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“Presence, behavior and risk assessment of pharmaceutical products in aquatic ecosystems”

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1. Introduction

1.1 Consumptions, sources and fate of pharmaceutical compounds in aquatic environment

The reduction of the severity of many pathologies due to the progress of medicine and the formulation of more effective pharmaceutical active principles, led to an enhancement of health quality, better disease prevention and an increase of life expectancy (Mezzelani et al., 2018a). All these aspects, along with the significant increase of the world population (UN World Population Prospects, 2022) caused a growing demand of drugs to prevent and cure diseases and age-related medical issues. Consequently, the global pharmaceutical market has quickly grown in terms of both total sales and the number of synthesized drugs (Eur. Comm. 2019, IQVIA Inst. Hum. Data Sci. 2019, Mezzelani and Regoli, 2022).

The consumption in Organization for Economic Co-operation and Development (OECD) countries of anti-hypertensive drugs, lipid regulators, antidepressants and antidiabetics has risen by 65%, 100% and 300%, respectively between 2000 and 2019 (OECD Health at a Glance, 2021).

In 2022 the North America market resulted to be the largest, representing the 52.3% of world pharmaceutical sales due to the leading role of the US pharmaceutical industry, followed by Europe with 22.4% of sales (Figure 1.1). However, during recent years (from 2017 to 2022), in emerging economies such as Brazil, India and China drug markets have grown by 13.0%, 11.0% and 5.3%, respectively, showing highest growth rates compared to an average market growth of 6.6% for the top 5 European Union markets (France, Germany, Italy, Spain and United Kingdom) and of 7.1% for the United States one (EFPIA The Pharmaceutical Industry in Figures, 2023; IQVIA MIDAS, May 2023). Among the European Union countries, Italy ranks first in terms of the incidence of territorial consumption of cardiovascular drugs (26.9%), followed by Germany (24.3%) and Portugal (23.5%). Conversely, regarding central nervous system drugs, the percentage of Standard Units (SU) consumed in Italy (15.1%) is lower than almost all other countries considered, except for Poland (14.8%). Among over-the-counter pharmaceuticals, the most consumed active principles in Italy are ibuprofen, with an increase of 51.9% between 2021 and 2022, and diclofenac, although the latter showed a decline in consumption of 9.8% between 2021 and 2022 (OSMED, 2022). Considering the number of synthesized active principles, the current version of DrugBank database (version 5.1.12, released on 14 March 2024) contains 17,166 pharmaceuticals, which include 2,787 approved small-molecule drugs, 1,636 approved biologics (proteins, peptides, vaccines, and allergenics), 135 nutraceuticals and over 6,722 experimental drugs.

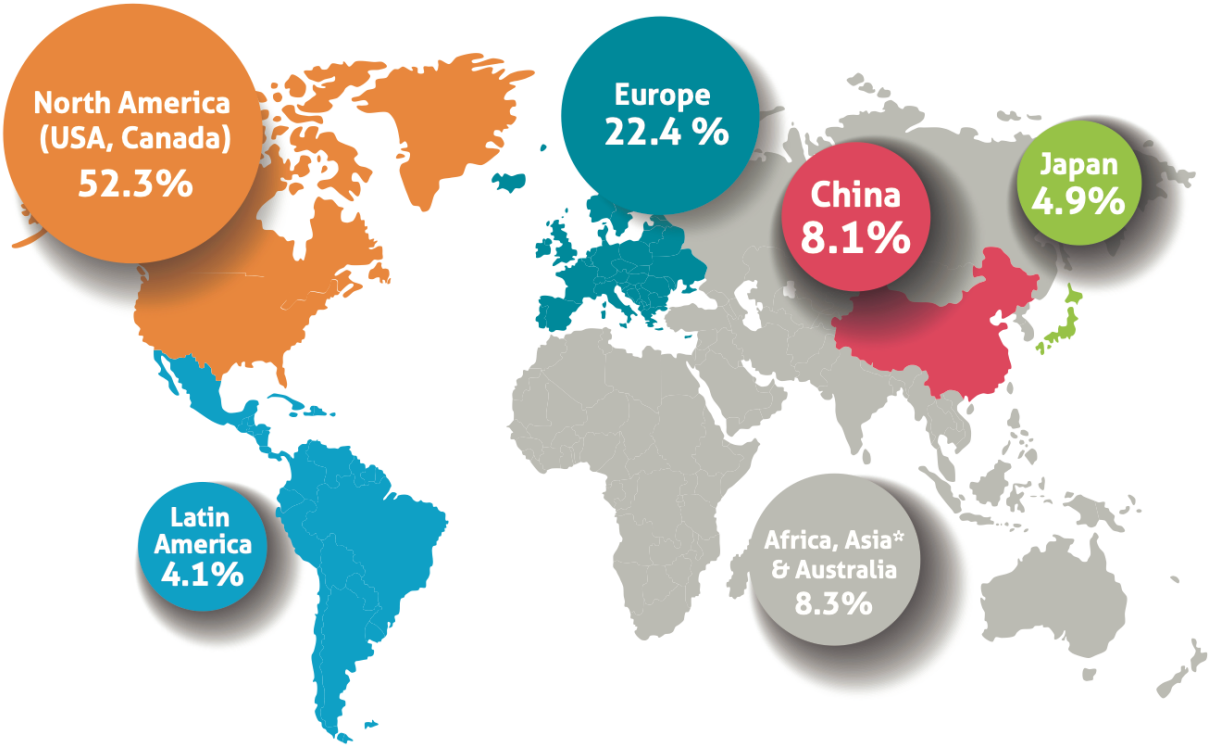


Figure 1.1 The image illustrates the world pharmaceutical (prescription) markets. (EFPIA The Pharmaceutical Industry in Figures, 2023)

Although their innumerous benefits in human society, the massive consumption of pharmaceuticals has become an environmental concern due to the ubiquitous occurrence of these molecules in aquatic ecosystems and their unknown adverse biological and ecotoxicological effects on non-target species. Detected for the first time in the early 1990s when traces of anticancer drugs were found in sewage effluents, rivers and drinking water, pharmaceuticals belonging to a variety of therapeutic classes have been identified in the marine environment (Aherne et al., 1990; Mezzelani et al., 2018a).

Nowadays, the most commonly detected pharmaceuticals therapeutic classes in surface waters include Non-Steroidal Anti-Inflammatory drugs (NSAIDs), analgesics, antibiotics, lipid regulators, steroids and related hormones, beta-blockers, as well as psychiatric and cytostatic drugs (Fatta-Kassinos et al., 2011; Mezzelani et al., 2018b). Pharmaceutical residues and their metabolites can reach the marine environment through various pathways, including hospital effluents, industrial wastewaters, landfill leachate, runoff from concentrated animal feeding operations and aquaculture, with wastewater treatment plant (WWTP) effluents being among the primary sources (Bagnis et al., 2019, Couto et al., 2019, de Oliveira et al., 2020, Mezzelani et al., 2018b). These facilities receive and treat sewage waters from various sources, including domestic households. Many individuals

improperly dispose unused or expired medications by discarding them into sinks and toilets, unaware of their fate (Petrovic et al., 2004; Prasad et al., 2019). Additionally, most ingested drugs are not fully absorbed and metabolized by the human or animal body; approximately 80% are excreted unchanged through urine and feces (Pandelani Munzhelele et al., 2024). The amount of pharmaceuticals released into the environment after human and/or veterinary usage depends on metabolism and excretion processes, which vary according to the type of ingested chemical and the posology.

