Multi-biomarker investigation to assess toxicity induced by two antidepressants on *Dreissena polymorpha*

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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 organisms. Thus, the aim of this study was the evaluation of sub-lethal effects on the freshwater mussel *Dreissena polymorpha* of 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 evaluated the sub-lethal effects in the mussel soft tissues by means of a biomarker suite: 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). The oxidative damage was evaluated by lipid peroxidation (LPO) and protein carbonylation (PCC), while genetic damage was tested on *D. polymorpha* hemocytes by Single Cell Gel Electrophoresis (SCGE) assay, DNA diffusion assay and micronucleus test (MN test). Finally, the functionality of the ABC transporter P-glycoprotein (P-gp) was measured in *D. polymorpha* gills. 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 on bivalves.

Keywords:
Antidepressants; sub-lethal effects; biomarkers; *Dreissena polymorpha*

1 INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) are considered emerging aquatic contaminants, because they are not included in any regulatory framework and their effects on human and aquatic community are largely 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 different groups of antidepressants, as the selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), selective serotonin-norepinephrine reuptake inhibitors (SSNRIs) and monoamine oxidase inhibitors (MAOIs; Fong and Ford, 2014). 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 such 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 ng/L (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. Regarding CT effects on mollusks, some studies reported the induction of foot detachment from the substrates in different species of snails. As showed by Fong and Hoy (2012), two different concentrations of CT caused foot detachment in *Leptoxis carinata* and *Stagnicola elodes* at 405 pg/L and 4.05 µg/L, respectively. Another study confirmed this effect of some antidepressants (CT and FLX included) in other species of snails (Fong and Molnar, 2013). In addition, Minguez and co-workers (2014) reported the cytotoxic and immunomodulatory effects of different antidepressants on hemocytes of *Haliotis tuberculate*, highlighting that CT was the less potent antidepressant in the alteration of immune mechanism. Thus, the aim of this study was the evaluation of sub-lethal effects induced by FLX and CT by means of the measure of biochemical endpoints, oxidative damage and genotoxicity on the zebra mussel *D. polymorpha*, one of the most useful biological models in freshwater ecotoxicology (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) and sub-lethal effects were assessed through a biomarker suite every three days. To assess the biochemical alterations, 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 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 and eventually rank the toxicity of FLX, CT and their MIX, the whole biomarker dataset was 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, during the post-reproductive period, 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 tanks 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 stock solutions (1 mg/L), which were then added in exact volume to exposure tanks to obtain the exposure concentrations of 500 ng/L administered alone and in MIX (500 ng/L FLX + 500 ng/L CT) to bivalves (final methanol concentration: 0.5 µL/L). Before the exposure we evaluated the baseline levels for all considered endpoints on bivalves taken from a single tank. Subsequently, we placed 70 specimens *per* tank (4 L) to perform the exposures (three tanks for each treatment). Exposures were performed in semi-static conditions, feeding bivalves 1 h before the daily renewal of the exposure solutions, for 14 days. We collected bivalves every three days (t=4, 7, 11 and 14 days) from each tank 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, we dissected gills from other 9 bivalves *per* treatment, from which 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, during the first day of exposure (t=0), 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 analyses 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 (2.1 x 150mm, 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 S1 (see supplementary information). The calibration curves, expressed as the 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% FLX and CT respectively).

2.4 *Biochemical biomarkers*

Since the methods and procedures of enzymatic 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 pools of three 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). A higher RhB accumulation within the biopsies indicates lower efflux functionality.

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 pools of three 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 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 endpoints 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 and data elaboration*

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), treatment (control, FLX 500 ng/L, CT 500 ng/L and their MIX) and their interaction 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). To make a toxicity comparison between tested molecules and other psychotropic substances, we calculated the percentage of alteration level (AL) compared to the corresponding control for each biomarker and for considered exposure times (t=4, t=7, t=11 and t=14); 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 biochemical alteration; 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:

