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EFFECTS OF ENVIRONMENTAL PHARMACEUTICALS ON PHYSIOLOGICAL PARAMETERS OF MARINE MUSSELS *MYTILUS GALLOPROVINCIALIS*

Thesis in: Physiology Applied to the Environment: Pollutant Impacts On Human Health and Ecosystem

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STATEMENT

I hereby declare that this work has been carried out by me and the thesis has been composed by me and has not been submitted for any other degree or professional qualification.

This work is presented to obtain a masters' degree in Water and Coastal Management (WACOMA).

Viergine Leopold

I dedicate this master thesis to my beloved Parents.

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ABSTRACT

The increasing consumption of pharmaceutical products is of environmental concern, as excreted parent compounds and their possibly active metabolites are not completely removed by wastewater treatment plants. Several studies have confirmed the occurrence of antidepressants in coastal waters in the ng/L concentration range. This research focuses on four antidepressants e.g. fluoxetine, FLX; sertraline, SERT; and citalopram, CITA (selective serotonin reuptake inhibitors, SSRIs) and venlafaxine, VEX (serotonin-norepinephrine re-uptake inhibitor, SNRI) and two metabolites norfluoxetine, NF and O-desmethylvenlafaxine, ODV, chosen because: serotonin and norepinephrine are the main invertebrate neuromodulators, thus changing their levels influences animal functions; antidepressants are among the top prescribed pharmaceuticals worldwide, biologically active at low concentrations with the potential to cause neuroendocrine disruption. This work aimed to observe difference in the toxicity among parent compounds, and the relative toxicity of metabolites. The effects have been measured on early life stages of Mediterranean mussel *Mytilus galloprovincialis* analyzing different end points after exposure to environmental concentration range (0.5 - 500 ng/L). SSRIs had greater effect on fertilization rate than SNRIs, with a similar effect by parent compound and metabolites. SERT caused the highest percentage of unfertilized eggs in a wide range of concentrations, from 10 ng/L to 500ng/L. The range of effect on embryo – larval development is as follows NF (5 -500 ng/L) > SERT (25-500 ng/L) > FLX (100-500 ng/L) > CITA (500 ng/L), while VEN and ODV were ineffective. Poor effects of antidepressants were observed on larvae motility and survival. In conclusion antidepressants and their metabolites affected mussel gamete fertilization and embryo development, thus representing a threat for the formation and maintenance of populations, disrupting the ecological system and biodiversity.

Keywords; Antidepressants, marine environment, Mytilus galloprovincialis, fertilization, embryo development

ACRONYMS

ANOVA	**Analysis of variance**
cAMP	**Cyclic Adenosine Monophosphate**
CITA	**Citalopram**
FSW	**Fresh seawater**
DMSO	**Dimethyl Sulfoxide**
DNA	**Deoxyribonucleic Acid**
ODV	**O-Desmethylvenlafaxine**
EC50	**Half Maximal Effective Concentration**
FLX	**Fluoxetine**
HQ	**Hazard Quotient**
NF	**Norfluoxetine**
PEC	**Predicted Environmental Concentration**
pf	**Post Fertilization**
PKA	**Protein Kinase A**
PNEC	**Predicted No Effect Concentration**
SEM	**Standard Error of Mean**
SERT	**Sertraline**
SNRI	**Serotonin Re-Uptake Inhibitor**
SSRI	**Serotonin Norepinephrine Reuptake Inhibitors**
STP	**Sewage Treatment Plant**
TCA	**Tricyclic Antidepressants**
VEN	**Venlafaxine**
WWTP	**Waste Water Treatment Plant**

AIM OF THE STUDY

Pharmaceuticals benefits for target organisms are not under dispute, however, due to their increased use by a growing global population, they have entered ecosystems as contaminants (Daughton and Ternes, 1999). Improper disposal and inefficiency of waste water treatment plants to eliminate these compounds contribute to their continuous discharge mainly in the aquatic ecosystems making them “pseudo-persistence” in the environment.

Among other pharmaceutical classes, the world consumption of antidepressants is increasing, and due to their capacity to act in low concentration, antidepressants represent a danger for non-targeted species (Canesi et al., 2022). Many studies have been done to evaluate the effect of these compounds on the ecosystem using fish, daphnia, and some invertebrates (mostly on fresh water species) and yet less on marine bivalves (Brodin et al., 2013, 2014; De Castro-Català et al., 2017; Lazzara et al., 2012; Schultz et al., 2011). Effects of fluoxetine have been investigated in marine invertebrate species with striking effects obtained from laboratory studies. Fluoxetine at 1-100 ng/L impacted learning and retention efficiencies in cuttlefish *Sepia officinalis* (Carole Di Poi et al., 2013a); at 1 ng/L a significantly reduced latency of burying after hatching in cuttlefish perinatally exposed to the drug was observed (Di Poi et al., 2014). At a concentration as low as 0.03 ng/L fluoxetine impaired neuroendocrine signaling in mussels M. galloprovincialis by decreasing cAMP levels and Protein Kinase A (PKA) activation (Canesi et al., 2022).

It has also been observed that few data are available on the potency of antidepressant metabolites (such as norfluoxetine and O-desmethylvenlafaxine) generating adverse effect on aquatic organisms. The suspicion that certain metabolites may be more toxic than the parent compounds urge in putting effort to enhance the understanding of the effects of these substances.

Early life stages of bivalves have been identified as an appropriate target to observe the effects of emerging pollutants as they are more sensitive to chemicals and represent the species recruitment. In such instance, we have selected the following objectives for our present study, by assessing the effect of four antidepressants fluoxetine

(FLX), sertraline (SERT), citalopram (CITA), and venlafaxine (VEN) and two metabolites norfluoxetine (NF) and O-desmethylvenlafaxine (ODV) on marine mussels *Mytilus galloprovincialis* during the early life stages with the endpoints of:

- Fertilization rate
- Embryotoxicy (Embryo-larvae development)
- D-shape veligers larvae-development (mortality and immobilization)

Chapter 1

1. Introduction

1.1. Pharmaceuticals in the environment – an overview

Pharmaceuticals are a group of biologically active emerging organic compounds that have played an important part in the improvement of life quality. They are developed to interact with physiological pathways of targeted organisms (Branchet et al., 2021; Fabbri and Franzellitti, 2016; González Peña et al., 2021; Kuemmerer, 2004; Kümmerer, 2008). They are widely used to prevent and/or treat human and animal diseases and are used in the food industry as growth promoters in intensive livestock farming. In recent years, pharmaceuticals and active pharmaceutical ingredients (API) have been the subject of extensive scientific research because of this use which may have potential adverse impact on non-target organisms (Christensen, 1998). Although their occurrence in the environment has been highlighted in the in United States in the 1970`s (Keith, 1976), due to lack of advanced analytical technologies for the detection of low concentrations of these compounds in water, the interest in the field only began to expand more recently (Santos et al., 2010). Compared to other environmental contaminants, pharmaceuticals have a high degree of stability and mediate their biological effects at low concentrations, which thus leads to ideal conditions for an environmentally persistence, bioaccumulation and potentially damage to aquatic organisms and ecosystems (Christensen, 1998; O'Flynn et al., 2021).

1.2. Pharmaceuticals and their mechanisms:

Drugs are defined as any substance that can change biological function through its chemical intervention. In many instances, the drug molecule interacts as an agonist (activator) or antagonist (inhibitor) with a specific target molecule (which are called receptors) that regulate the biologic system. To be able to interact with the receptors, drugs should have the right size (molecular weight), electrical charge (covalent, electrostatic, and hydrophobic), shape and atomic structure (Katzung, 2018). Drug body interaction is divided into two processes pharmacodynamics (action of drugs on the body)

and pharmacokinetics (action of the body on the drugs by absorption, distribution, metabolism and elimination). During pharmacokinetics there are two consecutive pathways for the metabolism of pharmaceuticals: phase I and phase II. Phase I are catabolic and involves addition of a new or existing functional group through oxidation (e.g. hydroxylation, N-oxidation, deamination) or hydrolysis reactions, whereas phase II are anabolic and includes conjugation (e.g. addition of a glucuronic acid, sulfate, acetate or amino acids) (Monteiro and Boxall, 2010). These processes play an important role on the bioavailability and bioactivity of pharmaceuticals after being released in the environment.

1.3. Consumption of pharmaceuticals

Approximately 4,000 pharmaceuticals are being used worldwide for medication, veterinary drugs and growth promoters with an annual worldwide consumption of about 100,000 tons/y (Boxall et al., 2012; Weber et al., 2014). The global, use of antibiotics alone in 2015 attained 34.8 billion defined daily doses as from year 2000 there has been an exponential increase of 65% (Klein et al., 2018). China, Brazil and India are the leaders in medical spending within these markets. It has been reported that the consumption pattern of medicine are affected by socioeconomic conditions and seasonal changes (Castiglioni et al., 2006). A drastic change in drugs consumption, especially in psychoactive drugs usage was noted during the economic crisis in Greece between 2010 and 2014 (Patel et al., 2019). According to the IQVIA institute for human science data (2019), worldwide expenses on medicines reached $1.2 trillion in 2018, and are set to exceed $1.5 trillion by 2023 with 4–5% growth globally. The quantity of new products launched every year is estimated to increase from an average of 46 in the past five years to 54 through 2023, which represent an augmentation of drugs release in the environment.

It is obvious that new COVID-19 pandemic is contributing to an exponential increase of new chemical and biological entities due to the energies to generate a greater amount and more effective pharmaceuticals and vaccines against the SARS-CoV-2 virus (OECD, 2021)

1.4. Sources and pathways of Pharmaceuticals and their metabolites to the Environment

1.4.1. Sources

Household pharmaceuticals discharges represent by far the major contribution to surface waters, followed locally by hospitals (Daughton, 2013). Pharmaceuticals are in fact poorly removed by waste water treatment plants (WWTPs) (Castiglioni et al., 2006). Moreover, some pharmaceuticals can be excreted as conjugates, and release the active moiety by cleavage during treatment in WWTPs (Heberer, 2002). We must not forget, however, that 44% of municipal wastewater produced across the world does not flow into treatment plants but remains untreated (UN-Water, 2021).

Seas and oceans are the ultimate sink for these compounds, and receive pharmaceuticals primarily from rivers or directly from raw or treated wastewaters, discharged from the coast through submarine pipelines (Feo et al., 2020). Aquaculture industries and run-off from farms play additional roles in the presence of veterinary pharmaceutical residues. Also contamination of groundwater has been shown worldwide (Anderson and Hakimian, 2014) and in this case pharmaceuticals can enter the sea directly via submarine groundwater discharge as a result of groundwater contamination; these affects coastal waters with a contribution that may be greater than the surface runoff (Szymczycha et al., 2020).

Illegal disposal and improper domestic disposal of unused pharmaceutical products contributes as well to the presence of drugs in the environment. Leaching of these products through soil can contaminate the groundwater which is then consumed by people and may cause health issues (Waleng and Nomngongo, 2022).

Finally, drug manufacturers and incorrect disposal of unused or expired medications significantly contribute to the total amount (Begum et al., 2021).

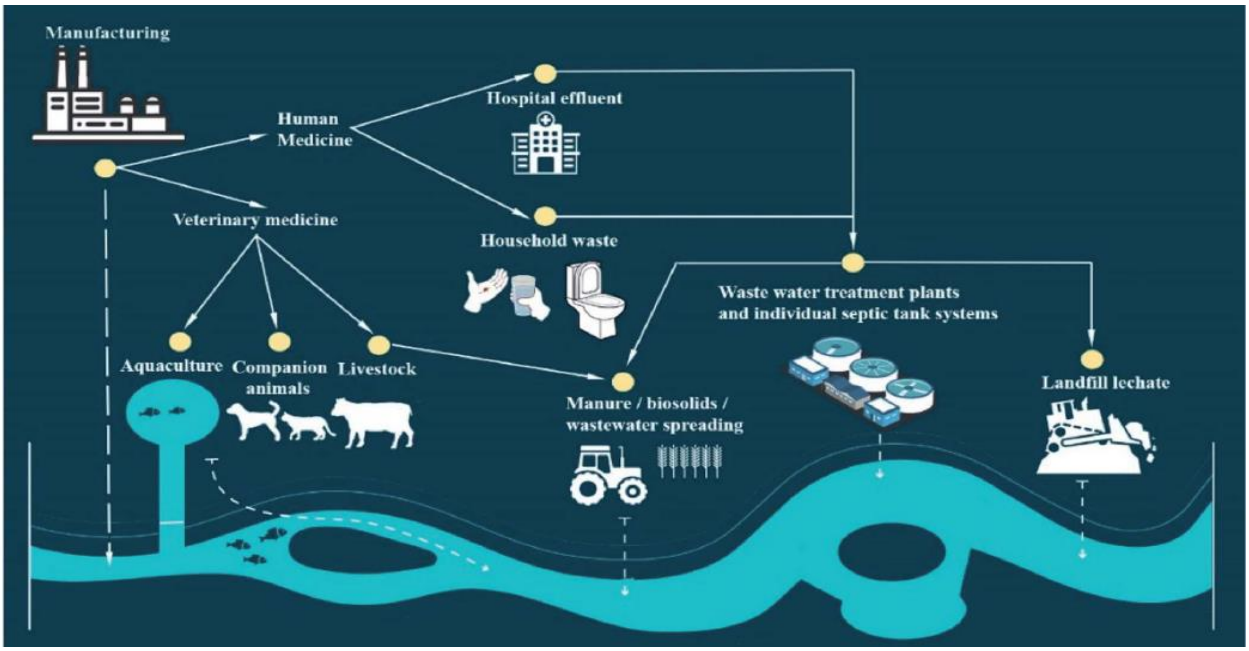

Figure 1-1. The pathways of pharmaceuticals to aquatic environment; adapted from (O'Flynn et al., 2021)

1.4.2. Pathways in the environment:

Pharmaceuticals and metabolites once arrived in the WWTPs are processed through different kind of reaction including homogenization of waste size, biological treatment of waste, final settlement of waste and return to lakes and sea. The type of treatment that is adsorption or biological treatment is selected according to the physicochemical properties of the drug. In line with their polarity, water solubility and persistence, some of these compounds may not be completely eliminated or transformed during sewage treatment, henceforth low concentration of pharmaceuticals are released in the environment. The elimination rate of pharmaceuticals depends on the construction and treatment technology, hydraulic retention time, season and performance of the sewage treatment plant. Studies show that excreted conjugated metabolites like estradiol can be cleaved in sewage treatment plants, resulting in the discharge of active parent compound. It is worth mentioning that new pilot plants are being tested for improving pharmaceutical removal, searching for the best balance between costs and benefits (Olasupo and Suah, 2021).

The occurrence of pharmaceuticals have been extensively described in fresh surface waters and in ground waters but their existence in the marine environment has

been less studied mostly because of the complexity of the matrices to investigate and the dilution of pharmaceutical in the marine waters (aus der Beek et al., 2016; Branchet et al., 2021). Pharmaceuticals have been detected in sediments, drinking water and have numerous routes by which they enter into the water cycle (González Peña et al., 2021).

Possibly the first research paper highlighting the presence of pharmaceuticals in the environment (sewage treatment effluent) was made over two decades by (Garrison et al., 1976). Clofibric acid (active compound from lipid regulators) has been reported at concentration of 0.8-2.0 pg/L in raw sewage and activated sludge effluent. Over the recent decades, beyond 100 different drugs have been detected in the aquatic environment at concentrations from the nanogram (ng) to the microgram (µg)/L range (Daughton and Ternes, 1999; Jørgensen and Halling-Sørensen, 2000; Kümmerer, 2001). Numerous attempts for detection of drugs in the environment was limited until 1990s as necessary chemical analysis tools with adequately elevated separatory efficiencies, to determine the drugs from the water matrices and low detection limits (nanograms per liters), were lacking (Daughton and Ternes, 1999).

