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Antidepressants and their metabolites primarily affect lysosomal functions in the marine mussel, *Mytilus galloprovincialis*

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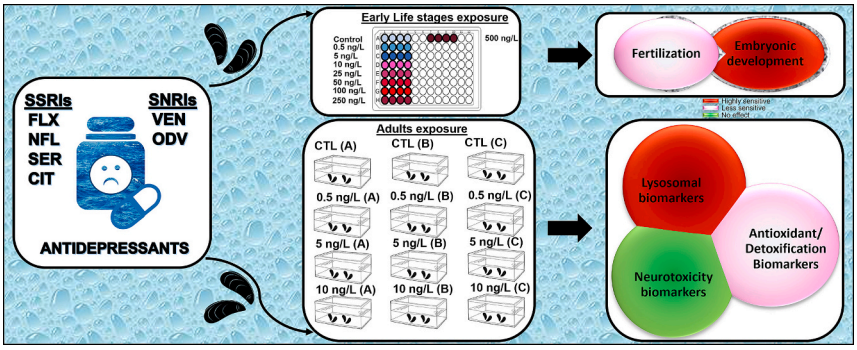
HIGHLIGHTS

- At the low environmental concentrations, antidepressants affect mussel health status.
- Lysosomes are the main target of all tested antidepressants.
- SSRIs, but not SNRIs, cause embryotoxicity.
- SSRIs, but not SNRIs, affect antioxidant/detoxification enzymes.
- Drug metabolites, NFL and ODV, are as effective as the parent compounds.

GRAPHICAL ABSTRACT



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ABSTRACT

Antidepressants widely occur as emerging contaminants in marine coastal waters, with concentrations reported in the low ng/L range. Although at relatively lower levels with respect to other pharmaceuticals, antidepressants – fluoxetine (FLX) in particular - have attracted attention because of their striking effects exerted at low doses on marine invertebrates. In this study, the effects of four antidepressants including FLX, sertraline (SER), and citalopram, as members of the selective serotonin reuptake inhibitor (SSRI) class, and venlafaxine (VEN) as a member of the serotonin and norepinephrine reuptake inhibitor (SNRI) class, were evaluated in the mussel *Mytilus galloprovincialis*. In addition, the effects of two main metabolites of FLX and VEN, i.e., norfluoxetine (NFL) and *O*-desmethylvenlafaxine (ODV) respectively, were compared to those of the parent compounds. Eight concentrations of each drug (0.5–500 ng/L range) were tested on the early life stage endpoints of gamete fertilization and larval development at 48 h post fertilization (hpf). Egg fertilization was reduced by all compounds, except for VEN. Larval development at 48 hpf was affected by all SSRIs, but not by SNRIs. The above effects were significant but never exceeded 20 % of control values. Adult mussels were exposed in vivo for 7 days to environmental concentrations of the drugs (0.5, 5, and 10 ng/L) and a battery of eight biomarkers was assessed. Antidepressants primarily targeted lysosomal functions, decreasing haemocyte lysosome membrane stability (up to 70 % reduction) and increasing of the lysosome/cytosol ratio (up to 220 %), neutral lipid (up to 230 %), and lipofuscin (up to 440 %) accumulation in digestive gland. Only SER and NFL significantly affected catalase and

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glutathione-S-transferase activities in gills and digestive gland. NFL and ODV, were effective and sometimes more active than the parent compounds. All compounds impaired mussel health status, as indicated by the low to high stress levels assigned using the Mussel Expert System.

1. Introduction

In the last decades, continuous improvements of analytical techniques have documented the worldwide occurrence of pharmaceuticals, personal care products, plastic additives, surfactants, flame retardants, etc. in the aquatic environment. These contaminants, referred to as contaminants of emerging concern (CEC), have the potential to pose risks to the ecosystem yet are not subjected to regular monitoring plans or legislative standards aimed at preventing their impacts (León and Bellas, 2023).

After consumption, pharmaceuticals are excreted by human patients or animals as the parent compound or metabolites which enter the environment. Being continuously released in wastewaters and only partially removed by most wastewater treatment-plants (WWTPs), they become pseudo-persistent contaminants within aquatic environments (Barceló and Petrovic, 2007). Pharmaceutical occurrence is reported in effluents from WWTPs (Paíga et al., 2019), as well as in surface (Zuccato et al., 2000) and drinking waters (Pereira et al., 2021). Investigations in the marine environment are relatively scarce, due to the complexity of the matrix, the low concentrations of chemicals, and the opinion that dilution was per se a safety factor (Fabbri et al., 2023).

However, in contrast to conventional pollutants, pharmaceuticals are designed to produce specific therapeutic effects at very low doses, by acting on specific targets (i.e., membrane receptors, transporters, etc.) that are possibly conserved during evolution (Fabbri et al., 2023). Therefore, concern for the health of aquatic animals exposed to environmental concentrations of pharmaceuticals cannot be ruled out.

Among the different classes of pharmaceuticals, antidepressant drugs are used primarily to treat or prevent mood disorders, and also administered for therapies against generalized anxiety, post-traumatic stress, obsessive and compulsive disorders, and even chronic pain (Maulvault et al., 2021). The average consumption of antidepressants in Europe has increased by about 2.5 times in the last two decades up to a maximum of 131 and 153 Daily Defined Dosage (DDD) per 1000 people in Portugal and Iceland, respectively (https://stats.oecd.org/Index.aspx?QueryId=30135 accessed on May 24, 2023). Antidepressants are included within the 45 top prescribed pharmaceuticals in the world (https://clincalc.com/DrugStats/Top200Drugs.aspx accessed on May 24, 2023). Fluoxetine (FLX) is the prototypical antidepressant being the first developed as a Selective Serotonin Reuptake Inhibitor (SSRI), and the most widely prescribed antidepressant under the brand name Prozac® (registered in 1985) (López-Muñoz and Alamo, 2009). Soon after, other SSRIs including fluvoxamine, paroxetine, sertraline (SER), citalopram (CIT), were introduced to the market (Vera-Chang et al., 2019). Despite SSRI specificity, not all patients experienced successful therapeutic results, this leading to new antidepressants with different pharmacodynamic properties. In this scenario, the serotonin and norepinephrine reuptake inhibitors (SNRIs) were designed including venlafaxine (VEN) and duloxetine (Ye et al., 2011).

FLX is metabolised by *N*-desmethylation in humans yielding mainly norfluoxetine (NFL), which also inhibits serotonin reuptake from the synaptic cleft (Silva et al., 2012). The excretion of FLX and its metabolites occurs approximately 80 % in the urine (containing about 20 % FLX and NFL, and 15 % glucuronide derivatives) and 15 % in the feces (Andrés-Costa et al., 2017; Silva et al., 2015). VEN is excreted in urine as the unchanged compound (about 5 %) and is transformed into metabolites in the liver, mainly *O*-desmethylvenlafaxine (ODV), which represents 56 % of the excreted form (Magalhães et al., 2014).

Wastewater discharges, consisting of untreated or incompletely treated sewage, are the main source of pharmaceuticals in surface waters, and marine waters represent the final sink (Fabbri et al., 2023). FLX concentrations in coastal environment are reported in the range of 1–10 ng/L (Biel-Maeso et al., 2018; Gonzalez-Rey et al., 2015; Gros et al., 2012; Vasskog et al., 2008) with highest values in urban estuaries (7.4–596 ng/L; Benotti and Brownawell, 2007). CIT has been reported at 0.6–8 ng/L in coastal waters from Santorini Island (Mezzelani et al., 2018b) and around the Fiji Islands (Dehm et al., 2021); paroxetine at 0.6–44 ng/L along Norway coast (Mezzelani et al., 2018b); SER at 22 ng/L and VEN at 7 ng/L (Dehm et al., 2021; Mole and Brooks, 2019). VEN was detected in European coastal waters with concentrations as high as 291 ng/L reported along the north western coast of Spain (Fernández-Rubio et al., 2019). In 4 Portuguese river estuaries, the sum of 11 antidepressants reached concentrations of 715 ng/L (Duarte et al., 2023).

NFL and ODV levels in the marine environment have rarely been reported. Although several attempts have been made, the lack of NFL detection in marine waters has been related to relatively high limits of quantification by the analytical methods (Wilkinson et al., 2022). Data on bioaccumulation in *M. galloprovincialis* sampled along the Portuguese Atlantic coast confirm the occurrence of NFL in seawater; average NFL concentrations of 13.5 ng/g d.w. compared to 4.8 ng/g d.w. FLX were reported (Silva et al., 2017). Higher bioaccumulation is in agreement with the low polarity of the metabolite with respect to the parent compound, being therefore more prone to bioaccumulation (Gelsleichter and Szabo, 2013). ODV and VEN were found at significant concentrations in seawater estuaries along the northeast coast of Scotland, with maximum values of 159 and 94 ng/L, respectively in the Clyde estuary, while concentrations were lower than 10 ng/L in samples from the Forth estuary (McKenzie et al., 2020).

