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Mechanistic insights into the effects of Fluoxetine in *Mytilus galloprovincialis* using *in vivo* and *in vitro* approaches

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ABSTRACT

Antidepressants such as selective serotonin reuptake inhibitors (SSRIs) are pseudo-persistent contaminants in aquatic ecosystems. While their effects and molecular targets are relatively well understood in vertebrates, less information is available in aquatic invertebrates. In this work, the effects and mechanisms of action of Fluoxetine (FLX), a widely prescribed SSRI, were investigated in the model marine bivalve *Mytilus galloprovincialis. In vitro* exposure of isolated hemocytes to FLX (0.03–0.3–3 μg/mL) induced significant alterations in immune and lysosomal parameters. In mussels exposed to FLX or its active metabolite norfluoxetine NFL (0.5–5–10–50 ng/L, 7 days), a concentration dependent accumulation of either compound was observed in the digestive gland. NFL was also detected in FLX-exposed mussels, indicating biotransformation. FLX and NFL affected transcription of monoamine receptors, and of lysosomal, autophagy, and ceramide related genes, with a distinct pattern for each compound, with FLX mainly inducing downregulation of gene expression. The results demonstrate that in mussels both FLX and NFL act through multiple molecular pathways, pointing at the lysosomal system as a main target for both compounds. These data provide novel mechanistic insights into antidepressant toxicity in a non-target marine invertebrate and contribute to draw Adverse Outcome Pathways for SSRIs in bivalves, that represent foundation species in coastal environments.

1. Introduction

Antidepressants rank among the most widely utilized pharmaceuticals globally. Their use parallels the increasing number of individuals diagnosed with depressive disorders, with estimates of about 4 % of the global population affected in 2021 (Drzymała, 2025). Following patient treatment, these drugs are excreted into the environment both as parent compounds and metabolites, which are potentially biologically active. Due to their incomplete removal by wastewater treatment facilities, different antidepressants are widely detected in different aquatic environments at concentrations ranging from ng/L to μg/L (Silva et al., 2012; Mole and Brooks, 2019; Puga et al., 2024; Drzymała, 2025).

Selective serotonin reuptake inhibitors (SSRIs), due to their efficacy and broad application, are among the commonly prescribed antidepressants: in US, sertraline, fluoxetine, citalopram, and venlafaxine were listed among the top 50 pharmaceuticals sold in 2022 (https://clincalc.com/DrugStats/Top200Drugs.aspx; accessed October 15th, 2025). Fluoxetine (FLX), a representative SSRI, is detected in different aquatic compartments at varying concentrations, from ng/L to μg/L (Mole and Brooks, 2019; Diaz-Camal et al., 2022; Fabbri et al., 2024; Drzymała, 2025); in coastal marine ecosystems, reported levels range between 0.6 ng/L and 90 ng/L (Cortez et al., 2019). Norfluoxetine (NFL), the main FLX metabolite, has also been detected in aquatic environments where, due to its structural similarity to the parent compound, it may pose an additional risk to aquatic organisms (Yan et al., 2023; Gozdzik et al., 2024).

The main therapeutic targets of FLX are the serotonin (5-HT) transporters (SERTs) in presynaptic neurons. Inhibition of re-uptake enhances the availability of 5-HT in the synaptic cleft, thereby augmenting serotonergic signalling. This mechanism appears to be conserved throughout vertebrate evolution: exposure to FLX has been demonstrated to affect various biological processes in fish, including neuronal

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and endocrine regulation (Vera-Chang et al., 2018; Yamindago et al., 2021; Correia et al., 2023). The serotonergic system is also physiologically active in invertebrate species, that exhibit high sensitivity to environmental concentrations of antidepressants (Fong and Ford, 2014; Canesi et al., 2022; Fabbri et al., 2024). Accordingly, in different aquatic invertebrates, FLX has been shown to affect reproduction, behavior, oxidative stress responses, morphology, gene expression, and developmental processes (Fong and Ford, 2014; Moreira et al., 2022; Fabbri et al., 2024; Drzymała, 2025).

In bivalve molluscs, a broad spectrum of sub-lethal effects of FLX has been described, including oxidative stress, biotransformation, lysosomal responses, genotoxicity, and alterations in neuroendocrine and growth parameters (reviewed in Fong and Ford, 2014; Canesi et al., 2022; Zhu et al., 2023). The marine mussel *Mytilus* spp, due to its worldwide distribution in coastal environments, sedentary and filter-feeding habits, knowledge on the biochemical and physiological responses to a wide variety of contaminants, has been long recognized as a "sentinel" species for the evaluation of responses to pollutants (reviewed in Tyler-Walters et al., 2022), including pharmaceuticals (Świacka et al., 2019).

In *M. galloprovincialis*, FLX is accumulated in the tissues where it is primarily metabolized into norfluoxetine (NFL) (Silva et al., 2016; Lemaire et al., 2024). Direct exposure to NFL also results in its accumulation and persistence in marine mussels (Hallmann et al., 2023).

The reported effects of FLX in bivalves often reflect general cellular stress responses rather than specific interactions with the serotonergic system. However, limited information on the mechanism of action of FLX is available in invertebrates. In *M. galloprovincialis*, FLX exposure has been correlated with decreased levels of cyclic adenosine monophosphate (cAMP) and reduced protein kinase A (PKA) activity in the digestive gland (Canesi et al., 2022; Fabbri et al., 2024). These changes were associated with increased occupation of 5-HT receptors, particularly the 5-HTR-1 subtype, and with overexpression of the 5-HT1R gene product (Canesi et al., 2022; Fabbri et al., 2024).

Identifying molecular targets of emerging contaminants is essential for establishing a mode-of-action (MOA)-driven approach to environmental risk assessment (Brooks et al., 2003). Recent findings indicate that lysosomes may serve as primary cellular targets for FLX in mussels: exposure to FLX (0.5, 5, and 10 ng/L for 7 days) resulted in decreased lysosome membrane stability in hemocytes, and increased lysosome/-cytosol ratio, neutral lipid and lipofuscin accumulation in the digestive gland (Rafiq et al., 2023). These data are in line with those obtained in mammalian cells, where, in addition to the well-established SERT inhibition as the primary target of FLX, the drug can also act as a lysosomotropic agent, inducing lysosomal activation and stress (Lu et al., 2017). Moreover, FLX has been shown to modulate autophagy pathways (Rein, 2019; Shu et al., 2019; Park et al., 2021), affect mitochondrial function (de Oliveira, 2016; Xu et al., 2023) and ceramide metabolism (Gulbins et al., 2015, 2018).

In this light, the present work aims at providing further insights into the mechanisms of action and related effects of FLX in the model marine bivalve *M. galloprovincialis*, utilising both *in vitro* and *in vivo* experimental settings. Cellular responses to FLX were first examined through short-term exposure of isolated immune cells, the hemocytes, where functional parameters were evaluated in order to clarify whether lysosomes and lysosomal-related functions represent a key target for FLX at the cellular level, and the potential effects on immune responses. The *in vivo* effects of FLX on digestive gland were further investigated in mussels exposed for 7 days to environmental concentrations (0.5, 5, 10, and 50 ng/L) of FLX. In these experimental conditions, FLX significantly affected a number of tissue biomarkers (Rafiq et al., 2023); however, FLX accumulation and its molecular targets were not investigated. In this work, the concentrations of FLX were quantified in both exposure media and tissue by U-HPLC-MS/MS. Moreover, expression of FLX target genes previously identified in mammalian systems, including those related to neuroendocrine signaling, antioxidant defences, detoxification, autophagy, lysosomal function, and ceramide metabolism was evaluated by qPCR. Since previous data also showed significant effects of NFL on mussel digestive gland (Rafiq et al., 2023), parallel experiments were carried out with NFL, in order to compare the *in vivo* effects of the parent compound with those of the main metabolite. Overall, the results will provide more detailed information on the mechanisms of action of FLX in the model bivalve *Mytilus* that can contribute in drawing Adverse Outcome Pathways (AOPs) for SSRIs in bivalves.

