Chronic toxicity induced by two antidepressants on freshwater mussels: a multi-biomarker investigation

Stefano Magni[1*], Marco Parolini[1], Camilla Della Torre[1], Luciana Fernandes de Oliveira[2], Martina Catani[3], Roberta Guzzinati[3,4], Alberto Cavazzini[3], Andrea Binelli[1**]

[1]Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy;
[2]Department of Physiological Sciences, State University of Londrina, C.P. 10011, Londrina, Paraná, Brazil;
[3]Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Via L. Borsari 46, 44121 Ferrara, Italy;
[4]ENEA SSPT-USER-R4R, Via Martiri Monte Sole 4, 40129 Bologna, Italy.

*Corresponding author. Tel.: +39 0250314729; fax: +39 0250314713; E-mail: stefano.magni@guest.unimi.it
**Co-corresponding author. Tel.: +39 0250314714; fax: +39 0250314713; E-mail: andrea.binelli@unimi.it

ABSTRACT

Antidepressants are one of the main pharmaceutical classes detected in the aquatic environment. Nevertheless, there is a dearth of information regarding their potential adverse effects on *non*-target organism in scientific literature. Thus, the aim of this study was the evaluation of chronic toxicity on the freshwater mussel *Dreissena polymorpha* induced by two antidepressants commonly found in the aquatic environment, namely Fluoxetine (FLX) and Citalopram (CT). *D. polymorpha* specimens were exposed to FLX and CT alone and to their mixture (MIX) at the environmental concentration of 500 ng/L for 14 days. We measured the sub-lethal effects by means of a biomarker suite: the cellular stress was evaluated by monitoring the activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and the activity of the phase II detoxifying enzyme glutathione-S transferase (GST), as well as the functionality of the ABC transporter P-glycoprotein (P-gp). Oxidative damage was evaluated by lipid peroxidation (LPO) and protein carbonylation (PCC), while genetic damage was tested by Single Cell Gel Electrophoresis (SCGE) assay, DNA diffusion assay and micronucleus test (MN test). Our results highlight that CT, MIX and to a lesser extent FLX, caused a significant alteration of the oxidative status of bivalves, accompanied by a significant reduction of P-gp efflux activity. However, only FLX induced a slight, but significant, increase in apoptotic and necrotic cell frequencies. Considering the variability in biomarker response and to perform a toxicity comparison of tested molecules, we

integrated each endpoint into the Biomarker Response Index (BRI). The data integration showed that 500 ng/L of FLX, CT and their MIX have the same toxicity level on exposed bivalves.

Keywords:
antidepressants, chronic toxicity, biomarkers, *Dreissena polymorpha*

Highlights:
-Antidepressants are considered emerging aquatic contaminants;
-Sub-lethal effects caused by 500 ng/L of FLX, CT and their MIX were investigated;
-Tested antidepressants caused an alteration of oxidative status of bivalves;
-Biomarker integration showed that FLX, CT and their MIX have the same toxicity.

1 INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) are considered emerging aquatic contaminants, because they are not included in any regulatory frame work and their effects on human and aquatic community are unknown (Deblonde et al., 2011). Among the plethora of PPCPs commonly found in the aquatic environment, antidepressants represent the 4% of total amount of pharmaceuticals (Santos et al., 2010) and are revealed at ng/L concentrations, similarly to other commonly used therapeutics, according to their worldwide use and the inability of traditional Wastewater Treatment Plants (WWTPs) in their removal from wastes (Heberer, 2002; Santos et al., 2010; Reungoat et al., 2011). A heterogeneous group of molecules belongs to the class of antidepressants, mainly used to contrast pathological phenomena such as dysthymia and depression. According to their mechanism of action (MOA), it is possible to distinguish five main groups of antidepressants: selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), tetracyclic antidepressants (TeCas), inhibitors of serotonin reuptake-norepinephrine (SNRIs) and monoamine oxidase inhibitors (MAOIs). The SSRIs, blocking the serotonin (5-hydroxytryptamine, 5-HT) reuptake from the pre-synaptic cleft, are among the most used antidepressants (Fong and Ford, 2014). In particular, Fluoxetine (FLX), the active principle of the well-known Prozac®, and to a lesser extent Citalopram (CT), are the most prescribed antidepressants worldwide. Although they are mainly metabolized in nor-fluoxetine and N-desmethyl-citalopram, respectively, about 20-30% of FLX and 26% of CT swallowed dose is excreted unaltered (Dalgaard and Larsen, 1999; Fong and Molnar, 2008) and released into the aquatic environment, where they are measured at concentrations ranging from 0.6 to 540 ng/L and from 9.2 to 382 ng/L, respectively (Santos et al., 2010; Fong and Ford, 2014). Despite the overt presence of antidepressants in freshwater

ecosystems, they are currently not included in regular monitoring surveys. However, an increasing number of studies is underlying the toxic effects of SSRIs on aquatic communities, since the modulation of 5-HT could have significant adverse effects on exposed organisms. As reported by Fong and Ford (2014), the antidepressants induce important alterations on aquatic invertebrates, interfering with major biological processes as metabolism, feeding behavior, locomotion and reproduction. FLX has been also demonstrated to be an endocrine disruptor: Fong (1998) observed an induction of spawning at FLX concentration of 50 nM in males of the freshwater mussel *Dreissena polymorpha*. Further research showed a decrease in oocytes and spermatozoa in *D. polymorpha* specimens after FLX exposure at concentrations as low as 20 nM (Lazzara et al., 2012), while Gonzalez-Rey and Bebianno (2013) reported effects on the endocrine system of *Mytilus galloprovincialis* exposed to 75 ng/L of FLX, accompanied by a tissue-specific antioxidant response. By contrast, to the best of our knowledge, the effects of CT on *non*-target aquatic organisms are still poorly investigated. Thus, the aim of this study was the evaluation of chronic toxicity induced by FLX and CT by means of the measure of cellular stress, oxidative damage and genotoxicity on the freshwater mussel *D. polymorpha*, one of the most useful biological models for ecotoxicological studies on freshwater ecosystems (Binelli et al., 2015). Bivalves were exposed to FLX, CT and their mixture (MIX) for 14 days at the environmental concentration of 500 ng/L (Santos et al., 2010; Fong and Ford, 2014). To assess cellular stress, we monitored on homogenates of the mussel soft tissue the activity of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as the activity of glutathione-S-transferase (GST), a phase II detoxifying enzyme, while the functionality of the P-glycoprotein (P-gp), an efflux pump acting as first defense towards contaminants, was also measured in mussel gills. Moreover, we measured the amount of protein carbonylation (PCC) and lipid peroxidation (LPO) to evaluate the oxidative damage. Lastly, the genotoxicity was assessed on *D. polymorpha* hemocytes by Single Cell Gel Electrophoresis (SCGE) assay, DNA diffusion assay and micronucleus test (MN test). In order to compare the toxicity of FLX, CT and their MIX, the whole biomarker dataset was then integrated into the Biomarker Response Index (BRI; Hagger et al., 2008).