238 $BRI=\Sigma(AL\ biomarker_x\ score_{t=4} + \ldots + biomarker_x\ score_{t=14})*biomarker_x\ weighting/\Sigma\ biomarker_x$
239 weighting
240
241 where AL=alteration level; x=considered endpoint; t=time of exposure (days).
242
243 3 RESULTS
244 During the 14 days of exposure we found a comparable and low mortality of bivalves in the control
245 and exposure tanks, showing that the antidepressants did not induce acute toxicity at the tested
246 environmental concentration, nor individually neither in MIX. Furthermore, the percentage of
247 hemocytes viability found in bivalves from the control tanks during the 2 weeks of exposure was
248 always higher than 70%, with a mean (± standard error of the means; SEM) of 80.2 ± 8.4%, as
249 required to perform genotoxicity tests (Kirkland et al., 2007). Mean baseline levels for each
250 considered endpoint, obtained at the beginning of the exposure (t=0 day), were as follows: 13.4 ±
251 1.2 U mg $prot^{-1}$ (SOD), 26.8 ± 1.2 mM min^{-1} mg $prot^{-1}$ (CAT), 11.3 ± 2.8 µM min^{-1} mg $prot^{-1}$
252 (GPx), 90.8 ± 2.1 mM min^{-1} mg $prot^{-1}$ (GST), 3021.2 ± 187.4 fluorescence arbitrary units (P-gp),
253 9.5 ± 1.6 nM g^{-1} $w.w.^{-1}$ (LPO), 5.7 ± 0.9 nM mg^{-1} $prot^{-1}$ (PCC), 2.1 ± 0.2% (DNA in the comet tail),
254 1.03 ± 0.01 (LDR), 0.7 ± 0.1‰ (MN frequency), 0.2 ± 0.1% (apoptotic cell frequency) and 0.3 ±
255 0.2% (necrotic cell frequency).
256
257 3.1 *FLX and CT in the exposure tanks*
258 To verify stability of FLX and CT stock solutions over the whole period of the exposures (14 days),
259 we measured the concentration of both the antidepressants at the moment of the dilution of FLX and
260 CT standard solutions in ultrapure water (t=0) and after 14 days. The concentration of FLX stock
261 solution at the beginning (t=0 day) and the end (t=14 day) of this period of time was 1.01 ± 0.02
262 mg/L and 0.97 ± 0.05 mg/L, respectively, while the concentration of CT stock solution was 0.97 ±
263 0.03 mg/L and 0.85±0.03 mg/L, respectively. In the control tanks the concentrations of FLX and CT
264 were below the detection limits. The FLX and CT concentrations measured in the exposure tanks 1
265 h after the spike of stock solutions were close to the nominal concentrations of 500 ng/L, since we
266 obtained mean values of 484.62 ± 1.17 ng/L for FLX and 595.95 ± 0.67 ng/L for CT. In the MIX
267 the concentrations of FLX and CT were 457.15 ± 2.66 ng/L and 575.81 ± 1.77 ng/L, respectively.
268 Since the method of quantification has a coefficient of variation of ± 20%, these data confirm the
269 reliability of our exposure conditions.
270
271