1.4.3. Distribution in the aquatic environment:

Active substances or their transformation products were identified in the environment of 89 countries of all five continents. To sum up, 992 different pharmaceutical substances were measured worldwide (Lehmphul, 2016). Pharmaceuticals are likely to have low vapor pressure which reveal that firstly they will be distributed in the aquatic environment, but also via food chain dispersal (Fent et al., 2006). The level of pharmaceuticals in the environment can fluctuate hourly, daily, seasonally, spatially and temporally. These changes depend upon consumption patterns, locations, with heavy inputs from manufacturing facilities and hospitals, degradation in sewers, rainfall, sampling uncertainties, and analysis techniques (Patel et al., 2019). According to the reviewed literatures the distribution of pharmaceuticals in same aquatic environment is not uniform, they can change from time to time. For example, diclofenac was detected at concentration ranging from 7.8–170 ng/L, 1.8–1300 ng/L and 1.8–121.6 ng/L , respectively (Dai et al., 2015; Ma et al., 2017; Yang et al., 2017). All these studies were done on the same Beivum River in Beijing (China) with temporal difference.

The spreading of pharmaceuticals in the aquatic environment is dependent of hydrodynamics resulting from their variable physicochemical properties. Drugs with high residence time tend to stay in high concentration at the site due to their persistent nature (Waleng and Nomngongo, 2022). Pharmaceuticals and their metabolites are considered as pseudo persistent because of their continuous input into environmental matrices despite their constant degradation and removal by various processes (Barceló and Petrovic, 2007; Bu et al., 2016; Patel et al., 2019).

Pharmaceuticals that have high surface water longevity (T1/2) tend to be more persistent and spread extensively (Waleng and Nomngongo, 2022). Photo degradation and biodegradation play key roles in these compound removal in the natural environment (Im et al., 2021; Kwon and Armbrust, 2005). It has been investigated that cloud cover affects the availability of contaminants in the environment (O'Flynn et al., 2021). According to studies made in Vaal River catchment (South Africa), estrone concentrations ranging from 0.0009–0.004 µg/L has been found out. In Periyah (India) collected surface water metoprolol (β-blocker) was detected at the concentration ranging up to 0.53 µg/L (Waleng and Nomngongo, 2022). An evaluation made by Küster and Adler (2014) with 650 human and 120 veterinary pharmaceuticals find out that only 10% are notable regarding their potential environmental risk. These drugs consisted of the following pharmaceutical classes: hormones, antibiotics, analgesics, antidepressants and antineoplastics.

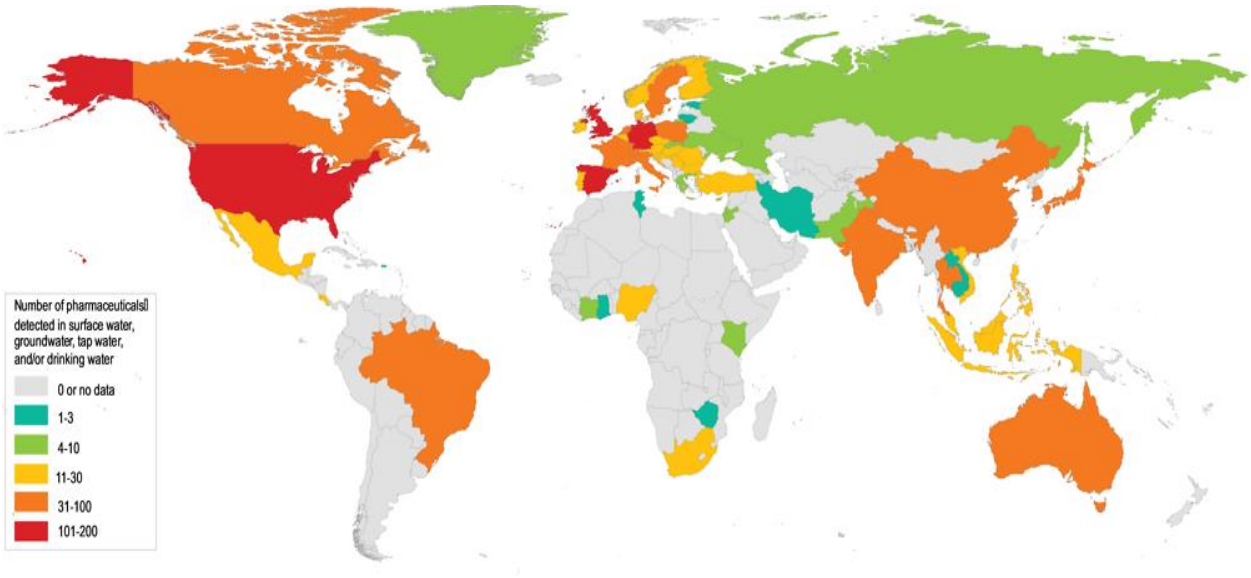

Figure 1-2. Number of pharmaceuticals detected in surface water, groundwater or drinking water globally (aus der Beek et al., 2016)

1.5. Impact of pharmaceuticals in the Environment:

Several studies have been done in view to evaluate the danger of these agents in the marine environment up to the human level. Pharmaceuticals are likely to bioaccumulate in the tissues of aquatic organisms (Almeida et al., 2020a, 2020b; Du et al., 2016; Feo et al., 2020). Flumequine and oxytetracycline were detected in marine invertebrates at levels ranging from 2500–2900 and 60–380 µg/kg, respectively. Pharmaceuticals can affect aquatic organisms directly by the biochemical interaction with receptor molecules (e.g. hormone or enzymes receptors) and interruption of cellular processes, as result of changes in gene expression, intracellular ion concentrations, cellular metabolism and the disruption of the endocrine system. The propagation of antimicrobial-resistance and the bio-accumulation of pharmaceuticals through trophic chain from the invertebrates to the higher level. Nevertheless, the associated risk by transfer of pharmaceuticals through the food chain is not fully understood (O'Flynn et al., 2021). The following has been find out according to the class of pharmaceuticals:

Table 1-1. Adverse effect of pharmaceuticals on aquatic organisms adapted from (OECD, 2019)

Pharmaceutical class	Impact on aquatic organisms	References
Psychiatric dugs	Behaviour changes - feeding, boldness, activity, sociality (fish) Disruption with hormones (fish) Behaviour changes - swimming and cryptic (invertebrates) Reproduction toxicity and disruption with hormones (invertebrates)	(Brodin et al., 2014, 2013; Kellner et al., 2016) (Schultz et al., 2011b) (De Castro-Català et al., 2017; Di Poi et al., 2014b) (Campos et al., 2016; Lazzara et al., 2012)
Antibiotics	Reduced growth (environmental bacteria, algae and aquatic plants) Indirect effects of antibiotic resistance (humans and animals)	(Brain et al., 2008; Guo et al., 2015; Roose-Amsaleg and Laverman, 2016)
Anti-cancer	Genotoxicity, Mutagenicity, carcinogenicity, toxicity to foetus	(Araújo et al., 2019; Česen et al., 2016; European Commission, 2016; Zounková et al., 2007)
Analgesics	Organ damage, reduced hatching success (fish) Genotoxicity, neurotoxicity and oxidative stress (mollusk) Disruption with hormones (frog)	(Mathias et al., 2018; Näslund et al., 2017; Xia et al., 2017) (Mezzelani et al., 2016) (Efosa et al., 2017)
Anti- epileptic	Reproduction toxicity (invertebrates), development delay (fish)	(Ferrari et al., 2003; Martinez et al., 2018)
Beta blockers	Reproduction behaviour (fish), reproduction toxicity (invertebrates)	(de Oliveira et al., 2016; Giltrow et al., 2009)
Endocrine disrupting pharmaceuticals	Disruption with hormones causing reproduction toxicity (fish, frogs).	(Armstrong et al., 2016; Gyllenhammar et al., 2009; Kidd et al., 2007; Kvarnryd et al., 2011; Moore et al., 2016; Nelles et al., 2011)

1.6. Antidepressants

Antidepressants are used to treat major depressive disorder as well as anxiety disorders, some chronic pain conditions, and also to help manage some drug addictions.

Antidepressants are medicines with the capacity to act on brain biochemistry through their interaction with neurotransmitters, such as dopamine, norepinephrine, and serotonin.

Antidepressants are classified in accordance with their pharmacological mechanism of action. Monoamine oxidase inhibitors (MAOIs) such as phenelzine blocks the enzyme that catalyze the oxidation of monoamines, thus preventing the metabolization of neurotransmitters e.g. serotonin and dopamine. Tricyclic antidepressants (TCA) such as clomipramine blocks serotonin and norepinephrine reuptake transporters. MAOI and TCA are the oldest discovered antidepressants however, due to the side effects the usage has been stopped except for TCAs in extreme case of psychiatric disorders. The above discoveries have contributed to the development of new psychotropics such as zimeldine first selective serotonin re-uptake inhibitor (SSRI) and serotonin norepinephrine reuptake inhibitors (SNRIs), they bind to and inhibit pre-synaptic reuptake transport proteins (recycle neurotransmitters back into the pre-synaptic terminal) (Fong and Ford, 2014).

Currently, the most widely prescribed antidepressants worldwide are fluoxetine and sertraline, acting as selective serotonin reuptake inhibitors (SSRIs), and venlafaxine and duloxetine, acting as serotonin-norepinephrine re-uptake inhibitors (SNRIs); these pharmaceuticals are in fact included within the 40 top prescribed pharmaceuticals (https://clincalc.com/DrugStats/ Top200Drugs.aspx). The inhibition of re-utpake substantially increases the permanence of serotonin (or norepinephrine) in the synaptic cleft, prolonging its action.

In US the usage of antidepressant has increased from 11.2 million in 1998 to 23.3 million by 2010 (Chalabi, 2013). According to Mikulic, 2021 the biggest numbers of individuals with anxiety lived in locations in South-East Asia and the Americas. The worldwide consumption of antidepressants has been increasing. As of 2020, within the select Organization for Economic Cooperation and Development (OECD) countries, Iceland, Portugal, and Canada were the major consumers of antidepressants. At that time, people in Iceland consumed antidepressants at a rate of about 153 defined daily doses (DDD) per 1,000 people (Mikulic, 2021). They are the biggest consumers of antidepressants in the world. A study showed that in Poland with the onset of Covid- 19,

pandemic a high purchasing of antidepressant was recorded in March 2020. The global market value of antidepressants was approximately USD 14,538 million in 2020 and expected to increase even more in combination with the Covid 19 pandemic by 2026 (Krupa et al., 2022).

1.6.1. Fluoxetine

Fluoxetine (well known as the active principle of Prozac®) is a selective inhibitor of serotonin re-uptake (SSRI). Fluoxetine is a combination of two lipophilic enantiomers compounds (Gram, 1994), used to treat depression, and is also prescribed for compulsive behavioral, eating and personality disorders (Brooks et al., 2003a). After metabolic reactions fluoxetine is converted to the active metabolite norfluoxetine and multiple other metabolites, of which less than 10% are excreted unchanged in the urine after oral administration (Hiemke and Härtter, 2000). The half-life of elimination of fluoxetine is between 1 and 4 days (mean 2 days) after a single dose and 2 to 7 days (mean 4 days) after multiple doses (Benfield et al., 1986).

1.6.2. Norfluoxetine

Norfluoxetine is the *N*-desmethyl metabolite of fluoxetine, and is alike fluoxetine in being a potent and selective inhibitor of the serotonin uptake carrier (Fuller et al., 1992). Norfluoexetine has longer a half-life than fluoxetine of 7–15 days (Hiemke and Härtter, 2000).

1.6.3. Sertraline

Sertraline remains the second most potent inhibitor of 5-HT reuptake and the second greatest selective blocker of serotonin over noradrenaline uptake (Hiemke and Härtter, 2000). Among the SSRI, it is the only one that binds to dopamine transporters (Richelson, 1994). The hepatic metabolism is the most important elimination pathway, with only 0.2% unchanged drug appearing in the urine within 48 hours of consumption with an elimination half-life of approximately 26 hours (Murdoch and McTavish, 1992).

1.6.4. Citalopram

Citalopram is a selective 5-HT reuptake inhibitor that has shown antidepressant success in several controlled clinical assays. The elimination half-life is 30 – 35 hours.

(Pollock, 2001). After an hepatic metabolism approximately 12% of citalopram is excreted unchanged in the urine after a single dose administration (Milne and Goa, 1991) .

1.6.5. Venlafaxine

Venlafaxine is a type of antidepressant in the drug class known as serotonin norepinephrine reuptake inhibitors (SNRI). Venlafaxine is the active principle of Effexor® which was the first SNRI approved by the U.S. Food and Drug Administration in 1993 to treat major depressive disorder in adults. Venlafaxine is approved to treat major depression, generalized anxiety disorder, panic disorder, and social phobia. The antidepressant inhibits the reabsorption of two chemicals in the brain that transmit nerve signals namely serotonin and norepinephrine. 5% of venlafaxine is excreted unchanged in the urine after administration. The biological half-life of venlafaxine is five hours (Levine et al., 1996; Sansone and Sansone, 2014; Turner, 2021).

1.6.6. O-desmethylvenlafaxine (ODV)

O-desmethylvenlafaxine is a single active metabolite of venlafaxine (Gasser et al., 2012). It is partially metabolized through conjugation and partially metabolized through the P-450 isoenzyme system, but, approximately 50 % of the excreted drug remains unchanged in urine. The half-life of desvenlafaxine is 11 hours (Sansone and Sansone, 2014).

1.7. Occurrence of Antidepressants in the Environment

Several publications have reported the occurrence of antidepressants in aquatic environments, both urban and natural (aus der Beek et al., 2016; EPA, 2007; Kolpin et al., 2002; Martin et al., 2019; Mole and Brooks, 2019; Schultz and Furlong, 2008). Citalopram and fluoxetine have been found in UK drinking water at concentrations of 2.26–2.80 ng/L and 0.27 ng/L, respectively (Peng et al., 2019). It has been shown that antidepressants occurring in the aquatic environment are seasonal with the highest levels in autumn. This may be related to increases in flow which reduce the removal efficiency (Ma et al., 2020). Studies made by Silva et al., (2014) in Canada shows that amount of discharge of the different compounds in influent wastewater follow a descending order from autumn (ranging between 14.6 and 20.11 mg/day/1000 inhabitants for fluoxetine and citalopram, respectively), spring (ranging between 1.35 and 15.63 mg/day/1000

inhabitants for sertraline and citalopram, respectively), winter (only citalopram was found in mass loads of 11.3 mg/day/1000 inhabitants) and summer (only citalopram was found in mass loads of 1.32 mg/day/1000 inhabitants). The removal efficiencies of fluoxetine, paroxetine and sertraline which occur in lower frequencies fluctuate between 80.37 and 100.00% (Silva et al., 2014). According to Evans et al. (2015) parents compounds compared to metabolites are better removed by WWTP. Consequently, it has been observed that metabolites may remain biologically active (Calisto and Esteves, 2009), for example norfluoxetine possesses substantial pharmaceutical activity in comparison with the parent compound fluoxetine (DeVane, 1999) and desmethylsertraline, the metabolite of sertraline, is around one-eighth as potent as the parent compound (Koe et al., 1983). Studies show that after excretion parent compounds of fluoxetine can be reactivated in the WWTP by cleavage of the glucuronides (Nałęcz-Jawecki, 2007). According to their physicochemical properties desvenlafaxine (ODV) is expected to adsorb to suspended solids and sediment (PubChem, 2005). The occurrence of pharmaceuticals in the aquatic environment depend on the effective removal of the contaminants from WWTP (Brooks et al., 2003a) and the analytical method for drugs detection (Evans et al., 2015).

Venlafaxine and its derivative O-desmethylvenlafaxine are the two antidepressants included in the latest updated Watch List (EU Commission, 2020) which also includes four antibiotics (amoxicillin, ciprofloxacin, trimethoprim, sulfametoxazole), and three antifungal medicines.

