Environmental concentrations of triclosan activate cellular defence mechanism and generate cytotoxicity on zebrafish (*Danio rerio*) embryos

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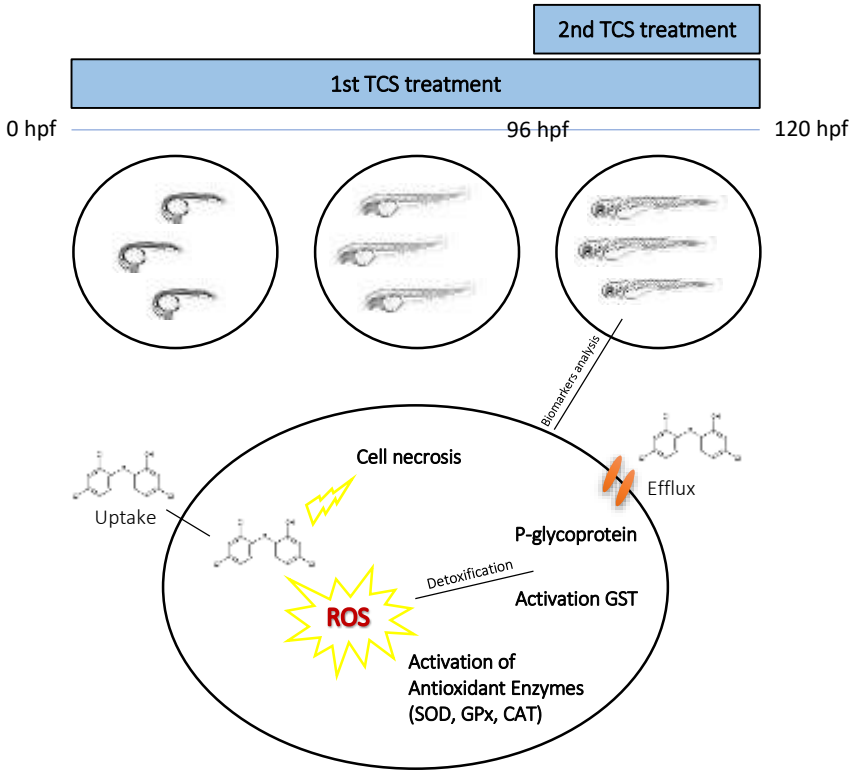
***Graphical Abstract**



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

- TCS exposure induces P-gp efflux functionality and oxidative stress enzymes.
- Embryos cellular defence system prevent the occurrence of oxidative damage by TCS.
- High levels of cell necrosis underline TCS cytotoxicity potential.
- TCS occurrence in the aquatic environment poses an actual risk for wildlife.

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ABSTRACT

Triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy) phenol) is becoming a major surface waters pollutant worldwide at concentrations ranging from ng L^{-1} to μg L^{-1}. Up to now, the adverse effects on aquatic organisms have been investigated at concentrations higher than the environmental ones, and the pathways underlying the observed toxicity are still not completely understood. Therefore, the aim of this study was to investigate the toxic effects of TCS at environmental concentrations on zebrafish embryos up to 120 hours post fertilization (hpf). The experimental design was planned considering both the quantity and the exposure time for the effects on the embryos, exposing them to two different concentrations (0.1 μg L^{-1}, 1 μg L^{-1}) of TCS, for 24 hours (from 96 to 120 hpf) and for 120 hours (from 0 to 120 hpf). A suite of biomarkers was applied to measure the induction of embryos defence system, the possible increase of oxidative stress and the DNA damage. We measured the activity of glutathione-*S*-transferase (GST), P-glycoprotein efflux and ethoxyresorufin-o-deethylase (EROD), the level of ROS, the oxidative damage through the Protein Carbonyl Content (PCC) and the activity of antioxidant enzymes. The genetic damage was evaluated through DNA Diffusion Assay, Micronucleus test (MN test), and Comet test. The results showed a clear response of embryos defence mechanism, through the induction of P-gp efflux functionality and the activity of detoxifying/antioxidant enzymes, preventing the onset of oxidative

damage. Moreover, the significant increase of cell necrosis highlighted a strong cytotoxic potential for TCS. The overall results obtained with environmental concentrations and both exposure time, underline the critical risk associated to the presence of TCS in the aquatic environment.