2. Materials and methods

2.1. Chemicals

Fluoxetine ((±)-NMethyl-γ-[4-(trifluoromethyl)phenoxy]benzenepropanamine) hydrochloride (FLX), deuterated Fluoxetine-d5 (FLX-d5) (Merck Life Science, Italy), and its metabolite norfluoxetine (γ-[4-(trifluoromethyl) phenoxy]-benzenepropanamine) monohydrochloride (NFL) (Cayman Chemical, USA) were prepared in stock solutions of 1 mg/mL in dimethyl sulfoxide (DMSO), aliquoted and stored at −20 °C until use. All other reagents were of the highest commercially available grade. For chemical analyses, stock solutions were prepared at 1 mg/mL in methanol and stored at −20 °C until use. Solvent reagents and formic acid (FA) (98 % purity) were of LC-MS analytical grade (Merck Life Science). Solid phase extraction (SPE) Oasis HLB™ (6 cc, 200 mg), Oasis PRiME HLB Plus Light (100 mg) cartridges, and QuEChERS salts (contents DisQuE™ pouch for CEN method) were purchased from Waters S. p.A. (Milan, Italy).

2.2. Animals

Mussels *M. galloprovincialis* (4–6 cm length), obtained from COPRALMO aquaculture farm (Cesenatico, Italy) were acclimatized for 5 days to laboratory conditions in filtered seawater (FSW), at 5 mussels/L, obtained from the same sampling site (pH 8.3, 33‰ salinity, 16 ± 2 °C). Mussels were kept under continuous aeration (>90 % oxygen saturation), with a 12 h light:12 h dark photoperiod. Animals were fed with *Nannochloropsis oculata* (BlueBiotech, Büsum, Germany) at 5×10^6 cells/L (Rafiq et al., 2023) before each seawater change.

2.3. In vitro effects of FLX on mussel hemocytes

The effects of FLX were evaluated in the hemocytes of control (untreated) mussels. Hemolymph was extracted from the posterior adductor muscle of 4 groups of at least 10 mussels and each pooled in 50 mL Falcon tubes at 18 °C (Ciacci et al., 2012; Balbi et al., 2018). Hemocyte monolayers or aliquots of whole hemolymph were incubated with FLX (0.03–0.3–3 µg/mL in artificial sea water-ASW) at 18 °C from 15 to 240 min, depending on the endpoint measured. Control samples in ASW and samples in ASW added with vehicle (DMSO, maximal final concentration 0.01 % v/v) were run in parallel.

2.3.1. Hemocyte functional parameters

Lysosomal membrane stability-LMS, phagocytosis, extracellular oxyradical production, nitric oxide production, lysozyme release were assessed as previously described (Ciacci et al., 2012; Balbi et al., 2018) (for details, see SI, Methods).

2.3.2. Confocal laser scanning microscopy (CLSM)

After exposure to FLX (0.03–0.3–3 µg/mL, 60 min), hemocytes were fixed with paraformaldehyde at 4 % for 10 min, washed two times for 2 min with TBS (Tris–HCl buffer) and permeabilized with 0.05 % NP-40 (Nonidet-40) for 10 min. Samples were incubated with different cell permeant fluorescent probes and then observed by CLSM using a Leica TCS SP5 confocal setup mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems, Heidelberg, Germany) as previously described (Ciacci et al., 2012; Balbi et al., 2018). Cells were stained with LysoTracker Green (LTG) for acidic compartments,

Monodansylcadaverine (MDC) for autophagosomes, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for intracellular oxyradical production, Tetramethylrhodamine ethyl ester perchlorate (TMRE) for mitochondrial membrane potential. Double staining with LTG and MitoTracker Deep Red (MTDR) was also performed to investigate lysosomal-mitochondrial co-localization (for details see SI, Methods).

2.4. In vivo exposure to FLX and NFL

After acclimatization, animals were randomly transferred into 5 plastic vessels (5 replicates) at a density of 1 mussel/L FSW, in the same conditions described for acclimatization, and exposed for 7 days to FLX or NFL at nominal final concentrations of 0.5, 5, 10, and 50 ng/L, obtained by serial dilution of aliquots of stock solutions in FSW. Chemicals were added at the beginning of the experiment (T0); sea water was changed every two days and concentrations were re-established after each water renewal (T1 = 2 days, T2 = 4 days, T3 = 6 days) as previously described (Rafiq et al., 2023). For each experimental condition and replicate, 5 mussels were utilized. Animals were fed before each water renewal. No mortality was observed along the whole experiment. At each time point (T0, T1, T2, T3 and at the end of exposure T4), aliquots of water (100 mL) were sampled from each vessel, pooled (total volume 500 mL), immediately frozen and kept at −20 °C for subsequent chemical analyses.

At the end of exposure (T4), digestive glands were dissected out, immediately frozen in liquid nitrogen and stored at −80 °C. Analysis of tissue FLX or NFL concentrations was performed on 5 tissue pools (1.5 g w.w. total) from 3 animals per vessel, for a total of 15 mussels per experimental condition. The remaining digestive glands were pooled (5 pools of 4 animals each) and stored in TRIzol reagent at −20 °C for subsequent RNA extraction and gene expression analysis.

2.4.1. Chemical analyses by U-HPLC-MS/MS

Extraction of FLX and NFL from water and digestive gland samples was carried out essentially following Profita et al. (2024) and Diallo et al. (2023), respectively, with some modifications. Details are reported in SI. Samples were analysed with an Ultra High-Performance Liquid Chromatography system (U-HPLC, 1290 series) coupled to a triple quadrupole mass spectrometer (MS/MS, 6465 series Ultivo) form Agilent Technologies Italia S.p.A. (Cernusco sul Naviglio, Milan, Italy). Chromatographic separation was performed using a reverse-phase C18 UPLC column (ZORBAX RRHD Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm, Agilent Technologies, Italy) and matching guard column maintained at 35 °C. The mobile phases consisted of water with 0.1 % formic acid (A) and methanol (B). Gradient elution started at 5 % B for 1 min, increased linearly to 100 % B over 6 min, held at 100 % B for 2 min, then returned to starting conditions and equilibrated for 2 min. The flow rate was 0.3 mL/min, and the injection volume was 3 µL. The MS/MS conditions were operated with Jet Stream electrospray ionisation (ESI) in positive mode and the spectra were acquired in multiple reaction monitoring mode (MRM). For quantification purposes the transition m/z 310.1→44.1, 316.1 → 44.1, and 297.1→134 were used for FLX, FLX-d5, and NFL, respectively. The amount of FLX and NLF recovered from the samples was estimated by comparison with a standard curve of seven concentration points (0.05, 0.1, 0.5, 1, 5, 10, and 50 µg/L). Data performance of the LC-MS method are reported in Table 1.

2.4.2. Tissue serotonin levels

Serotonin (5-HT) concentrations in the digestive gland of FLX and NFL exposed mussels were analysed by enzyme-linked immunosorbent assay (Serotonin/5-Hydroxytryptamine Competitive ELISA kit, EEL006; ThermoFisher). For details see SI, Methods.

2.4.3. Gene expression

Total RNA was extracted from 5 pools of tissues (about 100 mg each) obtained from each condition (n = 5) using TRIzol reagent (Sigma, Milan, Italy) according to the manufacturer's protocol. RNA concentration and quality were verified using the Qubit RNA assay (ThermoFisher, Milan, Italy) and electrophoresis on a 1.5 % agarose gel under denaturing conditions. Aliquots of 1 µg RNA were reverse transcribed into cDNA using a CFX96™ real-time PCR system (Biorad Italy, Segrate, Milan) as previously described (Balbi et al., 2023). Primers and conditions employed for qPCR analysis are listed in Table S1. A control lacking cDNA template (no-template) was included in the qPCR analysis to determine the specificity of target cDNA amplification. Amplification was performed in a CFX96™ Real-Time PCR system (Biorad Italy, Segrate, Milan) using a standard "fast mode" thermal protocol. Expression of 18S was evaluated as reference gene (Balbi et al., 2023). A comparative C_T method was utilized to calculate the relative expression of target mRNAs (Schmittgen and Livak, 2008). Data are reported as relative expression (mean ± SD) with respect to control samples.

