Science of the Total Environment 698 (2020) 134299

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Antagonistic effects in zebrafish (*Danio rerio*) behavior and oxidative stress induced by toxic metals and deltamethrin acute exposure

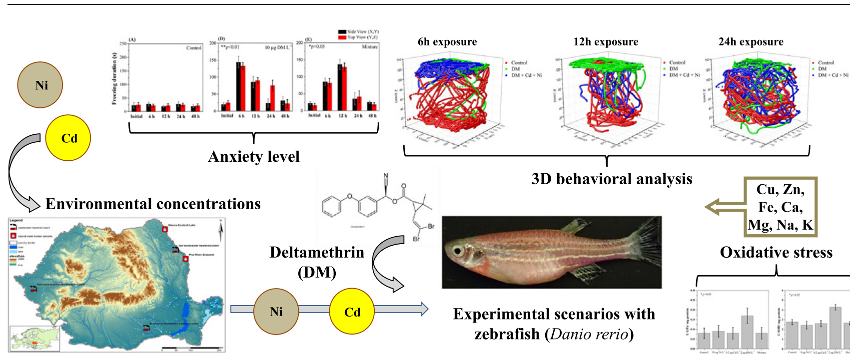
Roxana Jijie [a], Gheorghe Solcan [b], Mircea Nicoara [c,d,*], Dragos Micu [e], Stefan-Adrian Strungaru [a,c,*]

[a] *Alexandru Ioan Cuza University of Iasi, Department of Research, Faculty of Biology, Bd. Carol I, 20A, 700505 Iasi, Romania*
[b] *University of Agricultural Science and Veterinary Medicine "Ion Ionescu de la Brad", Department of Molecular Biology, Histology and Embriology, Faculty of Veterinary Medicine, 8, Mihail Sadoveanu Alley, 700489 Iasi, Romania*
[c] *Alexandru Ioan Cuza University of Iaşi, Doctoral School of Geosciences, Faculty of Geography-Geology, B-dul Carol I, 700505 Iasi, Romania*
[d] *Alexandru Ioan Cuza University of Iasi, Department of Biology, Faculty of Biology, Bd. Carol I, 20A, 700505 Iasi, Romania*
[e] *Romanian Waters National Authority, Dobrogea - Black Sea Basin Administration, Mircea cel Batran Blvd. 127, RO-900592 Constanta, Romania*

HIGHLIGHTS

- The acute combined effects of Cd, Ni and DM on zebrafish
- Cd and Ni had no effects for the studied concentrations.
- DM induced oxidative stress and behavior impairment.
- DM in mixture with heavy metals was less toxic for the studied variables.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 July 2019
Received in revised form 30 August 2019
Accepted 3 September 2019
Available online 04 September 2019

Editor: Henner Hollert

Keywords:
Zebrafish
Deltamethrin
Toxic metals
Joint effects
3D tracking analysis
Oxidative stress

ABSTRACT

In natural environments, the aquatic organisms are exposed to complex mixtures of chemicals which may originate from natural sources or from anthropogenic activities. In this context, the aim of the study was to assess the potential effects that might occur when aquatic organisms are simultaneously exposed to multiple chemicals. For that, we have studied the acute effects of cadmium ($0.2\ \mu g\ L^{-1}$), nickel ($10\ \mu g\ L^{-1}$) and deltamethrin ($2\ \mu g\ L^{-1}$) as individual toxicants and as mixture on the behavioral responses, oxidative stress (SOD and GPx), body electrolytes and trace metals profiles of zebrafish (*Danio rerio*). So far the scientific literature did not report about the combined effects of pesticides and toxic metals on zebrafish behavior using a 3D tracking system. Compared with other studies, in the present paper we investigated the acute effects of two heavy metals associated with a pesticide on zebrafish, in the range of environmentally relevant concentrations. Thus, the environmental concentrations of cadmium and nickel in three rivers affected by urban activities and one river with protected areas as background control were measured. The observations that resulted in our study demonstrated that deltamethrin toxicity was significantly decreased in some of the behavioral variables and oxidative stress when combined with Cd—Ni mixture. Consequently, our study supports previous works concerning the combined toxicity of environmental chemicals since their simultaneous presence in the aqueous environment may lead to higher or lower toxicological effects on biota than those reported from a single pollutant. Therefore, the evaluation of toxic effects of a single contaminant does not offer a realistic estimate of its impact against aqueous ecosystems.

* Corresponding authors at: Alexandru Ioan Cuza University of Iaşi, Doctoral School of Geosciences, Faculty of Geography-Geology, B-dul Carol I, 700505 Iasi, Romania.
E-mail addresses: mirmag@uaic.ro (M. Nicoara), stefan.strungaru@uaic.ro (S.-A. Strungaru).

https://doi.org/10.1016/j.scitotenv.2019.134299

This study also supports the idea that the interactions between different chemical compounds which do not exceed the maximum permitted limits in environment may have benefits for aquatic life forms or be more toxic.

1. Introduction

In recent years, heavy metals concentrations have exceeded the natural background levels in many surface waters, as a result of fast urbanization, agricultural and industrial practices (Fazio et al., 2014; Martínez-Guijarro et al., 2019; Strungaru et al., 2017; Vareda et al., 2019; Zhang et al., 2018). While, elements like Fe, K, Cu, Zn, Ca, and Mg, defined as essential because are necessary for maintenance of health, can be toxic to aquatic organisms when present in excessive concentrations, metals such as Hg, Cd, Ni, Pb pose a serious threat to the aquatic environment even in trace amounts. Thus, due to their toxicity even at low concentrations, chemical stability and affinity to accumulate in organisms, cadmium, nickel, lead, mercury and its components are listed as priority pollutants under the Environmental Quality Standards Directive (2008/105/EC).

In addition to the inorganic compounds, pesticide residues are a significant threat to aquatic ecosystems. Due to a wide spread use it is no surprise that they can be present in surface waters, and potentially affect non-target organisms (Allinson et al., 2015; Fang et al., 2019; Feo et al., 2010; Kapsi et al., 2019; Stara et al., 2019). Over the last two decades the use of pyrethroids has steadily increased and presently they comprise one-quarter of the pesticides sales all over the world (Huang et al., 2014). The pyrethroids are widely used for pest control in the agricultural fields, against crawling or flying home pests, animal and human parasites, as well as for public health concerns as a replacement of the more toxic organochlorines and organophosphates. According to their chemical structure they may be classified in type I (e.g. alletrin, permethrin, piretrin) and type II (deltamethrin, spermethrin, cypermethrin) α cyano group. Several monitoring studies have confirmed the presence of pyrethroid residues in surface waters and sediments, with concentrations ranging from 0.73 ng L^{-1} to 24 μg L^{-1} and from 8.27 to 473 ng g^{-1}, respectively (Allinson et al., 2015; Feo et al., 2010; Lao et al., 2010; Pawlisz et al., 1998; Turgut, 2003; Xue et al., 2005). Although because of their hydrophobicity (Laskowski, 2002), they have the tendency to sorb to any type of suspended solids rather than remain freely dissolved in water, the dissolved fraction may be much more mobile, making pyrethroids more available to aquatic organisms (Liu et al., 2004).

There is a considerable amount of research on the effects of exposure to a particular contaminant (Nabinger et al., 2018; Zheng et al., 2016b), although in the natural environment the massive exposure to a single pollutant is rare. The aquatic organisms are exposed not just to a single pollutant, but rather to multiple chemicals such as heavy metals, pesticides, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pharmaceuticals, personal care products and others (Boulanger et al., 2019; Fang et al., 2019; Feo et al., 2010; Lao et al., 2010; Turgut, 2003). Therefore, the evaluation of their acute and chronic effects individually does not offer a close to reality view of their impact on biological processes. Basically, the methods for mixture toxicity studies can be divided into two major classes, such as whole mixture approaches and component-based analyses. Further, to link the toxicity of the individual components with the effects of the mixture, several concept based on the three basic types of action for mixture of chemicals, like similar action, (also known as dose/concentration addition), dissimilar action (synonymous to independent action) and interactions, have been implemented. Briefly, dose/concentration addition and independent action are based on the assumption that chemicals in a mixture do not influence each other's toxicity, these can either have the same molecular site of action, or they act independently and may have different molecular target sites and modes of action. Whereas interaction describes the effect of multiple components as stronger (e.g. synergistic, potentiating, supra-additive) or weaker (e.g. antagonistic, inhibitive, subadditive, infra-additive) that would be expected on the basis of concentration addition, independent action or the combination index-isobologram models. (Godoy and Kummrow, 2017; Jonker et al., 2016; Scientific Committee on Consumer Safety, 2012). In general, the biochemical, immunohistochemical, histopathological and neurotoxic effects of pesticides, heavy metals and their mixtures are observed on the target organisms. The effects observed depend on the chemical nature of the pollutants involved, their concentration range, exposure time and studied species, and include changes of trace metals concentrations in the body (e.g. selenium) (Strungaru et al., 2019a) and of antioxidant levels (Kuder and Philip, 2017; Ling et al., 2011), inhibition of AChE (Pérez et al., 2013) and a significant impairment of the locomotory activity, such as hyperactivity, erratic swimming, muscle twitching, swimming at the water surface, paralysis, loss of the equilibrium and eventually death (Huang et al., 2014; Kienle et al., 2009; Pérez et al., 2013; Strungaru et al., 2019a).

Danio rerio (Hamilton, 1822), commonly known as the zebrafish, is widely used as vertebrate model for prediction of pollutants (neuro) toxicity in aquatic systems due to their complex behavioral responses. Behavioral tests appear ideal for monitoring and assessing the effects of aquatic pollutants on fish populations (Huang et al., 2014; Kienle et al., 2008, 2009; Kung et al., 2015; Nabinger et al., 2018; Strungaru et al., 2019a; Zhou et al., 2019). Because the animal behaviors integrate responses to internal (physiological and/or biochemical) and external (environmental, social) consequences, behavior endpoints are more sensitive and fast compared to traditional toxicological methods assessing the lethality, growth and reproductive effects (Melvin and Wilson, 2013). Thus, behavioral changes of an organism as effects to contaminants may be measured a short time after exposure and at relatively low concentrations. For example, swimming changes in zebrafish exposed to deltamethrin have been detected at a concentration as low as 1% of the LC50-24 h (~0.15 μg L^{-1}) within 5 h (Huang et al., 2014). In our previous study we demonstrated that chronic exposure to 0.5 μg L^{-1} deltamethrin may induce significant damage to zebrafish brain, resulting in a significant alteration of its behavior (Strungaru et al., 2019a). We have shown that behavior was the most sensitive parameter compared with developmental and survival parameters.