1. INTRODUCTION

Triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy) phenol) is an emerging contaminant included as antimicrobial agent in several personal care products (PCPs), such as soaps, deodorants, toothpastes, cosmetics and laundry detergents (Dann and Hontela, 2011), with a typical range of 0.1-0.3% of products weight (Montaseri and Forbes, 2016). The ubiquitous use of these products has drawn the attention of the research community, due to the well documented TCS toxicity in mammalians models, including irritation of eyes and skin, allergies, detrimental effects on development and reproduction, weakening of the immune system, inhibition of muscle function and genotoxicity (Barbaud et al., 2005; Binelli et al., 2009; Dann and Hontela, 2011; Cherednichenko et al., 2012; Savage et al., 2012; Halden, 2014). High levels of TCS were recently found in human urine, blood sample, liver, adipose tissue, brain and breast milk (Ruszkiewicz et al., 2017) and its structure is similar to the polychlorinated phenoxyphenols that, under oxidizing conditions, cyclize to other toxic byproducts, which present very harmful effects on human health (Solá-Gutiérrez et al., 2018). Furthermore, a recent proteomic study (Li et al., 2018) revealed a distinct evidence of the potential impact of TCS on human metabolic pathways.

Nowadays, there is growing awareness for TCS environmental implications so that the marketing of over-the-counter antibacterial soaps and body washes containing TCS was banned in the United States since 2016 (FDA, 2016), while the European Union (EU, 2016) has disapproved the use of TCS in human hygiene biocidal products (product-type 1). Nevertheless, the occurrence of TCS in the aquatic ecosystems is a worldwide issue, being commonly detected in surface waters (lake/river/streams with known input of raw wastewaters) with concentrations ranging from 1.4 to 40,000 ng L^{-1}, and in sea water from <0.001 to 100 ng L^{-1} (Dhillon et al., 2015). There is a scientific

evidence of TCS emission from Wastewater Treatment Plants (WWTPs) into the aquatic environment, due to their partial inability to remove it, with detected concentrations ranging from 10 to 2,210 ng L^{-1} in European WWTP effluents (Bedoux et al., 2012). TCS and mostly its methylated degradation product (methyl triclosan) are lipophilic and volatile (Wang and Kelly, 2017), causing a high tendency for bioaccumulation in aquatic organisms, which uptake them directly from food or water with bioconcentration factor (BCF) of 2.7-90 (Dhillon et al., 2015). Detected concentrations of TCS are reported in different wild organisms, such as algae and invertebrates, with concentrations up to 400 μg/kg (Coogan et al., 2008), and fish, with values up to 300 ng/g wet weight (ww) in muscle tissue (Yao et al., 2018).

The chronic and acute toxicity of TCS has been demonstrated in several aquatic models, which include green and blue algae (Orvos et al., 2002) and benthic invertebrates (Dussault et al., 2008), showing effects on biomass and growth rate, and generating oxidative stress and genotoxicity (Binelli et al., 2009; Riva et al., 2012; Martínez-Paz, 2018). In fish, TCS impacts equilibrium, swimming, spinal curvature and quiescence (Orvos et al., 2002; Ishibashi et al., 2004), as well as several studies showed the endocrine disruption effect of TCS, as demonstrated for the Japanese medaka *Oryzias latipes* (Ishibashi et al., 2004; Horie et al., 2018). Some recent studies performed on zebrafish *Danio rerio* embryos highlighted that TCS caused heart edema and slow heartbeat (Zhu et al., 2018), delayed hatching and increased mortality (Falisse et al., 2017), impaired lipid metabolism (Ho et al., 2016), and induced hepatotoxicity (Haggard et al., 2016) at concentrations up to 1.25 mg L^{-1}. Furthermore, Muth-Köhne et al. (2012) demonstrated that exposure to 3 μM TCS (870 μg L^{-1}) may have neurotoxic effects, delaying the development of zebrafish embryos motor neurons. Such results underline the TCS toxic potential for aquatic species, but do not represent a realistic scenario, since exposure concentrations are higher than those detected in aquatic ecosystems.

This study aimed to evaluate the environmental impact of TCS on aquatic ecosystem, assessing its toxic effects at environmentally relevant concentrations, and contribute to a better understanding of

the response of the organism detoxification systems to TCS exposure, using zebrafish embryos as experimental model. Embryos were exposed to two environmental concentrations (0.1 and 1 μg L^{-1}) of TCS for two different exposure time, from 0 to 120 hpf and from 96 to 120 hpf, to improve the understanding on cellular detoxification mechanisms and to investigate the temporal variation of chronic toxicity in terms of oxidative stress and cyto-genotoxicity. Actually, we used a biomarker suite based on the measure of reactive oxygen species (ROS) production and the antioxidant enzymes' activity, namely catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GP_x). We also measured the induction of detoxification activities by the glutathione-*S*-transferase (GST), P-glycoprotein and ethoxyresorufin-o-deethylase (EROD), as well as the potential oxidative damage through the Protein Carbonyl Content (PCC) and cyto-genotoxic effects through the DNA Diffusion Assay, Micronucleus test (MN test) and Comet test.

