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Abstract

Fluoxetine (FLX) the active pharmaceutical ingredient (API) in Prozac(®) is a widely prescribed psychoactive drug which ubiquitous occurrence in the aquatic environment is associated to a poor removal rate in waste-water treatment plant (WWTP) systems. This API acts as a selective serotonin reuptake inhibitor (SSRI) frequently reported to cause disrupting effects in non-target species. The objective of this study includes a multibiomarker response evaluation on mussel Mytilus galloprovincialis during two weeks exposure to 75 ng L(-1) FLX assessing antioxidant enzymes activities--superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST); lipid peroxidation (LPO), acetylcholinesterase (AChE) neurotoxic response and endocrine disruption through alkali-labile phosphates (ALP) indirect measurement of vitellogenin-like proteins. Results show transient tissue-specific enzymatic responses and damage affecting mostly mussel gills. However, the clear ALP levels inhibition throughout time in both sex-differentiated gonads gives evidence to FLX reinforced action as an endocrine disruptor rather than an oxidative or neurotoxic inducer.

1. Introduction

Ecotoxicological risks associated to the ubiquitous occurrence of active pharmaceutical ingredients (APIs) in aquatic ecosystems are far from known. As the detection technology improves, a larger variety of APIs are being detected in the aquatic environment (see reviews: Calisto and Esteves, 2009; Kümmerer, 2009; Li and Randak, 2009; Alonso et al., 2010; Pal et al., 2010; Kümmerer, 2010; Santos et al., 2010; Brausch and Rand, 2011). Waste water treatment plants (WWTPs) are still ill-equipped for the constant APIs discharge load removal, consequently these bioactive compounds end up entering surface waters (rivers, estuaries and lakes) particularly via household and hospital treated effluents, and from there often recycled to drinking water (Ternes, 2001; Ternes et al., 2002; Stackelberg et al., 2004; Jones et al., 2005; Gibs et al., 2007; Kim et al., 2007; Daughton, 2010) posing potential risks to non-target aquatic life (Fong, 2001; Brooks et al., 2005; Fent et al., 2006).

Fluoxetine (FLX) present in antidepressant Prozac® the most widely prescribed psychoactive drug in the market and like others (e.g. citalopram, fluvoxamine, paroxetine, and sertraline) act as selective serotonin reuptake inhibitor (SSRI) in the treatment of depression, and other mood disorders by increasing the serotonin (5-hydroxytrypamine – 5-HT) levels in neuron synaptic space (Brosen, 1993; De Vane, 1999; Hiemke and Härtter, 2000; Fent et al., 2006). Even though, FLX is excreted via urine approximately 10–30% as unchanged parent compound or metabolized to nor-fluoxetine (De Vane, 1999; Hiemke and Härtter, 2000; Fong and Molnar, 2008) is resilient to hydrolysis, photolysis and microbial degradation processes (Kwon and Armbrust, 2006) occurring in the aquatic environment at ng L^{-1} (Table 1). Since SSRIs alter neurotransmitter 5-HT regulation, which has been associated to the modulation of important functions in hormonal and neuronal mechanisms in both vertebrates and invertebrates (Fong, 2001; Fent et al., 2006; Stanley et al., 2007; Painter et al., 2009; Styrishave et al., 2011) most peer reviews on FLX exposure ecotoxicological effects focus on acute FLX toxicity and/or physiological, behavioral (mobility, feeding habits and aggression) and reproductive fitness alterations (Table 2). Nevertheless, FLX has also been related to affect antioxidant system in mice (Djordjevic et al., 2011). Oxidative stress is characterized by the imbalance when xenobiotic-mediated enhancement of reactive oxygen species (ROS) (e.g. superoxide anion ($OH^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals) exceed exposed aerobic organism's antioxidant defense mechanisms. One of these mechanisms involves the counteracting response of antioxidant enzymes activities such as, superoxide

Table 1
Concentrations of fluoxetine in the aquatic environment.

Country	Concentration (ng L^{-1})	Environment	Reference
Canada	46	Surface water	Metcalfe et al., 2003
	99	WWTP effluent	
	509	WWTP effluent	Chen et al., 2006
Croatia	66	Surface water	Gros et al., 2006
South Korea	2–7	River water and creek water	Yoon et al., 2010
	2	WWTP effluent	Trenholm et al., 2006; Kim et al., 2007
Spain	18–66	River water	Fernández et al., 2010
	100	River water	Gros and Ginebreda, 2010
	8–44	WWTP downstream	Alonso et al., 2010
	19–299	WWTP effluent	Martínez et al., 2007
	70	WWTP effluent	Gros et al., 2006
Sweden	225	WWTP effluent	Zorita et al., 2007
USA	5	Reclaimed water facility effluent	Kinney et al., 2006
	12–20	Stream	Schultz and Furlong, 2008
	2.6	Surface water	Vanderford and Snyder, 2006
	12	Surface water	Kolpin et al., 2002
	111	Surface water	Bringolf et al., 2010
	14	Water	Stackelberg et al., 2007
	17–25	WWTP effluent	Vanderford and Snyder, 2006
	18	WWTP effluent	Stackelberg et al., 2004
	21	WWTP effluent	Glassmeyer et al., 2005
	58	WWTP effluent	Schultz and Furlong, 2008
	540	WWTP effluent	Weston et al., 2003
	560	WWTP effluent	Benotti and Brownawell, 2007

dismutase (SOD) and catalase (CAT) and glutathiones (peroxidase − GPx and reductase − GR) (Livingstone, 2001; Regoli et al., 2002a,b; Valavanidis et al., 2006). Additionally, Phase II glutathione S-transferase enzyme also enables detoxification, acting as a catalyst in conjugation reactions between glutathione with xenobiotic compounds electrophilic centers (Regoli and Principato, 1995). When the antioxidant system response is compromised by an ROS excess, lipid peroxidation (LPO) occurs, resulting in the damage of phospholipids membrane (Valavanidis et al., 2006). The fluctuation of antioxidant enzymes parallels to lipid peroxidation generation due to contaminant exposure have been used successfully as oxidative stress and damage biomarkers in mussel species (Regoli and Principato, 1995; Regoli et al., 2002a; Santovito et al., 2005; Bebianno et al., 2005).