2 MATERIALS AND METHODS

2.1 *Sampling and maintenance of bivalves*

D. polymorpha specimens were collected in September 2015 from Lake Lugano (North Italy) that is considered a reference site according to its low level of PPCP contamination (Zuccato et al., 2008). Bivalves were then transported in bags filled with lake water to laboratory and placed in tanks (15

L) with a mixture of tap and deionized water (50:50 v/v) and maintained at 20 ± 1 °C with a natural photoperiod, pH=7.5 and oxygen saturation. Water was changed every two days during the following two weeks to purify the bivalves by possible contaminants previously accumulated in their soft tissues. Bivalves were fed daily with a suspension of the blue-green alga *Spirulina* spp. Only animals attached to the tank supports from their byssi and with a shell length of about 15 ± 4 mm were selected for the subsequent exposure tests.

2.2 *Experimental design*

The standards of FLX (Fluoxetine hydrochloride solution; CAS number 59333-67-4) and CT (Citalopram hydrobromide solution; CAS number 59729-32-7) were purchased from Sigma-Aldrich (Steinheim, Germany); both standards were certified as single component solutions. Each standard (1 mg/mL in methanol) was diluted in ultrapure water to obtain the working solutions (1 mg/L), which were then used to obtain the exposure concentrations of 500 ng/L administered alone and in MIX (500 ng/L FLX + 500 ng/L CT) to bivalves. Before the exposure we evaluated the baseline levels for all considered endpoints on bivalves taken from a single tank; subsequently, we placed 70 specimens in 4 L tanks to perform the exposures (three tanks for each treatment). Exposures were performed in semi-static conditions, feeding bivalves 1 h before the daily renewing of the exposure solutions, for 14 days. We collected 11 bivalves every three days from each tank (33 individuals for each treatment) to be used for biomarker analyses. We collected the hemolymph from 9 bivalves to evaluate genotoxicity on hemocytes and to contemporarily assess the cell viability through the Trypan blue exclusion method. Subsequently, the soft tissues from the same bivalves were frozen in liquid nitrogen and stored at -80 °C for further analyses of oxidative damage. In addition, the soft tissues of other 15 bivalves for each treatment were frozen in liquid nitrogen and stored at -80 °C until the measurement of the enzymatic activities. Lastly, from other 9 bivalves *per* treatment, gills were gently dissected and a 4 mm circular portion of tissue was removed using a skin biopsy punch (Acuderm® inc., USA) to carry out the measurement of the P-gp efflux activity. Furthermore, 1 h after the contaminant spike, we sampled a 100 mL aliquot of water from both the control and the exposure tanks, which were stored at -20 °C until the quantification of antidepressant concentrations.

2.3. *Identification and Quantification of antidepressants in the exposure tanks*

2.3.1 *Sample pretreatment and solid phase extraction*

Chemicals used in this section were of LC-MS grade (Sigma-Aldrich), water was of Milli-Q grade (Merck Millipore).

All water samples were filtered through 0.22 µm nylon filters (GVS) and stored at -20°C until analysis.

Extraction of the target compounds was performed by adjusting the Offline Solid Phase Extraction (Offline-SPE) procedures already described in literature (Schultz, 2008; Demeestere et al., 2010).

Waters Oasis® HLB (150 mg, 6 mL) cartridges were firstly washed with 5 mL of methanol and preconditioned with 5 mL of milli-Q water acidified with 0.1% (v/v) formic acid.

Acidified water samples (100 mL + 0.1% (v/v) formic acid) were loaded onto each cartridge and then washed with 1 mL of 70% methanol in 2% (v/v) ammonium acetate.

The analytes of interest were eluted with 4 mL methanol in 2% (v/v) acetic acid; each extract was dried under a gentle nitrogen stream and then reconstituted with 1 mL of acetonitrile/water 15/85 +0.1% (v/v) formic acid containing 15 ppb of the internal standard fluoxetine-D6.

2.3.2 Detection of antidepressant by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) analysis

An LC/MS instrument composed of a micro-HPLC (Finnigan Surveyor Plus) interfaced to a LTQ-XL linear ion-trap MS detector (Thermo Scientific) was used for all measurements.

HPLC separation was performed on a Symmetry C18 column (2.1x150mm, 3.5µm , Waters) at 30°C with mobile phase A water+0.1% (v/v) formic acid and B acetonitrile+0.1% (v/v) formic acid and at a flow rate of 0.15 mL/min. The chromatographic run was set as follows:

0-8.00 min: gradient from A/B 85/15 (v/v) to 20/80 (v/v)

8.10-10.00 min: isocratic A/B 5/95 (v/v)

10.10- 18.00 min: isocratic 85/15 (v/v)

An electrospray ionization interface in positive mode ESI(+) was employed for detection of all compounds. The capillary voltage was set to 31.97 V and the source temperature was 275°C. For the quantification of the analytes the transition between the precursor ions $[M+H]^+$ and the most abundant product ions was observed, as summarized in table…

Table…

Optimized conditions of LC-MS/MS analysis

Compound	t_R (min)[a]	Precursor ion (m/z)	Product ion (m/z)	CID CE (%)[b]
Fluoxetine	6.7	310.2	147.9	16
Citalopram	5.9	325.2	262.1; 108.9	16
Fluoxetine-D6	6.7	316.2	154.0	18

[a] retention time.