2. MATERIALS AND METHODS

2.1 *Preparation of TCS solutions*

Triclosan (TCS, CAS 3380-34-5, purity 97%) was purchased from Sigma-Aldrich (Milan, Italy). Firstly, a stock solution of TCS, 1 g L^{-1} in dimethyl sulfoxide (DMSO), was prepared and stored at 4°C, while two TCS working solutions were prepared successively diluting the stock solution in ultrapure water at 0.1 mg L^{-1} and 1 mg L^{-1}, respectively. The maximum percentage of DMSO was lower than 0.0001%, and not produce any changes on hemocyte viability, DNA damage or enzyme activity (Parolini et al., 2011). These working solutions were then properly diluted to reach the selected exposure concentrations of 0.1 μg L^{-1} and 1 μg L^{-1}, which fall in the range of the current environmental freshwater levels of TCS (Ho et al., 2016; Zhou et al., 2017). These concentrations also fall within the Predicted No Effect Concentration (PNEC) range (70 ng L^{-1} to 1,550 ng L^{-1}) reported by Capdevielle et al. (2007), which made a Species Sensitivity Distribution (SSD) analysis based on pre-existing chronic toxicity data of TCS for 14 different aquatic species.

2.2 *Zebrafish maintenance and embryos exposure*

Adult zebrafish of the AB strain are raised in the facility of the Department of Biosciences, University of Milan, where they are maintained in tanks into a thermostatic chamber (28 °C) with 14-h light/10-h dark cycle. The facility follows Italian laws, rules and regulations (Legislative Decree No. 116/92), as confirmed by the authorization issued by the municipality of Milan (PG 384983/2013).

Different groups of embryos were collected by means of natural spawning, following the procedure for maximal embryo production described by Westerfield (2007). Control embryos were maintained in zebrafish water (Instant Ocean, 0.1% methylene blue), while others were exposed to TCS (0.1 μg L^{-1} and 1 μg L^{-1}) dissolved in zebrafish water.

We planned two different exposures in order to evaluate the potential temporal variations, one from 0 hpf to 120 hpf (120 h of exposure) and the other one from 96 hpf to 120 hpf (24 h of exposure). Biochemical analysis exposures were performed in 90 x 15 mm petri dish with a maximum of 70 embryos per petri and two replicates per experimental group, adding 25 mL of the appropriate TCS working solution to each treatment group and 25 mL of zebrafish water to control groups (Fig. S1a). ROS level and cyto-genotoxic biomarkers were performed in multi-well plates with 12 and 5 embryos per well, respectively, and three replicates per experimental group, adding 2 mL of the appropriate TCS working solution to each treatment group and 2 mL of zebrafish water to control groups (Fig. S1b). Both exposures (120 h and 24 h) proceeded at 28 °C under semi-static conditions, replacing the exposure medium every 24 h at the longer exposure. Embryo mortality and effects on morphology, developmental delay and behavioural alterations (occurrence of edema, weak or no pigmentation, malformed or underdeveloped embryos, deformed spine and no or malformed fins) were systematically assessed during the experiment, and no significant effects were observed at chosen TCS concentrations. At the end of both exposures, embryos were processed immediately for the cyto-genotoxicity endpoints or collected and stored at -80 °C until further analysis.

2.3 *Biochemical analysis*

Pools of 60 embryos from each exposure was collected and homogenized, using a motorized pestle mixer, in 800 µL of 100 mM phosphate buffer (pH=7.4), with 100 mM KCl, 1 mM EDTA, 100 mM dithiothreitol (DTT), and protease inhibitors (1:100 v/v). The homogenates were centrifuged at 12,000 r.p.m. for 10 min at 4 °C. The supernatants were collected, pooled together and immediately used for the measure of glutathione-*S*-transferase (GST), catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities in triplicate, using a 6715 UV/Vis spectrophotometer (Jenway, USA), following the protocol described by Della Torre et al. (2017). ROS production was assessed following the method described by Parolini et al. (2017), using dichlorofluorescein-diacetate (DCFH-DA). Briefly, 12 embryos from each experimental group were washed with PBS and homogenized as described above. The homogenates were centrifuged at 15,000 r.p.m. for 20 min at 4 °C and then twenty microliters of the homogenate were added to a 96-well plate and incubated for 5 min at 37 °C. We added 100 µL of PBS and 8.3 µL of DCFH-DA (10 mg/mL in DMSO) in each well before incubated the plate 30 min at 37 °C. At the end, we measured the fluorescence intensity by the EnSight™ multimode plate reader (PerkinElmer) at λ_{ex} 485 nm and λ_{em} 530 nm. The ROS concentration was expressed in fluorescence units (FU) mg protein^{-1}. Protein carbonyl content (PCC) was measured forming protein hydrazone derivatives with 2,4-dinitrophenylhydrazine (DNPH) (10 mM in 2M HCl). Proteins were then precipitated and the pellet was washed and resuspended in guanidine hydrochloride (6 M). The absorbance of protein-hydrazone was measured spectrophotometrically at 375 nm (Della Torre et al., 2018). A pool of about 30 embryos from each exposure was used for the ethoxyresorufin-o-deethylase (EROD) activity measure, which followed the same procedure described above. The EROD assay was performed using the EnSight™ multimode plate reader (PerkinElmer) at λ_{ex} 535 nm and λ_{em} 590 nm and temperature of 37 °C. The incubation mixture contained zebrafish embryo homogenates (4 replicates) in 50 mM Tris buffer pH 7.4 with BSA (5.32 mg/mL in 50 mM Tris) and NADPH (6.7

