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

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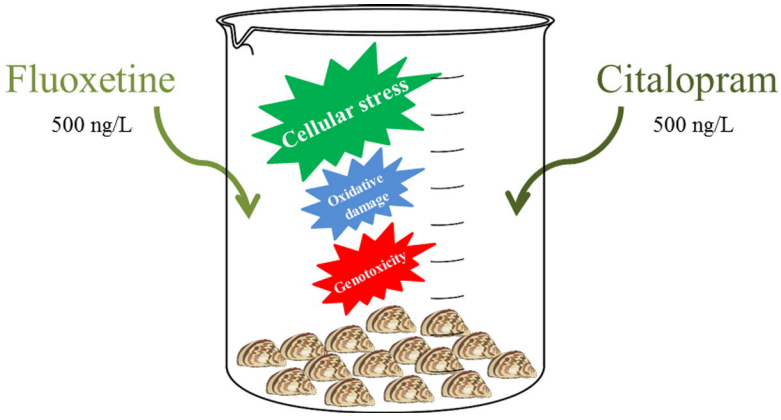
## HIGHLIGHTS

- 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.

## GRAPHICAL ABSTRACT

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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

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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.



## 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 et al. (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. 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

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