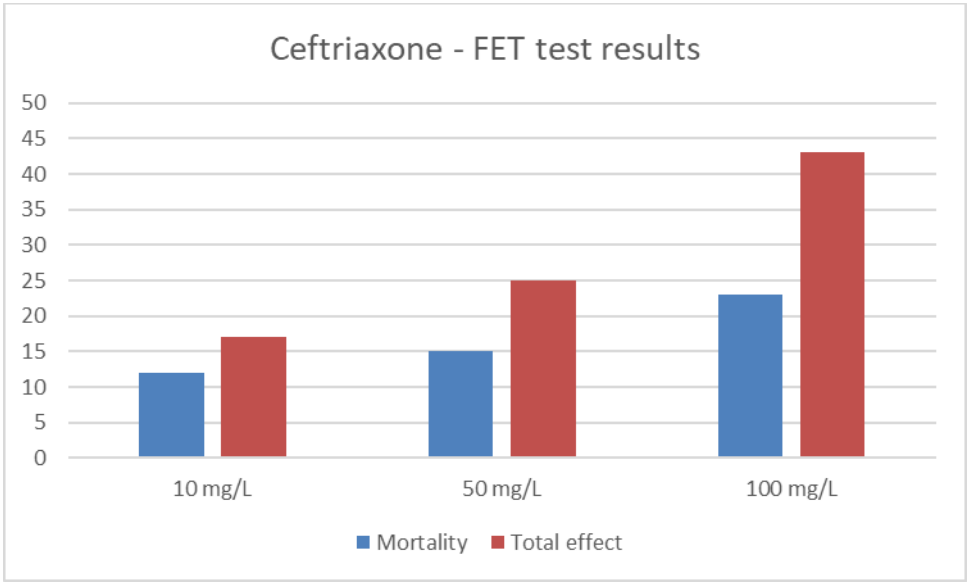

Figure 16: Ceftriaxone FET test results

	Zebrafish (OECD 236)			
Ceftrixone Concentration	% Mortality	% Total Subl. Effect	% Depigmentation	% Late hatching
5 mg/L	12	17	5	0
10 mg/L	15	25	0	10
100 mg/L	23	43	10	10

Figure 17: Ceftriaxone mortality and sublethal effects

Even in this case a low percentage of sublethal effects were observed, although here depigmentation and late hatching ere the most commonly occurring endpoints (Figure 17).

3.3.2 CAT test

The CAT test show a different trend compared to Amoxicillin. As shown in Fig. 18 and 19, a general increase in burst count, activity and duration is observed in relation to the increase of the chemical concentration. However it has to be noted that the higher values are observed by far in the intermediate concentration, showing higher activity, count and total movement duration, while the mean burst duration increase in a dose-dependent manner.

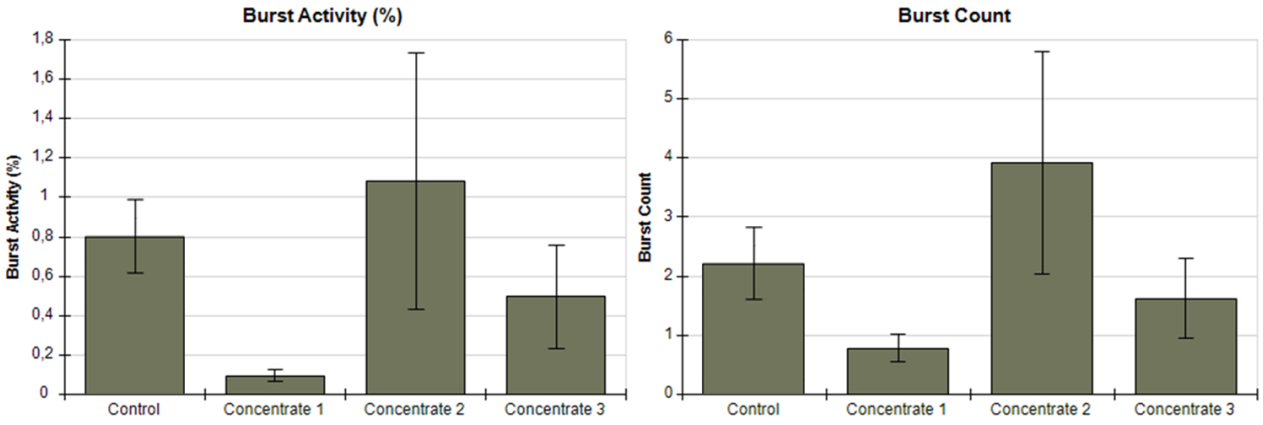

Figure 18: the tests performed with the CAT test show hypoactivity compared to the control, with the exception of the intermediate concentration, that shows hyperactivity.

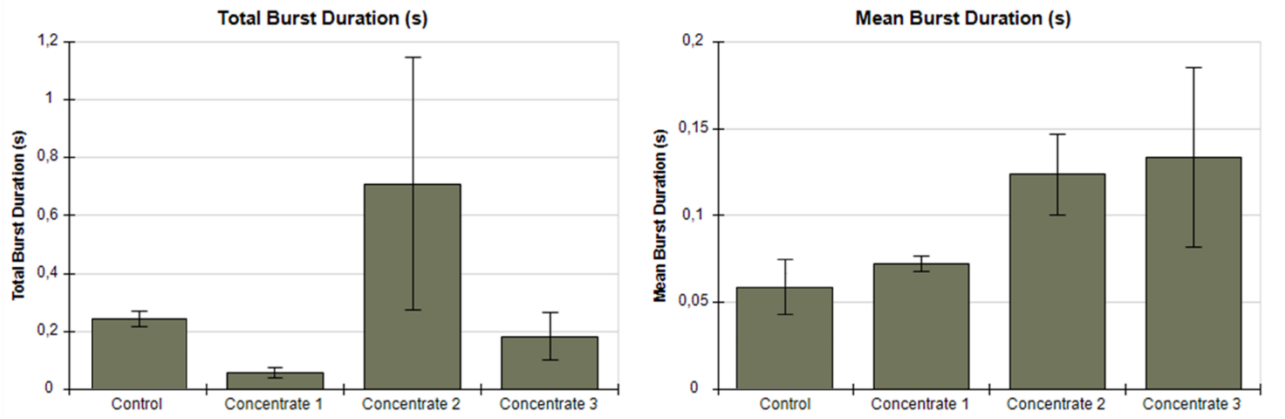

Figure 19: the tests performed with the CAT test shows hypoactivity compared to the control, with the exception of the intermediate concentration. The mean burst duration however shows an increase in dose-dependent manner.