mM in 50 mM Tris), and the reaction substrate ethoxyresorufin (100 μM in 15% MeOH). Standard concentrations of resorufin, ranging from 0 to 0.1 μM, were made by diluting the stock solution in 15% MeOH. EROD activity was calculated from the relative fluorescence units as the product of resorufin, quantified with the resorufin standard curve (R^2 = 0.99), in pmol min^{-1} mg $prot^{-1}$. All analyses were normalized on the total protein content of each sample (Table S2) measured according to Bradford method using bovine serum albumin as standard (Bradford, 1976).

2.4 *Quantification of P-glycoprotein activity*

The efflux functionality of the P-glycoprotein (P-gp) was measured according to the procedure described by Fischer et al. (2013), using Rhodamine B (RhB) as fluorescent substrate. A pool of 30 embryos per treatment was collected from two independent exposures and incubated for 90 minutes in zebrafish water with 1 μM RhB. For quantification of RhB uptake, embryos were homogenized in 350 μL lysis buffer (pH= 7.4) containing 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, and centrifuged for 10 minutes at 12000 x g. One hundred μL of the supernatant (3 replicates for each treatment) were transferred to a multi-well plate and the RhB fluorescence was measured using the EnSight™ multimode plate reader (PerkinElmer) at λ_{ex} 530 nm and λ_{em} 595 nm. The amount of RhB was quantified with a RhB standard curve, ranging from 0 to 0.1 μM (R^2 = 0.99).

2.5 *Cyto-genotoxicity*

Biomarkers of cyto-genotoxicity were assessed on cells obtained by mechanical dissociation as described in Parolini et al. (2017), from a pool of five embryos collected from three independent exposures (three pool per treatment). Cells viability was evaluated by Trypan Blue dye exclusion method. The Comet assay (SCGE assay) was performed following the protocol of Kosmehl et al. (2008). One hundred cells per slide (6 slides per each exposure) were analysed with the Comet Score® image analysis software. DNA damage was determined by the percentage of DNA observed in the comet tail and the ratio between the migration length and diameter of the comet head (LDR).

The same diffusion assay was used to evaluate the percentage of apoptotic/necrotic cells. Slides were labelled with DAPI in order to observe DNA with a fluorescence microscope (Leica DM2000). One hundred cells per slide (6 slides per each exposure) were counted, among which apoptotic cells were recognized as a dense central zone surrounded by a large DNA halo, while necrotic cells were visualized just as an appearance of a light DNA halo. The frequency of micronuclei (MN) was evaluated on 400 cells per slide (n=9; 3 slides per pool), as reported in our previous studies (Magni et al., 2016; 2017).

2.6 *Statistical analysis*

Data normality and homoscedasticity were verified using Shapiro-Wilk and Levene tests, respectively. The differences between treatments and control were identified performing a one-way analysis of variance (one-way ANOVA), where treatment (control, TCS 0.1 μg L^{-1} and TCS 1 μg L^{-1}) was the predictor factor, and each biomarker was a dependent variable. For both exposure times, separately, the differences between treated and control were evaluated using Duncan's Multiple Range *post-hoc* Test (DMRT), taking $p < 0.05$ as significant cut-off. To observe the eventual covariation between tested biomarkers, we performed the Pearson's correlation considering all end-points. Statistical analyses were carried out using STATISTICA 7.0 software package.

3. RESULTS AND DISCUSSION

Cellular response to TCS uptake was primarily evaluated through the quantification of P-gp activity, which is an efflux transporter protein identified in fish and involved in detoxification mechanisms, preventing the entrance of xenobiotics or eliminating them from cells (Jackson et al., 2017). We observed a significantly ($F_{2,9} = 75.05$ for 24 h and $F_{2,9} = 93.39$ for 120 h; $p < 0.01$) lower RhB accumulation in all treatment groups compared to the level of RhB measured in controls (Fig. 1a), indicating an induction of P-gp efflux functionality, probably related to active elimination of TCS or its by-products from cells. Another important role in the detoxifying mechanism is due to