2.5. Data analysis

Data from *in vitro* experiments are the mean ± SD of at least 4 independent experiments, with each assay performed in triplicate. Data on chemical analyses on digestive gland are the mean ± SD of 5 samples (each representing the pool of 3 mussels). Statistical analyses were performed ANOVA plus Dunnett's post-test (* = $p \leq 0.05$).

For gene transcription, data are the mean ± SD of at least 5 samples in triplicate ($n \geq 5$), and statistical differences were evaluated by the Mann-Whitney U test (* = $p \leq 0.05$).

All statistical calculations were performed using the PRISM 7 software (GraphPad Prism 5 software package, GraphPad Inc.).

3. Results

3.1. In vitro effects of FLX on mussel hemocytes

Short-term *in vitro* experiments were carried out in isolated mussel hemocytes exposed to FLX (0.03–0.3–3 µg/mL), from 15 to 240 min, depending on the endpoint measured.

3.1.1. Effects on immune parameters

Data on hemocyte functional immune parameters are reported in Figs. 1 and 2. FLX induced a large inhibition of phagocytic activity (≤50 % of control values, $p \leq 0.05$) at all the concentrations tested (Fig. 1A). Extracellular ROS production was not significantly affected, except for a modulation at 3 µg/mL ($p \leq 0.05$) (Fig. 1B). However, FLX induced a progressive increase in intracellular ROS production at increasing concentrations, as indicated by DCF-DA fluorescence (Fig. 1C).

FLX also induced significant increases ($p \leq 0.05$) in NO production, to a different extent at different concentrations and times of exposure

Table 1
Data performance of the LC-MS method. SW: seawater; DG: mussel digestive gland. LOD: Limit of detection. LOQ: Limit of quantification. RSD: Relative standard deviation.

SSRI	matrix matched linearity (r2)	LOD	LOQ		% matrix effects		% recovery		% RSD	
		pg (injected)	SW ng/L	DG ng/g w.w.	SW	DG	SW	DG	SW	DG
FLX	0.998	0.003	0.03	0.02	103.70	96.44	109.53	116.11	7.14	6.53
NFL	0.998	0.05	1.07	0.07	84.74	97.22	92.63	98.12	19.97	10.51

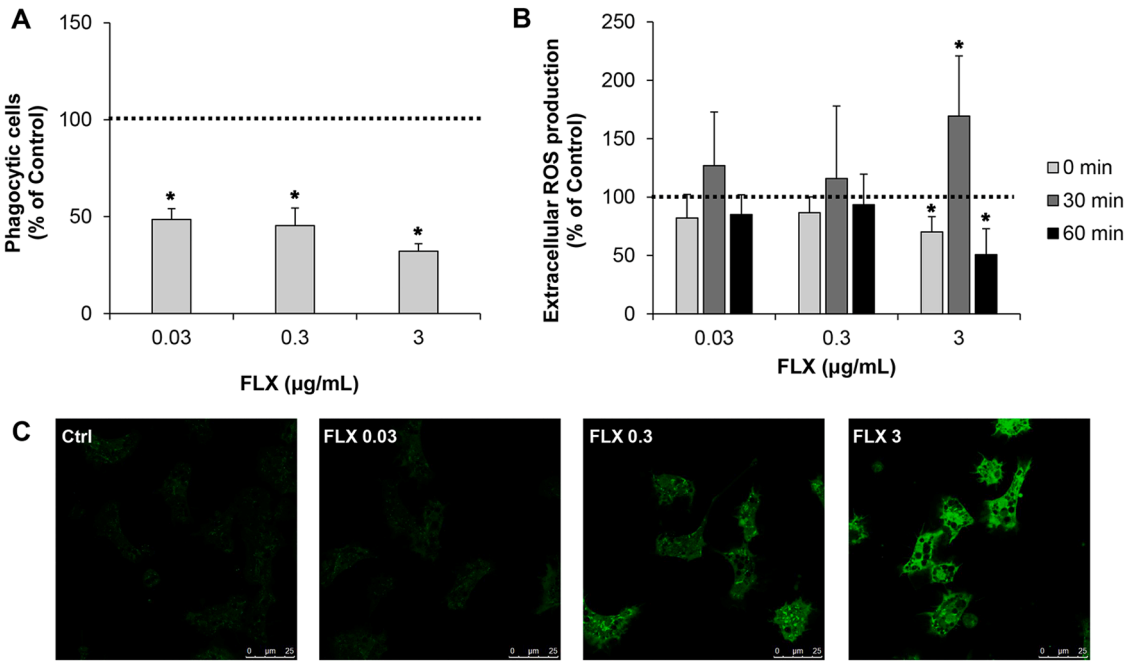

Fig. 1. *In vitro* effects of FLX (0.03, 0.3 and 3 µg/mL) on mussel hemocytes. A) Phagocytic activity was evaluated by optical microscopy using the uptake of neutral Red-conjugated zymosan particles; B) Extracellular ROS production was measured spectrophotometrically as cytochrome *c* reduction at 550 nm. Data, representing the mean ± SD of at least 4 independent experiments, are reported as percent values with respect to controls. * = $p \leq 0.05$, ANOVA + Dunnett's post-test. C) Intracellular ROS production was monitored using the cell-permeant probe 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA). Representative confocal images of DCF-DA fluorescence are shown. For determination of different parameters, incubation with FLX was carried out at 60 min, unless otherwise indicated.

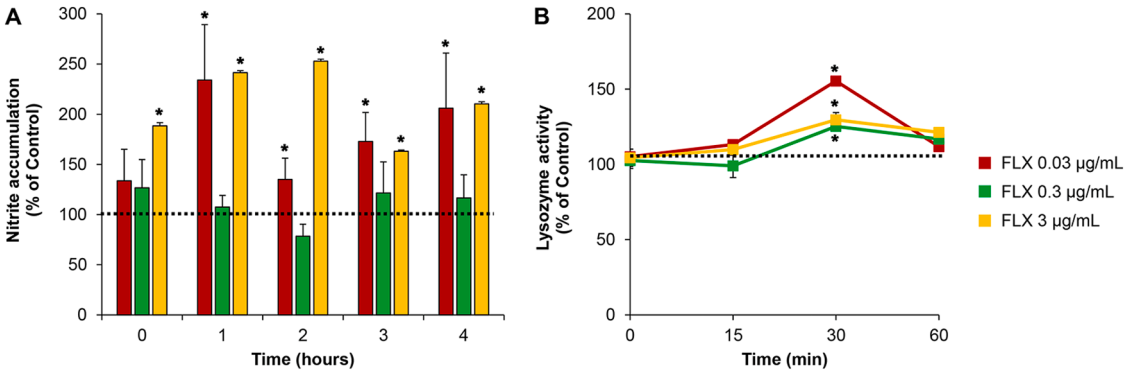

Fig. 2. *In vitro* effects of FLX (0.03, 0.3 and 3 µg/mL) on mussel hemocytes. A) NO production was evaluated by the Griess reaction, which quantifies the nitrite (NO_2^-) content of supernatants; B) Lysozyme release was determined spectrophotometrically at 450 nm using a suspension of *Micrococcus lysodeikticus*. Data, representing the mean ± SD of at least 4 independent experiments, are reported as percent values with respect to controls. * = $p \leq 0.05$, ANOVA + Dunnett's post-test.

(Fig. 2A), however a clear concentration or time dependent trend could not be identified. A transient stimulation of lysozyme release was observed only at 30 min incubation at all concentrations tested (Fig. 2B) with the highest effect at the lowest concentration.

3.1.2. *Effects on lysosomal and mitochondrial compartments*

Hemocyte incubation (60 min) with FLX induced a dose and time dependent lysosomal membrane destabilization, with a large decrease in LMS from the lowest concentration tested (−50 % with respect to controls, $p \leq 0.05$) (Fig. 3A). Confocal microscopy also showed a progressive increase in LTG fluorescence, indicating vacuole acidification, lysosomal fusion and enlargement (Fig. 3B). Fluorescence of MDC, a marker for late-stage autophagosomes, showed a decrease at increasing FLX concentrations (Fig. 3C).