Despite the relatively low levels of antidepressants compared with other pharmaceuticals (Mezzelani et al., 2021), the occurrence of antidepressants in coastal waters has attracted attention because of their reported effects on marine invertebrates (Ford and Fong, 2016; Franzellitti and Fabbri, 2014; Sumpter et al., 2014), which are exerted at very low doses and over short exposure times. Di Poi et al. (2014) reported that cognitive abilities were impaired at 1 ng/L FLX in young cuttlefish (*S. officinalis*), and juveniles exposed to a mixture of FLX/VEN (2.5 ng/L of each substance) showed decreases in sand-digging behaviour within a week. At concentrations as low as 0.3 ng/L FLX decreased cAMP levels and protein kinase A activities in digestive gland and mantle/gonads of mussels exposed for 7 days (Franzellitti et al., 2014).

Since most studies carried out in marine invertebrates focused on FLX, the aim of the present study was to compare the biological impacts of four antidepressants including FLX, CIT, and SER as members of the SSRI class, and VEN as member of the SNRI class; in addition, the potential effects of two main metabolites of FLX and VEN, i.e. NFL and ODV respectively, were evaluated since metabolites are excreted together with the parent compounds and may contribute to the cumulative toxicity of antidepressants toward aquatic organisms (Ma et al., 2018). VEN and ODV were chosen because they have recently been included as new compounds in the revised Watch List for priority substances (EU Commission, 2020) requiring further monitoring data, in freshwater. Although we are aware that drugs occur in the environment as mixtures, and that their combination can produce unexpected effects (Mezzelani et al., 2023), single compounds were applied here to better understand and compare their individual contributions.

The Mediterranean mussel, *M. galloprovincialis*, was selected as the model species. *Mytilus* spp. live in intertidal zones across the world's coasts. As a filter-feeding, sessile organism *Mytilus* can bioaccumulate dissolved substances in gills and digestive gland and the species has been

recognized as ideal for assessing the impact of pollutants in the seawater column (Viarengo and Canesi, 1991).

The effects of several concentrations of the six compounds were assessed for possible impairment of mussel gamete fertilization and embryonic development, at 48 h post fertilization (hpf) (Fabbri et al., 2014). Exposure concentrations spanned the range reported for marine coastal waters (0–500 ng/L).

A set of cellular, biochemical, and physiological responses, i.e., biomarkers (OSPAR Commission, 2013; Viarengo et al., 2007) was investigated to evaluate adult mussel health status after in vivo exposure to the six compounds at three different concentrations within the low environmental range of antidepressants (0.5, 5 and 10 ng/L). The applied battery of biomarkers included (i) lysosomal parameters of general stress, such as the lysosomal membrane stability (LMS), the lysosome/cytosol volume ratio (LYS/CYT), and neutral lipids (NL) accumulation; (ii) oxidative stress and biotransformation biomarkers, including the activities of the enzymes glutathione S-transferase (GST) and catalase (CAT), as well as malondialdehyde (MDA) and lipofuscin (LF) contents; and (iii) response to neurotoxic stimuli as assessed using acetylcholinesterase activity (AChE).

Although antidepressants likely interact with specific receptors/transporters in mussels, biomarker evaluation was preferred in this experiment to assess possible effects of pharmaceuticals beyond their mechanism of action, and the potential of relatively easy and cost-effective methodologies for biomonitoring.

2. Materials and methods

2.1. Chemicals

Mussels were exposed to the SSRI antidepressants fluoxetine ((±)-N-Methyl-γ-[4-(trifluoromethyl)phenoxy]benzenepropanamine) hydrochloride (FLX) and its metabolite norfluoxetine (γ-[4-(trifluoromethyl) phenoxy]-benzenepropanamine) monohydrochloride (NFL), sertraline ((1*S*,4*S*)-4-(3,4-Dichlorophenyl)-1,2,3,4-tetrahydro-*N*-methyl-1-naphthalenamine) hydrochloride (SER) and citalopram (1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobensofurancarbonitrile) hydrobromide (CIT) and to the SNRI antidepressant venlafaxine ((+/−)-1-[2-(Dimethylamino)-1-(4-methoxyphenyl) ethyl] cyclohexanol) hydrochloride (VEN), and its metabolite *O*-desmethylvenlafaxine (4-[2-dimethylamino)-1-(1-hydroxycyclohexyl) ethyl] phenol) (ODV). The four antidepressants and the metabolite ODV were purchased from Merck Life Science, Italy, while NFL was purchased from Cayman Chemical, USA. A stock solution of each chemical was prepared at a concentration of 0.2 mg/mL using the organic solvent dimethyl sulfoxide (DMSO), since the chemicals were poorly soluble in water, then aliquoted and stored at −20 °C until use. In preliminary trials, the effects of DMSO at the highest concentrations used (i.e., 0.00025 and 0.000005 % DMSO for early life stages and adult mussels, respectively) were tested in parallel with seawater controls and no effect of DMSO was observed in any of the parameters investigated. All other reagents were of the highest commercially available grade.

2.2. Animal holding and experimental procedures

Adult marine mussels *M. galloprovincialis* (4–6 cm length), were obtained from COPRALMO aquaculture farm (Cesenatico, Italy). After collection, mussels were immediately acclimatized for five days under laboratory conditions (filtered seawater - FSW, 12 h light:12 h dark photoperiod, 16 ± 2 °C) at 5 mussels/L before being exposed to experimental conditions at 1 mussel/L. For early life stages investigations, sexually mature mussels were purchased and kept at 16 °C for 4 to 6 h until spawning (ASTM, 2004).

2.2.1. Early life stage tests

This study evaluated the effects of four antidepressants and two metabolites on mussel early life stage parameters, including gamete fertilization and embryonic development at 48 hpf. To investigate these effects, in vitro testing of 8 different concentrations of each chemical (in the range 0.5–500 ng/L) was conducted in quadruplicate ($n = 4$) using 96-well microplates. Controls containing only FSW were run in parallel to each assay.

2.2.1.1. Fertilization and embryotoxicity tests. Sexually mature specimens of *M. galloprovincialis* were purchased during the spawning season (November–February), transferred to the laboratory and distributed in tanks containing aerated FSW. Gamete collection, oocyte fertilization and sample handling were performed following the procedure described by Capolupo et al. (2020). When mussels began to spontaneously spawn, each individual was immediately transferred to a 250 mL beaker containing 200 mL of aerated FSW until complete gamete emission. Gametes used in the following tests were always collected from 5 parental pairs and combined. After checking egg quality (shape, size) and sperm motility, sperms (250 per well) were exposed to different concentrations of each chemical for 1 h, then oocytes were added in a 1:5 egg:sperm (50 eggs per well). After 30 min, the fertilization process was stopped by adding calcium buffered formalin (4 %) and rate of fertilization examined under an inverted microscope at 40× magnification. The criteria for fertilization success were the appearance of polar lobe or the cleavage stage (Capolupo et al., 2018). Average fertilization success in controls was >95 %.

Embryonic development was assessed following the ASTM acute embryotoxicity test (ASTM, 2004) adapted for 96-well microplates (Fabbri et al., 2014). Untreated oocytes and spermatozoa (50:250) were exposed to different concentrations of chemicals into wells, then incubated for 48 h. At 48 hpf, the experiment was stopped by adding calcium buffered formalin (4 %) and larvae examined under the inverted microscope. Larvae were considered normal when the shell was D-shaped (straight hinge) and the mantle did not protrude out of the shell, and abnormal if the 48 hpf larvae had not reached the typical stage (D-veliger) or when showing developmental defects (concave, convex or damaged shell, protruding mantle). The recorded endpoint was the percentage of normal D-veliger in each well compared to the total number.

The acceptability of test results was based on controls for a percentage of normal D- veligers >70 % (ASTM, 2004; Fabbri et al., 2014). Details on methodologies are reported in Supplementary Material.

2.2.2. Adult mussels

Acclimatized adult mussels were treated with the four antidepressants and two metabolites at three different concentrations within the low environmental range (0.5, 5, 10 ng/L). For each treatment, 45 mussels were randomly divided in 3 tanks (3 replicates), at a density of 1 mussel/L, for 7 days, as previously described (Franzellitti et al., 2015). In parallel with the treatment groups, a group of unexposed mussels was also maintained as a control. Exposures were conducted under controlled laboratory conditions i.e., temperature (16 ± 2 °C), photoperiod (12 h light:12 h dark), and feeding of mussels (5×10^6/L of green algae *Nannochloropsis oculata,* BlueBiotech, Büsum, Germany*)* in line with previous experiments (Capolupo et al., 2021). FSW, chemicals, and food were renewed daily.

2.2.2.1. Biomarker analysis. Exposed adult mussels were assessed using a battery of eight biomarkers, as suggested by the OSPAR guidelines (OSPAR Commission, 2013). Tissues of choice were haemolymph, gills, and digestive gland. Haemolymph was collected from the posterior adductor muscle using a sterile 1-mL syringe containing physiological saline solution and used to evaluate lysosomal membrane stability (LMS) in haemocytes. These cells exert an immune function in mussels and contain a significantly high number of lysosomes making them suitable for assessing this highly sensitive biomarker (Viarengo et al.,

2007). Gills are responsible for respiration through gas exchange and nutrition through the uptake of food particles (Viarengo and Canesi, 1991), so this tissue represents an interface between the external and internal environment. Digestive gland has proven to be a suitable tissue for identifying the impacts of pharmaceuticals as previous studies have shown (Cortez et al., 2019; Franzellitti et al., 2014, 2015). Bioaccumulation and metabolism of hydrophobic compounds have also been reported in this tissue (Venier et al., 2006).