[b] collision induced dissociation energy

The calibration curves, expressed asthe ratio between the peak areas of the two antidepressant and that of the internal standard, fluoxetine-D6, versus the concentration of each drug, exhibited linearity, with R^2>0.99 for both analytes, over the concentration range 0–50 µg/L.
All the samples were injected twice and the measured analytes concentration were adjusted with the offline-SPE estimated percentage of recovery (65% and 89% Fluoxetine and Citalopram respectively).

2.4 *Biomarkers of cellular stress*

Since the methods and procedures of all the biomarkers applied in the present study are described in detail elsewhere (Parolini et al., 2010), we reported here just their brief description. We measured the activity of antioxidant enzymes SOD, CAT and GPx, as well as the activity of phase II detoxifying enzyme GST in homogenates from *D. polymorpha* whole soft tissues. These endpoints were measured in triplicate on cytosolic fraction from a pool of 3 mussels for each tank (n=3; 9 specimens *per* treatment). The soft tissue of bivalves was homogenized in 100 mM of phosphate buffer (pH 7.4) containing KCl 100 mM, EDTA 1 mM, dithiothreitol (DTT) 100 mM and a protease inhibitors cocktail (1:100 v/v). The homogenate was ultra-centrifuged at 20,000 g for 1 h at 4 °C. The obtained supernatant was processed for protein determination according to Bradford (1976), while the enzymatic activity was measured following the methods reported by Orbea et al. (2002). Briefly, CAT activity was determined evaluating the consumption of 50 mM hydrogen peroxide (H_2O_2) at 240 nm. SOD activity was determined evaluating the reduction of cytochrome c (10 µM) inhibition by the superoxide anion ($\bullet O_2^-$) at 550 nm generated by the reaction of xanthine oxidase (1.87 mU/mL) and hypoxanthine (50 µM). The GPx activity was evaluated measuring the NADPH consumption at 340 nm using H_2O_2 0.2 mM as substrate with glutathione (2 mM), sodium azide (NaN_3; 1 mM), glutathione reductase (2 U/mL), and NADPH (120 µM). Lastly, the activity of GST was measured at 340 nm in presence of reduced glutathione (1 mM) and 1-chloro-2,4-dinitrobenzene (CDNB) as co-substrate. The efflux functionality of P-gp was evaluated in gills as described by Navarro et al. (2012). 12 gill biopsies from 3 animals *per* each tank, were placed in Petri dishes with tap and deionized water mixture (50:50 v/v) and the fluorescent substrate rhodamine B (RhB; 1 µM). Samples were then incubated for 90 min at room temperature in dark condition with gentle shaking. The P-gp inhibitor verapamil (10 µM) was used as positive control. After the incubation, the biopsies were washed twice and stored at -80 °C. Subsequently, 300 µL of tap and deionized water mixture (50:50 v/v) were added to each biopsy, which were then homogenized and centrifuged for 10 min at 14,000 rpm. The amount of RhB in biopsies was

measured in fluorescence through the multi-well reader Infinite® F200 PRO from Tecan Trading AG (excitation = 545 nm; emission = 575 nm).

2.5 *Biomarkers of oxidative damage*

We measured in triplicate the levels of LPO and PCC in homogenates of 3 *D. polymorpha* specimens collected from each exposure tank (n=3; 9 specimens *per* treatment), obtained pottering mussel soft tissues in phosphate buffer 100 mM (pH 7.4) containing KCl 100 mM, EDTA 1 mM, DTT 1 mM and a protease inhibitors cocktail (1:100 v/v). The obtained homogenate was processed for protein quantification using the Bradford method (1976) and for measurement of LPO and PCC levels. The PCC was evaluated exploiting the reaction between the 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl groups of protein and reading the absorbance at 370 nm (Mecocci et al., 1999), while LPO content was assessed by the measurement of thiobarbituric acid-reactive substances (TBARS) at 535 nm according to Ohkawa (1979).

2.6 *Biomarkers of genotoxicity*

The SCGE assay was performed on hemocytes of *D. polymorpha* according to Parolini et al. (2010). 50 cells for each slide (8 slides for each treatment) were analyzed using a specific software (Comet Score®). Two end-points were evaluated: the ratio between length and comet head diameter (LDR) and the percentage of DNA in the comet tail. The apoptotic and necrotic frequencies were evaluated using the method suggested by Singh (2000); we considered 300 cells for each slide (5 slides for each treatment). Lastly, the MN test was conducted as described in Pavlica et al. (2000): 400 cells were counted for each slide (8 slides for each treatment). The micronuclei were identified according to the criteria purposed by Kirsch-Volders et al. (2000).

2.7 *Statistical analyses*

The statistical analyses were performed using STATISTICA 7.0 software package. Data normality was verified using Shapiro-Wilk test while homoscedasticity was evaluated through the Levene tests. To identify the difference between treated samples and related controls we conducted a two-way analysis of variance (two-way ANOVA), where time (t=4; t=7; t=11 and t=14) and treatment (control, FLX 500 ng/L, CT 500 ng/L and their MIX) were categorical predictor factors, while the measured biomarkers were considered as dependent variables. This analysis was followed by a Fisher LSD post-hoc test to evaluate significant differences (*$p<0.05$; **$p<0.01$) between treated samples and the corresponding controls (time *versus* time).

3 RESULTS

During the 14 days of exposure we found a comparable and low mortality of bivalves in the control and exposure tanks, showing that the antidepressants did not induce acute toxicity at the tested environmental concentration, nor individually neither in mixture. Furthermore, the percentage of hemocytes viability found in bivalves from the control tanks during the 2 weeks of exposure was always higher than 70%, with a mean of 80.2 ± 8.4%, as required to perform genotoxicity tests (Kirkland et al., 2007). Mean baseline levels (± standard error of the means; SEM) for each considered endpoint, obtained at the beginning of the exposure (t=0 day) are the following: 13.4 ± 1.2 U mg $prot^{-1}$, 26.8 ± 1.2 mM min^{-1} mg $prot^{-1}$, 11.3 ± 2.8 µM min^{-1} mg $prot^{-1}$ and 90.8 ± 2.1 mM min^{-1} mg $prot^{-1}$ respectively for SOD, CAT, GPx and GST activities, 3021.2 ± 187.4 fluorescence units for P-gp functionality, 9.5 ± 1.6 nM g^{-1} $w.w.^{-1}$ and 5.7 ± 0.9 nM mg^{-1} $prot^{-1}$ for LPO and PCC and, regarding genotoxicity, 2.1 ± 0.2% for DNA in the comet tail, 1.0 ± 0.0 for LDR parameter, 0.7 ± 0.1‰ for MN frequency, 0.2 ± 0.1% and 0.3 ± 0.2% for apoptosis and necrosis frequencies.