Although zebrafish exhibit complex 3D swimming patterns, most of the behavioral studies rely on 2D data, in which the zebrafish behavior analysis is based on videos recorded using a single camera (Strungaru et al., 2019a). In the past years, considerable efforts have been devoted to the development of high-speed 3D automatic tracking systems that allow the analysis of zebrafish behavior in a 3D space (Cachat et al., 2011; Maaswinkel et al., 2013b; Qian and Chen, 2017; Rosemberg et al., 2011; Stewart et al., 2015). The 3D approach offers a marked advance in the existing 2D methods, providing a more comprehensive and accurate description and analysis of fish locomotory patterns. According to a prior study compared to a 3D observation, the 2D data may be flawed by over- or under-reporting of locomotory differences (Macrì et al., 2017). The 3D analysis shows great potential in many applications, include neurophenotyping, drugs screening and the study of anxiety-like behavioral responses in zebrafish (*Danio rerio*) (Cachat et al., 2010, 2011; Green et al., 2012; Maaswinkel et al., 2012, 2013a; Stewart et al., 2015).

In this context, the aim of our study was to assess the potential effects that occur when aquatic organisms are simultaneously exposed to multiple chemicals. For that we have studied the acute effects of cadmium, nickel and deltamethrin, as single chemicals and as mixture, on the behavioral responses, on the superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and on the body electrolytes and trace metals profiles of zebrafish (*Danio rerio*). Compared with other studies, in the present work we studied the acute effects of two heavy metals associated with a pesticide on zebrafish, in the range of environmentally relevant concentrations. In addition, no data are available on combined effects of pesticides and heavy metals on zebrafish behavior measured using a 3D tracking system.

2. Materials and methods

2.1. Ethical note

The animals were strictly maintained and treated according to EU Commission Recommendation (2007) on guidelines for the accommodation and care of animals used for experimental and other scientific purposes, Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. This experiment has been approved by Ethical Commission from Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Iasi, with the registration number 113/04.02.2019.

2.2. Chemicals

Nickel standard solution (1000 mg L^{-1}, 119792), cadmium standard solution (1000 mg L^{-1}, 119777), nitric acid 65% Suprapur® (HNO_3, 1004411000), hydrogen peroxide 30% Perhydro® (H_2O_2, 1072101000) were purchased from Merck, Germany. Superoxide Dismutase Determination Kit (SOD, 19160-1KT-F), Glutathione Peroxidase Cellular Activity Assay Kit (GPx, CGP1-1KT), Total Protein Kit, Micro Lowry, Peterson's Modification (TP0300-1KT) were from Merck, Germany. Physiological saline (0.90% NaCl) was purchased from Hemofarm (Romania). The deltamethrin used in the study is the active compound of a well-known insecticide (100 g L^{-1} DM) purchased from local market with quality certification. According to the producer, it had a DM purity of minimum 985 g kg^{-1}. To avoid any conflict of interest for this product, the brand name of the producer was kept under anonymity. We chose to use it in the study in order to investigate a scenario similar to a real case.

2.3. Assessment of Ni and Cd concentrations in Romanian treated urban wastewater and in freshwater

The presence of heavy metals has been investigated in 3 rivers (Bahlui, Bega and Dambovita), 400 m downstream from the emissary of three wastewater treatment plants located in Romania's largest cities (Iasi, Timisoara and Bucharest), and also in two control areas (to set the natural background concentrations) located on Prut River and Stanca-Costeşti Lake - a protected Romanian area (ROSPA0058) (Fig. 1). The results revealed higher concentrations of Ni and Cd in all samples near cities, wastewater treatment plants and protected areas (not published data). The treated urban wastewater and the freshwater samples were collected in October 2018, ten replicates per site in 250 mL polyethylene (PE) sterile bottles, prewashed with sampling water and acidified to a pH < 2 with HNO_3 for preservation. In the laboratory, each water samples was filtered and measured a volume of 50 mL that was mixed in TFM pressure vessels with 1.5 mL HNO_3. This was prepared by a 3 steps program of the Berghof's microwave digestion system speedwave MWS-2 (Strungaru et al., 2017). After mineralization, the samples were kept in sterile 50 mL PE bottles for metal analysis. Metal concentrations were measured with an atomic absorption spectrometer (HR-CS GF-AAS, ContrAA 600-Analytik Jena, Germany) calibrated with certified nickel (1000 mg L^{-1}) and cadmium (1000 mg L^{-1}) standard solutions that were diluted with ultrapure water produced by the LabosStar (Siemens). The final validation of the methods and results was done with European Reference Materials with certificate of analysis ERM-CA713 wastewater. This is already prepared by the producer according to its certification. A number of 6 samples were done by dilution 1:3 of reference material in a 1.5 mL sampling cup with 0.05% (HNO_3) acidified ultrapure water.

2.4. Experimental animals

Adult zebrafish (8–12 months, mass = 0.48 ± 0.03 g, body length = 28.5 ± 0.7 mm) were obtained from local suppliers and maintained in 50 L glass tanks containing 100 fish at 26 ± 1 °C under a 10 h light/14 h dark regime. Fish were fed with TetraMin Tropical Flakes twice a day, a 1% body mass daily ration. The aquarium was equipped with trickling filters and constantly aerated. The medium was renewed every 24 h to avoid the intoxication with harmful nitrogen compounds resulted from degradation of organic matter (e.g. NH_4^+, NO_2^- and NO_3^-). In addition, the water parameters were daily measured by using the HI 9828 Multiparameter Portable Meter. The water parameters in housing aquarium were similar to the experimental aquariums: pH 7.6 ± 0.1, conductivity 542 ± 3 μS cm^{-1}, salinity 0.26 ± 0.05, TDS 265 ± 3 (mg L^{-1}), ORP (+362 ± 5).

2.5. Acute toxicity tests with nickel, cadmium, deltamethrin and their mixture

In order to understand the combined toxic effects of environmental chemicals on aquatic organisms, as well as the potential interactions among chemicals, zebrafish were exposed to two heavy metals associated with a pesticide. The experimental setup was designed according to OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems. Fish acute toxicity test No. 203. After the acclimatization for 1 month, zebrafish were randomly separated into five groups of 7 animals and housed in a 7 L glass tanks with the respective treatment. The tanks have been filled with dechlorinated tap water that was constantly aerated by an air pump. One group served as control and the other four as exposed groups (Fig. 2). Zebrafish were exposed for a period of 48 h to 10 μg Ni L^{-1}, 0.2 μg Cd L^{-1}, 2 μg DM L^{-1} and to their mixture. The concentration of heavy metals was chosen according to the highest concentrations measured in the studied areas presented in Table 1 while DM concentration was chosen based on the values reported in the literature by (Feo et al., 2010; Pawlisz et al., 1998) and the results provided by our previous studies (Strungaru et al., 2019a, b), from which we already known that it is a non-lethal concentration. Water samples (50 mL) were collected from each aquarium at the beginning and at the end of experiments, acidified with 500 μL of 65% nitric acid and kept for total metal measurements.

Feeding was suspended 24 h prior to and also during the experiment to avoid any effects resulting from the feed. In addition, before starting the behavioral tests, during 4 days zebrafish were gently hand-netted from their experimental tank to a temporally tank in order to get used with the stress of being caught and transferred in the novel tank for 3D observations.

After the behavior testing was completed, fish from the control and the exposed groups were decapitated and stored frozen until the enzyme activities, body electrolytes and metal profiles analysis. Three replicated experiments were carried out for the control and for each test concentration (7 zebrafish with 3 experimental replicas).

2.6. 3D analyses of zebrafish behavioral responses

The behavioral measurements were performed in a 7 L glass trapezoidal tank filled with 6 L of dechlorinated tap water by using the Track3D module of EthoVision XT 14 video tracking software (Noldus Information Technology, Holland). The 3D approach combines the information resulted from two cameras positioned orthogonal to one-another to provide a top view (X,Y) and a front view (Y,Z) of fish