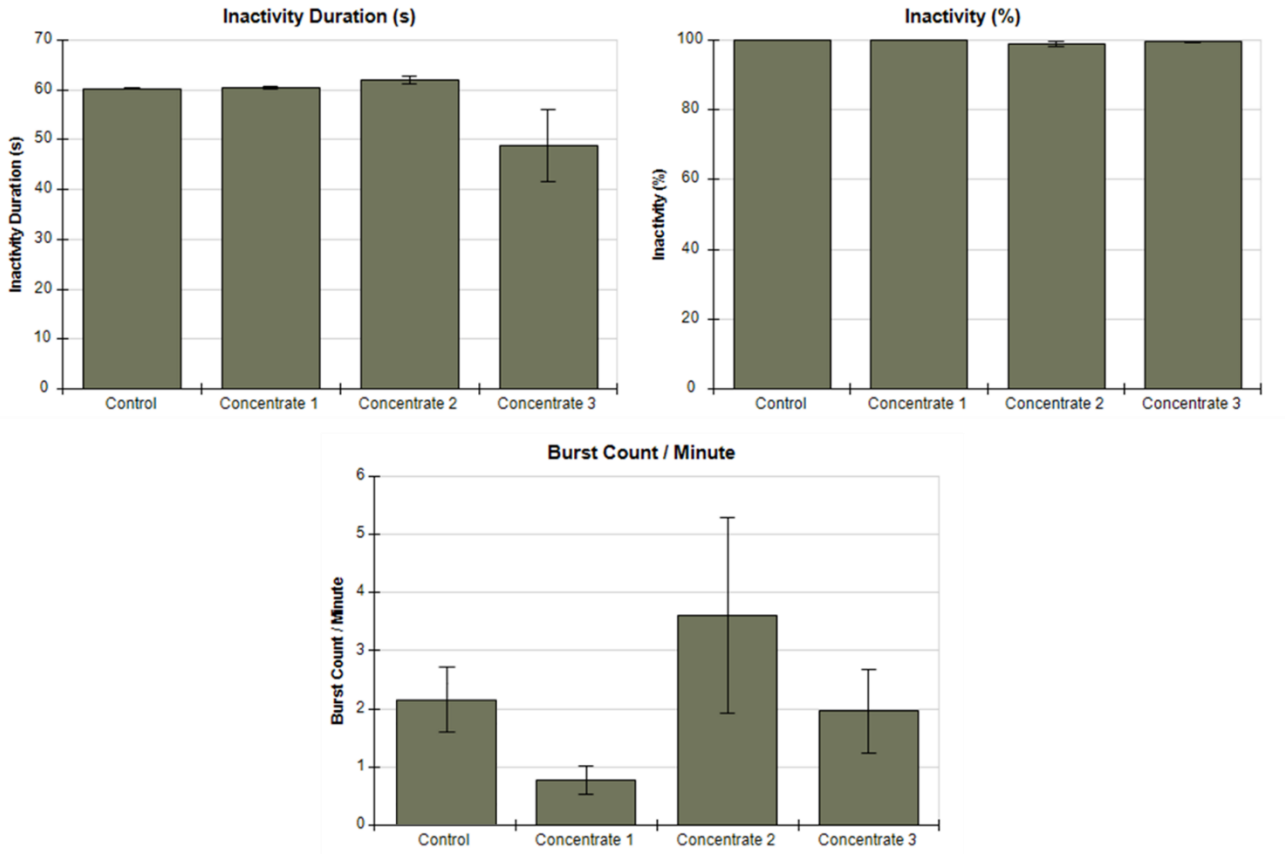

Figure 20: there is no clear difference in inactivity values between treated and untreated individuals.

3.4 Tiber river

3.4.1 FET test

The Fish Embryo Acute Toxicity (FET) test was conducted according to the OECD 236 (OECD, 2013). At the end of the test, four lethality endpoints indicating acute toxicity were recorded: coagulation of the embryo, non detachment of the tail, lack of somite formation and lack of heartbeat.

Furthermore, the following sublethal endpoints were also recorded: depigmentation, cardiac edema, and spine deformation, i.e., scoliosis and lordosis. We also analyzed body length and eye size with Danioscope software to determine possible deviations from normal development caused by chemical substances; these sublethal endpoints were investigated sincevthey can reveal potential toxicity due to several chemical compounds (160). Each test was performed in three replicates for a total of 60 embryos per sample. Statistical analysis was performed: the effect percentage was calculated normalizing the sample values with the control, applying the following formula:

$$(\%)\ \text{Effect} \frac{X_{\text{control}} - X_{\text{exposed}}}{X_{\text{control}}}$$

$X_{Control}$ represents the mean value of the single parameter (body length and eye size) calculated on the control, and X_{Sample}, the mean value on the samples separately. Furthermore, the statistical analysis was performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, California, USA). Kruskal–Wallis test (ANOVA-on-ranks) was used in combination with Dunn's test for analysis of effects on body length and eye size.

The results highlight the presence of acute toxicity in both samples analyzed. Specifically, TB20 shows average mortality of 35%, while TB21 shows an average mortality of 40% (Fig. 3). No effects of hatching delay were recorded, and all the surviving larvae hatched within the time required by FET test conditions. All validity criteria required by the guideline, such as a ≤ 10% mortality rate in the control plate and in the internal controls and a mortality percentage ≥ 30% in the positive control, have been met at the end of the exposure.