3.2 *Toxicity of FLX*

SOD activity (Fig. 1A) showed a particular trend characterized by a significant ($p<0.01$) decrease in early exposure times (t=4 and 7 days) compared to corresponding control, followed by a clear raise at the end of exposure, where a significant ($p<0.05$) difference with control level was measured. We also recorded a significant effect of time ($F_{3,63}=44.40$; $p<0.01$) and interaction time to treatment ($F_{3,63}=8.00$; $p<0.01$). CAT activity (Fig. 1B) highlighted a significant increase ($p<0.01$) at the end of exposure up to about 27% compared to control. Significant effects of treatment ($F_{1,64}=20.84$; $p<0.01$), time ($F_{3,64}=43.95$; $p<0.01$) and their interaction ($F_{3,64}=5.34$; $p<0.01$) were found. The activity of GPx (Fig. 1C) showed a significant effect of treatment ($F_{1,62}=19.80$; $p<0.01$), time ($F_{3,62}=32.00$; $p<0.01$) and their interaction ($F_{3,62}=11.51$; $p<0.01$), with a significant ($p<0.01$) increase only at the end of experiment (t=14 day). Although a significant effect of FLX ($F_{1,63}=5.00$; $p<0.05$) and time ($F_{3,63}=11.27$; $p<0.01$) on GST activity was noticed, we did not obtained a significant effect of their interaction (Fig. 1D). A significant inhibition of RhB efflux comparing to control by the inhibitor verapamil (-36%) was observed, confirming the P-gp functionality in our experimental model. A significant effect of treatment ($F_{1,88}=4.58$; $p<0.05$), time ($F_{3,88}=3.08$; $p<0.05$) and their interaction ($F_{3,88}=4.44$; $p<0.01$) was observed in the modulation of P-gp efflux functionality, which resulted significantly inhibited (-38%; $p<0.01$) 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 *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 ($F_{1,64}=57.80$; $p<0.01$), time ($F_{3,64}=86.11$; $p<0.01$), and their interaction ($F_{3,64}=33.14$; $p<0.01$), 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. A significant effect of time ($F_{3,64}=58.71$; $p<0.01$) and interaction time to treatment ($F_{3,64}=23.98$; $p<0.01$) on CAT activity was observed (Fig. 1B), highlighting 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 ($F_{3,61}=23.61$; $p<0.01$) and time *per* treatment interaction ($F_{3,61}=76.02$; $p<0.01$), 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 ($F_{1,64}=21.87$; $p<0.01$), time ($F_{3,64}=33.44$; $p<0.01$) and their interaction ($F_{3,64}=8.61$; $p<0.01$) 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 ($F_{1,88}=11.50$; $p<0.01$), time ($F_{3,88}=3.59$; $p<0.05$) and their interaction ($F_{3,88}=3.88$; $p<0.05$), with a significant reduction of pump functionality (-43%; $p<0.01$) after 4 days compared to control. Furthermore, despite no significant increase of lipid peroxidation was found (Fig. 3B), a significant effect of time ($F_{3,64}=8.50$; $p<0.01$) and interaction time to treatment ($F_{3,64}=8.79$; $p<0.01$) 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 increase 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 increase ($p<0.01$) from the seventh day of exposure, with a significant effect of treatment ($F_{1,63}=58.71$; $p<0.01$), time ($F_{3,63}=77.82$; $p<0.01$) and their interaction ($F_{3,63}=40.29$; $p<0.01$). A significant effect of treatment ($F_{1,64}=24.74$; $p<0.01$), time ($F_{3,64}=132.52$; $p<0.01$) and interaction time to treatment ($F_{3,64}=31.76$; $p<0.01$) 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 compared to control. A significant effect of time ($F_{3,63}=30.73$; $p<0.01$) and interaction time to treatment ($F_{3,63}=100.58$; $p<0.01$) was observed on GPx (Fig. 1C), disclosing a significant inhibition ($p<0.01$) at 4 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 time ($F_{3,63}=22.79$; $p<0.01$) and interaction time to treatment ($F_{3,63}=7.06$; $p<0.01$) was observed for GST. As in bivalves exposed to CT, a significant effect of treatment ($F_{1,87}=4.24$; $p<0.05$), time ($F_{3,87}=5.15$; $p<0.01$) and their interaction ($F_{3,87}=3.19$; $p<0.05$) was observed for P-gp efflux activity; we registered a significant reduction in its activity (-30%; $p<0.01$) compared to control after 4 days of exposure (Fig. 2). Whilst LPO did not show significant differences between treated and control, except at 7 days (Fig. 3B), a significant effect of time ($F_{3,64}=11.10$; $p<0.01$) and treatment *per* time interaction ($F_{3,64}=5.69$; $p<0.01$) on PCC was noted, reaching a 24% significant increase ($p<0.01$) at t=11 days (Fig. 3A). No significant genotoxic effect was found in zebra mussel MIX-treated specimens compared to controls, with the exception of a single significant increase of MN frequency ($p<0.05$) at 7 exposure days (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 biological processes conserved in many organism, often target of pharmaceuticals, could increase the potential toxicity associated with the environmental presence of these 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 from the present study revealed a significant depression of cellular response already at 4 days of exposure, mainly in mussels exposed to CT and MIX, as pointed out by the significant inhibition of the activity of SOD, CAT, GPx, GST, as well as in P-gp functionality, compared to background levels (Figs. 1 and 2). Considering that neurotransmitters, such as 5-HT and noradrenaline, are implicated both in depression and chronic pain, antidepressants are also used in medicine as analgesics (Rodieux et al., 2015); for this reason, the early inhibition of cellular response could be associated to the analgesic effect of considered molecules. In fact, although the analgesic potential have been especially observed for SSNRIs and TCAs (Fishbain, 2000; Fishbain et al., 2000; Dworkin et al., 2010), it cannot be excluded that some SSRIs may act in the same way also in *non*-target organisms. Therefore, this pharmacological effect, in *non*-target species, can be consider a potential toxic effect, which is manifested already in early days of exposure. Protracting the exposure, we observed a raise of antioxidant activity for all treatments (Fig. 1), as already reported in previous studies aimed at assessing the effects of FLX on *Mytilus galloprovincialis* (Gonzalez-Rey and Bebianno, 2013; Franzellitti et al., 2014) and *Crassostrea gigas* (Di Poi et al., 2014). In spite of increasing trends in SOD and CAT activities after FLX exposure, a clear *non*-monotonic response was observed for all enzyme activities in bivalves exposed to CT and MIX (Fig. 1A, B, C, D). The significant inhibition of CAT at the end of treatment in the bivalves exposed to CT suggests a substrate inhibition phenomenon caused by an excess of H_2O_2 (Vlahogianni and Valavanidis, 2007). Other bell shaped trends (Fig. 1A, B, C, D) could then confirm the overproduction of reactive oxygen species (ROS) and it should not be excluded that, prolonging the exposure time, a significant inhibition in treated mussels compared to relative controls could be achieved. In addition, the significant ($p<0.01$) increase of protein carbonylation after 11 days noticed only for bivalves exposed to CT and MIX, when antioxidant enzymes showed bell-shaped trends (Fig. 3A), could be induced by the $\bullet O_2^-$ and H_2O_2 overproduction. In fact, these pro-oxidant molecules, as well as the hydroxyl radical (•OH) formed in the Fenton and Haber-Weiss reaction, are able to cause an