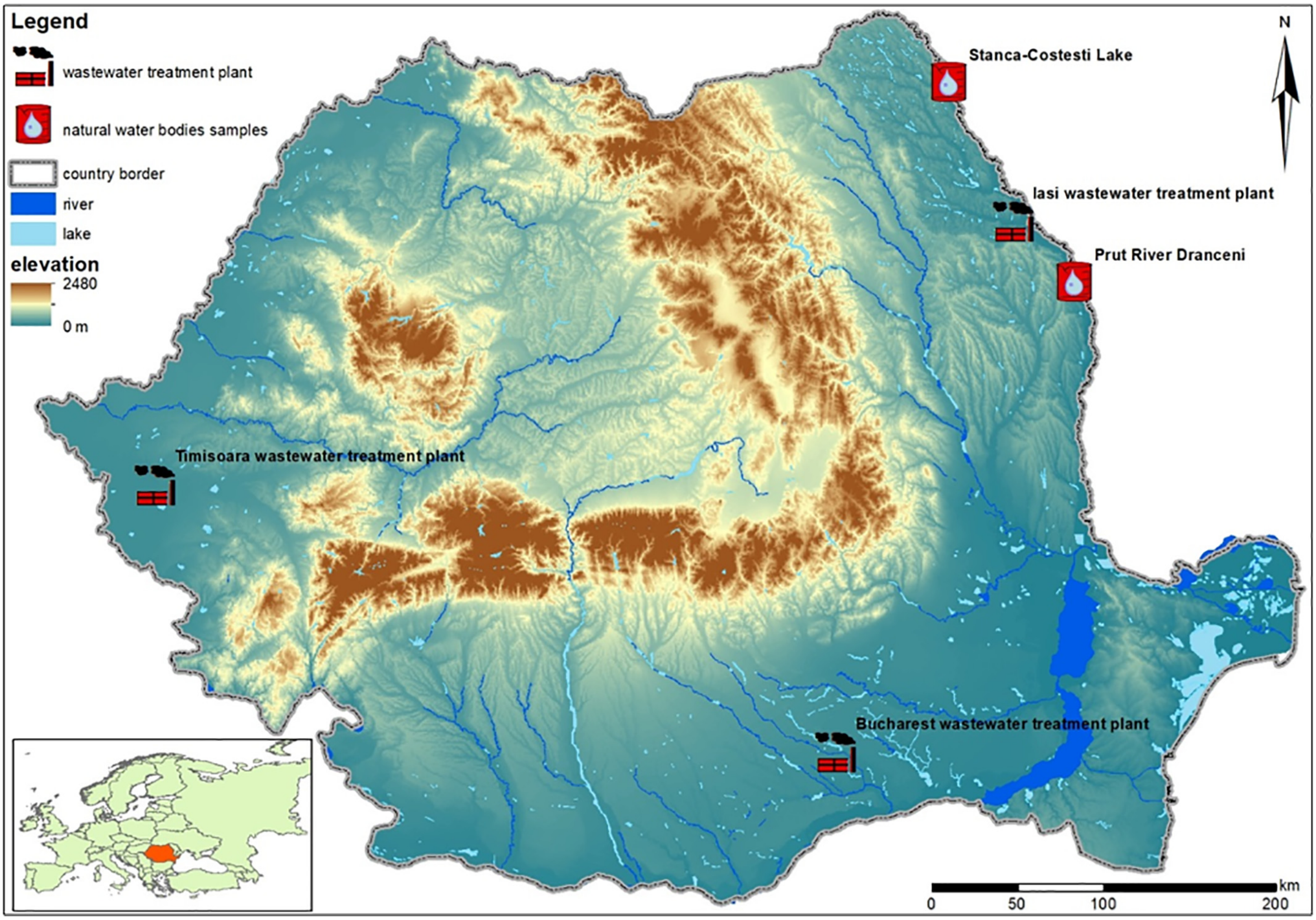


Fig. 1. Map of Romania with the different sampling sites (realized with ArcGIS 10.2 software).

swimming (Fig. 2). Following the synchronization of the two videos, this method integrates the position and information from the top and front views, providing a wide range of kinematics parameters as well as the fish trajectory resulting in a 3D image. The video files recorded with the two cameras were analyzed with EthoVision XT 14 software, generating the 2D coordinates of the traces on a track file. Then, the track files (one file for each camera) were imported into Track3D for analysis and visualization of the traces in 3 dimensions. In addition, prior the first recording, it is necessary to calibrate the 3D space in which the fish is tracked (Stewart et al., 2015). The time length of the trial was 4 min for each individual and experimental condition such as exposure scenario (control, 10 μg Ni L^{-1}, 0.2 μg Cd L^{-1}, 2 μg DM L^{-1}

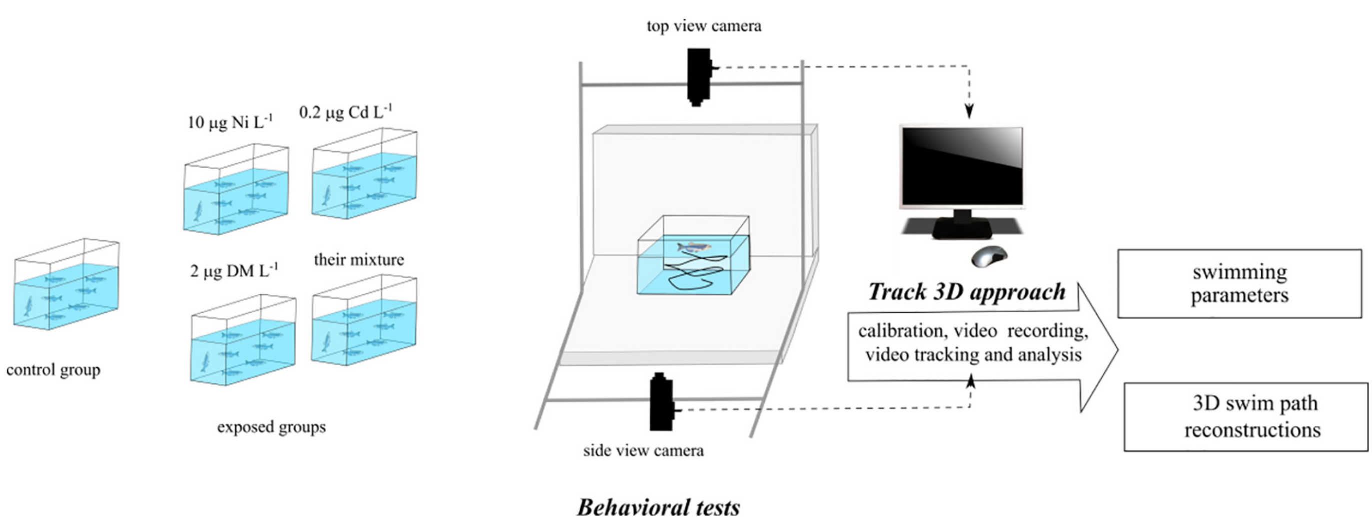


Fig. 2. Illustration of experimental setup for zebrafish behavioral measurements.

Table 1
Nickel and cadmium concentrations (average ± SD, n = 10) measured in the water sampling sites.

	Sampling 400 m downstream the wastewater treatment plants			Control area (protected area)	
	Bahlui River Iasi	Bega River Timisoara	Dambovita River Bucharest	Stanca-Costesti Lake	Prut river Dranceni
Ni ($\mu g\ L^{-1}$)	4.02 ± 0.21	2.1 ± 0.17	**9.39 ± 0.6**	2.03 ± 0.4	2.67 ± 0.18
Cd ($\mu g\ L^{-1}$)	0.065 ± 0.019	0.061 ± 0.009	**0.25 ± 0.027**	0.068 ± 0.01	0.09 ± 0.005

Reference material ERM-CA713 wastewater (n = 6): Cd certified value 5.09 ± 0.2 $\mu g\ L^{-1}$, Cd obtained value 5.12 ± 0.14 $\mu g\ L^{-1}$; Ni certified value 50.3 ± 1.4 $\mu g\ L^{-1}$, Ni obtained value 49.8 ± 1.7 $\mu g\ L^{-1}$.
The concentration of heavy metals used in acute toxicity tests were shown by bold font.

and to their mixture) and exposure time (control, 6 h, 12 h, 24 h and 48 h). Initially, each experimental group is studied to set the baseline behavior. This was presented in our study as initial behavior of the group. Each group required an experimental accommodation time before experiment for 96 h. After each 24 h every specimen was tested using the 3D approach. The average values of these tests before treatment (including control) represent the initial behavior. The discussions on behavioral response results were done for each group with comparisons between initial behavior and treatments based on the averages resulted after experimental repetition. Further, to assess the antagonistic responses offered by swimming behavior of the zebrafish, the following behavioral endpoints were analyzed: the total distance moved in the 3D space (mm), velocity (mm s^{-1}) and freezing duration (s). Freezing duration was defined as the absence of body movements excluding opercular movements.

2.7. Whole body ions and trace metals analysis

Each fish (3 specimens per experimental group) was washed several times with ultrapure water (Siemens Labostar water purification system), grinded, weighed (0.4 g) and microwave digested with 4 mL 65% HNO_3 and 2 mL 30% H_2O_2 (Plavan et al., 2017). After mineralization, the samples were transferred into 50 mL flasks and diluted with ultrapure water until was reached a 50 mL volume. Ten samples were prepared for each studied group. Copper (Cu), cadmium (Cd) and nickel (Ni) concentrations were measured by HR-CS GF-AAS (ContrAA 600-Analytik Jena, Germany) following the method described by Plavan et al. (2017) and sodium (Na), calcium (Ca), magnesium (Mg), potassium (K), iron (Fe) and zinc (Zn) using air-acetylene FL-ASS with hollow-cathode lamps (GBC Avanta, Australia). The equipment was calibrated with certified standard solutions (1000 mg L^{-1} from Merck, Germany) for each investigated element. The methods were validated with a reference material for fish muscle (ERM-BB422), certified by the EU Joint Research Center Institute for Reference Materials and Measurements. They were prepared from this material 6 samples with same digestion protocol. All concentrations were reported as $\mu g\ g^{-1}$ wet weight ± SD.

2.8. Enzyme activities assay

On completion of exposure, each fish (4 specimens per experimental group) was homogenized in 10 volumes of ice-cold saline (0.90% NaCl) and centrifuged at 5500 rpm for 10 min at 4 °C, according to a previously reported protocol (Jin et al., 2015; Ni et al., 2019). Subsequently, the supernatant of each sample was transferred to a clean tube and was divided into aliquots for SOD, GPx and total protein analysis. Ten samples were prepared for each studied group. The SOD and GPx levels, as well as the protein concentrations of tissues suspensions, were determined using the assay kits from Merck with their protocols. Briefly, the SOD activity assay was based on the use of Dojindo's highly water-soluble tetrazolium salt (WST-1). The rate of WST-1 reduction by superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, the IC50 of SOD can be determined by a colorimetric method. One unit of SOD activity was defined as the amount of the enzyme in 20 μL of sample solution that inhibits reaction of WST-1 with superoxide anion by 50% at 450 nm per mg protein in the fish. GPx activity was determined by quantifying the oxidizing rate of glutathione (GSH) to oxidized glutathione (GSSG), catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ is indicative of GPx activity. One unit of GPx will cause the formation of 1 μmol of $NADP^+$ from NADPH in 1 min per mg protein in the fish at pH 8 at 25 °C in the presence of reduced glutathione, GR and tert-butyl hydroperoxide. A procedure based on Peterson's modification of the micro Lowry method was used for quantification of total proteins in fish. The procedure is based on two chemical reactions, biuret reaction which is followed by the reduction of the Folin & Ciocalteu's phenol reagent, which yields a purple color. Absorbance at 750 nm recorded by the Specord 210 Plus from Analytik Jena (Germany) was used to determine protein concentration using bovine serum albumin as a standard. Data were expressed as average ± SD.

