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CELLULAR AND MOLECULAR BIOLOGY

Multibiomarker responses in *Danio rerio* after exposure to sediment spiked with triclosan

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Abstract: Triclosan (TCS) is an antimicrobial and antimycotic agent widely used in personal care products. In aquatic environments, both TCS and its biomethylated more persistent form, methyl-triclosan (MeTCS), are usually detected in wastewater effluents and rivers, where are commonly adsorbed to suspended solids and sediments. The aim of this study was to evaluate biochemical and physiological effects in *Danio rerio* after a short term (2 days) and prolonged (21 days) exposures to sediment spiked with TCS acting as the source of the pollutant in the assay. The activities of catalase (CAT), glutathione-s transferase (GST) and superoxide dismutase (SOD), lipid peroxidation levels (LPO), total capacity against peroxyl radicals (ACAP), and acetylcholinesterase enzymatic activity (AChE) were measured in liver, gills, and brain. Most of TCS on the spiked sediment was biotransformed to MeTCS and promoted different adverse effects on *D. rerio*. Gills were the most sensitive organ after 2 day-exposure, showing lipid damage and increased SOD activity. After 21 days of exposure, liver was the most sensitive organ, showing lower ACAP, increased LPO levels, and SOD and CAT activities. This is the first study reporting the effects on biochemical markers in *D. rerio* from a MeTCS sink resulting from sediment spiked with TCS.

Key words: Methyl-triclosan, multivariate analysis, oxidative stress, whole sediment test, zebrafish.

INTRODUCTION

Personal care products (PCPs) are one of the main emerging urban pollutants that were proved to have a negative impact on the environment. Their increasing production, because of their wide spectra of applications, inevitably leads to their uncontrolled release into aquatic ecosystems. Moreover, the lack of efficient urban wastewater treatment leads to the presence of these compounds and their metabolites in these ecosystems, all the while their toxic effects on biota remain unclear (Montes-Grajales et al. 2017). Among PCPs, triclosan (TCS) is widely used as antimicrobial ingredient in toothpastes, soaps, and medical disinfectants (Chen et al. 2018). After its use, TCS is washed or rinsed off and may enter the aquatic environment via local wastewater treatment plants where it is partially removed as result of biodegradation, photolysis, and sorption (Capdevielle et al. 2008). Methyl-triclosan (MeTCS), one degradation product of TCS, is a more stable and persistent compound, with a much slower kinetic degradation rate (Lindström et al. 2002, Bedoux et al. 2012). The fate of this kind of compounds in environment compartments depends on their physicochemical properties, which in turn impact on their degradation and transformation (Singh et al. 2013). Because of their high hydrophobicity, TCS and MeTCS would be expected to be adsorbed to organic matter and sediment. Currently,

they have been detected not only in surface or ground water, but also in sludges, biosolids, and aquatic sediments (Wang et al. 2019).

Several studies have shown that TCS is toxic to many aquatic organisms, such as algae, insect larvae, benthic organisms, and fish (Orvos et al. 2002, Dussault et al. 2008, Oliveira et al. 2009, Perron et al. 2012). Deleterious effects of TCS have been reported in *Danio rerio* in terms of gene expression (Wang et al. 2019), metabolism (Falisse et al. 2017, Macedo et al. 2017, Fu et al. 2019), histological alterations (Gyimah et al. 2020), metamorphosis, and reproductive fitness (Stenzel et al. 2019). However, although MeTCS is more persistent in aquatic environments, little information is available about its adverse effects on aquatic biota. MeTCS mechanism of action is similar to TCS and can occur at quantifiable concentrations even when TCS is below the limit of detection (Lindström et al. 2002). In this context, MeTCS cytotoxicity was evaluated in invertebrates (DeLorenzo et al. 2007, Gaume et al. 2012) and bacteria (Farré et al. 2008), meanwhile when *D. rerio* was exposed via waterborne to it, malformations in the cardiovascular system, spinal curvature, disrupted nitrogen metabolism pathways, altered energetic metabolisms, and fatty acid synthesis were registered (Macedo et al. 2017, Fu et al. 2019). However, it still exists a lack of knowledge about the MeTCS effects on redox balance and neurotoxicity, especially after other kind of exposures rather than the waterborne one.

Assays conducted with whole sediment samples are a more realistic approach and have become a useful tool when assessing toxicity on aquatic organisms. Sediment is an inherently complex, heterogeneous geological matrix, with a number of routes by which biota is exposed to its associated contaminants (Davoren et al. 2005). Ecotoxicological sediment assessments have been focused on many invertebrate benthic organisms (Giusto et al. 2012, Perron et al. 2012, Ho et al. 2013). However, apart from representing a major sink of persistent of toxic substances, sediments are considered a source of contaminants that could reach the water column, and then, those substances became available to non-benthonic organisms (Rocha et al. 2011). Exposure to sediment spiked with TCS generated deleterious effects on embryo-larval development in sea urchin *Lytechinus variegatus* and in the bivalve *Perna perna* (Pusceddu et al. 2018), and also changed the composition of meio and macrofauna in marine benthic communities (Ho et al. 2013). To the best of our knowledge, no studies employing fish as test species have been developed.

Fish are organisms widely used for measurement of biomarkers and their size allows obtaining several tissues that provide a more systemic scenario of the toxicity of a compound (Ale et al. 2018a). *Danio rerio* is a cyprinid species that inhabits and feeds in whole of the vertical water column (Spence et al. 2007). This fish species is broadly used as a model organism to assess ecotoxicological research because its easy accessibility, widely known farming characteristics, and its sensitivity to contaminants. As the impact of TCS on fish is poorly understood, this study aimed to investigate biochemical and physiological effects of sediment spiked with TCS after a short term (2 days) and prolonged (21 days) exposures in the standardized fish species *D. rerio*. We analyzed multiple biomarker responses including morphological indices, neurotoxicity (acetylcholinesterase enzymatic activity) and oxidative stress (superoxide dismutase, catalase, glutathione-S-transferase antioxidant enzymatic activities, lipid peroxidation levels, and total antioxidant competence against peroxyl radicals) in liver, gills, and brain of fish, and, finally, we performed a multivariate

analysis to obtain a holistic view of the effects generated by this emergent pollutant.