To our knowledge, this is the first study focused on the potential antioxidant alteration status of an environmental realistic concentration of FLX (75 ng L^{-1}) exposure in mussels *M. galloprovincialis* through the assessment of antioxidant enzyme activities: SOD, CAT; phase II GST activity, and LPO in mussels' gills and digestive gland. In parallel, SSRI FLX potential to cause neurotoxic effects response was tested by assessing the activity of an essential neurotransmission modulator, enzyme acetylcholinesterase (AChE) in mussel gills. AChE activity has been reported to be inhibited in the presence of several organic contaminants (such as pesticides, detergents and pharmaceuticals) (Almeida et al., 2010; Solé et al., 2010). Finally, alkali-labile phosphate (ALP) method was applied on sex-differentiated mussel gonads to assess FLX as an endocrine disruption inducer, since ALP levels are positively correlated with those from vitellogenin-like proteins which are naturally synthesized in females and inactive in males (Blaise et al., 1999; Gagné et al., 2002; Matozzo et al., 2008).

2. Materials and methods

2.1. Chemicals

R-(-) fluoxetine hydrochloride (F1678, >98%, CAS: 114247-09-5); 1.1.3.3. tetramethoxypropane (MDA) (108383, CAS: 102-52-3); 1-methyl-2-phenylindole (99%, CAS: 3558-24-5); 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (D8130, ≥98% TLC, CAS: 69-78-3); acetyl thiocholine iodide (ATC) (A5751, ≥98% TLC, CAS: 1866-15-5); bovine albumin serum (BSA) (A9418, >98%, CAS: 9048-46-8); butylated hydroxytoluene (BHT) (B1378, ≥99.0% GC, CAS: 128-37-0); cytochrome c from equine heart (C7752, >95%, CAS: 9007-43-6); diethylenetriaminepentaacetic acid dianhydride (DTPA) (D6148, CAS: 23911-26-4); ethylenediaminetetraacetic acid (EDTA) (ED, ≥99%, CAS: 60-00-4); Fiske and Subbarow Reducer (F5428); glutathione reductase (G3664, CAS: 9001-48-3); HEPES (H3375, >99.5%, CAS: 7365-43-9); hydrogen peroxide solution (H1009, 30% w/w, CAS: 7722-84-1); hypoxanthine (H9377, >99%, CAS: 68-94-0); L-glutathione oxidized (GSSG) (G4501, >98%, CAS: 27025-41-8); L-glutathione reduced (GSH) (G4251, >98%, CAS: 70-18-8); methanesulfonic acid (>99.5%, CAS: 75-75-2); triton x-100 (X6878, CAS: 9002-93-1) xanthine oxidase from bovine milk (X1875, CAS: 9002-17-9); β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADPH) (N8129, ≥97%, CAS: 606-68-8) were purchased from Sigma–Aldrich (Germany). Protein-assay dye reagent concentrate (phosphoric acid CAS: 7664-38-2 + methanol CAS: 67-56-1) was obtained from Bio-Rad Laboratories, Inc. (USA). 1,4-dithiothreitol (DTT) (≥99%, CAS: 3483-12-3); acetonitrile (99.8%, CAS 75-05-8); methanol (99.9%, CAS 67-56-1); natriumazide (sodium azide) (106688, ≥99%, CAS: 26628-22-8); potassium chloride (KCl) (104936, 99.5%, CAS: 7447-40-7); trichloroacetic acid (TCA) (100807, CAS: 76-03-9); TRIS-(hydroxymethyl)-aminomethane (≥99%, CAS: 77-86-1) were acquired from Merck (Germany). 1-chloro-2,4,-dinitrobenzene (CDNB) (24440, ≥98.0% GC, CAS: 97-00-7); molybdate reagent solution (puriss p.a.) and potassium dihydrogen phosphate (60218, 99.5%, CAS: 7778-77-0) acquired from Fluka. D(+) saccharose extra pure (16104, 99%, CAS: 57-50-1); sodium chloride (NaCl) (puriss p.a., CAS: 7647-14-5); sodium hydroxide (NaOH) (purified, CAS: 1310-73-2) obtained from Riedel-de-Haën (Germany).

2.2. Fluoxetine exposure assay

Mussels *M. galloprovincialis* (n = 245, average shell length size: 67 ± 2 mm, width: 37 ± 1 mm) were collected in 2010 from Ria Formosa Lagoon in Portugal. These specimens were transported alive to the laboratory, subjected to shell cleaning and finally placed in separate aquaria (n = 35, 1 mussel L^{-1}) concerning control and exposure treatment to 75 ng L^{-1} of FLX. Previously to the 75 ng L^{-1} of FLX exposure all mussels were kept for 7 days acclimatizing in aerated natural seawater. The aquaria were kept at constant temperature (18.6 °C ± 1), salinity (33 ± 0.4), pH (8.1 ± 0.2) and oxygen saturation (>98% ± 2). Mussels were not fed until the end of the experiment. Water was changed and FLX concentration was re-established at every 48 h.

At each set up time (0, 3, 7, and 15 days), mussels (n = 20) were removed from control and exposure aquaria, individual shell biometric data was measured (length, width) and dissection performed, separating gills, digestive gland and gonads. Each tissue sample was immediately frozen in liquid nitrogen and stored individually at −80 °C prior to analysis. For condition index (CI) estimation, 15 mussels of each aquarium, where individual weighted regarding the ratio (Eq. (1)):

$$\mathrm{CI}\ (\%) = \left[\text{whole soft tissue (w.w)} \cdot \text{whole body tissue with shell (w.w)}^{-1}\right] \cdot 100 \quad (1)$$

2.3. Tissue preparation for antioxidant enzyme activities analysis

Antioxidant enzyme analysis was performed using previously dissected gills (n = 5) and digestive glands (n = 5) separately. Each tissue sample was homogenized on ice individually with 20 mM TRIS buffer (containing 1 mM of EDTA + 0.5 M of saccharose + 0.15 M of KCl + 1 mM of DTT) at pH 7.6. Homogenates were centrifuged for 15 min at 500 × g at 4 °C and resulting supernatants recentrifuged for 45 min at 12,000 × g at 4 °C. After cytosolic fraction volume measurement, Sephadex® G-25 gel columns were applied to further purify the sample removing low molecular weight proteins. Prior to the purification a 100 μl aliquot was collected for total protein quantification using bovine serum albumin (BSA) as a standard according to Bradford's method (Bradford, 1976).