3.1 *FLX and CT in the exposure tanks*

To verify stability of FLX and CT working solutions over the whole period of the exposures (14 days), we measured the concentration of both the antidepressants at the moment of the dilution of FLX and CT stock solutions and after 14 days. The concentration of FLX at the beginning (t=0 day) and the end (t=14 day) of exposure was 1.01 ± 0.02 mg/L and 0.97 ± 0.05 mg/L, respectively, while the concentration of CT was 0.97 ± 0.03 mg/L at t=0 day and 0.85±0.03 mg/L at t=14 day of the exposure. In the control tanks the concentrations of FLX and CT were below the detection limits. The FLX and CT concentrations measured in the exposure tanks were close to the nominal concentrations of 500 ng/L, since we obtained a mean values of 484.62 ± 1.17 ng/L for FLX and 595.95 ± 0.67 ng/L for CT. In the MIX the concentrations of FLX and CT were 457.15 ± 2.66 ng/L and 575.81 ± 1.77 ng/L, respectively. Since the method of quantification has a coefficient of variation of ± 20%, these data confirm the reliability of our exposure conditions.

3.2 *Chronic toxicity of FLX*

SOD activity (Fig. 1A) showed a particular trend characterized by a significant ($p<0.01$) decrease in the early exposure time (t=4 and 7 days) compared to control, followed by a clear raise at the end of exposure, where a significant ($p<0.01$) difference with baseline level was measured. We also obtained a significant effect of time ($F=68.591$; $p<0.01$) and interaction time to treatment ($F=12.760$; $p<0.01$). CAT activity (Fig. 1B) also highlighted a significant ($p<0.01$) early drop in its activity and a great increase at the end of exposure up to about 27% compared to control.

Significant effects of treatment ($F=35.186$; $p<0.01$), time ($F=74.180$; $p<0.01$) and their interaction ($F=9.014$; $p<0.01$) were found. The activity of GPx (Fig. 1C) showed a significant effect of treatment ($F=38.614$; $p<0.01$), time ($F=63.568$; $p<0.01$) and their interactions ($F=21.727$; $p<0.01$), with a significant ($p<0.01$) increase only at the end of experiment (t=14 day), while a significant effect ($F=21.285$; $p<0.01$) of FLX on GST was noticed only after 11 days of treatment (Fig. 1D). A significant inhibition of P-gp functionality by the inhibitor verapamil (-36%) was observed. In this context, a significant effect of treatment ($F=6.644$; $p<0.05$), time ($F=4.470$; $p<0.01$) and their interaction ($F=6.441$; $p<0.01$) was observed in the modulation of P-gp efflux functionality, which resulted significantly inhibited (-38%) compared to control after 11 days of FLX exposure (Fig. 2). Regarding the oxidative damage, no increase in protein carbonylation and lipid peroxidation (Fig. 3A, B) was found. The SCGE assay did not show any increase of primary DNA damage due to FLX exposure (Table 1). Despite no increase in MN frequency was found (Table 1), we observed a significant ($p<0.01$) enhancement of apoptotic and necrotic cells after 4 and 7 days of exposure, respectively (Table 1).

3.3 *Chronic toxicity of CT*

An alteration of oxidative status in the bivalves exposed to CT was observed; in particular, each considered enzyme activity showed a bell-shaped trend (Fig. 1A, B, C, D). SOD activity (Fig. 1A) showed a significant effect of treatment ($F=94.835$; $p<0.01$), time ($F=141.282$; $p<0.01$), and their interaction ($F=54.376$; $p<0.01$), with a significant inhibition ($p<0.01$) at 4 days of exposure followed by a significant increase ($p<0.01$) compared to control from 7 days till the end of exposure. CAT activity (Fig. 1B) showed a significant effect of treatment ($F=6.176$; $p<0.05$), time ($F=113.801$; $p<0.01$) and their interaction ($F=46.480$; $p<0.01$) and highlighted a significant increase at 7 and 11 days of exposure, with a significant inhibition ($p<0.01$) after 4 days of exposure and at the end of treatment (t=14 days). GPx activity (Fig. 1C) showed a significant effect of time ($F=33.491$; $p<0.01$) and time *per* treatment interaction ($F=106.613$; $p<0.01$), with a significant inhibition ($p<0.01$) at 4 days of exposure and a significant increase ($p<0.01$) at 11 days of exposure. A significant effect of treatment ($F=41.917$; $p<0.01$), time ($F=64.105$; $p<0.01$) and their interaction ($F=16.497$; $p<0.01$) was observed for GST (Fig. 1D), which also showed a significant inhibition ($p<0.01$) at 4 and 14 days. The efflux activity of P-gp (Fig. 2) showed a significant effect of treatment ($F=12.772$; $p<0.01$), time ($F=3.993$; $p<0.01$) and their interaction ($F=4.310$; $p<0.01$), with a significant reduction of pump functionality (-43%, $p<0.01$) after 4 days compared to baseline levels. Furthermore, despite no significant increase of lipid peroxidation was found (Fig. 3B), a significant effect of time ($F=10.371$; $p<0.01$) and interaction time to treatment ($F=10.721$; $p<0.01$)

was measured, resulting in a significant increase (27%; $p<0.01$) of protein carbonylation at 11 days of exposure (Fig. 3A). Regarding the genotoxicity, no significant effects on the considered endpoints were found (Table 1).

