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RESEARCH ARTICLE

Emerging contaminant occurrence and toxic effects on zebrafish embryos to assess the adverse effects caused by mixtures of substances in the environment

Gisela de Assis Martini[1] · Cassiana Carolina Montagner[2] · William Viveiros[3] · Gilson Alves Quinaglia[3] · Daniela Dayrell França[3] · Nívea Cristina Guedes Munin[2,5] · Mônica Lopes-Ferreira[4] · Sizue Ota Rogero[1] · José Roberto Rogero[1]

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Abstract

The contaminants of emerging concern (CECs) have been receiving global attention due to their worldwide presence in water bodies. The CECs could be originated from synthetic or natural sources, and they are not commonly monitored, although these substances are continuously reaching the aquatic environment. The main goal of this study was to determine the occurrence of some target CECs in São Paulo state surface water, once there is practically no information on the presence and concentration range of these substances at the studied sites. In addition, the present study aimed to assess adverse effects in the non-target fish embryo of *Danio rerio* (zebrafish) after exposure to surface water organic extract samples during 96 h using FET test. The CECs in surface water samples were determined by solid-phase extraction and liquid chromatography coupled by mass spectrometry. A 2-year study was assessed in 7 rivers and 3 reservoirs at São Paulo state, where 25 of the 30 analyzed substances were quantified, being caffeine the substance with the highest concentration range (5.5 ng L^{-1} to 69 μg L^{-1}) and detected in 95% of analyzed samples, followed by bisphenol A (6.5–1300 ng L^{-1}) and carbendazim (4.7–285 ng L^{-1}), found in 50% and 85% of the analyzed samples, respectively. The chemical analysis and biological test were not performed in order to show a direct relationship between concentrations and observed effects on embryos; however, the combined approach can provide a better understanding of the adverse effects caused by mixtures of substances at relevant environmental concentrations. Regarding the adverse effects, it was observed that in the samples from sites with higher anthropogenic activity in the surroundings, there was also a higher mortality rate in organisms. At the Ribeirão Pires River and Sapucaí-Guaçu River, the mortality rate during the 2-year study was 21.6% and 9.3%, respectively. The morphological abnormality rates were higher at Ribeirão Grande (21.4%) and Ribeirão Pires (29.5%) Rivers. The obtained results aim to show that even in low concentrations (ng–μg L^{-1}) the CECs can cause adverse effects on non-target species, and because of that, new chemical indicators would be important to monitor the water quality and protect the aquatic biota.

Keywords Surface water · Contaminants of emerging concern · Chemical analysis · FET · *Danio rerio* embryos

Introduction

In the last decades, the occurrence of contaminants of emerging concern (CECs) in the environment has become a matter of worldwide concern, as these substances reach the environment from various anthropogenic sources and can achieve different environmental matrices. The CECs consist of a variety of chemicals, such as pesticides, hormones, personal care products, pharmaceuticals, plasticizers, and antibacterial compounds, among other substances within origin from industrial, agricultural, and domestic use (Ekman et al. 2013; Nilsen et al. 2019). Most of these substances are not commonly regulated and can reach the aquatic environment when they are not

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✉ Gisela de Assis Martini
giselaamartini@gmail.com

1 Centro de Química e Meio Ambiente, Instituto de Pesquisas Energéticas e Nucleares, São Paulo, Brazil

2 Instituto de Química, Universidade Estadual de Campinas, São Paulo, Brazil

3 Companhia Ambiental do Estado de São Paulo, São Paulo, Brazil

4 Laboratório Especial de Toxinologia Aplicada, Instituto Butantan, São Paulo, Brazil

5 Universidade Federal do Amazonas, Manaus, Brazil

completely removed by water and wastewater treatment processes, or through other anthropogenic sources, and can cause adverse effects to human and ecological health (Gavrilescu et al. 2015; USGS 2017; Deere et al. 2020). When these contaminants enter in the environment, they can compose complex mixtures of different substances classes', and although most of these compounds are present in low concentrations, they are capable of causing adverse effects to the biota (Aeppli et al. 2008). It is a worldwide challenge to identify the presence of potentially harmful compounds in the environment and even more difficult to assess the effects caused by these contaminants to aquatic biota. This scenario is even more tough in developing countries, such as Brazil, due to poor sanitation coverage, with discharge of sewage into water bodies, irregular human occupation near rivers and reservoirs, runoff of agricultural products, and other anthropogenic sources that lead to continuous discharge of contaminants into the aquatic environment (Starling et al. 2019; Frena et al. 2016; Jardim et al. 2012; Sodré et al. 2010). In order to acquire a more comprehensive knowledge of some of the target CECs in Brazilian surface waters, chemical analysis was performed to verify the occurrence of pesticides, hormones, and alkylphenols, as well as bisphenol A, triclosan, and caffeine in seven rivers and three reservoirs of the São Paulo state, the most populous and industrialized state in Brazil, and also with large agro-industry activities (Albuquerque et al. 2016; Caldas et al. 2013). Although the environmental concentrations of these CECs are low, many of them are considered to cause toxic effects, which are a serious environmental concern, since it can cause changes in the organisms' development (Oviedo and Aga 2016; Escher et al. 2013; Schwarzenbach et al. 2006). In addition, some CECs, as fungicides, herbicides, and insecticides, are designed to exert biological effects in target organisms; therefore, adverse effects in non-target organisms can be induced after the exposure to these substances (Nilsen et al. 2019). In this context, biological tests could provide information on the effects caused by these substances in different biological organization levels. As aquatic organisms are exposed to a variety of substances throughout their life cycle, adverse effects such as morphological abnormalities, behavioral changes, and changes in reproductive patterns can occur; these substances are still capable of interacting with each other and affecting aquatic life (Schmidt et al. 2016). Among the various biological techniques for assessing effects on non-target organisms, the Fish Embryo Acute Toxicity (FET) test is an established bioassay and has been used in environmental and human toxicological studies worldwide (Embry et al. 2010; OECD 2013; Braunbeck et al. 2015). The acute toxicity test with zebrafish embryos can provide comprehensive and realistic information on about the potential for toxicity in water (Rocha et al. 2011), and for this reason was chosen to evaluate the effects after exposure to surface water organic extract samples. Since contaminants rarely occur individually in the environment, and most of the time they are a mixture of thousands of compounds that can interact with each other, studies evaluating the toxicity of mixtures have shown that effects can occur even when all of these substances occur in concentrations below which no effect is observed, or with mixtures of compounds that do not cause effects when evaluated individually (Smith et al. 2013; Neale et al. 2015). There is not much available information regarding the presence of CECs in rivers and reservoirs of the São Paulo state or about the toxicity and effects of their mixtures in non-target organisms. Due to this, the present study aimed to determine whether CECs are present in some important rivers and reservoirs across the state and to evaluate the possible effects of these contaminant mixtures in environmentally relevant concentrations using zebrafish embryos as the sentinel organism in order to obtain a better scenario of the contamination by these compounds.

Materials and methods

Sampling sites

The chosen surface water sampling sites (Fig. 1) were distributed throughout the São Paulo state, in order to obtain representative data from eight of the twenty-two state's hydrographic units. During 2 years, bimonthly samplings were carried out in seven rivers and three reservoirs, obtaining one hundred and twelve (112) surface water samples. A brief description of the sampling sites is presented in Table 1.