some isoforms of the cytochrome P450 superfamily, such as EROD, which catalyse the transformation of several planar compounds upon the aryl hydrocarbon receptor mediated regulation (Van Der Oost et al., 2003). Our results showed that TCS was not able to induce significant EROD activity for both tested concentrations at the development stages analysed (Fig. 1b). On the other hand, the involvement of EROD activity in TCS detoxification in aquatic species is still controversial, as recently described by Zhou et al. (2017), who showed the inhibition of EROD upon exposure to levels of TCS up to 0.7 mg L^{-1}. Another hypothesis to explain the lack of EROD modulation is related to the low inducibility of cytochrome P4501A (CYP1A) in developing embryos. Indeed, a recent study in which embryos (96 hpf) were exposed to different concentrations of the CYP1A inducer benzo(α)pyrene (B(α)P) showed only a low increase of *cyp1a* gene transcription at the concentration of 20 μg L^{-1} (Della Torre et al., 2017). Moreover, a previous study by Saad et al. (2016), which aimed to determine the biotransformation potential of zebrafish embryos during their critical window for teratogens, observed a very low CYP1A activity during organogenesis. Our results seem to suggest that other enzymes (for instance CYP3A) could be involved in the first phase of TCS metabolism/detoxification. The assessment of detoxification activity was also evaluated through the analysis of phase II detoxifying enzyme GST, which showed the same trend at the two different exposure times (Fig. 1c). The induction of GST activity resulted significantly higher at the low TCS concentration ($F_{2,9}$ = 91.49 and $p < 0.001$ at 24 h; $F_{2,9}$ = 6.55 and $p < 0.01$ at 120 h). Our results are in line with findings of Oliveira et al. (2009), who reported an increase of GST activity in zebrafish embryos exposed to TCS (0.25-0.35 mg L^{-1}), underlying the key role of GST in the detoxification response to TCS. The trend observed for GST was also obtained for the activities of other antioxidant enzymes (Fig. 2a, b, c), suggesting the induction of embryos antioxidant protective response upon TCS exposure. Specifically, during the 120 h exposure, SOD was significantly induced at 0.1 μg L^{-1} TCS ($F_{2,9}$ = 10.895; $p < 0.01$), while no changes in enzymatic activity were noticed for the shorter exposure time (Fig. 2a). GPx activity was again significantly induced at lower TCS concentration during both exposure times ($p < 0.01$

and $p < 0.05$, respectively; Fig. 2b2b). Concerning CAT, this enzyme showed a significantly higher activity at lower TCS dose during the 24 h exposure ($F_{2,9} = 16.21$; $p < 0.01$; Fig. 2c), while after the 120 h at both tested concentrations ($F_{2,9} = 7.8609$; $p < 0.05$ and $p < 0.01$, respectively; Fig. 2c). The absence of a significant modulation of detoxifying/antioxidant enzymes activity after 24 h of exposure can explain the significant higher level of ROS measured at the higher TCS concentration, respect to control ($F_{2,9} = 12.07$; $p < 0.01$; Fig. 2e). In the short exposure time, the first cellular defence mechanism (P-gp efflux functionality) is significant activated, while the second line of defence against ROS did not have enough time to be activated. On the contrary, after the longer exposure time, the antioxidant enzyme activities are induced to counteract the formation of ROS and restore the cellular homeostatic condition, as shown in Fig. 2e, where ROS levels are even below the control (120 h exposure). In summary, our results pointed out the ability of embryos cellular defence system to prevent the occurrence of oxidative damage caused by TCS exposure, as it is confirmed by PCC analysis, that did not show any significant oxidative damage at all exposure conditions (Fig. 2d).

Concerning cyto-genotoxic end-points, we verified that all treatment conditions showed a cells viability higher than 90% (Table 1), a percentage well above the minimum required to perform genotoxicity tests, according to the IV International Workshop on Genotoxicity Test Procedures (Kirkland et al., 2007). Nevertheless, a significant decrease of cells viability was recorded in TCS 1 $\mu g\ L^{-1}$ treatment after 120 h exposure ($F_{2,9} = 5.11$; $p < 0.05$). This result can be correlated to the observed percentage of necrotic cells, which was significantly different from controls at the higher concentration (Table 1) at both exposure times ($F_{2,9} = 21.733$ for 24 h and $F_{2,9} = 92.238$ for 120 h; $p < 0.001$). Moreover, we also noticed a remarkable increase of necrosis at 0.1 $\mu g\ L^{-1}$ TCS ($F_{2,9} = 92.24$; $p < 0.05$) after 120 h. The induction of cytotoxic effects by TCS was expected, since it has antiseptic properties causing cell lysis by lipid, RNA and protein synthesis inhibition, and membrane perturbations (Schweizer, 2001). The biomarkers of genotoxicity (SCGE assay and MN test) revealed that exposures to environmental concentrations of TCS induced only low genotoxic