Wastewater treatment plants are often inefficient in removing pharmaceuticals, which typically exhibit biorefractory behaviors and are only minimally removed by primary and secondary treatment processes (Peake et al., 2016). These facilities are designed to remove easily or moderately biodegradable carbon, nitrogen, and phosphorus compounds, as well as microbiological organisms, which generally arrive at the WWTP in concentrations of mg/L and at least 10^6 MPN/100 mL, respectively. Consequently, they are not equipped to handle complex compounds at low concentrations, such as pharmaceuticals (Cuoto et al., 2019). Additionally, their removal capacity can be affected by fluctuations in sewage volume, such as during heavy rainfall or seasonal population increases in touristic areas, which significantly reduce the system's optimal operating capacity (Lorenzo-Toja et al., 2015; Sala-Garrido et al., 2012).Primary treatment processes include physical procedures (flocculation, coagulation, sedimentation) that have the objective of removing suspended solids mainly through sorption. Removal efficiency at this stage for pharmaceuticals with octanol-water partition coefficient (log K_{ow}) values less than 3.0 (*e.g.* the NSAIDs diclofenac and ibuprofen, the lipid lowering agent atenolol and the antiepileptic carbamazepine) range up to only 28%, indicating insignificant adsorption of these compounds to the particles removed in this type of facility (Behera et al., 2011; Cuoto et al., 2019). Secondary treatment involves biological processes where the removal of chemicals can occur through partition, adsorption, biotransformation, and biodegradation. Biodegradation, a key process in biological treatment systems, breaks down complex molecules into simpler, often less toxic products via microorganism activity, utilizing these compounds as electron donors for energy production. Two main mechanisms of pharmaceuticals biodegradation in WWTPs are co-metabolism and substrate sludge degradation. In co-metabolism, compounds are degraded by enzymes secreted by microorganisms in the biological sludge. In substrate sludge degradation, drugs serve as the carbon and energy source for the microbial mass (Jelić et al., 2011). Jiang et al. (2014) found that the fungus *Trametes versicolor* effectively removed the antiepileptic carbamazepine by secreting laccase and peroxidase enzymes. The removal mechanisms, degradation rates, and efficiencies can vary significantly even among pharmaceuticals within the same therapeutic class, depending on whether aerobic or anaerobic digestion is used, as well as the compounds' molecular structures and functional groups. For instance, a comparison of co-

metabolic and single substrate degradation processes revealed that co-metabolic biodegradation was the main removal mechanism for the NSAIDs ibuprofen and naproxen, whereas ketoprofen was only partially degraded as a single substrate (Quintana et al., 2005). Furthermore, the study of Salgado et al. (2012) highlighted that ibuprofen has a higher tendency to be biologically removed in the sludge tank (> 75%) compared to diclofenac (< 25%).

In aerobic digestion was found a faster degradation of chlorinated compounds compared to polyhalogenated compounds. Moreover, a long-chain aliphatic compounds degraded more easily than aromatic ones with sulfate or halogen groups due to their complex ring structures (Schwarzenbach et al., 2005). Besides that, some drugs, as carbamazepine, are highly persistent and have been shown to be inert to the biological treatment process (Clara et al., 2005); while others as the antidiabetic metformin showed high rates of removal (93–97 %) probably due to the microbiological transformation into its major metabolite guanylurea (Trautwein et al., 2014). Tertiary treatment processes, which are based on more advanced technologies such as finer filtration techniques (sand filters, microfiltration, ultrafiltration, nanofiltration, reverse osmosis and adsorption by activated carbon), offer enhanced pharmaceutical removal capabilities but are still limited in their applications due to higher costs (Mezzelani and Regoli, 2022). Literature studies have reported higher concentrations of certain pharmaceuticals, such as the antiepileptic carbamazepine and the NSAID diclofenac, in WWTP effluents compared to their influents (Nivala et al., 2019; Martínez-Alcalá et al., 2021). This increase may be due to microbial activities or hydrolysis within WWTPs, which can convert drug metabolites or conjugates back into their parent compounds (Kruglova et al., 2014), or to the initial adsorption of these molecules onto fecal particles in the sewage, which are subsequently released after passing through the facility.

Pharmaceuticals from WWTP effluents are transported and accumulate in various environmental matrices, including surface water, groundwater, sediments, and biota (Parra-Saldivar et al., 2021). The distribution of each active principle in these matrices is primarily influenced by its physical and chemical properties (*e.g.*, the octanol/water partition coefficient, dissociation constants, vapor pressure or Henry's Law constant). Hydrophilic molecules tend to persist in the water column, whereas lipophilic molecules are more readily bioaccumulated by organisms or may settle in sediments (Peake et al., 2016; Mezzelani et al., 2018a). Lipophilicity is evaluated using the Octanol/Water partition coefficient (log K_{ow}), which compares the concentration of the chemical in n-octanol (a surrogate for lipids) to its concentration in water, indicating the tendency of an organic compound to partition into lipids (Kümmerer, 2008). Organic compounds are categorized based on their log K_{ow} values: those with log $K_{ow} < 2.5$ are more hydrophilic, those with values between 2.5 and 4 are moderately lipophilic, and those with log $K_{ow} > 4$ are highly lipophilic (Korkmaz et al.,

2022). Generally, it is assumed that chemicals with a log K_{ow} value of 3 or higher can bioaccumulate in biota. However, accumulation in biological tissues also depends on other factors, such as uptake and depuration kinetics, which are crucial for understanding the biodegradation and biotransformation pathways of these substances, particularly in non-target species (Huerta et al., 2012). Drugs belonging to the same therapeutic class do not always exhibit the same log K_{ow} values. NSAIDs have log K_{ow} values ranging from 1.90 (diclofenac) to 3.18 (naproxen) (Swiacka et al., 2022). Antidepressants generally have a log $K_{ow} > 3$, with exceptions such as venlafaxine at 2.8, sertraline at 1.37, and citalopram at 1.39 (Kwon and Armbrust, 2008; Minguez et al., 2014). Cardiovascular drugs can have either low or high log K_{ow} values depending on the active principles, such as ACE inhibitors ramipril (0.70) and captopril (0.34), and β-blockers atenolol (3.97), propranolol (2.60), telmisartan (7.7) (Tong et al., 2015; Zhang et al., 2020; Swiacka et al., 2022). Lipid-lowering agents generally exhibit higher log K_{ow} values, ranging between 2.57 and 6.36, such as fibrate gemfibrozil (4.39) and statin atorvastatin (6.36) (Capolupo et al., 2018; Zhang et al., 2020).

Pharmaceuticals distribution in environmental matrices is significantly influenced also by their pKa values. Hydrophilic compounds, characterized by low pKa values, tend to remain in the aqueous phase, thereby accumulating in surface waters and groundwater (Fent et al., 2006; Ternes, 1998). For instance, acidic pharmaceuticals such as the NSAIDs ibuprofen, ketoprofen, naproxen and diclofenac having pKa values ranging from 4.9 to 4.1, occur as ion at neutral pH, and have little tendency of adsorption to the sediment (Fent et al., 2006). Conversely, lipophilic compounds, with high pKa values, are more likely to adsorb onto sediments or bioaccumulate in aquatic organisms (Daughton & Ternes, 1999; Kümmerer, 2009). This behavior is influenced by environmental conditions such as salinity, temperature, pH, the presence of reactive oxygen species, and dissolved organic matter (DOM), which can alter the solubility and stability of these pharmaceuticals (Heberer, 2002; Schwarzenbach et al., 2006; Peake et al., 2016). For instance, the widely used antibiotic amoxicillin degrades at a faster rate in basic waters (Braschi et al., 2013; Mezzelani et al., 2018b). Atmospheric conditions also affect the persistence and diffusion of drugs in aquatic environments, with solar irradiation potentially causing photodegradation and rainfall impacting concentrations through dilution or increased runoff from sewer overflows (Luo et al., 2014). For example, the intensity of solar irradiation can lead to the environmental photolysis of the antiepileptic carbamazepine into acridine, a highly toxic, mutagenic, and carcinogenic compound (Chitescu et al., 2015; Ebele et al., 2017).

Despite the inherent degradability of pharmaceuticals, their continuous usage and release into the environment impart a pseudo-persistent behavior to these compounds. This highlights the need to

characterize their bioaccumulation and biological effects in non-target organisms to evaluate their potential environmental risks.