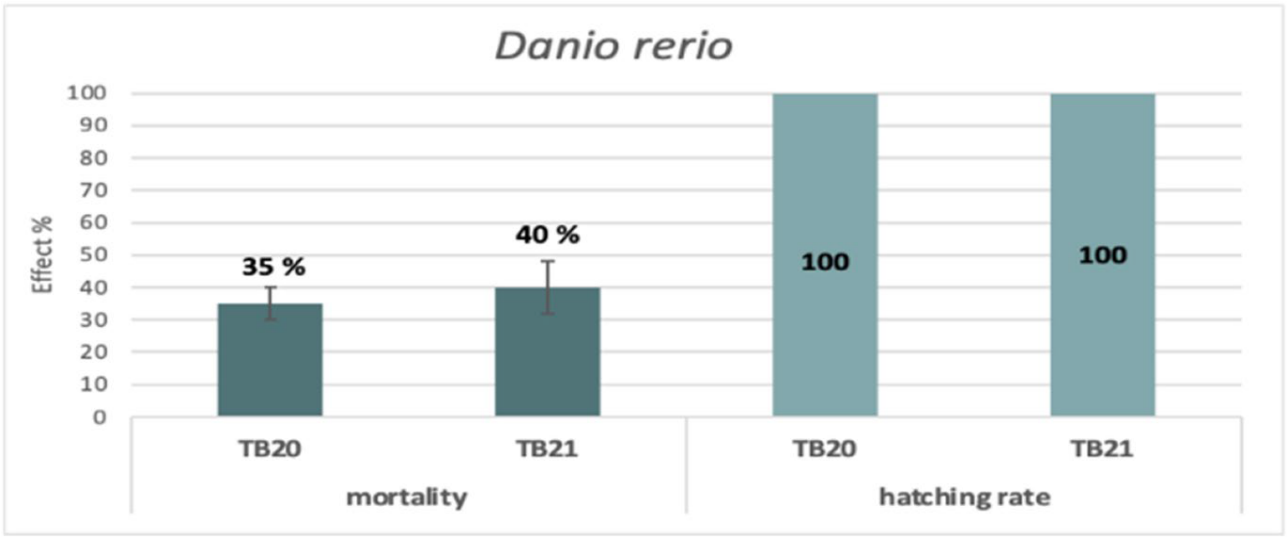

Fig. 21: Results of the Fish Embryo Acute Toxicity (FET) test—OECD 236: mortality and hatching rate expressed as mean + / − SD

Several sublethal endpoints have been observed: depigmentation, spine deformation (scoliosis and lordosis), cardiac edema, body length, and eye-size variation. Depigmentation, spine deformation, and cardiac edema have been found in TB20; spine deformation and cardiac edema in TB21 (Fig. 4). The most recurring effects in TB20 were depigmentation and spine deformity (45%), while in TB21, the most commonly recorded sub-lethal effect was spine deformation, with a value of 75% (all the deformations were attributable to lordosis).

Sample	Depigmentation	Spine deformation		Cardiac Edema
		scoliosis	lordosis	
TB20	45%	15%	30%	10%
TB21	0	0	75%	25%

Fig. 4 Sublethal effects recorded in TB20 and TB21

3.4.2 Body Length and Eye Size

The surviving individuals were analyzed with Danioscope software for the measurement of two morphological parameters: body length and eye size (Fig. 22). Both samples showed a toxic effect that is reflected in the length of the total body of the larvae and the total surface of the eyes, which appeared statistically significantly smaller in size (Fig. 22). Particularly, zebrafish larvae exposed to

TB21 showed the main effects. The effect percentages of the two samples related to the control were 7.2% (TB20) and 11.9% (TB21) for body length; 13.4 (TB20) and 25.3 (TB 21) for eye size.

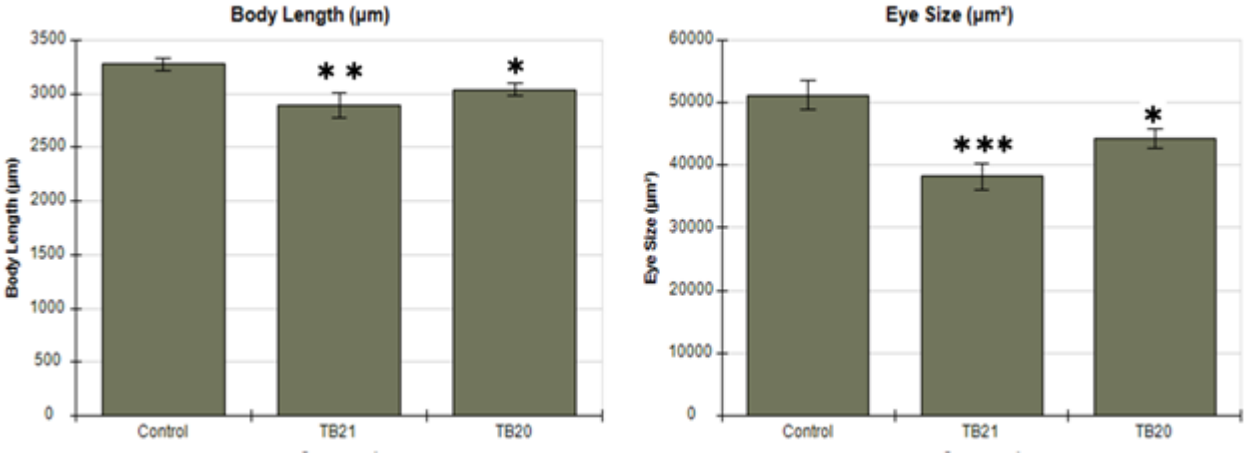

Fig. 22: Body length and eye size (mean ± SE.; n = 35 per group) in zebrafish larvae exposed to river samples TB20 and TB21 (ANOVA-on-ranks, Dunn's test). The symbol * indicates a significant difference between tested samples and negative control (*p < 0.01, **p < 0.001, ***p < 0.0001)

4.Discussion

The literature that was included in the review show numerous good quality studies published in peer-reviews journals. 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 more 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 (161), whose inhibition can be correlated with alterations of behaviour (162). In our case, all papers that tested AChE activity also observed behavioural alterations, and these two parameters showed a high correspondence (87.5%)

The results from our query highlight a growing number of research papers that fit our inclusion criteria (Figure 2). This confirms the growing popularity that the zebrafish has gained throughout the years (163); there is also evidence of the increase of the use of zebrafish for chemical screening for several classes of chemicals, such as environmental pollutants and pharmaceuticals (164).