Table 1-2: Occurrence of antidepressants in the aquatic environment

Antidepressant	***Amount***	***Media detected***	***Country***	***References***
Fluoxetine	-	Surface water	Portugal	(Fernandes et al., 2020)
	33.7 - 42.9 ng/L	River	Shanghai,China	(Wu et al., 2017)
	600(±280) and 560(±250) ng/L	Wastewater influent and effluent	Jamaica Bay, USA	(Benotti and Brownawell, 2007)
	34–152 µg/kg	Sludge	Canada	

	0.14–1.02 mg/kg	Fish meat	Canada	(Lajeunesse et al., 2012) (Chu and Metcalfe, 2007)
Norfluoxetine	0.15–1.08mg/kg	Fish meat	Canada	(Chu and Metcalfe, 2007)
	51.2 ng/L	WWTP influent	Portugal	(Paíga et al., 2016)
	0.011 µg/L	WWTP	Ontario,	(Metcalfe et al., 2010)
	0.77 ng/L	Tap water	Canada	(Benotti et al., 2009)
	8.9–60 µg/kg	Sediment (sludge)	USA	(Lajeunesse et al., 2012)
Sertraline	33 - 49 ng/L	River	Texas, USA	(Schultz and Furlong, 2008)
	203–528 µg/kg	Sludge	Canada	(Lajeunesse et al., 2012)
	<3.1 ng/L	Tap water	Warsaw	(Giebułtowicz and Nałęcz-Jawecki, 2014)
Citalopram	8000 ng/L	Surface water	India	(Fick et al., 2009)
	37 - 120 ng/L	River	Madrid,Spain	(González Alonso et al., 2010)
	95–1381 µg/kg 121 µg/kg	Sludge Sludge	Canada UK	(Evans et al., 2015; Lajeunesse et al., 2012)
	up to 1.5 ng/L	Tap water	Warsaw	(Giebułtowicz and Nałęcz-Jawecki, 2014)
	13.0–612 ng/L	STP influent	Norway	(Vasskog et al., 2006)
	9.2–382 ng/L	STP effluent	Norway	(Vasskog et al., 2006)
Venlaflaxine	55,000 ng/L	River (urban zone)	Ecuador	(Voloshenko-Rossin et al., 2015)
	641 ng/L	River	Leça, Portugal	(Fernandes et al., 2020)
	159 ng/L	River	Lis, Portugal	(Castillo-Zacarías et al., 2021)
	0.251 ng/g	Sediment	Douro,Portugal	(Fernandes et al., 2020)
	220 ng/L	WWTP effluent	Colorado,USA	(Schultz et al., 2010)
	210 ng/L	WWTP effluent	Lowa,USA	(Schultz et al., 2010)
	30.3 – 63.7 ng/L	WWTPs	Beijing, China	(Sheng et al., 2014)

	0.195 – 0.213 µg/L	WWTP influent	Canada	(Lajeunesse et al., 2008)
	0.176–0.214 µg/L	WWTP effluent	Canada	(Lajeunesse et al., 2008)
	0.013–0.045 µg/L	Streams	Canada	(Lajeunesse et al., 2008)
O-desmethylvenlafaxine	173.68 ng /L	Wastewater	Shanghai,China	(Ma et al., 2020)
	4.53 ng/L	River	Shanghai,China	(Ma et al., 2020)
	121.12 ng/L	Influent	Shanghai,China	(Ma et al., 2020)
	173.68 ng/L	Effluent	Shanghai,China	(Ma et al., 2020)
	21-68.7 ng/L	River	Canada	(Lajeunesse et al., 2008)

1.8. Impact of antidepressants on the environment:

Antidepressants have effect at very low environmental relevant concentration (Fong and Ford, 2014). It has been studied that they can induce spawning in bivalves (Fong, 1998; Lazzara et al., 2012), alter cell signaling mediator namely cyclic adenosine 3′,5′-monophosphate/protein kinase A pathway and serotonin (5-hydroxytryptamine [5-HT]) in mussels (Franzellitti et al., 2013), cause foot detachment from substrate in snails (Fong and Hoy, 2012), decrease in memory capacity, altered cognitive function, and decrease in the ability to protect from predators in cuttlefish (Di Poi et al., 2013; Di Poi et al., 2014), changed reproduction (Schultz et al., 2011), activity (Barry, 2013), and embryonic/development endpoints (Yang et al., 2014) in fish. Brooks et al. (2005) found out that in brain and liver the concentration of antidepressant and their metabolites was higher than in muscles. SSRI was found at value greater than 0.1ng/g in all fish tissues residing in a municipal effluent-dominated stream in Texas, USA

Brown trout juveniles and larvae exposed to citalopram (1mg/L) results in changes of swimming activity (Ziegler et al., 2020). Brooks et al. (2003b) observed that algae were sensitive to fluoxetine with a median effect concentration (EC50) of 0.039 mg/L. It has been noted that algae and invertebrates show toxic effects that are additive to mixtures of antidepressants (Christensen et al., 2007; Henry and Black, 2007). An experiment run on microbial communities exposed to sertraline hydrochloride showed that

antidepressant inhibited the growth of *C. vulgaris* and *M. aeruginosa* and decreased the *Chl-a* quantity in the microcosm, which has an effect on the photosynthetic efficiency of the microcosm. It has been concluded that sertraline hydrochloride disrupts the ecological equilibrium in microcosms and represents an ecological risk (Yang et al., 2019). Flaherty and Dodson. (2005) observed that fluoxetine can interacts with clofibric acid to induce sub lethal responses in *Daphnia magna* (mortality significant deformities, including malformed carapaces and swimming setae). A study made by Bossus et al. (2014) revealed that there is behavioral effect (on velocity) on amphipod after 1 h exposure to sertraline at 0.01 µg/L and after 1 day exposure to fluoxetine as low as 0.001 µg/L It was also shown that *D. magna* exposed to fluoxetine increase their offspring production. Like fluoxetine, norfluoxetine can as well induce parturition in fingernail clams, *Sphaerium striatinum* (Fong and Molnar, 2008). However, some studies show that norfluoxetine is tenfold more toxic to aquatic protists and crustacea, i.e., *Spirostomum ambiguum*, *Tetrahymena thermophila*, and *Thamnocephalus platyurus*, than fluoxetine (Andrés-Costa et al., 2017; Nałęcz-Jawecki, 2007).

Best et al. (2014) performed experiments with rainbow trout and observed that venlafaxine disrupted the interrenal steroidogenic capacity, including altered handling of stressor-mediated changes in mRNA abundances of steroidogenic acute regulatory protein and cytochrome P450 side chain cleavage. It also brought alterations in the gill of trout. The results showed that venlafaxine can compromise the adaptive responses of rainbow trout to an acute stressor. It has been demonstrated that venlafaxine at measured concentrations in the aquatic environment has an effect on the behavior and can compromise the adaptive responses to the environment in zebrafish larvae (Tang et al., 2021).

1.9. *Mytilus galloprovincialis* and toxicity tests

Most of antidepressant ecotoxicity tests have been carried out using freshwater molluscs, fish and algae and little is known on marine bivalves. *Mytilus spp*. are considered as good bio-indicators (Goldberg et al., 1978; Libralato et al., 2013; OECD, 2010). The sessile nature of mussels increases the exposure to toxic substances. In fact, they have contact with benthic substrate and sediments, have a widespread distribution,

have high filtration/accumulation rate and are able to response to physical and chemical stressors. Molluscs, such as *Mytilus galloprovincialis*, are used as indicator organisms for environmental quality assessments (Capolupo et al., 2021; Faucet et al., 2004; Mulcahy, 2000; Warren et al., 1995).

1.9.1. Early life stage testing

Due to their sensitivity to contaminants more than adults and their role as a good indicator of the bioavailability of pollutant concentrations in seawater, early life stages of marine invertebrate have long served as a model system for marine ecotoxicology (Bay et al., 1993; Byrne, 2012; Hagger et al., 2005; Libralato et al., 2013; Mai et al., 2012; Valenti et al., 2006). Planktonic larvae are a key stage in the completion of life cycle, in addition of playing a crucial role in the recruitment and dissemination of populations (Cormier et al., 2021).

Toxicity tests on molluscs are usually performed easily, are also highly sensitive, cost-effective and timesaving, as meaningful results can often be obtained in 24 to 48 hours. The different endpoints such as fertilization, embryotoxicity and larvae survival/immobilization tests are done to evaluate acute, sub-chronic or chronic toxicity of contaminants (His et al., 1997; Libralato et al., 2013). Bioassays mostly those based on echinoderm and molluscan development are strategic regulatory tools for monitoring toxicity of point source pollution discharges (ASTM, 2004; EPA, 2002)

1.10. Ecological risk assessment and management

Ecological risk assessment is a mean to assess the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors (toxicity test). Hazard quotient (HQ) is used to characterize the ecological risk of pharmaceutical to aquatic organism. The latter is expressed as the relationship between a predicted environmental concentration (PEC) and a predicted no effect concentration (PNEC). If a HQ resultant from a PEC/PNEC ratio is >1, then it is considered as a high risk to the environment (Brooks et al., 2003a). Research done according to the risk assessment standard (EU and US Food and Drug Administration (FDA)) enables to create policy instruments in view to protect the environment from

harmful contaminants like the creation of surface water watch list for the European Commission (EC) from Directive 2013/39/EU.

Bioassays have been well developed to study about subtle effects of drugs on organisms in the environment using biomarkers and other molecular technologies, e.g., proteomic and genomic techniques. However, little are known on the effect of metabolites in the environment and the meaning of risk assessment data in terms of ecological functioning. Toxicity test on single-species and extrapolation alone is not fully representative of the possible effect of pharmaceuticals to the aquatic communities. Current knowledge is most often based on single substances, but in the real world multiple factors work in concert and may affect the availability and toxicity of these substance. For instance, risks of mixtures and effects of environmental factors, the effects of mixtures (drug and drug interaction) are an important area of study (Boxall, 2004). The OECD report (2019) recommended reduction of the excessive and inappropriate use of pharmaceuticals from physicians, veterinarians, pharmacists, patients and farmers for cost-effective management of pharmaceuticals for protection of water quality and aquatic ecosystem.

Chapter 2

2. Methodology

2.1 Chemical handling

The tested chemicals were four antidepressants i.e. Fluoxetine hydrochloride (FLX), Venlafaxine hydrochloride (VEN), Citalopram hydrobromide (CITA), Sertraline hydrochloride (SERT); and two metabolites O-desmethylvenlafaxine (ODV), and Norfluoxetine (NF). The four antidepressants and ODV were purchased from Sigma–Aldrich, Milan, Italy (purity ≥98%) and NF was purchased from Cayman Chemical, US (purity ≥98%). A stock solution of each chemical was prepared at the concentration of 0.2 mg/ml using the organic solvent dimethyl sulfoxide (DMSO) since the chemicals were poorly soluble in water; stock solutions were aliquoted and stored at -20ºC until use. At use, the serial dilutions were performed in fresh seawater (FSW) to obtain final concentrations of 0.5, 5, 10, 25, 50, 100, 250 and 500 ng/L.

2.2 Animal holding and experimental design:

Adult Mediterranean mussels *Mytilus galloprovincialis*, were collected from an aquaculture body “COPRALMO” in Cesenatico, Italy (NW Adriatic coast) and transported immediately to wet laboratory for 5-days acclimatization in tanks at a density of 5 mussels/L in continuously aerated filtered FSW (0.22 µm) at 16 ±1 ºC. The effects induced by antidepressants and metabolites were investigated on mussel early life stages through fertilization toxicity test, embryotoxicity, larvae motility and mortality tests (eggs, spermatozoa, embryos, and D-shaped veliger larvae). Control tests were done in parallel to each test and performed number of replicates (N) were specific to each test.

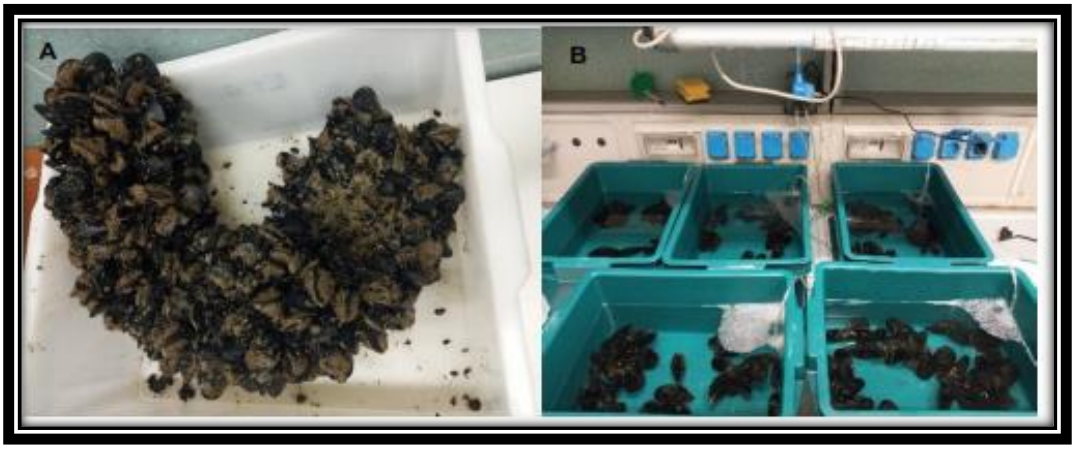

Figure 2-1 : (A) Collected mussels *M. galloprovincialis* from the aquaculture farm in Cesenatico, Italy, and (B) acclimatization of mussels for 5 days

2.3 Gamete collection:

Mussels were induced to spawn by providing them thermal shock of 10 °C (16 to 26 °C) according to the ASTM standard protocol (ASTM, 2004) in FSW filtered using nylon meshes (50 µm and 100µm) to remove debris (**Error! Reference source not found.**). Once they started to spawn, each mussel was separated and collected in 250 mL beaker containing 200 mL filtered FSW (0.22µm). After complete spawning, sperms were sieved through 50 µm and eggs through 100 µm nylon meshes; then maturity of sperms (motility) and viability of eggs (shape/color/size) were checked using inverted optical microscopy at 40x magnification (OPTECH-IB series: Munich, Germany). Good quality gametes were selected to perform tests of early life stages.

Figure 2-2 Incubation of mussels *M. galloprovincialis* to induce spawning providing thermal shock of 10ºC (temperature)

2.4 Fertilization assay:

The fertilization toxicity test was carried out to analyze the effects of antidepressants and derivatives on mussels' gamete fertilization according to a protocol defined by Capolupo et al., (2018) with slight modifications. Spermatozoa were exposed to a range of nominal concentrations of each chemical into 96-well microplates. After 60-min exposure, eggs were introduced at a 1:3 ratio of eggs to spermatozoa. After 60 min the experiment was stopped by adding 40% calcium-buffered formalin. Successful fertilization was assessed by inverted microscopy (40 x magnification). The criteria of fertilization success were the appearance of the polar lobe or cleavage stage (Capolupo et al., 2018). Data were recorded as mean ± SEM (N=5) of the percentage of fertilization success. The assay validity was set at a mean fertilization rate of >60% and ≤ 98%% (Environment Canada, 2011)

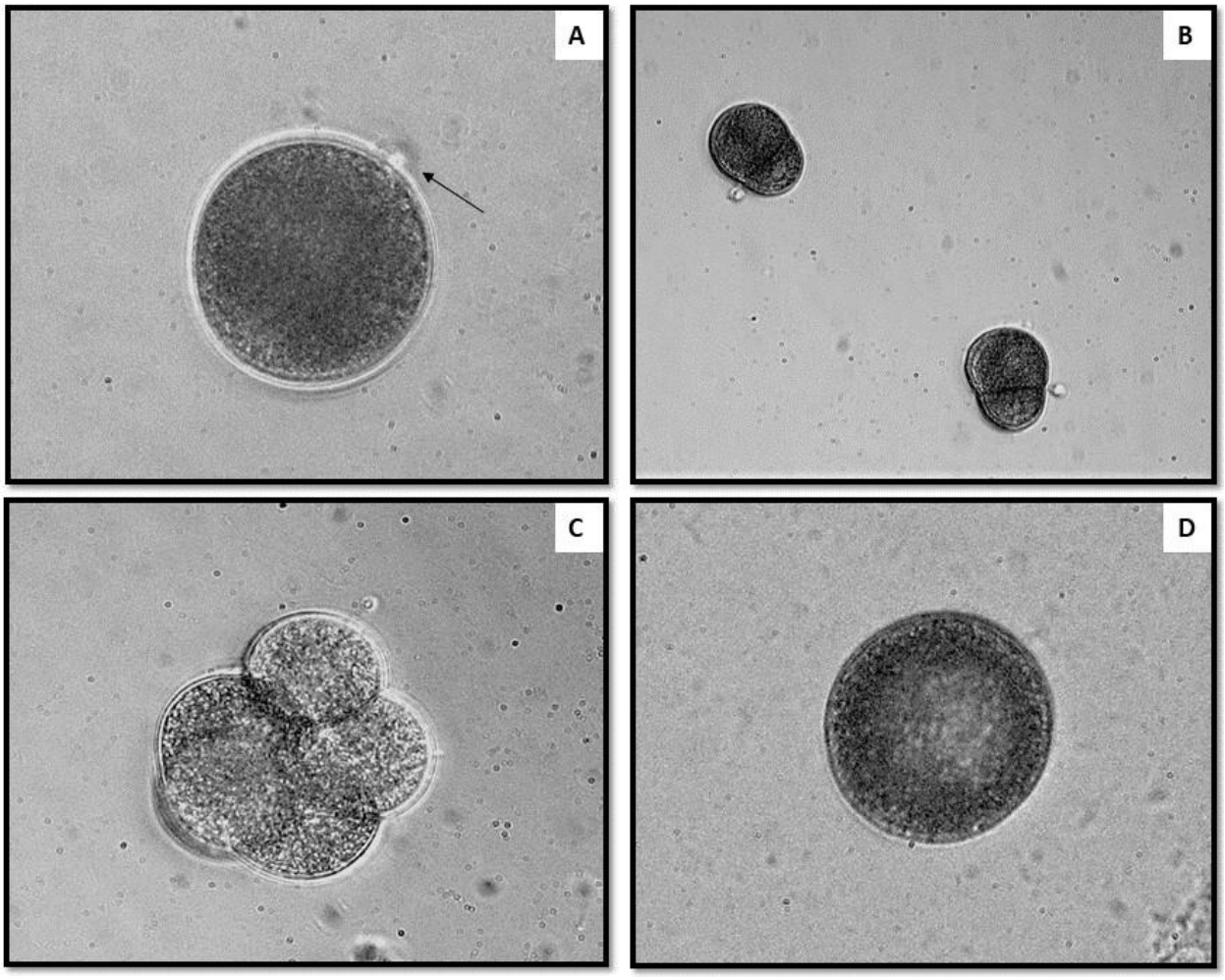

Figure 2-3 fertilized and unfertilized eggs; A. fertilized egg with polar lobe, B. fertilized egg at 2- cell division stage, D. 5 – cell division stage (cleavage) and D. unfertilized egg (UF).