1.2 Presence, distribution and ecotoxicological effects of pharmaceuticals in aquatic ecosystems

Active pharmaceutical ingredients (APIs) represent emerging contaminants of particular concern for their ubiquitous presence and potential impact on the environment. More than 300 active ingredients, have been detected in different environmental matrices, including seawater and sediments at concentrations ranging from nanograms to micrograms per liter or kilogram (Almeida et al., 2021; Madikizela et al., 2020; Mezzelani and Regoli, 2022; Pereira et al., 2015). APIs, designed to permeate biological membranes, have been identified in various wild freshwater and marine species, such as macroalgae (*Saccharina spp., Laminaria spp.*), mollusks (*Mytilus spp., Cassostrea spp., Dreissena spp., Elliptio spp*.), crustaceans (*Gammarus spp.*), and fish from different ecological categories (*Liza aurata, Platichthys flesus, Pleuronichthys verticalis, Hemiculter leucisculus, Carassius auratus*) (Martínez-Morcillo et al., 2020; Swiacka et al., 2019; Mezzelani et al., 2018b; Zenker et al., 2014; Ali et al., 2018; Elizalde-Velázquez and Gómez-Oliván, 2020; Mezzelani et al., 2020; Almeida, Soares, et al., 2021; Sharma, Thakur, and Kaushik, 2021; Mezzelani and Regoli, 2022). These chemicals exert their biological activity by targeting metabolic, enzymatic, or cell signaling processes through well-characterized mechanisms of action in human and animals. Since some molecular receptors are evolutionarily conserved in many aquatic species, the same pathways can also be altered in organisms with identical or similar target organs, tissues, cells, or biomolecules (Fent et al., 2006; Mezzelani and Regoli, 2022). While in other species these molecular targets can differ or even totally lack, with different and unknown modes of action (Fent et al., 2006).

The following subsections provide an overview of the main analytical techniques used for evaluating the ecotoxicological effects of pharmaceuticals, as well as the current knowledge of the common pathways of action, metabolism, and toxicity in non-target organisms for the most frequently detected therapeutic classes in marine environments, including non-steroidal anti-inflammatory drugs (NSAIDs), psychiatric compounds, antidiabetics, cardiovascular drugs, and lipid-lowering agents.

1.2.1 Analytical approaches for the ecotoxicological assessment of pharmaceuticals

Considering the wide range of potential effects caused by pharmaceutical compounds in non-target organisms, their intricate network of molecular, biochemical, and cellular responses can be assessed at different levels of biological complexity by using several analytical methodologies. Among these, *in vitro* assays with fish cells have been widely applied by the scientific community due to their advantages in minimizing animal use, enabling the testing of a wide range of chemicals and

concentrations, as well as their high reproducibility and low cost (Eisenbrand et al., 2002). Several studies have concluded that this approach is well-suited for the first screening of acute toxicity of xenobiotics, including pharmaceuticals (Caminada et al., 2006, 2008; Laville et al., 2004; Rodrigues et al., 2019). Furthermore, the selection of targeted endpoints enables the investigation of specific metabolic pathways, providing valuable insights into the mechanisms of action of the molecules under examination, making this approach a valuable tool in ecotoxicological research. However, these systems are not able to predict the physiological response of organs or tissues, as they lack cell-cell and cell-extracellular matrix interactions (Cho & Yoon, 2017). In this context, *ex vivo* approaches, which use organs, tissues, or tissue fragments rather than isolated cells, aim to preserve the tissue's original architecture, offering a model that more closely resembles *in vivo* conditions (El Haj et al., 2019; Giuliani et al., 2019). Indeed, tissue slices contain various cell types, with intact intracellular and cell-matrix interactions (de Kanter et al., 2002). This technique is widely used in various applications, including the study of biochemical functions such as endogenous metabolism, biotransformation, drug induction and transport, as well as toxicological research and the evaluation of drug efficacy in diseased tissues (De Graaf et al., 2010). Although it is well-established and validated approach in medical research, its application in ecotoxicology remains limited, despite its significant potential to elucidate the mechanisms of action and metabolic pathways of environmental pollutants (Schmieder et al., 2004; Yadetie et al., 2018; Giuliani et al., 2019).

In vivo approaches involve exposing whole, living organisms to specific concentrations of contaminants under controlled laboratory conditions, allowing for a comprehensive assessment of the toxic effects of a compound without excluding any metabolic pathways. Several research on pharmaceutical toxicity focus on acute toxicity tests in variety of species from different trophic levels, including bacteria, algae, invertebrates, and vertebrates, in which are measured endpoints related to multiple biological processes as mortality, reproduction, growth, and behavioral changes. However, many of these studies have been conducted at concentrations several orders of magnitude higher (mg/L) than those typically found in aquatic ecosystems (Ferrari et al., 2003; Nunes et al., 2006; Ribeiro et al., 2015). While such high doses can effectively reveal immediate toxic effects and mechanisms of action, they often do not reflect the real-world risk posed by pharmaceuticals in environmental concentrations (Binelli et al., 2009; Brausch & Rand 2011).

Chronic *in vivo* exposures, particularly in fish and invertebrates, are increasingly employed in ecotoxicology to investigate the sublethal effects of drugs. These long-term studies are crucial for assessing the risks associated with the persistent, low-concentration presence of these substances in the environment (Pastorino et al., 2024). These approaches typically rely on biomarker-based methodologies to evaluate a broad range of biochemical and cellular responses, including those

related to immune function, oxidative stress, lysosomal dysfunction, lipid peroxidation, genotoxicity, histological alterations, and various physiological indices (Mezzelani et al., 2016a; 2016b; 2023). Furthermore, alterations at lower levels of biological organization are usually identified through advanced molecular techniques, which provide insights into gene expression profiles, regulatory pathways, and the molecular mechanisms affected by pharmaceuticals (Milan et al., 2013; Maria et al., 2016; Hampel et al., 2017). Although the significant utility of all the techniques previously mentioned is undeniable, the use of a single approach may sometimes be insufficient to fully understand the real ecotoxicological impact of a chemical substance. For this reason, integrating multiple methodologies is crucial for a more comprehensive environmental risk assessment. In support of this, several studies have demonstrated that the integration of acute and chronic exposures and/or analysis of responses at different levels of biological organization, can provide a deeper understanding of the complex array of molecular, biochemical, and cellular responses that modulate the mechanisms of action of these molecules, potentially helping to clarify the onset of adverse long-term effects in aquatic non-target species (Milan et al., 2013; Vitale et al., 2018; Mezzelani et al., 2018b, 2023, 2024).

1.2.2 Non-Steroidal Anti-Inflammatory Drugs (NSADs)

NSAIDs are extensively used globally to treat pain, inflammation, fever, rheumatoid arthritis, and musculoskeletal disorders. These drugs act by inhibiting the cyclooxygenase (COX) enzymes, which play a crucial role in the metabolism of arachidonic acid. Arachidonic acid, a polyunsaturated fatty acid derived from cell membranes through the action of cytosolic phospholipase A2 (PLA2), is converted via the COX pathway into prostaglandins, a group of lipid inflammatory mediators collectively known as eicosanoids. The COX pathway initially converts arachidonic acid into prostaglandin G2 (PGG2), which is then reduced to prostaglandin H2 (PGH2), and subsequently transformed into five primary biologically active prostaglandins: PGD2, PGE2, PGF2, PGI2 (prostacyclin), and thromboxane A2 (TxA2). These eicosanoids modulate (either increasing or decreasing) intracellular levels of cyclic AMP (cAMP) and calcium in various ways, influencing critical neurophysiological functions, the regulation of inflammation and immune responses, protein metabolism, and the contraction and relaxation of muscles and blood vessels (Burian, 2007). NSAIDs inhibit the conversion of arachidonic acid to prostaglandins and thromboxanes by blocking one or both isoforms of the cyclooxygenase (COX) enzyme: COX-1, which is constitutively expressed in various tissues and plays a role in maintaining homeostatic cytoprotection, and/or COX-2, which is primarily involved in mediating pain and inflammation (Bacchi et al., 2012; Ghosh et al., 2015). Based on their mechanisms of action, NSAIDs are classified into three categories: nonselective,

which inhibit both COX-1 and COX-2 (*e.g.,* ibuprofen, naproxen, ketoprofen); selective, which target only COX-2 (*e.g.,* celecoxib and nimesulide); and semi-selective, which predominantly inhibit COX-2 but can also affect COX-1 (*e.g.,* diclofenac) (Mezzelani and Regoli, 2022).