Surface water sampling and sample preparation

The water samples were collected in 1-L amber glass bottles which were previously heated to 400 °C in order to eliminate organic residues. The surface water samples were kept refrigerated (4 °C) without preservatives, for a maximum holding time of 7 days, until solid-phase extraction (SPE). The samples were extracted using an SPE method accredited under ISO/IEC 17025, capable of reproducibly concentrating organic pollutants from 1 L of surface water in 1 mL of the eluate. The SPE was performed using high-capacity hydrophilic/lipophilic balanced (HLB) disks from Atlantic in a SPE-DEX 4790 extractor system from Horizon Technology. Before the sample extraction, HLB disk with AP20 membrane and Fast Flow pre-filter were conditioned using 15 mL of methanol and 10 mL of ultrapure water. The disks were dried for 30 min, and then, samples were eluted with 15 mL of methanol. After SPE, the extracts were dried by vacuum evaporation using the EZ-2 Genevac and kept below 0 °C until being analyzed.

Table 1 Sampling site information regarding the Hydrographic Units of Management of the State of São Paulo (UGRHI), geographical coordinates, and land use in the surrounding region

Hydrographic unit	Sampled sites	Location	Description of land use
Alto Tietê	Guarapiranga Reservoir	23° 45′ 15″ S 46° 43′ 37″ W	High population density. The reservoir is used as a source for public supply
	Ribeirão Pires River	23° 42′ 52″ S 46° 25′ 45″ W	High population density, urbanized area, domestic and industrial sewage discharge
Mogi Guaçu	Araras River	22° 16′ 46″ S 47° 13′ 23″ W	Agricultural, urban, industrial use of water/soil
Tietê Jacaré	Ribeirão Grande River	22° 15′ 39″ S 48° 48′ 35″ W	Agricultural, urban, and industrial use of water, domestic and industrial sewage discharge
Mantiqueira	Sapucaí-Guaçu River	22° 42′ 58″ S 45° 33′ 36″ W	Conservation Unit, the water main use is for public supply
Piracicaba, Capivari, and Jundiaí	Piracicaba River	22° 41′ 51″ S 47° 23′ 14″ W	Industrial and agricultural uses of water, high population density
	Jaguari River	22° 52′ 39″ S 46° 36′ 26″ W	Water designated for public and industrial supply, wastewater discharge and agricultural irrigation
Alto Paranapanema	São Miguel Arcanjo River	23° 53′ 18″ S 48° 01′ 32″ W	Water for public and industrial supply, wastewater discharge
Paraíba do Sul	Jaguari Reservoir	23° 17′ 38″ S 46° 14′ 02″ W	Water for public supply to São Paulo Metropolitan Area
Peixe	Cascata Reservoir	22° 12′ 48″ S 22° 12′ 48″ W	Water to public supply. Wastewater discharge

Chemical analysis

The occurrence of 30 target compounds such as hormones, pharmaceuticals and personal care products, pesticides, and industrial compounds in surface water samples was performed using liquid chromatography coupled to mass spectrometry (LC–MS/MS) analysis. The selected LC–MS/MS experimental parameters for each compound are presented in Table S1 of

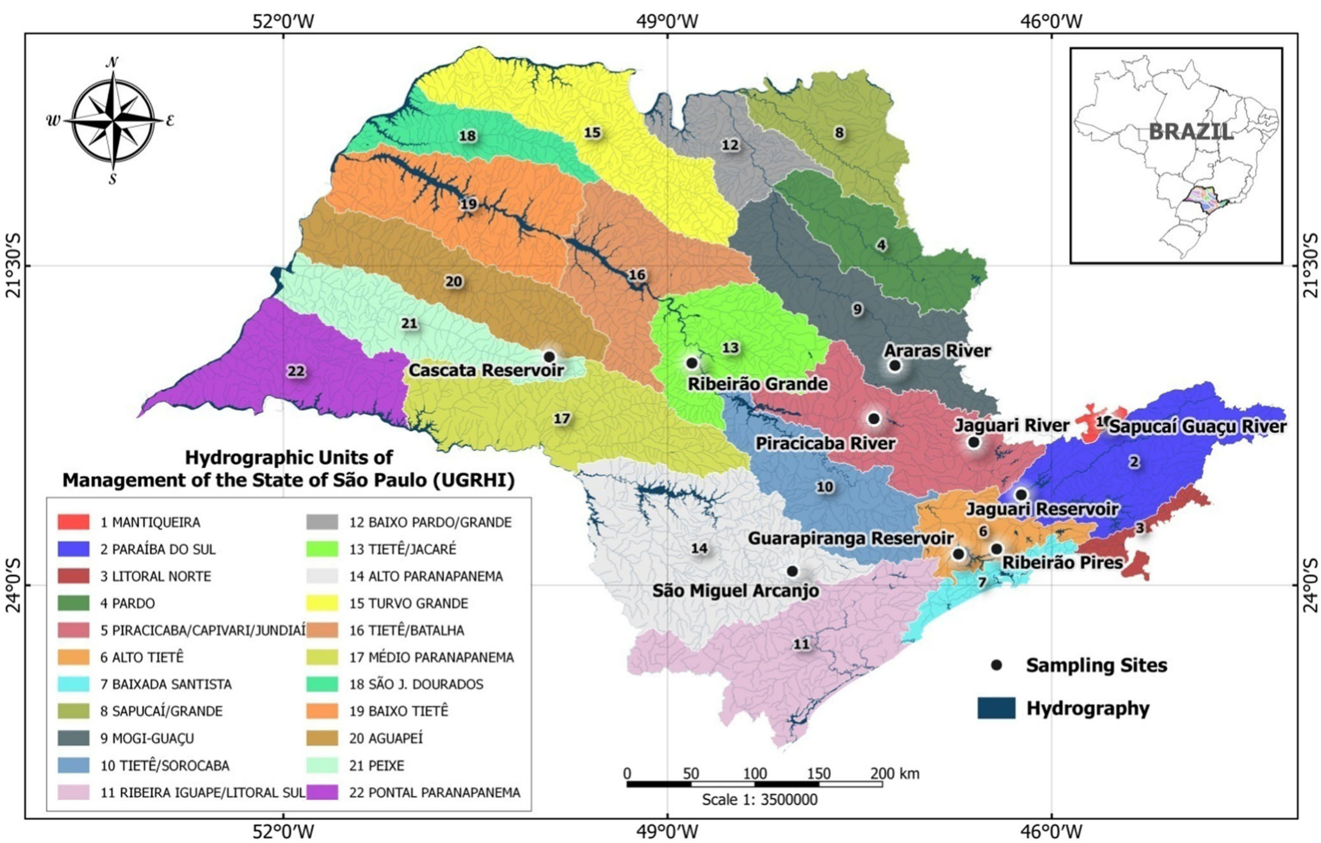

Fig. 1 Sampling sites according to Hydrographic Units of Management of the State of São Paulo (UGRHI)