This same growing trend can be also observed by considering the 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 (165). 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 a growing concern for the issue on a global level (Fig. 23).

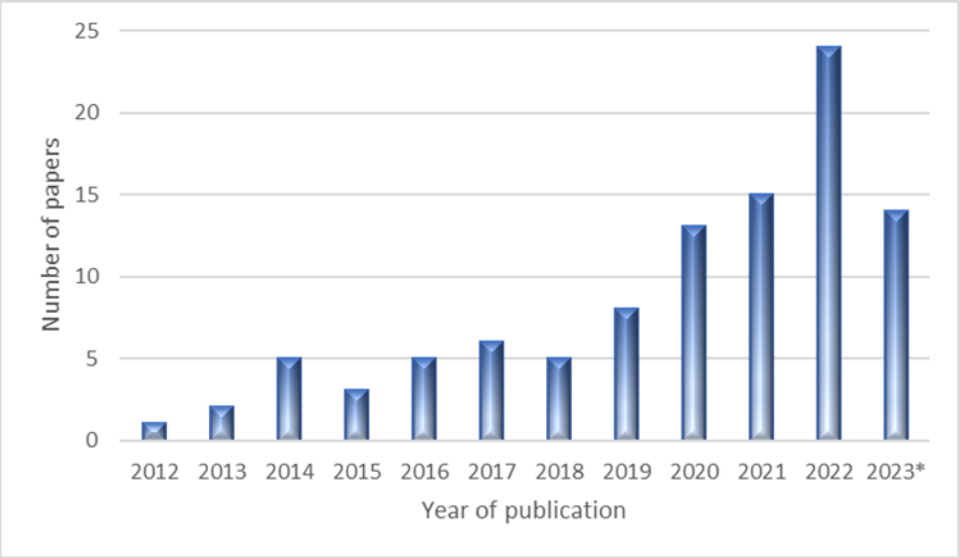

Figure 23: Year of publication of the selected papers included in this study, following the screening processes. The research included papers published between January 2012 and September 2023. (only papers included in this study are showed).

*: until September 2023

In the first 5 days 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 development of the embryo does have also practical consequences on the planning of the study, 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, and can be an interesting alternative to the classic animal testing (165). This is particularly relevant in the view of the recent "3R" strategy (Replacement, Reduction, Refinement) (166) on animal studies, since the use of zebrafish embryos could allow to reduce the use or even be a replacement to some *in vivo* studies, and provide a refined model that makes possible a quick and easy observation of the internal effects on the organism (165).

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. In relation to chemical mixtures, the study from Martin et al. (167) highlighted the possibility of detecting synergistic effects with zebrafish

embryos. Moreover, Jakobs et al. (168) compared measured with predicted toxicity of nine mixtures in zebrafish embryos to investigate the applicability of the CA and IA models for the prediction of phenotypic effects in a whole organism through a short-term acute assay, also suggesting the importance of using *in vivo* bioassays to confirm the predictivity of models such as the CA.

However, it has to be noted that the use of embryos comes with 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, although not so frequently (169). For this reason, however, in some studies the chorion is forcefully removed before the exposure (135). Moreover, the behavioural patterns of the younger stages might be not so sophisticated when compared to the adults (170). This might at least partially explain our results, where despite the large number of papers focused on short or very short exposures (less than five days, Figure 24), 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. 25).

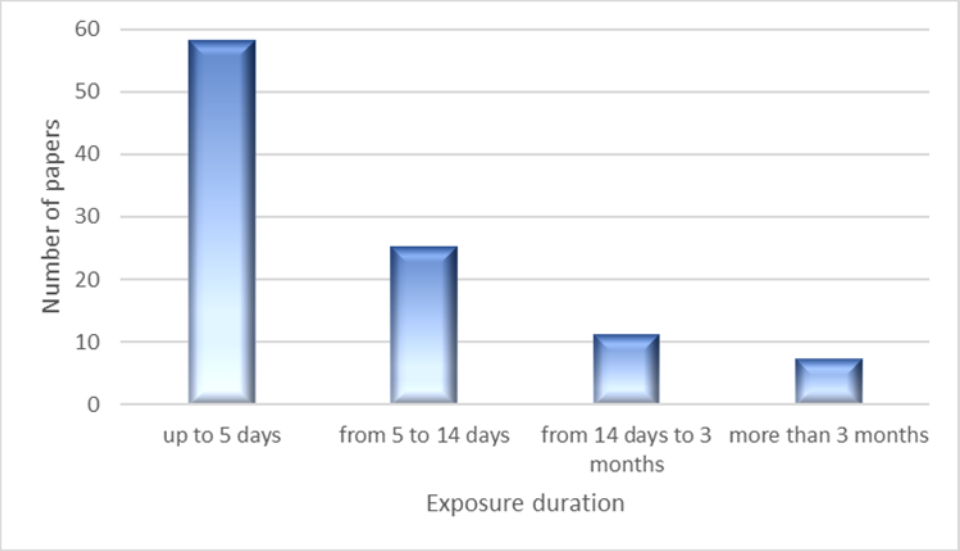

Figure 24: Categorisation according to exposure duration. The papers selected for this review are categorized according to the duration of the exposure of the chemical mixture to zebrafish. The most prevalent exposure window is “up to 5 days” (58 papers).