374 elevated protein oxidation, as suggested by Verlecar and co-workers (2008). The lack of significant
375 protein carbonylation in mussels exposed to FLX could be associated to the complete lack of *non*-
376 monotonic response in the antioxidant enzyme activities (Fig. 1A, B, C, D). Similar results were
377 also obtained by Di Poi and co-workers (2014) in the mollusk *Crassostrea gigas* exposed to 1, 10
378 and 100 ng/L of FLX that did not produce significant oxidative damage probably because of the
379 efficacy of the cellular antioxidant mechanisms. At the same time, in all treatment, we did not
380 observed a significant increase in LPO levels. In this context, the P-gp is one of the most relevant
381 ABC transporters, involved in the defense mechanism towards a wide variety of anthropogenic
382 contaminants (Della Torre et al., 2014). The P-gp activity and its role in the tolerance to
383 environmental pollution has been well characterized in *D. polymorpha* (Faria et al., 2011; Navarro
384 et al., 2012). Our results showed a significant reduction of the efflux functionality, similar to that
385 produced by the inhibitor verapamil, by CT and MIX after 4 days and by FLX after 11 days of
386 exposure probably due to the analgesic effects mentioned above. Several pollutants including
387 PPCPs, pesticides and hydrocarbons are known to suppress the activity of P-gp, through a chemo-
388 sensitization mechanism (Smital et al., 2004). Such effect has severe ecotoxicological consequences
389 as it might reduce the detoxifying capacity of the organism, thereby increasing the accumulation
390 and toxicity of other pollutants. Either FLX and CT are well known substrates of P-gp *in vitro*,
391 while their interaction *in vivo* is still controversial (O'Brien et al., 2012). The observed inhibitory
392 effect suggested a potential chemo-sensitizing effect for FLX, CT and their MIX to *D. polymorpha*,
393 which might affect the susceptibility of bivalves towards other toxic chemicals. The low effect of
394 FLX, CT and MIX on oxidative stress and cellular biomarkers was also confirmed by genotoxicity
395 assays, as shown by the lack of DNA damage to zebra mussel hemocytes (Table 1). These results
396 could be probably due to the inability of these chemicals to directly induce DNA injuries and/or to
397 the slight oxidative stress situation experienced by zebra mussels, which was efficiently
398 counteracted by the activation of antioxidant defense mechanism. Since the adverse effects
399 observed in bivalves exposed to MIX were similar and showed overlapping trends to those from CT
400 exposure, we could suppose that CT was the main responsible of MIX toxicity. However, the wide
401 variability in biomarker responses prevents to accurately support this suggestion. For this reason,
402 each biomarker response for each molecule was integrated into the BRI, to make a toxicity
403 comparison of tested antidepressants. The BRI results (Fig. 4A) suggested that FLX, CT and MIX
404 had the same toxicity on *D. polymorpha*. Considering the contribution of each single biomarker in
405 the toxicity BRI values (Fig. 4B), it was possible to point out that the toxicity of FLX is exactly
406 divided between genotoxicity and biochemical alterations/oxidative damage (Fig. 4B). In contrast,
407 the toxicity induced by CT and MIX was mainly associated to biochemical alterations and oxidative

damage (for 60% of the total effect), confirming the main role played by CT in the MIX toxicity, as previously suggested. 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 BRI approach (Fig. 4C) highlighted that FLX (BRI=5.43) and CT (BRI=5.79) were, with AMPH, the molecules showing the lowest toxicity towards the zebra mussel, while Δ-9-THC and BE were the most toxic ones. However, it is important to consider that the evaluation of other endpoints could modify this toxicity ranking. In fact, some evidences showed that FLX negatively affected endocrine (Fong and Ford, 2014) and nervous systems (Munari et al., 2014), as well as control and storage of energy in *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 to endpoints described in the present study.