the Supplementary Materials. The chromatographic conditions, data processing, and validation of the methodology have been described in Montagner et al. (2014) and Jardim et al. (2012). The method used an Agilent 1200 LC system coupled to an Agilent 6410 Triple-Quad mass spectrometer. The volume of 500 μL of the water/methanol 70:30 (v/v) solution which was the same composition of the initial mobile phase of the chromatography method was added in the dried eluate containing the target compounds and quantitatively transferred to a vial for LC–MS/MS analysis. The chromatographic separation was performed with a Zorbax SB-C18 column (2.1 × 30 mm, particle size of 3.5 μm) in a thermostatized column compartment at 30 °C and the injection sample volume of 10 μL. After the chromatographic separation, the compounds were ionized using an electrospray ionization source (ESI) operating in the positive and negative ion mode. The mobile phase components were (A) ultra-pure water with 0.1% v/v formic acid (for positive ionization) and ultra-pure water with 0.01% v/v ammonium hydroxide (for negative ionization) and (B) methanol, with elution in the gradient mode. Stepwise gradient elution at a flow rate of 0.3 mL min^{-1} was programmed by increasing B concentration from 30 to 60% in 3 min maintaining for 7 min, followed by an increase to 67% in 10 min, and held constant for another 5 min, re-adjusting to the initial conditions, totaling 18 min of analysis for positive ions. The stepwise gradient elution for negative ions was increasing B concentration from 30 to 70% in 3 min maintaining for 3 min, followed by an increase to 90% in 6 min, and held constant for another 6 min, re-adjusting to the initial conditions, totaling a 15-min analysis. Data acquisition was performed by multiple reactions monitoring (MRM), recording the transitions between the precursor ion and at least two product ions for each target analyte. The external calibration prepared with analytical standards with purity higher than 97% purchased from Sigma-Aldrich (Steinheim, Germany), Riedel-de Haën (Seelze, Germany) or Fluka Analytic (Milwaukee, USA) in the solid form. The calibration solutions (500, 300, 200, 100, 50, 25, 10, 5, and 1 μg L^{-1}) were prepared by adding different volumes of working solutions to 70:30 (v/v) H_2O–MeOH solution. The analytical curves were obtained in triplicate, for each ionization type, for the year of analyses and included measurement of three independent standards (quality control—QC) at about the middle of the calibration range, each eighty analysis. The smallest analytical linearity was obtained of the calibration range, with $r^2 > 0.989$ for atrazine. The limit of quantification (LOQ) of the analytical method was obtained statistically from a calibration curve according to Miller and Miller (2005). The LOQ ranged from 0.33 to 74.93 ng L^{-1} for hexazinone and mestranol, respectively. The mass spectrometer parameters for each compound and the respective LOQ of the analytical methodology are described in Table S1.

Fish Embryo Acute Toxicity (FET) test

Bioassays using *Danio rerio* (zebrafish) are a well-described model, in which the development, differentiation, and animal growth occur in parallel; it is considered an alternative and efficient method in evaluating a range of effects caused by chemical stressors (Scholz et al. 2013; Wirbisky et al. 2016; Zoupa et al. 2020). The surface water organic extracts previously prepared using SPE were reconstituted with reconstituted water and dimethylsulfoxide (DMSO at 200 μL L^{-1} to the same concentration as the original sample (1 L), to evaluate apical effects (mortality) and morphological abnormalities in zebrafish embryos after 96 h of exposure. The reconstituted water is deionized water to which reagent-grade chemicals have been added. The resultant synthetic fresh water is free from contaminants and has the desired characteristics of pH, alkalinity, and hardness (EC 2014). In the present study, reconstituted water for FET tests was prepared according to Keating (1985). The tests were carried out according to the OECD Test Guideline 236: Fish Embryo Acute Toxicity (FET) Test (OECD 2013). Although the FET test was designed to assess the acute toxicity of chemicals and mixtures, a variety of sublethal, morphological, and behavioral changes can also be evaluated during embryo development (Di Paolo et al. 2016; Kovács et al. 2016). In the present study, FET test was performed to assess mortality (apical effects) and sublethal effects (morphological abnormalities) in zebrafish embryos after 96 h of exposure at organic extracts of surface water samples. The fish maintenance and egg production were carried out according to Lammer et al. (2009). The egg selection was performed using a stereomicroscope, and tests were conducted when the batch fertilization rate was ≥ 80%. After egg selection, two zebrafish eggs were placed per well within a maximum of 4 h post-fertilization (hpf) in 24-well plates containing 2 mL samples/well. To assure test quality, a solution of 4 mg L^{-1} of 3.5-dichloro aniline, reconstituted water, and a solution of DMSO at 200 μl L^{-1} were used respectively as positive control, negative control, and solvent control. All the plates were incubated for 96 h at 26 ± 1 °C with a 12:12 light/dark photoperiod. Subsequently, the endpoints described in Table 2 were assessed using an inverted microscope AXIOcam ERc 5s Vert. A1—Zeiss. The tests were considered valid when the survival of the embryos in the negative control and in the solvent control was ≥ 90%, and the positive control had a mortality rate ≥ 30%. According to EU Directive 2010/63/EU and the Commission Implementing Decision 2012/707/EU, fish are non-protected animals until the stage of free feeding. This limit was set at 120 hpf for zebrafish. In the present study of FET, tests did not exceed 120 hpf, but were nevertheless part of a larger project for which approval by the Ethical Committee for Animals from Nuclear Energy Research Institute (CEUA/IPEN) was registered under number 207/18 and obtained in February 15th 2018.

Table 2 Endpoints assessed in zebrafish embryos after 96 h of sample exposure

Apical effects (mortality)	Non-lethal effects (morphological abnormalities)
Coagulated embryo	Column malformations
Non-formation of somites	Pericardial edema
Non-detachment of the tail	Yolk sac edema
Non-detection of heartbeat	Tail malformation
	Reduced organism size

Statistical analysis

All the observed effects of apical effects and morphological abnormalities obtained by FET test with zebrafish embryos were assessed using the software Sigma Plot 14.0. The data were evaluated for normality (Shapiro–Wilk) and equal variance (Brown–Forsythe). When the data showed normal distribution, one-way ANOVA was performed; if the data failed normality, Kruskal–Wallis one-way analysis of variance on ranks method was used. Furthermore, Bonferroni *t* test was performed to analyze statistical difference ($P \leq 0.05$) between the treated group (bimonthly samples) and control (DMSO 200 μl L^{-1}) for each category of evaluated effects (apical or morphological abnormalities). In order to meet the hypothesis test criteria, a sample was classified as toxic when *P* value was ≤ 0.05 and non-toxic if *P* value was > 0.05. The data set used for the statistical assessment is presented in Table S2 of the Supplementary Materials.

Results and discussion

Chemical analysis

The chemical analysis of the surface water samples has resulted in quantification of twenty-five from the thirty selected target compounds (Table S1). The results were confirmed using external analytical reference standards, according to the retention times, mass accuracy, isotopic pattern, and fragment ions. The obtained concentration range according to each substance category is summarized in Table 3. The concentrations of each compound quantified in the samples are described in Table S3 of the Supplementary Materials.

The presence of contaminants of emerging concern (CECs), such as endocrine-disrupting compounds, pharmaceuticals, personal care products, and many other substances, is a worldwide concern once these substances are not completely removed during conventional water and wastewater treatment processes, including coagulation, flocculation, sedimentation, and filtration, and even on biologically activated sludge processes (Heo et al. 2019). Nowadays, successful technologies for the removal of CECs from water and wastewater are available; however, these technologies are dependent on the very specific properties of some target compounds, being not totally effective for a comprehensive range of CECs. In addition, effective treatments such as the design and use of adsorbents, membranes, and UV/oxidation processes have a much higher cost compared to the current conventional technologies (Hernández-Maldonado and Blaney 2019).

Caffeine

As observed in Table 3, caffeine has been detected in 95% of all analyzed samples from all sampling sites, within a wide concentration range (5.5 ng L^{-1} to 69.6 μg L^{-1}). The caffeine presence in water is directly related to domestic sewage; therefore, this substance is used worldwide as an anthropogenic activity indicator (Bahlmann et al. 2012; Li et al. 2020; Qian et al. 2020). High concentrations of caffeine have been found at rivers Ribeirão Grande (69.6 μg L^{-1}) and Ribeirão Pires (64.1 μg L^{-1}) (Fig. 2), which have highly urbanized surrounding areas. Several other studies have been verified the caffeine occurrence at high concentrations in Brazilian aquatic matrices (Montagner et al. 2019; Sposito et al. 2018; Machado et al. 2016; Pereira et al. 2016). López-Doval et al. (2017) quantified caffeine at Guarapiranga Reservoir whose concentrations (0.006–4.8 μg L^{-1}) were at the same range as the measured concentrations reported in this study at the same site. Moreover, some studies correlated the caffeine concentrations with the presence of other emerging contaminants and estrogenicity (Buerge et al. 2003; Montagner et al. 2014), which suggests that caffeine analysis could be an advisable alternative to indicate anthropogenic pollution caused by raw and treated sewage in water bodies.