Haemolymph was analyzed immediately after being collected, while other tissues (gills and digestive gland) were stored at −80 °C for biomarker analysis. LMS was assessed according to the Neutral Red Retention Assay (Martínez-Gómez et al., 2015; UNEP/RAMOGE, 1999), using 4 mussels/replicate for each treatment.

The protocol of Capolupo et al. (2021) was used to assess histological biomarkers namely lysosome/cytosol ratio (LYS/CYT), neutral lipid (NL), and lipofuscin (LF) accumulation. In order to determine these parameters, 10 μm thick cryo-sections of digestive glands from 4 mussels per replicate were prepared according to a protocol defined in UNEP/RAMOGE (1999). Spectrophotometric measurements of three enzymes glutathione S-transferases (GST), catalase (CAT), and acetylcholinesterase (AChE) and content of malondialdehyde (MDA) were conducted on supernatants after homogenizing and centrifuging tissues (gills and/or digestive glands) from 6 mussels per replicate of each treatment, for a total of 18 mussels per experimental conditions. The activity of GST and CAT was measured in gills and digestive gland of mussels, as previously reported by Capolupo et al. (2017). Gill AChE activity was assessed as described by Valbonesi et al. (2003). MDA content was assessed in digestive glands as reported by Franzellitti et al. (2014). Details on methodologies are reported in Supplementary Material.

2.3. Statistical analysis

Data were analyzed using SigmaPlot 12 (Systat Software Inc. San Jose, CA, USA) and Primer v6 (PRIMER-ELtd., Albany, New Zealand). All data were tested for normality using the Shapiro-wilk test and for variance equality using the Levene's test. The data were then subjected to a One-way Analysis of Variance followed by Bonferroni *post-hoc* test to assess for statistical significance. The pairwise multiple comparison test (Suppl Mat, Table S1) was run to compare the dose-dependent responses in mussels after exposure to the chemicals. Potential relationships between the analyzed biomarkers were assessed using the Pearson's correlation test. Statistical significance for the above tests was accepted when $p < 0.05$. Biomarker data were subjected to a Principal Component Analysis (PCA) to allow for a comparable interpretation of the obtained results. Based on the responses at the cell and tissue levels (biomarkers), and organism level (embryos), the Mussel Expert System (Dagnino et al., 2007) was applied to integrate the results and quantify the degree of stress induced syndrome by pharmaceutical exposure.

3. Results

3.1. Early life stages tests

3.1.1. Fertilization test

The effects of the tested antidepressants and metabolites on mussel gamete fertilization are illustrated in Fig. 1. The mean fertilization success of mussels ranged from 96.0 to 99.7 % in FSW controls, which is within the acceptable range for test acceptance (Environment Canada, 2011). SSRIs and SNRIs had significant effects on fertilization success when compared with controls. SER significantly reduced gamete fertilization in the widest concentration range, from 5 to 500 ng/L reaching about 15 % inhibition. FLX showed small but significant effects at 100, 250, and 500 ng/L (about 4 % inhibition), while CIT only caused effects at the highest tested concentration (about 10 % at 500 ng/L). In addition, the SSRI metabolite NFL and SNRI metabolite ODV induced significant effects within a wide range of concentrations, with maximum inhibition of about 6 %. The success of gamete fertilization was not affected by exposure to VEN.

3.1.2. Embryotoxicity

The effects on embryonic development observed after exposure to antidepressants and their metabolites are shown in Fig. 2. In the control groups, the mean percentage of normally developed D-veliger ranged from 95.8 to 97.9 %, i.e., within accepted test values (ASTM, 2004). With respect to controls, SSRIs (FLX, NFL, SER and CIT) exposure significantly inhibited embryonic development. FLX induced significant reduction (about 11 %) at the highest concentrations (100, 200 and 500 ng/L), whereas NFL significantly affected the normal development of embryos over a wider range (10 to 500 ng/L) with maximum reduction of normal D-veliger by about 15 %; effects on embryonic development were also noted by SER (in the range 25–500 ng/L) and CIT (25, 100 and 500 ng/L), never exceeding 20 % reduction versus controls. Embryos treated with FLX and SER showed primarily a delayed development, remaining at the trocophore stage at 48 hpf, while CIT caused mainly abnormalities (data not shown). The SSRI VEN and its metabolite ODV did not show any significant effect on embryonic development.

Overall, the compounds tested in the range 0.5–500 ng/L induced significant but rather small effects on fertilization and larval development at 48 hpf, with lower than 20 % difference versus control values. Furthermore, VEN did not affect egg fertilization and VEN and ODV did not affect larval development.

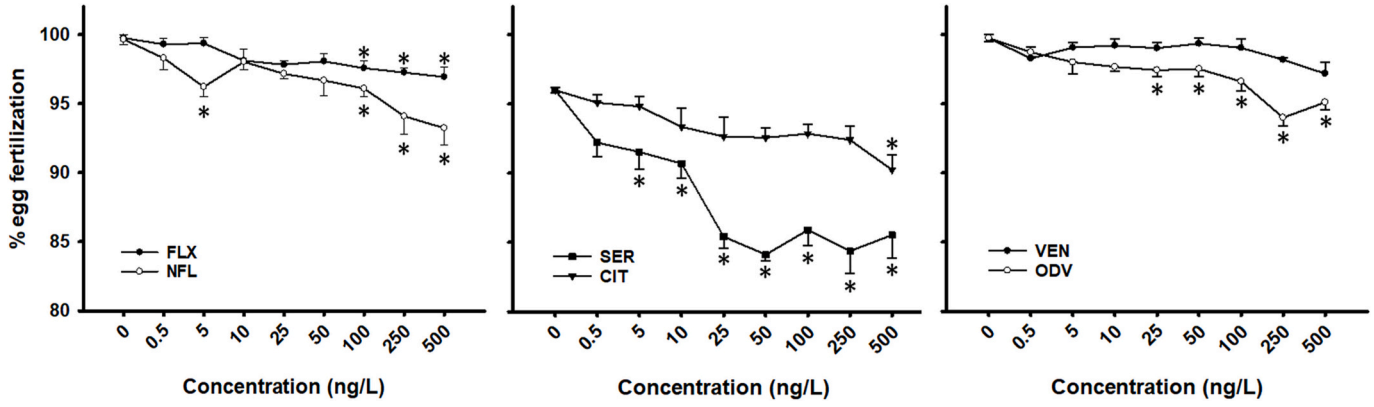

Fig. 1. Percentage of gamete fertilization of *M. galloprovincialis* after exposure to SSRI antidepressants (fluoxetine, FLX and its metabolite norfluoxetine, NFL; sertraline, SER; and citalopram, CIT) and to SNRI antidepressants (venlafaxine, VEN and its metabolite *O*-desmethylvenlafaxine, ODV). Data represent the mean ± SEM ($n = 4$). Asterisks indicate significant differences compared to control ($p < 0.05$).

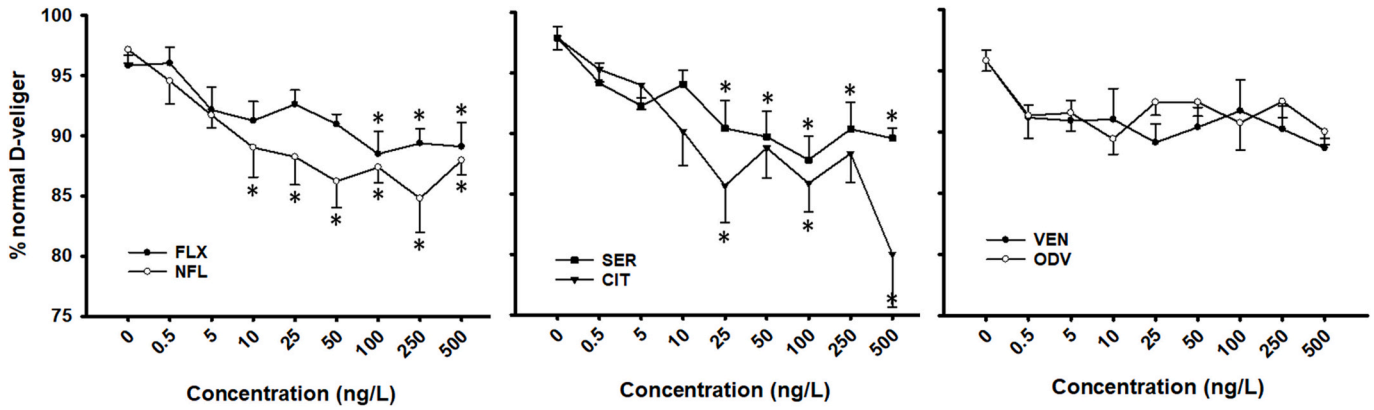

Fig. 2. Percentage of normally developed D-veliger of *M. galloprovincialis* at 48 hpf after exposure to SSRIs (fluoxetine, FLX and its metabolite norfluoxetine, NFL; sertraline, SER; and citalopram, CIT) and to SNRIs (venlafaxine, VEN and its metabolite *O*-desmethylvenlafaxine, ODV). Data represent the mean ± SEM (n = 4). Asterisks indicate significant differences compared to control ($p < 0.05$).