2.5 Embryotoxicity assays:

The embryotoxicity test was performed as described by Fabbri et al., (2014) to analyze the effects of tested chemicals on embryonic development of D-veliger. Mussel oocytes and spermatozoa (1:3 egg to spermatozoa ratio) were exposed to a range of nominal concentrations of each chemical into 96-well microplates. After exposure, the fertilization success (>80%) was checked under microscope. Then, the plates were incubated for 48-h at 16ºC ± 2 ºC with a photoperiod of 16-h Light and 8-h Dark. At the end, the experiment was stopped by adding 4% calcium-buffered formalin. The criteria to assess the normal development of larvae was fully developed D-shape shell (straight hinge) and malformed when development abnormalities were observed such as

concave/convex shape, smaller size, protruding velum or assessed as delayed development such as trocophore or earlier stages (Capolupo et al., 2020). The test acceptability was based on >75% normal D-shell shaped veliger in controls (ASTM, 2004). Data were recorded as mean ± SEM (N=5) of percentage of normally developed larvae (D-veliger).

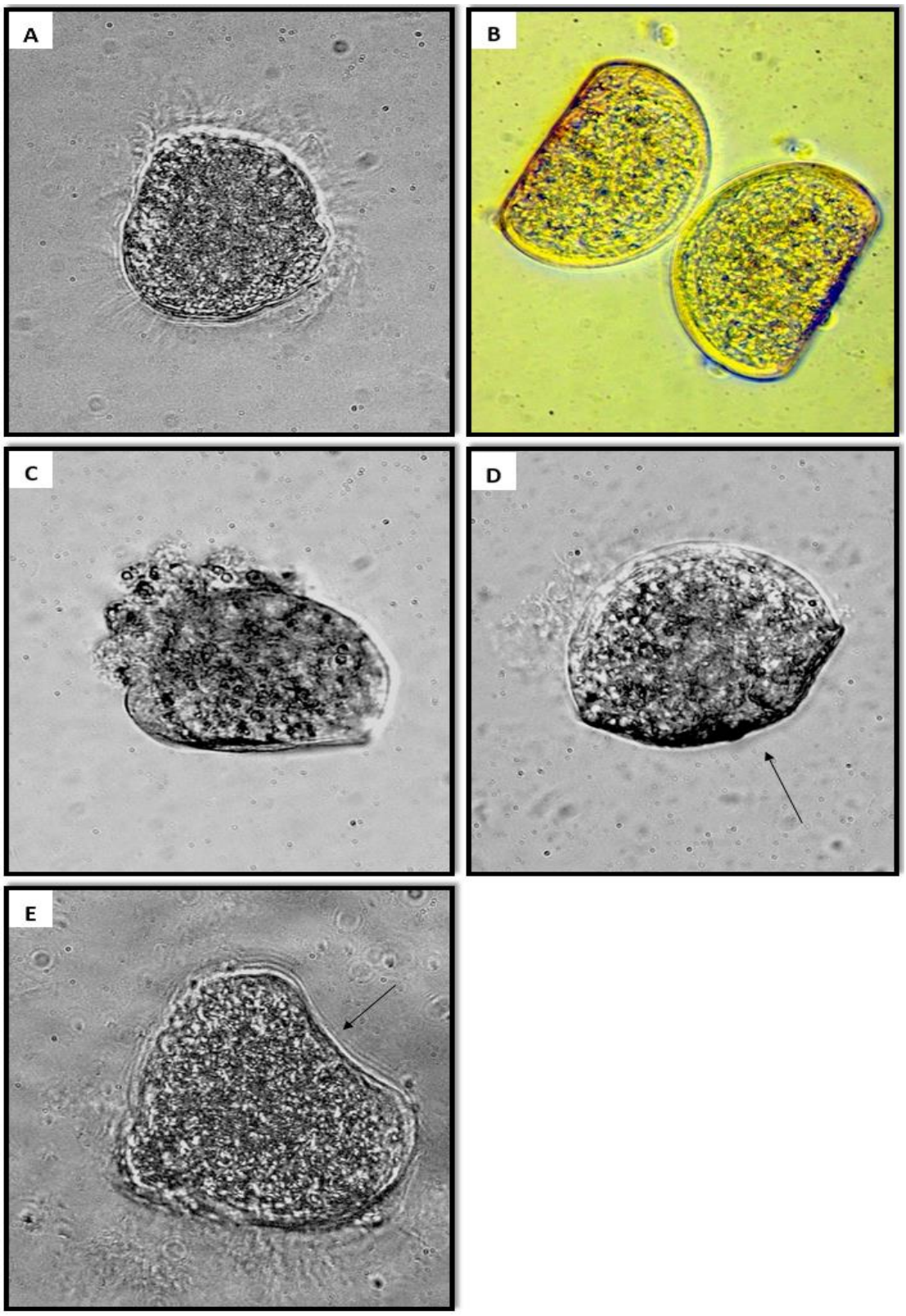

Figure 2-4 D-shaped larval development of mussel; A. Trocophore stage, B. Normal D-shell shape larvae, C. Abnormal larvae with mantle protuberances, D. larvae with convex hinge, and E. larvae with protruding mantle and concave hinge.

2.6 Larvae immobilization and Survival assay:

This test was performed according to (Capolupo et al., 2020), where the effect of antidepressants and the derivatives was analyzed on D- shape veligers larvae with the endpoint survival and immobilization. Oocyte fertilization was done in 2 L glass beakers having 2 L filtered seawater; larvae from 48 h post fertilization (pf), were fed day-to-day with a suspension of microalgae (*Nannochloropsis oculata*; 1,200 cell/mL) until 5-day pf. At 6-day pf, reared larvae were sieved through a 20 µm pore nylon mesh and collected. Then they were exposed to nominal concentrations of each tested chemical in 96-well microplates (~50 individuals/well).

The number of motile and viable larvae was recorded after 48 h, 96 h, 168 h and 264 h of exposure, using an inverted optical microscope (40-100 × magnification). Mortality criteria assessment consisted of the clearly visible degradation/decomposition of the soft tissue and/or the absence of visceral movement and heart/cilia beating (Capolupo et al., 2020). The larvae was considered immobilized when it showed no swimming or spiral/circulatory movements typical of mussel D-shaped veliger (Sprung, 1984), though there was sign of visceral, heart and/or cilia beating. Data for survival were expressed as the mean ± SEM of the percentage of viable larvae, while immobilization was expressed as the mean ± SEM of the percentage of non-motile larvae over the proportion of viable larvae.

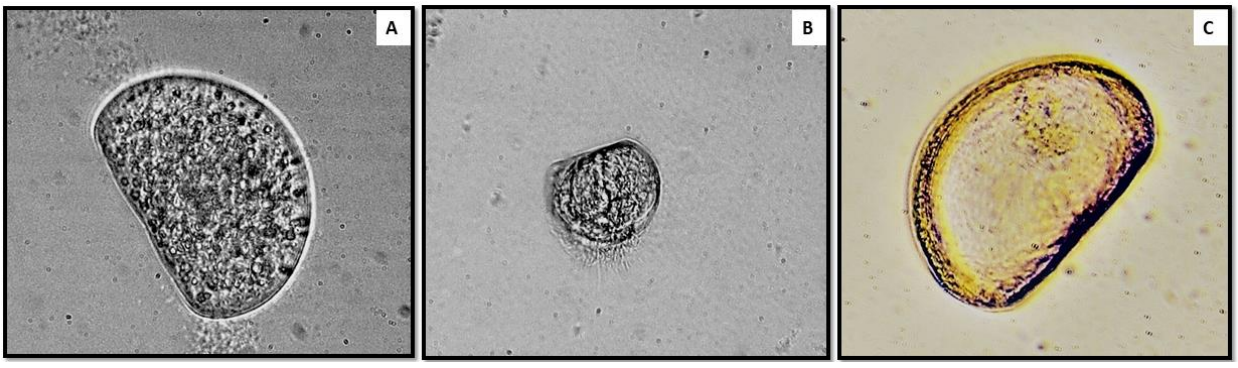

Figure 2-5 Alive and dead larvae; A. Alive mussel D-shape veliger, B. Alive larvae with cilia beating and C. Dead mussel larvae.

2.7 Statistical Analysis:

Data were analyzed by using statistical package SigmaPlot 12 (Systat Software Inc. San Jose, CA, USA). One-way Analysis of Variance (ANOVA), followed by the Dunnett's test were applied to assess statistically significant variations between controls and treatments. Data were considered significant for $p < 0.05$.

CHAPTER 3

3. Results

3.1 Fertilization assays

Figure 3-1 presents the effects of the antidepressants and derivatives tested on mussel gamete fertilization**.** In controls, the mean fertilization success of mussels ranged from 95.99 to 99.74 %, which is within the acceptable range for test acceptance (Environment Canada, 2011). Compared to controls, the effects of 60min exposure to SERT, FLX, ODV, and NF on fertilization success were significant. SERT altered fertilization at a higher number of treatments than other tested substances. FLX caused significant reduction of fertilization success at 100, 250, and 500 ng/L; SERT significant effects were observed at concentrations of 10, 25, 50, 100, 250, and 500 ng/L **(Figure 3-1 e, c)**. The metabolite ODV induced the inhibition at concentrations of 25, 50, 100, 250, 500 ng/L and NF at concentrations of 0.5, 100, 250, 500 ng/L, significantly **(Figure 3-1 b, d)**. As shown in **Figure 3-1 a, f**, exposure to VEN and CITA did not significantly affect fertilization success.

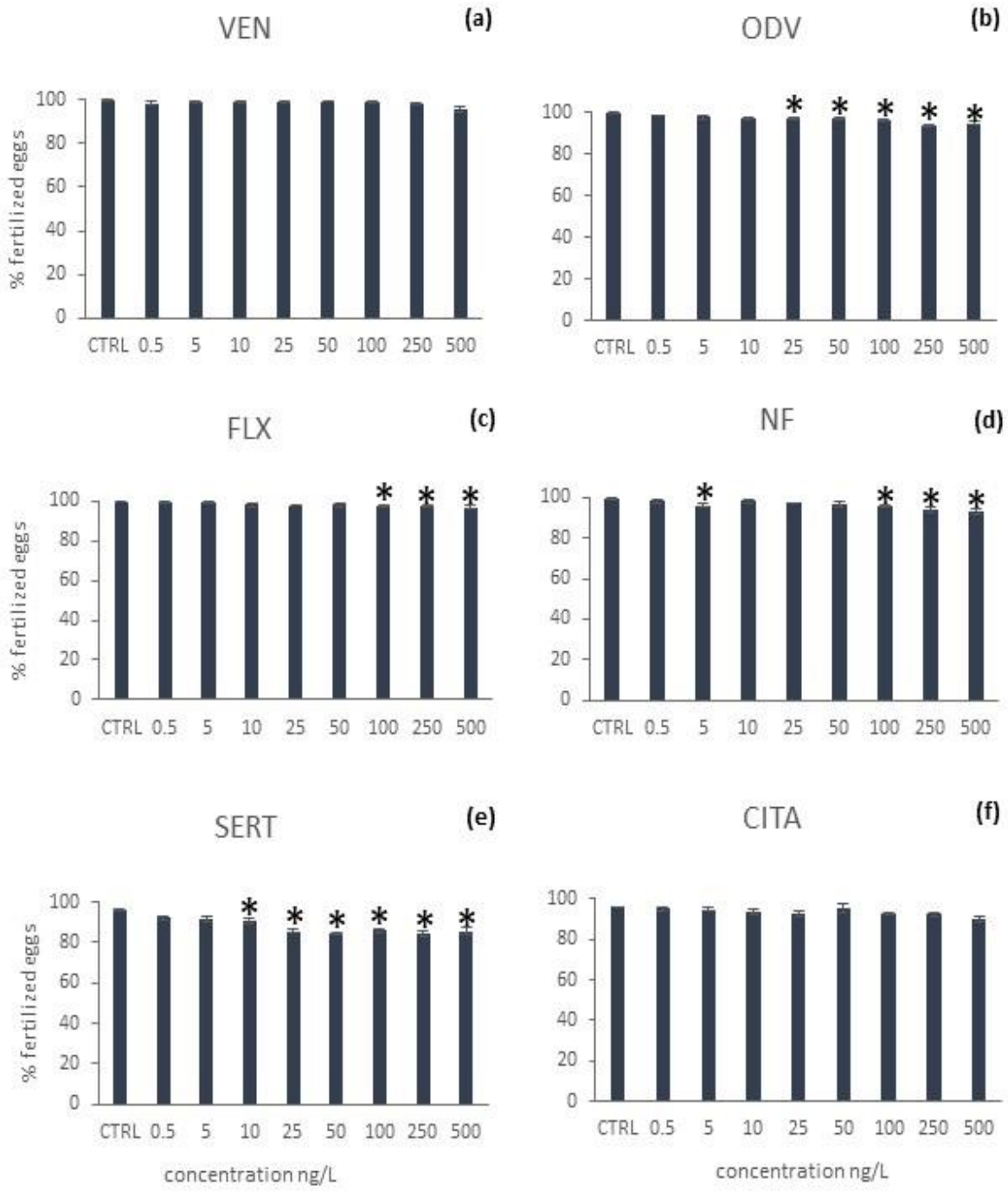

Figure 3-1 Percentage of fertilization of *Mytilus galloprovincialis* gametes after 60 min exposure to venlaflaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluexetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 5. Asterisks indicate significant differences compared to the control ($p < 0.05$, One-way ANOVA, Dunnett's post-hoc comparison).

In **Figure 3-2**, percentage of unfertilized eggs (UF) and fertilized eggs with polar lobe (PL) or under cell division (D) are reported. Controls showed a higher mean percentage of cell division; the polar lobe stage was dominant for all tested substances as compared to cleavage stage. The prevalence of unfertilized eggs (UF) is evident at the lower concentrations of all tested substances, but a higher percentage of non-fertilized eggs was detected in case of SERT compared to controls.