2.9. Statistical analysis

The Shapiro-Wilk test, for the data distribution, was firstly applied for all sets before conducting any comparison test. The one-way ANOVA test, followed by the Tukey HSD test, was performed in order to demonstrate the significant variance of the investigated behavioral variables, from the initial condition of the subjects (initial behavior) to the end of exposure (Strungaru et al., 2019a). For metals and oxidative stress was analyzed the variance and differences between control and treatments groups with one-way ANOVA test, followed by the Tukey HSD test. The statistical analyses were carried out using OriginPro v.9.3 (2016) software designed and created by OriginLab Corporation, USA. The results were presented as average ± SD and statistical significance was assumed at $p < 0.05$. The video trials resulted from the behavioral tests were sorted in files for each group. These were reused and combined in EthoVision XT 14 to generate average heat maps for experiments. We combined the results of all 7 specimens per group to obtain the average heat maps. With the Track3D module were combined the data for each group for the averages that were represented in 3D figures.

3. Results and discussion

3.1. The environmental pollutants concentrations

As showed in Table 1, the values that we measured for Ni and Cd for treated urban wastewater and freshwater samples were below the annual average values (AA-EQS) or maximum allowable concentrations (MAC-EQS) defined in the Annex 1 of the Directive 2008/105/EC. An annual average value (AA-EQS) of 20 $\mu g\ L^{-1}$ is reported for Ni and a maximum allowable concentration (MAC-EQS) ranging from 0.45 to 1.5 $\mu g\ L^{-1}$, depending on the hardness of the water, is proposed for Cd.

In surface waters, heavy metals are often mixed with other pollutants, such as pesticide residues that can reach the aquatic environment through direct runoff, leaching or improper practices such as careless washing of equipment and disposal of empty containers. For examples, Turgut (2003) proved the presence of organochlorinated pesticides and metals in the Küçük Menderes River from Turkey, despite the fact that

Table 2
Examples of acute and chronic toxicity tests on zebrafish for the selected aquatic pollutants reported in the literature.

Classes	Exposure	Animals	Concentrations	Exposure time	Endpoints	Reference
Heavy metals	Cd	Adult female zebrafish	1 mg L^{-1}	24 h and 96 h	Oxidative stress and immunotoxicity	(Zheng et al., 2016b)
	Cd	Adult male zebrafish	2 μg L^{-1} 10 μg L^{-1}	7 and 21 days	Gene expression	(Gonzalez et al., 2006)
	$CdCl_2$	Adult zebrafish	1 mg L^{-1}	16 days	Accumulation and cytotoxicity in the brain	(Favorito et al., 2011)
	$CdCl_2$ $K_2Cr_2O_7$	Zebrafish embryo/larvae	1–10 μM Cd 3–30 μM Cr	96 hpf	Oxidative stress, immunotoxicity and behavioral assessment	(Jin et al., 2015)
	Cd, Zn	Adult zebrafish	30 μg Cd L^{-1} 180 μg Zn L^{-1}	5 weeks	Growth performance, survival rate, histological observations and oxidative stress	(Zheng et al., 2016a)
	Cd, Cu and their mixture	Adult zebrafish	0.025 μM Cd 0.05–0.5 μM Cu	48 h	Uptake routes	(Komjarova and Bury, 2014)
	mixture of Cd, Cu, Ni, Pb and Zn	Adult zebrafish	0.0125–0.2 μM Cd 0.025–0.4 μM Cu 0.1 μM Ni 0.025 μM Pb 0.1 μM Zn	46 h	Uptake routes	(Komjarova and Blust, 2009)
	$ZnCl_2$, $Cd(CH_3COO)_2$, $HgCl_2$, $Pb(CH_3COO)_2$	Adult zebrafish	5 mg Zn L^{-1} 100 μg Cd L^{-1} 20 μg Hg L^{-1} 20 μg Pb L^{-1}	24 h, 96 h and 30 days	AChE activity and gene expression	(Richetti et al., 2011)
	Cd, Ni, Cu	Zebrafish larvae and adults	30–3000 μg Cd L^{-1} 3–300 μg Cu L^{-1} 0.9–15 mg Ni L^{-1}	~40 h	Uptake routes	(Alsop and Wood, 2011)
	$NiCl_2$	Zebrafish embryos/larvae	Acute test: 0.25–15 mg L^{-1} Subchronic test: 0.25–15 mg L^{-1}	2 h 11 dpf	Locomotory activity, survival and vitality	(Kienle et al., 2008)
	$NiCl_2$	Zebrafish larvae Zebrafish adults	0.025, 2, 5 and 15 mg L^{-1}	2 h or 11 days 12 h and 96 h	Morphological alterations, survival and locomotory activity	(Nabinger et al., 2018)
Pyrethroid	DM	Zebrafish embryos/larvae	2.5, 10, 25 and 50 μg L^{-1}	96 h	Apoptosis, oxidative stress, AChE activity and morphological abnormalities	(Parlak, 2018)
		Adult zebrafish	0.15, 1.5, 3.75, 7.5 and 15 μg L^{-1}	24 h	Behavioral assessment (swimming speed and depth)	(Huang et al., 2014)
		Adult zebrafish	0.25, 0.5, 1 and 2 μg L^{-1}	15 days	Aggressive behavior and swimming performance, histological observations and changes of the body elements concentrations	(Strungaru et al., 2019a)
		Zebrafish embryos/larvae	0.25–0.5 μg L^{-1}	3–72 hpf	Locomotory activity	(Kung et al., 2015)
		Adult zebrafish	2–6 μg L^{-1}	96 h	Antioxidant enzymatic activities and lipid peroxidation	(Kuder and Philip, 2017)
		Adult zebrafish	0.29 μM	96 h	bdnf and c-fos genes	(Özdemir et al., 2018)
Heavy metals and organic pollutants	NiCl, CHP and their mixture	Zebrafish embryos/larvae	Acute test: 0–1 mg CHP L^{-1} 0–15 mg Ni L^{-1} Subchronic test: 0–0.01 mg CHP L^{-1} 0–15 mg Ni L^{-1}	2 h at 5 dpf 11 dpf	Locomotory activity, morphological abnormalities and mortality	(Kienle et al., 2009)
			0.5–15 mg Ni L^{-1} 0.1–1000 μg CHP L^{-1}	Up to 168 hpf	Development, histological alterations and the stress protein (Hsp70)	(Scheil et al., 2010)
	Cd, Zn, MP and Cd + MP Zn + MP	Adult zebrafish	5 or 2 mg MP L^{-1} 5 mg Cd L^{-1}, 15 mg Zn L^{-1}	96 h	Activities of SOD, CAT and AChE	(Ling et al., 2011)
	Cd, MP and their mixture	Adult zebrafish	5 mg L^{-1}	96 h	Differential expression proteins in the brain?	(Ling et al., 2012)
	$NiSO_4$ and buprofezin	Zebrafish embryos	2.5–600 mg L^{-1}	24 h	CA model prediction and oxidative stress	(Ku et al., 2015)
	Ni, Cd, DM and their mixture	Adult zebrafish	10 μg Ni L^{-1} 0.2 μg Cd L^{-1} 2 μg DM L^{-1}	48 h	Behavioral responses with Track 3D, activities of SOD and GPx and the body elements concentrations	Present study

CHP: chlorpyrifos; DM: deltamethrin; SOD: superoxide dismutase; CAT: catalase; AChE: acetylcholinesterase; MP: methyl parathion.

pesticides have not been used for a long time. Allinson et al. (2015) have detected twenty-three different pesticide residues in water samples from 24 urban wetlands in Melbourne, Australia (April 2010). The simazine has been observed in every sample with a concentration ranging from 0.43 μg L^{-1} (min) to 4.8 μg L^{-1} (max). According to Lao et al. (2010) permethrin, bifenthrin and deltamethrin were the most abundant pyrethroids in Ballon estuary (CA, USA) sediments (June 2008). Pyrethroids, such as cypermethrin, fenvalerate and deltamethrin, have been also detected in the water and sediment samples from Ebro River Delta (NE Spain) (Feo et al., 2010) and from Guanting reservoir (Beijing) (Xue et al., 2005). Deltamethrin was the pesticide found most frequently in Guanting reservoir, while in Canadian agricultural areas its concentrations ranged between 0.04 and 24 μg L^{-1} in water samples (Pawlisz et al., 1998). Further, 2.5 μg L^{-1} is recommended by a water quality guideline for the protection of livestock (CCME 1997). In the light of these observations and available information concerning the occurrence of pyrethroids, the concentration of heavy metals was chosen according to what we measured at Bucharest wastewater sector and 2 μg L^{-1} for DM. As is described by the literature, the maximum admissible concentrations should be established in order to protect against short-term exposure.