Due to their massive consumption, NSAIDs are detected in natural environments worldwide: in fresh and seawater these drugs have been detected ranging from the limit of detection (LOD) to several hundred nanograms per liter (ng/L), with occasional peaks reaching tens of micrograms per liter (µg/L) (Fernandes et al., 2021; Madikizela et al., 2020; Blasco et al., 2023). These higher levels have made NSAIDs one of the most extensively studied therapeutic classes.

Specifically, in surface waters, diclofenac was found in Brazil, Spain, and China at concentrations of up to 2625, 650, and 201 ng/L, respectively. Ibuprofen was detected in India, Pakistan, and China with levels reaching 2302, 2720, and 320 ng/L, respectively. Naproxen concentrations in China, Mexico, and South Korea were up to 125.7, 4880, and 527 ng/L, respectively (Hernández-Tenorio et al., 2022). In marine waters, due to the dilution effect, the concentrations are slightly lower: diclofenac was detected in Baltic Sea, Atlantic Ocean, Red Sea and Noth Sea in levels up to 196, 343.6, 10221.1 and 10 ng/L, respectively; ibuprofen was found in Baltic Sea, Antartic Penisula, Atlantic Ocean and Red Sea in concentrations up to 48, 10053, 222 and 508.7 ng/L, respectively (Hernández-Tenorio et al., 2022). Studies investigating bioaccumulation in marine organisms have reported concentrations up to several hundred nanograms per gram (ng/g) (Mezzelani et al., 2020; Fernandes et al., 2021; Blasco et al., 2023). For instance, diclofenac was detected in bivalves of Italy (171.1 ng/g d.w., Mezzelani et al., 2020) and Portugal (4.5 ng/g d.w., Cunha et al., 2017), in fish of Sweden (5.2 ng/g d.w., Swaicka et al., 2022) and Malaysia (10.76 ng/g d.w., Omar et al., 2019) and in gastropods of Malaysia (7.41 ng/g d.w., Omar et al., 2019). Ibuprofen and nimesulide were measured in bivalves of Italy at concentrations of 143.7 and 80.6 ng/g d.w., respectively (Mezzelani et al., 2020). Naproxen was found in bivalves of U.S.A. at levels of 3.39 ng/g d.w. (Granek et al., 2016).

COX-mediated metabolism of arachidonic acid and its derivatives, such as PGD2, PGE2, PGF2, and PGI2, has been observed in marine invertebrates (*e.g.,* sponges, corals, and mollusks) where these compounds perform similar functions as in mammals (Di Costanzo et al., 2019). Environmental NSAIDs have been demonstrated to affect arachidonic acid metabolism and COX pathways in aquatic organisms. Exposure to ibuprofen, ketoprofen, and nimesulide (from few to hundreds µg/L) has been shown to affect genes related to arachidonic acid metabolism, such as PLA2, TBXAS1, PGR1, EP4, and PTGER4, in *Mytilus spp*. and *Ruditapes philippinarum* (Almeida et al., 2020b; Mezzelani et al., 2018a; Milan et al., 2013). Similarly, exposure to diclofenac has been reported to inhibit PGE2

synthesis in Mediterranean mussel *Mytilus galloprovincialis* (1–100 µg/L) and alter COX activity in in brown mussel *Perna perna* (0.5 µg/L) (Sathishkumar et al., 2020).

Due to their role in mediating both innate and adaptive immune responses, NSAID inhibition of prostaglandins can lead to significant immune system modulation, potentially resulting in severe inflammatory responses, macrophage accumulation, and ulceration (Mezzelani and Regoli, 2022). For instance, the study of Zhang et al. (2021) revealed that environmentally relevant concentrations of ibuprofen (4.8, 48.0 and 480.0 ng/L) can affect the immune parameters (such as serum lysozyme activity, and mRNA levels of interleukin 6, interleukin-1-beta, and tumor necrosis factor alpha) of juvenile grass carp after 14 days exposures. Although invertebrates lack adaptive immunity, they possess a highly effective and complex innate immune system comparable to that of vertebrates. Invertebrate free-circulating hemocytes have a crucial role in cell-mediated immunity functions, including phagocytosis, the production of reactive oxygen species (ROS), and the secretion of hydrolytic enzymes and antimicrobial peptides.

Both functional and molecular immune responses are shown in marine mollusks (*Mytilus spp.* and *Ruditapes spp*.) treated with NSAIDs, including a significant impairment of hemocyte responsiveness, compromised lysosomal membrane stability, inhibition of phagocytic activity, altered granulocyte/hyalinocyte ratios, reduced immune surveillance against pathogens, and the modulation of immune response-related genes, such as those coding for lysosomal enzymes, chitinases, Toll-like and NOD-like receptors, lectins, and scavenger receptors (Almeida et al., 2020b; Mezzelani et al., 2018a; Milan et al., 2013). In mammals and fish, pharmaceuticals such as NSAIDs are primarily metabolized by Phase I (Cytochrome P450, CYP450) and II enzymes. Although the CYP450 biotransformation pathway in marine invertebrates is not fully understood, the modulation of transcriptional levels of phase I and phase II-related genes in response to environmental concentrations of NSAIDs has been observed (Mezzelani et al., 2018a, b; Milan et al., 2013). Bivalves (*M. galloprovincialis* and *R. philippinarum*) exposed to diclofenac, ibuprofen, ketoprofen, and nimesulide showed changes of drug metabolism genes, including CYP1A, phase II-related proteins (glutathione S-transferase A4, glutathione S-transferase P2, and sulfotransferase family 1B member 1), and heat shock proteins (HSP70L and HSP70I). Other cytotoxic effects of NSAIDs primarily derived from their ability to increase reactive oxygen species (ROS) production (Ghosh et al., 2015). These pro-oxidant effects include the generation of nitric oxide (NO•) due to the dissociation of nitric oxide synthase (iNOS), enhanced production of NADPH oxidases (NOXs) and xanthine oxidoreductase (XOR) with an increase in oxyradicals and lipid peroxidation products (*e.g.*, malondialdehyde and lipofuscin), the modulation of antioxidant enzymes (such as catalase, superoxide dismutase, glutathione reductase, and glutathione S-transferase), the increase in

micronuclei frequency and DNA damage, and the ROS-mediated apoptosis by inhibiting protein kinase B (Akt) signaling pathways, downregulating the transcription factor nuclear factor κB (NF-κB), and altering the p53 pathway (Bebianno & Gonzalez-Rey 2015; Mezzelani et al. 2018a,b; Mezzelani and Regoli, 2022). Supporting this, elevated mRNA levels of the NF-κB gene have been detected in *Ruditapes philippinarum* exposed to 100 and 1000 μg/L of ibuprofen (Milan et al., 2013). NSAIDs also exhibit a high affinity for peroxisome proliferator-activated receptors (PPARs), which may affect lipid and fatty acid metabolism in non-target species. This effect is supported by Mezzelani et al. (2018a), who reported that a 60-day exposure to ibuprofen, naproxen, and diclofenac (at 2.5 μg/L) led to the upregulation of genes involved in lipid metabolism, the decreases in neutral lipids content and the inhibition of acyl-CoA oxidase activity in the mussel *Mytilus galloprovincialis*.

1.2.3 Psychiatric drugs

Pharmaceuticals targeting the central nervous system include antidepressants acting as selective serotonin reuptake inhibitors (SSRIs) (*e.g.* fluoxetine, paroxetine, sertraline, citalopram) and as serotonin-norepinephrine reuptake inhibitors (SNRIs) *(e.g.* venlafaxine), and the antiepileptic carbamazepine (Hillhouse & Porter 2015).