Purified aliquots of gills and digestive glands were individually analyzed in triplicate for the quantification of several antioxidant enzymes by spectrophotometric analysis applying the following methodology. In order to determine SOD activity in mussel tissues, 100 μl of each purified aliquot was evaluated measuring the cytochrome c absorbance reduction by 50% at 550 nm wavelength generated by xanthine oxidase/hypoxanthine system according to McCord and Fridovich (1969) method. SOD activity is expressed by arbitrary units (U) per minute of mg^{-1} of total protein. For CAT activity assessment 100 μl of each purified tissue aliquot was used to measure the absorbance decrease related to hydrogen peroxide (H_2O_2) consumption at 240 nm (Greenwald, 1985) and expressed as μ moles mg^{-1} of total protein concentration min^{-1}. GST activity analysis was performed quantifying the reaction of 50 μl of each purified sample with CDNB at 340 nm, following an adaptation of Habig et al. (1974) method and expressed as μmol of resulting CDNB conjugate formed mg^{-1} of total protein min^{-1}.

Table 2
Fluoxetine effects in aquatic mollusks and fishes.

Species name	Exposure concentration	Exposure time	Test	Biological end-points	Reference
Mytilus edulis (common mussel)	0.15 mg L^{-1}		Reproduction	• Induction of spawning	Hazardous Substances Databank (HSDB), 2005
Sphaerium striatum (fingernail clam)	5 μM FLX + 5 − 500 μM 5-HT	3 h	Reproduction	• Induction of parturition	Fong et al., 1998
Dreissena polymorpha (zebra mussel)	5×10^{-7} from 5×10^{-4} M	1 h	Reproduction	• Induction of spawning in males	Fong, 1998
	5×10^{-6} from 5×10^{-5} M	2 h		• Induction of spawning in females	
	>100 nM	4 h		• Induction of spawning and parturition	Fong and Molnar, 2008
	20−200 ng L^{-1}	6 d		• Induction of spawning • Dose dependent decrease oocytes (40−70%) and spermatozoan (21−25%)	Lazzara et al., 2012
Elliptio complanata (freshwater mussel)	300 μg L^{-1}	96 h	Reproduction	• Induction of parturition of nonviable larvae from female	Bringolf et al., 2010
	3000 μg L^{-1}	48 h		• Induction of spermatozeugmata release	
	Caged freshwater mussels at stream sites near a municipal WWTP		Bioaccumulation	• 79.1 ng g^{-1} ww (bioaccumulation)	
	Injection 2.5 μg		Metabolism	• Increase of 5-HT response factor	Gagné and Blaise, 2003
Anodonta cygnea (freshwater mussel)	1×10^{-6}M + presence of light	24 h	Reproduction	• Induction of parturition	Cunha and Machado, 2001
Potamopyrgus antipodarum (New Zealand mudsnail)	3.7, 11.1 and 100 μg L^{-1}	42 day	Reproduction	• Decrease of neonates number per living adult at 100 μg L^{-1} FLX • Increase of shelled embryos and embryos total number at 3.7 and 11.1 μg L^{-1} FLX • Increase of unshelled embryos number at 3.7 μg L^{-1} and decrease at 100 μg L^{-1}) • Thinner gonad tissues at 100 μg L^{-1}	Gust et al., 2009
	69 μg L^{-1}		Reproduction	• Decrease of reproduction (number of newborns per individual)	Péry et al., 2008
	0.64, 3.2, 16, 80, and 400 μg L^{-1}	14−56 days	Behavior and reproduction	• Reduction of the mean embryo number at 80 and 400 μg L^{-1}	Nentwig, 2007
	400 μg L^{-1}		LC50		
Oncorhynchus mykiss (rainbow trout)	1545 mg L^{-1}	24 h	EC50	• Induction of hepatocyte cytotoxicity	Laville et al., 2004
	0−140 μg L^{-1}	4 h		• Induction of ROS production in fish hepatocytes PLHC-1	
	2 mg L^{-1}	48 h	LC50		Ferrari et al., 2004
Pimephales promelas (fathead minnow)	705 mg L^{-1}	48 h	LC50		Brooks et al., 2003
	212 μg/L R-FLX	48 h	LC50		Stanley et al., 2007
	16.1 (±20.2) μg L^{-1} R-FLX	15 min after 7 d exposure	EC10	• Decrease of feeding rate	
	132.9 (±21.2) μg L^{-1} R-FLX	7 d		• Decrease of growth rate	
	125 ng L^{-1}	12 d post-hatched	Behavior	• Slower predator avoidance behaviors in larvae	Painter et al., 2009
Oryzias latipes (Japanese medaka)	0, 0.1, 0.5, 1.0 and 5.0 μg L^{-1}	4 weeks		• Abnormalities in embryo development (edema, curved spine, no pectoral fins, reduced eyes) • Increase of female circulating estradiol levels 0.1 and 0.5 μg L^{-1} FLX	Brooks et al., 2003; Foran et al., 2004
	0.2 (pH 9), 1.3 (pH 8), 5.5 (pH 7) μg L^{-1}	96 h	LC50		Nakamura et al., 2008
Danio rerio (zebrafish)	0.32, 3.2, 32 μg L^{-1}	7 d	Reproduction	• Ovarian levels of 17 β-estradiol (E2) decrease • Average eggs spawning reduction • Reduction of ovarian aromatase, follicle stimulating hormone receptor (FSHr), and luteinizing hormone receptor (LHr) gene expression	Lister et al., 2009
Thalassoma bifasciatum (bluehead wrasse)	Injection 6 μg g^{-1} day^{-1}	2 weeks	Behavior	• Decrease of territorial aggression	Perreault et al., 2003; Semsar et al., 2004
Ictalurus punctatus (channel catfish); *Pomoxis nigromaculatus* (black crappie); *Lepomis macrochirus* (bluegill)	Collected from 100 m downstream from the effluent discharge (Pecan Creek, USA)		Bioaccumulation	• In brain (1.58 ± 0.74 ng g^{-1}); liver (1.34 ± 0.65 ng g^{-1}) and muscle (0.11 ± 0.0365 ng g^{-1})	Brooks et al., 2005