MATERIALS AND METHODS

Tests organisms

Wild-type adults of *D. rerio* (n=128; 0.50 ± 0.05 g body weight and 3.6 ± 0.2 cm total length) were reared in the GECAP (Grupo de Estudios de Contaminación Antrópica en Peces) laboratory. During the acclimation period, fish were daily fed with commercial pellets and maintained in aquaria with continuous flow of unchlorinated freshwater (dissolved oxygen 8.0 ± 0.6 mg L^{-1}, pH 8.2 ± 0.4, and conductivity 1000 ± 6 µS cm^{-1}), controlled temperature of 25 ± 2 °C and photoperiod 12:12 light-dark cycles.

Sediment preparation and TCS spiking

Surface sediment (up to 10 cm depth) was taken from an unpolluted site located in Las Flores stream (59° 07´W and 34° 29´S) (Buenos Aires, Argentina). Due to its low anthropic intervention, this site has been assigned as a reference by other authors (Ronco et al. 2008, Giusto et al. 2012, Peluso et al. 2013). Sediment grain size distribution (sand 61%, clay 12% and silt 27%) has been previously characterized (Giusto et al. 2012, 2014, Scarcia et al. 2014). Subsamples from the sediment were taken to determine humidity and total organic matter by combustion at 500 °C for 5 hours (modified from Gordon 2000).

Triclosan (Irgasan, 5-chloro-2-(2, 4-dichlorophenoxy) phenol, ≥97.0% purity, HPLC) was purchased from Sigma-Aldrich. According to previous works (Chen et al. 2011, Ho et al. 2013) degradation processes of TCS can occur during spiking sediment procedure; then, the nominal concentration was set as 2.9 mg kg^{-1} dry weight (dw). This concentration was 3 times greater than the maximum values of TCS reported in the United States: 0.7-0.8 mg kg^{-1} (Miller et al. 2008).

Spiked sediment was prepared by plating 2 mL of acetone TCS concentrated solution onto a glass mortar and thoroughly mixed with 1 kg dry sediment and transferred into a dark amber glass bottle which was kept in a continuous rolling system for 4 days (Pusceddu et al. 2018). Finally, the sediment was stored at 4 °C in the dark for 2 days to establish a chemical equilibrium between TCS and the sediment (Löffler et al. 2005).

Experimental design

Whole sediment static bioassays were conducted in glass aquaria according to the following conditions: 15 L of unchlorinated tap water and 1 kg of unspiked sediment (serving as the control, Ctrl) or TCS spiked sediment. After the addition of the sediment, the system was allowed to stabilize for 24 h before adding the fish. The animals were randomly distributed to each experimental treatment (n=32) and remained exposed for 2 and 21 days under constant aeration and photoperiod (12:12). In addition of aeration, aquaria design included a closed water circuit in order to recirculate and mix the water column of aquaria. Fish were daily fed with commercial pellets, and after the times of exposures the organisms were anesthetized in ice chilled water, weighed, measured, and sacrificed by incision behind the operculum. Gills, liver, and brain were excised and kept in -80 °C until further determinations. This method follows the recommendation of the local and National Institutes of Health Guidelines (Resolution 672-15, National University of Lujan). All experiments were conducted in accordance with national and institutional guidelines (CONICET 2005) for the protection of animal welfare.

Physicochemical parameters in water

Dissolved oxygen (OD), pH, conductivity and temperature were registered with a

multiparametric Hach (HQ30D) probe with OD laser probe; measurements were made in triplicate and data were taken three times during the 2 days period and four times for the 21 days of exposure (0, 7, 14 and 21 days).

Ammonium (µg $N\text{-}NH_4^+$ L^{-1}) present in water was determined during the exposure period according to the phenol method based on the formation of indophenol from the reaction of ammonium with hypochlorite and phenol in an alkaline medium. The determinations were made at 635 nm (APHA 2005). For this purpose, water samples were collected three times during the test and filtered with fiberglass filters Munkell® MF/C, which were previously weighed for the determination of suspended particulate matter (SPM).

Quantification of TCS and Me-TCS in water, sediment, and suspended particulate matter.

For the determinations of TCS and MeTCS concentrations in water, sediment and suspended particulate matter, samples were taken in triplicate at each time of exposure during the test. Also, two samples of TCS spiked sediment were collected to determine initial sediment concentrations of TCS and MeTCS.

Extractions

For MeTCS and TCS analysis in water, samples were extracted by passage through a Waters® Oasis C18 HLB 60 mg cartridge (Canosa et al. 2005, Elorriaga et al. 2013). The SPE cartridges were first conditioned with 5 mL of methanol, followed by 5 mL of nanopure water. Water samples were extracted at a 5 mL min^{-1} flow rate under moderate vacuum in a Visiprep™ SPE Vacuum Manifold (Supelco, Bellefonte, PA). After the extraction, the cartridges were washed with 5 mL of nanopure water, and then air dried for 20 min under vacuum. Retained analytes were eluted with 5 mL of methanol. The elutes were dried under a gentle stream of nitrogen, reconstituted in acetonitrile, passed through 0.45 µm filters, and transferred to amber chromatographic vials.

Suspended particulate matter was extracted by placing the filters in 15 mL polypropylene tubes and adding 5 mL of acetonitrile. Two 10 min sonication cycles were performed, and then centrifuged for 10 min at 3000 *g*. Lastly, 1 mL of the extract was filtered through 0.45 µm membranes and then placed into amber vials.

Sediments were extracted using a modified *QuEChERS* procedure (Mac Loughlin et al. 2017). Briefly, 7 g of wet sediment were weighed into a 50 mL polypropylene tube and extracted with 15 mL of acetonitrile. After adding solvent, the tubes were vigorously shaken and then sonicated for 10 min, shaken for 1 min and sonicated again. Extraction salts (2 g of NaCl and 6 g of anhydrous $MgSO_4$) were added and shaken manually for 2 minutes. Finally, the tubes were centrifuged for 10 min at 3000 *g*. From the supernatant (acetonitrile), 1 mL aliquots were filtered through 0.45 µm filters, and placed in amber chromatographic vials. One was stored at -20 °C until MeTCS analysis by GC-µECD, while the other was immediately analyzed for TCS by HPLC-MS.