SSRIs are the most commonly prescribed therapeutic class for the treatment of clinical depression, obsessive-compulsive disorder (OCD), panic disorders, social anxiety, and attention deficit hyperactivity disorder (ADHD) (Mezzelani et al., 2018a). SSRIs act by increasing extracellular levels of serotonin [or 5-hydroxytryptamine (5-HT)] in the synaptic cleft. Serotonin, synthesized from tryptophan and stored in synaptic vesicles, is released into the cleft upon vesicle fusion with the presynaptic membrane. It then binds to 5-HT receptors, modulating the associated signaling pathway. Excess 5-HT is either broken down by monoamine oxidase or taken back into the presynaptic terminal by the 5-HT transporter (5-HTT). SSRIs act by binding and distorting the tertiary structure of the 5-HTT, leading to increased serotonin levels in the synaptic cleft inhibiting its re-uptake and enhanced 5-HT receptor interaction (Nichols & Sanders-Bush, 2003).

The prolonged presence of serotonin in the extracellular space activates 5-HT1 and 5-HT5 receptors, which inhibit adenylate cyclase, leading to a reduction in cyclic adenosine monophosphate (cAMP) and cAMP-dependent enzymes. Conversely, 5-HT3, 5-HT6, and 5-HT7 receptors increase cAMP levels and activate downstream signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway, extracellular signal-regulated kinase 1 and 2 (ERK1/2), and the cAMP response element-binding protein (CREB) cascade. This cascade induces the expression of brain-derived neurotrophic factor (BDNF), a neurotrophin involved in neurogenesis, synaptic plasticity, and neuroimmune regulation (Faure et al., 2006).

The antidepressants belonging to the serotonin-norepinephrine reuptake inhibitors (SNRIs) have a dual-function, affecting both serotonin (5-HT) and norepinephrine (NE) neurotransmission. Noradrenergic neurotransmission has been linked to lack of motivation and loss of interest. In general, this class of antidepressants is more effective than SSRIs in relieving various types of chronic pain; while they have less ability in reducing irritability and anxious symptoms (Sussman et al., 2003). In aquatic ecosystems, detected concentrations of antidepressants range from a few ng/L to hundreds of ng/L. Specifically, in coastal areas, fluoxetine values are reported to range from 1 - 10 ng/L (Biel-Maeso et al., 2018; Gonzalez-Rey et al., 2015; Gros et al., 2012; Vasskog et al., 2008), with peaks in urban estuaries reaching 596 ng/L (Benotti and Brownawell, 2007). Citalopram concentrations in coastal waters from Santorini Island and around the Fiji Islands range from 0.6 - 8 ng/L (Mezzelani et al., 2018b; Dehm et al., 2021). Paroxetine concentrations range from 0.6 - 44 ng/L along the Norwegian coast (Mezzelani et al., 2018b), while sertraline and venlafaxine are found at 22 ng/L and 7 ng/L, respectively, with the highest venlafaxine concentrations (291 ng/L) detected along the northwestern coast of Spain (Fernández-Rubio et al., 2019).

An investigation on Mediterranean mussels (*Mytilus galloprovincialis*) revealed the presence of paroxetine in 43 % of analyzed samples, with concentrations up to 50 ng/g, making it the third most frequently detected pharmaceutical (Mezzelani et al., 2020). Additional data on bioaccumulation in *M. galloprovincialis* sampled along the Portuguese Atlantic coast confirmed the occurrence of fluoxetine and its metabolite, norfluoxetine, at concentrations of 4.8 ng/g and 13.5 ng/g, respectively (Silva et al., 2017). Furthermore, fluoxetine was detected in fish from Saudi Arabia, Sweden, the USA, and Poland at concentrations of 9.4, 4.0, 4.9, and 0.57 ng/g d.w., respectively. Paroxetine was measured in fish from Sweden at a level of 5.0 ng/g, while sertraline concentrations reached 17 ng/g d.w. in fish from the USA (Swaicka et al., 2022).

Although simpler than in mammals, the serotonergic signaling pathway is conserved in marine organisms, both vertebrates and invertebrates (Sumpter et al., 2014). In these species, serotonin is involved in many neurophysiological processes, such as feeding behavior, locomotion, the sensitization and facilitation of withdrawal reflexes, and reproduction (Franzellitti et al., 2014). Specifically, the 5-HT1 receptor is the only serotonin receptor that has been pharmacologically identified in invertebrates (Franzellitti et al. 2014; Mezzelani and Regoli, 2022). Environmental concentrations of fluoxetine (0.03 - 300 ng/L) significantly decrease cAMP levels and PKA activity in the digestive gland and mantle/gonads of mussels, consistent with an increased occupation of 5-HT1 receptors and consequently a serotonin accumulation in the postsynaptic cleft (Franzellitti et al., 2014).An inhibition of acetylcholinesterase activity was measured in bivalves (*Mytilus spp*. and *R. philippinarum*) treated with the SSRI fluoxetine (0.03 - 5 µg/L) (Cortez et al., 2018, Franzellitti et al.,

2014, Gonzalez-Rey & Bebianno, 2013; Munari et al., 2014). Neurotoxic effects, including decrease in norepinephrine and in number of NMDA-like receptor binding sites, and impairment of the camouflage ability, were also observed in cuttlefish *S. officinalis* by Bidel et al. (2016) after 20 days-exposure to SNRI venlafaxine (5 ng/L - 100 ng/L). Besides their effects on the serotonergic pathway, both SSRIs and SNRIs also have prooxidants function in non-target species. For instance, modulation of antioxidant responses (activity of enzymes as catalase, glutathione reductase, glutathione peroxidase, superoxide dismutase, and glutathione S-transferase), alteration of immune parameters, decreased lysosomal stability, and increased levels of malondialdehyde measured in *M. galloprovincialis*, *P. perna*, and *R. philippinarum*, revealed an onset of oxidative insult in response to fluoxetine exposure (0.03 - 5 µg/L) (Franzellitti et al., 2014, Munari et al., 2014). Furthermore, 7 days-exposure to environmental concentrations (0.5, 5, and 10 ng/L) of sertraline and norfluoxetine (metabolite of fluoxetine) significantly affected catalase and glutathione-S-transferase activities in gills and digestive gland of *M. galloprovincialis* (Rafiq et al., 2023). Enhancing antioxidant mechanisms including modulation of the catalase, glutathione S-transferase, and superoxide dismutase activity, were also reported in fish *Argyrosomus regius* after an exposure of 28 days to 20 µg /L of venlafaxine (Maulvault et al., 2019).

Given their characteristics as a weakly basic drugs with an amino group, antidepressants can be effectively sequestered by acidic subcellular compartments such as mammalian lysosomes where they are protonated and are no longer able to return to the cytosol, resulting in accumulation and loss of lysosomal functions (Daniel, 2003; Daniel and Wójcikowski, 1997). This mechanism is also facilitated by the single phospholipid layer which characterizes the lysosome membrane, and by the cation-π interactions that can develop between the aromatic rings of antidepressants with the ammonium group of glycerophospholipids, main components of eukaryotic membranes (Miguez et al., 2014). According to the biological read-across theory, which states that a drug can elicit similar effects in non-target species by acting through MOAs conserved with humans (Seiler, 2002; Brooks et al., 2003a; Huggett et al., 2003; Ankley et al., 2007; Gunnarsson et al., 2008), it is possible to hypothesize that lysosomal sequestration also occurs in marine invertebrates. In support of this, the ability of SSRIs and SNRIs to significantly alter lysosome functions in *M. gallorpovincialis* was highlighted by Rafiq et al. (2023), in whose study exposure to environmentally realistic concentrations (0.5 - 5 - 10 ng/L) of fluoxetine, sertraline, citalopram, and venlafaxine, caused a decrease in haemocyte's lysosome membrane stability (up to 70 % reduction) and increase of the lysosome/cytosol ratio (up to 220 %), neutral lipid (up to 230 %), and lipofuscin (up to 440 %) accumulation in digestive gland.