2.4. LPO analysis

Dissected gills ($n = 10$) and digestive glands ($n = 10$) were homogenized on ice individually with 20 mM TRIS–HCl buffer and butylated hydroxytoluene (BHT) in a 100:1 µl ratio at pH 8.6. In order to precipitate the cytosolic fraction, the homogenates were centrifuged at 30,000 × *g* for 45 min at 4 °C. An aliquot was set aside for total protein quantification according to Bradford's method (Bradford, 1976). Resulting cytosolic fraction was used for the measurement of LPO levels by the quantification of by-products malondialdehyde (MDA) and (2E)-4-hydroxy-2-nonenal (HNE) formation absorbance at 586 nm following an adaptation of Erdelmeier et al. (1998) method. LPO levels are expressed as µmol MDA g^{-1} total protein.

2.5. Acetylcholinesterase (AChE) analysis

Dissected gills ($n = 5$) were homogenized in 100 mM TRIS–HCl buffer and 100:1 µl of Triton at pH 8.0 on ice. The homogenates were centrifuged at 12,000 × *g* for 30 min at 4 °C. Resulting supernants were separated in aliquots, one for total protein determination (Bradford, 1976) and the other for AChE activity analysis according to an adaptation of Ellman et al. (1961) method. AChE activity was measured through the increase of yellow color resulting from the production of 5-mercapto-2-nitrobenzoate at 405 nm ($\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$) by the reaction of substrate thiocholine with DTNB. AChE activity is expressed nmol mg^{-1} total protein min^{-1}.

2.6. Alkali-labile phosphates (ALP) concentrations

Sex-differentiated gonads ($n = 10$) were homogenized in 25 mM Hepes–NaOH buffer (containing 125 mM NaCl + 1 mM DTT + 1 mM EDTA) at pH 7.4 in ice following an adaptation of Blaise et al. (1999) method. Homogenates were centrifuged at 12,000 × g at 2 °C for 30 min, resulting pellets were discarded. An aliquot of each supernant was reserved to determine total protein content (Bradford, 1976). The remaining cytosolic fractions were adjusted with 35% acetone and centrifuged at 10,000 × g for 5 min. Resulting pellets were dissolved with 1 M NaOH and placed for 30 min in a 60 °C heating bath. Phosphomolybdenum method (Stanton, 1968) was used to calculate inorganic phosphate (KH_2PO_4) concentration at 660 nm wavelength. ALP concentrations are expressed as µg [PO_4] mg^{-1} total protein.

2.7. Statistical analysis

All biomarkers results are presented as mean ± standard deviation corresponding to each set of time. Two-way ANOVA was performed using SIGMAPLOT® to test differences between non- and mussels treated with FLX on each tissue at each set of time. Ad-hoc Holm-Sidak was used on single biomarker difference discrimination over time, within and when applicable between tissues. Pearson correlation was used to verify the dependency between biomarkers. Principal Component Analysis (PCA) were performed with XLSTAT® 2012 to assess each biomarker responsibility on the variability at each set of time in non- and FLX exposed 1) gills, 2) digestive glands and 3) gills vs. digestive glands. AChE activity was only considered in gills' PCA, and ALP was not considered as it was analyzed in gonads. Statistical significance was defined at $p < 0.05$ level.

3. Results

3.1. CI

A significant decrease was observed in control mussels from the beginning (26.0 ± 2.6%) to the 3rd day (22.2 ± 3.3%), remaining unchanged until the end of the experiment (min. 19.5 ± 4.4%). Additionally, FLX exposed mussels' condition index was not affected throughout the exposure (day 3: 21.9 ± 4.0; day 7: 22.0 ± 3.3; day 15: 20.0 ± 3.8%) being not significantly different from respective controls.

3.2. Antioxidant enzymes

SOD activity is significantly higher in gills than in digestive glands for both non- and FLX exposed mussels throughout the experiment. Exposed gills showed a SOD activity inhibition trend over time only significant after two weeks (Fig. 1A) ($p < 0.05$), whereas in the exposed mussels' digestive glands no significant differences with controls exist (Fig. 1B) ($p > 0.05$).

Contrarily to SOD, CAT activity was higher in mussels' digestive glands than in gills. Even though, no significant differences between control and FLX exposed gills were established, it was noticeable a slight CAT activity increase in exposed gills, which was related to the SOD activity inhibition over time in this tissue ($r = -0.921$, $p < 0.05$) (Fig. 1C and D). This was further related with an inverse relationship between these antioxidant enzymes. In digestive gland, controls mussels showed a slight, but significant CAT decrease over the experiment duration, while exposed mussels showed a transient pattern, in which CAT activity fluctuates to significantly higher activity levels than controls after 3 and 15 days of FLX exposure ($p < 0.05$).

Regarding phase II enzyme GST (Fig. 1E and F), both tissues exhibit similar activities, although with irregular patterns. In controls, GST activities in both tissues decreased; after the first week in gills and immediately after the 3rd day in digestive gland. In exposed mussels, GST activity remained unaltered in gills (Fig. 1E), whereas in digestive gland (Fig. 1F) it fluctuates decreasing to control levels after the first week of exposure ($p < 0.05$) and varying to significantly higher activities than controls after the 3rd and 15th day of exposure. Furthermore, GST and CAT activities are directly related in this tissue ($r = 0.791$, $p < 0.05$).

3.3. LPO levels

LPO levels (Fig. 1G and H) were higher in gills than in digestive glands in both mussels groups. In gills (Fig. 1G), while controls showed no differences over time ($p > 0.05$), exposed mussels exhibit a significantly higher LPO levels than controls after one week ($p < 0.05$), recovering to control levels by the end of the experiment ($p > 0.05$). In the digestive gland (Fig. 1H), controls remained unaltered after a significantly decrease at the 3rd day, whilst exposed mussels LPO levels increased but only significantly after two weeks of exposure (4-fold higher than controls) ($p < 0.05$).