FLX exposure did not induce changes in TMRE fluorescence, indicating that the mitochondrial membrane potential was unaffected (Fig. 4). However, TMRE staining showed the presence of mitochondria within thin intercellular connections. At the highest concentration tested, a strong TMRE signal was associated with large vacuoles, suggesting the possible colocalization of mitochondria within the lysosomal system.

Hemocytes were thus double-stained with LTG and MitoTracker Deep red (MTDR). In control cells, no merged signal (yellow) was observed (Fig. 5, first row, last column), indicating the absence of colocalization. In contrast, FLX-exposed hemocytes showed a progressive increase in yellow puncta within large lysosomal vacuoles, that was evident from 0.03 µg/mL. At higher concentrations (Fig. 5, third and fourth row, last column), a significant correlation between LTG and the MTDR signals was obtained (Pearson's correlation coefficient = 0.6). The results indicate lysosomal internalization of mitochondria induced by FLX exposure.

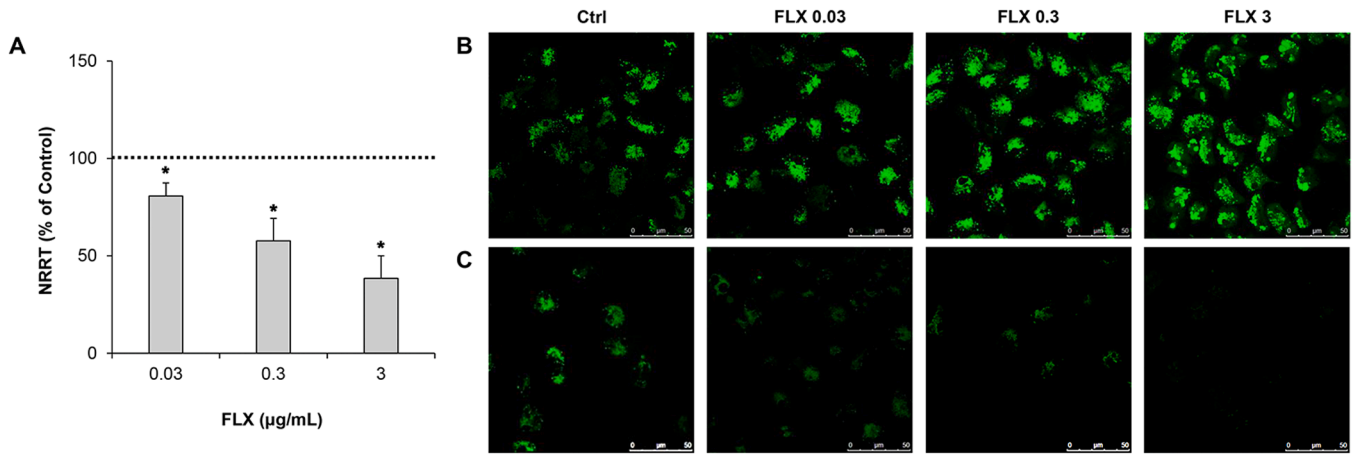

Fig. 3. *In vitro* effects of FLX (0.03, 0.3 and 3 µg/mL, 60 min) on lysosomal compartments of mussel hemocytes. A) Lysosomal membrane stability was evaluated in hemocytes monolayers by the Neutral Red Retention Time (NRR time). Data, representing the mean ± SD of at least 4 independent experiments, are reported as percent values with respect to controls. * = $p \leq 0.05$, ANOVA + Dunnett's post-test. B) and C) Representative confocal images of Lysotracker Green-LTG (A) and Monodansylcadaverine-MDC (B) fluorescence at increasing FLX concentrations.

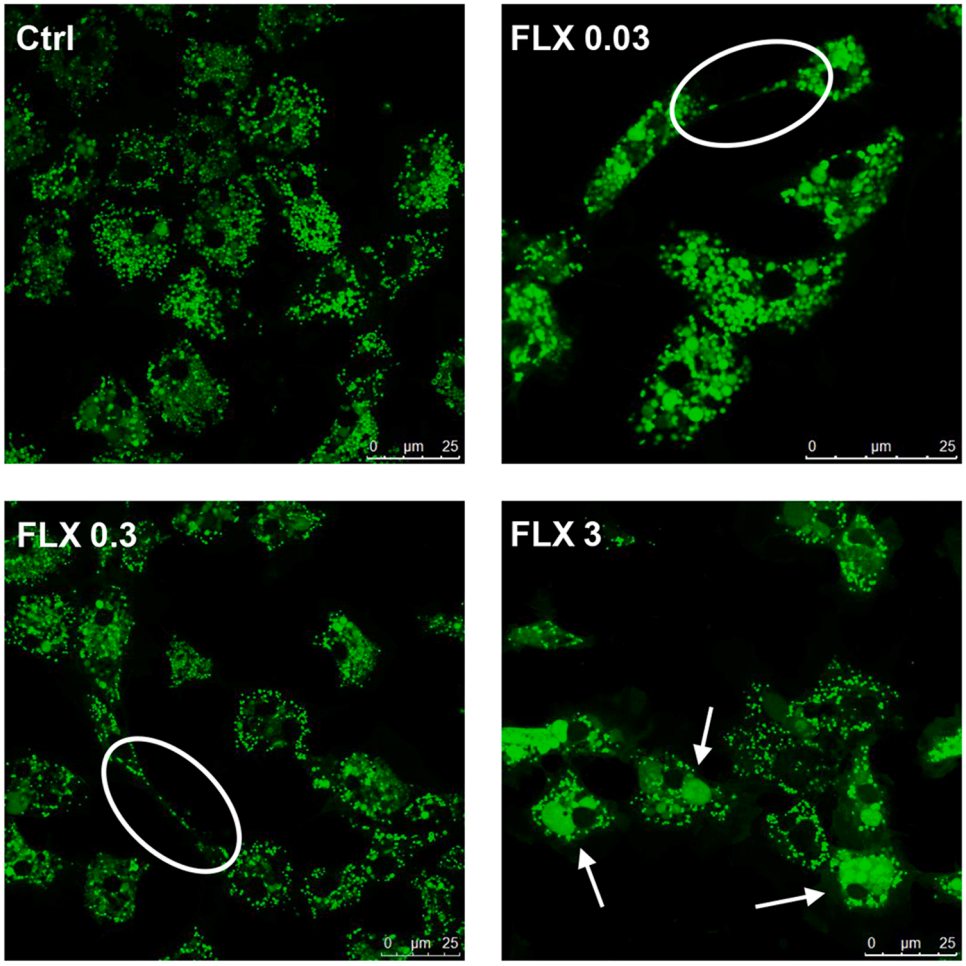

Fig. 4. *In vitro* effects of FLX (0.03, 0.3 and 3 µg/mL, 60 min) on hemocyte mitochondria. Representative confocal images of Tetramethylrhodamine ethyl ester perchlorate-TMRE fluorescence. Circles indicate the presence of mitochondria within thin intercellular connections. Arrows indicate high TMRE fluorescence within large intracellular vacuoles.

3.2. *In vivo exposure to FLX and NFL*

The effects of FLX were investigated in *in vivo* exposure experiments focusing on mussel digestive gland as previously described (Rafiq et al., 2023). Mussels were exposed for 7 days to different environmental concentrations of FLX (0.5, 5, 10, 50 ng/L). Parallel experiments were performed using NFL, the main FLX metabolite.