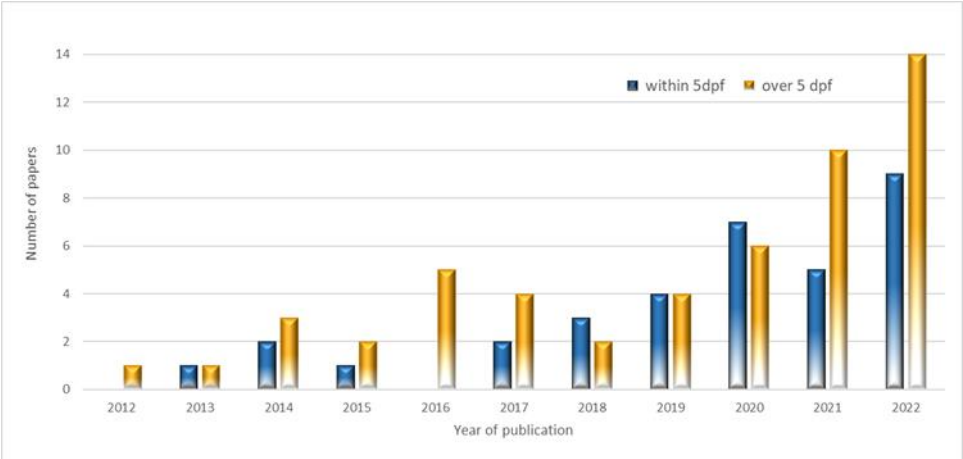

Figure 25: Categorisation based on year of publication and duration of exposure. The chart shows the number of works that conducted the chemical mixture exposures within (blue bars) or over (orange bars) 5 days post fertilisation, between 2012 and September 2023.

Finally, there is a wide variety of different behaviours that the zebrafish exhibit at every developmental stage, so 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 even in our review, where several different assays were performed. Among the behavioural endpoints, the more 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 (Figure 26). 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 (171), while the tactile and the visual startle response appear earlier, at 2 and 3 dpf respectively (53). The coiling emerges at around 17 hpf but only last a few hours, while social behaviours, such as shoaling tend to develop and gain complexity in juveniles and adults (172). 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. Very common are also the assays based on different forms of swimming activity, and this might be explained by the possibility to consider this behaviour at almost every developmental stage, since it firstly appears in a clear manner at around 4 dpf (173).

In the environmental context, the 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 also leading to health decline and vulnerability to

infections. 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 (174).

As a general statment, the studies included in this work are all published on peer-reviewed journals and show a high overall quality. However, as highlighted in a 2021 paper by Martin et al. (167), for future studies a better knowledge and description of the concepts of synergism and additivity might be desirable.

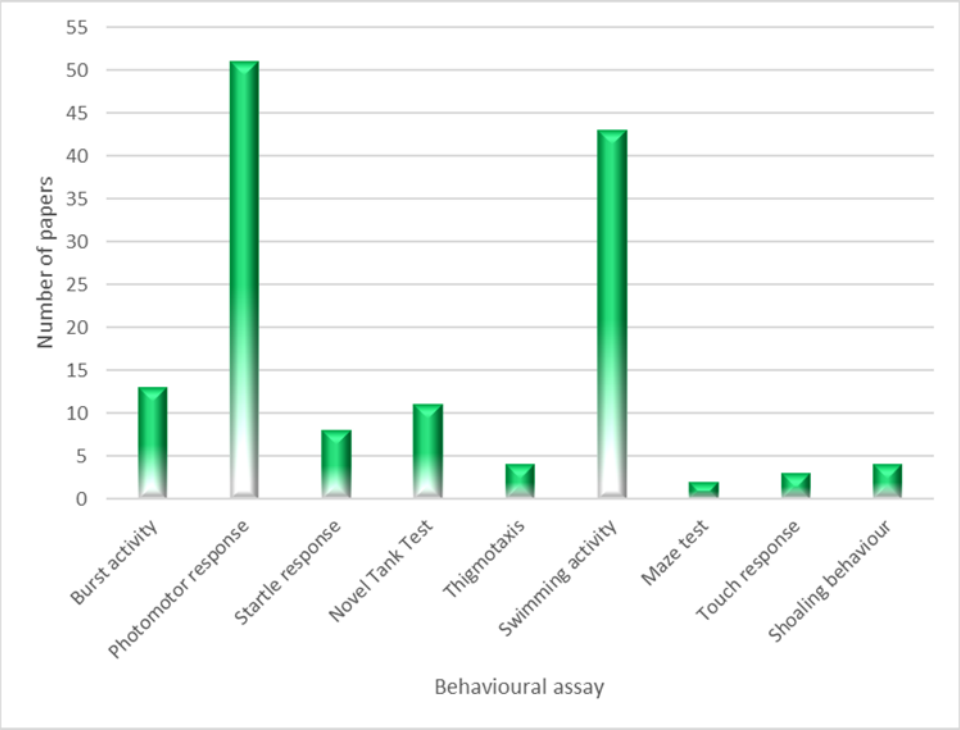

Figure 26: Categorisation of the papers based on the selected behavioural assay/endpoint. Among the assays/endpoints considered, swimming activity without stimuli (swimming activity) and swimming activity under different light conditions (photomotor response) were the most widely applied.