3.2. Combined acute toxic effects of Ni, Cd and DM on zebrafish (Danio rerio)

As it is presented in Table 2, there is a considerable amount of research regarded the effects of single chemical at elevated concentrations, which were measured near industrial areas or near agriculture regions. For example, the most industrially contaminated waters have Ni concentrations ranging between 50 and 2000 μg L^{-1} (Kienle et al., 2008; Kienle et al., 2009), whereas the highest environmental concentration of Cd (about 1 mg L^{-1}) was measured in some industrialized areas in China (Jin et al., 2015; Zheng et al., 2016b). Previous studies concerning the acute and chronic Cd exposures have shown the high toxicity of Cd on fish species (e.g. zebrafish). Even at low doses, cadmium exposure induced oxidative stress and inflammatory responses, behavioral alterations, inhibited the growth and reduced the survival rate in zebrafish (Jin et al., 2015; Zheng et al., 2016a,b). Another metal to which the aquatic organisms are exposed is Ni. Compared to cadmium, the acute and chronic toxicity of nickel has received a low research attention, although Ni exposure have been associated with a wide range of toxic effects on fish, including delayed embryo hatching, effects on the respiration, behavior and survival (Kienle et al., 2008, 2009; Ku et al., 2015; Nabinger et al., 2018; Pane et al., 2003; Scheil et al., 2010). It is well known that the heavy metals toxicity is influenced by water hardness, pH, total suspended solids, salinity and fish species. In general, metals are more toxic in soft water than in hard water (Komjarova and Blust, 2009). Furthermore, present as a mixture in ambient waters, the trace metals may enter in aquatic organisms via common uptake routes and interact with each other affecting the uptake, bioaccumulation and toxicity. Thus, the uptake of metals in mixture may demonstrate the competitive and noncompetitive inhibition, as well as enhanced uptake in some cases. For example,

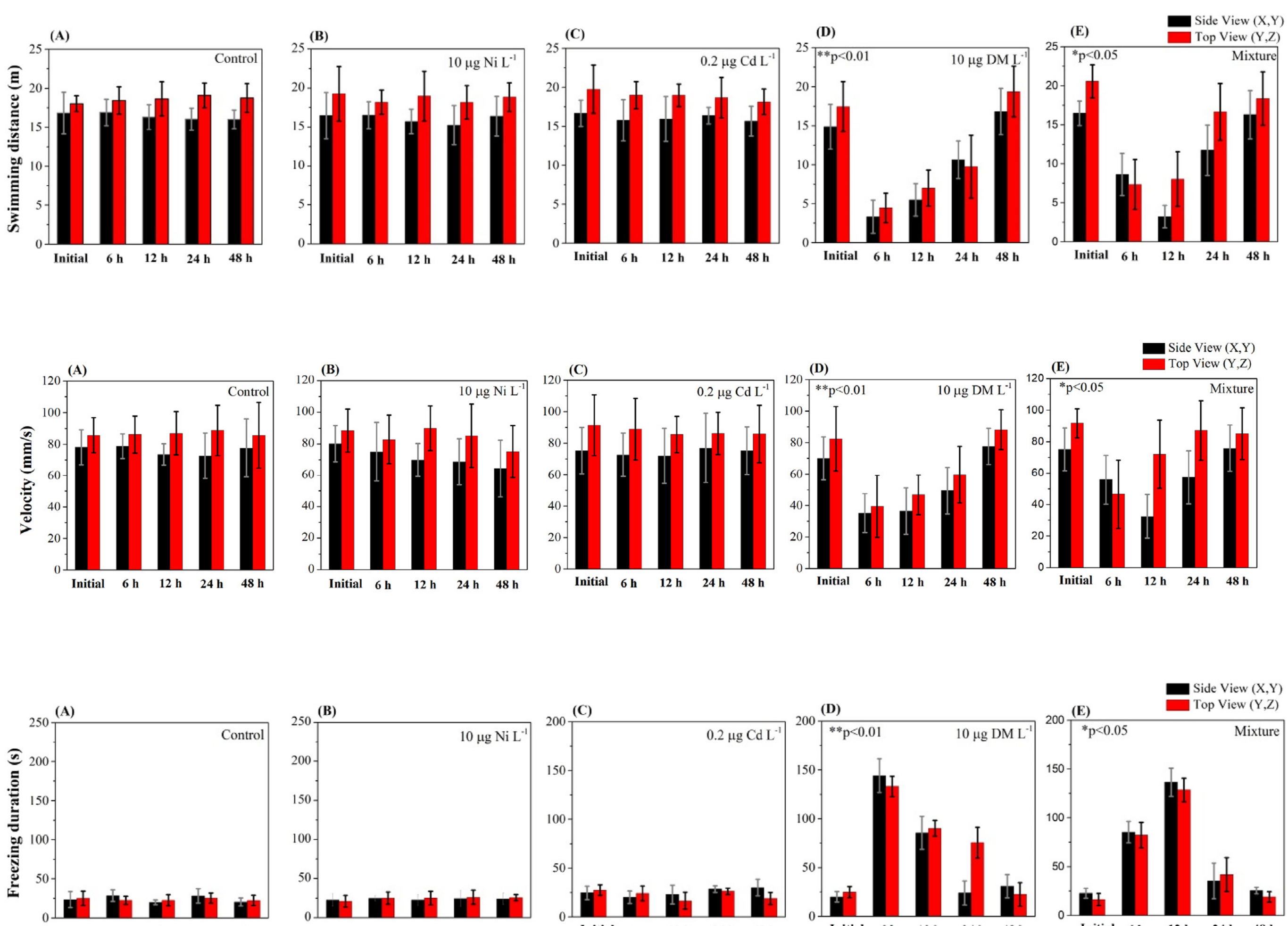


Fig. 3. Basic behavioral endpoints of the control (A) and exposed groups (B–E) obtained with Track3D approach. The graphs show the total distance in the 3D space covered by the zebrafish, the velocity and freezing duration under the condition of no exposure (A) and exposure to 10 μg Ni L^{-1} (B), 0.2 μg Cd L^{-1} (C), 2 μg DM L^{-1} (D) and their mixture (E). Data represent average ± SD evaluated by one way ANOVA (n = 0.21,*p < 0.05 and **p < 0.01).

the uptake of Cd was inhibited by Cu, Pb and Zn and enhanced by Ni at concentrations above 5 μg L^{-1} (Komjarova and Blust, 2009). In another paper, Komjarova associated the decrease in Cd accumulation rate with an increase in transcript abundance of ECaC at 24 h and DMT1 at 48 h and a decrease in Zip8 transcript levels (Komjarova and Bury, 2014). An increasing number of studies pointed that exposure to DM may cause neurobehavioral deficits. Kung et al. (2015) observed that a developmental exposure of zebrafish embryos to low doses of DM results in an increase of locomotory activity following a transition into darkness at the larval stage. They related these locomotory effects with changes in dopaminergic gene expression and of neurochemistry. Özdemir et al. (2018) showed that DM poses a great danger to the zebrafish brain tissues, resulting in an increase of the protein levels of bdnf and c-fos and of their mRNA transcription levels. Other surveys reported the oxidative stress as one of the mechanisms involved in the reported neurotoxicity of DM (Kuder and Philip, 2017; Parlak, 2018). Additionally, the formation of a complex between a heavy metal and a pesticide, which can enhance metal bioaccumulation and may cause serious injury compared with individual treatments, has been reported (Chen et al., 2013; Ku et al., 2015; Steevens and Benson, 1999). The tests performed on *D. rerio* larvae and embryos by Scheil et al. (2010) revealed that both $NiCl_2$ and CHP exhibit different mode of action and exerted additive actions. Synergistic patterns were observed when zebrafish larvae were exposed to chlorpyrifos mixtures containing atrazine and terbuthylazine. Pérez et al. (2013) assumed that the presence of these herbicides accelerated the transformation of chlorpyrifos in its oxon form, thus increasing the toxicity by inhibiting AChE activity. Although, there has been a considerable amount of research done on the effects of environmental chemicals in fish, the mechanisms underlying the toxic effects observed still remain poorly understood.

No mortality was recorded in all the treatment and control groups during the acute exposure experiments. 3D tracking was used for