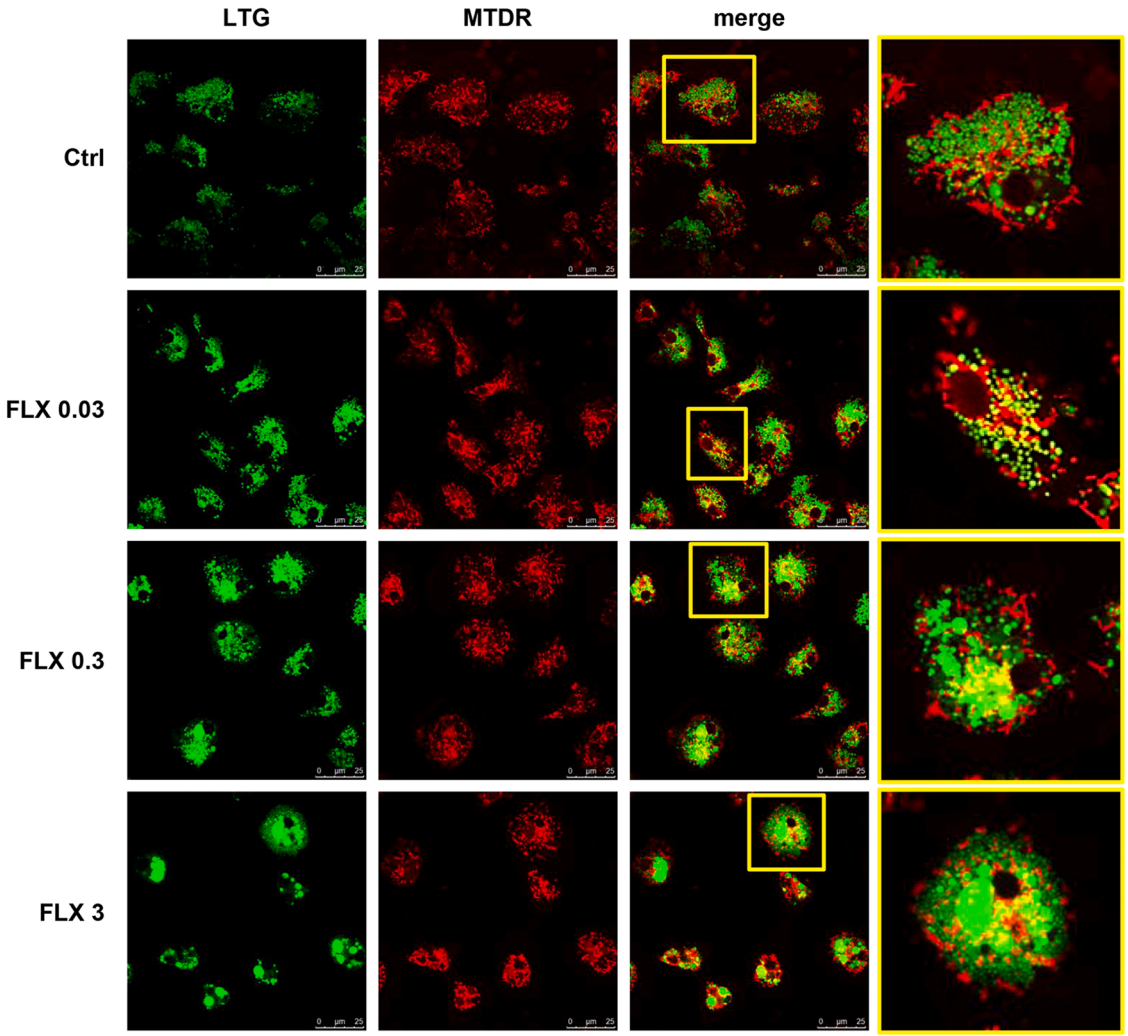

Fig. 5. Confocal images of hemocytes exposed to FLX (0.03, 0.3 and 3 µg/mL, 60 min) after double staining with LTG for lysosomes (green, first column) and MTDR for mitochondria (red, second column). The third column shows the merged signal (yellow squares). In the fourth column, details of hemocytes showing the merged signal that confirms the co-localization of mitochondria within lysosomes are reported.

3.2.1. FLX and NFL concentrations in exposure media

Concentrations of FLX and NFL in sea water and in digestive gland were measured by U-HPLC-MS/MS. Details of quality assurance and performance of the method are provided in Table 1 and SI.

Data obtained in seawater at T0 (after first chemical administration), before each subsequent addition T1 (2 days), T2 (4 days), T3 (6 days) and at the end of experiment T4 (7 days), are reported in Fig. 6 (A and B, respectively) and in Table S2. At T0, the measured concentrations of each compound in exposure medium reflected the nominal added concentrations, with differences for FLX <10 % from 5 to 50 ng/L and of 20 % at 0.5 ng/L. For NFL, differences were from <10 % to 30 % from 5 to 50 ng/L and measured values at 0.5 ng/L were below LOQ (Table S2). At

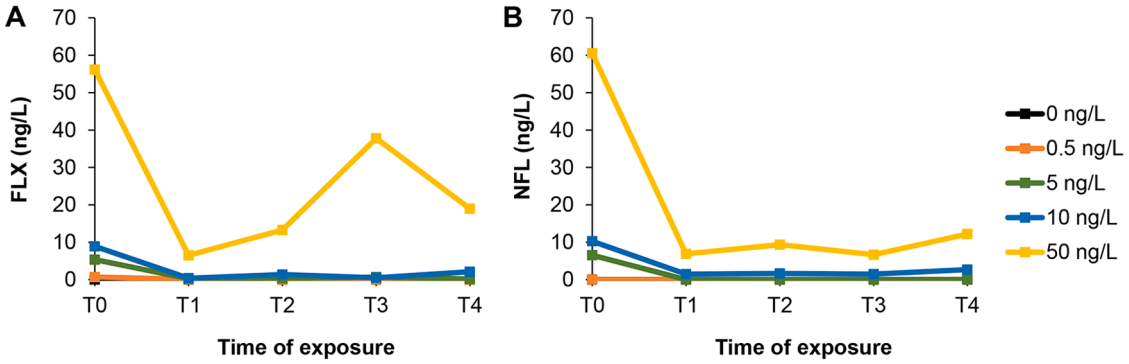

Fig. 6. FLX (A) and NFL (B) concentrations measured in exposure media (ng/L) by U-HPLC-MS/MS. Water samples were collected at T0 (immediately after first chemical administration), and before each water and chemical renewal T1 (2 days), T2 (4 days), T3 (6 days) and at the end of exposure T4 (7 days). Data are the mean of 3 analytical replicates.

lower exposure concentrations, progressive decreases in both FLX and NFL levels were observed from T0 to T4. However, at the highest exposure concentration (50 ng/L), a distinct trend was observed for FLX and NFL. After the large decrease observed after the 2 days of exposure (T1) for both compounds (about 90 %), higher FLX levels were measured at T3 and T4 (up to about 40 and 20 ng/L, respectively), whereas NFL levels remained below about 12 ng/L until the end of exposure. No signal of FLX or NFL was detected in control water samples, nor signal of NFL in water samples from FLX exposure experiments.

3.2.2. FLX and NFL concentrations in digestive gland

In FLX-exposed mussels, a concentration dependent accumulation of FLX was observed, up to about 8 ng/g tissue (w.w.) at 7 days (Fig. 7A); a comparable accumulation of NFL was observed in NFL-exposed mussels (Fig. 7B). Interestingly, in the digestive gland of mussels exposed to higher FLX concentrations, the presence of NFL was also detected, up to about 0.6 ng/g tissue (w.w.) at 50 ng/L (Fig. 7A, inset).

3.2.3. Tissue serotonin levels

Data on 5-HT levels in the digestive gland, evaluated by ELISA and expressed as ng 5-HT/mg protein, are reported in Fig. S1. FLX induced a general increase in 5-HT concentrations from 5 ng/L (about +20 % with respect to controls), although not significant, due to high data variability. No changes in 5-HT levels were observed in mussels exposed to NFL (not shown).