Equipment

TCS was identified and quantified by HPLC-MS through the use of an Agilent model 1100 liquid chromatograph coupled to an Agilent model VL single quadrupole mass spectrometer (Agilent Technologies Inc., Miami, FL, USA). For the ionization, an electrospray source was used in negative mode with selective ions m/z 287.7 and 288.7. The chromatograph was equipped with a C_{18} X-SELECT™ column (75 mm × 4.6 mm, and 3 mm pore size, from Waters Corp., Milford), separation was run in isocratic condition of

methanol (HPLC grade, J.T. Baker, USA) and nanopure water (formic acid 0.1%, analytical quality, Merck, Germany), with a flow of 0.5 mL min^{-1}. Detection instrumental limits were 0.1 µg L^{-1} and quantification limit was 0.5 µg L^{-1}.

MeTCS was identified and quantified by GC-µECD through the use of an Agilent 6890N gas chromatograph. The system was equipped with a HP-5 (15 m × 0.53 mm i.d. × 1.5 µm film thicknesses) column. A volume of 3 µL was injected in splitless mode, with the injector temperature at 250 °C. The oven ramp was set to an initial temperature of 80 °C, increased to 180 °C at 20 °C min^{-1}, and then to 250 °C at 10 °C min^{-1}, with a total acquisition program of 15 min. Hydrogen was used as carrier gas and nitrogen as make-up gas. The detector was set at 250 °C. Instrumental detection and quantification limits were 0.5 and 1 µg L^{-1}, respectively.

Biomarker determinations

The condition factor (CF) index was calculated as body weight (g)/total body length $(cm)^3$ (Bagenal & Tesch 1978) and the hepatosomatic index (HSI) was determined as liver weight (g) × 100 x total fish weight $(g)^{-1}$ (Sloof et al. 1983).

D. rerio liver, gills and brain were pooled processed (n= 4 tissues per pool; n= 8 pool per treatment). Tissues were homogenized on ice until total disintegration with buffer pH 7.4 (0.1 M NaH_2PO_4; 0.15 M KCl; 1 mM EDTA; 1 mM DTT; 10% v/v glycerol) according to Nilsen et al. (1998). An aliquot was used for the determination of lipid peroxidation levels and the remaining homogenate was centrifuged at 20000 *g* for 20 min at 4 °C. The obtained supernatant fraction was reserved for the quantification of enzyme activities and protein content.

Lipid peroxidation (LPO) was determined in liver, gills and brain by measuring the formation of thiobarbituric reactive substances under acidity and heat conditions according to Ohkawa et al. (1979) and Oakes and van der Kraak (2003).

Catalase (CAT, EC 1.11.1.6) activity was determined in liver, gills and brain following the method described by Beutler (1982), and Glutathione S-transferase (GST, EC 2.5.1.18) activity was determined according to Habig and Jakoby (1981).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was evaluated in liver and gills following an indirect method involving the inhibition of cytochrome c reduction by the competition with SOD for the superoxide anion radical formed by the xanthine/xanthine oxidase system (McCord & Fridovich 1969). The activity was expressed as units of SOD*mg^{-1} protein, where 1 SOD unit (U) is defined as the enzyme quantity that causes 50% of inhibition of reduction of cytochrome c per minute.

Total antioxidant competence against peroxyl radicals (ACAP) was determined in liver, gills and brain according to Amado et al. (2009) and further modifications adopted by Monserrat et al. (2014). The measure of antioxidant capacity is given by difference in the fluorescence of the samples after 30 min with and without ABAP and is calculated by the following expression: (FU 30 $min_{with\ ABAP}$ - FU 30 $min_{without\ ABAP}$)/FU 30 $min_{without\ ABAP}$. As high fluorescence levels are obtained after adding ABAP, a high difference is considered to indicate a low antioxidant capacity suggesting a low ability to neutralize peroxyl radicals.

Acetylcholinesterase activity (AChE) (EC 3.1.1.7) was measured in brain according to Ellman et al. (1961) modified for microplate measurement.

The protein content of the supernatant fractions was measured according to Lowry et al. (1951).

Statistical Analysis

Data distributions were tested for normality and variance homogeneity with Kolmogorov–Smirnov and Levene's tests, respectively. Significant differences between each Ctrl group and exposure treatments were assessed by t-Student with $p<0.05$. Also, principal component analysis (PCA) was performed to get an overall view of biological responses and to define the most important parameters involved in TCS-MeTCS toxicity for both time of exposure. After excluding outliers, multivariate analysis was carried out considering seven cases per treatment and twelve variables. All statistical analysis was performed by the InfoStat software (Di Rienzo et al. 2020).

RESULTS

No mortality was registered along the bioassay, neither in the control group nor in TCS exposed groups.

Water quality parameters in aquaria and effective concentration of TCS and MeTCS

Water physicochemical parameters are detailed in Table I. Dissolved oxygen, pH, and conductivity were the parameters that remained with similar values throughout the experimental periods. However, ammonium concentrations between the Ctrl treatments and TCS-MeTCS were variable at both times of exposure.

Effective concentrations of TCS and MeTCS are presented in Table II. Both compounds were present in sediment, however only MeTCS was detected in water. About 1% of the MeTCS detected in the sediment mobilized to the water column. No differences were observed in the concentration of MeTCS in water during exposure times, with average exposure value 12.3 ± 1.1 µg L^{-1} (Fig. 1). Neither TCS nor MeTCS were detected both (sediment and water) on the Ctrl.

Sediment humidity was 43 ± 2.4 % and total organic matter was 3.1 ± 0.4 %.

Biomarkers

No significant changes were observed in the CF and HSI of *D. rerio* (N= 32) after acute and prolonged exposure to sediment spiked with TCS (Table III).

Liver

Effects of TCS-MeTCS on liver biomarkers of *D. rerio* are presented in Fig. 2. After 2 days, SOD activity increased 36% and ACAP decreased 48% in respect of Ctrl. On the other hand, after the 21 days exposure with sediment spiked with TCS, all liver biomarkers evaluated were altered

Table I. Water physicochemical parameters measured on aquaria under control conditions (Ctrl) or exposed to TCS spiked sediment (TCS-MeTCS). Values are expressed as mean ± standard deviation. (2 days n= 3 and 21 days n=4).