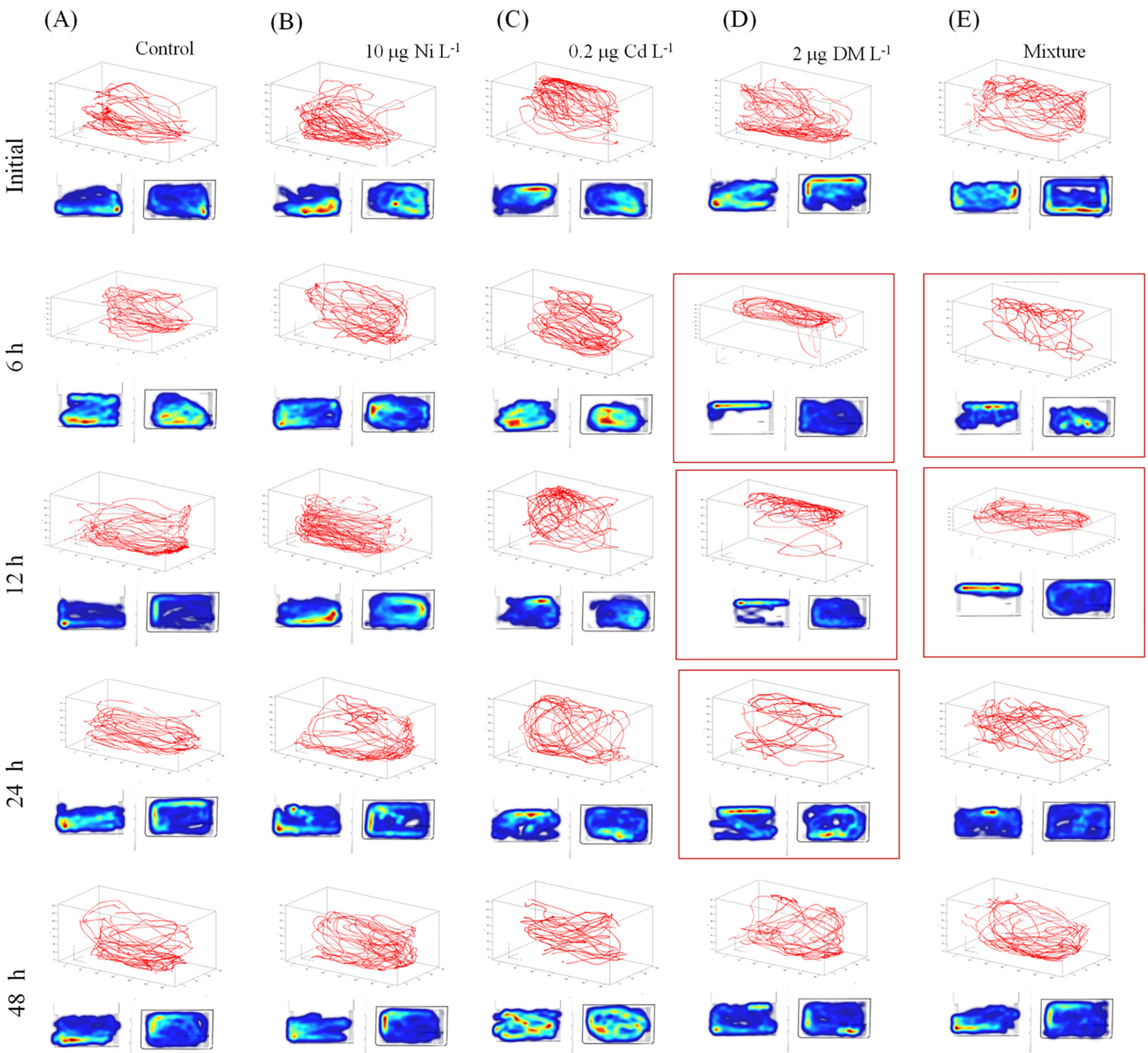


Fig. 4. The 3D swim traces results for zebrafish control group (A) and groups treated with 10 μg Ni L^{-1} (B), 0.2 μg Cd L^{-1} (C), 2 μg DM L^{-1} (D) and their mixture (E) at 0, 6, 12, 24 and 48 h (n = 7). An automated integration of traces using Track3D software results in 3D swim tracks (red color). The down panel shows generation of top (X, Y) and side (Y, Z) views of zebrafish in the 4 min test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

assessing the individual and joint action on the zebrafish behavior of two heavy metals and a pesticide. We performed a quantitative analysis for each fish in order to calculate the basic behavioral endpoints and to determine the fish motion trajectory in the 3D space. An analysis of the fish behavioral endpoints reveals that there is no significant difference between the locomotory activity of the zebrafish exposed to heavy metals and control (Fig. 3A–C). Instead, some different behavioral responses of the zebrafish were noticed immediately after exposure to DM as a single chemical and as a mixture with Ni and Cd (Fig. 3D–E). Therefore, a significant decrease in the travel distance and velocity as well as an increase in freezing was measured in the first hours of exposure, which may indicate a possible energy reallocation from locomotion to detoxification process. It is very interesting to note the different behavioral responses of the zebrafish under sudden stress from DM alone and from DM associated with Ni and Cd. The presence in mixture elicited an antagonistic deviation; immediate and more pronounced effects in zebrafish were observed for the DM treated groups, whereas the significant decrease/increase in the value of kinematic parameters was measured 6 h after of exposure for DM treated group and 12 h after exposure for chemicals mixture. The exposure to pesticide and pesticide associated with heavy metals increased duration freezing from on average 9% of the time for control group to an average of 55% of the time, also noted previously by Maaswinkel et al. (2013a). On the other hand, an adaptive response of zebrafish under acute exposure has been observed, after 48 h no significant differences between the exposed groups and control group regarding the behavioral responses were recorded.

Therefore, the data generated for zebrafish behavioral endpoints, such as the total distance covered, velocity and freezing duration demonstrate overt differences between control and animals treated with DM, and DM associated with Ni and Cd, as well as between two exposure scenarios, which are also consistent with the 3D swim trace reconstructions. Although variations existed between individuals, distinct spatial differences between treatments can easily be visualized and detected using the Track 3D method. As showed in Fig. 4 for different exposure times, the DM single or in mixture significantly reduced the zebrafish exploratory behavior and increased freezing duration, erratic movements with more transition in the upper half. Compared with the zebrafishes exposed to DM, Ni and Cd in mixture, the specimens exposed to DM have displayed an immediate and long-term top swimming, also observed by Stewart et al. (2015) for nicotine-treated zebrafishes and by Maaswinkel et al. (2013a) for buspirone and 0.5%-ethanol treated groups. We assume that zebrafish swim in the horizontal plane, near to the water surface, as a result of breathing problems or of increased anxiety level. The results reveal that the effects expected for mixture of chemicals, such as Ni, Cd and DM, deviate from the concept of independent action, the behavioral responses of zebrafish being delayed and reduced by the antagonistic interactions. A similar result was reported by Kienle et al. (2009), when CHP and $NiCl_2$ mixture elicited antagonistic deviation from the concept of independent action, in the case of locomotory activity. This finding agrees well with their previous results when studied the effects of $NiCl_2$ and oxygen depletion on the behavior and vitality of zebrafish embryos and larvae; a significant antagonistic action between the O_2 deficiency and Ni treatment was detected (Kienle et al., 2008). Similarly, Ling et al. (2011) reported an antagonistic toxic response when zebrafish were simultaneously exposed to methyl parathion and Zn or Cd.

Moreover, several studies have shown that oxidative stress may be induced by Cd and DM exposure on zebrafish, at the origin of reactive oxygen species (ROS) production, such as superoxide anions (O_2^-), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) (Kuder and Philip, 2017; Ling et al., 2011; Zheng et al., 2016a,b). To counteract the action of the ROS, organisms have developed an efficient antioxidant defense system at different levels such as preventive, radical scavenging, repair and de novo, and the adaptation, that includes both enzymatic and non-enzymatic antioxidants (Lobo et al., 2010).

In the present study we investigated the superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in order to assess the oxidative stress in zebrafishes, as a result of their acute exposure to Cd, Ni, DM and their mixture. Both, SOD and GPx are considered to be the first line of defense against oxidative stress, since SOD helps in conversion of O_2^- to O_2 and H_2O_2, while GPx can effectively catalyze the reduction of H_2O_2 to water and a variety of organic peroxides to alcohols. As showed in the Fig. 5, the activities of SOD and GPx remained unchanged in the zebrafish upon treatment with Ni and Cd, but were significantly elevated after DM exposure. Similar results have been reported by Kuder and Philip (2017): exposure to DM increased SOD and GPx activities. This change could be a mechanism adopted by the zebrafish to compensate the oxidative damage induced by DM. Contrarily, the exposure to a mixture of Cd, Ni and DM elicited an antagonistic response in the oxidative stress of the zebrafish, in accordance with behavior results. Similarly, Ling et al. (2011) noted that the SOD activity rapidly increased in the first hours after zebrafish were exposed to a single pollutant, respectively decreased and remained stable when exposed to a mixture of contaminants. The competitive uptake of Ni, Cd and DM from the surrounding environment, their biotransformation and elimination within zebrafish may be a possible explanation for the different trend observed for ternary mixture. For example, Mehler

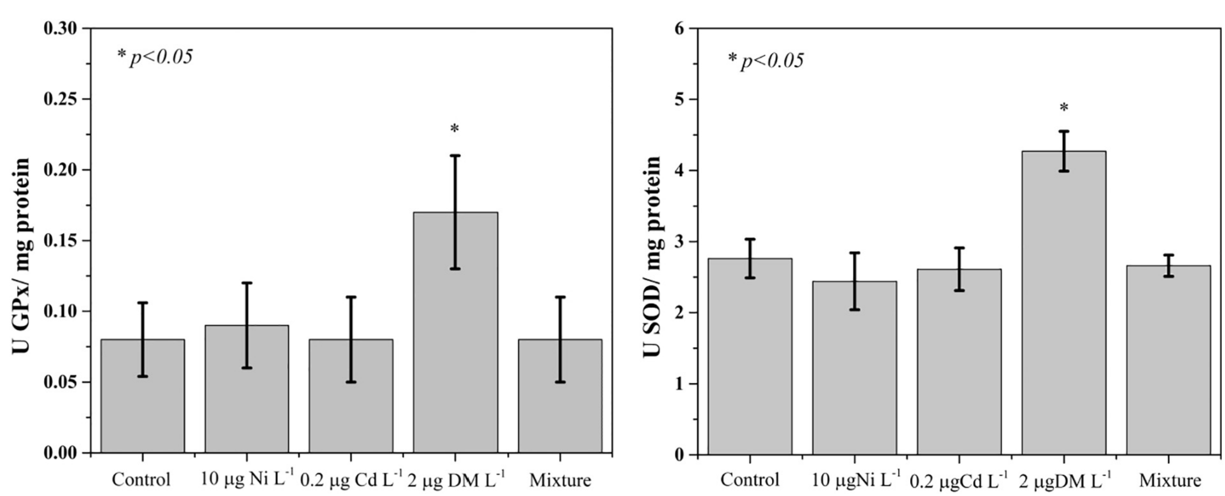


Fig. 5. Activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) levels in zebrafish exposed to environmental chemicals. Data were evaluated as average ± SD. Differences were considered statistically significant when $^*p < 0.05$.(one-way ANOVA, n = 12).

Table 3
Total electrolytes and trace metal profiles reported in zebrafish body measured for wet weight (average ± SD, n = 9).

Elements (μg g^{-1})	Exposure				
	Control	10 μg Ni L^{-1}	0.2 μg Cd L^{-1}	2 μg DM L^{-1}	Mixture
Cu	0.977 ± 0.125	1.033 ± 0.096	0.973 ± 0.223	1.064 ± 0.008	1.071 ± 0.01
Zn	2046 ± 11.9	2088 ± 19	2080 ± 57	2034 ± 19	2109 ± 0.558
Fe	19.3 ± 0.5	18.4 ± 1.6	18.7 ± 0.6	18.1 ± 1.7	19.2 ± 0.08
Ca	5556 ± 200	5481 ± 462	5768 ± 465	5641 ± 188	5517 ± 129
Mg	370.7 ± 10	382 ± 23	349 ± 35	371 ± 2	364 ± 17
Na	877 ± 50	910 ± 105	894 ± 107	949 ± 90	864 ± 78
K	2392.7 ± 50	2223 ± 121	2467 ± 232	2386 ± 188	2442 ± 116

The results of the fish muscle reference material (n = 6) that was prepared and analyzed were: Na (certified 2.8 g kg^{-1} and obtained 2.65 ± 1.3 g kg^{-1}), K (certified 21.8 g kg^{-1} and obtained 21.1 ± 0.65 g kg^{-1}), Ca (certified 0.342 g kg^{-1} and obtained 0.343 ± 0.002 g kg^{-1}), Mg (certified 1.37 g kg^{-1} and obtained 1.38 ± 0.03 g kg^{-1}), Zn (certified 16 ± 1.1 mg kg^{-1} and obtained 15.3 ± 1.2 mg kg^{-1}), Fe (certified 9.4 ± 1.4 mg kg^{-1} and obtained 9.3 ± 0.96 mg kg^{-1}) and Cu (certified 1.67 ± 0.16 mg kg^{-1} and obtained 1.64 ± 0.32 mg kg^{-1}).

et al. (2011) and Wang et al. (2015) showed that two chemicals may react to form a less toxic product. The bioavailability of cypermethrin may be reduced by forming a Pb^{2+}-cypermethrin complex through the CN group of the pyrethroid insecticide, whereas antrazine due to the five electron-donor atoms can potentially complex with Cd to form antrazine-Cd complexes which might reduce the amount of exposed chemicals. It should be pointed out that little information is available on the effects and mechanism of chemical mixtures on zebrafish and these merits further investigation.