3.2.4. Gene expression

Transcription of 16 selected genes involved in different functions (monoamine signaling, antioxidant and biotransformation response, lysosomal function, autophagy, ceramide metabolism) was evaluated by qPCR. Exposure to FLX and NFL resulted in distinct transcriptional responses, as shown by the heat-maps reported in Fig. 8. As a general trend (Fig. 8A), FLX mainly induced downregulation of transcription. In contrast, in NFL-exposed samples (Fig. 8B), upregulation of several genes was observed from 0.5 to 10 ng/L, followed by downregulation at the highest concentration. However, a clear concentration dependent effect could not be observed for either compound.

Details on the expression level of individual genes are reported in Fig. S2 and S3. FLX exposure did not result in significant effects at the lowest concentration tested (0.5 ng/L) (Fig. S2, A-E). However, significant changes ($p \leq 0.05$) were observed from 5 ng/L: *HEX, GC2* and *aCDase* were downregulated at 5 ng/L (Fig. S2C and E), and *CAT* and *GC2* at 10 ng/L (Fig. S2E). At 50 ng/L, the lysosomal enzyme *CTSL* was the only upregulated gene (Fig. S2C), whereas significant decreases were observed in transcription of both monoamine receptors *5-HTR1* and *DR1* (Fig. S2A), autophagy related genes (*mTor, Atg13, Atg2*) (Fig. S2D), and the ceramide related genes *SP2* and *GC2* (Fig. S2E).

In contrast, as shown in Fig. S3 (A-E), NFL exerted significant effects from 0.5 ng/L, with upregulation of *CAT* (Fig. S3B) and *CTSL* (Fig. S3C) and downregulation of *SP2* and *KDSR2* (Fig. S3E). At 5 ng/L, significant increases in transcription of *DR1, CAT, CTSL* and *GUSB* were observed (Fig. S3A, B, C), and decreases in *Atg2* (Fig. S3D) and *SP2* (Fig. S3E). At 10 ng/L several genes were upregulated, including *DR1, GST*, and all lysosomal genes (Fig. S3A, B, C), whereas *GC2* and *aCDase* were downregulated. At the highest concentration tested (50 ng/L) NFL induced significant downregulation of *mTor* and *Atg13* (Fig. S3D), and of all ceramide related genes (Fig. S3E). Upregulation was observed only for the biotransformation gene *ABCB* (Fig. S3B).

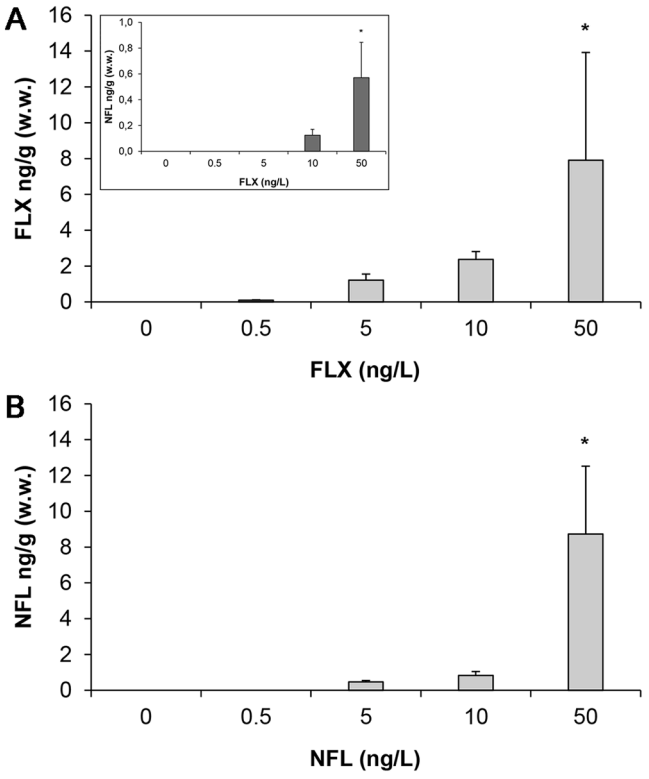

Fig. 7. Concentrations of FLX (A) and NFL (B) in the digestive gland of mussels exposed to either compound (0.5, 5, 10 and 50 ng/L) for 7 days were measured by U-HPLC-MS/MS. A) Bioaccumulation of FLX. Inset: NFL concentrations (dark grey) in FLX exposed mussels. B) Bioaccumulation of NFL. Data are expressed as mean ± SD of 5 samples (each from a pool of 3 mussels). * = $p \leq 0.05$, ANOVA + Dunnett's post-test.

4. Discussion

SSRIs represent significant contaminants of the aquatic environments, whose effects on marine invertebrates, bivalves in particular, are widely documented (Fong and Ford, 2014; Canesi et al., 2022; Zhu et al., 2023). However, little information is still available on their mechanisms of action in these non-target organisms. The results of the present work, obtained utilizing both *in vitro* and *in vivo* approaches, provide novel mechanistic insights into potential toxicity of the model SSRI FLX in the mussel *M. galloprovincialis*.

4.1. In vitro effects of FLX on mussel hemocytes

For a mechanistic exploration of the effects of FLX at the cellular level, short term responses to FLX were first investigated in the *in vitro* model of freshly isolated hemocytes, using a concentration range (0.03, 0.3 and 3 μg/mL, corresponding to 0.1, 1 and 10 μM), similar to those previously utilized both in mammalian cells (Shu et al., 2019; Park et al., 2021) and mussel hemocytes (Franzellitti et al., 2016). FLX induced a dramatic decrease in hemocyte phagocytic activity, paralleled by an increase in both extra and intracellular ROS production, NO production and lysozyme release. The results indicate that FLX induced frustrated phagocytosis and inflammatory processes; however, a concentration-dependent trend could not be observed.

In mammalian neuronal and glial cells, pleiotropic effects of FLX and other antidepressants have been essentially ascribed to the activity of these drugs as lysosomotropic agents. Lysosomotropic compounds are weak lipophilic bases that enter cells in their neutral form and become protonated and therefore trapped within the acidic lysosomes, leading to lysosomal membrane permeabilization and disruption of lysosomal function (Ashoor et al., 2013; Lu et al., 2017). Mussel hemocytes are characterized by an extremely developed lysosomal system, in relation to their role in innate immune response (de la Ballina et al., 2022). Previous data showed that FLX did not induce Multidrug Xenobiotic Resistance (MXR) efflux, resulting in cytotoxicity, although modulation of MRX transporter transcription was observed (Franzellitti et al., 2016). The lack of effect of FLX on MXR activity was partly ascribed to its

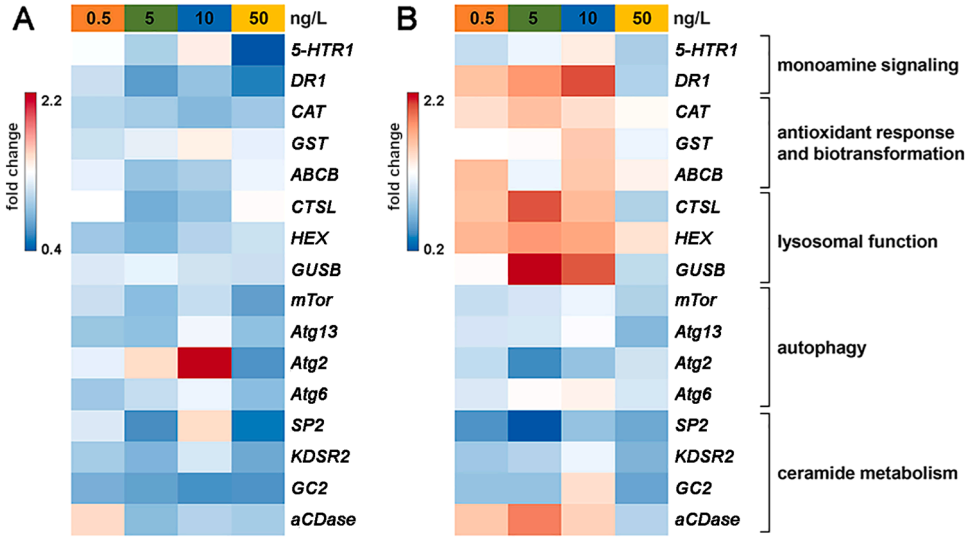

Fig. 8. Effects of *in vivo* exposure to FLX or NFL (0.5, 5, 10, 50 ng/L for 7 days) on gene transcription in mussel digestive gland, evaluated by qPCR. Data are reported as fold-changes with respect to controls. A) FLX; B) NFL. Data are reported as heatmap of transcription levels of genes involved in monoamine signaling (*5-HTR1* and *DR1*), antioxidant and biotransformation response (*CAT, GST, ABCB*), lysosomal function (*CTSL, HEX, GUSB*), autophagy (*mTor, Atg13, Atg2, Atg6*), ceramide metabolism (*SP2, KDSR2, GC2, aCDase*).