3.4 *Combined effects of FLX and CT*

As obtained for the CT exposure, in the bivalves exposed to MIX we observed for all enzyme activities a bell-shaped trend (Fig. 1A, B, C, D). SOD activity (Fig. 1A) showed a significant inhibition ($p<0.01$) after 4 days followed by a significant raise ($p<0.01$) from the seventh day until the end of exposure, with a significant effect of treatment ($F=68.792$; $p<0.01$), time ($F=93.312$; $p<0.01$) and their interaction ($F=47.978$; $p<0.01$). A significant effect of treatment ($F=30.796$; $p<0.01$), time ($F=164.971$; $p<0.01$) and interaction time to treatment ($F=39.539$; $p<0.01$) on CAT (Fig. 1B) was induced by MIX, showing a significant inhibition after 4 days followed by an activity increase at 7 and 11 days of exposure. A significant effect of treatment ($F=7.869$; $p<0.01$), time ($F=73.062$; $p<0.01$) and their interaction ($F=244.356$; $p<0.01$) was observed also on GPx (Fig. 1C), disclosing a significant inhibition ($p<0.01$) at 4 and 7 days of exposure and a significant increase ($p<0.01$) at 11 days of treatment. The GST activity (Fig. 1D) showed a significant inhibition ($p<0.01$) at 4 days and the end of exposure ($t=14$), but a significant increase ($p<0.01$) at 11 days of treatment. A significant effect of treatment ($F=4.773$; $p<0.05$), time ($F=29.574$; $p<0.01$) and their interaction ($F=9.209$; $p<0.01$) was observed for GST. As in bivalves exposed to CT, a significant effect of treatment ($F=4.334$; $p<0.05$), time ($F=5.860$; $p<0.01$) and their interaction ($F=3.507$; $p<0.05$) was observed for P-gp efflux activity; we registered a reduction in its activity (-30%) compared to control after 4 days of exposure (Fig. 2). Whilst LPO did not show significant differences between treated and control (Fig. 3B), a significant effect of time ($F=14.203$; $p<0.01$) and treatment *per* time interaction ($F=7.281$; $p<0.01$) on PCC was noted, reaching a 24% increase at $t=11$ days (Fig. 3A). No significant genotoxic effect was found in zebra mussel MIX-treated specimens compared to controls (Table 1).

4 DISCUSSION

Pharmaceutical compounds, being synthesized to affect specific cellular structures and metabolic processes on specific targets, may also cause adverse effects on *non*-target organisms. In this context, it is important to bearing in mind that many conserved biological processes could increase the risk associated with the presence of aquatic pollutants (Huggett et al., 2003; Gunnarsson et al., 2008). In particular, the SSRIs, interfering with the 5-HT metabolism, modulate important biological activities in aquatic invertebrates (Fong and Ford, 2014; Ford and Fong, 2016). Results

337 from the present study revealed a significant depression of cellular response at 4 days of exposure,
338 mainly in mussels exposed to CT and MIX, as pointed out by the significant inhibition of the
339 activity of SOD, CAT, GPx, GST, as well as in P-gp functionality, compared to background levels
340 (Figs. 1 and 2). Considering that antidepressants, besides their use in the therapy of depression, are
341 also used to treat chronic pain (Rodieux et al., 2015), the early inhibition of cellular response could
342 be associated to their analgesic effect. Although the therapeutic effects have been especially
343 observed for SNRIs and TCAs (Fishbain, 2000; Fishbain et al., 2000; Dworkin et al., 2010), it
344 cannot be excluded that some SSRIs may act as analgesic also on *non*-target organisms. However,
345 starting from the seventh day of exposure, such potential pharmacological/analgesic effect of
346 antidepressants seems to be replaced by an increase in their toxic behavior, as shown by the raise of
347 antioxidant activity observed for all the treatments (Fig. 1), as reported in previous studies aimed at
348 assessing the effects of FLX on *Mytilus galloprovincialis* (Gonzalez-Rey and Bebianno, 2013;
349 Franzellitti et al., 2014) and *Crassostrea gigas* (Di Poi et al., 2014). In view of an increasing trends
350 for SOD and CAT activities after FLX exposure, a clear bell-shaped trend was observed for CT and
351 MIX (Fig. 1A, B). This latter trend can suggest an overproduction of H_2O_2 that determined the
352 observed slight CAT decrease at the end of exposure, likely due to the substrate inhibition
353 (Vlahogianni and Valavanidis, 2007). Furthermore, this effect could be also responsible for the final
354 reduction of SOD activity instead mediated by the product inhibition. This different behaviour is
355 further shown for GPx, even if the increasing trend for FLX was only outlined (Fig. 1C). This
356 explanation was confirmed by the significant ($p<0.01$) increase of protein carbonylation noticed
357 only for CT and MIX treatments after 11 days of exposure (Fig. 3A), which could be induced just
358 by the $\bullet O_2^-$ and H_2O_2 overproduction. In fact, these oxygen radicals, as well as the hydroxyl radical
359 (•OH) formed in the Fenton and Haber-Weiss reaction, are able to cause an elevated protein
360 oxidation, as suggested by Verlecar and co-workers (2008). Furthermore, the lack of significant
361 protein carbonylation in mussels exposed to FLX could be associated to the complete lack of
362 antioxidant enzyme inhibition (Fig. 1A, B, C, D). Similar results were also obtained by Di Poi and
363 co-workers (2014) in the mollusk *Crassostrea gigas* exposed to 1, 10 and 100 ng/L of FLX that did
364 not produce significant oxidative damage probably because of the efficacy of the cellular
365 antioxidant mechanisms. In this context, the P-gp is one of the most relevant ABC transporters,
366 involved in the defense mechanism towards a wide variety of anthropogenic contaminants (Della
367 Torre et al., 2014). The P-gp activity and its role in the tolerance to environmental pollution has
368 been well characterized in *D. polymorpha* (Faria et al., 2011; Navarro et al., 2012). Our results
369 showed a significant reduction of the efflux functionality, similar to that produced by the inhibitor
370 verapamil, by CT and MIX after 4 days and by FLX after 11 days of exposure maybe due to the