5 CONCLUSIONS

This study attempted to investigate the potential sub-lethal effects 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 alteration of the bivalve oxidative status. Indeed, the integration of single biomarker results into the BRI showed how these molecules can be placed at the end of a decreasing toxicity scale in comparison with other psychotropic substances. 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. Despite the results obtained in this research, 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 an exhaustive toxicity data not subject to a reductionist approach of single or few endpoints.

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582

583 Figure Captions:

584

585 Figure 1: Effects of 500 ng/L of FLX, CT and their MIX on the activity (mean ± SEM) of SOD (A),
586 CAT (B), GPx (C) and GST (D) in *D. polymorpha* soft tissues (n=3 pools of three specimens *per*
587 treatment) during 14 exposure days. Asterisks indicate the significant differences (two-way
588 ANOVA, Fisher LSD post-hoc test: *<0.05, **<0.01), time *versus* time, between treated and
589 control. The red lines indicate the baseline level of each biomarker calculated as the mean of values
590 measured at t=0 (see results).

591

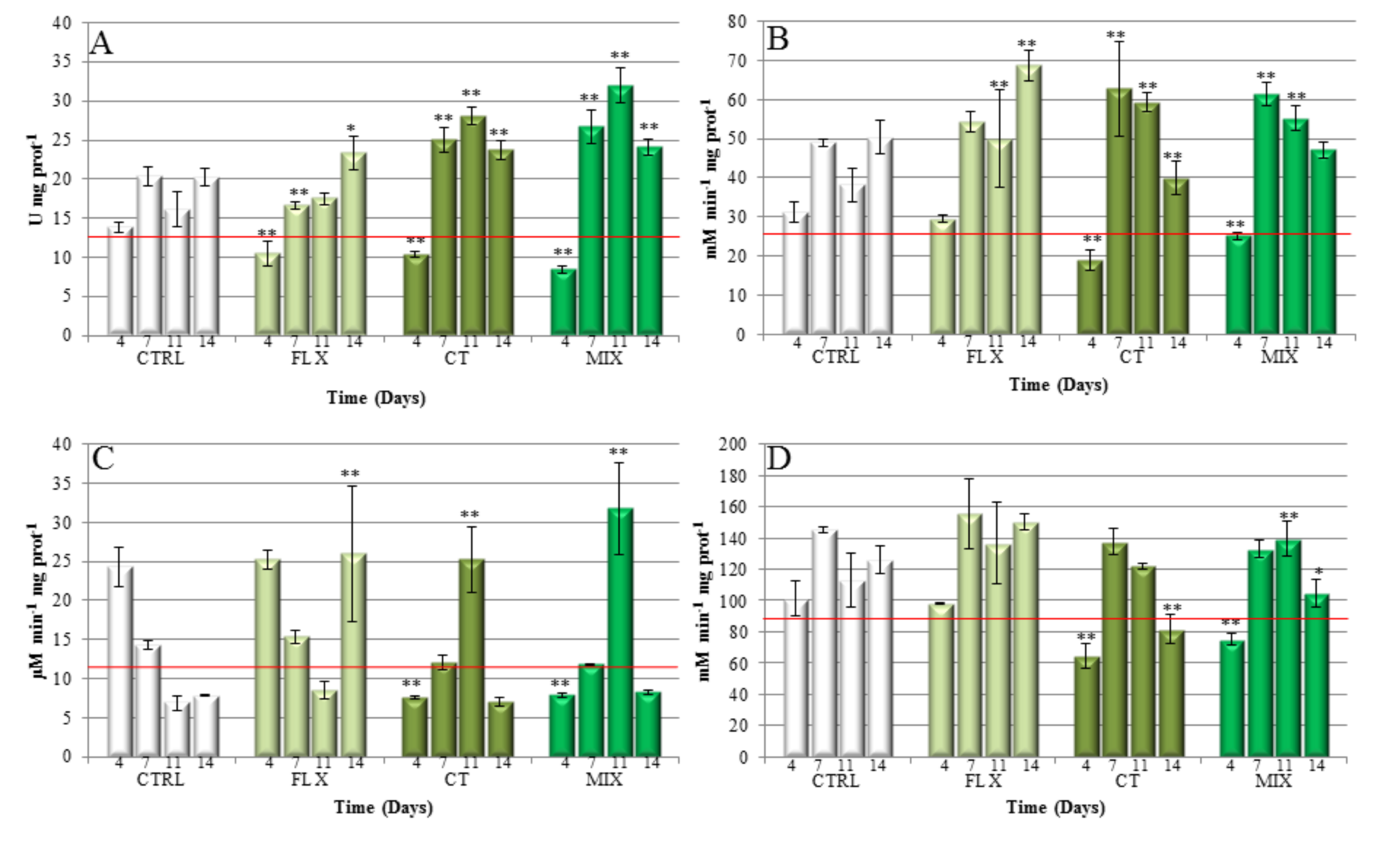
592 Figure 2: Effects of 500 ng/L of FLX, CT and their MIX on the efflux activity (mean ± SEM) of P-
593 gp in *D. polymorpha* gills (n=12 gill biopsies *per* treatment) during 14 exposure days. RhB retained
594 into gill biopsies is expressed as arbitrary fluorescence units; higher RhB is indicative of reduced
595 efflux activity. Asterisks indicate the significant differences (two-way ANOVA, Fisher LSD post-
596 hoc test: *<0.05, **<0.01), time *versus* time, between treated and control. The red line indicates the
597 baseline level of P-gp calculated as the mean of values measured at t=0 (see results).