effects in zebrafish embryos. The primary genetic damage measured by the SCGE assay was evaluated both through the percentage of DNA tail and the LDR. Although both of them mostly remained within the physiological range, the percentage of DNA tail showed a significant ($F_{2,9}$ = 29.32; $p < 0.01$) frequency of DNA damage at the end of the 120 h exposure, revealing a slight primary genotoxic effect caused by the higher TCS concentration (Table 1). The mild level of genotoxicity was confirmed by the lack of chromosomal damage, highlighted by the MN test, that showed no significant differences between treatments and control during both exposure tests (Table 1). This result seems to be in contrast with the high MN frequency observed in zebra mussel specimens exposed to TCS (Binelli et al., 2009), although the previous studies tested concentrations were at least three times higher than those used in this study. The low genetic damage observed might be correlated to embryos high resistance to the genotoxic injury, as well to the short time of exposure, in agreement with a previous study (Della Torre et al., 2017) in which zebrafish embryos exposed to the carcinogen B(α)P up to 96 hpf did not show any significant induction of primary DNA damage and occurrence of MN. The overall results obtained by both biochemical and cyto-genotoxicity analyses suggest that the observed cyto-genotoxic effects were produced directly by TCS and not indirectly related to an increase of oxidative stress.

Finally, all the investigated endpoints were combined together to evaluate their relationship through the Pearson's correlation statistic test (Table 2). The positive significant correlation between GST and antioxidant enzymes suggests that also this enzyme is actively involved in preventing oxidative stress, as also supported by the negative significant correlation with ROS level. These results are similar to those reported for the catfish *Pelteobagrus fulvidraco* (Ku et al., 2014), in which TCS exposure induced a significant increase of CAT activity, and for zebra mussel that showed a rise of GST and CAT activities in response to TCS (Binelli et al., 2011). The positive correlation highlighted between SOD and GPx (Table 2; Fig. S3), but not with CAT, confirmed the slight oxidative stress generated by environmental concentrations of TCS. Indeed, GPx is more sensitive to low H_2O_2 concentrations generated by SOD, while CAT is induced only by high levels of this

oxidative by-product (Powers and Lennon, 1999). Lastly, the cell viability was negatively correlated ($p < 0.01$) with necrosis suggesting the independent cytotoxic effect of environmental levels of TCS on zebrafish embryos.

CONCLUSIONS

This study is the first attempt to investigate the potential toxicity of TCS administered at environmental concentrations on zebrafish embryos. TCS exposure elicited the induction of oxidative stress response in embryos, preventing the occurrence of oxidative damage. Though, the pollutant generated a significant cytotoxicity, which subsequently drives to an increase of cellular necrosis.

Overall results highlight the concern related to the presence of TCS in the aquatic environment, suggesting the need to assess the effective exposure of aquatic wildlife to this pollutant. Further studies focusing on understanding the toxicity mechanisms of TCS on other aquatic models and including the investigation of other endpoints (i.e. transcription levels of stress related genes, proteomics) at environmental concentrations are also recommended.

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FIGURE CAPTIONS

Fig. 1. Activity of P-glycoprotein (a), EROD (b) and GST (c) in zebrafish embryos exposed for 24 h and 120 h to TCS at 0.1 μg L^{-1} and 1 μg L^{-1}, compared to relative control (mean ± SD).

One-way ANOVA, Duncan's Multiple Range post-hoc test, $^{**}p < 0.01$, $^{***}p < 0.001$.

Fig. 2. Activity levels of antioxidant enzymes (SOD, GPx and CAT) in zebrafish embryos exposed for 24 h and 120 h to TCS at 0.1 μg L^{-1} and 1 μg L^{-1}, compared to relative controls (mean ± SD).

One-way ANOVA, Duncan's Multiple Range post-hoc test, $^{*}p < 0.05$, $^{**}p < 0.01$.

Table
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Table 1. Biomarkers of cyto-genotoxicity in zebrafish exposed for 24 h and 120 h to TCS at 0.1 µg L^{-1} and 1 µg L^{-1} (mean ± SD). One-way ANOVA, Duncan's Multiple Range post-hoc test, *p < 0.05, **p < 0.01, ***p < 0.001.

TREATMENT	CELL VIABILITY (%)	DNA TAIL (%)	LDR (%)	NECROSIS (%)	MICRONUCLEI (‰)
24 h					
ctrl	96.94 ± 0.52	1.92 ± 0.49	1.03 ± 0.01	2.37 ± 0.93	3.61 ± 1.16
0.1 µg L^{-1}	97.18 ± 1.27	1.87 ± 0.39	1.03 ± 0.00	1.91 ± 0.71	4.45 ± 0.73
1 µg L^{-1}	95.19 ± 3.77	1.55 ± 0.49	1.03 ± 0.01	***5.76 ± 1.22	5.40 ± 2.49
120 h					
ctrl	98.70 ± 1.33	2.07 ± 0.20	1.03 ± 0.00	0.90 ± 0.74	5.03 ± 2.78
0.1 µg L^{-1}	97.16 ± 2.66	*1.43 ± 0.39	1.02 ± 0.01	*3.61 ± 1.22	7.25 ± 2.04
1 µg L^{-1}	*94.69 ± 2.36	**2.80 ± 0.34	1.04 ± 0.00	***12.83 ± 2.28	9.51 ± 7.39

Table 2. Matrix of Pearson's correlation, showing r and p-level, for all biomarkers examined in zebrafish exposed for 24 h and 120 h to TCS at 0.1 µg L^{-1} and 1 µg L^{-1}. Significant correlations (p < 0.05) are reported in bold.