3.4. AChE activity

AChE (Fig. 2) activity remained consistent in control individuals throughout the experiment ($p > 0.05$). Conversely AChE activity was significantly incremented in mussels' gills after the 3rd day of FLX exposure comparing to controls, followed by a progressive inhibition, reaching a significantly lower activity than controls by the end of the experiment ($p < 0.05$).

3.5. ALP concentration

ALP levels (Fig. 3) decreased significantly in control females after the first week ($p < 0.05$), while in males' remained unchanged throughout the experiment duration ($p > 0.05$). Nevertheless, in exposed gonads, after 3 days of exposure, ALP levels decreased significantly in both females and males though more markedly in males comparing to controls. After this, ALP levels were significantly incremented after one week of exposure in both genders, decreasing again to levels significantly lower than controls by the end of the experiment ($p < 0.05$).

3.6. Principal component analysis (PCA) in gills and digestive gland

PCA was applied to all parameters measured in the gills (Fig. 4A) revealing 72% of total variance (TVar). PC1 represents 52% of variance, highlighting the separation between non- and FLX exposed gills by the opposition in the factorial weight distribution of SOD activity with the remaining enzymes activities and LPO levels. The second component (explaining only 19% of the variance) shows the further influence of CI in the separation of day 0 (control) from the remaining controls, and day 15 FLX exposed gills from the other exposure-day groups in opposition to LPO levels and AChE activities. Furthermore, PCA reveals that SOD and CAT activities exhibit opposing factorial distribution, corroborating with the above mentioned inverse relationship.

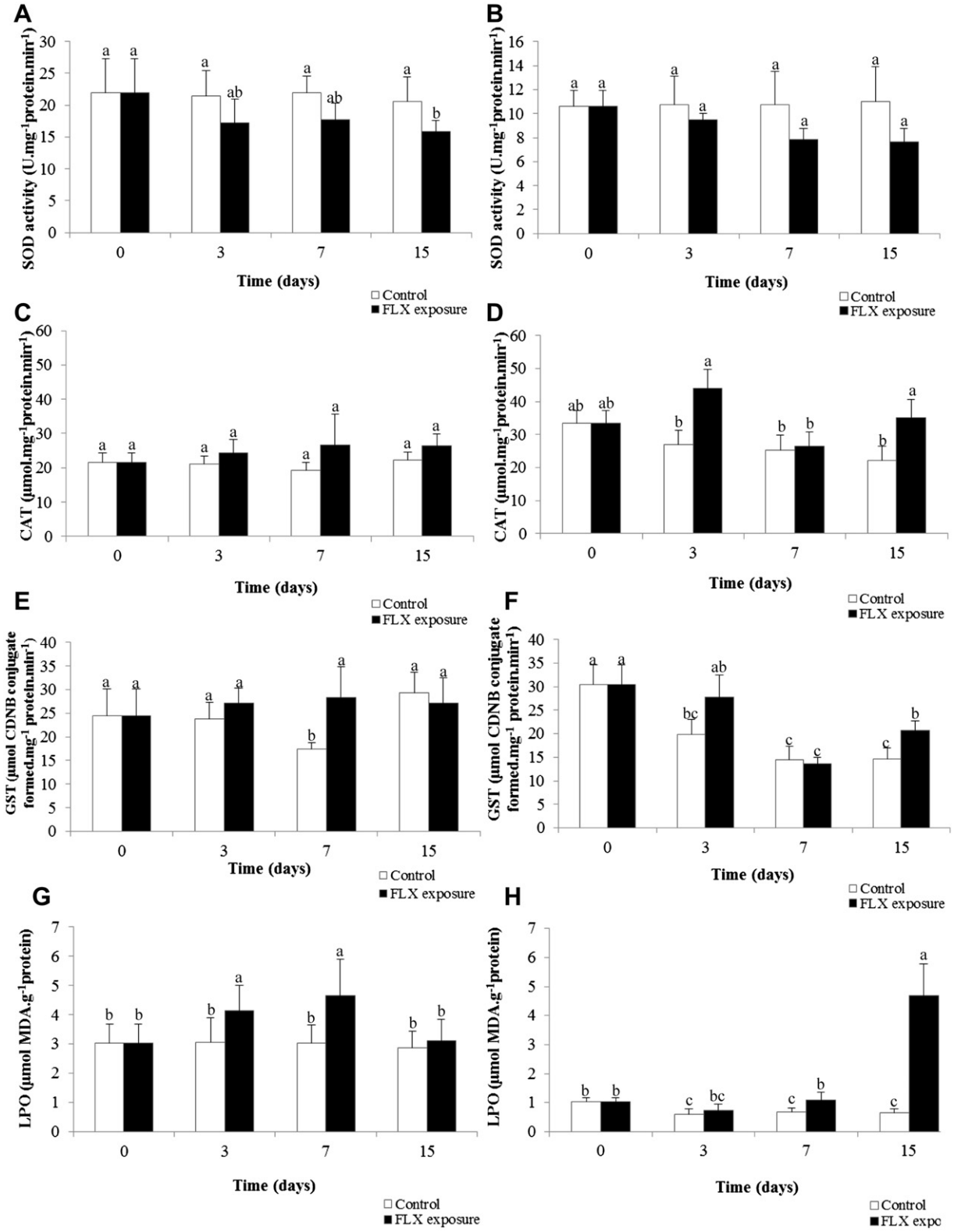

Fig. 1. Antioxidant enzyme activities and LPO levels (mean ± standard deviation) in control and FLX exposed *M. galloprovincialis* tissues. SOD activity in gills (A) and in digestive gland (B); CAT activity in gills (C) and in digestive gland (D); phase II GST activity in gills (E) and in digestive gland (F); LPO levels in gills (G) and in digestive gland (H). Different letters express significant differences ($p < 0.05$).