598

599 Figure 3: Measure (mean ± SEM) of protein carbonylation (A) and lipid peroxidation (B) levels in
600 *D. polymorpha* soft tissues (n=3 pools of three specimens per treatment). Asterisks indicate the
601 significant differences (two-way ANOVA, Fisher LSD post-hoc test: *<0.05, **<0.01), time *versus*
602 time, between treated and control. The red lines indicate the baseline level of each biomarker
603 calculated as the mean of values measured at t=0 (see results).

604

605 Figure 4: Toxicity comparison, through integration of considered endpoints into the BRI, between
606 FLX, CT and their MIX in *D. polymorpha* (A); schematic contribution of each considered endpoint
607 in the histogram of antidepressant toxicity (B); toxicity comparison between FLX, CT and other
608 psychotropic substances as Δ-9-tetrahydrocannabinol (Δ-9-THC), ecgonine methyl ester (EME),
609 benzoylecgonine (BE), morphine (MOR), 3,4-methylenedioxymethamphetamine (MDMA) and
610 amphetamine (AMPH) tested on *D. polymorpha* at the same concentrations of 500 ng/L (C). For
611 this comparison we considered only common endpoints used for the toxicity evaluation of
612 abovementioned molecules as SOD, CAT, GPx and GST activities, PCC and LPO levels,
613 percentage of DNA in the comet tail and apoptotic and MN frequencies.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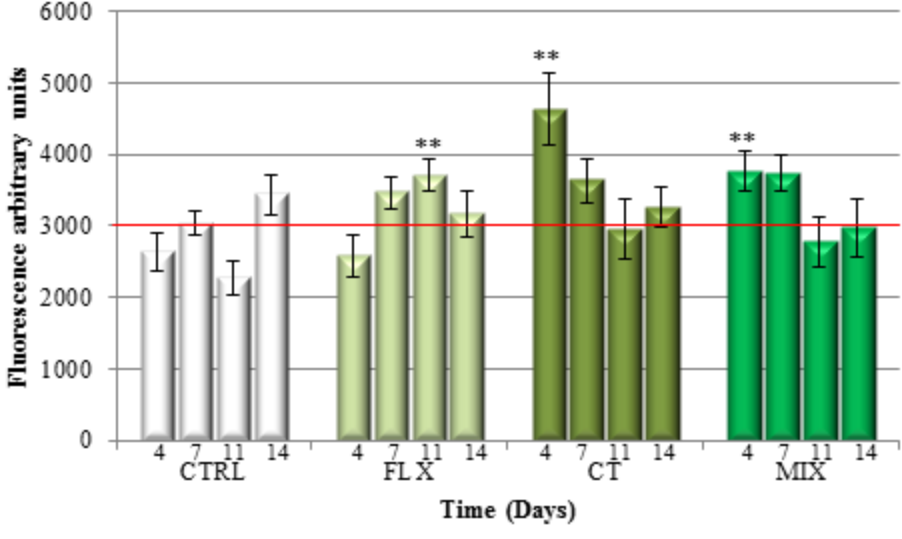
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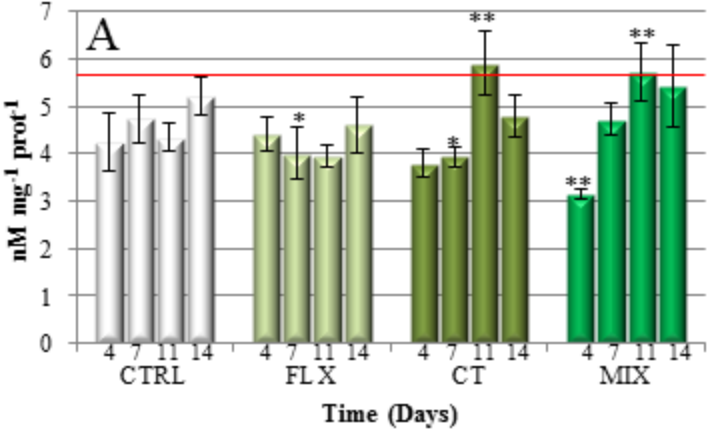
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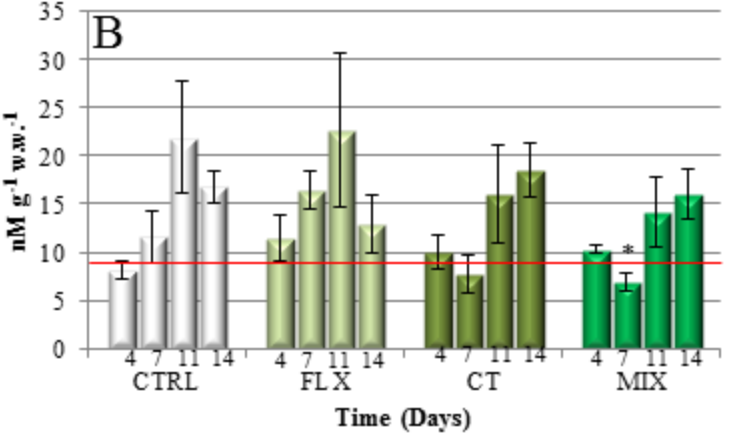
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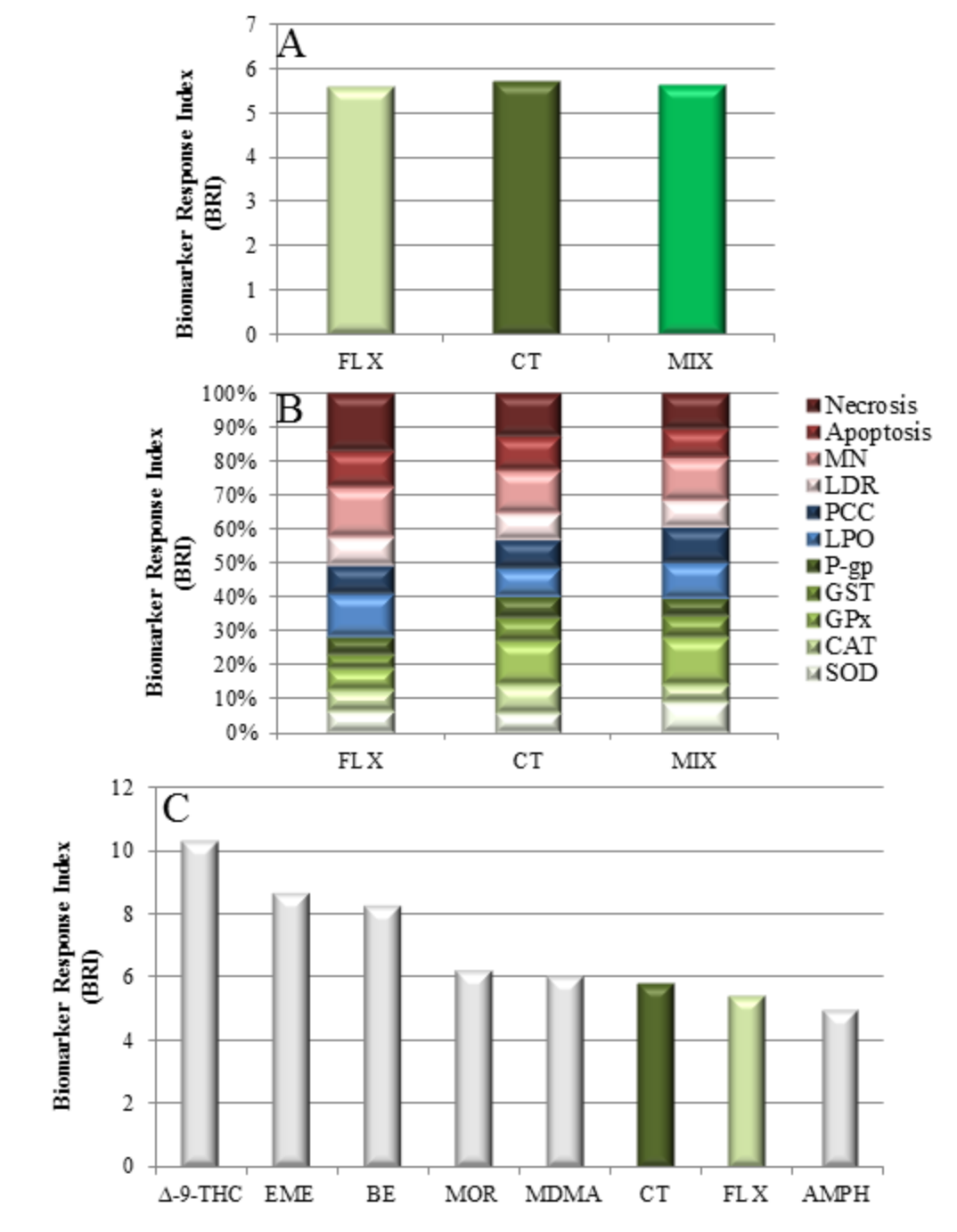
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70%
80%
90%
100%
FLX
CT
MIX
Necrosis
Apoptosis
MN
LDR
PCC
LPO
P-gp
GST
GPx
CAT
SOD
C
Biomarker Response Index (BRI)
0
2
4
6
8
10
12
Δ-9-THC
EME
BE
MOR
MDMA
CT
FLX
AMPH