analgesic effects mentioned above. Several pollutants including PPCPs, pesticides and hydrocarbons are known to suppress the activity of P-gp, through a chemo-sensitization mechanism (Smital et al., 2004). Such effect has severe ecotoxicological consequences as it might reduce the detoxifying capacity of the organism, thereby increasing the accumulation and toxicity of other pollutants. Either FLX and CT are known substrates of P-gp *in vitro,* while their interaction *in vivo* is still controversial (O'Brien et al., 2012). The observed inhibitory effect suggested a potential chemo-sensitizing effect for FLX and CT and their MIX to *D. polymorpha*, which might affect the susceptibility of bivalves towards other toxic chemicals. The low effect of FLX, CT and MIX on oxidative stress and cellular biomarkers was also confirmed by genotoxicity assays as shown by the lack of DNA damage to zebra mussel hemocytes (Table 1). These results could be probably due to the inability of these chemicals to directly induce DNA injuries and/or to the slight imbalance of oxidative stress, which was efficiently counteracted by the activation of antioxidant defense mechanism. Since the adverse effects observed in bivalves exposed to MIX were similar and showing overlapping trends to those from CT exposure, we could assume that CT was the main responsible of MIX toxicity. However, the wide variability in biomarker responses prevents the accurately support this suggestion. For this reason, each biomarker response for each molecule was integrated into the BRI, to make a toxicity comparison of tested antidepressants. Briefly, we calculated for each biomarker and for considered exposure times (t=4, t=7, t=11 and t=14 days) the percentage of alteration level (AL) compared to the corresponding control; according to the obtained AL value, we attributed a score to each endpoint and multiplied this value for the biological weigh of considered biomarkers (score = 1 for cellular stress; score = 2 for oxidative and genetic damage; Hagger et al., 2008; Parolini et al., 2013; Magni et al., 2016). Subsequently, we calculate the BRI according to the following algorithm:

$$BRI=\Sigma(AL\ biomarker_x\ score_{t=4} + \ldots + biomarker_x\ score_{t=14})*biomarker_x\ weighting/\Sigma\ biomarker_x\ weighting$$

where AL = alteration level; x = considered endpoint; t = time of exposure (days).

The integration of biomarker endpoints into the BRI (Fig. 4A) suggested that FLX, CT and MIX had the same toxicity on *D. polymorpha*. Considering the contribution of each biomarker in the toxicity histogram (Fig. 4B), it was possible pointing out that the slight toxicity of FLX was exactly divided between genotoxicity and cellular stress/oxidative damage, accounting for about 50% of the whole toxic effect each one. The toxicity induced by CT and MIX, accounting for about 60% of the total, was instead mainly associated to cellular stress and oxidative damage, confirming the main role played by CT in the MIX toxicity, as previously described. Considering that in aquatic

environment, in addition to antidepressants, there are other psychotropic substances as illicit drugs, to rank their potential toxicity we made a comparison between FLX, CT and the following compounds previously tested at the same concentration of 500 ng/L: the two main cocaine metabolites benzoylecgonine (BE; Parolini et al., 2013) and ecgonine methyl ester (EME; Parolini and Binelli, 2013), Δ-9-tetrahydrocannabinol (Δ-9-THC; Parolini and Binelli, 2014), morphine (MOR; Magni et al., 2016), 3,4-methylenedioxymethamphetamine (MDMA; Parolini et al., 2014) and amphetamine (AMPH; Parolini et al., 2016). Therefore, we recalculated the BRI considering only biomarkers used in common to all abovementioned studies. The endpoint integrations (Fig. 4C) highlighted that FLX (BRI=5.43) and CT (BRI=5.79) were, with AMPH, the molecules showing the lowest toxicity; however, it is important to consider that evaluating other end-points could modify this toxicity ranking. In fact, some evidences showed that FLX has effects on endocrine (Fong and Ford, 2014) and nervous systems (Munari et al., 2014), as well as in the control and storage of energy on *non*-target organisms (Franzellitti et al., 2014; Hazelton et al., 2014). Therefore, it is possible that the toxicity of FLX, and likely of CT, can be mostly associated to these effects, rather than endpoints described in the present report.

5 CONCLUSIONS

This study attempted to investigate the potential chronic toxicity of antidepressants, whose effects on *non*-target organisms are still poorly understood. The obtained results suggest that FLX, CT and their MIX at environmental concentration of 500 ng/L did not cause evident damage on exposed organisms, despite the significant increase of cellular stress. The integration of single biomarker results into the BRI allowed to compare the toxicity of the single molecule and to show their similar individual contribution to the MIX toxicity. Considering that some studies reported that FLX has effects on endocrine and nervous systems of *non*-target organisms, it is possible that tested molecules show this low toxicity because the endpoints tested in the present study do not fall in their specific toxicity mechanism. However, taking into account that aquatic organisms are exposed to contaminants throughout their life, it is plausible that, by increasing time of exposure, oxidative and genetic damage could also be enhanced. Therefore, further studies are needed to define the mechanism of toxicity of FLX and CT on *non*-target organisms, confirming the importance to use a wide battery of biomarkers to obtain a realistic toxicity data not subject to a reductionist approach of single or few endpoints.

6 ACKNOWLEDGEMENTS

We want to thank the Fondazione Fratelli Confalonieri (Milan, Italy) who financed the post-doc fellowship of Dr. Stefano Magni.

7 REFERENCES

Binelli, A., Della Torre, C., Magni, S., Parolini, M., 2015. Does zebra mussel (*Dreissena polymorpha*) represent the freshwater counterpart of *Mytilus* in ecotoxicological studies? A critical review. Environ. Pollut. 196, 386-403.

Bradford, MM., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Dalgaard, L., Larsen, C., 1999. Metabolism and excretion of citalopram in man: identification of O-acyl-and N-glucuronides. Xenobiotica 29, 1033-1041.

Deblonde, T., Cossu-Leguille, C., Hartemann, P., 2011. Emerging pollutants in wastewater: a review of the literature. Int. J. Hyg. Environ. Health 214, 442-448.

Della Torre, C., Bocci, E., Focardi, S., Corsi, I., 2014. Differential expression and efflux activity of ABCB and ABCC transport proteins in gills and hemocytes of *Mytilus galloprovincialis* and their involvement in cadmium response. Mar. Environ. Res. 93, 56-63.