Hormones

The presence of estrogenic substances has been reported in several studies worldwide, and one of the major concerns is the occurrence of hormones in environment correlated to domestic wastewater and other anthropogenic sources (Xu et al. 2019; Huang et al. 2016; Bergman et al. 2015; Kolpin et al. 2004). The hormones 17α-ethinylestradiol, 17β-estradiol,

Table 3 Concentration range (ng L^{-1}), limit of quantification (LOQ), and frequency of quantification (%) of the target substances in the studied surface water sites (the occurrence of the substance at least once in each of the 10 sampled sites is shown at the last column)

Substance category	Target substances	LOQ (ng L^{-1})	Concentration range (ng L^{-1})	Frequency of quantification (%)	Number of positive sampled sites
Stimulant	Caffeine	1.80	5.5–69,585.0	95	10
Hormones	17α-Ethinylestradiol	6.6	50.0–68.0	28	10
	17β-Estradiol	4.6	27.0–57.0	24	10
	Estriol	1.1	5.9–224.0	17	8
	Estrone	3.8	3.8–77.0	19	8
	Testosterone	1.28	8.4–12.5	3	2
	Diethylstilbestrol	2.0	-	0	0
	Mestranol	74.93	-	0	0
	Levonorgestrel	15.01	-	0	0
	Progesterone	0.58	-	0	0
Antibacterial	Triclosan	3.6	5.6–7.2	3	1
Plastificant	Bisphenol A	6.5	6.5–1300.0	50	10
Alkylphenols	Octylphenol	2.2	68.0–70.0	26	10
	Nonylphenol	1.6	58.0–59.0	24	10
Fungicides	Azoxystrobin	2.1	2.8–3.1	4	10
	Carbendazim	4.7	4.7–285.0	85	6
	Tebuconazole	0.5	0.5–14.0	35	10
Insecticides	Carbofuran	1.7	1.7–107.0	9	6
	Imidacloprid	2.7	2.7–46.0	22	5
	Fipronil	1.0	1.0–4.0	13	7
	Malathion	0.7	3.1–55.0	49	10
Herbicides	2,4-D	1.2	1.4–260.0	44	6
	Ametryn	0.5	1.2–43.0	23	9
	Atrazine	0.7	3.1–86.0	32	10
	Simazine	0.5	0.5–44.0	15	6
	Hexazinone	0.3	0.4–41.0	27	9
	Hydroxiatrazine	1.6	17.0–298.0	29	7
	Clomazone	12	31.0–46.0	5	3
	Diuron	12	26.0–134.0	39	4
	Tebuthiuron	4.7	9.9–229.0	37	6

estriol, estrone, and testosterone evaluated in this study were quantified in a range from 1.1 to 224 ng L^{-1}. The hormones progesterone, mestranol, and levonorgestrel were also analyzed in this study; however, they were not quantified in any samples. Figure 3 shows the occurrence of hormones (sum of the analyzed compounds) in the bimonthly samples for each sampled site. According to this assessment, hormones have been quantified in less than 30% of samples, where the highest concentrations were at Araras River (117 ng L^{-1}), Ribeirão Grande River (122 ng L^{-1}), and Ribeirão Pires River (224 ng L^{-1}). These obtained concentrations are in agreement with other studies carried out in Brazilian environmental matrices (Jardim et al. 2012; Weber et al. 2017; Sposito et al. 2018; Nascimento et al. 2018) that also analyzed other impacted rivers in the country.

Pesticides

As agriculture has economic and social impact in Brazil, several studies aiming the presence and toxicity of pesticides in Brazilian environment have been conducted throughout the country (Albuquerque et al. 2016; Vieira et al. 2016; Vale et al. 2019; Souza et al. 2020; Severo et al. 2020). Complex mixtures of pesticides are found in a variety of environmental compartments, being a unique combination that depends on the environmental conditions to which these mixtures are exposed (Ryberg and Gilliom 2015). Furthermore, the mixture can cause more adverse effects on exposed organisms than the exposure to a single pesticide with known toxicity (Gandar et al. 2017; van de Merwe et al. 2018; Xie et al. 2019). The compounds analyzed in the present study belong to three of the main classes of pesticides: herbicides, fungicides, and

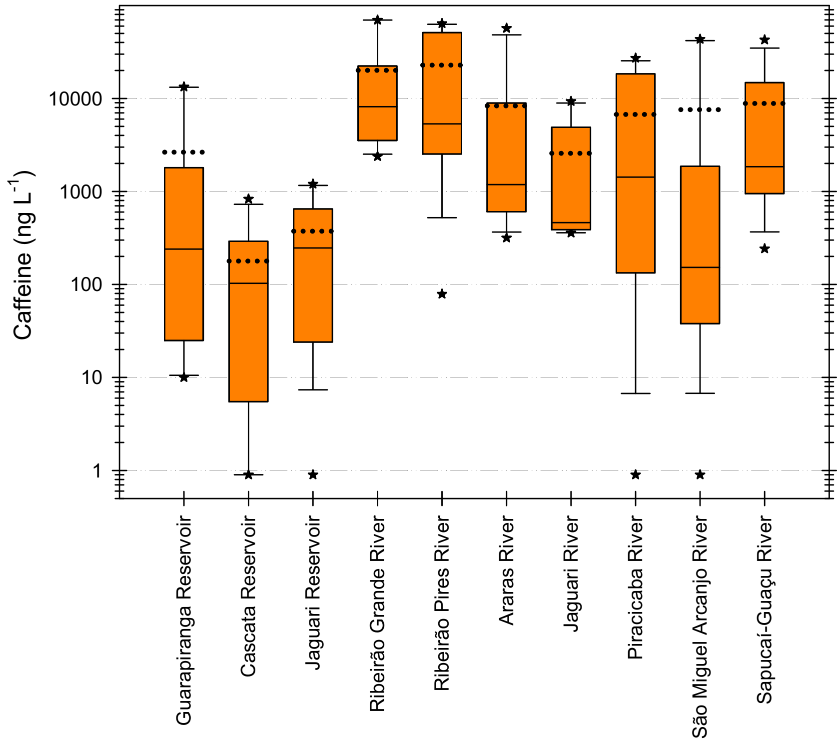

Fig. 2 Caffeine concentration (ng L^{-1}) during the 2-year study in a box plot graph with standard deviation for each sampled site; the dotted line (…) refers to the mean, solid line (—) refers to the median, and asterisk (*) is the 5th/95th percentile

insecticides. Among pesticides, the herbicides were the substance class with the highest frequency of quantification (Fig. 4), where the highest concentrations were found at Piracicaba River (285 ng L^{-1}) and Araras River (261 ng L^{-1}), which is associated with the intense agricultural activity at their vicinities. The herbicides with higher detected concentrations were hydroxyatrazine, atrazine, and tebuthiuron. The fungicide carbendazim was found in 85%