Among drugs targeting the central nervous system, the anticonvulsants as carbamazepine are used to treat epilepsy, neuropathic pain, and manic disorders in humans. In mammals, carbamazepine reduces the frequency of impulses during epileptic seizures by blocking voltage-gated sodium channels, thereby preventing the generation of action potentials and cell depolarization (Ambrosio et al., 2002; Siebel et al., 2010). Additionally, it also provokes the same effect by acting as an agonist at gamma-aminobutyric acid (GABA) receptor, inhibiting glutamate release and chloride entry into cells (Mezzelani and Regoli, 2022). Carbamazepine has been detected in European waters with average levels ranging from below the limit of detection (< LOD), up to hundreds of ng/L, with peaks of 1410 ng/L in the east coast of Ireland (Almeida et al., 2021). Bioaccumulation data indicate that carbamazepine is in more than 90 % of mussels collected from Mediterranean coasts with the highest concentration of 299.7 ng/g d.w (Mezzelani et al., 2020) and in various fish and shellfish sampled in the Red Sea (Ali et al., 2018). The ubiquitous presence of this drug in all environmental matrices can be attributed to its bio-refractory properties, resulting in an average half-life of over 200 days in aquatic ecosystems (Martin-Diaz et al., 2009). Several studies have demonstrated similarities in the mechanisms of action of carbamazepine between mammals and non-target marine species. For instance, Fabbri et al. (2015) reported inhibition of adenylyl cyclase and PKA activities in *Mytilus spp.* exposed to carbamazepine, potentially linked to the reduction of ion-channel opening caused by this drug. Additionally, the effects of carbamazepine on mussel voltage- and ligand-gated channels and ion transporters (the Na^+/K^+ pump and Na^+/Ca^{2+} exchanger) were suggested by a significant downregulation of a sodium-dependent serotonin transporter (SLC6A4) in response to its environmental concentrations (1 μg/L, 28 days) (Mezzelani et al., 2021). The mechanism of action of carbamazepine appears to be conserved in marine vertebrates as well. In the fish *Sparus aurata*, low doses of this pharmaceutical (6.95 μg/L, 28 days) resulted in the modulation of genes involved in the transmembrane transport of Ca^{2+}, including calcium-binding protein v2-like, calcium calmodulin-dependent serine protein kinase (CASK), and potassium intermediate/small conductance calcium-activated channel (KCNN4) (Hampel et al., 2017).

Like most pharmaceuticals, carbamazepine is metabolized through the cytochrome P450 biotransformation pathway, which consequently enhances ROS production leading to several side effects. Similar fate has been reported in non-target marine species exposed to this drug. For instance, up-regulation of drug metabolism related-genes (cytochrome P450 enzymes, as CYP4F8, CYP3A2, CYP3A29; sulfotransferase, SULT1B1), oxidative stress related-genes (protein sestrin 3, SEST3) and immune response related-genes (GIMAP4, GIMAP7,MYNA, LYZ1, DEFI, MRC1, IRG1, LITAF, and PLA2) was reported in *M. galloprovincialis* exposed for 28 days to 1 μg/L of carbamazepine (Mezzelani et al., 2021). Cellular effects of this drug in bivalves (*M. galloprovincialis*, *R.*

philippinarum, *Venerupis decussata*, and *Scrobicularia plana*) include oxidative stress responses as modulation of antioxidant enzymes (catalase, superoxide dismutase, glutathione reductase, and glutathione S-transferase) and accumulation of lipid peroxidation products (malondialdehyde, lipofuscin, and neutral lipids), immune responses as decrease of lysosomal membrane stability and inhibition of phagocytosis capacity, and neurotoxic responses as increase of acetylcholinesterase activity (Martin-Diaz et al., 2009; Almeida et al., 2020b, Freitas et al., 2016, Hampel et al., 2017, Mezzelani et al., 2021). Analogous effects were observed by Santos et al. (2018), who reported neurotoxic and oxidative responses in *Danio rerio* following 63 days of exposure to 10 μg/L of carbamazepine.

At the organismal level, alterations in mussels' physiological condition (cardiac index) and gonadosomatic index following 28 days-exposure to environmental concentrations (0.3, 3.0, 6.0 and 9.0 μg/L) of carbamazepine were observed, suggesting potential impairments in reproductive capacity and detrimental effects on feeding (Oliveira et al., 2017). These physiological responses may lead to a slowdown in metabolism, resulting in decreased reproductive performance and energy reserves, with long-term adverse consequences for population sustainability.

1.2.4 Antidiabetics

Among antidiabetics, metformin is the most widely prescribed drug for the treatment of type 2 diabetes (T2D) due to its robust glucose-lowering effects. In addition to effective hypoglycaemic effects, metformin may also be able to treat other basic metabolic diseases, including cancer (Ann et al., 2020; Chen et al., 2020), ageing (Konopka et al., 2019), cardiovascular disease (Charytan et al., 2019), neurodegenerative disease (Mor et al., 2020) and polycystic ovary syndrome (Fruzzetti et al., 2017). In mammals, the liver is the main target of metformin, where it enters hepatocytes via the organic cation transporter 1 (OCT1) (Ambrosio-Albuquerque et al., 2021). The metabolism of metformin has not been fully elucidated, but recent evidence suggests that it exerts its biological effects primarily by acting on mitochondria (Feng et al., 2022). Indeed, it inhibits the complex I in the mitochondrial electron transport chain, leading to glucose and adenosine triphosphate (ATP) depletion, which are necessary for hepatic gluconeogenesis (Foretz et al., 2014). As consequence it increases AMP (adenosine monophosphate) levels by activating 5' AMP-activated protein kinase (AMPK), which inhibits the Target of Rapamycin Complex 1 (TORC1), thereby affecting cell growth, proliferation, and metabolism. AMPK suppresses Fru-1,6-bisphosphatase (FBPase), a critical enzyme in gluconeogenesis that converts fructose-1,6-bisphosphate to fructose-6-bisphosphate, thus blocking the gluconeogenic pathway. Additionally, AMPK reduces the activity of sterol regulatory element-binding protein 1 (SREBP1), a key transcription factor involved in lipid and cholesterol synthesis.

This inhibition of mitochondrial activity results in increased glycolysis and lactate accumulation (Ambrosio-Albuquerque et al., 2021).

Despite the measured high removal rates by WWTPs (Trautwein et al., 2014), metformin has been detected at relatively high concentrations (peaks of 9200 ng/L in the USA) in effluent and surface waters due to its high influent load (Oosterhuis et al., 2013; Ambrosio-Albuquerque et al., 2021). Furthermore, high levels of this pharmaceutical (27.8 ng/g) were observed in sculpin fishes (*Leptocottus armatus*) from the Nisqually estuary (Meador et al., 2016). Given the evolutionary conservation of molecular drug targets and the established genetic and functional similarities between mammalian and zebrafish mitochondria (Pinho et al., 2013), it is reasonable to hypothesize that the role of mitochondria in the mechanism of action of metformin extends to both aquatic invertebrates and vertebrates. In support of this, Barros et al. (2022) demonstrated that environmentally relevant concentrations of metformin (ranging from 390 to 14,423 ng/L) disrupted the activity of mitochondrial complex I (COX I) in *Danio rerio* following a full life-cycle exposure. Although it does not structurally resemble hormone-like compounds, several studies have reported that metformin can act as an endocrine disruptor (Viollet et al., 2012). For instance, Niemuth et al. (2015a) reported an up-regulation of vitellogenin mRNA levels, which is an egg yolk protein that is normally expressed only in females, in adult male fish (*Pimephales promelas*) exposed to 40 µg/L of metformin for 28 days. The same authors also reported in the same species a development of intersex gonads in males, reduced size of treated male, and a decrease of fecundity for treated pairs after 320 days of exposure to metformin (40 µg/L) (Niemuth et al., 2015b). Additionally, a long-term and short-term exposure experiments showed that metformin (1 - 10 - 100 - 1000 nM) have an impact on locomotor activity of zebrafish, and several genes involved in neurological and cardiovascular development (Phillips et al., 2021).