Demeestere, K., Petrović, M., Gros, M., Dewulf, J., Van Langenhove, H., Barceló, D., 2010. Trace analysis of antidepressants in environmental waters by molecularly imprinted polymer-based solid-phase extraction followed by ultra-performance liquid chromatography coupled to triple quadrupole mass spectrometry. Anal. Bioanal. Chem. 396, 825-837.

Di Poi, C., Evariste, L., Séguin, A., Mottier, A., Pedelucq, J., Lebel, J. M., Serpentini, A., Budzinski, H., Costil, K., 2014. Sub-chronic exposure to fluoxetine in juvenile oysters (*Crassostrea gigas*): uptake and biological effects. Environ. Sci. Pollut. Res. 23, 5002-5018.

Dworkin, R.H., O'Connor, A.B., Audette, J., Baron, R., Gourlay, G.K., Haanpää, M.L., et al., 2010. Recommendations for the pharmacological management of neuropathic pain: an overview and literature update. In Mayo Clin. Proc. 85, S3-S14. Elsevier.

Faria, M., Navarro, A., Luckenbach, T., Pina, B., Barata, C., 2011. Characterization of the multixenobiotic resistance (MXR) mechanism in embryos and larvae of the zebra mussel (*Dreissena polymorpha*) and studies on its role in tolerance to single and mixture combinations of toxicants. Aquat. Toxicol. 101, 78-87.

Fishbain, D., 2000. Evidence-based data on pain relief with antidepressants. Ann. Med. 32, 305-316.

Fishbain, D.A., Cutler, R., Rosomoff, H.L., Rosomoff, R.S., 2000. Evidence-based data from animal and human experimental studies on pain relief with antidepressants: a structured review. Pain. Med. 1, 310-316.

Fong, P. P., Ford, A.T., 2014. The biological effects of antidepressants on the molluscs and crustaceans: a review. Aquat. Toxicol. 151, 4-13.

Fong, P.P., 1998. Zebra mussel spawning is induced in low concentrations of putative serotonin reuptake inhibitors. Biol. Bull. 194, 143-149.

Fong, P.P., Molnar, N., 2008. Norfluoxetine induces spawning and parturition in estuarine and freshwater bivalves. Bull. Environ. Contam. Toxicol. 81, 535-538.

Ford, A.T., Fong., P.P., 2016. The effects of antidepressants appear to be rapid and at environmentally relevant concentrations. Environ. Toxicol. Chem. 35, 794-798.

Franzellitti, S., Buratti, S., Capolupo, M., Du, B., Haddad, S.P., Chambliss, C.K., Brooks, B.W., Fabbri, E., 2014. An exploratory investigation of various modes of action and potential adverse outcomes of fluoxetine in marine mussels. Aquat. Toxicol. 151, 14-26.

Gonzalez-Rey, M., Bebianno, M.J., 2013. Does selective serotonin reuptake inhibitor (SSRI) fluoxetine affects mussel *Mytilus galloprovincialis*? Environ. Pollut. 173, 200-209.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol. 42, 5807-5813.

Hagger ,J.A., Jones, M.B., Lowe, D., Leonard, D.R.P., Owen, R., Galloway, T.S., 2008. Application of biomarkers for improving risk assessments of chemicals under the Water Framework Directive: a case study. Mar. Pollut. Bull. 56, 1111-1118.

Hazelton, P.D., Du, B., Haddad, S.P., Fritts, A.K., Chambliss, C.K., Brooks, B.W., Bringolf, R.B., 2014. Chronic fluoxetine exposure alters movement and burrowing in adult freshwater mussels. Aquat. Toxicol. 151, 27-35.

Heberer, T., 2002. Tracking persistent pharmaceutical residues from municipal sewage to drinking water. J. Hydrol. 266, 175-189.

Huggett, D.B., Cook, J.C., Ericson, J.F., Williams, R.T., 2003. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. Hum. Ecol. Risk Assess. 9, 1789-1799.

Kirkland, D.J., Hayashi, M., Jacobson-Kram, D., Kasper, P., MacGregor, J.T., Müller, L., 2007. Summary of major conclusions from the 4th IWGT, San Francisco, 9-10 September, 2005. Mutat. Res. 627, 5-9.

Kirsch-Volders, M., Sofuni, T., Aaderma, M., Albertini, S., Eastmond, D., Fenech, M., Ishidate Jr, M., Kirchner, S., Lorge, E., Morita, T., Norppa, H., Surrallés, J., Vanhauwaet, A., Wakata A., 2000. Report from the in vitro micronucleus assay working group. Environ. Mol. Mutagen. 35, 167-172.

Lazzara, R., Blázquez, M., Porte, C., Barata, C., 2012. Low environmental levels of fluoxetine induce spawning and changes in endogenous estradiol levels in the zebra mussel *Dreissena polymorpha*. Aquat. Toxicol. 106, 123-130.

Magni, S., Parolini, M., Binelli, A., 2016. Sublethal effects induced by morphine to the freshwater biological model *Dreissena polymorpha*. Environ. Toxicol. 31, 58-67.

Mecocci, P., Fano, G., Fulle, S., MacGarvey, U., Shinobu, L., Polidori, M.C., Cherubini, A., Vecchiet, J., Senin, U., Flint Beal, M., 1999. Age-dependent increases in oxidative damage to DNA, lipids, and proteins in human skeletal muscle. Free Radic. Biol. Med, 26, 303-308.

Munari, M., Marin, M. G., Matozzo, V., 2014. Effects of the antidepressant fluoxetine on the immune parameters and acetylcholinesterase activity of the clam *Venerupis philippinarum*. Mar. Environ. Res. 94, 32-37.

Navarro, A., Weißbach, S., Faria, M., Barata, C., Piña, B., Luckenbach, T., 2012. Abcb and Abcc transporter homologs are expressed and active in larvae and adults of zebra mussel and induced by chemical stress. Aquat. Toxicol. 122, 144-152.

O'Brien, F.E., Dinan, T.G., Griffin, B.T., Cryan, J.F., 2012. Interactions between antidepressants and P-glycoprotein at the blood–brain barrier: clinical significance of in vitro and in vivo findings. Brit. J. Pharmacol. 165, 289-312.