	2 days		21 days	
	Ctrl	**TCS-MeTCS**	**Ctrl**	**TCS-MeTCS**
Dissolved Oxygen (mg O_2 L^{-1})	8.1 ± 0.1	7.9 ± 0.6	7.6 ± 0.3	7.2 ± 0.5
pH	8.4 ± 0.1	8.5 ± 0.1	8.5 ± 0.1	8.3 ± 0.4
Conductivity (µS cm^{-1})	959 ± 49	935± 51	874 ± 81	910 ± 110
Ammonium (µg N-NH_4^+ L^{-1})	700 ± 60	300 ± 200	400 ± 10	110 ±100

Table II. Effective concentrations of TCS and MeTCS in the spiked sediment, water and suspended particulate matter (SPM) in different exposure times. Values are presented by mean ± standard deviation. <LD: below detection limits. (0 days n= 2; 2 and 21 days n=3).

	0 days		2 days		21 days	
	TCS	**MeTCS**	**TCS**	**MeTCS**	**TCS**	**MeTCS**
Sediment (μg kg^{-1} dw)	226 ± 8	2832 ± 216	220 ± 6	2488 ± 602	65 ± 9	2173 ± 658
Water (μg L^{-1})	<LD	11.4 ± 2.0	<LD	11.9 ± 2.5	<LD	13.6 ± 3.4
SPM	<LD	<LD	<LD	<LD	<LD	<LD

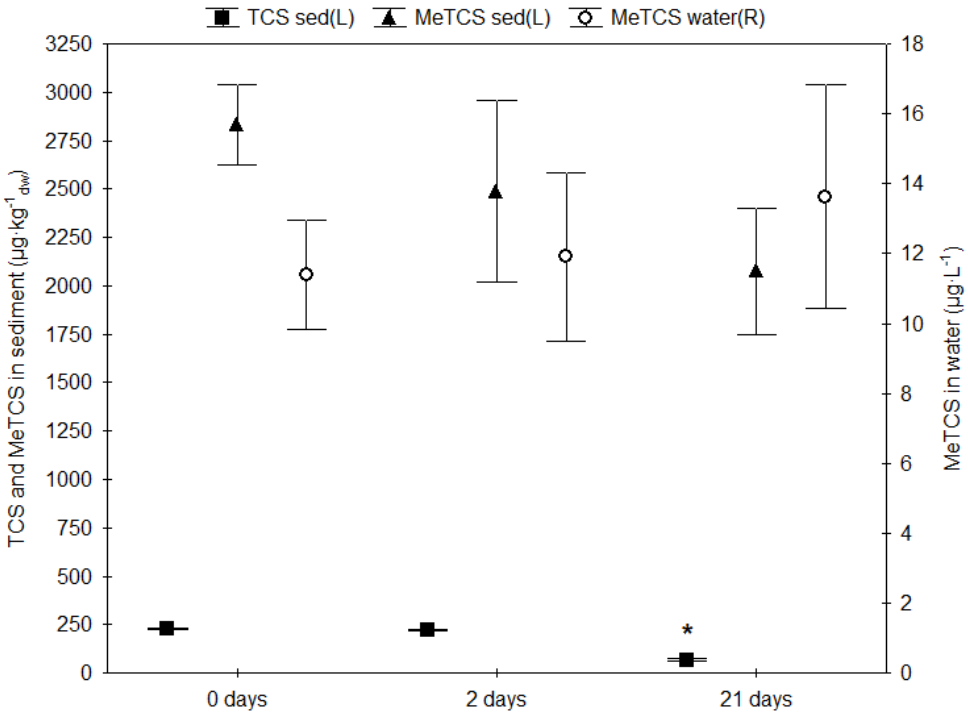

Figure 1. Concentrations (mean ± standard error) of TCS and MeTCS in sediment (left y-axis) and water (right y-axis). * Denoted the existent of significant differences (p < 0.05) between concentrations across the time.

in respect of the Ctrl: SOD and CAT activity increased (58% and 35% respectively), GST was inhibited (12%), ACAP decreased in 40% and lipid damage increased in 57%.

Gills

The results of acute and prolonged effects in gills of *D. rerio* are presented in Fig. 3. After 2 days of exposure, LPO levels increased 75% respect to Ctrl. This change was joined with an increased in SOD activity (12%) and inhibition of GST (36%). In prolonged exposure, lipid damage and SOD activity increased 48% and 50% respectively, and ACAP decreased in 68% respect to Ctrl.

Brain

The effects produced in brain by sediment spiked with TCS are presented in Fig. 4. CAT activity decreased 56% in fish after being exposed during 2 days to TCS-MeTCS, and GST

Table III. Condition Factor (CF) and hepatosomatic index (HSI) of *D. rerio*. Results are expressed as mean ± standard deviation. (n=32 per treatment).

	2 days		21 days	
	Ctrl	TCS-MeTCS	Ctrl	TCS-MeTCS
CF	1.1 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.2
HSI	2.2 ± 0.9	1.9 ± 0.8	1.7 ± 0.7	2.1 ± 0.9

increased after 2 and 21 days of exposure (23 and 26% respectively). AChE activity showed no differences between treated organisms and Ctrl after both exposure times.

Principal component analysis

The first and second component of principal component analysis (PCA) accounted 29.9% and 18.4% of the total variance, respectively (Fig. 5). Control group were grouped and differed mainly from fish treated by the principal component (PC) 1. On the other hand, fish that were in short term exposure were grouped and differentiated from fish exposed for 21 days by the PC2. PC1 focuses on ACAP in liver and gills and LPO in gills, while PC2 focuses on the activation of antioxidant enzymes (GST and CAT) in the 3 evaluated organs. Organisms that were exposed for 21 days to sediment with TCS differed from fish exposed for 2 days primarily due to CAT activity in the brain and liver and GST in gills.