Although there is significantly less information available in the literature, similar estrogenic effects have been reported in invertebrates. *Mytilus edulis* exposed to environmental concentrations of metformin showed increased vitellogenin mRNA expression, onset of severe gonadal pathologies and destabilization of lysosomal membranes in hemocytes (Koagouw and Ciocan, 2018).

Additional metformin effect in non-target species include the onset of oxidative stress and the impairment of immune system. Concentrations of metformin (40 - 80 µg/L) led to increased reactive oxygen species and free radical generation in fish *Labeo rohita* exposed for 28 days. This resulted in alterations in immune parameters (*e.g.*, total hemoglobin, red and white blood corpuscles), oxidative stress, lipid peroxidation, and genotoxic damage (Sibiya et al., 2023).

Moreover, an exposure of high doses of metformin (10 mg/L, 7 days) in catfish (*Clarias gariepinus*) induced cell oxidative stress through the inhibition of super oxide dismutase (SOD) antioxidant

enzyme and the reduction of the total antioxidant capacity (TAC), and increased the expression of inflammatory mediators as interleukin-6 (IL-6) and interleukin-1 beta (IL-1 β) (Taher et al., 2022). Metformin's mode of action includes the repression of lipogenesis and activation of catabolic processes like fatty acid oxidation through a mechanism involving the activation of AMP-activated protein kinase (AMPK) and the downregulation of sterol regulatory element-binding protein 1c (SREBP1c), a key transcription factor for de novo lipid synthesis (Fryer and Carling, 2005). Given that AMPK has been reported in several fish species, including blunt snout bream (*Megalobrama amblycephala*) (Xu et al., 2017), rainbow trout (*Oncorhynchus mykiss*) (Polakof et al., 2011b), and turbot (*Scophthalmus maximus*) (Zeng et al., 2016), it is possible to hypothesize that metformin may also play a role in regulating lipid metabolism in non-target species. Supporting this hypothesis, Zhou et al. (2019) demonstrated that metformin (200 μM for 48 hours) activated AMPK in hepatocytes of blunt snout bream (*Megalobrama amblycephala*), which contributed to enhanced lipid metabolism and reduced lipid deposition in cells exposed to a high-fat medium.

1.2.5 Cardiovascular drugs

Cardiovascular drugs are used for the treatment of hypertension, myocardial infarction, heart failure, and coronary artery disease in humans. This therapeutic class includes a wide panel of compounds that directly modulate cardiac and vascular function, such as beta-blockers (*e.g.* atenolol, carazolol, metoprolol, propranolol), Angiotensin Converting Enzyme (ACE) inhibitors (*e.g.* captopril, ramipril), angiotensin II receptor blockers, ARBs (*e.g.* losartan, valsartan), and calcium channel blockers (*e.g.* diltiazem, norverapamil, verapamil) (Fuentes et al., 2018).

Beta-blockers target β-adrenergic receptors, which, upon stimulation by endogenous catecholamines, activate adenylyl cyclase by converting ATP to cAMP. The latter is used by cAMP-dependent PKA to phosphorylate calcium channels, thus increasing the cellular calcium influx available for muscle contraction (Mezzelani and Regoli, 2022). By binding to β-adrenergic receptors, beta-blockers prevent their interaction with norepinephrine and epinephrine, thereby decreasing cAMP and PKA levels. This inhibition of sympathetic stimulation reduces heart rate, cardiac contractility, conduction velocity, and relaxation rate, thereby decreasing myocardial oxygen demand and enhancing exercise tolerance (Smith and Ashiya, 2007; de Oliveira et al., 2016). Furthermore, due to their high affinity for serotonin receptors, this class of drugs can also affect some central nervous mechanisms, inducing behavioral side effects and depressive moods (Alhayek & Preuss, 2021).

ACE inhibitors and ARBs interfere with various stages of the renin-angiotensin-aldosterone system, which plays a crucial role in controlling blood pressure, electrolyte balance, and systemic vascular

resistance (Zhang et al., 2020). These classes of drugs lower angiotensin II hormone levels through distinct mechanisms: ACE inhibitors target the enzyme renin, which catalyze its formation, while ARBs bind to angiotensin type 1 (AT1) receptors (Mezzelani and Regoli, 2022). Finally, calcium channel blockers limit the influx of calcium into cardiac and smooth muscle cells by inhibiting voltage-gated calcium channels. This leads to vasodilation, enhancing oxygen delivery to the heart and reducing blood pressure (Shahzad et al., 2019).

Due to incomplete elimination in WWTPs, residues of various cardiovascular drugs occur in aquatic environment in the range of low ng to several µg per L or g concentrations. The beta-blocker atenolol has been detected in surface waters in Spain, the USA, and South Africa at concentrations of up to 6.0, 1.1, and 39.1 µg/L, respectively (Muñoz et al., 2009; Cantwell et al., 2018; Agunbiade and Moodley, 2014). In the USA, the angiotensin receptor blocker (ARB) valsartan exhibited the highest detection frequency (100 %) with concentrations up to 2.0 µg/L (Cantwell et al., 2018), whereas in coastal areas, levels ranged from below the limit of quantification (LOQ) to 92 ng/L (Mezzelani et al., 2023). Additionally, in surface waters of the USA, the beta-blocker metoprolol and the ARB losartan were detected at concentrations up to 3.8 and 1.7 µg/L, respectively (Cantwell et al., 2018).

Although bioaccumulation data on cardiovascular drugs are limited, existing studies reported levels of valsartan in mussels (up to 6.7 ng/g d.w.) from the Mediterranean sea (Mezzelani et al., 2020); of atenolol in bivalves (up to 13.0 ng/g d.w.), crustaceans (up to 21.1 ng/g d.w.) and fish (up to 63 ng/g d.w.) from U.S.A, China and Saudi Arabia, respectively (Dodder et al., 2014; Ruan et al., 2020; Ali et al., 2018); and of metoprolol in crustaceans (up to 3.82 ng/g d.w.) from China (Ruan et al., 2020).

Beta blockers are primarily metabolized by cytochrome P450 enzymes (CYP1A2 and CYP2D6) as well as through phase II glucuronidation reactions. In contrast, angiotensin receptor blockers (ARBs) are less affected by cytochrome P450 enzymes and predominantly undergo direct de-ethylation and glucuronidation (Mezzelani and Regoli, 2022). Some β-type adrenoceptors similar to those of their mammalian counterparts have been identified in marine species (Fabbri et al., 2015), indicating a potentially analogous mechanism of action for these drugs in non-target organisms. To support this, the antagonistic effects of propranolol on β-adrenergic and serotonin receptors have been demonstrated in the mussel *Mytilus galloprovincialis*, which exhibited significantly reduced cAMP levels and PKA activities following exposure to environmentally relevant concentrations (0.3 - 300 ng/L) (Fabbri et al., 2015). Additionally, in zebrafish embryos, propranolol and metoprolol (⩾10 µg/L, 96 h) induced transcriptional changes in β-adrenergic receptors, antioxidant responses (catalase), and apoptosis pathways (p53 and PUMA) (Sun et al., 2014). In the same work, high doses of metoprolol (16 mg/L) caused also a decrease of the heart rate and hatching rate (Sun et al., 2014). Propanolol (5 µg/L) also altered the embryonic development of the sea urchin *Paracentrotus lividus*

and caused a reduction in fecundity, growth inhibition, and changes of heart and metabolic rates in *Daphnia magna*, with reported multigenerational effects (Zhang et al., 2020). Furthermore, a growth reduction, alteration in gill histopathology and gene expression (gonadotropins and vitellogenin) were observed in Nile tilapia (*Oreochromis niloticus*) exposed to metoprolol (107 μg/L) from fertilized egg until 80 days post-hatch (Gröner et al., 2017). Some studies also reported the ability of environmental concentrations of beta-blockers to alter non-target organisms' behaviors. For instance, propanolol exposure (1 μg/L and 4.11 μg/L) caused a reduction of anxiety as reflected in swimming behavior in fish *Phalloceros harpagos* (Matus et al., 2018); and changes in in the number of visits to the nest in male fathead minnows (Lorenzi et al., 2012). The metabolism of β-blockers in non-target species also contributes to oxidative stress. For instance, mussels treated with propranolol exhibited significant modulation of antioxidant enzymes (catalase and glutathione S-transferase), induction of carboxylesterase, decreased lysosomal membrane stability, and inhibition of acetylcholinesterase (Fabbri et al., 2015; Solé et al., 2010).