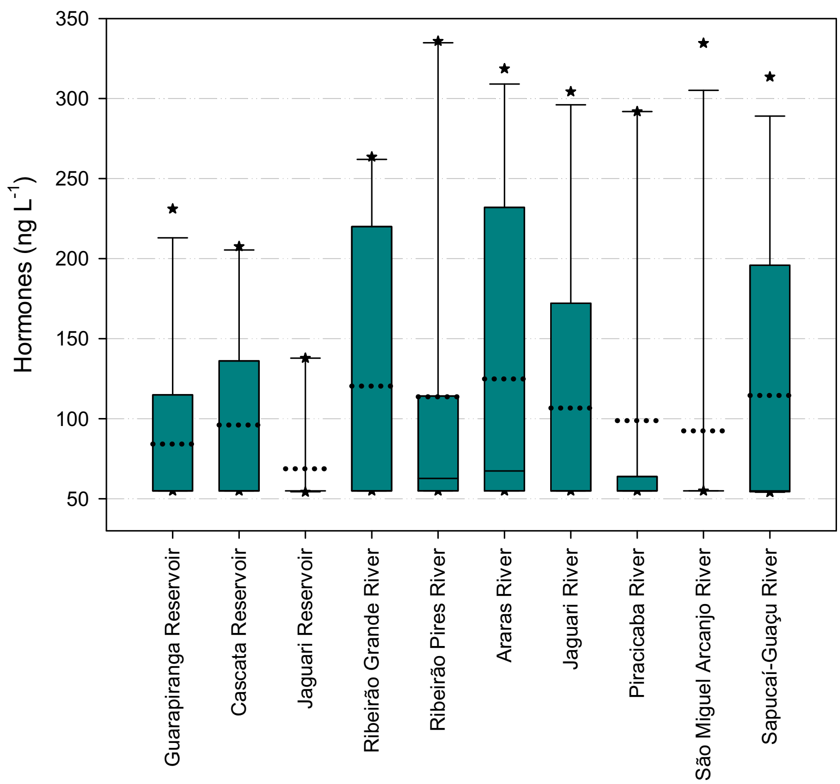

Fig. 3 Hormone concentration (ng L^{-1}) during the 2-year study in a box plot graph with standard deviation for each sampled site; the dotted line (…) refers to the mean, solid line (—) refers to the median, and asterisk (*) is the 5th/95th percentile

of the analyzed samples (from 4.7 to 285 ng L^{-1}) which corroborates with Montagner et al. (2019), where it was found in 90% of the samples in concentrations from 0.8 to 4520 ng L^{-1} in other Brazilian rivers. The use of pesticides in urban areas such as lawns, gardens, and impermeable surfaces is a serious concern, as they can reach reservoirs, lakes, and other aquatic matrices used as public supplies (Meftaul et al. 2020). As a result of this urban application, concentrations of pesticides have been found at Ribeirão Pires River (0.4–224 ng L^{-1}), Guarapiranga Reservoir (0.4–68 ng L^{-1}), Cascata Reservoir (0.5–117 ng L^{-1}), and Jaguari River (0.3–233 ng L^{-1}), which have highly urbanized surrounding areas.

Bisphenol A (BPA)

The plastificant BPA can enter aquatic matrices through a variety of routes, including industrial operations and the disposal of effluents showing concentrations in surface waters ranging from nanograms per liter to micrograms per liter in distinct locations throughout the world (Bilal et al. 2019; Wang et al. 2017; Esteban et al. 2014; Barceló et al. 2004), whereas regions with industrial activity have shown considerably higher BPA concentrations (88–637 ng L^{-1}) (Kim et al. 2014; Barnes et al. 2008; Kolpin et al. 2004). It was found in this study a BPA concentration range of 82 ng L^{-1} to 2.5 μg L^{-1}, which was present in 50% of analyzed samples in all sites (Fig. 5). These high concentrations were also found in Brazilian environmental matrices by Montagner et al. (2019) and Souza et al. (2011) in levels of 2 ng L^{-1}–13 μg L^{-1} and 0.6–12 μg L^{-1}, respectively. However, it is not known whether BPA contamination of aquatic bodies in Brazil is related to punctual or diffuse sources.

Alkylphenols

The environmental occurrence of alkylphenols is continuously monitored regarding the concern about their potential impact on ecosystem and human health (Acir and Guenther 2018; Priac et al. 2017; Careghini et al. 2015; Brix et al. 2010). The alkylphenols (4-*n*-nonylphenol and 4-*n*-octylphenol) occurred in all sampling sites of this study within 66 ± 5 ng L^{-1}, however in less than 30% of analyzed samples. Environmental concentrations have been reported between 0.1 ng L^{-1} and 15.0 μg L^{-1} in river waters worldwide (Petrovic et al. 2002; Zhao et al. 2009; Meffe and Bustamante 2014) showing why those contaminants have been considered as a priority contaminant in water matrices.

Triclosan

The daily personal and household uses of products containing the antibacterial triclosan have been one of the main reasons of its occurrence in the environment (Zhao et al. 2013). In the present study, triclosan was quantified in 3% of the analyzed samples, in Araras River within 6.4 ± 0.8 ng L^{-1}. Even in low concentrations, triclosan can cause adverse effect in aquatic organisms and its occurrence has also been reported in a variety of environmental matrices worldwide (Guo and Iwata 2017; Pintado-Herrera et al. 2014; Cortez et al. 2012).

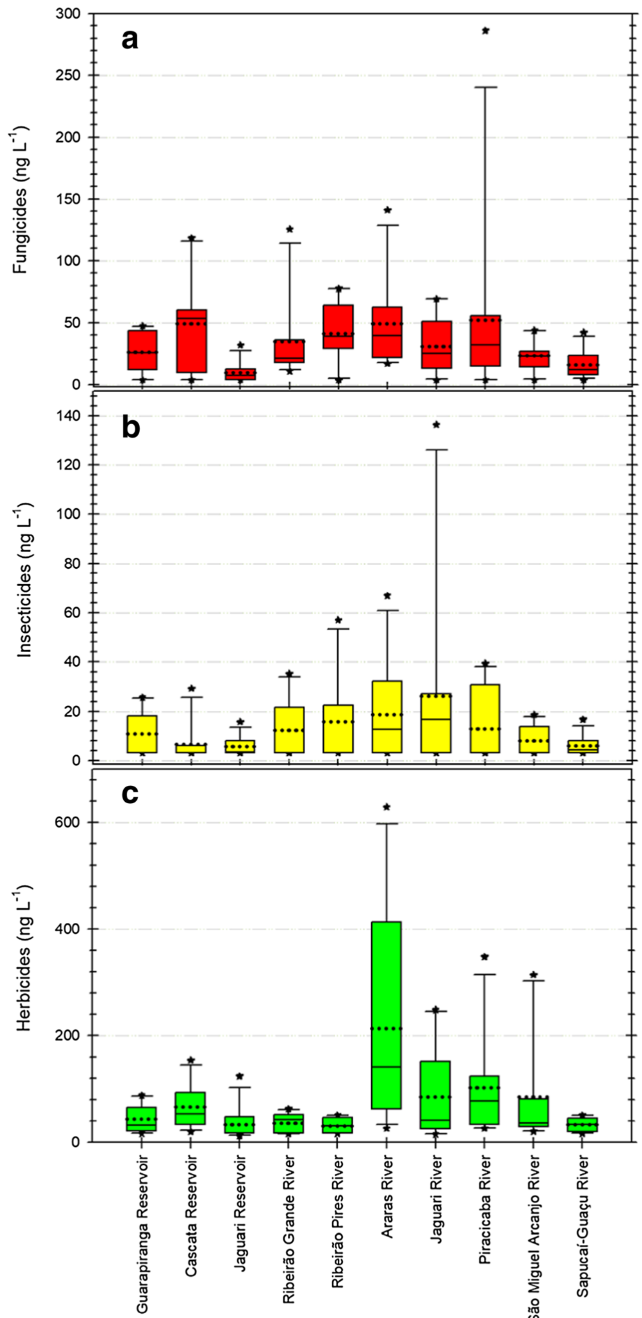

Fig. 4 Pesticide concentration (ng L^{-1}) during the 2-year study, presented by their classes: **a** fungicides, **b** insecticides, and **c** herbicides for each sampled site in a box plot graph with standard deviation; the dotted line (…) refers to the mean, solid line (—) refers to the median, and asterisk (*) is the 5th/95th percentile