In the Tiber river work, EBMs and targeted chemical analysis were performed on samples in correspondence with two significant weather events that were cause of the death of thousands of fish in the years 2020 and 2021. The chemical analysis showed low concentrations of chemical pollutants for both years. Some of these compounds might show toxicity on fish, but the concentrations that were found resulted too low to hypothesize a key role of such pollutants in the aforementioned events. Among the pharmaceuticals, carbamazepine, an antidepressant widely found in aquatic environments, has been detected; this substance can cause several effects on zebrafish embryos and also on *Cyprinus carpio* (175), a fish species that has been found in the river along the course of this campaign; one of the main MoA of this contaminant is neurotoxicity. The

antibacterial sulfapyridine has also been already found in previous studies in the river (176); terbutylazine, an insecticide widely present in the Italian aquatic environments due to his large use, has also been found, but its concentrations are in compliance with the EQS defined by the Italian legislation (Dgls 172/2015). It is also evident that to show relevant effects on fish the chemical concenetrations would require values several orders of magnitude higher compared to the levels found in the river. The PFAS chemical analyses showed the presence of several congeners, but still at concentrations clearly lower than those required to have such an evident effect on the fish population. The levels detected for this class of compounds are however an indicator of the anthropogenic pressure on the area sorrounding the river basin. PFAS are considered ubiquitous contaminants, and the concentrations found are comparable to those previously detected in other projects carried out in the same river. It is also important to mention that PFAS have several chronic effects on fish, even at low concentrations. Some of the chemical contaminants detected in this study were also found in a previous study conducted in the urban part of the Tiber river (177), although the sampling sites were located in slightly different areas. The concentrations of the antidepressant carbamazepine and the PFAS were comparable with those previously found, while the levels of the insecticide DEET (10–200 ng/L) and of the anticonvulsant Gabapentin-Lactam were higher than those previously found (300 ng/L). An interesting difference compared to the previous study is the detection of the plastic additive Bisphenol S. It is also relevant to highlight that the Regional Environmental Agency ARPA Lazio (ARPA Lazio, 2021) performed monitoring campaigns. In the context of the chemical analysis carried out in 2020, the pesticide cypermethrin (0.014 µg/L) and clothianidin (0.75 µg/L), a neonicotinoid substance have been detected. In 2021, the same contaminants were found at lower levels (178).

The acute effect concentrations on fish for these substances are 104.2 mg/L for clothianidin and 0.00151 mg/L for cypermethrin (Pesticide Properties Database (herts.ac.uk). The levels of macrodescriptors (e.g., BOD5, COD) detected by ARPA Lazio immediately after the events are in the range of the normal values for the river. Furthermore, the levels of heavy metals (lead, nickel, chromium, arsenic) did not increase in comparison to previous campaigns carried out by ARPAL. The samples analyzed with the FET test show mortality values up to 40%, with several sublethal effects that occurred during the exposure: spine deformation (scoliosis or lordosis), which has been the most recurring effect, low pigmentation, variations of body length and eye size, and heart edema. Spine deformations and the other morphological malformations detected could be associated with several classes of contaminants (179) such as pharmaceuticals (ibuprofen) also in mixtures,

pesticides, PAHs, and heavy metals; also, contaminants detected in this study (e.g., carbamazepine) can give the same effects. It is also important to mention that the EBMs results are consistent with those recorded in previous sampling campaigns in the urban stretch of the river (177), in which water samples were collected also under different weather conditions. The high mortality of riverine fish concerned almost all the species and the different ecological guilds (e.g., habitat, tolerance vs intolerance, reproductivity, autochthonous vs allochthonous) that live in this stretch of the river. The fish-kills events might be caused by several abiotic and biotic factors. Godinho et al., (180) recently investigated factors related to fish-kills events in 67 Mediterranean reservoirs near the Iberian Peninsula, and they also reported a change in the likelihood of fish-kills event with the foreseen climate change. The authors outlined that such events occurred more often in shallow reservoirs with particular conditions such as lower oxygen content, larger surface area, and higher chlorophyll levels. Other factors related to mortality, such as toxic spills, were not taken into consideration by the authors, but their role cannot be excluded. In the Pacific Northwest, coho salmon (*Oncorhynchus kisutch*) inhabiting the USA, acute mortality is annually related to polluted rainfall exposure when adult individuals migrate to urban areas for reproduction. Through the application of effect-directed analysis, some researchers (181) recently identified a tire rubber antioxidant, a highly toxic quinone transformation product of N-(1,3-dimethylbutyl)-N'-phenyl-pphenylenediamine (6PPD) as the main cause.

The concentrations of chemical contaminants found in the river study are not sufficient however to justify the fish-kills events, although it has to be pointed out that it was not possible to analyze all chemical pollutants that might be have been present in the water. On the other hand, based on the results obtained with the EBMs (Fish Embryo Toxicity test), it was possible to hypothesize a role for some chemical contaminants or some specific mixtures in the fish-kills, and there are several examples in literature where chemical analysis showed low levels of contamination, while EBMs detected toxicity effects on fish (145). The results from the examinations on the fish species affected in both events suggest the involvement of the inlet water that from the Aniene river flows directly in the Tiber river a few kilometers up to the sampling site. According to the technical report published by the Regional Environmental Agency (178), this hypothesis is also supported by the fact that no dead fish was found upstream of the Aniene river inlet. Also, other water additions (i.e., drainage channels, run-off from paved urban surfaces, other untreated waters bypassing sewage plants) may have contributed to the injection of several other stressors in the river. In addition,

taking into account the analysis of the dead species, their ecological characteristics, and the proposed classification in ecological guilds, further considerations can be done: in particular, the discovery of many species classified as very tolerant (i.e., *S. glanis*) and relatively tolerant, in both the benthic area (i.e., *C. carpio, T. tinca, Barbus* spp.) and in the water-column (i.e., *C. ramada, Squalus* spp.), may suggest that a state of acute hypoxia and anoxia involved both the deepest layers of the waters (5–6 m of depth) and the entire water column, although at the same time, it is important to consider that the levels of macrodescriptors detected are in a normal range for the river. What can be assumed is that the fish-kills events can be caused by several factors, all generated by anthropogenic stress: the impact of the massive input of organic materials, the possible effects caused by specific chemical contaminants, and the combined action of biotic and abiotic factors may have contributed (182). The strong rainfall, following a prolonged period of drought, may have resulted in localized floods in small tributaries with the leaching of run off from the main water passages in the urban stretches of the Aniene-Tiber rivers; these floods mobilized the sediments which had previously accumulated at the bottom of the tributary watercourses (additionally, the sediments had been enriched by a substantial amount of organic matter as well as chemical run-off from paved urban surfaces).