3.2. Biomarker analysis in adult mussels

3.2.1. Lysosomal parameters

3.2.1.1. Lysosomal membrane stability (LMS). Fig. 3 shows a significant reduction ($p < 0.05$) in LMS after mussel exposure to all antidepressants and their metabolites at all concentrations tested (0.5, 5 and 10 ng/L). Average destabilization time in control mussels was 100 min, which is in accordance with the threshold value defined by UNEP/RAMOGE (1999), while it was reduced up to 30 min after exposure to antidepressants. Fig. 3 shows an overall trend of decreasing LMS with increasing exposure concentrations, however statistical analysis conducted on single compounds indicated that the effects of antidepressants on LMS were not dose- dependent except for VEN and SER. At the lowest

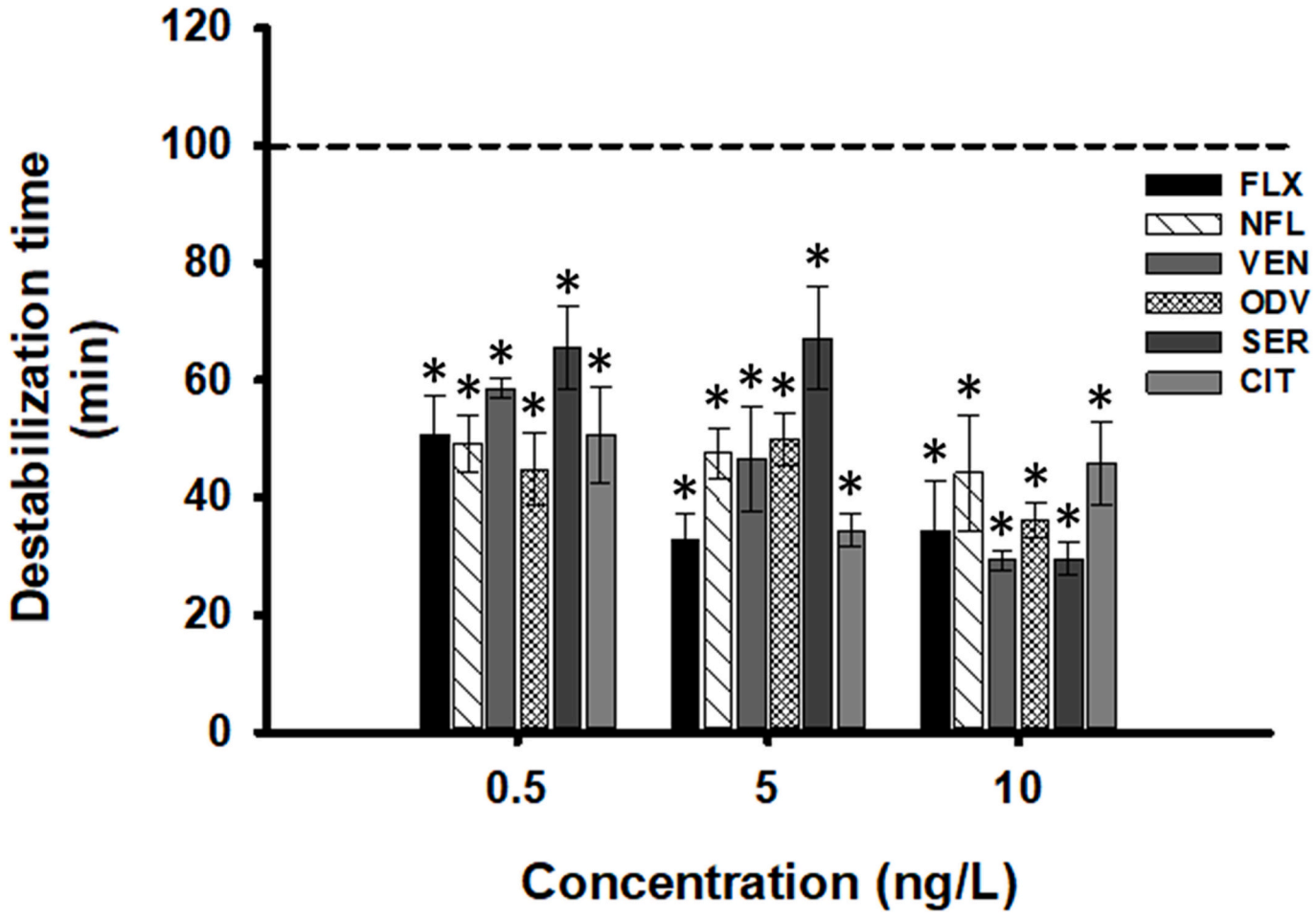

Fig. 3. Lysosomal membrane stability (LMS) assessed in haemocytes from mussels after a 7-day exposure to SSRIs (FLX and its metabolite NFL; SER; and CIT) and SNRIs (VEN and its metabolite ODV) at three tested concentrations (0.5, 5, and 10 ng/L). Data represent the mean ± SEM (n = 3). Average control destabilization time is 100 ± 2.89 min. Asterisks indicate significant difference compared to control ($p < 0.05$).

concentration VEN and SER reduced LMS by about 40 and 35 %, respectively, while at the highest concentration the pharmaceuticals strongly reduced LMS by about 70 % with respect to the control values. The effects on LMS caused by the parent compounds FLX and VEN (up to about 65 and 70 % reduction with respect to controls, respectively) were not significantly different from those of their corresponding metabolites, NFL and ODV. Overall, compounds of the SSRI and SNRI classes reduced LMS to about the same extent.

3.2.1.2. Histological biomarkers. The effects of antidepressants and their metabolites on further lysosomal parameters were evaluated in mussel digestive gland including lysosome/cytosol ratio (LYS/CYT), neutral lipid (NL) and lipofuscin (LF) accumulation, compared to control values (Fig. 4). The LYS/CYT was significantly increased by antidepressants (Fig. 4A). Among SSRIs, CIT exposure resulted in significant effects at all concentrations, with maximum effects of about 200 % at 10 ng/L; FLX and its metabolite NFL significantly increased LYS/CYT at 5 and 10 ng/L, with maximum effects of about 195 % and 150 % at 10 ng/L FLX and NFL, respectively. SER-treated mussels showed significant effects only at the highest concentration (10 ng/L; about 160 %). In the case of SNRIs, the parent compound VEN significantly increased LYS/CYT by about 150 % at all concentrations, while ODV significantly affected LYS/CYT only at 5 (about 180 %) and 10 (about 220 %) ng/L. The effects on LYS/CYT were similar between SSRIs to SNRIs, including metabolites. NFL and ODV were ineffective at 0.5 ng/L, while significantly increased LYS/CYT at 5 and 10 ng/L, to a similar extent as the parent compounds.

NL levels in lysosomes of the mussel digestive gland are reported in Fig. 4B. Among the SSRIs, FLX resulted in a significant NL accumulation (up to about 205 % with respect to control values), while its metabolite NFL did not show any effect. SER was effective only at the highest concentration (10 ng/L) resulting in an increase of about 130 %, whilst CIT did not induce any significant change in NL content. The SNRI VEN increased NL accumulation at all tested concentrations up to a maximum of about 230 %, whereas its metabolite ODV acted only at 5 and 10 ng/L reaching the same maximum increase of about 230 % vs control values. The only significant difference among concentrations was observed for ODV, which showed no effects at the lowest concentration, while significantly increased NL accumulation at 5 and 10 ng/L. No significant differences were noted on NL accumulation between the effects of the two class representatives, i.e. FLX and VEN. However, VEN induced significantly higher effects on NL compared to all concentrations of the SSRIs SER and CIT. As to the metabolites, NFL did not affect NL levels, while ODV was ineffective at 0.5 ng/L but induced significant accumulation of NL at 5 and 10 ng/L, similarly to the parent compound VEN.