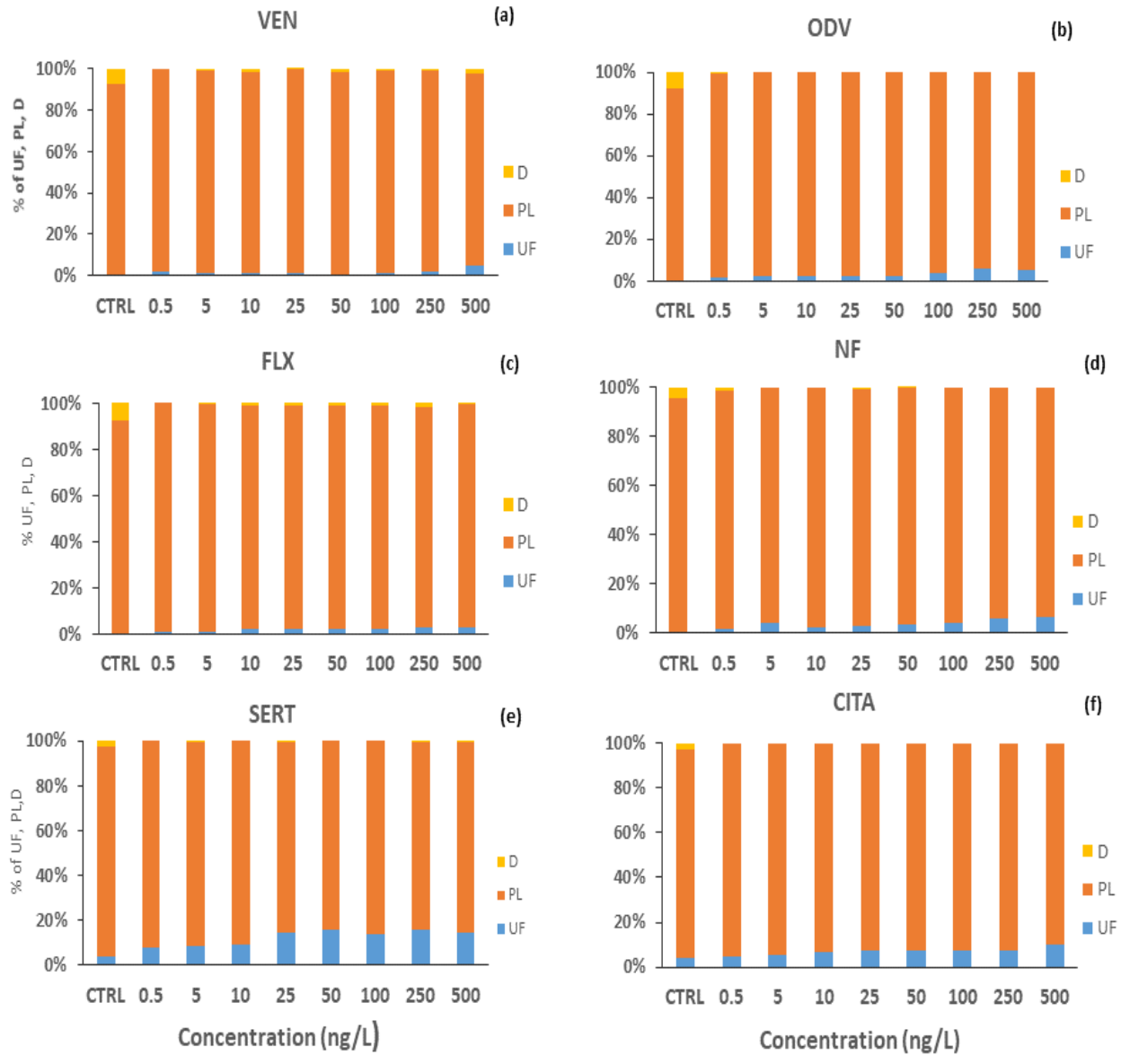

Figure 3-2. Percentage of unfertilized eggs (UF), egg with polar lobe (PL) and cell division stage (D) after 60 min exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 5.

3.2 Embryo-larval development assays

Figure 3-3 shows the effects of antidepressants and derivatives tested on mussel embryos. In the control groups, the mean percentage of normally developed D-veliger ranged from 95.84 to 97.90 %, i.e., within accepted test values (ASTM, 2004). Compared to controls, 48-h exposure of mussel embryos to FLX and its derivative NF, SERT and CITA caused significant reduction in the number of normally developed D-veligers. NF inhibited normal embryonic development at all concentrations except the lowest (0.5 ng/l), whereas FLX exhibited significant effects at concentrations of 100, 250 and 500 ng/l (**Figure 3-3 c, d**). The effects on normally developed D-veliger were also reported at SERT concentrations of 25, 50, 100 and 500 ng/L **(Figure 3-3 e)**. CITA inhibited the normal development of embryos only at a higher concentration of 500 ng/L **(Figure 3-3 f)**. No significant reduction of normally developed larvae was observed for VEN and ODV **(Figure 3-3 a, b)**.

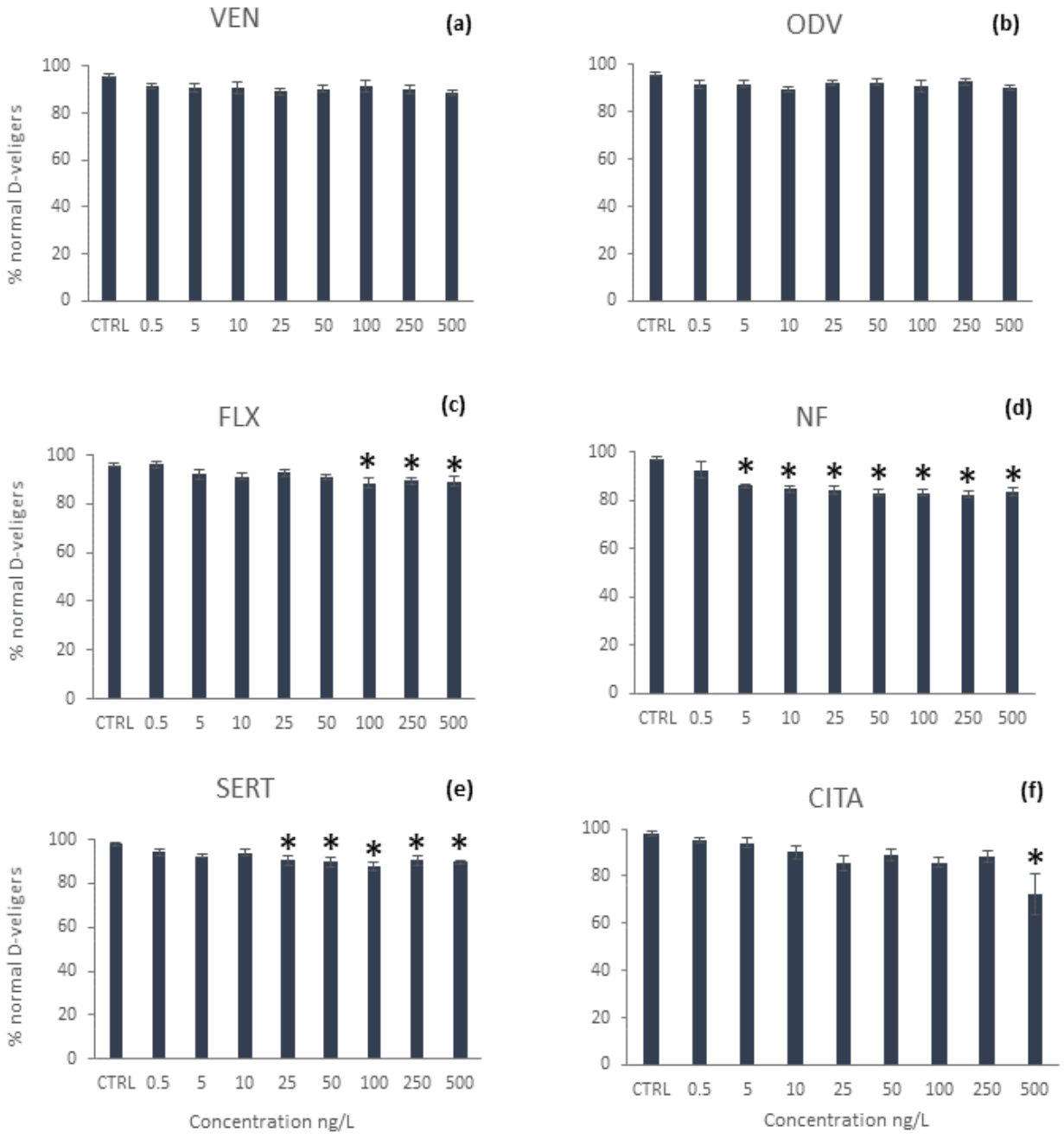

Figure 3-3. Percentage of normally developed of *M. galloprovincialis* larvae after 48 h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 5. Asterisks indicate significant differences compared to the control ($p < 0.05$, One-way ANOVA, Dunnett's post-hoc comparison).

Embryotoxicity assay was analyzed according to three categories: normally developed D-shaped veliger (ndv), abnormally developed D-shaped veliger (adv) and delayed development that is trocophore stage (troc) (**Figure 3-4**). SERT and CITA caused a greater percentage of abnormally developed D-shaped veliger rather than delayed development (Figure 3-4 e, f) which account for the effects on embryonic development. However, significant effects by FLX and NF (Figure 3-4 c, d) are supported by a higher mean percentage of delayed trocophore stage instead of abnormally developed D-shape veliger. VEN and ODV (Figure 3-4 a, b) have also been observed to induce a higher mean of trocophore stages than abnormal D-shaped veliger.

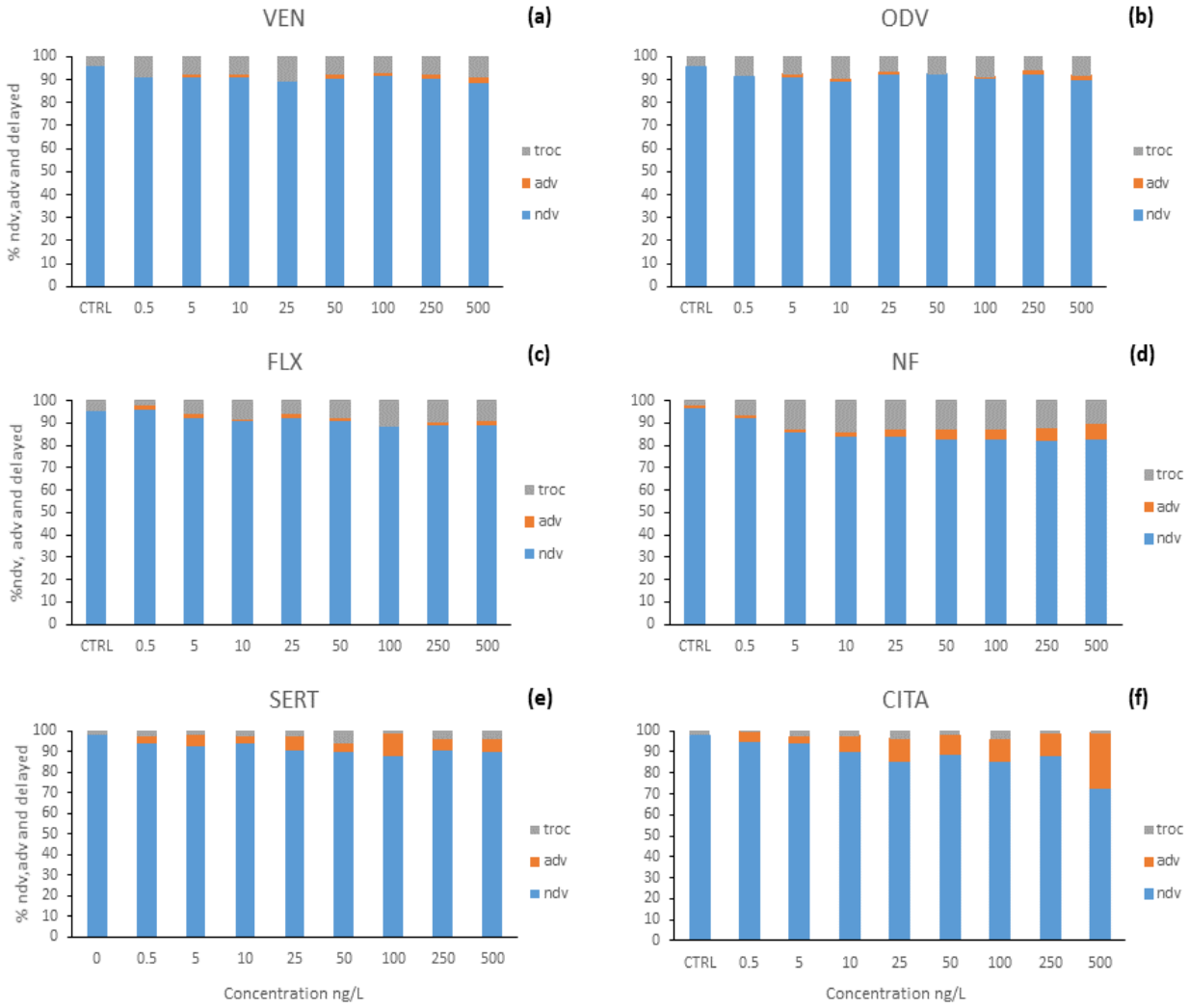

Figure 3-4 Percentage of normally developed D-shaped veliger (ndv), abnormal D-shaped veliger (adv) and trocophore stage (troc) of *M. galloprovincialis* larvae after 48 h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 5.

3.2 Larvae immobilization assay:

Results of 48-h, 96-h, 168-h, and 264-h motility test performed on *M. galloprovincialis* larvae exposed to antidepressants and derivatives are reported in **Figure 3-5**, **Figure 3-6**, **Figure 3-7**, and **Figure 3-8**. A significant reduction in larvae movement after 264-h has been observed after exposure to ODV at 0.5 ng/L and to FLX at 500 ng/L. No significant reduction in larvae motility has been noted after exposure to VEN, SERT, CITA and NF.

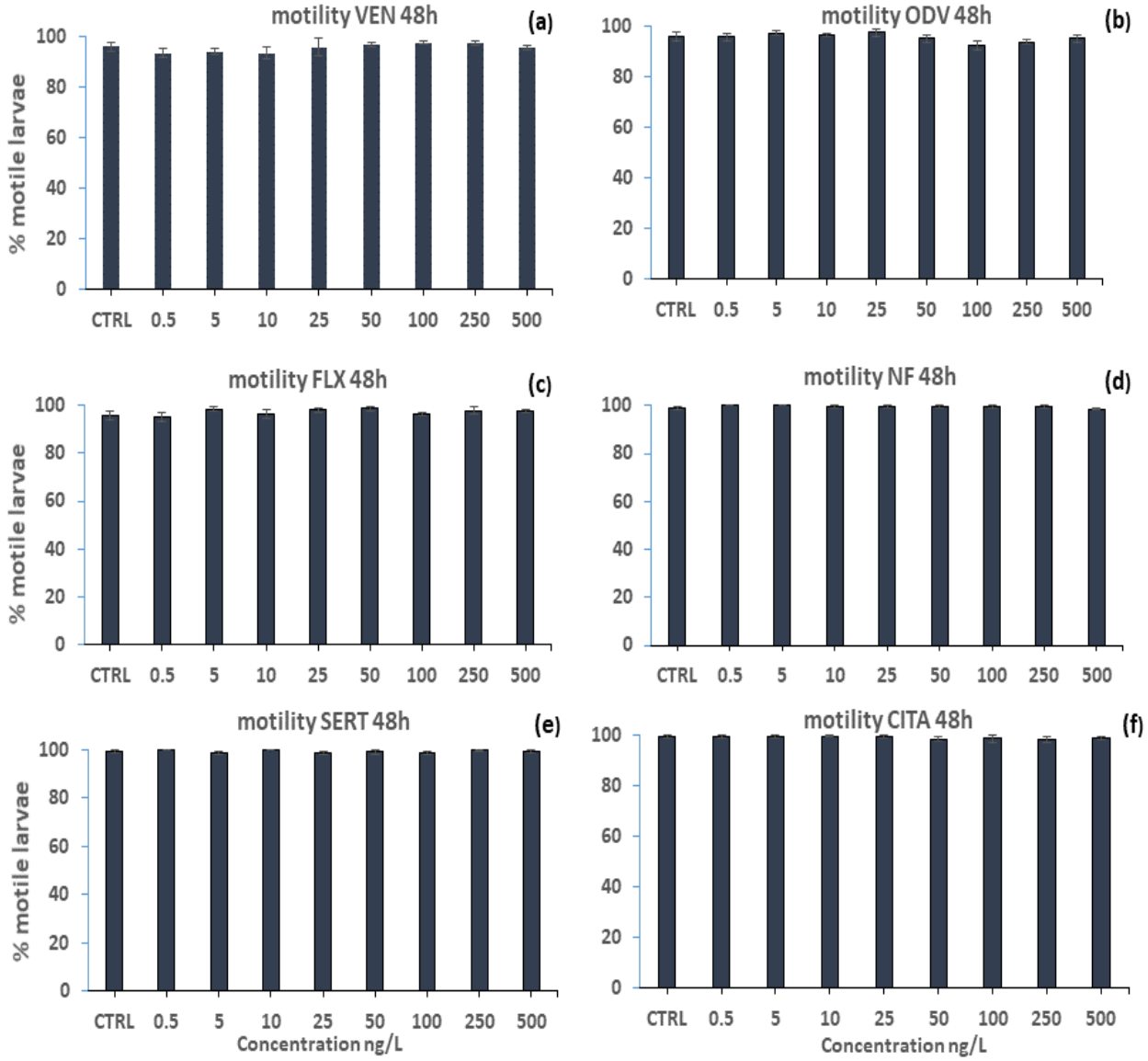

Figure 3-5. Percentage of motile larvae of *M. galloprovincialis* larvae after 48-h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 4.