5. Discussion about EBMs results and applications

The application of the FET test has been proved useful in several scientific studies in the detection of the effects of chemical pollution on living organisms, even for substances at very low concentration (183). The experimental approach of this work could higlight the presence of effects induced by single chemicals or by chemical mixtures that may not be easily assessed only through the record of lethal endpoints. Moreover, the detection of sublethal effects allows to understand the Modes of Action (MoA) of the involved substances, e.g. DNA toxicity, neurotoxicity, developmental toxicity, cardio-circulatory toxicity (184).

Underdevelopment is commonly observed in chemicals such as dioxins and pesticides (185). Two very common sublethal endpoints were spine deformities (186), that are shared effect by almost every group of dangerous substances, these effects are also linked to neurotoxic substances (49). The head portion is also injured along with eye malformations that could be potentially induced by the heavy metals (187) and biocides (188). Other typical malformations involve fin deformation and tail defects as underlined at least in studies that investigated the effects of pesticides,

organobromides, PFAS and heavy metals (189). Such effects are strongly connected to the alterations in locomotor activity and consequently to the survival rate.

There are also substances able to advance or delay the hatching time, such as organobromides (190), heavy metals (191), pesticides and the perfluorinated alkylated substances as the PFOS (192). Also, hypopigmentation or absence of pigmentation (193) have been observed in embryos exposed to alkylphenols, heavy metals and pesticides.

These substances include pharmaceuticals, pesticides, plasticizers, antibiotics, cosmetic product, personal care product and solvents.

The fish kill event at the end of May 2020 showed the death of thousands of fish of different species (e.g. *Barbus barbus, Squalius squalus, Silurus glanis, Bramis brama, Cyprinus carpio, Carassius* sp.) in the stretch between Ponte Milvio and the Ponte Marconi the 31th of May.

The analysis performed with the test FET (Fish Embryo Toxicity Test) showed embriological and neurological effects. In particular, the results of the sample taken at Ponte Milvio showed high acute toxicity with a 45% of mortality in zebrafish ebryos at 96 hpf. Furthermore, many sublethal effects occurred after the exposure; in particular, 30.3% of embryos showed deformities with spine deformations (scoliosis or lordosis) and 17.7% appeared with scarce pigmentation. Also the swimming behavior appeared abnormal, with the 24,4% of the individuals that showed swimming alterations. In the Isola Tiberina sample similar effects have been reported.

These effects can be potentially linked to the exposure to chemical substances such as pesticides. In particular, it is relevant to highlight that the chemical analysis performed by the regional local authority detected the pesticides cypermethrin (0.014 µg/L) and Clothianidin, a neonicotinoid substance (0.67 µg/L). In particular the values reported for Cypermethrin are higher compared to the river average levels. Both these substances are also included in the EU WFD list. Cypermethrin is a synthetic pyrethroid which has been used widely as pesticides/insecticide. It has been classified as Priority Substance for the WFD and for this reason its use should be reduced whenever possible. The EQS included in the Directive 2013/39/UE for this substance are the following: AA (Annual Average): 8 × 10–5 µg/L MAC (Maximum Allowable Concentration): 6 × 10–4 µg/L. It is evident that the concentrations detected in the Tiber river of Cypermethrin are much higher also of the Maximum Allowable Concentration that should protect from acute effects. In an other study (194) Zebrafish embryo toxicity test was used to determine the toxic effects of cypermethrin in the present study. Zebrafish embryos were exposed to various concentration of cypermethrin (0.001, 0.003, 0.01, 0.03 and 0.05 µg/l) and the observations on the lethal, sub lethal endpoints were

recorded. The results showed that the sub lethal and lethal effects of the zebrafish embryos increased with respect to an increase in the concentration of the cypermethrin. It is evident from this study that even low levels of Cypermethrin contamination in the aquatic environment would affect the developmental stages of fishes inhabiting the aquatic systems. In another study (195) Cypermethrin induced several abnormalities including bodyaxis curvature, pericardial edema and enlarged yolk sac in zebrafish embryos. The teratogenic lesions induced by Cypermethrin in zebrafish embryos were in accordance with the results reported.

It is probable that the overall effects detected by the EBMs are caused also by the mixtures of all these pollutants, since also before the fish kills similar effects have been observed with the use of the FET test and the chemical analysis detected several compounds. Furthermore, similar phenomena can also be caused by suspended material that may reduce the oxygen levels in the water. This hypothesis may also be plausible, since the the fish kill has been preceded by a flash storm in the area sorrounding the city of Rome and the city itself and that may have leached chemical substances and wastes in the river.

The Tiber River might be used in the near future as a reservoir for drinking water, especially considering the effects of the climate change and the consequent water scarcity (196). Water scarcity that can also increase the concentration and the effects of pollutants. The Tiber river is also affected by floodings that might be cause of cross-contamination of rivers/soil/lands and increasing run-off, remobilization of sediment chemical contaminants. Therefore, every new result on the water quality of the river should be carefully considered. Integrating EBMs, e.g. FET test into investigative monitoring for water quality management is an important tool to evaluate the risk posed by chemical substances in surface water bodies.

In general, in the Tiber river basin different ecotoxicological effects have been observed and the results can be a support for the local authorities for the application of the best measures aimed at protecting the aquatic ecosystem. The study is also a contribution to the knowledge on the quality status of the river and will help to better comprehend the sources of pollution along with some of the effects induced by chemical mixtures. A better comprehension of the chemical pollution levels in the basin, including emerging substances not included in the current legislation, would be fundamental to prevent risks for human health when there is reuse of water for agricultural and drinking purposes.

As a general comment, EBMs may reveal risks for natural communities working as environmental early warning systems. It is of major importance to highlight effects related to chemical substances

before significant effects on population level occur, because damage at the population and ecosystemic level can take a long time to be reversed. The detection of effects through the use of EBMs is indicative of the presence of bioactive compounds. The selection of EBMs to use in a particular case needs to consider case-specific circumstances. If compounds present are largely unknown (not monitored), a whole battery of EBMs, covering different trophic levels is needed. Also, knowledge about the source/s (type of pressure) is valuable in selecting a suitable battery of EBMs. In this sense, the use of zebrafish as a vertebrate could be useful, as highlighted in both the river and the veterinary pharmaceuticals studies.