Time (Days)	CTRL	FLX	CT	MIX
% Necrosis				
4	0.70 ± 0.34	1.60 ± 0.45	0.90 ± 0.27	1.40 ± 0.19
7	1.40 ± 0.22	** 3.20 ± 0.70	1.80 ± 0.31	1.40 ± 0.19
11	2.00 ± 0.68	1.40 ± 0.22	* 0.90 ± 0.25	1.40 ± 0.29
14	0.73 ± 0.34	0.27 ± 0.12	0.87 ± 0.27	0.53 ± 0.08
% Apoptosis				
4	3.30 ± 0.80	** 6.10 ± 0.95	1.70 ± 0.67	3.10 ± 0.70
7	4.90 ± 0.36	3.50 ± 0.72	4.60 ± 1.37	4.10 ± 0.79
11	4.27 ± 0.74	4.10 ± 0.57	3.50 ± 1.12	3.50 ± 1.08
14	1.67 ± 0.45	0.33 ± 0.18	2.27 ± 0.71	1.40 ± 0.43
‰ Micronuclei (MN)				
4	0.63 ± 0.41	0.63 ± 0.41	0.31±0.31	0.63 ± 0.41
7	0.31 ± 0.31	0.31 ± 0.31	0.30±0.30	* 1.25 ± 0.67
11	0.94 ± 0.46	3.13 ± 1.48	2.19±0.88	0.31 ± 0.31
14	0.90 ± 0.50	1.25 ± 0.70	0.30 ±0.30	0.90 ± 0.70
Length and comet head diameter (LDR)				
4	1.04 ± 0.00	1.06 ± 0.01	1.05 ± 0.00	1.07 ± 0.01
7	1.06 ± 0.01	1.05 ± 0.01	1.06 ± 0.01	1.05 ± 0.01
11	1.05 ± 0.00	* 1.07 ± 0.02	1.06 ± 0.01	1.07 ± 0.02
14	1.04 ± 0.00	* 1.07 ± 0.02	1.06 ± 0.01	* 1.07 ± 0.01
% DNA in comet tail				
4	3.16 ± 0.71	3.34 ± 0.81	2.52 ± 0.69	3.56 ± 0.63
7	2.94 ± 0.34	3.08 ± 1.01	3.18 ± 1.04	3.08 ± 0.54
11	1.95 ± 0.39	2.05 ± 1.08	1.51 ± 0.59	1.68 ± 1.09
14	0.89 ± 0.42	1.53 ± 1.07	1.14 ± 0.70	1.51 ± 0.93

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.