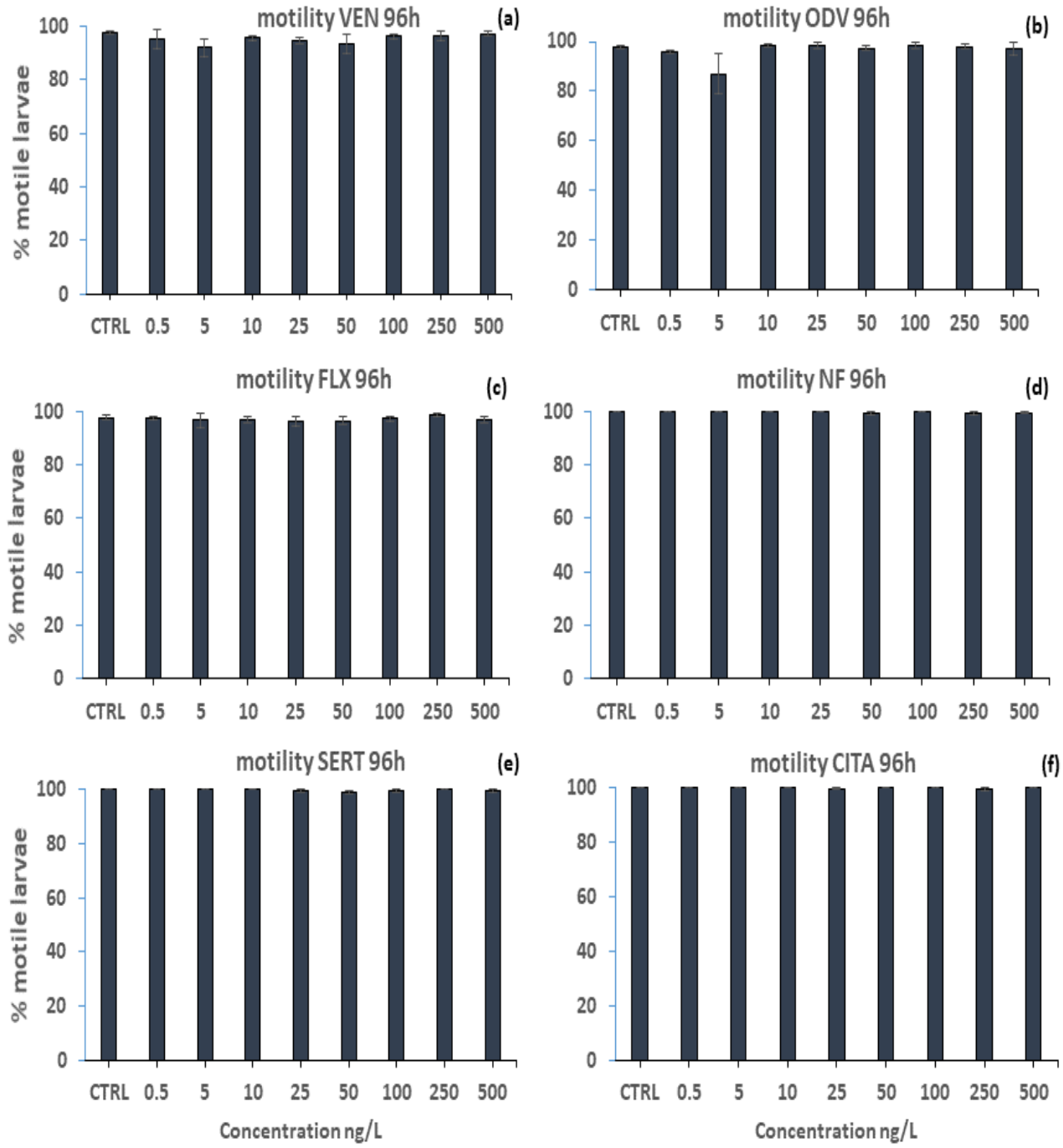

Figure 3-6. Percentage of motile larvae of *M. galloprovincialis* larvae after 96-h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 4.

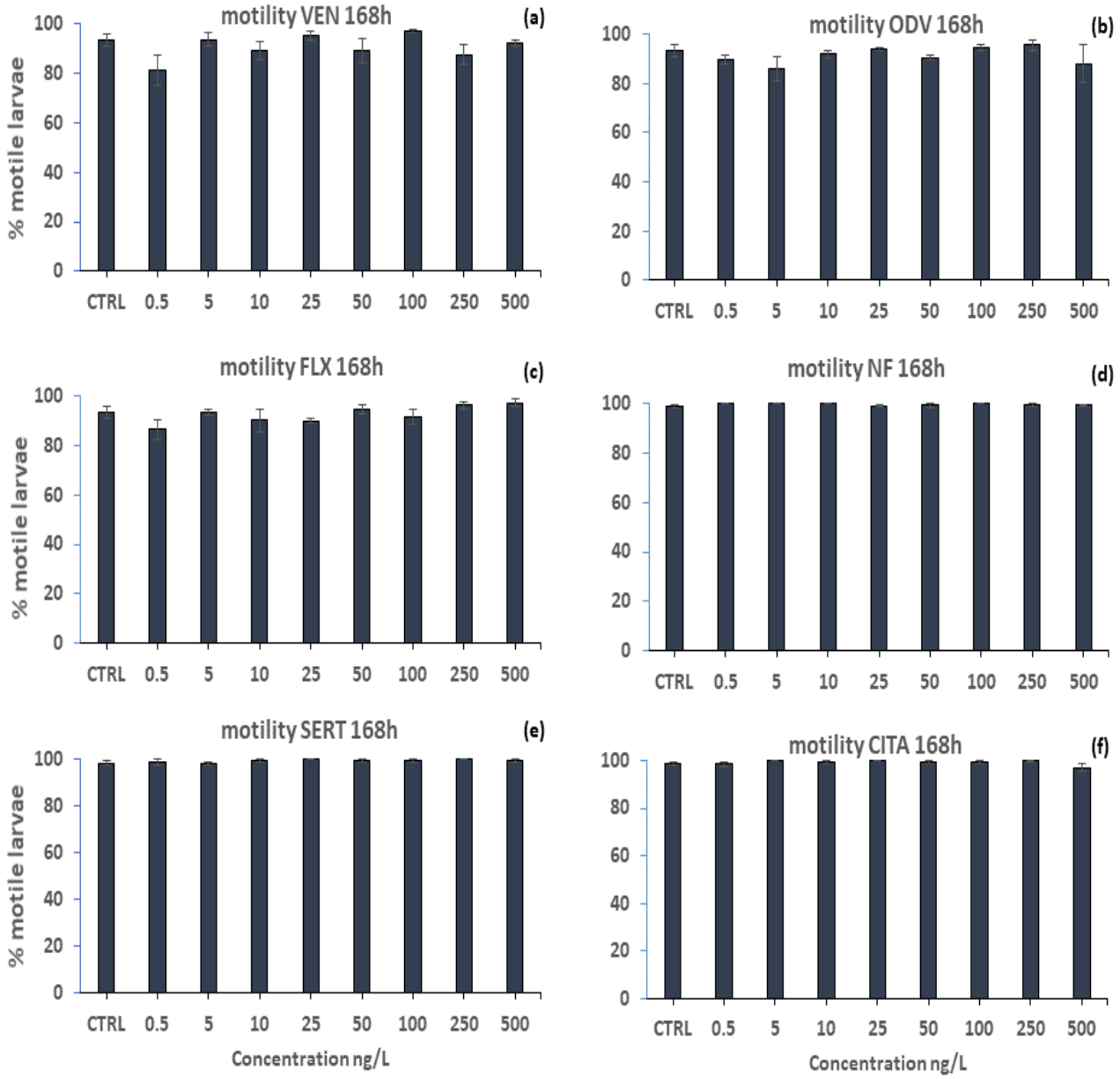

Figure 3-7. Percentage of motile larvae of *M. galloprovincialis* larvae after 168 h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 4.

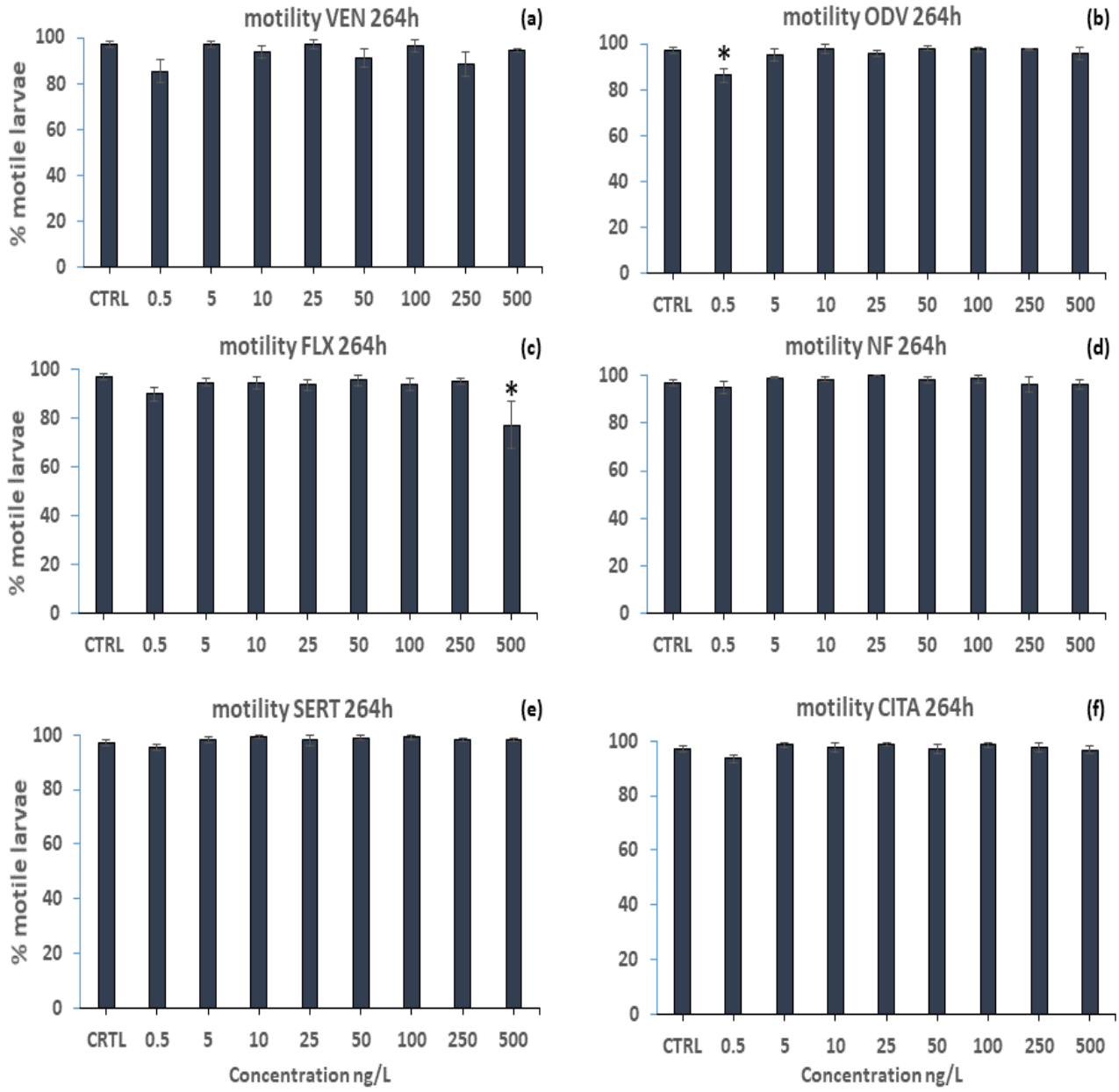

Figure 3-8. Percentage of motile larvae of *M. galloprovincialis* larvae after 264 h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 4. Asterisks indicate significant differences compared to the control ($p < 0.05$, One-way ANOVA, Dunnett's post-hoc comparison).

3.3 Larvae survival assay:

Results of 48-h, 96-h, 168-h, and 264-h survival test performed on *M. galloprovincialis* larvae exposed to antidepressants and derivatives are reported in **Figure 3-9, Figure 3-10, Figure 3-11**, and **Figure 3-12**. Compared to controls there is a significant reduction in larvae survival when exposed to FLX after 48-h at 10, 25 and 100 ng/L; after 96-h at 10 ng/L; and after 168-h at 10 and 25 ng/L. No significant decrease in larvae survival has been observed when exposed to VEN, ODV, SERT, CITA and NF after 48-h, 96-h, 168-h and 264-h.

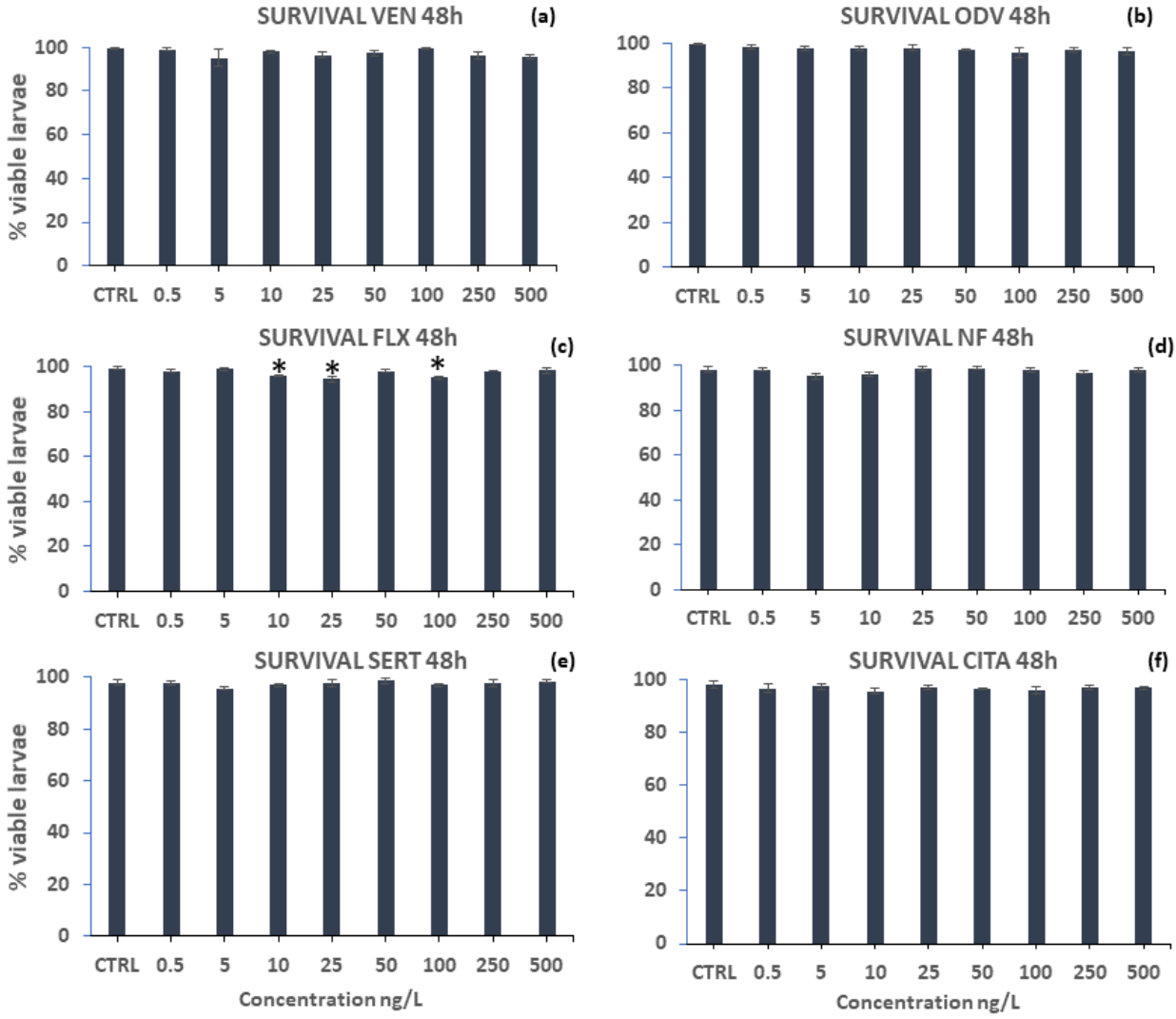

Figure 3-9. Survival percentage of *M. galloprovincialis* larvae after 48-h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 4. Asterisks indicate significant differences compared to the control ($p < 0.05$, One-way ANOVA, Dunnett's post-hoc comparison).