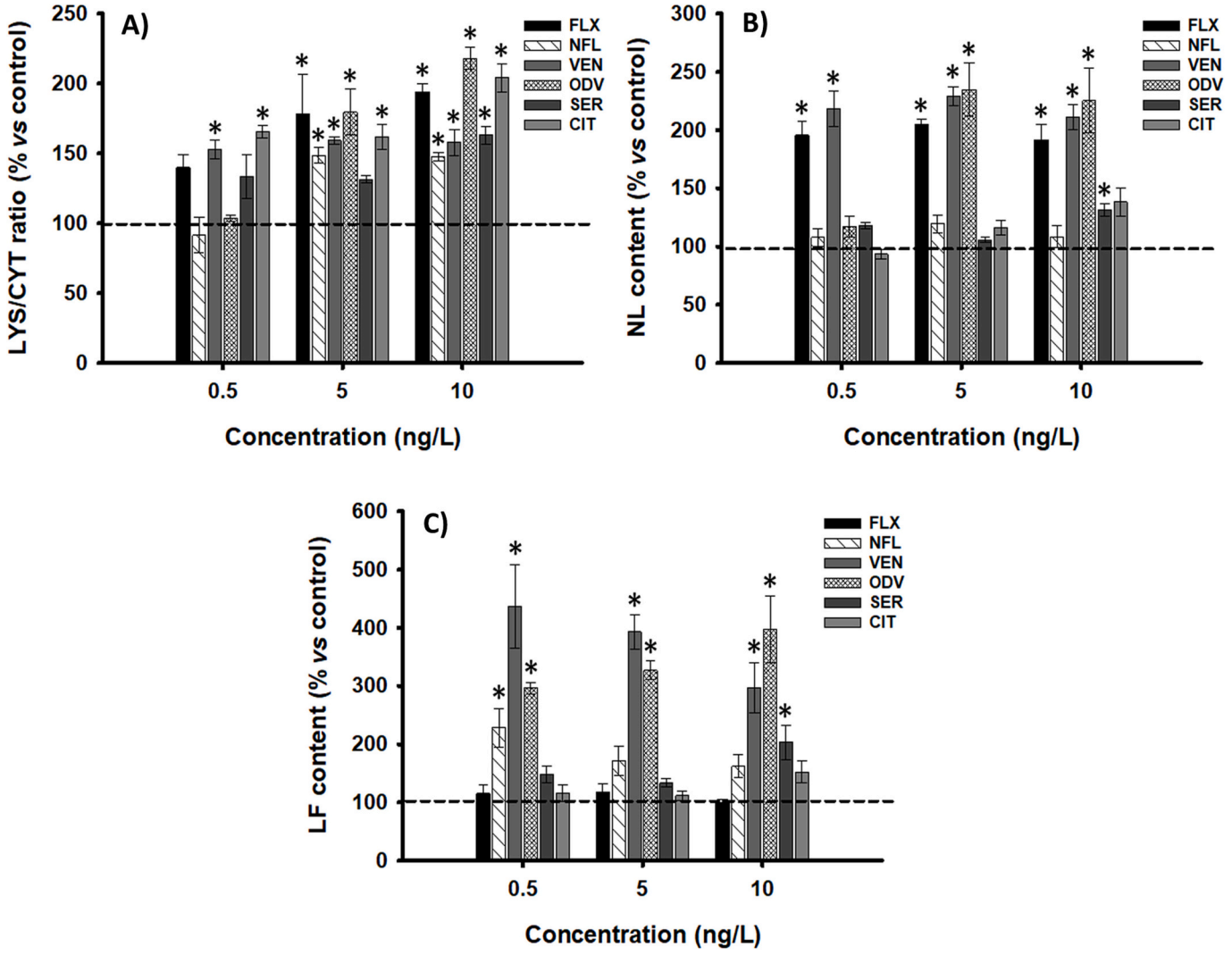

Fig. 4. Lysosome to cytoplasm ratio, LYS/CYT (A); accumulation of neutral lipid, NL (B) and lipofuscin, LF (C) in digestive glands of mussels after a 7-day exposure to SSRIs (FLX and its metabolite NFL; SER; and CIT) and SNRIS (VEN and its metabolite ODV) at three tested concentrations (0.5, 5, and 10 ng/L). Data represent the mean ± SEM ($n = 3$) of the percent variations versus controls represented by the dashed line (LYS/CYT 100 ± 2.48; NL 100 ± 6.99; LF 100 ± 5.45). Asterisks indicate significant differences compared to control ($p < 0.05$).

The SSRIs FLX and CIT resulted in no significant effects on LF (Fig. 4C). NFL enhanced LF accumulation at the lowest concentration (0.5 ng/L; about 230 % vs control), while SER did it at the highest concentration (10 ng/L; about 200 % vs control). The highest LF accumulation was induced by SNRIs at all concentrations tested, with maximum effects of about 440 % versus controls induced by 0.5 ng/L VEN, and of about 400 % induced by 10 ng/L ODV.

Overall, SSRIs and SNRIs behaved differently on LF content: no effects were induced by FLX, and small effects by NFL (SSRIs), while VEN and ODV (SNRIs) at all concentrations potently induced LF accumulation. The effects of VEN and ODV were always significantly higher than those due to the other SSRIs, SER and CIT.

3.2.2. *Oxidative stress and detoxification biomarkers*

The activities of GST enzymes in gills and digestive gland were assessed in mussels treated with antidepressants and their metabolites at three concentrations (0.5, 5, 10 ng/L), and compared to respective controls (Fig. 5 A, B). Among SSRIs, GST activity was significantly increased in gills at 5 and 10 ng/L SER, and in digestive gland at 0.5 and 5 ng/L NFL. None of the other chemicals, including the SNRIs VEN and ODV, showed any significant effects compared to controls. CAT activities were also analyzed in both tissues (Fig. 5 C, D). SER (at 5 and 10 ng/L) was the only antidepressant which significantly increased CAT activity in digestive gland. NFL reduced CAT activity in gills (at 10 ng/L) while increased it in digestive gland (at 5 ng/L). Comparing parent compounds with metabolites, only NFL affected GST and CAT activities at some concentrations, while FLX showed no effects. As for the SNRIs, VEN and ODV never affected GST and CAT activities.

MDA content was unaffected in digestive glands of mussels exposed to any of the tested compounds (Suppl Mat, Fig. S1A).

3.2.3. *Neurotoxicity biomarker*

AChE activities were unaffected in gills of mussels after 7 days of exposure to the antidepressants (SSRIs and SNRIs) and their metabolites at the three concentrations tested (0.5, 5, 10 ng/L), compared to the controls (Suppl Mat, Fig. S1B).

3.2.4. *Correlation among biomarker responses*

Pearson's correlation among biomarkers tested in adult mussels exposed to SSRIs and SNRIs is shown in Table 1. As expected LMS in haemolymph cells was inversely correlated to the lysosomal enlargement in digestive gland. Moreover, among histological parameters, LF content and the LYS/CYT were found to be positively correlated to NL content. CAT and GST in digestive gland were positively correlated. Activities of CAT in digestive gland and GST in gills were negatively correlated with NL. Digestive gland GST was positively correlated with MDA, and both were negatively correlated with LF content.

3.2.5. *Principal Component Analysis (PCA)*

PCA analysis performed on data from adult mussels exposed to SSRIs (FLX and its metabolite, NFL; SER; CIT) and SNRIs (VEN and its metabolite, ODV) is presented on Fig. 6. The first two components, PC1 and PC2, accounted for 86.4 % of the cumulative variation. Data from control has been scaled at PC1 < 0/PC2 > 0. All concentrations of SSRIs