In summary, all these results indicate that acute exposure to DM not only affected the zebrafish behavior but also induced significant oxidative stress. Furthermore, our results highlight the complexity of the joint toxicity of organic and inorganic contaminants in aquatic system as well as their competitive uptake from the surrounding environment. It should be also pointed out that the behavioral studies on animal models are particularly useful for assessing the possible effects of various pollutants on aquatic populations. The rapid and sensitive behavioral responses seen in zebrafish turn the behavioral approaches into an early warning tool for detecting accidental pollution.

Although in the literature is indicated that the uptake of Ni and Cd is mediated by Ca^{2+} transport pathways (Komjarova and Bury, 2014), in our study the acute exposure to Cd and Ni for up to 48 h had no visible effect on the total calcium from the body. Our results are comparable to those reported by others authors (Alsop and Wood, 2011). The results presented in Table 3 showed that the studied element concentrations were not affected by the exposures performed in this study. One of the reasons may be duration of exposure that was short and limitation of the study in measuring the elements in different organs.

It should be noted that the above approach has been developed considering the exposure to and effects of two metals and a pesticide on individual organisms and an additional level of complexity can be introduced by increasing the number of contaminants, as well as to investigate their effects on multiple organisms simultaneously over a wide range of doses, exposure durations and dosage regimes. All studies conducted in laboratory conditions have limitations because in real situation there is an enormous number of possible chemical combinations and biological interactions at different levels.

4. Conclusions

The study proved a direct relationship between impairment of zebrafish swimming behavior and the activities of antioxidant enzymes such as SOD and GPx, whose role in zebrafish is to prevent or reduce the effects of ROS. Significant changes in antioxidant enzymes activities were recorded after exposure a 48 h to DM but not in the case for DM associated with Ni and Cd. This is supported by the behavioral analyses variables that showed a significant faster recovery, due to a decreased toxicological intensity of DM. These indicate that the ternary mixture of Cd, Ni and DM did not display higher toxicity than the sum of the individual treatments, resulting antagonistic effects. Thus, how pollutants behave in mixture is strongly influenced by their physical and chemical properties, the type of toxic action and potential interactions.

Consequently, our study supports previous works concerning the combined toxicity of environmental chemicals since their simultaneous presence in the aqueous environment may lead to higher or lower toxicological effects on biota than those reported from a single pollutant. Therefore, the evaluation of toxic effects of a single contaminant does not offer a realistic estimate of the impact against aquatic ecosystems. The study also supports the idea that the environmental interaction between different chemical compounds which do not exceed the maximum permitted limits, may have benefits for aquatic life forms or may be more harmful. It is important for future studies to run an evaluation of the environmental level of pollutants and to test them in mixtures in laboratory conditions to understand what is happening in the environment. The development of behavioral applications on aquatic organisms will improve in future the understanding of antagonistic or synergistic effects of aquatic pollutants.

Declaration of competing interest

All authors declared that present study did not involve any conflict of interest.

Acknowledgements

This work was financially supported partially by the grant of the Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI, project number 26PCCDI/01.03.2018, “Integrated and sustainable processes for environmental clean-up, wastewater reuse and waste valorization” (SUSTENVPRO), within PNCDI III and partially by the grant “Developing innovation capacity and increasing the impact of excellence research at UAIC” funded by the Romanian Ministry of Research and Innovation within Program 1 - Development of the national RD system, Subprogram 1.2 - Institutional Performance - RDI excellence funding projects, Contract no. 34PFE/19.10.2018.

References

2008/105/EC D, 2008. Environmental Quality Standards in the Field of Water Policy, Amending and Subsequently Repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and Amending Directive 2000/60/EC. European Parliament and the Council of the European Union.

Allinson, G., Zhang, P., Bui, A., Allinson, M., Rose, G., Marshall, S., et al., 2015. Pesticide and trace metal occurrence and aquatic benchmark exceedances in surface waters and sediments of urban wetlands and retention ponds in Melbourne, Australia. Environ. Sci. Pollut. Res. 22, 10214–10226.

Alsop, D., Wood, C.M., 2011. Metal uptake and acute toxicity in zebrafish: common mechanisms across multiple metals. Aquat. Toxicol. 105, 385–393.