lysosomotropic behavior, possibly resulting in its sequestration into lysosomes. Our data show that FLX induced a concentration dependent lysosomal membrane destabilization and acidification, as shown by LMS data and LGT fluorescence, resulting in high lysosomal stress at the highest concentration tested. These results are in line with previous data obtained *in vivo*, indicating that mussel exposure to FLX induced lysosomal destabilization in hemocytes (Rafiq et al., 2023). Overall, the results demonstrate that the lysosomal system represents a direct target for FLX in mussel hemocytes, resulting in altered immune function. All the effects of FLX on functional hemocyte parameters were extremely rapid, in line with the fast response of these cells to both chemical exposure and bacterial challenge (Balbi et al., 2021).

In mammalian cells, FLX also modulates autophagy pathways (Rein, 2019; Shu et al., 2019; Park et al., 2021). The observed decrease in MDC fluorescence suggests that FLX might affect the formation, or decrease the half-life, of late-stage autophagolysosomes; however, MDC alone is not a reliable marker of autophagy (Klionsky et al., 2021). When more specific markers such as LC3II/LC3I and p62 were evaluated by Western blotting as previously described (Balbi et al. 2018), only slight, not significant increases were observed in response to FLX up to 60 min incubation (data not shown), indicating that in mussel hemocytes FLX cannot significantly affect autophagy pathways within short times of exposure.

FLX can also affect the mitochondrial function through different mechanisms (de Oliveira, 2016). In mussel hemocytes, TMRE fluorescence did not indicate changes in mitochondrial membrane potential induced by FLX, but showed the presence of mitochondria in intercellular connections, suggesting mitochondrial transfer between cells, a process through which some cell types (including *Mytilus* immune cells) export their mitochondria to other recipient cells (Auguste et al., 2020). Moreover, at the highest FLX concentrations, TMRE fluorescence was associated with vacuolar structures. Double staining with lysosomal and mitochondrial markers LGT and MTDR confirmed the co-localization of mitochondria within lysosomes, suggesting that FLX promote the lysosomal clearance of damaged mitochondria as previously observed in mammalian astrocytes (Shu et al., 2019).

4.2. *Effects of in vivo exposure to FLX and NFL in mussel digestive gland*

Previous studies showed that mussel exposure to environmental concentrations of FLX affected the function of the digestive gland (Rafiq et al., 2023) a key tissue in metabolism, whose digestive cells are endowed with an extremely developed lysosomal system for intracellular digestion of food particles (Izagirre et al., 2014). Similar results were obtained with the main metabolite NFL (Rafiq et al., 2023). In this work, in the same experimental conditions, tissue accumulation of chemicals was evaluated, and the possible molecular targets underlying the observed effects were investigated.

Measured FLX and NFL concentrations in exposure media reflected nominal exposure concentrations and showed a decrease over time. However, at the highest concentration, after a first large decrease at T1 (corresponding to 2 days of exposure), FLX levels remained higher than those of NFL until the end of exposure. This may reflect differences in the bioaccumulation potential between NFL and FLX observed in whole soft tissues of mussels (Hallmann et al., 2023).

In the digestive gland, a similar concentration dependent accumulation of FLX and NFL was observed. The results are in line with those obtained in mussels exposed to either compound (Silva et al., 2016; Hallmann et al., 2023; Lemaire et al., 2024). The presence of NFL, the main FLX metabolite produced by phase I de-methylation reactions involving CytP450 (Hallmann et al., 2023) in FLX exposed mussels, also confirms previous data (Silva et al., 2016; Lemaire et al., 2024). However, this is the first report of FLX metabolism into NFL in mussel tissues from exposure concentrations as low as 10 ng/L.

The results obtained on gene expression show that exposure to both FLX and NFL affected almost all the selected genes involved in different biological processes, although with no clear concentration dependent effects. However, a distinct transcriptomic response was observed for the parent compound and its metabolite: FLX mainly induced downregulation of gene transcription, whereas with NFL several upregulations were observed at lower concentrations.

These data provide a further insight into the mechanisms of action of FLX in mussel tissues. The serotonin receptor *5-HTR1* showed a bell-shaped trend, with upregulation at lower concentrations followed by downregulation at the highest concentration tested. In these conditions, also the dopamine receptor *DR1* was significantly downregulated. Both monoamine receptors thus appear significant targets for FLX in mussel digestive gland. The effect of FLX on *5-HTR1* expression may be related to its main/classical mechanism of action, via inhibition of 5-HT reuptake by SERT, consequent increase in 5-HT concentration, and negative feedback on receptor expression (Dwyer et al., 2014; Fong and Ford, 2014). Actually, FLX exposure increased 5-HT levels in the digestive

gland; the amount and variability of measured 5-HT concentrations could be ascribed not only to the indirect method utilized, but also to the presence in tissue samples of variable portions of serotonergic neurons of the visceral ganglia (Canesi et al., 2022; Fabbri et al., 2024; Risso et al., 2025). The results support the hypothesis that inhibition of 5-HT uptake represents a conserved target of FLX (and possibly other SSRIs) in mussels. Moreover, in vertebrates, SSRIs can also act on other components of 5-HT signalling, as well as of dopaminergic transmission (Dwyer et al., 2014). Although NFL is more potent than the parent compound (Krout et al., 2017), NFL exposure did not affect *5-HTR1* expression, whereas it induced significant upregulation of *DR1*, indicating dopaminergic signaling as a potential target for NFL in mussel digestive gland. Chronic administration of FLX was shown to increase *DR1* receptor expression in mature granule cells of mice dentate gyrus (Shuto et al., 2020), possibly due to NFL accumulation: however, to our knowledge, no data are available on the effects of NFL on *DR* expression in invertebrate systems.

Based on the effects here obtained in isolated hemocytes, and previous data obtained *in vivo* on changes of hemocyte and digestive gland lysosomal biomarkers (Rafiq et al., 2023), lysosomes appear to represent another direct target of both FLX and NFL, acting as lysosomotropic compounds in mussel cells. In hemocytes, the endo-lysosomal system is involved in innate immunity (Balbi et al., 2021; de la Ballina et al., 2022). In digestive cells, it plays a key role in the uptake and digestion of food materials, as well as in xenobiotic accumulation and detoxification (Izagirre et al., 2014). Among lysosomal genes, only Cathepsin L (*CTSL*) was significantly upregulated by FLX exposure, whereas N-acetyl-β-hexosaminidase (*HEX*), and β-glucuronidase (*GUSB*) were unaffected; in contrast, NFL induced a significant increase in expression of all three genes, to a different extent at different concentrations.

Lysosomal genes also participate in regulation of autophagic processes. Cathepsins, including CTSL, catalyze the cleavage of peptide bonds of different autophagy substrates, thus disposing of the autophagic flux (Kaminskyy and Zhivotovsky, 2012). HEX and GUBS are involved in breakdown of glycolipids, glycoproteins, and glycosaminoglycans (GAGs), and represent key enzymes in lysosomal degradative capacity (Izagirre et al., 2014), which is critical for autophagic flux. Both FLX and NFL induced downregulation of *mTor, Atg2* and *Atg13* transcripts, that are key components in the control of dynamic membrane events during autophagosome biogenesis and have been suggested as molecular markers of autophagy in mussels (Xie et al., 2022). The results indicate that interference with autophagic pathways can represent a novel action for both FLX and NFL in mussel cells. In invertebrate systems, perturbation of autophagic pathways has been linked to modulation of cAMP/PKA signaling, where the interplay between cAMP and autophagy represents a significant regulatory network influencing immunity, development, and metabolic responses (Yamada et al., 2019). A rising body of literature points towards a conserved mechanism across species, where cAMP acts not only as a second messenger but also as a facilitator of key cellular processes such as autophagy, which is critical for the survival and adaptation of invertebrates under various physiological conditions (Yamada et al., 2019). In this light, the observed changes in expression of autophagy related genes in mussel digestive gland in response to FLX may be related to the effects of this compound previously observed on cAMP signaling in mussel cells (Canesi et al., 2022; Fabbri et al., 2024).