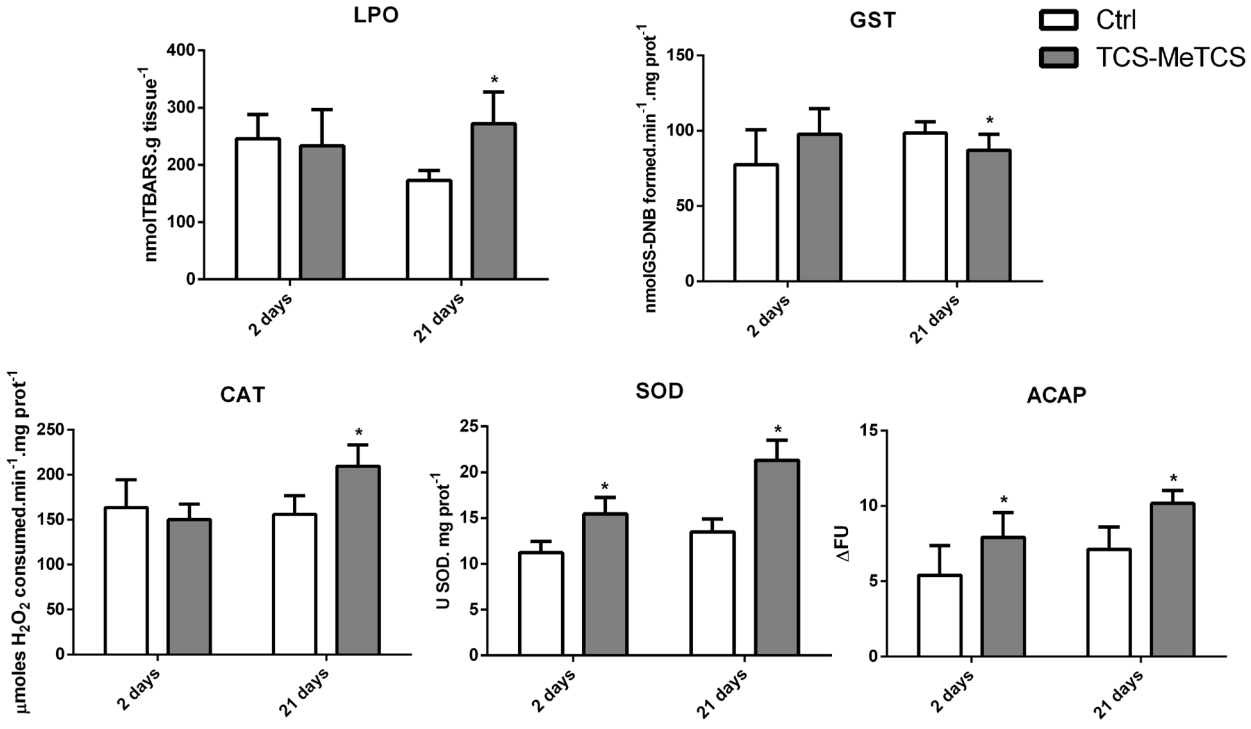

Figure 2. Liver biomarkers of *Danio rerio* after 2 and 21 days of exposure to sediment spiked with TCS. GST, Glutathione S-transferase; CAT, catalase; SOD, superoxide dismutase; LPO, lipid peroxidation and ACAP, total capacity against peroxyl radicals (lower height of the bars (ΔFUs) represents a higher antioxidant capacity and vice versa). *Denoted the existent of significant differences with Ctrl group ($p < 0.05$). Results are expressed as mean ± standard error.

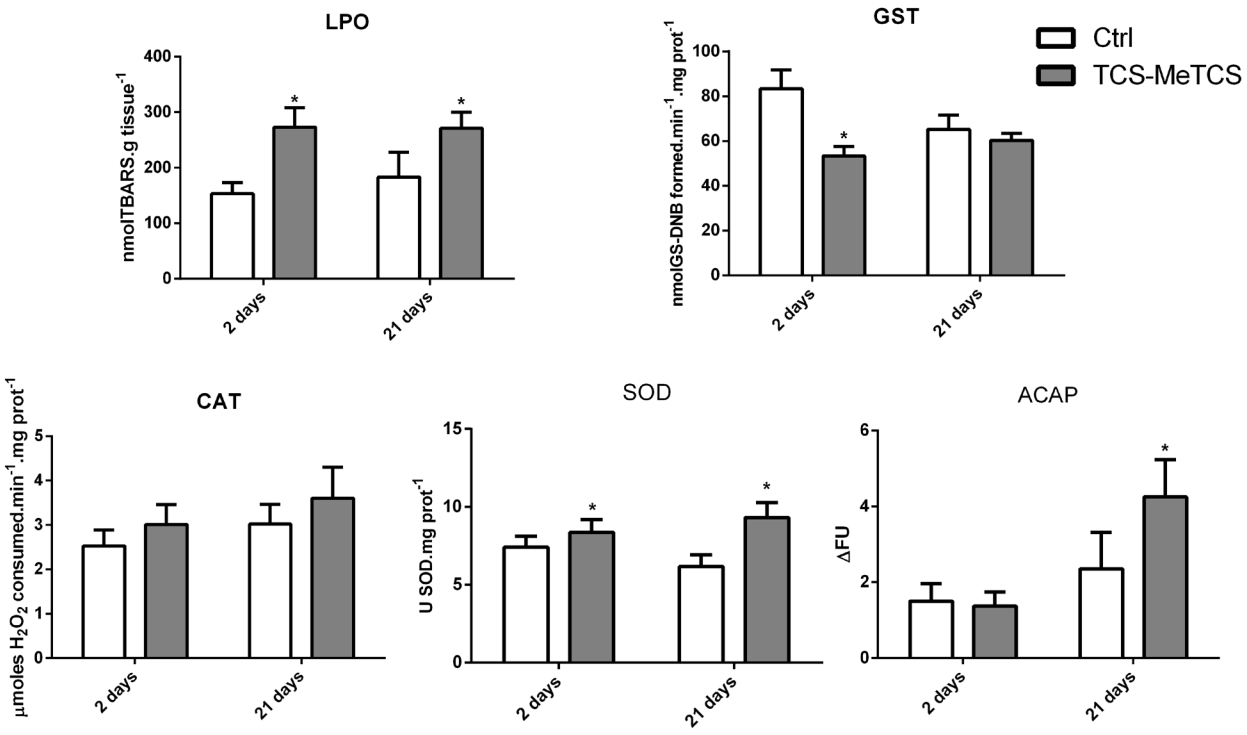

Figure 3. Gills biomarkers of *Danio rerio* after 2 and 21 days of exposure to sediment spiked with TCS. GST, glutathione S-transferase; CAT, catalase; SOD, superoxide dismutase; LPO, lipid peroxidation and ACAP, total capacity against peroxyl radicals (lower height of the bars (ΔFUs) represents a higher antioxidant capacity and vice versa). *Denoted the existent of significant differences with Ctrl group ($p < 0.05$). Results are expressed as mean ± standard error.