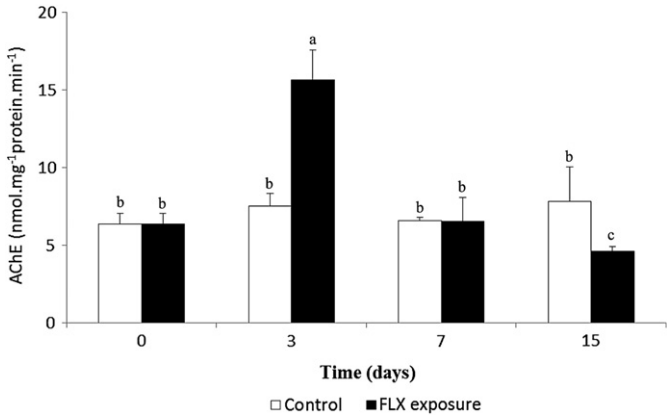

Fig. 2. AChE activity (mean ± standard deviation) in non- and exposed mussel's *M. galloprovincialis* gills to 75 ng L^{-1} of FLX along 15 days. Different letters express significant differences ($p < 0.05$).

Digestive gland PCA (Fig. 4B) represents almost 84% of TVar, in which both components had similar influence on the overall groups' distribution (PC1, 46% and PC2, 38%). AChE activity was not considered since it was performed only in gills. PC1 shows the cluster of controls (exception for day 0) particularly associated to the opposition of SOD activities with the remaining variables; and the separation of day 7 FLX exposed digestive glands from the other exposure days. As observed in gills PC2, CI is the highest factor explaining the variability of day 0 control group from remaining groups and in this case, day 15 FLX exposed digestive glands were further influenced by the LPO levels. The digestive gland PCA also corroborates with the direct relationship between CAT and GST activities in this tissue.

PCA (Fig. 5) is related with tissue-specific integration of oxidative stress biomarkers (SOD, CAT, GST and LPO), explaining approximately 85% of TVar (PC1, 51% and PC2, 34%). The first component clearly separates non- and FLX exposed gills from digestive glands treatment groups, particularly regarding gills to LPO levels and GST and SOD activities, while digestive glands are more related to the activity of CAT. The second component highlights the separation of both exposed gills and digestive glands from controls groups, with the exception of day 0 control and day 7 exposed digestive glands and day 15 gills control regarding to levels of CAT, LPO and GST in opposition to SOD. In overall PCA shows the aggregation of: 1) gills controls relating to higher SOD activities; 2) FLX exposed gills with the increment of LPO levels; 3) FLX exposed digestive glands with higher CAT activities; and finally 4) digestive glands' controls in opposition to all integrated biomarkers.

4. Discussion

4.1. Oxidative stress

To our knowledge this is the first study concerning the effect of FLX as a potential oxidative stress inducer applying antioxidant enzymes response activities in mussels. The results reveal a transient antioxidant status alteration in both mussels' tissues. As the first organ in direct contact with the contaminant, the significant SOD activity downregulation was inversely related only in the gills, the concomitant inhibition tendency of SOD activity in digestive glands resulted in a significant enhancement of CAT after 3 and two weeks of FLX exposure (Figs. 1 and 5). The exposure of rainbow trout (*Oncorhynchus mykiss*) hepatocytes to FLX has shown to induce an enhancement of ROS production (Laville et al., 2004). Considering SOD primarily role in the dismutation of $OH^{\bullet-}$ into H_2O_2, its inhibition in presence of FLX is associated to a higher presence of ROS particularly in the gills and since digestive gland has a higher participation on general redox-cycling and biotransformation processes (Livingstone et al., 1992) CAT's catalytic action is later triggered in this tissue (Regoli and Principato, 1995). An inhibition of SOD activity was also found by Djordjevic et al. (2011) in mice exposed to 5 mg/kg FLX body mass, although no enhancement of CAT activities was observed. Additionally, higher CAT activities were reported in digestive glands than in gills in the same mussel species exposed to carbamazepine (Martín-Díaz et al., 2009). Overall PCA clustering (Fig. 5) provides a further validation for the discrepancy between the activity levels of SOD in gills and CAT in digestive gland.

Phase II GST promotes reduced glutathione (GSH) conjugation with parental electrophilic compounds enabling its transformation to more extractable hydrophilic metabolites (Halling-Sørensen et al., 1998). Although, GST activity was generally transitory between treatments in both tissues (Fig. 1E and F) giving evidence to the actuation of other affecting variables rather than exposure itself, this enzyme was more responsive in FLX-exposed digestive gland showing simultaneously higher activity than controls (except after one week) and was directly related with CAT activity. A positive relationship between the enhancement of GST and CAT

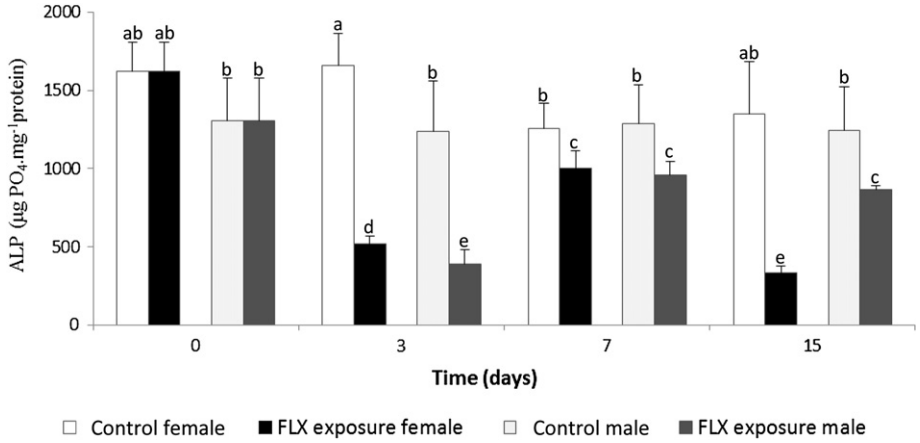

Fig. 3. ALP level (mean ± standard deviation) in non- and exposed mussel's gonads *M. galloprovincialis* to 75 ng L^{-1} of FLX throughout 15 days. Different letters express significant differences ($p < 0.05$).