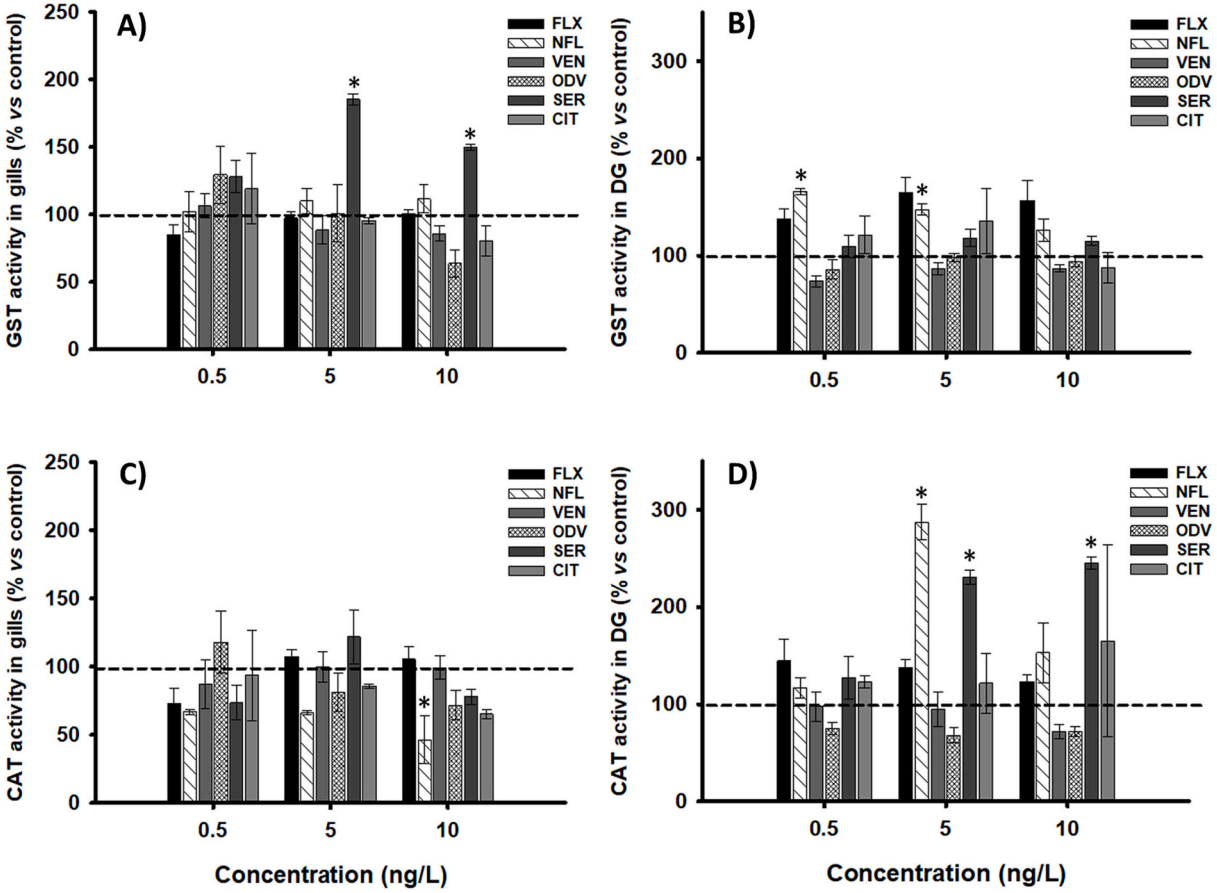

Fig. 5. Glutathione-S-transferases (GST) (A,B) and catalase (CAT) (C,D) activities in gills and digestive gland (DG) of mussels after a 7-day exposure to SSRIs (FLX and its metabolite NFL; SER; and CIT) and SNRIs (VEN and its metabolite ODV) at three tested concentrations (0.5, 5, and 10 ng/L). Data represent the mean ± SEM (n = 3) of the percentage of variations versus respective controls represented by the dashed line (GST control values were 535.67 ± 51.76 and 115.54 ± 4.88 mU/mg of protein in gills and DG, respectively; CAT control values were 46.96 ± 1.99 and 37.42 ± 4.41 mU/mg protein in gills and DG, respectively. Asterisks indicate significant differences compared to control ($p < 0.05$).

Table 1
Pearson's correlation among measured biomarkers after 7-day in-situ exposure of mussels to SSRI antidepressants (fluoxetine, FLX and its metabolite norfluoxetine, NFL; sertraline, SER; and citalopram, CIT) and to SNRI antidepressants (venlafaxine, VEN and its metabolite *O*-desmethylvenlafaxine, ODV). The *p*-value associated with each pairwise correlation is shown in brackets. Significant correlation ($p < 0.05$) is indicated by asterisks. g: gills; dg: digestive gland.

	LYS/CYT	NL	LF	MDA	CATg	CATdg	GSTg	GSTdg	AChE
LMS	−0.552* (0.014)	−0.363 (0.127)	−0.175 (0.473)	−0.113 (0.646)	0.120 (0.625)	−0.025 (0.917)	0.239 (0.324)	−0.214 (0.378)	−0.338 (0.158)
LYS/CYT		0.553* (0.014)	0.149 (0.541)	0.253 (0.295)	−0.134 (0.584)	−0.189 (0.439)	−0.417 (0.076)	−0.103 (0.675)	−0.338 (0.158)
NL			0.615* (0.005)	−0.111 (0.652)	0.104 (0.671)	−0.504* (0.027)	−0.537* (0.017)	−0.262 (0.278)	0.337 (0.175)
LF				−0.557* (0.013)	−0.002 (0.992)	−0.439 (0.060)	−0.261 (0.28)	−0.639* (0.003)	0.224 (0.357)
MDA					0.062 (0.801)	0.112 (0.647)	−0.191 (0.432)	0.482* (0.037)	−0.006 (0.981)
CATg						−0.336 (0.159)	0.346 (0.147)	−0.122 (0.619)	0.010 (0.967)
CATdg							0.389 (0.099)	0.570* (0.010)	−0.218 (0.370)
GSTg								0.0693 (0.778)	−0.238 (0.326)
GSTdg									−0.206 (0.399)
AChE									

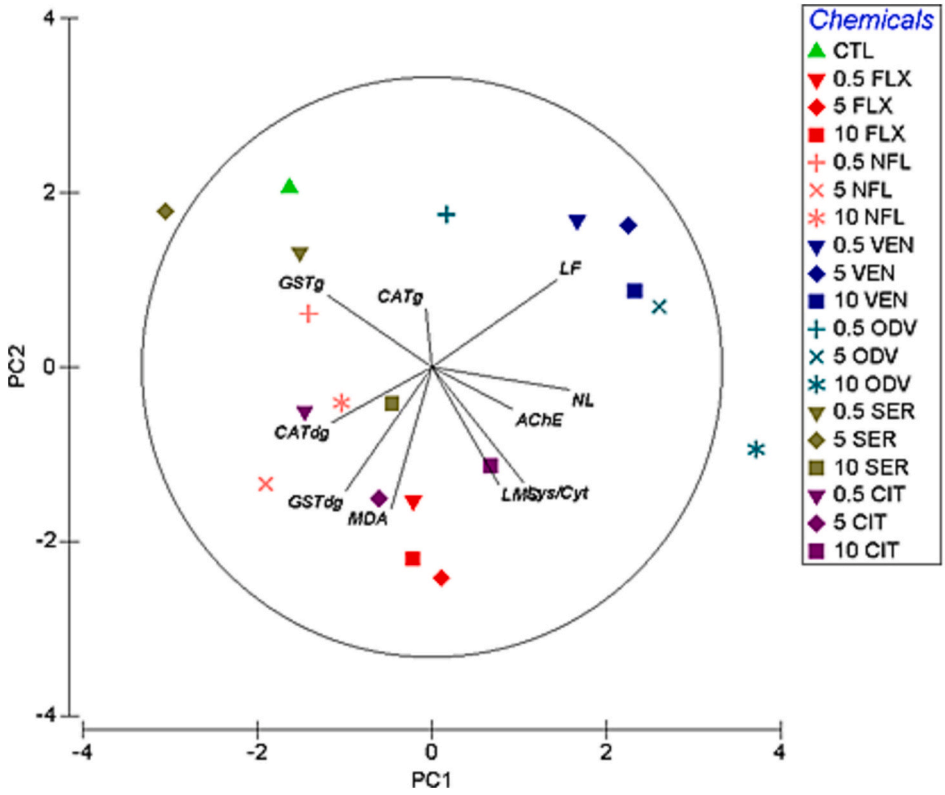

Fig. 6. Biplot of Principal component analysis (PCA) showing output of tested biomarkers in adult mussels after a 7-day exposure to SSRIs (FLX and its metabolite NFL; SER; and CIT) and SNRIs (VEN and its metabolite ODV) at three tested concentrations (0.5, 5, and 10 ng/L). g: gills; dg: digestive gland.

are coordinated at PC1 < 0 except for 5 ng/L of FLX and 10 ng/L of CIT (scaled at PC1 > 0), meanwhile, PC2 is also scaled at <0 for the treatments treated with all concentrations of SSRIs except for 0.5 ng/L of NFL; and 0.5 and 5 ng/L of SER (scaled at PC2 > 0). The exposure at all concentrations of SNRIs are scaled at PC1 > 0, differently, PC2 is <0 for the highest concentration (10 ng/L) of ODV and > 0 for all concentrations of VEN and two concentrations (0.5 and 5 ng/L) of ODV. The coordinates of vectors categorized the variable data into 4 spatial directions. Lysosomal parameters, except LF content, and AChE activity are coordinated around PC1 > 0/PC2 < 0, meanwhile, LF content is coordinated at >0 for both PC1 and PC2 axes. For antioxidant/detoxification enzymes (CAT, GST) activity in digestive glands and MDA content are clustered at <0 for both PC1 and PC2, while vectors for the activity of enzymes in gills are oriented at PC1 < 0/PC2 > 0.

3.2.6. *Mussel Expert System (MES)*

The health status index (HSI) derived from the Mussel Expert System (MES) is presented in Table 2. Rankings are based on responses of cellular/tissue biomarkers (Suppl Mat, Table S2) in adult mussels and of whole organisms from early life stages at 48 hpf, after exposure to SSRIs

Table 2
Health status index (HSI) as elaborated by the Mussel Expert System (MES) which integrated the biomarker responses in mussels exposed to SSRI (FLX and its metabolite NFL, SER, CIT) and SNRI (VEN and its metabolite ODV) for 7 days and the data from the embryo larval stages exposed to the same compounds. HSI is a health status Index which indicates stress level. A) healthy, B) low stress C) moderate stress D) high stress.