DISCUSSION

Research about the toxicity of contaminated sediments has been limited by the complexity of sediment-water column. Whole sediment bioassays are necessary to assess the toxicity of total sediment, including both soluble and solid phases. In this way, they represent the most realistic exposure scenario for considering bioavailability (Hallare et al. 2011). In addition, it is relevant to employ test species that are not in direct contact with the sediment but may be targeted by contaminants released from it.

Dynamic to sediment spiked with TCS

Total organic matter and sediment humidity found in this work were consistent with values reported by Giusto et al. (2012, 2014) for the sediment in "Las Flores" stream (Buenos Aires, Argentina). The fate and effects of an organic pollutant are related to its bioavailability. In sediment, these compounds become more stable and persistent over time, increasing their potential adverse effects on biota (Ronco et al. 2008, Boulanger et al. 2019).

TCS presents a water solubility of 12 mg L^{-1} and an octanol-water partitioning coefficient log Kow=3.5-4.8 at neutral pH. The half-life of TCS ranges from 4 to 60 days in sediment and depending on the initial concentration and environmental factors such as the pH, oxygen and light (Bedoux et al. 2012). In addition, TCS could be susceptible to degradation via aqueous photolysis, with a half-life of <1 hour (SCCS 2010). On the other hand, MeTCS has lower water solubility than TCS (0.4 mg L^{-1}), but a higher octanol-water partitioning (log Kow=5.34), so

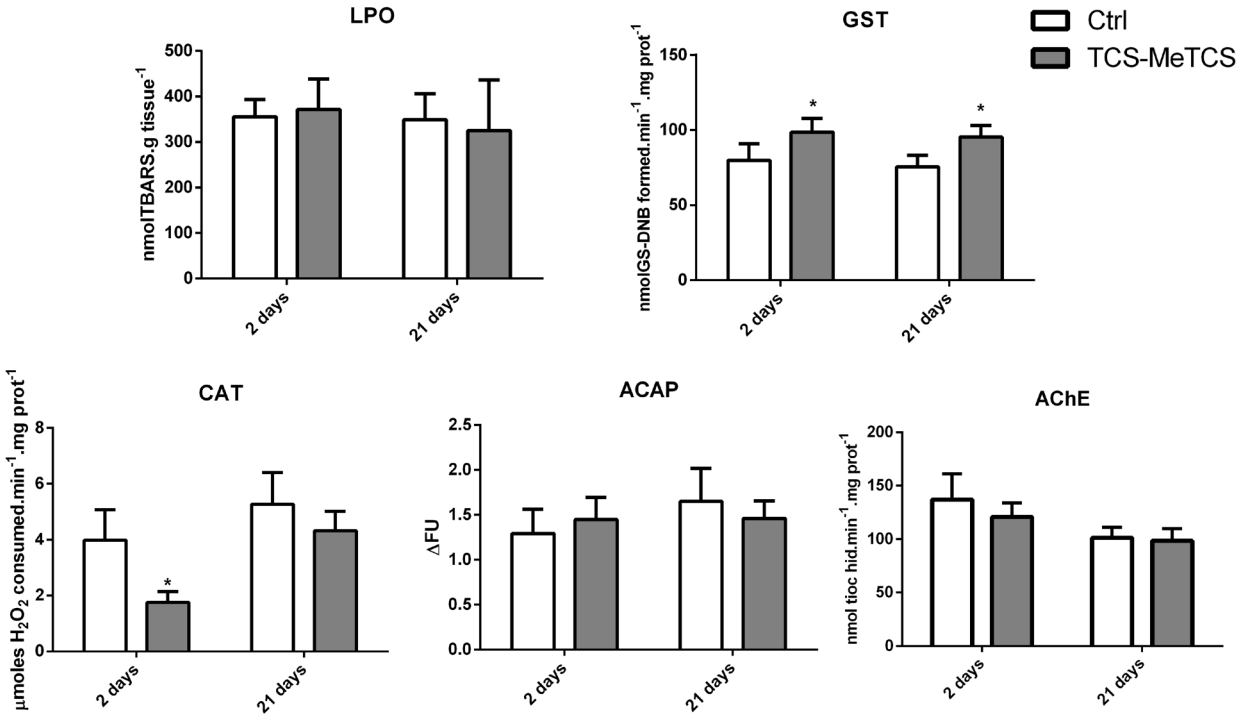

Figure 4. Brain biomarkers of *Danio rerio* after 2 and 21 days of exposure to sediment spiked with TCS. GST, Glutathione S-transferase; CAT, catalase; LPO, lipid peroxidation; ACAP, total capacity against peroxyl radicals (lower height of the bars (ΔFUs) represents a higher antioxidant capacity and vice versa); AChE, acetylcholinesterase. * Denoted the existent of significant differences with Ctrl group ($p < 0.05$). Results are expressed as mean ± standard error.

it is more stable associated to organic matter and in sediments than TCS (Balmer et al. 2005, Chen et al. 2011). These characteristics led to the observed less persistent presence of TCS in sediment and the higher persistence and stability of MeTCS.

Sediment preparation with TCS involved an initial interaction of the toxicant in order to favor adsorption and stabilization processes. In this work, 6 days after spiking, more than 90% of TCS had degraded and biotransformation to MeTCS occurred in sediment with non-sterile conditions. In agreement with our results, Chen et al. (2011) reported that 75% of TCS was removed in sediment within 6 days under aerobic conditions. In addition, Ho et al. (2013) showed that TCS in sediment declined to 46 to 60% of nominal concentration after 3 days. In this context, bacteria from the sediment metabolized TCS to MeTCS, further biomethylating it throughout, maintaining the MeTCS concentrantion constant for 21 days. The sediment acted as a contaminant source, continuously releasing them into the water, and keeping the MeTCS concentration stable throughout the exposure period. Likewise, the effective concentration of TCS in sediment tested in our work is environmentally relevant (Davis et al. 2012, Pusceddu et al. 2018).