Boulanger, E., Barst, B.D., Alloy, M.M., Blais, S., Houde, M., Head, J.A., 2019. Assessment of environmentally contaminated sediment using a contact assay with early life stage zebrafish (Danio rerio). Sci. Total Environ. 659, 950–962.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K.M., et al., 2010. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat. Protoc. 5, 1786.
Cachat, J., Stewart, A., Utterback, E., Hart, P., Gaikwad, S., Wong, K., et al., 2011. Three-dimensional neurophenotyping of adult zebrafish behavior. PLoS One 6, e17597.
Chen, L., Qu, G., Sun, X., Zhang, S., Wang, L., Sang, N., et al., 2013. Characterization of the interaction between cadmium and chlorpyrifos with integrative techniques in incurring synergistic hepatoxicity. PLoS One 8, e59553.
Fang, W., Peng, Y., Muir, D., Lin, J., Zhang, X., 2019. A critical review of synthetic chemicals in surface waters of the US, the EU and China. Environ. Int. 131, 104994.
Favorito, R., Chiarelli, G., Grimaldi, M.C., De Bonis, S., Lancieri, M., Ferrandino, I., 2011. Bioaccumulation of cadmium and its cytotoxic effect on zebrafish brain. Chem. Ecol. 27, 39–46.
Fazio, F., Piccione, G., Tribulato, K., Ferrantelli, V., Giangrosso, G., Arfuso, F., et al., 2014. Bioaccumulation of heavy metals in blood and tissue of striped mullet in two Italian lakes. J. Aquat. Anim. Health 26, 278–284.
Feo, M.L., Ginebreda, A., Eljarrat, E., Barceló, D., 2010. Presence of pyrethroid pesticides in water and sediments of Ebro River Delta. J. Hydrol. 393, 156–162.
Godoy, A.A., Kummrow, F., 2017. What do we know about the ecotoxicology of pharmaceutical and personal care product mixtures? A critical review. Crit. Rev. Environ. Sci. Technol. 47, 1453–1496.
Gonzalez, P., Baudrimont, M., Boudou, A., Bourdineaud, J.P., 2006. omparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (Danio rerio). BioMetals 19, 225–235.
Green, J., Collins, C., Kyzar, E.J., Pham, M., Roth, A., Gaikwad, S., et al., 2012. Automated high-throughput neurophenotyping of zebrafish social behavior. J. Neurosci. Methods 210, 266–271.
Huang, Y., Zhang, J., Han, X., Huang, T., 2014. The use of zebrafish (Danio rerio) behavioral responses in identifying sublethal exposures to deltamethrin. Int. J. Environ. Res. Public Health 11, 3650–3660.
Jin, Y., Liu, Z., Liu, F., Ye, Y., Peng, T., Fu, Z., 2015. Embryonic exposure to cadmium (II) and chromium (VI) induce behavioral alterations, oxidative stress and immunotoxicity in zebrafish (Danio rerio). Neurotoxicol. Teratol. 48, 9–17.
Jonker, M., van Gestel, C.A., Kammenga, J.E., Laskowski, R., Svendsen, C., 2016. Mixture Toxicity: Linking Approaches From Ecological and Human Toxicology. CRC Press.
Kapsi, M., Tsoutsi, C., Paschalidou, A., Albanis, T., 2019. Environmental monitoring and risk assessment of pesticide residues in surface waters of the Louros River (N.W. Greece). Sci. Total Environ. 650, 2188–2198.
Kienle, C., Köhler, H.-R., Filser, J., Gerhardt, A., 2008. Effects of nickel chloride and oxygen depletion on behaviour and vitality of zebrafish (Danio rerio, Hamilton, 1822)(Pisces, Cypriniformes) embryos and larvae. Environ. Pollut. 152, 612–620.
Kienle, C., Köhler, H.-R., Gerhardt, A., 2009. Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (Danio rerio) embryos and larvae. Ecotoxicol. Environ. Saf. 72, 1740–1747.
Komjarova, I., Blust, R., 2009. Multimetal interactions between Cd, Cu, Ni, Pb, and Zn uptake from water in the zebrafish Danio rerio. Environ. Sci. Technol. 43, 7225–7229.
Komjarova, I., Bury, N., 2014. Evidence of common cadmium and copper uptake routes in zebrafish Danio rerio. Environ. Sci. Technol. 48, 12946–12951.
Ku, T., Yan, W., Jia, W., Yun, Y., Zhu, N., Li, G., et al., 2015. Characterization of synergistic embryotoxicity of nickel and buprofezin in zebrafish. Environ. Sci. Technol. 49, 4600–4608.
Kuder, R.S., Philip, G.H., 2017. Antioxidant enzymatic activities and lipid peroxidation in liver and ovary of zebrafish (Danio rerio) exposed to deltamethrin. Chem. Ecol. 33, 739–749.
Kung, T.S., Richardson, J.R., Cooper, K.R., White, L.A., 2015. Developmental deltamethrin exposure causes persistent changes in dopaminergic gene expression, neurochemistry, and locomotor activity in zebrafish. Toxicol. Sci. 146, 235–243.
Lao, W., Tsukada, D., Greenstein, D.J., Bay, S.M., Maruya, K.A., 2010. Analysis, occurrence, and toxic potential of pyrethroids, and fipronil in sediments from an urban estuary. Environ. Toxicol. Chem. 29, 843–851.
Laskowski, D.A., 2002. Physical and chemical properties of pyrethroids. Reviews of Environmental Contamination and Toxicology. Springer, pp. 49–170.
Ling, X., Zhang, Y., Lu, Y., Huang, H., 2011. Superoxide dismutase, catalase and acetylcholinesterase: biomarkers for the joint effects of cadmium, zinc and methyl parathion contamination in water. Environ. Technol. 32, 1463–1470.
Ling, X.P., Lu, Y.H., Huang, H.Q, 2012. Differential protein profile in zebrafish (Danio rerio) brain under the joint exposure of methyl parathion and cadmium. Environ. Sci. Pollut. Res. 19, 3925–3941.
Liu, W., Gan, J.J., Lee, S., Kabashima, J.N., 2004. Phase distribution of synthetic pyrethroids in runoff and stream water. Environ. Toxicol. Chem. 23, 7–11.
Lobo, V., Patil, A., Phatak, A., Chandra, N., 2010. Free radicals, antioxidants and functional foods: impact on human health. Pharmacogn. Rev. 4, 118.
Maaswinkel, H., Zhu, L., Weng, W., 2012. The immediate and the delayed effects of buspirone on zebrafish (Danio rerio) in an open field test: a 3-D approach. Behav. Brain Res. 234, 365–374.
Maaswinkel, H., Le, X., He, L., Zhu, L., Weng, W., 2013a. Dissociating the effects of habituation, black walls, buspirone and ethanol on anxiety-like behavioral responses in shoaling zebrafish. A 3D approach to social behavior. Pharmacol. Biochem. Behav. 108, 16–27.
Maaswinkel, H., Zhu, L., Weng, W., 2013b. Using an automated 3D-tracking system to record individual and shoals of adult zebrafish. J. Vis. Exp. 82, 1–27.
Macrì, S., Neri, D., Ruberto, T., Mwaffo, V., Butail, S., Porfiri, M., 2017. Three-dimensional scoring of zebrafish behavior unveils biological phenomena hidden by two-dimensional analyses. Sci. Rep. 7, 1962.
Martínez-Guijarro, R., Paches, M., Romero, I., Aguado, D., 2019. Enrichment and contamination level of trace metals in the Mediterranean marine sediments of Spain. Sci. Total Environ. 693, 133566.
Mehler, W.T., Du, J., Lydy, M.J., You, J., 2011. Joint toxicity of a pyrethroid insecticide, cypermethrin, and a heavy metal, lead, to the benthic invertebrate Chironomus dilutus. Environ. Toxicol. Chem. 30, 2838–2845.
Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93, 2217–2223.
Nabinger, D.D., Altenhofen, S., Bitencourt, P.E.R., Nery, L.R., Leite, C.E., Vianna, M.R.M.R., et al., 2018. Nickel exposure alters behavioral parameters in larval and adult zebrafish. Sci. Total Environ. 624, 1623–1633.
Ni, H., Peng, L., Gao, X., Ji, H., Ma, J., Li, Y., et al., 2019. Effects of maduramicin on adult zebrafish (Danio rerio): acute toxicity, tissue damage and oxidative stress. Ecotoxicol. Environ. Saf. 168, 249–259.
Özdemir, S., Altun, S., Özkaraca, M., Ghosi, A., Toraman, E., Arslan, H., 2018. Cypermethrin, chlorpyrifos, deltamethrin, and imidacloprid exposure up-regulates the mRNA and protein levels of bdnf and c-fos in the brain of adult zebrafish (Danio rerio). Chemosphere 203, 318–326.
Pane, E., Richards, J., Wood, C., 2003. Acute waterborne nickel toxicity in the rainbow trout (Oncorhynchus mykiss) occurs by a respiratory rather than ionoregulatory mechanism. Aquat. Toxicol. 63, 65–82.
Parlak, V., 2018. Evaluation of apoptosis, oxidative stress responses, AChE activity and body malformations in zebrafish (Danio rerio) embryos exposed to deltamethrin. Chemosphere 207, 397–403.
Pawlisz, A., Busnarda, J., McLauchlin, A., Caux, P.Y., Kent, R., 1998. Canadian water quality guidelines for deltamethrin. Environ. Toxicol. Water Qual. 13, 175–210.
Pérez, J., Domingues, I., Monteiro, M., Soares, A.M., Loureiro, S., 2013. Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early-life stages of the zebrafish Danio rerio. Environ. Sci. Pollut. Res. 20, 4671–4680.
Plavan, G., Jitar, O., Teodosiu, C., Nicoara, M., Micu, D., Strungaru, S.-A., 2017. Toxic metals in tissues of fishes from the Black Sea and associated human health risk exposure. Environ. Sci. Pollut. Res. 24, 7776–7787.
Qian, Z.-M., Chen, Y.Q., 2017. Feature point based 3D tracking of multiple fish from multi-view images. PLoS One 12, e0180254.
Richetti, S.K., Rosemberg, D.B., Ventura-Lima, J., Monserrat, J.M., Bogo, M.R., Bonan, C.D., 2011. Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. NeuroToxicol 32, 116–122.
Rosemberg, D.B., Rico, E.P., Mussulini, B.H.M., Piato, Â.L., Calcagnotto, M.E., Bonan, C.D., et al., 2011. Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. PLoS One 6, e19397.
Scheil, V., Zürn, A., Köhler, H.R., Triebskorn, R., 2010. Embryo development, stress protein (Hsp70) responses, and histopathology in zebrafish (Danio rerio) following exposure to nickel chloride, chlorpyrifos, and binary mixtures of them. Environ. Toxicol. 25, 83–93.
Scientific Committee on Consumer Safety, Scientific Committee on Health and Environmental Risks and Scientific Committee on Emerging and Newly Identified Health Risks, 2012. Toxicity and Assessment of Chemical Mixtures. European Commission, Brussels.
Stara, A., Bellinvia, R., Velisek, J., Strouhova, A., Kouba, A., Faggio, C., 2019. Acute exposure of common yabby (Cherax destructor) to the neonicotinoid pesticide. Sci. Total Environ. 665, 718–723.
Steevens, J.A., Benson, W.H., 1999. Toxicological interactions of chlorpyrifos and methyl mercury in the amphipod, Hyalella azteca. Toxicol. Sci. 52, 168–177.
Stewart, A.M., Grieco, F., Tegelenbosch, R.A., Kyzar, E.J., Nguyen, M., Kaluyeva, A., et al., 2015. A novel 3D method of locomotor analysis in adult zebrafish: implications for automated detection of CNS drug-evoked phenotypes. J. Neurosci. Methods 255, 66–74.
Strungaru, S.-A., Nicoara, M., Teodosiu, C., Micu, D., Plavan, G., 2017. Toxic metals biomonitoring based on prey-predator interactions and environmental forensics techniques: a study at the Romanian-Ukraine cross border of the Black Sea. Mar. Pollut. Bull. 124, 321–330.
Strungaru, S.-A., Plavan, G., Ciobica, A., Nicoara, M., Robea, M.A., Solcan, C., et al., 2019a. Toxicity and chronic effects of deltamethrin exposure on zebrafish (Danio rerio) as a reference model for freshwater fish community. Ecotoxicol. Environ. Saf. 171, 854–862.
Strungaru, S., Radojković, P., Dumitru, G., Nicoara, M., Plavan, G., Todirascu-Ciornea, E., 2019b. Oxidative stress and changes in swimming performances at zebrafish model (Danio Rerio H. 1822) produced by acute exposure to deltamethrin. Surv. Fish. Sci. 5, 121–137.
Turgut, C., 2003. The contamination with organochlorine pesticides and heavy metals in surface water in Küçük Menderes River in Turkey, 2000–2002. Environ. Int. 29, 29–32.
Vareda, J.P., Valente, A.J.M., Durães, L., 2019. Assessment of heavy metal pollution from anthropogenic activities and remediation strategies: a review. J. Environ. Manag. 246, 101–118.
Wang, Y., Chen, C., Qian, Y., Zhao, X., Wang, Q., 2015. Ternary toxicological interactions of insecticides, herbicides, and a heavy metal on the earthworm Eisenia fetida. J. Hazard. Mater. 284, 233–240.
Xue, N., Xu, X., Jin, Z., 2005. Screening 31 endocrine-disrupting pesticides in water and surface sediment samples from Beijing Guanting reservoir. Chemosphere 61, 1594–1606.

Zhang, Z., Lu, Y., Li, H., Tu, Y., Liu, B., Yang, Z., 2018. Assessment of heavy metal contamination, distribution and source identification in the sediments from the Zijiang River, China. Sci. Total Environ. 645, 235–243.
Zheng, J.-L., Yuan, S.-S., Wu, C.-W., Li, W.-Y., 2016a. Chronic waterborne zinc and cadmium exposures induced different responses towards oxidative stress in the liver of zebrafish. Aquat. Toxicol. 177, 261–268.
Zheng, J.-L., Yuan, S.-S., Wu, C.-W., Lv, Z.-M., 2016b. Acute exposure to waterborne cadmium induced oxidative stress and immunotoxicity in the brain, ovary and liver of zebrafish (Danio rerio). Aquat. Toxicol. 180, 36–44.
Zhou, S., Chen, Q., Di Paolo, C., Shao, Y., Hollert, H., Seiler, T.-B., 2019. Behavioral profile alterations in zebrafish larvae exposed to environmentally relevant concentrations of eight priority pharmaceuticals. Sci. Total Environ. 664, 89–98.