The effects of antidepressants on autophagic pathways have been also linked to ceramide metabolism (Gulbins et al., 2015, 2018; Rein, 2019; Shu et al., 2019; Li et al., 2023). Ceramides are membrane sphingolipids that play key roles in the complex mechanisms (membrane adhesion, fusion, and lipid exchange events) occurring during autophagy (reviewed in Varela et al., 2024). The ceramide pathway plays a central role in the pathogenesis of major depressive disorders and has been proposed as a new target for antidepressants (Kornhuber et al., 2014). Our data, indicating a general interference of both FLX and NFL on expression of ceramide related genes, possibly leading to changes in ceramide levels, are in line with these observations. In particular, FLX induced downregulation of *GC2* and *aCDase*, enzymes involved in ceramide biosynthesis and breakdown, respectively. A distinct effect was observed with NFL, that induced downregulation of *SP2* and *KSDR2*, the key limiting enzymes in *de novo* synthesis of ceramides, and increases in *GC2* and *aCDase*. Actually *aCDase*, together with *DR1*, was the most upregulated gene by NFL.

Finally, with regards to transcription of antioxidant and biotransformation enzymes, the results do not indicate induction of oxidative stress or phase II conjugation and efflux activities by FLX, in line with previous data obtained in mussel hemocytes (Franzellitti et al., 2016). In contrast, NFL increased expression of *CAT, GST* and *ABCB*; accordingly, in the same experimental conditions, NFL, but not FLX, induced CAT activity in mussel digestive gland (Rafiq et al., 2023).

Overall, the results provide additional, novel insights into the mechanistic basis for FLX action in mussels. In bivalves, Fay et al. (2017) outlined 4 different Adverse Outcome Pathways (AOPs) for SSRIs (AOPs 97, 195, 203, 204, https://aopwiki.org), leading to population changes (increase/decrease) and decreased reproductive success. Inhibition of 5-HT transport was utilized as the presumptive Molecular Initiating Event (MIE), based on the assessment of the high degree of conservation of the SERT target across different taxa. Key Events (KE) and Adverse Outcomes (AO) were established from data retrieved from USEPA ecotoxicology database (http://cfpub.epa.gov/ecotox). With the increase in available data on components of the serotonergic system and the identification of additional molecular targets for SSRIs, more detailed AOPs for FLX in bivalves can be drawn (Fig. 9).

As to the MIE, SERT orthologs have been phylogenetically inferred in few molluscs, including bivalves (Canesi et al., 2022; Goulty et al., 2023) and, more recently, they have been identified in *M. galloprovincialis* larvae, where their expression increases throughout development (Risso et al., 2025). Moreover, the results of the present work support a direct lysosomotropic action of FLX in bivalve cells, indicating an additional MIE for this compound. At the cellular level, changes in *5-HTR1* expression, modulation of [cAMP]/PKA signalling and lysosomal stress can result in altered immune function in the hemocytes (this work; Canesi et al., 2022; Rafiq et al., 2023; Fabbri et al., 2024). In the digestive gland, changes in lysosomal biomarkers (lysosomal destabilization, neutral lipids and lipofuscin accumulation) (Rafiq et al., 2023) and of expression of lysosomal, autophagy and ceramide related pathways (this work), can ultimately affect physiological processes at tissue level (digestion and detoxification). These effects would potentially result in immunodepression and altered metabolism at the individual level. At population level, possible increases in susceptibility to disease and alterations of metabolic processes supporting growth/reproduction can be envisaged. In this context, the obtained results underscore the importance of generating novel mechanistic insights into antidepressant toxicity in non-target marine invertebrates for improvement of ecological risk assessments in coastal ecosystems.

However, in realistic environmental conditions, FLX does not occur as an isolated compound, but rather as part of complex mixtures with other SSRIs or other antidepressants, their metabolites, and other pollutants, possibly resulting in complex interactive effects (Drzymała, 2025; Liu et al., 2025). Understanding these interactions is therefore essential for ecological risk assessment and development of strategies to mitigate the effects of these contaminants (Liu et al., 2025). Increasing knowledge on the possible multiple mechanisms of action of different model antidepressants, based on their frequency of detection in aquatic compartments, on key non target species, may contribute to identify and predict the biological pathways most affected by each compound, alone and in combination. Moreover, in a global change scenario, changes in sea water pH and temperature may affect antidepressant behaviour, their consequent uptake, biotransformation and bioaccumulation, and related physiological responses (Lo et al., 2021; Aulsebrook et al., 2022; Li and Gaitan-Espitia, 2024; Liu et al., 2025). Since the sensitivity of different invertebrate groups to antidepressants has been increasingly

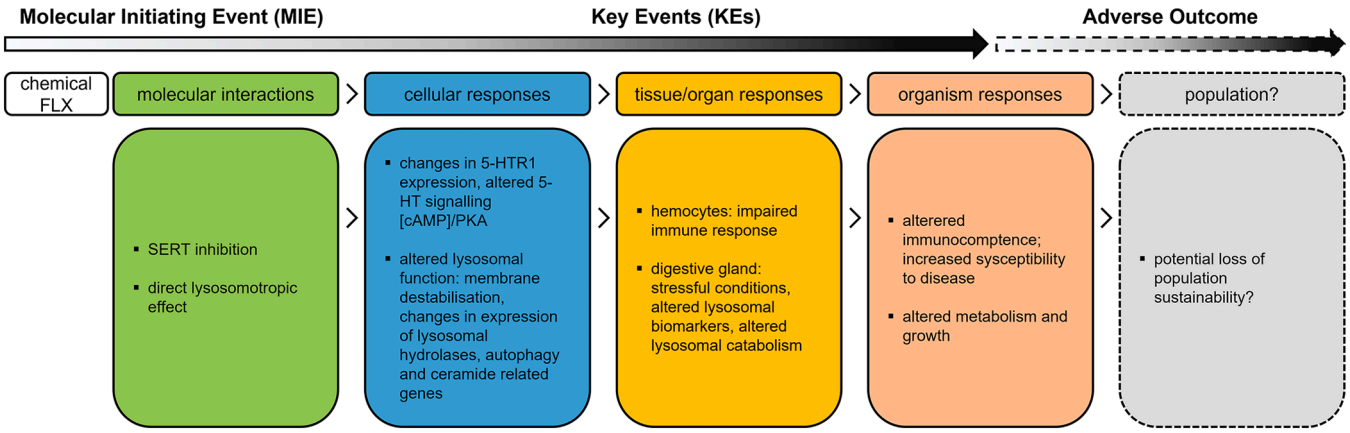

Fig. 9. Potential Adverse Outcome Pathway (AOP) of model SSRIs in bivalve molluscs, based on data obtained with environmental concentrations of FLX.

recognized, further research is needed to explore the effects of SSRI mixtures, their combination with other contaminants, and in the presence of predicted conditions of seawater warming and acidification, in representative species of coastal environments (Franzellitti et al., 2020; Sokolova et al., 2026).

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CRediT authorship contribution statement

Teresa Balbi: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Paola Valbonesi:** Writing – original draft, Methodology, Investigation, Data curation. **Marilin Profita:** Writing – original draft, Methodology, Investigation, Data curation. **Caterina Ciacci:** Writing – original draft, Methodology, Investigation, Data curation. **Elena Fabbri:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Laura Canesi:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2026.107717.

Data availability

Data will be made available on request.

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