Even though TCS has been reported in the particulate material (Kumar et al. 2010), the lack of TCS detection in the matrix in this study could be due to the low resuspension of the sediment provoked by *D. rerio*. The presence of benthic species could generate greater movement of

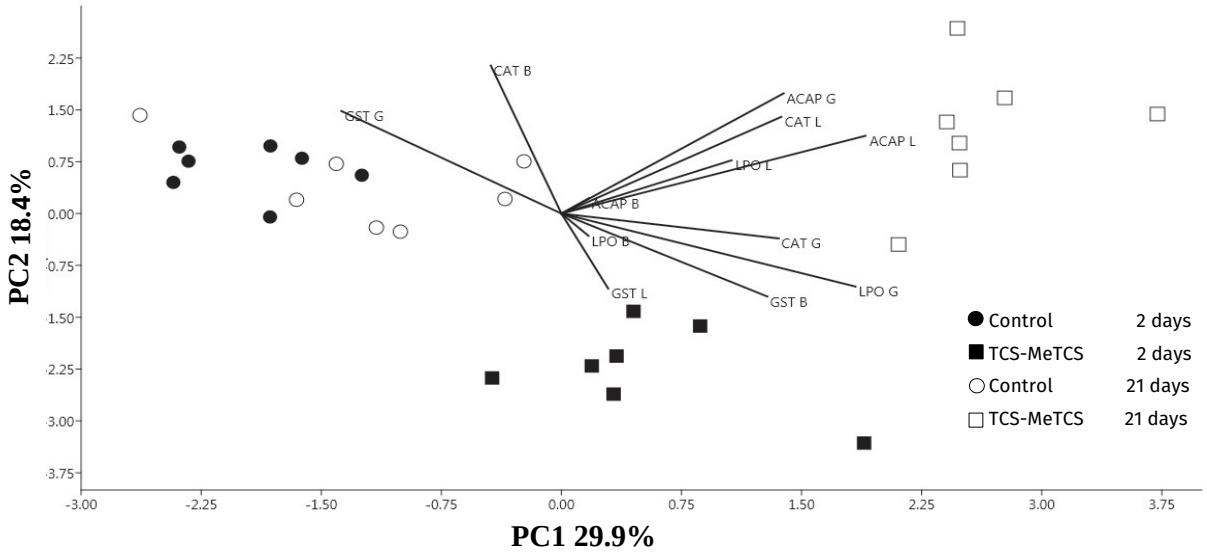

Figure 5. Representation of the biochemical markers in liver (L), gills (G) and brain (B), and individuals (symbols indicate different treatments; n=7) onto the principal components analysis (PCA). All markers abbreviations are explained in the text. Filled symbols represent short term exposure to sediment spiked with TCS and blank symbols to prolonged exposure.

particulate matter in water, and therefore higher amounts of pollutants for fish.

Physicochemical parameters

Although ammonium showed variability even in the control group, these values were lower than the maximum permitted quantity for protection of freshwater life (1130 µg N-NH_4^+ L^{-1}) according to recommended aquatic life prolonged criteria by USEPA (1999). The experimental conditions (e.g. permanent nutrient enrichment by fish and presence or not of TCS) could explain the variability of ammonium values observed in our work (Stief et al. 2003). Also, the pH and conductivity were similar to the values of fish breeding and maintenance, and the conductivity was found in the optimal range (300-1500 µS cm^{-1}) for *D. rerio* (Avdesh et al. 2012).

Biomarkers

Taking into account that 1% of MeTCS became available in the water column, our results showed that TCS spiked sediment was able to exert adverse effects on *D. rerio* biochemical parameters.

One of the main functions of liver is to metabolize lipophilic substances, including xenobiotics (Di Giulio & Hinton 2008), so it is an organ of interest to evaluate toxic effects generated by pollutants. On the other hand, the first enzyme involved in antioxidant defense mechanism is SOD, which catalyzes the dismutation of superoxide anion to hydrogen peroxide and molecular oxygen. The increased activity of SOD observed in liver after the short term exposure to sediment spiked with TCS showed that antioxidant defenses were activated to counteract the superoxide anion and may prevent lipid damage in this tissue. However, the total antioxidant capacity against peroxyl radicals decreased. Besides, the greatest liver damage was observed after 21 days, where activation of both antioxidant enzymes, SOD and CAT, were not sufficient to prevent lipid damage. In addition, the total capacity against peroxyl radicals continued to decrease, evidencing an

overall decreased liver antioxidant capacity after a prolonged exposure to sediment spiked with TCS.

Another important detoxification enzyme is CAT, and its activity has been used as a potential biomarker of fishes exposed to toxicants (Zhang et al. 2004). Because CAT degrades H_2O_2 in water and oxygen, the increase in H_2O_2 content generated by SOD activity could explain the increase in CAT activity observed in our study. Similar results were observed in liver of *Oreochromis niloticus* and *Clarias gariepinus* fish after exposure to organic pollutants such as butachlor (herbicide) and azinphos-methyl (insecticide) (Oruc et al. 2004, Farombi et al. 2008).

Additionally, as GST has a critical role against oxidative damage (Elia et al. 2003), the lower level of this enzyme in fish liver suggests a significant reduction of fish capacity to withstand oxidative stress following 21 days of exposure. In this way, *GST* mRNA expression decreased over time after TCS exposure in liver of the catfish *Pelteobagrus fulvidraco* (Ku et al. 2014). This inhibition could be due to an incomplete degradation of TCS absorbed by the fish. Due to the structural similarity of TCS to polychlorobiphenols, bisphenol A and dioxins, it is thought that TCS could act as a selective inhibitor, as well as a substrate, for phase II enzymes (Wang et al. 2004).