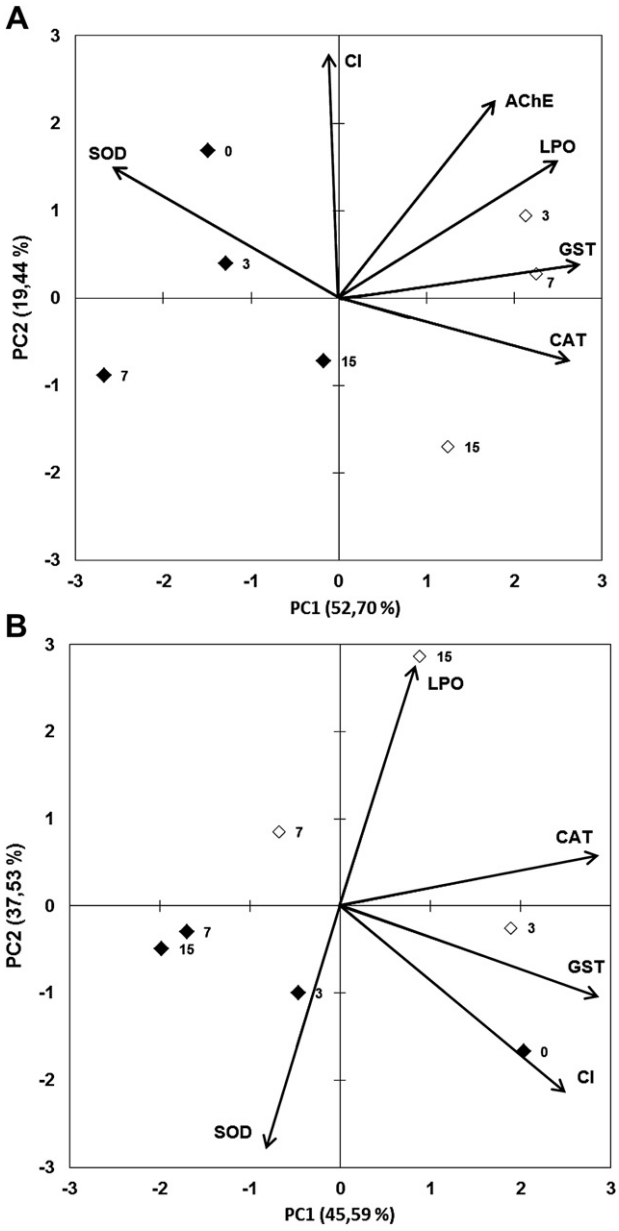

Fig. 4. PCA equivalent to the integration (A) of SOD, CAT and GST, LPO, AChE and CI on 77% of total variance in gills; (B) of SOD, CAT and GST, LPO and CI on 83% of total variance in digestive glands of mussel *M. galloprovincialis* between treatments (◆ – control, ◇ – FLX exposed mussels) over exposure time (days: 0, 3, 7 and 15).

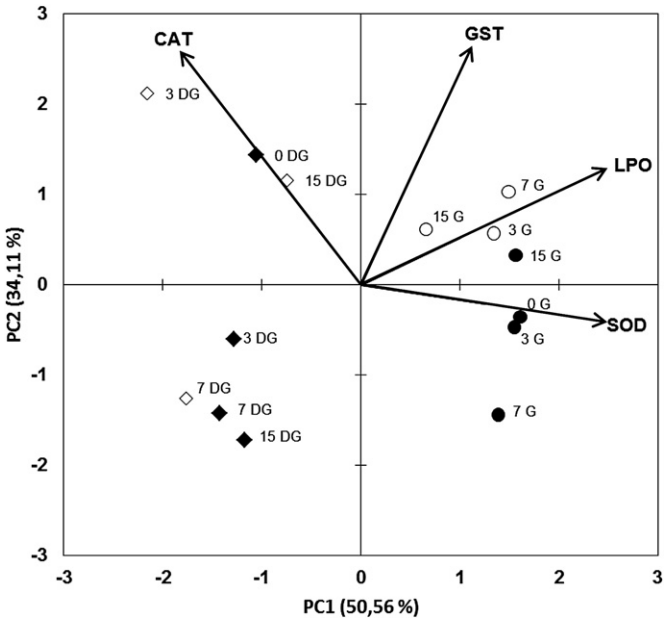

Fig. 5. PCA related to the integration of SOD, CAT and GST, LPO on 85% of total variance in mussels' *M. galloprovincialis* tissues between treatments (● – gills controls, ○ – FLX exposed gills, ◆ – digestive gland controls, ◇ – FLX exposed digestive glands) over exposure time (days: 0, 3, 7 and 15).

was also observed in *M. galloprovincialis* digestive glands exposed to 250 ng L^{-1} ibuprofen for the same time (Gonzalez-Rey and Bebianno, 2012) alike for bezafibrate (Canesi et al., 2007), carbamazepine (Martín-Díaz et al., 2009) and propranolol exposure (Franzellitti et al., 2011). Finally, GST enhancement confirms, as stated by Canesi et al. (2007) after 38.2 ng g^{-1} bezafibrate injection in same species mussel, that FLX is partially metabolized in mussels' digestive glands.

The alteration of antioxidant enzymes was unable to prevent and counter-act FLX-exposure as there was a clear tissue-specific response of membrane damage in which gills showed higher vulnerably at the beginning of FLX-exposure until the end of the first week while LPO enhancement in exposed digestive glands (7-fold higher) continued until the end of the experiment (Fig. 1G and H). Even though, exposed gills seemed to recover to control levels after 2 weeks, PCA (Fig. 5) suggests that FLX has a higher impact in this tissue than in digestive gland over time. Higher levels of LPO were also observed after 250 ng L^{-1} ibuprofen exposure in the same mussels' tissues particularly in gills (max: 19 μmol MDA mg^{-1} protein) than the ones induced by FLX (Gonzalez-Rey and Bebianno, 2011, 2012).