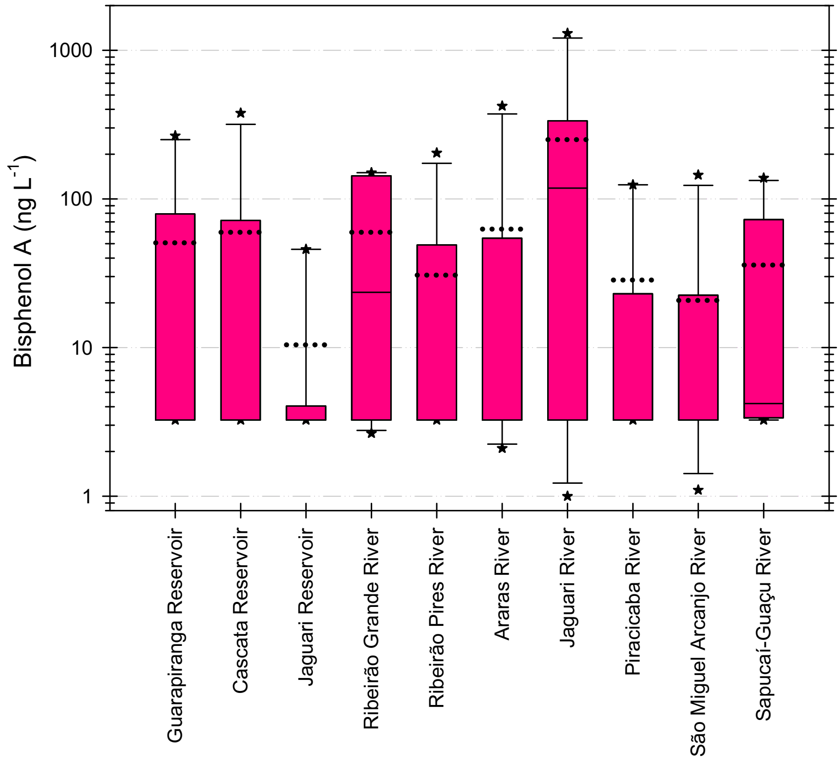

Fig. 5 Bisphenol A concentration during the 2-year study in a box plot graph with standard deviation for each sampled site; the dotted line (…) refers to the mean, solid line (—) refers to the median, and asterisk (*) is the 5th/95th percentile

Fish Embryo Toxicity Acute (FET) test

One hundred and twelve (112) organic extracts of surface water samples were analyzed for the occurrence of apical effects (mortality) and for non-lethal effects (morphological abnormalities) in zebrafish embryos after 96 h of exposure to the samples. All the tests meet the requirements described at the OECD No. 236 (2013): embryo survival rate was ≥ 90% in negative control and in solvent control; mortality was ≥ 30% in positive control; also mortality on internal plate controls were not observed in any analyzed sample. The FET results according to each sampled site are described in Table S4 of the Supplementary Materials.

Mortality and morphological abnormality evaluation

In general, embryos and larvae are considered to be the most sensitive stage in the life cycle of zebrafish (Jiang et al. 2016; Schulte and Nagel 1994), being more vulnerable to contaminants because their vital biological systems are in development. Consequently, the effects that occur during this phase are ideal for studies of acute and chronic toxicity evaluation (Wagner et al. 2017; Kurobe et al. 2018). Also, these life stages seem to experience less or no pain, suffering, or stress, and are less invasive than using adult individuals to achieve the same scientific purpose (EC 2010; EFSA 2005). Table 4 summarizes the evaluated effects with FET test in this study, and the percentage (%) of the effects observed according to each sampled site.

As the environmental samples are highly complex, a direct relationship between the compounds detected by chemical analysis and the effects measured in FET test had not been inferred. The mortality (apical effects) rate, malformation (morphological abnormalities) rate, and normality (normal embryos) rate are presented in Fig. 6, according to each sampled site and with the negative control (reconstituted water) and solvent control (DMSO).

The mortality rate (apical effects) was higher in embryos exposed to organic extracts derived from the sites with greater anthropogenic activity in surrounding areas and with domestic sewage input, such as Ribeirão Pires River (17.3%) and Sapucaí-Guaçu River (10.5%). However, no apical effects were measured at São Miguel Arcanjo River and Jaguari Reservoir, which have public supply of water as one of the main purposes. Regarding the adverse effects, it was observed that the coagulated embryos and the lack of heartbeat were the most frequently measured in all samples during the study (Fig. 7).

In respect to the morphological abnormalities, embryos with pericardial and/or yolk edema were the most recurrent observed effects in all analyzed samples, followed by reduced organism size (Fig. 8). Samples from Ribeirão Pires and Ribeirão Grande rivers showed the highest percentage of

Table 4 Lethal and the morphological abnormality endpoints evaluated in surface water samples and the percentage (%) of general effects in all analyzed samples

Sampling sites	Apical effects				Morphological abnormalities					Percentage (%) of the total observed effects in analyzed samples
	Coagulated embryo	Non-formation of somites	Non-detachment of the tail	Non-detection of heartbeat	Column malformations	Pericardial edema	Yolk sac edema	Tail malformation	Reduced organism size	
Araras River	•			•	•	•	•	•	•	15
Cascata Reservoir	•					•	•			8
Guarapiranga Reservoir	•			•		•	•	•	•	7
Jaguari Reservoir						•	•			1
Jaguari River	•			•	•	•	•	•	•	19
Piracicaba River	•			•	•	•	•	•	•	15
Ribeirão Grande	•		•			•	•	•	•	26
Ribeirão Pires	•	•	•	•	•	•	•	•	•	53
São Miguel Arcanjo River	•					•	•			2
Sapucaí-Guaçu River	•		•	•	•	•	•	•	•	23

morphological abnormalities, 28.5% and 24.4%, respectively; and Jaguari Reservoir had the lowest rate of observable morphological abnormalities (0.7%).

Embryotoxicity on zebrafish

Although the concentrations of a single individual substance present in the aquatic environment are low to show an effect, several substances can result in significant toxicity for non-target aquatic species (Altenburger et al. 2004; Walter et al. 2002). Therefore, in several scenarios, mixtures of substances and their possible interactions with other contaminants present in the environment can be underestimated, which demands the toxicity evaluation of mixtures for a better understanding on how the contaminants affect the aquatic ecosystem. In Fig. 9 is shown the percentage (%) of samples with acute toxicity, chronic toxicity, and non-toxic samples, according to the sampled sites throughout the 2 years of study.

Among the toxic effects on embryos, the obtained results showed that environmentally relevant concentrations of a variety of substances present in aquatic matrices are capable of causing adverse effects, which can also affect the normal development of organisms. As shown in Fig. 9, São Miguel Arcanjo River and Jaguari Reservoir were the only sites of this study that did not show toxicity in any of the samples during the 2 years of study; and at Ribeirão Pires and Ribeirão Grande rivers were observed the highest percentage of samples with toxicity, 92% and 80%, respectively. The present study evaluated adverse effects on embryos after exposure to water samples; however, these effects are not related directly to one single substance, neither to a substance group. Nevertheless, it should be noted that studies have already evaluated the effects on zebrafish embryos related to CECs that have also been found in surface waters of the São Paulo state. Several studies have shown that exposure to trace concentrations of caffeine in early developmental stages of zebrafish can cause cell damage, stimulation of the central nervous system, morphological abnormalities, and mortality (Qian et al. 2020; Zhou et al. 2019; Rah et al. 2017; Pruvot et al. 2012; Chen et al. 2008). Pesticides are formulated to affect target species; however, they can reach aquatic matrices and therefore affect non-target organisms. Studies have shown that pesticides such as atrazine, diuron, and malathion can cause toxic effects of endocrine pathways and cell damage to morphological abnormalities and mortality in aquatic organisms (Cleary et al. 2019; Kao et al. 2019; Shen et al. 2020). In the study conducted by Severo et al. (2020), twenty-four pesticides were reported in surface waters in southern Brazil, and alterations in zebrafish embryos spontaneous movement, heart rate, and hatching rate were observed after exposure to these water samples. Even though environmental concentrations of pesticides are low (ng–μg L^{-1}), it has been shown that they can affect normal functions in aquatic