	GST	CAT	GPx	SOD	P-gp	EROD	MN	VIAB	NECRO	COMET	PCC	ROS
GST	1.0000	**0.8028** **p=0.000**	**0.6000** **p=0.003**	**0.4428** **p=0.039**	-0.3059 p=0.166	0.4078 p=0.060	0.2069 p=0.355	0.1975 p=0.378	0.0093 p=0.967	0.1135 p=0.615	0.3700 p=0.090	**-0.5425** **p=0.009**
CAT		1.0000	**0.5988** **p=0.003**	0.2084 p=0.352	-0.2091 p=0.350	0.3875 p=0.075	0.3761 p=0.085	0.0223 p=0.921	0.3072 p=0.164	0.2782 p=0.21	-0.1322 p=0.557	**-0.4991** **p=0.018**
GPx			1.0000	**0.5134** **p=0.015**	0.1423 p=0.528	**0.8040** **p=0.000**	0.4211 p=0.051	0.1339 p=0.552	0.2523 p=0.257	0.2642 p=0.235	-0.4003 p=0.065	**-0.7595** **p=0.000**
SOD				1.0000	**-0.4429** **p=0.039**	**0.4839** **p=0.023**	0.1515 p=0.501	0.1527 p=0.498	-0.1501 p=0.505	-0.3685 p=0.092	**-0.6590** **p=0.001**	-0.3667 p=0.093
P-gp					1.0000	0.0218 p=0.923	-0.2057 p=0.358	0.2084 p=0.352	-0.0226 p=0.920	**0.4904** **p=0.02**	0.2748 p=0.216	-0.1732 p=0.441
EROD						1.0000	**0.4904** **p=0.020**	-0.0211 p=0.926	0.1501 p=0.505	0.1516 p=0.501	-0.3429 p=0.118	**-0.6262** **p=0.002**
MN							1.0000	0.0694 p=0.759	0.0736 p=0.745	-0.2301 p=0.303	0.1678 p=0.455	-0.2041 p=0.362
VIAB								1.0000	**-0.6239** **p=0.002**	-0.0537 p=0.812	0.2747 p=0.216	0.0962 p=0.0670
NECRO									1.0000	**0.6020** **p=0.003**	-0.3295 p=0.134	-0.3709 p=0.089
COMET										1.0000	-0.0553 p=0.807	-0.3663 p=0.094
PCC											1.0000	**0.5256** **p=0.012**

Figure
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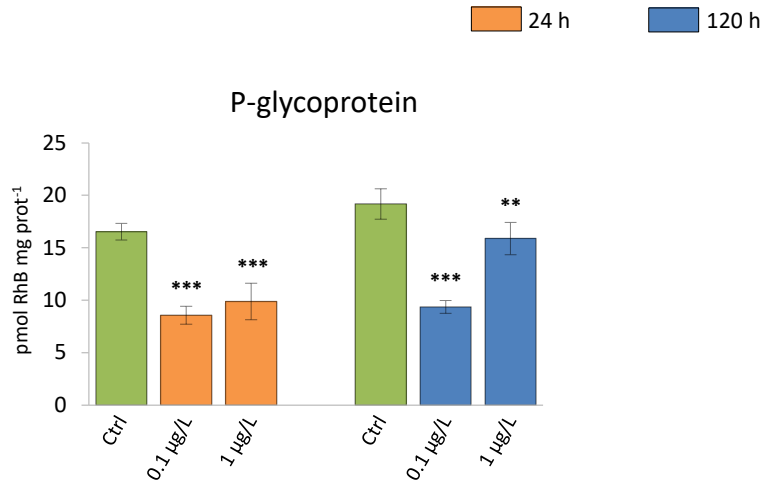

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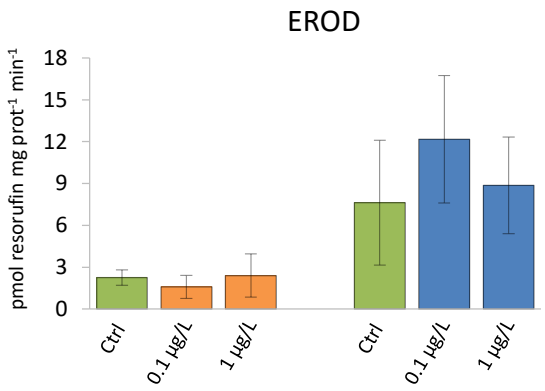

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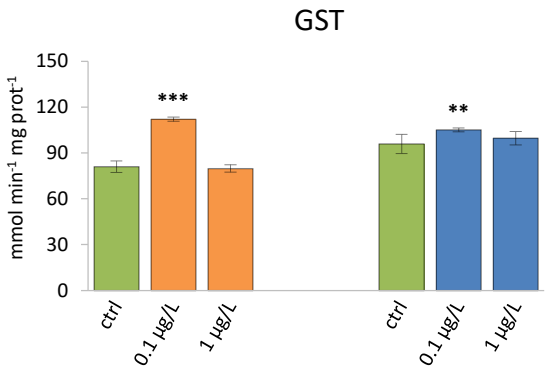

c.