4.2. Neurotoxic effect

AChE is responsible for the hydrolysis of neurotransmitter acetylcholine (ACh) to choline and acetic acid and has an important role in cholinergic nervous function (Tsuchiya et al., 2004), as the neurotransmitter serotonin (5-HT) has in serotonergic neurotransmission. These neurotransmitters are critical in the control of many physiological processes like e.g. cardiac regulation in bivalves, though their roles may be inverted regarding excitation and inhibition in different species (Kuwasawa and Hill, 1997). Since SSRI FLX induces an increase in extracellular 5-HT by the serotonin transport protein inhibition at nerve synapses (Lister et al., 2009) is not illicit to think that AChE may also be affected by FLX presence, further considering the evidences of 5-HT increase in mussel *Elliptio complanata* after injection with FLX (Gagné and Blaise, 2003).

Even though AChE activity has been reported to be inhibited in mussels by APIs namely by acetaminophen (23 and 403 μg L^{-1}) in *M. galloprovincialis* gills (Solé et al., 2010) and by diazepam (4, 20 and 100 nmol per mussel) in *Elliptio complanata* visceral mass (Gagné et al., 2011) in FLX-exposed *M. galloprovincialis* gills the significant downregulation of AChE activity was only observed at the end of the 15th day, being firstly preceded by a clear upregulation of AChE after 3 days of FLX exposure. Few other studies have shown the induction of AChE namely, in mussels placed in the final aeration lagoon (Gagné et al., 2010) and in crabs in an impacted stream (Nieto et al., 2010) with no noticeable explanation for this fact rather than antagonistic effects between contaminants. The enhancement of AChE activity implies the depletion of ACh, we hypothesize several possible explanations for the AChE activity temporary enhancement at the beginning of the experiment: 1) the enhancement of neurotransmitter 5-HT concentration by FLX

action at nerve endings may have been competing with ACh and therefore causing its depletion, even though the receptor activation mechanism of 5-HT in mussels is still unknown as stated by Gagné and Blaise (2003); 2) AChE increase is associated to cell apoptosis (Zhang et al., 2002), FLX presence may promote gills' cell apoptosis at the beginning of the experiment followed by the mussels' recovery to controls' AChE activity; 3) a relationship between estradiol levels and AChE activity regulation in mice brain cortex cells was reported noting that with high levels of 17β −estradiol (E2) AChE activity was suppressed whereas without E2, it increased (Tsuchiya et al., 2004). The alteration of endogenous levels of E2 may explain these AChE activity alterations in FLX exposed mussels at the beginning of the exposure, even though AChE activity was not measured in sex-separated gills the results indicate by the concomitant ALP downregulation over the 3rd day of exposure that FLX has the ability to interfere with estrogen receptors.

4.3. Endocrine disruption

ALP levels are released by vitellogenins (Vt) after alkali hydrolysis (Porte et al., 2006), which precede egg-yolk protein vitellin (Vn) in oviparous species (Matozzo et al., 2008). In bivalves, vitellogenesis is induced by estradiol (E2) and a neuropeptide (Matozzo et al., 2008). ALP levels enhancement is a sign of endocrine disruption (ED) in males (Blaise et al., 1999; Gagné et al., 2002; Matozzo et al., 2008). In FLX exposure, ALP levels were generally high (max. observed in females 1600 μg $[PO_4]$ mg^{-1} total protein) and similar to the ones in *M. edulis* in anthropogenic influenced sites (Gagné et al., 2008). However FLX exposure did not induce an enhancement of males ALP levels, but rather a downregulation in both sex-differentiated gonads particularly in females over the 3rd day. A similar experimental design with the same species exposed to 250 ng L^{-1} of ibuprofen showed both: lower basal ALP values (max. in exposed males: 117 μg $[PO_4]$ mg^{-1} total protein) and significant induction in exposed mussels (Gonzalez-Rey and Bebianno, 2012).

ALP downregulation was also observed in females exposed to North Sea crude oil (NSO) which in turn was accompanied by the decreased of gonads development suggesting that ALP levels influence on gonad development in adult blue mussels *Mytilus edulis* through an antiestrogenic effect of polycyclic aromatic hydrocarbon (PAHs) and oils (Ortiz-Zarragoitia and Cajaraville, 2006). As also stated by these authors it is important to consider 5-HT function in bivalves on gonad development and reproduction, for this reason a histological study of FLX-exposed gonads would greatly complement the data on mussels' reproduction fitness alteration.

Furthermore, the ALP downregulation in both FLX-exposed female and male gonads may be associated to E2 inverse relationship with 5-HT levels as reported in freshwater mussels *Eliptio complanata* and gonad 5-HT concentration increase during spawning (Gagné and Blaise, 2003) since by opposition vitellogenin synthesis induction is related to gonad serotonin levels decrease (Matozzo et al., 2008). Also, recently Lazzara et al. (2012) reported the increase of 1.5-fold of E2 after 6 days of exposure to 200 ng L^{-1} of FLX in mussel *Dreissena polymorpha*. Nevertheless, along with the decrease of oocytes and spermatozoan, esterified E2 levels decreased approximately 3−8-fold in this species between spawning and after-spawning phases after 20 and 200 ng L^{-1} of FLX exposure thus confirming this SSRI to be an endocrine disruptor.

5. Conclusions

FLX exposure to mussels for two weeks induced a transient antioxidant enzyme activities alteration particularly in gills. However these alterations were not as severe as overall ALP sex-differentiated downregulation in gonadal tissues, highlighting a higher endocrine disruption effect of FLX rather than an oxidative stress inducer. Furthermore, resulting from the influence of FLX as a SSRI, on 5-HT levels increase as mentioned before, AChE activity was clearly altered throughout the experiment and possibly results on cholinergic neurotransmission functions breakdown. The measurement of FLX concentration in tissues, as well as, serotonin and estradiol level alteration in both non- and exposed mussels' tissues should clearly complement these findings. Finally, FLX presence, even at a relevant environmental concentration, clearly has potential to induce ecotoxicological effects in mussel *M. galloprovincialis* particularly affecting its reproduction fitness, although the mechanisms of 5-HT receptor activation in mussels are still unknown.

Conflict of interest statement

The authors declare the inexistence of any conflicts of interest.

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