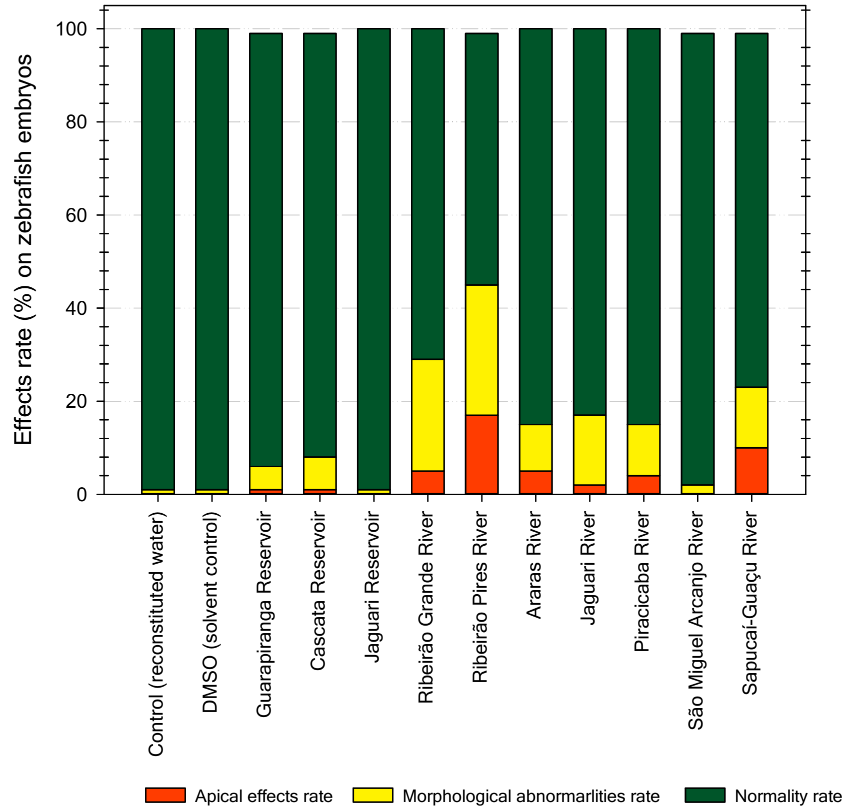

Fig. 6 Apical effect rate (%), morphological abnormality rate (%), and normality rate (%) on zebrafish embryos after 96 h of exposure to organic extracts of surface water samples

organisms. In the present study, pesticides were widely detected among the sampled sites. The highest concentrations and frequencies of detection were in the Araras and Piracicaba rivers, which, as previously mentioned, are located in regions of high agricultural activity. It was also observed at the same locations a high incidence of zebrafish embryos with pericardial edema, and the percentage of samples with toxicity in these sites was 64% and 36%, respectively. Regarding the steroid hormones, the exposure has been linked to neurotoxicity, teratogenic effects, and changes in gene expression in the early stages of zebrafish development (Schmid et al. 2020; Silva et al. 2019; Petersen et al. 2013; Colman et al. 2009). These substances are generally found in aquatic matrices at very low levels; however, trigger concentrations for the appearance of anatomical and physiological changes, between 10 and 20 ng L^{-1}, have been reported by Silva et al. (2019). These concentration ranges were found in all sampled sites (Table 3) over the 2 years of this study. Although the observed effects on zebrafish embryos in this study were related to the exposure of surface water samples, such findings show that the environmental concentrations of the CECs, as steroid hormones, deserve attention regarding their detection in the aquatic environment. Recent research has assessed the adverse effects of BPA on zebrafish in a variety of systems, such as early embryogenesis and cardiac malformation (Brown et al. 2019; Tse et al. 2013), and also as an endocrine disruptor, this substance is capable of increasing the vitellogenin level on fish due to its estrogenicity (Huang et al. 2020; Song et al.

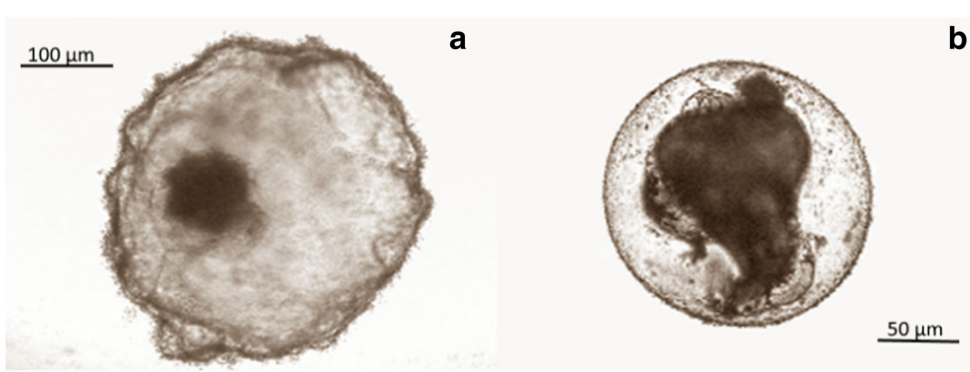

Fig. 7 Acute toxic effects observed in embryos after surface water exposure. **a** Coagulated embryo. **b** Lack of heartbeat and developmental effects

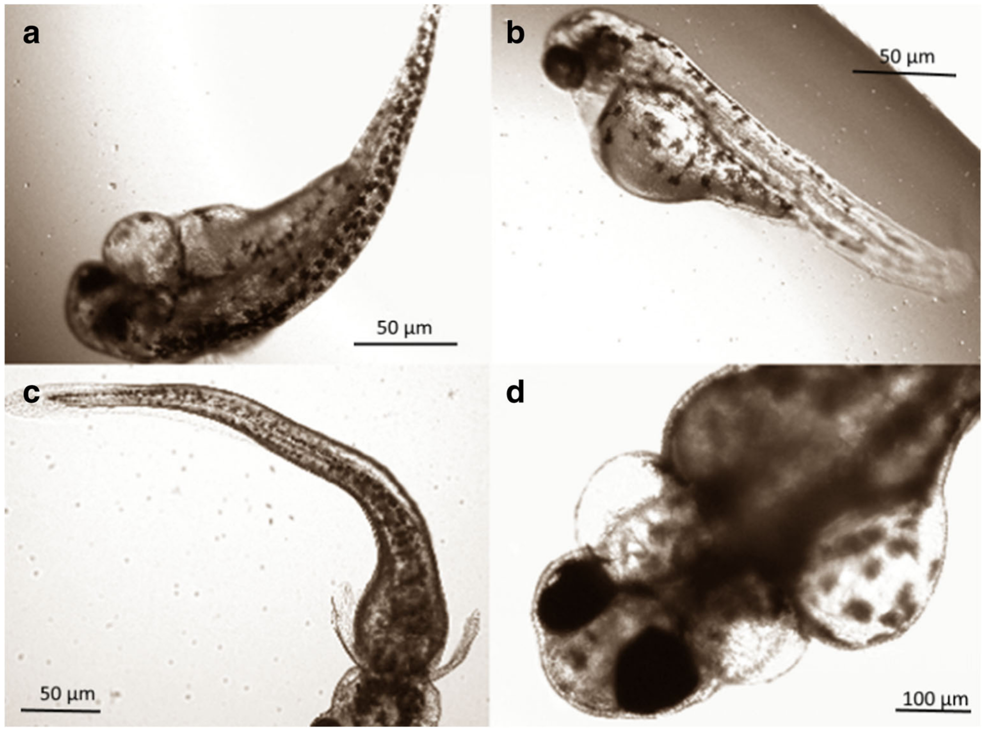

Fig. 8 Morphological abnormalities in zebrafish embryos after 96 h of exposure to organic extract of surface water samples. **a** Reduced size organism, spine curvature, and pericardial and yolk sac edema. **b** Reduced size organism. **c** Tail malformation. **d** Pericardial and yolk sac edema