ACAP levels allow to emphasize the importance of understanding how antioxidants interact with reactive oxygen species (ROS) through the determination of the total antioxidant capacity, instead of the measurement of limited number of antioxidants (Amado et al. 2009, Ale et al. 2018b). As was mentioned before, ACAP decreased in liver after 2 and 21 days of exposure to sediment spiked with TCS. This could indicate that MeTCS in water impaired the ability of liver to cope with ROS and therefore, leads to a decreased capacity of cells to neutralize peroxyl radicals. This reduction of antioxidant capacity could have led this organ to suffer from oxidative stress. In this sense, lipid damage was found in liver after 21 days, which confirmed that spiked sediment with TCS induced disrupted the redox balance.

Sediment spiked with TCS promoted the same responses in liver of fish as TCS present in water after prolonged exposure. Paul et al. (2020) found in *P. hypophthalmus* a significant increase of SOD, CAT and GST activity in liver, after 30 days exposure to 91 and 182 μg L^{-1} of TCS. Likewise, Li et al. (2018) observed increased liver CAT activity and lipid damage in *Carassius auratus* after 15 days of exposure to 50 μg L^{-1} of TCS. Hemalatha et al. (2019) reported the same effect in the liver of *Labeo rohita* after a prolonged exposure to TCS.

Short term toxicity in gills is attributable to TCS entering fish body mainly through passive diffusion across the gill membrane, resulting in an enhanced toxicity in this organ (Khatikarn et al. 2018). In our study, the gills were the only organ that showed increased lipid peroxidation levels in *D. rerio* after both times of exposure to sediment spiked with TCS. Gills are the major site of uptake for most waterborne toxicants and the main site of toxic impact for many of them (Ale et al. 2018b). The enzyme GST participates in the defense against oxidative stress detoxifying endogenous harmful compounds like hydroxyalkenals, breakdown products of lipid peroxidation (Cnubeen et al. 2001). In this sense, the increased lipid damage could have generated a great number of metabolites of lipid peroxidation producing the observed GST inhibition after the short term exposure (Farombi et al. 2008). Similar GST responses were reported after 48-hour *Channa punctatus* exposure to deltamethrin (Sayeed et al. 2003).

The higher SOD activity, observed in gills after both periods of exposure, suggests an increase in superoxide anion and a response to counteract them by the cells. However, it was not enough to prevent lipid damage in this tissue in neither of the two exposure periods tested. Similar results were reported by Paul et al. (2020) in gills of *P. hypophthalmus* after different TCS concentrations and pH values.

Likewise, the decrease in the total capacity against peroxyl radicals after 21 days would allow asserting that after prolonged exposure antioxidant response in gills of *D. rerio* was decreased. Therefore, the sediment enriched with TCS (and/or MeTCS) produced a decrease ability of the branchial cells to counteract peroxyl radicals. In agreement with our results, Wang et al. (2017) showed in *Carassius auratus* a decreased in capacity against radicals after 14 days of exposure to TCS. Furthermore, Wang et al. (2019) found decreased antioxidant defenses in *D. rerio* after 42 days of exposure to TCS.

The brain was the less sensitive organ in *D. rerio* exposed to sediment spiked with TCS. GST activity was increased in both exposure periods and similar results were reported by Araújo et al. (2019) in larvae of *D. rerio* after 22 days of TCS exposure. This mechanism could explain the absence of lipid damage in this tissue. In agreement with our results, Paul et al. (2020) showed an increased GST activity in the brain of *P. hypophthalmus* after 30 days of exposure to TCS, meanwhile Gyimah et al. (2020) found inhibition of CAT activity in brain of *D. rerio* after exposure to TCS. Additionally, Gyimah et al. (2020) showed absence of brain lipid damage after prolonged exposure to TCS. Overall, contradictory results were shown in brain of fish, therefore more studies considering neurological biomarkers should be carried out.

Furthermore, potential changes occurring in brain could elicit alteration in fish behavior, including their ability to escape from predators, reproduce and compete with other fish (Araújo et al. 2016). In this sense, AChE is a useful biomarker of neurotoxic effects of chemicals on organisms. In the present study no differences have been observed among treatments. Although several works reported inhibitions of AChE after TCS exposure (Sahu et al. 2018, Paul et al. 2019, 2020, Li et al. 2018), these studies were carried out using higher concentrations than the one used in the present work.

According to the principal component analysis, the first principal component (PC1) clearly separated fish exposed to sediment with TCS from the Ctrl, regardless of exposure time, while the effect of exposure time was explained (PC2) by oxidative damage protective enzymes. After the two times of exposure, the organs that were most affected were liver and gills, however the biomarkers that explained the variability were respectively different. After 2 days of exposure, CAT and GST enzymatic activities were the most important biomarkers on the evaluated organs which mean that after the short term exposure *D. rerio* was able to respond and counteract oxidative damage in liver and brain.

On the other hand, after 21 days of exposure to sediment spiked with TCS, the major changes were observed in ACAP and LPO, so after a prolonged period of exposure organisms lost the ability to counteract ROS, leading to lipid damage.

Carrying out prolonged studies reflecting an environmental scenario such as whole sediment exposures with TCS can be considered as a useful tool in environmental risk assessment. In agreement with our results, Pusceddu et al. (2018) observed an inhibition of the embryo-larval development in *Perna perna* after exposure to environmental concentrations (7.5-750 μg kg^{-1}) of TCS in sediment. Besides, prolonged effects

could be related to a multitude of sublethal more sensitive endpoints such as antioxidant responses, ROS generation, behavior and metabolism (Kar et al. 2020). Thus, TCS and MeTCS need to be monitored continuously along with their chronic toxicity.

CONCLUSIONS

The battery of biochemical markers assessed in *Danio rerio* provided a greater understanding of the ecotoxicity of sediments contaminated with TCS. The characterization of compounds such as TCS and MeTCS in the matrices, allowed to make direct inferences about the contaminant and their effects on *D. rerio*. Liver and gills were the most sensitive organs; while SOD activity, lipid damage, and ACAP levels were the most sensitive biomarkers. Whole sediment exposure assay conducted with a non-benthic model organism also highlighted effects elicited from an important sink of TCS metabolite (MeTCS). To our knowledge, this is the first attempt to evaluate the exposure of sediment spiked with this emergent pollutant and provide a more realistic scenario of exposure, whereas non-target organisms may be threatened and therefore also the whole ecosystem integrity.

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