	SSRI antidepressants and metabolite (ng/L)															
	FLX				NFL				SER				CIT			
Output (HSI)	CTL A	0.5 A	5 C	10 C	CTL A	0.5 B	5 C	10 C	CTL A	0.5 A	5 B	10 D	CTL A	0.5 A	5 C	10 C
	SNRI antidepressants and metabolite (ng/L)															
	VEN								ODV							
Output (HSI)	CTL A	0.5 B	5 C	10 C	CTL A	0.5 B	5 B	10 C								

(FLX and its metabolite NFL, SER, CIT) and SNRIs (VEN and its metabolite ODV) at 0.5, 5 and 10 ng/L. The LMS was used as the guide biomarker for MES evaluation (Dagnino et al., 2007). The MES categorized control mussels as healthy (HSI = A). Similarly, mussels were considered healthy after 7 days of exposure to 0.5 ng/L FLX, SER, and CIT. A low stress level (HSI = B) was attributed to mussels exposed to 0.5 ng/L NFL, VEN and ODV and to 5 ng/L SER and ODV. A moderate stress level (HSI = C) was assigned to mussels treated with 5 and 10 ng/L FLX, NFL, CIT, and VEN, and 10 ng/L ODV. A high stress level (HSI = D) was attributed to mussels exposed to 10 ng/L SER.

4. Discussion

Pharmaceuticals are designed to resist chemical degradation during therapeutic treatments (Fabbri et al., 2023). Half-lives of antidepressants once consumed by patients range between hours (5 h VEN; 10 h ODV; 26 h SER; 36 h CIT) to a few days (1–4 days FLX; 7–15 days NFL) (Renoir, 2013; Shelton, 2019). In the aquatic environment, though variable depending on temperature and pH, the pharmaceutical half-life is much longer. FLX half-life is reported to range between 277 and 102 days at pH 7 and 9, respectively (Kwon and Armbrust, 2006). NFL half-life is reported as 60 days in 25 °C water; SER half-life is around 6 days (Lam et al., 2004) and CIT ranges between 14 and 65 days (Jiménez-Holgado et al., 2021). The half-life of VEN is reported to range between 9 and 62 days (de Souza et al., 2022) whereas that of its metabolite ODV was found to be about 18 h (Rúa-Gómez and Püttmann, 2013).

Antidepressants, and other pharmaceuticals, are considered to be pseudo-persistent contaminants of emerging concern in the aquatic environment, as they are continuously released (Fabbri et al., 2023). It is important therefore to learn more about the impact of pharmaceutical contamination on marine organisms. Recent actions were taken by the EU to monitor several pharmaceuticals, including VEN and ODV (EU Commission, 2020). These are generally limited to freshwater, while more information is needed to support the "zero pollution" action (https://environment.ec.europa.eu/strategy/zero-pollution-action-plan_en accessed on 24 May 2023) in the marine environment. Specifically regarding marine mussels, these investigations are also important in light of the significant decrease in aquaculture mussel production across Europe that has been related to environmental stress factors including pollutants (Avdelas et al., 2021).

The present work aimed to answer some main questions related to antidepressants as contaminants of emerging concern occurring in the marine environment.

1. Do a variety of antidepressants affect mussel health status at concentrations found in seawater?

Embryo-larval development at 48 hpf is the most sensitive endpoint among early life stage tests (Balbi et al., 2016; Capolupo et al., 2020). It contributes to understanding the morphological and functional effects of environmental stressors (Latchoumycandane et al., 2018). Possible effects of antidepressants were hypothesized based on the fact that serotonin modulates key physiological functions in bivalves (Fabbri and Capuzzo, 2010) starting from early development. Moreover, serotonin immunoreactive neurons are detected in mussel embryos in the region of the developing apical organ as early as 24 hpf (Canesi et al., 2022).

Detrimental effects of pharmaceuticals on embryo-larval stages are reported (e.g., Balbi et al., 2018). At all concentrations tested (0.5–500 ng/L), embryotoxicity was noted for all tested SSRIs, but not for SNRIs. The effects at 48 hpf even when significant never exceeded 20 % of control values. Small reductions in egg fertilization were also noted. These results lead us to conclude that embryo-larval stages are weakly affected by antidepressants within the applied range of concentrations, although an impact on regulatory mechanisms that we did not examine cannot be ruled out. However, it must be considered that in nature embryos are likely exposed to combinations of antidepressants and other marine contaminants (Duarte et al., 2023), thus even small though significant effects should not be overlooked.

The most relevant response measured in adult mussels after a 7-day exposure, was an impaired lysosome membrane integrity, with up to a 70 % LMS reduction. LMS in haemocytes is the most representative biomarker of general stress in marine bivalves (Martínez-Gómez et al., 2015; Viarengo et al., 2007). LMS reduction reflects an impaired integrity and functionality of the lysosomal compartment and represents a predictive indicator for cell and tissue injuries in *Mytilus* spp. (Moore et al., 2006; Viarengo et al., 2007). A 15 % reduction of LMS is already considered a significant indication of stress syndrome development in mussels (Dagnino et al., 2007).

Lysosome membrane damage by antidepressants has been documented in abalone haemocytes and correlated with Log Kow coefficients, thus with the hydrophobicity of the compounds (Minguez et al., 2014). Molecular modelling also highlighted interactions between the antidepressants and phosphatidylcholine, a major component of cell membranes. Crucial players in the interaction and acute toxicity were the distance between the ammonium groups of phosphatidylcholines and the aromatic rings of the antidepressants as well as the angle between the aromatic rings in the different drugs. The molecular modelling supported a greater ecotoxicity displayed by paroxetine and a lower ecotoxicity by CIT and VFX (Minguez et al., 2014). In the present work, no significant difference among antidepressant effects was found on LMS. We may notice that exposures were performed at much lower concentrations, i.e., in the low ng/L range. Thus, the contribution by the above-mentioned molecular features may occur, but not be the most relevant.

All pharmaceuticals also increased the LYS/CYT reaching 220 % of controls. The lysosomal enlargement is an advanced physiopathological condition observed in the digestive gland of mussels, well correlated with the reduction of LMS, and is thought to be detrimental to the viability of digestive cells and whole gland functions (Orbea et al., 2006).

All compounds, except for NFL and CIT, increased NL levels up to 230 %. NL accumulation is a biomarker of lipidosis that can result from

either an increased cytosolic lipid content or a decrease in fatty acid processing capability (Viarengo et al., 2007). NL alterations were identified in mussels exposed to organic pollutants in field conditions (Capolupo et al., 2017), as well as to emerging contaminants in experimental settings, including bisphenol A (Canesi et al., 2007) and plastic leachates (Capolupo et al., 2020, 2021).

All compounds, except for FLX and CIT, increased LF contents up to 440 % with respect to controls. LF are peroxidation end-products accumulated in lysosomes as insoluble granules containing autofluorescent pigments. The increase of these pigments in digestive gland cells of mollusks is an indication of the oxidative stress level in the cells and is related to the level of membrane lipid peroxidation (Viarengo et al., 2007).

Membranes of cell organelles are permeable to non-ionized compounds, such as FLX and possibly other antidepressants (Moore et al., 2006). Once inside the lysosomes, they are protonated and are no longer able to return to the cytosol, so they accumulate. This phenomenon, called lysosomotropism, results in an increase in volume and a loss of lysosome function and is an indicator of cytotoxicity in mussels (Cortez et al., 2019).

Only NFL and SER significantly changed antioxidant (CAT) and detoxification (GST) enzyme activities, with increasing or decreasing effects, depending on tissue and exposure concentrations. No significant change was observed in the presence of SNRIs. In line with the lysosomotropic behaviour, antidepressants may enter and accumulate in the lysosomes, thus partially bypassing the antioxidant/detoxification defenses that reside in the cytosol but are absent within the organelles (Terman and Brunk, 2006). A previous report, showed no clear antioxidant responses to FLX in *M. galloprovincialis* digestive gland in the range 0.03 to 300 ng/L, although significant elevation of CAT activity was detected at 0.3 ng/L FLX (Franzellitti et al., 2014).

Highly variable and non-significant effects were observed regarding MDA accumulation, in line with previous data for FLX (Franzellitti et al., 2014). A negative correlation between MDA and LF content was found, that is consistent with the role played by the two molecules in lipid peroxidation processes, where MDA acts an intermediate product (Terman and Brunk, 2006) converted to LF within one week of exposure (Viarengo et al., 2007). This relationship was also confirmed by previous studies (Capolupo et al., 2023; Franzellitti et al., 2014).

No effect was observed on AChE activity, by any chemical tested. AChE inhibition results primarily from the exposure to neurotoxic compounds (Viarengo et al., 2007), while an increase has been reported to oxidative stress (Melo et al., 2003). In response to pharmaceuticals, AChE activity changes are variable in bivalves. Inhibition was reported in gills of *M. galloprovincialis* exposed to FLX (Franzellitti et al., 2014). AChE increased in mussels (Trombini et al., 2016), clams (Munari et al., 2014) and marine worms (Castro-Aguirre et al., 2016; Gonzalez-Rey and Bebianno, 2013) after exposure to several pharmaceuticals. No significant variations were measured in haemolymph and gills of mussels exposed to anti-inflammatory drugs (Mezzelani et al., 2018a). Overall, we may conclude that AChE is not a significant target of antidepressants, with responses being related with non-specific effects due to animal conditions.