2014). Environmental concentrations of BPA (0.03 and 0.1 mg L^{-1}) caused effects such as pericardial edema and spinal malformation in zebrafish embryos (Gyimah et al. 2021). The BPA highest concentration (1.3 μg L^{-1}) was found in Ribeirão Grande River, and it was observed at this sampling site a high percentage of samples with toxicity on apical effect evaluation (20%) and toxicity on morphological abnormalities (60%). According to Chen et al. (2017) and Xu et al. (2019), the exposure to BPA at concentrations ≥ 10 μg L^{-1} was able to cause adverse effects in zebrafish embryos including developmental abnormalities and cell damage. Other substances that have been found in the environment and their presence that were related to adverse effects on biota were the alkylphenols. The study by Xia et al. (2010) indicates that exposure to

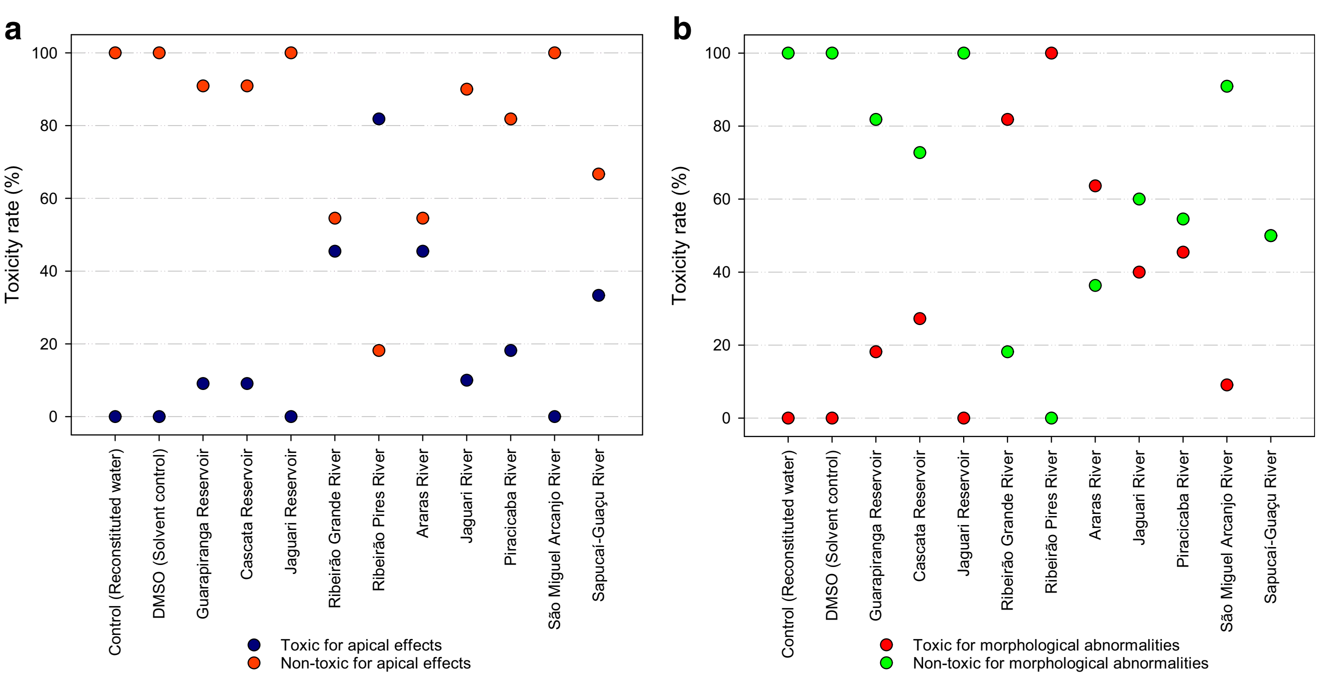

Fig. 9 Obtained toxicity rate (%) for **a** apical effects and **b** morphological abnormalities during the 2-year study according to each sampling site

nonylphenol alters locomotor activity and promotes behavioral changes in zebrafish. After exposure to alkylphenols, it was observed that there were changes in DNA toxicity and endocrine alterations in zebrafish embryos (Vosges et al. 2012). All the sampling sites of this study had alkylphenol concentrations around 50 ng L^{-1}. Triclosan is widely used as an antimicrobial agent and has been found continuously in environmental matrices, thus being able to affect the normal functions of organisms. According to Pullaguri et al. (2020), exposure to triclosan may alter the behavior of adult zebrafish, interfering with AChE activity and expression. In addition, acute toxicity effects on zebrafish embryos and morphological effects, such as malformations, spinal curvature, pericardial edema, and delayed hatching, were observed after exposure to 0.42 mg L^{-1} (Oliveira et al. 2009). This substance was found in a few number of analyzed samples (3%) on this study; however, it is of great importance to be monitored in the environment.

Conclusion

A great concern with the occurrence of CECs in the aquatic environment and their effects on biota has been highlighted in recent years. Several classes of CECs have been quantified simultaneously in this study, which shows that organisms have been continuously exposed to these substances' mixtures. Even though concentrations below trigger concentrations were found in the rivers and reservoirs of this study, several adverse effects were assessed in zebrafish embryos, which show that interactions between CECs and the effects of mixtures can affect non-target organisms. However, the environmental implications of the occurrence of contaminants in the aquatic environment and their effects on aquatic biota are challenging, and since mixtures of substances and their interactions are rarely evaluated, it is necessary to develop new methodologies and approaches to improve the knowledge of these substances in aquatic matrices, in order to protect the environment and human health. Therefore, providing CEC occurrence data in environment is essential and serves as a basis for prioritizing substances that must be monitored and, consequently, future regulated. Further studies on distribution and occurrence of CECs in aquatic environment and their toxic effects, including synergistic effects of mixtures of these compounds, toward aquatic biota, appear even more necessary due to the extremely limited availability of high-quality datasets. Finally, the improvement of scientific knowledge about sources, pathways, fate, and toxic effects related to the contaminants of emerging concern, specifically in the aquatic matrices in Brazil, must have adequate strategies to monitor environmental risk and ecosystem protection.

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Authors' contributions I declare that all the authors of this manuscript contributed to the methodological and theoretical development of this study. The sample collection and preparation were carried out by Gisela de Assis Martini, Gilson Alves Quináglia and Daniela Dayrell França; chemical analysis was carried out by Cassiana Carolina Montagner and Nívea Cristina Guedes Munin; the biological tests with zebrafish embryos were carried out by Gisela de Assis Martini, William Viveiros, and Mônica Lopes-Ferreira; José Roberto Rogero, Sizue Ota Rogero, and Cassiana Carolina Montagner contributed to the manuscript structure and development. The present version of the manuscript was written by Gisela de Assis Martini, as well as the statistical analysis. Lastly, it is true that all authors have read and approved the submitted version of this manuscript.

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Data availability The obtained and analyzed data of this study are included in this article and on the supplementary information files.

Compliance with ethical standards

Ethics approval and consent to participate Ethical Committee for Animals from Nuclear Energy Research Institute (CEUA/IPEN) registered under number 207/18 and obtained in February 15th 2018.

Consent for publication Not applicable to this manuscript.

Competing interests The authors declare that they have no conflict of interest.

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