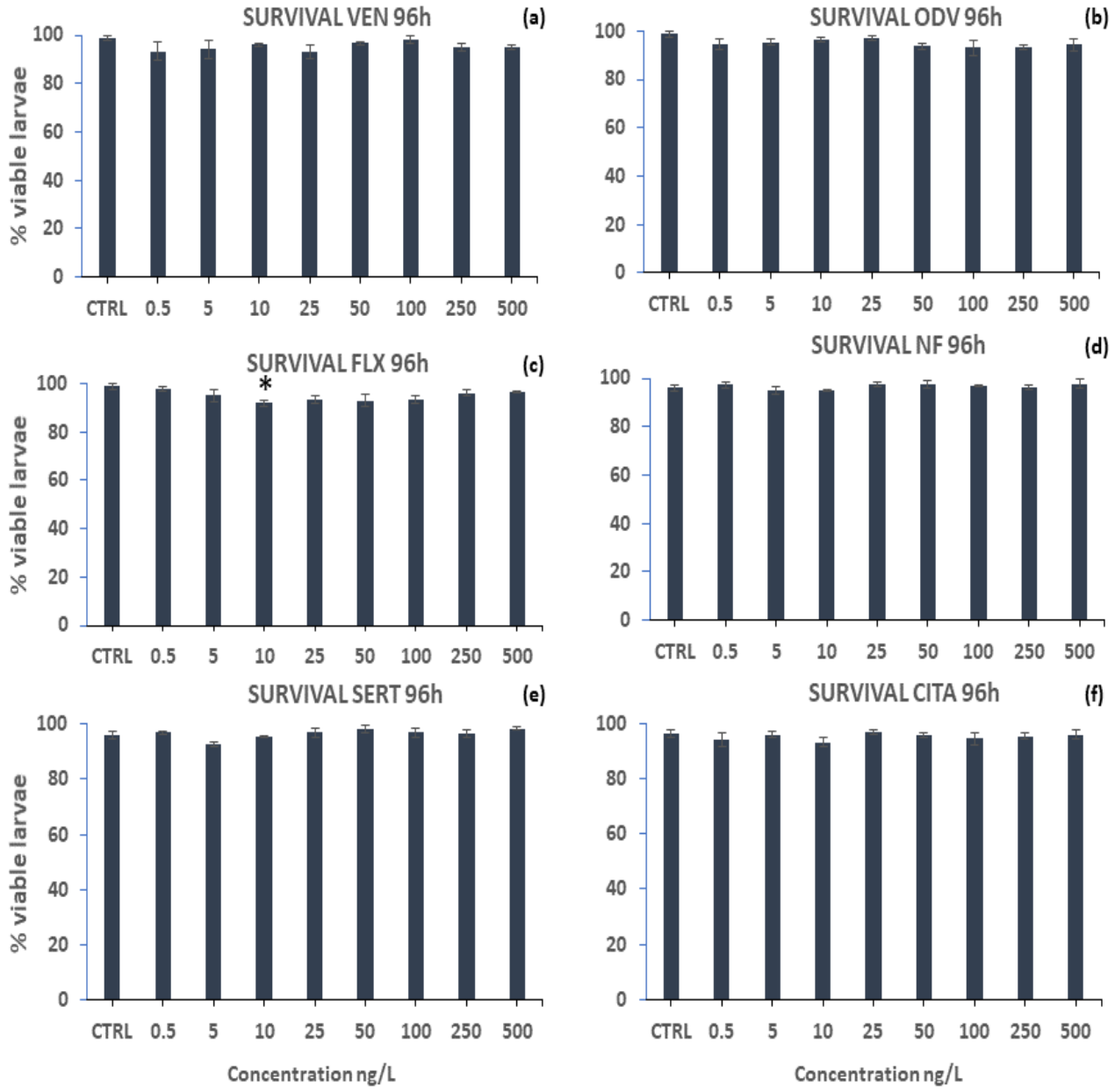

Figure 3-10. Survival percentage of *M. galloprovincialis* larvae after 96-h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 4. Asterisks indicate significant differences compared to the control ($p < 0.05$, One-way ANOVA, Dunnett's post-hoc comparison).

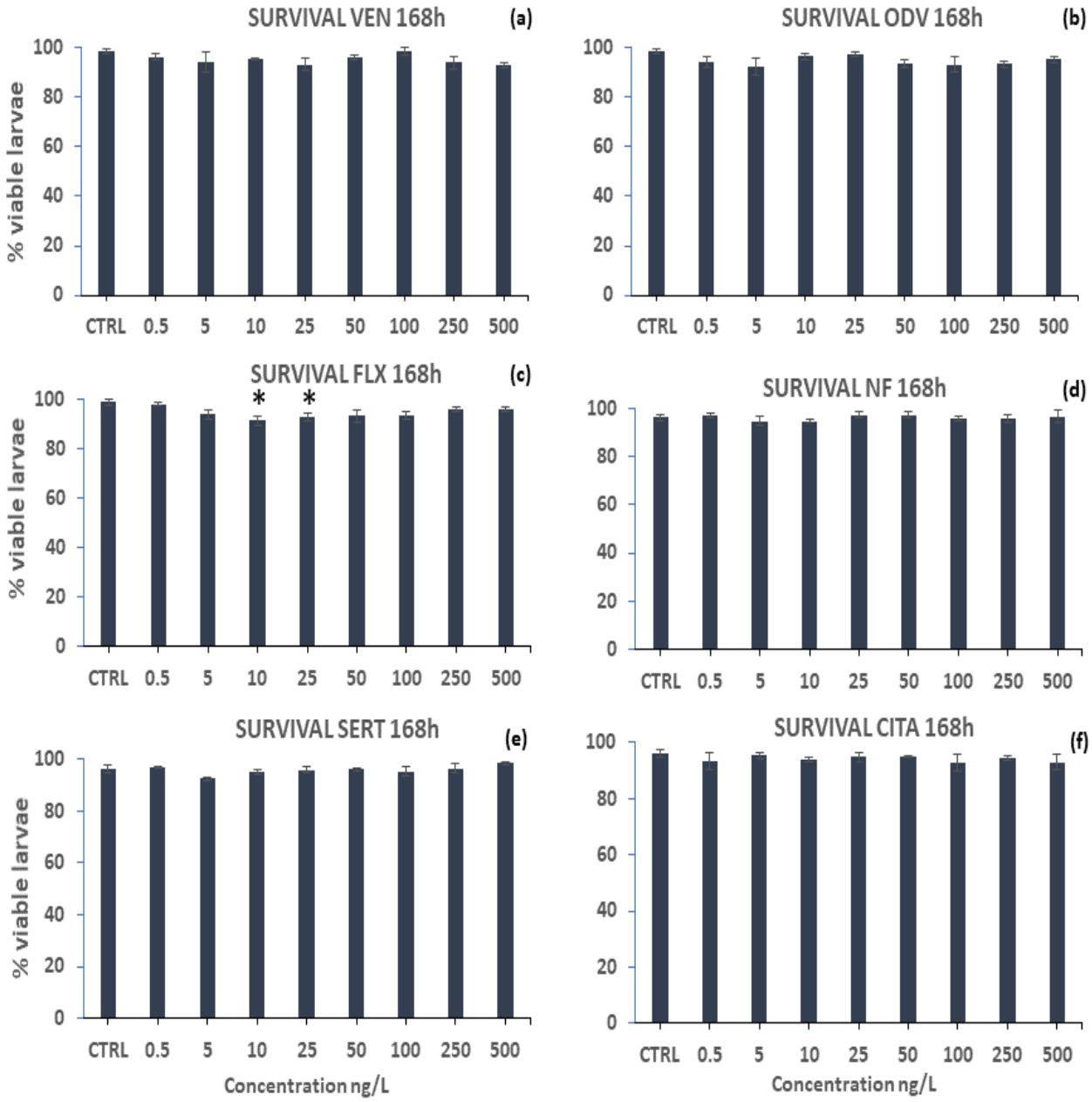

Figure 3-11. Survival percentage of *M. galloprovincialis* larvae after 168-h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 4. Asterisks indicate significant differences compared to the control ($p < 0.05$, One-way ANOVA, Dunnett's post-hoc comparison).

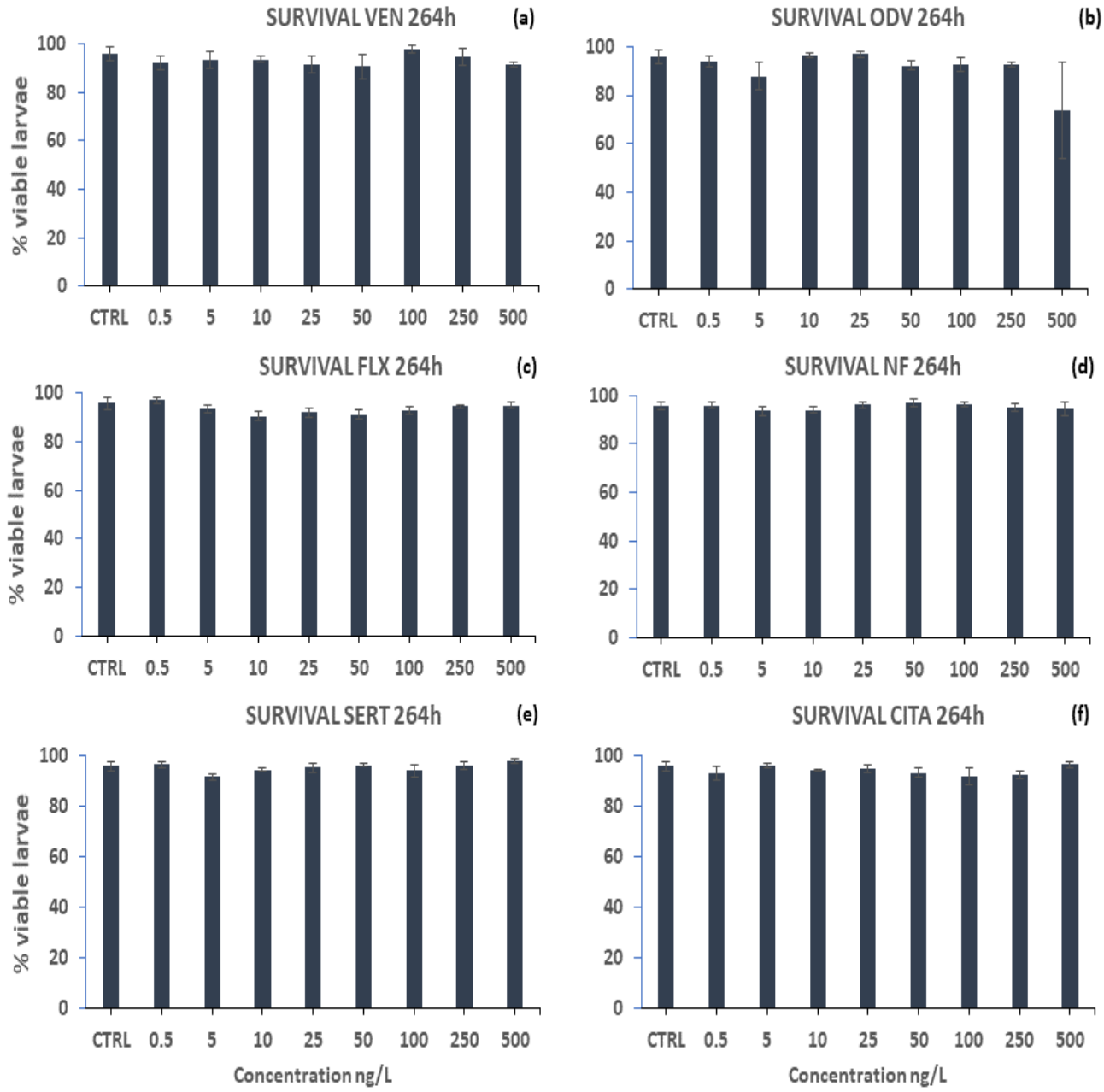

Figure 3-12. Survival percentage of *M. galloprovincialis* larvae after 264-h exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV), fluoxetine (FLX), norfluoxetine (NF), sertraline (SERT) and citalopram (CITA). Data are expressed as mean ± SEM; n = 4.

Chapter 4

4. Discussion

Previous studies have shown effects of antidepressants and metabolites mainly on adult mussels, in this study a battery of bioassays on early life stages (important in reproduction cycle and sustainability of the population) using the Mediterranean mussels *Mytilus galloprovincialis* has been done in the effort to evaluate the effect of four antidepressants and two metabolites. Fluoxetine (FLX), sertraline (SERT), citalopram (CITA) and norfluoxetine(NF) (SSRIs) are known to increase the extracellular concentrations of serotonin (known as 5-hydroxytryptamine or 5-HT), furthermore, venlafaxine (VEN) and O-desmethylvenlafaxine (ODV) (SNRI) raise the levels of norepinephrine and serotonin in the brain. Studies have been done to understand the action of 5-HT on mussels; it has been observed that it increases cAMP in gills epithelium, control ciliary motility, stimulates sodium uptake, muscle relaxation, rhymic activities, spawning and heart contraction (Gosselin, 1961; Scheide and Dietz, 1984). The mode of action of these neurotransmitters in exogenous form has been evaluated, and it has been found that they produce action on ovaries and testis in a dose dependent manner, with less sensitiveness on females than males (Alavi et al., 2017), FLX exposure lead to increase Glutathione-S-transferases (GST) activity in brown mussels after 48 and 96 h (from range 3 to 30 ng/L) and DNA damage in digestive gland after 48h (Cortez et al., 2019), moreover, long term exposure (107 days) of *M. californianus* to FLX (0.3-300 ng/L) decline shell growth (length and biomass), clearance rate of algal, and reproduction period (Peters and Granek, 2016).

4.1 Effects on fertilization rate:

Successful fertilization is related with the quality of the resulting zygote, which sequentially depend upon the health of the egg and sperm (Gallo et al., 2020). Sperm motility is an important feature that enables sperm travel to the oocyte and subsequently process fertilization; a major cause of male infertility is poor sperm motility (McLaren, 2012). It has been observed that *M. galloprovincialis* eggs release chemical cues that guide sperm result in higher fertilization rates and larval survival toward them (Evans et

al., 2012; Oliver and Evans, 2014) –which is an indication that the species are capable to detect and react to genetic compatibility signals prior to gamete contact (Eads et al., 2016). In this series of experiments, the effect of antidepressants and metabolites has been observed on fertilization rate. Exposure to SERT caused the highest percentage of unfertilized eggs in a wide range of concentrations, from 10 ng/L to 500 ng/L; the same pattern from lowest to highest concentrations has been noted for ODV (25 - 250 ng/L) and NF (5, and 100-500 ng/L). FLX significantly decreased fertilization rate in the range 100-500 ng/L. VEN and CITA did not have effect on fertilization rate.