Only some of the pharmaceuticals demonstrated significant dose dependency when examined on single effects. The three concentrations selected for the biomarker exposure within the low environmental range were possibly too similar to indicate dose-dependent effects. However, biomarkers responses often present non-monotonic curves (Mezzelani et al., 2018b), and the principle of using a suite of biomarkers is that their contribution is evaluated as an overall response and not as single analysis (Dagnino et al., 2007). Some biomarkers may be extremely sensitive and develop rapid responses, in comparison to others during stress development; also, some may depend more than others on the physiological state of the organism.

Interestingly, the elaboration by MES, that translates complex biological responses into an objective evaluation of the changes in organism physiology due to contaminant exposure, was able to differentiate among treatments. Overall, among the 18 exposure conditions (6 compounds at 3 concentrations), the output of MES differentiates 15 different degrees of stress and indicates higher stress levels in line with concentration increase. It is worth noting that MES integrated all responses at cell, tissue and organism levels obtained in this study within the concentration range 0.5–10 ng/L. Therefore, this result is extremely relevant in the light of sublethal effects at low environmental concentrations of antidepressants, in a rather narrow range. These estimates may represent a more sensitive tool for future contaminant studies using mussels.

The answer to the initial question is therefore that the antidepressants tested, including two main metabolites, do affect mussel health status at marine environmental concentrations. Our data confirm previously reviewed investigations on biological effects of antidepressants in non-target aquatic organisms, where non-monotonic dose responses and significant effects at low concentrations of exposure were reported (Mezzelani et al., 2018b). Our findings indicate lysosomes as a primary target of antidepressants in the mussel *M. galloprovincialis*.

2. Are the effects of the parent compounds and metabolites comparable?

Specific transporters or cell signalling pathways modulated by antidepressants were not addressed in this study. One objective of this work was to evaluate an additional contribution of metabolites to the overall toxicity of pharmaceuticals released in the marine environment.

FLX is transformed into its active metabolite NFL by *N*-demethylation performed by several cytochrome enzymes (Margolis et al., 2000). NFL has recently been reported to operate with a similar mode of action and induce comparable and even more toxic effects than the parent compound in zebrafish, even though it shows a slightly lower affinity for the serotonin transporter (Yan et al., 2023). About 70 % of the mussels collected along the Portuguese Atlantic coast were contaminated with SSRIs and NFL was the compound with highest mean levels in the tissues (Silva et al., 2017).

In our experiment, NFL had higher effects than the parent compound FLX on the percent egg fertilization and also caused embryotoxicity over a wider range of concentrations (10–500 ng/L) with respect to FLX (100–500 ng/L). On adult mussel biomarkers, NFL did not affect NL accumulation whereas FLX did. NFL increased LF accumulation and affected CAT and GST activities at selected concentrations while FLX was inefficacious.

Demethylation of VEN to its main metabolite, ODV, is the primary metabolism route in humans (Magalhães et al., 2014). ODV acts as an SNRI, with higher binding affinity for serotonin and norepinephrine reuptake compared with VEN, and it is approved for therapeutic treatments (Lieberman and Massey, 2009). In our experimental trials, ODV significantly reduced egg fertilization while the parent compound VEN was ineffective. Similarly to VEN, ODV did not induce embryotoxicity. On adult mussel biomarkers, VEN caused significant accumulation of NL at all tested concentrations, while ODV was active only at 5 and 10 ng/L. Both VEN and ODV greatly increased LF content at all tested concentrations. VEN significantly increased LYS/CYT and NL content, while ODV was not effective.

Overall, the answer to the above question is that the metabolites NFL and ODV are biologically active also in mollusks, and according to the parameters measured, their effects are similar or even greater than that of the corresponding parent compound. This highlights the risk posed by pharmaceutical metabolites as contaminants of emerging concern in the marine environment so that total pharmaceutical content must include their metabolites.

3. Do the effects of SSRIs differ from SNRIs?

These two classes of antidepressants were designed to act as specific

inhibitors of the serotonin- and the serotonin/norepinephrine-reuptake transporters, respectively. Therefore, they do have different specific targets in humans. There is support for the occurrence of specific targets for FLX, and presumably for other SSRIs in mussels, namely the serotoninergic system (Canesi et al., 2022), but to the best of our knowledge there is poor evidence for the noradrenergic system, on which SNRIs partially act. Nevertheless, the two classes of compounds may have non-specific effects that deserve investigation.

No significant difference can be ascribed *apriori* on the basis of the lipophilicity of these compounds, because both classes have log Kow values between 3 and 4, with a maximum value of 5.1 for SER (Minguez et al., 2014). Most pharmaceuticals, however, are ionizable, so the log Dow (Kow influenced by the pH dependency of the ionizable form) instead of log Kow should be considered (Maulvault et al., 2018). Also, further mechanisms influence bioavailability and toxicity, including the intermolecular interactions of the aromatic rings with the membrane phospholipids (Minguez et al., 2014) as well as their diverse metabolic potential and rates of elimination (Fabbri et al., 2023).

From our data, the clearest response difference was that SNRIs did not affect larval development. We may hypothesize that the interaction with the developing serotoninergic system is lower than for SSRIs; also, the (nor)adrenergic system is poorly studied in bivalves (Canesi et al., 2022), and possibly the absence of targets may justify the lack of effect. Further clear evidence is that antioxidant/detoxification biomarkers (GST and CAT) appeared affected only by SSRIs. PCA analysis scaled the two classes broadly into two spatial orientations on PC1. SSRIs were categorized at PC1 < 0 based on antioxidant/detoxification biomarkers, in fact these enzymes were mainly affected by SSRIs compared to SNRIs.

However, LMS and LYS/CYT were affected similarly by the two antidepressant classes, and SNRIs had a significantly higher effect on NL and LF contents than SSRIs. SNRIs were categorized around lysosomal biomarkers by the PCA analysis, specifically around the vector of LF content where they showed higher effects compared to SSRIs. We may hypothesize that these greater effects are consistent with the lack of antioxidant/detoxification enzyme activations observed for SNRIs.

4. Can any major sublethal effects of antidepressants at concentrations observed in coastal waters be identified?

Taken together, our data highlight that both classes of antidepressants (SSRIs and SNRIs) have similar and significant effects on lysosomal functions compared to all other tested parameters. Lysosomes are multifunctional organelles found in eukaryotic cells, involved in the degradation of other damaged or aged organelles, and in the process of autophagy, i.e., the ability to break down macromolecules and other cell organelles to enable the maintenance of the cell during periods of environmental stress (Moore et al., 2006). The functional efficiency of the haemocyte lysosomal compartment is important in mussels, since they have an open circulatory system with haemolymph contents directly reaching all cells (Martínez-Gómez et al., 2008).

To our knowledge, the evidence that antidepressants target primarily mussel lysosome functions is supported for the first time by our data. However recent investigations in humans are in line with this conclusion. A study to assess the side effects of antidepressant administration (Gulbins et al., 2018) revealed that autophagy is crucially involved, leading to accumulation of sphingomyelin in hippocampal neuron lysosomes after mice treatment with FLX and amitriptyline. Also, drug-induced phospholipidosis was recently reviewed (Breiden and Sandhoff, 2020) and concluded that several drugs, including FLX and other antidepressants, resulted in excessive accumulation of non-degraded phospholipids in patient lysosomes. Entry and accumulation of antidepressants into the lysosomes reduced the digestive potential of the organelles for phospholipids and neutral lipids, leading to dysfunction (Breiden and Sandhoff, 2020). Psychotropic drugs with cationic amphiphilic structures showed cytotoxicity on cancer cells, where they caused mitochondrial and lysosomal disruption and consequent induction of cell death (Varalda et al., 2020). The present investigation on mussels supports the accumulation of lipids as a common consequence of antidepressant treatments in different organisms.

5. Conclusions

In this work, antidepressants were examined as emerging contaminants of the marine environment and investigated for their effects on early stages endpoints and adult biomarkers in an intertidal mussel. At concentrations widely reported in coastal waters, all tested antidepressants impaired mussel health status, as indicated by the low to high stress levels assigned by the MES to animals exposed at different concentrations of the compounds. The effects of antidepressants primarily targeted lysosomal functions. Embryo development was hampered by SSRIs, and not by SNRIs. Antidepressant metabolites, namely NFL and ODV, were biologically effective at environmental concentrations.

Nonetheless, it is important to consider that pharmaceuticals are designed to interact with specific receptors or cell pathways to ensure therapeutic effects at the lowest possible doses. Further studies are therefore advisable on the interaction of SSRIs and SNRIs with target receptors/pathways in marine invertebrates. Such studies should address the serotonin and norepinephrine reuptake transporters, aimed to provide further details on the mechanisms of action of these compounds, leading to a better knowledge on neuro-endocrine regulation of physiological functions and a greater understanding of neuro-endocrine disruption in marine bivalves to these emerging pharmaceutical contaminants.

CRediT authorship contribution statement

A. Rafiq: Conceptualization, Investigation, Data analysis, Writing original draft; M. Capolupo: Methodology, Data analysis, Writing review; G. Addesse: Methodology, Formal analysis; P. Valbonesi: Investigation, Data analysis, methodology supervision; E. Fabbri: Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166078.

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