In Italy the application of EBMs has been recently adopted for the Health Impact Assessment of large activities such as incinerators or refineries. The EBMs in this context have a role of screening and monitoring and can be the trigger for the adoption of further measures. If EBMs suggest an unacceptable risk, decisions on further measures might be necessary.

6. Overall Conclusions

As highlighted in the OH concept, human health largely depends upon ecosystem products and services (such as availability of fresh water, food and fuel sources) which are requisite for good human health and productive livelihoods. Significant impacts on human health can occur if ecosystem services are no longer provided.

Human activities are altering the ability of ecosystems to provide their services (e.g. food, pharmaceutical products, purification of air, water, soil, sequestration of pollutants, etc). The introduction of chemical pollutants into the ecosystem as a result of anthropic activities and global and socio-economic environmental changes gives rise to "emerging problems" (197) whom affects the environment and, directly or indirectly, human health. Therefore, the development of efficient and rapid methods (198) of screening is fundamental for the reduction of their effects and for the implementation of preventive measures. Ecosystemic approaches explore how changes in the ecosystem can have adverse impacts on human health and implement practical solutions to address these challenges.

The results of this work highlight the importance of the application of Effect Based Methods in the evaluation of the effects of chemical pollution thanks to their versatility. Among the methods that can be useful in such approach, those involving zebrafish show potential for future applications. EBMs employing zebrafish can be applied with several advantages:

- Screening Function: the detection of toxicity may suggest the presence of environmental pollution caused by chemicals, and can thus trigger further investigative activities such as source identification, analysis of the pressure and impacts, chemical monitoring.
- Early Warning: EBMs are versatile tools for early warning assessment in the before impact at population levels occur (precautionary principle).
- Detection of chemical mixtures toxicity: EBMs can detect the overall effects of chemical mixtures in the ecosystems, effects that it might be otherwise difficult to highlight through chemical analysis.
- Neurotoxicity detection: neuroactive chemicals commonly occur in the ecosystem as a result of different human activities forming simple and complex mixtures. Thus, the application of EBMs, based on behavioural analysis, is useful to improve the knowledge of the neurotoxic effects in the environment.

Also, some further recomendations can be given on the use of zebrafish for neurotoxicity assesment:

- As highlighted in our review the use of additional parameters on 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, it emerges the necessity of the development of more standardized and shared methods for neurotoxicity assessment that could improve the replicability and reproducibility.

7.Bibliography

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Abstract: Robbiati C, Milano A, Declich S, Riccardo F, Mancini L, di Domenico K, Scavia G, Dente MG. Building a European One Health workforce forprevention and preparedness to health threats. 16th European Public Health Conference 2023. Dublin, 8 – 11 november 2023

Poster: di Domenico K, Mancini L, D'Amelio S. Evaluation of the ecotoxicity induced by veterinary pharmaceuticals on the aquatic vertebrate danio rerio through a one health approach. PhD day 2023, Roma. 6 november 2023

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Poster: Walter Cristiano W, Chiara De Marchi C, Kevin di Domenico K, Ornella Punzo O, Aurora Mancini A, Laura Mancini L. The Elephant In The Room In Greenhouse Gases Emisions: Rethinking Healthcare Systems To Face Climate Change. Abstract inviato alla Setac Europe 2024, Siviglia, 5 - 9 may 2024.

Poster: Ines Lacchetti I, Giulia Gatti G, Kevin di Domenico K, Walter Cristiano W, Melissa Barra M, Aurora Mancini A, Mario Carere M, Laura Mancini L. Neurotoxic effects of antidepressants on zebrafish embryos. Abstract inviato alla Setac Europe 2024, Siviglia, 5 - 9 may2024.

Poster: Carere M, Lacchetti I, Cristiano W, di Domenico K, Mancini L. Investigative monitoring with Zebrafish embryo test in the urban part of a river after a fish-kill event in central Italy. SETAC Europe 31th Annual Meeting, 3-6 may 2021

Poster: Cristiano W, Lacchetti I, Mancini L, di Domenico K, Corti M, Di Paolo C, Hollert H, Carere M. Alternative methods to animal testing: the zebrafish earliest life stages as a tool for the detection of chemical pollution effects in the context of Water Framework Directive. SETAC Europe 30th Annual Meeting, 3-7 may 2020

Poster: Lacchetti I, Cristiano W, di Domenico K, Corti M, Mancini L, Carere M. Application of effect-based methods (EBMs) in a river basin: a preliminary study in Central Italy. SETAC Europe 30th Annual Meeting, 3-7 may 2020

Poster: Lacchetti I, Cristiano W, di Domenico K, Carere M, Mancini L. Spontaneous tail movement in zebrafish embryo as indicator of neutoroxic substances in the environment. 20th MESAEP International Symposium, 25-28 april 2020

Poster: di Domenico K, Carere M, Lacchetti I, Cristiano W, Mancini L. Plants biodiversity in relation to medicine and human well-being. 20th MESAEP International Symposium, 25-28 april 2020

Acknowledgements

A special thanks goes to the head of my unit, Laura Mancini, who gave me the motivation to participate in this PhD course and that has always been a great example of experience and competence. I also would like to thank her for all the opportunities she gave me throughout the years that allowed me to further develop myself as a researcher.

I would also like to thank my colleagues of the Ecosystems and Health Unit of the ISS, and especially Mario Carere and Ines Lacchetti for all the support and the supervision throughout the years and in particular during this thesis. Thanks to them I was able to approach the world of the ecotoxicology in all its complexity.

Finally, I would like to thank Professor Stefano D'Amelio of the University "La Sapienza" in Rome for all the support along these 3 years of Doctorate.