Figure
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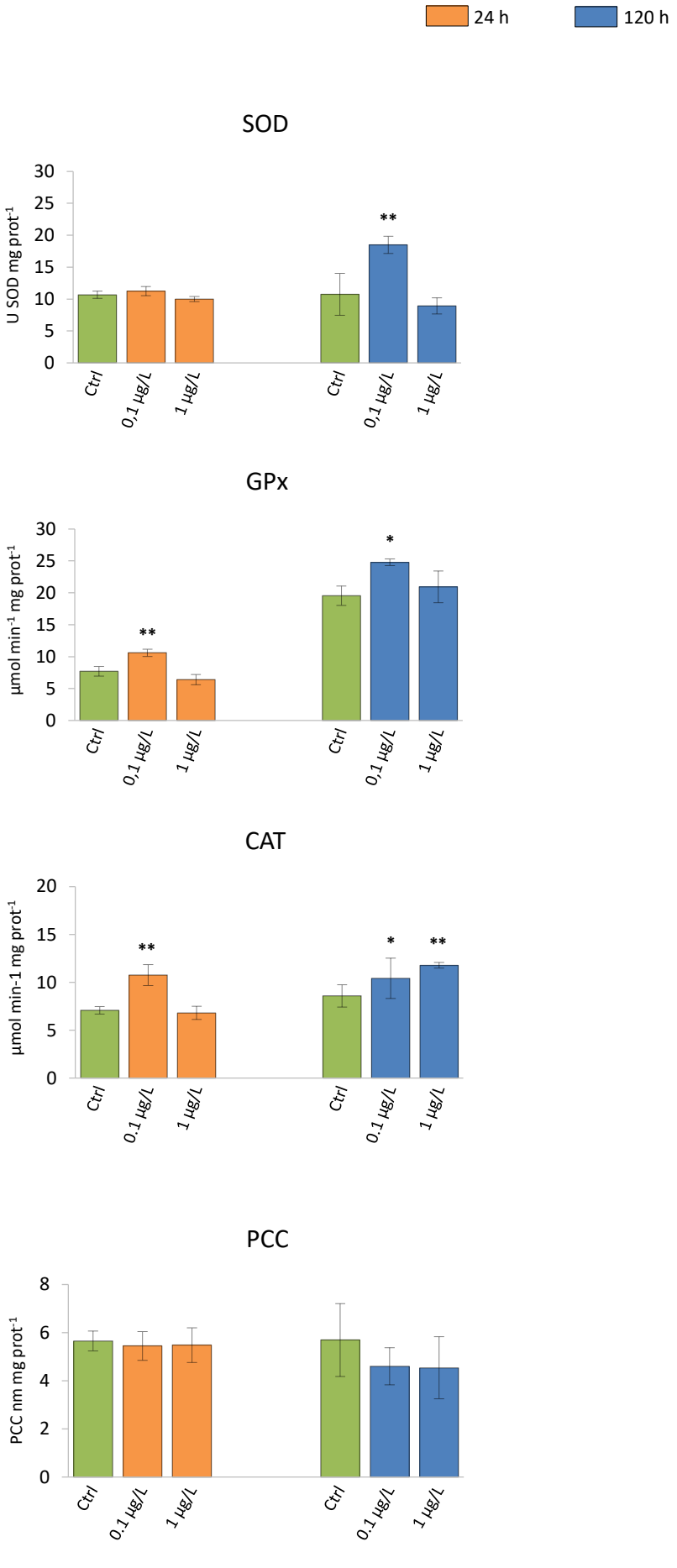

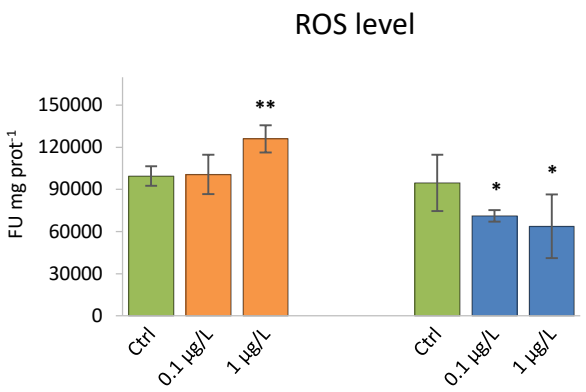
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