Ohkawa, H., Ohisi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351-358.

Orbea, A., Ortiz-Zarragoitia, M., Solé, M., Porte, C., Cajaraville, M.P., 2002. Antioxidant enzymes and peroxisome proliferation in relation to contaminant body burdens of PAHs and PCBs in bivalve molluscs, crabs and fish from the Urdaibai and Plentzia estuaries (Bay of Biscay). Aquat. Toxicol. 58, 75-98.

Parolini, M., Binelli, A., 2013. Adverse effects induced by ecgonine methyl ester to the zebra mussel: a comparison with benzoylecgonine. Environ. Pollut. 182, 371-378.

Parolini, M., Binelli, A., 2014. Oxidative and genetic responses induced by Δ-9-tetrahydrocannabinol (Δ-9-THC) to *Dreissena polymorpha*. Sci. Total Environ. 468-469, 68-76.

Parolini, M., Binelli, A., Cogni, D., Provini, A., 2010. Multi-biomarker approach for the evaluation of the cyto genotoxicity of paracetamol on zebra mussel (*Dreissena polymorpha*). Chemosphere 79, 489-498.

Parolini, M., Magni, S., Binelli, A., 2014. Environmental concentrations of 3,4-methylenedioxymethamphetamine (MDMA)-induced cellular stress and modulated antioxidant enzyme activity in the zebra mussel. Environ. Sci. Pollut. Res. 21, 11099-11106.

Parolini, M., Magni, S., Castiglioni, S., Binelli, A., 2016. Amphetamine exposure imbalanced antioxidant activity in the bivalve *Dreissena polymorpha* causing oxidative and genetic damage. Chemosphere 144, 207-213.

Parolini, M., Pedriali, A., Riva, C., Binelli, A., 2013. Sublethal effects caused by the cocaine metabolite benzoylecgonine to the freshwater mussel *Dreissena polymorpha*. Sci. Total Environ. 444, 143-150.

Pavlica, M., Klobucar, G.I.V., Vetma, N., Erben, R., Papes, D., 2000. Detection of micronuclei in haemocytes of zebra mussel and ramshorn snail exposed to pentachlorophenol. Mutat. Res. 465, 145-150.

Reungoat, J., Escher, B.I., Macova, M., Keller, J., 2011. Biofiltration of wastewater treatment plant effluent: effective removal of pharmaceuticals and personal care products and reduction of toxicity. Water. Res. 45, 2751-2762.

Rodieux, F., Piguet, V., Berney, P., Desmeules, J., Besson, M., 2015. Pharmacogenetics and analgesic effects of antidepressants in chronic pain management. Pers. Med. 12, 163-175.

Santos, L.H.M.L.M., Araújo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M.C.B.S.M., 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J. Hazard. Mater. 175, 45-95.

Schultz, M. M., Furlong, E. T., 2008. Trace analysis of antidepressant pharmaceuticals and their select degradates in aquatic matrixes by LC/ESI/MS/MS. Anal. Chem. 80, 1756-1762.

Singh, N.P. , 2000. A simple method for accurate estimation of apoptotic cells. Exp. Cell. Res. 256, 328-337.

Smital, T., Luckenbach, T., Sauerborn, R., Hamdoum, A., Vega, L., Epel, D., 2004. Emerging contaminants-pesticides, PPCPs, microbial degradation products and natural substances as inhibitors of multixenobiotic defense in aquatic organisms. Mutat. Res. 552, 101-117.

Verlecar, X.N., Jena, K.B., Chainy, G.B.N., 2008. Modulation of antioxidant defences in digestive gland of *Perna viridis*, on mercury exposures. Chemosphere 71, 1977-1985.

Vlahogianni, T.H., Valavanidis, A., 2007. Heavy-metal effects on lipid peroxidation and antioxidant defence enzymes in mussels *Mytilus galloprovincialis*. Chem. Ecol. 23, 361-371.

Zuccato, E., Castiglioni, S., Bagnati, R., Chiabrando, C., Grassi, P., Fanelli, R., 2008. Illicit drugs, a novel group of environmental contaminants. Water. Res. 42, 961-968.

Figure Captions:

Table 1: Genotoxic effects (mean ± SEM) of 500 ng/L of FLX, CT and their MIX on *D. polymorpha* hemocytes. Asterisks indicate the significant differences (two-way ANOVA, Fisher LSD post-hoc test: *<0.05, **<0.01), time *versus* time, between treated and control.

Figure 1: Effects of 500 ng/L of FLX, CT and their MIX on the activity (mean ± SEM) of SOD (A), CAT (B), GPx (C) and GST (D) in *D. polymorpha* specimens (n=3; 9 specimens *per* treatment) during 14 exposure days. Asterisks indicate the significant differences (two-way ANOVA, Fisher LSD post-hoc test: *<0.05, **<0.01), time *versus* time, between treated and control.

Figure 2: Effects of 500 ng/L of FLX, CT and their MIX on the efflux activity (mean ± SEM) of P-gp in *D. polymorpha* specimens (n=12) during 14 exposure days. Asterisks indicate the significant differences (two-way ANOVA, Fisher LSD post-hoc test: *<0.05, **<0.01), time *versus* time, between treated and control.

Figure 3: Measure (mean ± SEM) of protein carbonylation (A) and lipid peroxidation (B) levels (n=3; 9 specimens *per* treatment). Asterisks indicate the significant differences (two-way ANOVA, Fisher LSD post-hoc test: *<0.05, **<0.01), time *versus* time, between treated and control.

Figure 4: Toxicity comparison between 500 ng/L of FLX, CT and their MIX in *D. polymorpha* (A); schematic contribution of each considered end-point in the histogram of antidepressant toxicity (B); toxicity comparison between FLX, CT and other psychotropic substances tested on *D. polymorpha* at the same concentrations of 500 ng/L (C; Δ-9-THC, EME, BE, MOR, MDMA and AMPH). For this comparison we considered only common endpoints used for the toxicity evaluation of abovementioned molecules as SOD, CAT, GPx and GST activities, PCC and LPO levels, percentage of DNA in the comet tail and apoptotic and micronucleus frequencies.