Overall, FLX and its derivative NF have similar effects on this parameter, with NF active also at one of the lowest concentrations (5 ng/L); VEN has no effects, while its derivative ODV significantly decreased fertilization rate in a wide range of concentrations (25-250 ng/L). Comparing FLX (SSRIs) with VEN (SNRI), we could suggest that serotonin is more involved than norepinephrine in this process, however we cannot exclude that the compounds have a direct effect on gametes, independently on their therapeutic mechanism of action.

Observing the results obtained, it appears that 1hour post-fertilization (pf) polar lobe stage was dominant compared to cleavage stage. This result can be the effect of a direct alteration of the eggs and/or sperms by the antidepressants through possible mechanisms disputed below. A previous study noted alteration of oocyte and spermatozoa densities in zebra mussels following several days of exposure to FLX at 20 ng/L and 200 ng/L (Lazzara et al., 2012). Another study run at higher concentrations than the ones used in our work showed that VEN affected the fertilization rate 0.54 µg/L of FLX (VEN & FLX) and 5 µg/L VEN (Vera-Chang et al., 2019) in the zebrafish. This study also demonstrated that mussels exposed to FLX (filial generation 4) sensitized the descendants to VEN. Pereira et al., (2017) reported that environmental stressors affect the quality of gamete by alteration of various calcium and cAMP-dependent protein kinase and phosphatases pathways, production of reactive oxygen species (ROS) and dysregulation of cell volume and osmolality.

The mechanisms of action of either oxidative stress, endocrine disrupting chemicals (EDCs) and metabolic disorders by which contaminant affect the gamete

quality and reproduction is still under evaluation, however several hints on epigenesis and imprinting via alterations in DNA methylation of gene promoters is growing (Gallo et al., 2020). It is suggested that environmental stressors can act directly on eggs and/or sperm. For instance, exposure to chemical- dispersing agents in capelin fish (*Mallotus villosus*) can alter the fertilizing capacity of sperm (Beirão et al., 2018), and decrease of sperm motility has been observed in fish exposed to heavy metals (Abascal et al., 2007), endocrine-disrupting chemicals (Carnevali et al., 2018) and in invertebrates exposed to xenobiotic (Gallo, 2018). It has been found by Han et al., (2019) that in broadcast-spawning bivalves (*Tegillarca granosa*), titanium dioxide can attach to the oocyte surface and damage the plasma membrane, which is thought to contribute to decrease successful gamete fusion. We have found that SSRIs and SNRIs affects fertilization of *M. galloprovincialis*, however, the mode of action of these antidepressants and their metabolites on fertilization is still under elucidation, and further study should be done in this way.

4.2 Effect on embryotoxicity (embryo-larval development):

M. galloprovincialis shows high tolerance to chemical pollutants throughout the life stages, but embryo-larval stages are particularly sensitive to toxicant exposure (Boukadida et al., 2016). Study conducted on mussels by Alavi et al., (2017) showed that 5-HT content increases from embryonic development to metamorphosis, decreases after metamorphosis, and contribute to development of embryos.

In this research, FLX inhibits *M. galloprovincialis* embryos development at high concentration (100-500 ng/L) with higher percentage at delayed stage rather than abnormal formation, its metabolite NF affect embryos development with effects observed at lower concentration (5-500ng/L); the effects are similar to FLX, with high percentage in delayed stage. This data suggests that NF is more toxic than FLX. However, study conducted by Rodrigues et al., (2022) on zebrafish (*Dano rerio*) embryos disputed that FLX is more toxic than NF by enhancing abnormal pigmentation through gene expression alterations (possibly as markers genes drd2b, 5-ht2c and abcc2).

Di Poi and Bellanger, (2014) pointed out that the severity of the effects could be species and age-dependent, and related to the mode and duration of exposure. Another study of fluoxetine-exposed zebrafish embryos, show transcriptional change related with biological process (e.g., circadian rhythm-related genes and early growth response factor gene) in early development (Wu et al., 2017). Blahova et al., (2021) recorded high level of malformation of zebrafish embryo exposed to fluoxetine hydrochloride at high concentration (1000 µg/L and 10,000 µg/L). It has been found that after 48-h exposure of *Mytilus trossulus* embryo to NF (at 100 ng/L) there is significant effect on embryo development with higher average size at trocophore stage compared to control, underlying possible impact on either growth hormones or the whole growth metabolism (Świeżak et al., 2022).

The present investigation has also shown that SERT has effect on embryo-larvae development from low to high concentration (25 - 500 ng/L) producing higher mean of abnormally developed D-shape veligers rather than delayed/arrested development. However, a study made by Estévez-Calvar et al., (2017) on *M. galloprovincialis* embryos exposed to SERT (at 0.1 to 50 mg/L) resulted in higher percentage of arrested development than abnormal development, with most individuals remaining at the trochophora stage. At 300 mg/L no normal fully developed D-larvae larvae were observed. As proposed by Akcha et al. (2012) this variability may be elucidated by the dissimilar sensitivities of the genitors to pollutants, which may underlie differences in the physiological status of the larvae batches and/or may be due to genetic differences. Another reason can be room temperature factor as a previous study confirms that 18 °C is an optimum temperature for the embryonic development of this mussel species (His et al., 1997).

Differently, CITA produced effects only at the highest concentration tested (500 ng/L). In the present study CITA effect produced more abnormal D-shape veligers than delays in development. Statistically significant difference has previously been noted in embryonic toxicity test of zebrafish exposed to CITA. A recent investigation reported that combination of low concentration of FLX (100 ng/L) and CITA (10 ng/L) significantly increase D-shape malformations (Blahova et al., 2021).

Overall, we observe different effects of the pharmaceutical tested on embryotoxicity. FLX has effect at higher concentrations (100-500 ng/L) than its metabolite NF (5 -500 ng/L); SERT is efficacious in the range 25-500 ng/L while CITA shows effects only at 500 ng/L, Finally, the SNRI VEN has no effects and the same is true for its derivative ODV. Comparing SSRIs (FLX, NF, SERT and CITA) with SNRIs (VEN and ODV), data seem to clearly indicate that serotonin is more involved than norepinephrine in this process. As above reported, however, we cannot exclude that the compounds have a direct effect on embryos, independently on their therapeutic mechanism of action.

The observed abnormalities in D-shape veligers in this present investigation was due to shell deformation and mantle protruding protuberances. A study done by Di Poi et al., (2013) on Pacific oyster, *C. gigas* exposed to antidepressants noted reduction in the rates of normal D-shaped larvae with an increase in the antidepressant concentrations, abnormal development was mainly related to the abnormalities affecting the mantle than to the shell abnormalities. According to EC50 comparison, it was observed that SSRI was slightly more toxic than the other antidepressants (SNRI and TCA).

In the present study, no effect has been noted in *M. galloprovincialis* embryos exposed to VEN and ODV. However, VEN exerted effects on exposed zebrafish (at early life stages) by the disruption of the metabolic and behavioral performance across their ontogeny, behavioral disorders when zebrafish come to be free swimming and reduction growth in juveniles (Thompson et al., 2022), depigmentation and spinal deformities at dose 16 –10,000 ng/L after exposure for 80 h (Rodrigues et al., 2020). Fish at early life stages exposed to SSRIs and SNRIs were reported to develop delays in development and this was related to endocrine disruption (Thompson and Vijayan, 2022). The comparison between molluscs and fish is not always straightforward, because different control mechanisms may be involved in the different functions; therefore, more data are needed on VEN, that is in fact included in the latest Watch List for aquatic contaminants (EU commission, 2020)

The embryonic stage of *M. edulis* has been demonstrated to be the most sensitive to copper (Hoare et al., 1995), which has been explained, partly, by induction of high metabolic rate and the high susceptibility of enzymes to oxidative damage. In fact, any

change in enzyme activity can radically impair certain metabolic pathway and decline development and survival (Fitzpatrick et al., 2008). At early embryo stage, the few-celled rudiments do not own sophisticated defense systems compared to adults, for example the immune system or the nervous system which represent survival factor. Under extreme environmental conditions, embryos of many species temporarily slow or suspend development (Hamdoun and Epel, 2007) which may be an another explanation of arrested development observed after exposure to some SSRIs. Calcification begins as the precipitation of a calcium carbonate shell onto an organic template during the trochophore larval stage which is characterized by a free-swimming, ciliated larva (Bayne, 1976). Calcifying larvae of marine invertebrates in particular have been shown to be particularly sensitive to environmental pollutants and stressors (Canesi et al., 2022; Hamdoun and Epel, 2007; Przeslawski et al., 2015) which may result in the abnormal formation of shell. In particular, Miglioli et al., (2021) demonstrated that Bisphenol A reduced the transcription of the genes coding for the enzyme tyrosinase, that plays a key role in remodeling of the shell organic matrix, and for HOX1, a member of homeobox genes involved in larval shell formation and neurogenesis, and affected the time course of mRNA levels for tyrosinase, shifting from 24 to 48 hpf. In the same experiments, BPA also reduced the development of serotonin-5-HT-immunoreactive neurons interfering with key processes occurring during the first developmental stages of mussels, such as the valve construction. This latter effect hampering the serotonin control of mussel development caused by BPA, could be even more target of serotonin re-uptake inhibitors, with similar effects on embryo development.

4.3 Effect on D- shape Larvae (immobilization and mortality assays):

Differences in morphological defense mechanisms of mussels against environmental stressor make the difference in sensitivity between embryos and larval stages (Fitzpatrick et al., 2008). Investigation shows that veligers are able to capture food and modify swimming behavior and settle in response to neuroactive substances (Beiras and Widdows, 1995; Coon et al., 1985).

The present research shows that antidepressants have poor or no effect on larvae motility. FLX induced significant immobilization of *M. galloprovincialis* D-shape veligers,

and it was observed only after 264-h exposure at the highest concentration (500 ng/L) compared to control. At 264-h pf was also observed an effect of ODV at a single concentration of 0.5 ng/. A similar experiment run on *M. edulis* larvae exposed to serotonin and norepinephrine observed the following: at 10^{-6} M serotonin a small number of larvae exhibited abnormal rotational movement; at higher concentration (i.e. > 3 x 10-6 M), 25% of the larvae accumulated in the surface water layer their velum directed upwards and actively beating cilia. At a higher concentration high percentage of larvae were immobilized and a large percentage of larvae showed abnormal rotating movements around the superior-inferior axis and occasionally around the antero-posterior axis. Larvae exposed to high serotonin concentration were not able to recover once the stress was removed and showed abnormal swim. Approximately 70% of the larvae exposed to norepinephrine at concentration 3×10^{-6} M were non-motile. After being exposed to higher norepinephrine concentrations (> 3×10^{-6} M), 80 to 90% of the larvae were immobilized with the valves closed, a small number exhibited abnormal movements rotating around the antero-posterior axis. It has been concluded that norepinephrine has a cilio-inhibitory effect on *M. edulis* veliger larvae at relatively low concentration, resulting in a decline in the rates of food-particle clearance and ingestion rates, as well as inhibiting swimming (Beiras and Widdows, 1995). This may justify the high immobilization rate of *M. galloprovincialis* exposed to low concentration ODV during this study. Moreover, it has been observed that neural control of cilia on the velum of the larvae is similar to the dual innervation of gill cilia in adult mussels, with serotonin and dopamine acting as cilio-excitatory and cilio-inhibitory neurotransmitters, respectively (Beiras and Widdows, 1995). Also, experiment run on zebrafish exposed to VEN in a light and dark behavioral test, demonstrated that the organisms were less active and covered shorter distance compared to the controls (Thompson et al., 2022).

In the present study, only FLX caused a significant increase in larvae mortality at 48-h, 96-h and 168-h of exposure at low concentrations, compared to control. It has been observed that some of the larvae first became non-active and then died. Multi-cellular organisms may have a self-defensive mechanism against foreign toxic molecules, such as immunity and detoxification. Diffusion of toxicant through the shell depends on time and concentrations of exposure. Toxicant can be adsorbed or absorbed onto the flesh

surface of larvae and lead to larvae death through complex biological mechanisms (Haque et al., 2014). Moreover, it has been demonstrated that after exposure to high (1 mM) concentration of serotonin, the siphons in zebra mussel opens and the muscles contracts, however at low (1 µM) concentrations the muscles relax. Additionally, the mechanism of closing and withdrawal of siphons into the shells may have a significant role in defense and avoidance of toxic substances (Ram et al., 1999). A key component in multixenobiotic resistance mechanism of cytoprotection is P-glycoprotein, which naturally avoids accumulation of detrimental xenobiotic in cells by active extrusion. This mechanism is considered essential for aquatic organisms to survive in polluted environments (Bard, 2000). Taking into account this function, the inhibition of cAMP/PKA pathway and P-glycoprotein expression caused by FLX at environmental concentrations may critically affect the animals capacity to develop strategic defenses against chemical exposure (Canesi et al., 2022).

Chapter 5

5. Conclusion

In conclusion, this research confirms that three antidepressants fluoxetine, sertraline and citalopram and the two metabolites norfluoxetine and O-desmethylvenlafaxine have impact at the different end points of *M. galloprovincialis* early life stages tested. FLX has effect at the three different endpoints that is fertilization rate, embryo-development larvae and larvae immobilization and survival. NF and SERT show high effect on embryotoxicity and fertilization rate. In contrary to its parent compound, the derivative ODV induce effect on fertilization rate and larvae motility. CITA has effect only on embryotoxicity. Although no effect has been induced by VEN, its impact on other aquatic organisms early life stages, i.e. fish, has been reported by other authors. The high sensitivity of the early developmental stages to these compounds highlight the relevance of including these stages when evaluating the toxicity of chemicals where little information is available. Although older life stages may be more tolerant to toxicants, the population survival will be compromised if new recruits are not viable, with implications to the whole ecosystem health and functioning of the impacted area. Due to the complexity and cost in the elimination of antidepressants from WWTP, reduction of the excessive and inappropriate use of pharmaceuticals from source is an important step in decreasing the occurrence of pharmaceuticals in the environment.

Future intervention and research are needed:

Regulations and Management:

Improvement of treatment plants is needed to ensure surface and drinking water quality. Innovation to support the development of “greener” pharmaceuticals that degrade more readily to harmless substances needs to become a priority.

Regarding pharmaceuticals currently in use, their ultimate environmental fate, degradation products, partioning and binding to marine particles, need further studies. The EU Watch List is a valid approach, but extension to marine waters is strongly recommended. Thus, enhanced monitoring of pharmaceuticals in marine waters is necessary to ensure water quality and allow effective future regulations and management.

Owing that pharmaceuticals display biological effects at very low doses that are not necessarily detected by standard ecotoxicity tests, more sensitive tests need to be developed, assessing both embryo/larval development and adult responses in marine species, able to provide regulators with consistent and effective data.

Awareness must be raised in the population, to favor appropriate disposal of unused drugs.

Research:

Antidepressants are not the pharmaceuticals showing the highest concentrations in marine waters, however they showed striking effects at very low concentrations in marine invertebrates. Several hypotheses have been proposed regarding exposure and interactions with different protein targets, and specific investigations are needed to clarify their validity. Data cannot be fully extrapolated from fresh- to salt-water, because the different environmental features (especially pH and salinity) may greatly influence the fate of pharmaceuticals. In general, the potential of pharmaceuticals, including antidepressants, to significantly impair physiological functions at environmentally relevant concentrations cannot be ignored.

More insights on toxicokinetics and toxicodynamics of pharmaceuticals in non-target organisms, and in particular on their biotransformation, are needed to have an integrated overview of environmental exposure and risk.

Knowledge of their mechanism of action may allow grouping pharmaceuticals into homogeneous classes exploiting the same pathway, thus understanding the importance of the pathways affected. This may also help to address the challenging and complex question of mixture effects, since even low, individually non-toxic concentrations might combine to produce substantial mixture effects.

The relative importance of pharmaceuticals amongst all other environmental pollutants cannot be predicted at this time, however, pharmaceuticals act as additional stressors on marine ecosystems already impacted by many factors. Modification of pH and temperature associated with climate change influence the chemical properties of most pharmaceuticals, which therefore may contribute differently to their global effect.

Finally, little data are available to eliminate the possibility that any amount of pharmaceuticals is transferred back from the sea to humans through seafood. The probability is estimated to be very low, however, all possibilities need to be considered.

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