Less information on ARBs effects in non-target organisms are available in literature. Cyto-genotoxic and oxidative effects and the induction of metabolization processes (cytochrome P450–like activity, glutathione S-transferases, and glutathione peroxidases) were reported in mussels exposed to 300–3,000 ng/L of lorsartan (Zhang et al., 2020). Mezzelani et al. (2023) observed an induction of acetylcholinesterase (AChE) activity in mussels exposed for 14 days to 0.5 μg/L of valsartan, indicating the drug's potential to interact with neuronal mechanisms and exhibit neuroprotective properties in non-target species. Additionally, the study demonstrated that valsartan impacts lipid metabolism, as evidenced by a significant increase in acyl-CoA oxidase activity, and affects cell-mediated immunity, as indicated by the loss of lysosomal membrane integrity, inhibition of granulocyte phagocytosis, and alterations in Toll-like receptor and NF-kappa B signaling pathways (Mezzelani et al., 2023).

1.2.6 Lipid lowering agents

Lipid regulators, typically prescribed in combination with cardiovascular drugs, are used to treat dyslipidemias and can be divided into two major subclasses, the statins (*e.g.* atorvastatin) and fibrates (*e.g.* gemfibrozil, fenofibrate). Statins function by inhibiting β-hydroxy β-methylglutaryl–coenzyme A (HMG-CoA) reductase, the principal enzyme involved in cholesterol biosynthesis. Thereby, they suppress cholesterol biosynthesis (Pahan, 2006). Fibrates, on the other hand, stimulate the peroxisomal β-oxidation pathway mainly in peroxisomes and partly in mitochondria, leading to reduction in fatty acids, triglycerides, and low-density lipoprotein (LDL) levels (Pahan, 2006). In particular, the degradation pathway of fatty acids involves the activation of acyl-CoA oxidase, an

enzyme that oxidizes very long-chain fatty acids. This process leads to the release of hydrogen peroxide, resulting in modulation of cell oxidative homeostasis and formation of hydroxyl radicals (Gonzalez, 1998). Furthermore, fibrates are characterized by a pronounced induction of hepatic peroxisome proliferation, mediated via peroxisome proliferator-activated receptor alpha (PPARα). This leads to an increase in the number and size of peroxisomes, contributing to further oxidative stress, and an enhance of lipid catabolism (Barreto et al., 2017).

In surface waters, the highest concentrations of gemfibrozil were detected in North America, Europe, and Turkey around 0.75, 1.5 and 9.71 μg/L, respectively (Sanderson et al., 2003; Korkmaz et al., 2022); while atorvastatin reached concentrations of 15 ng/L in Canadian surface waters (Metcalfe et al., 2004). In the Mediterranean Sea, gemfibrozil was found at levels ranging from 3.3 to 5.7 ng/L (Biel-Maeso et al., 2018). Bioaccumulation data highlighted the presence of atorvastatin in fish from Sweden, with concentrations up to 2 ng/g d.w. (Swaicka et al., 2022).

A few studies carried out on lipid regulators indicate that they can modulate lipid metabolism also in non-target species. For instance, a significant reduction of triglycerides and an increase in fatty acids were observed in mussels exposed to environmentally realistic concentration of clofibrate (0.2 μg/L) (Lazzara et al., 2012). While variations of plasma triglycerides, cholesterol levels, and changes of genes involved in lipid metabolism, such as FABP, APOA1, APOEB, FASN, SREBP2, HMGCR1, PPARA, and SREBP1, were showed in fish exposed to gemfibrozil (Al-Habsi et al., 2016, Skolness et al., 2012). Furthermore, 1.2 μg/L of atorvastatin caused a depletion of lipid, carbohydrate, and protein pools and suppressed the transcription of key enzymes involved in mitochondrial biogenesis and fatty acid metabolism in *Mytilus edulis* (Falfushynska et al., 2019). Other side effects were highlighted for this class of drugs, including modulation of redox homeostasis and genotoxicity. Gemfibrozil exposures induced oxidative responses, including modulation of glutathione-S-transferase and catalase activity, increase of lipid peroxidation (LPO), lysosomal destabilization, and DNA damage in *Mytilus spp*. (Schmidt et al., 2011; Canesi et al., 2007). Additionally, gemfibrozil and atorvastatin caused a significant loss of DNA integrity in the teleost *Danio rerio* after 7 days of exposure to 380 and 13 ng/L, respectively (Rocco et al., 2010). Standard ecotoxicological bioassays were less sensitive in detecting acute effects of environmental concentrations of lipid regulators, which caused toxicity only at doses higher than those typically found in marine environments. For example, the EC50 values for bezafibrate were 172.73 mg/L in *A. fisheri*, 7.62 mg/L in *Anabaena*, and 240.4 mg/L in *Daphnia magna*. Similarly, the EC50 values for fenofibric acid were 1.72 mg/L in *A. fisheri*, 10.85 mg/L in Anabaena, and 4.90 mg/L in *Daphnia magna* (Rosal et al., 2010).

1.3 Interactive effects in complex scenarios: pharmaceutical mixtures and climate change-related stressor

Non-steroidal anti-inflammatory drugs, psychiatric drugs, antidiabetics, cardiovascular agents, and lipid regulators are among the most common classes of pharmaceuticals occurring in marine ecosystems and accumulated by aquatic non-target species. Each active ingredient, even within the same therapeutic class, possesses distinct chemical structures and physical characteristics, leading to different environmental fates. For an accurate assessment of the potential ecotoxicological effects and environmental risks, it is essential to consider that wild organisms are continuously exposed to complex cocktails of chemical pollutants, including pharmaceutical compounds. The effects of these mixtures can sometimes differ from those of individual compounds due to interactions among their components (Patel et al. 2019). Furthermore, as previously described, different therapeutic classes can sometimes target the same cellular components and metabolic pathways, resulting in potential interactions with additive, synergistic, or antagonistic effects (Mezzelani et al., 2022). To date, significant scientific efforts have been dedicated to investigate the biological impacts of individual pharmaceutical compounds. However, there is a relative scarcity of studies examining drug mixtures, particularly those involving more than two compounds from different therapeutic classes. Among them, several studies demonstrated that even if a single active ingredient is present at a concentration too low to cause an effect on its own, it can still contribute to the overall toxicity of a mixture. For instance, Cleuvers (2004) showed that a mixture of NSAIDs, consisting of diclofenac, ibuprofen, naproxen, and acetylsalicylic acid, at six different concentrations (1, 3.2, 10, 32, 100, and 320 mg/L) induced toxicity in *Daphnia magna*, whereas the individual compounds did not. Similarly, Cleuvers (2005) observed that the effect of a mixture of cardiovascular drugs, including atenolol, metoprolol, and propranolol (different concentrations, from 0.5 to 1000 mg/L), in immobilization tests using *D. magna* and growth inhibition tests using *Desmodesmus subspicatus* was greater than the sum of the effects of the individual compounds in both bioassays. The combination of ibuprofen (NSAID) and omeprazole (proton-pump inhibitor) increased toxicity and enhanced growth inhibition of bacterium *Aliivibrio fischeri* (Zuriaga et al., 2019). Additionally, a 7-day exposure to binary mixtures of fluoxetine (SSRI) and roxithromycin (antibiotic), and fluoxetine and propranolol (beta-blockers), caused significantly enhanced variations in CYP-associated enzymes and oxidative effects in *Carassius auratus* compared to the same drugs administered alone (Ding et al., 2016). Rodrigues et al. (2020) observed that a mixture of the antidepressants venlafaxine (2000 ng/L) and norfluoxetine (3.2 ng/L) led to increased mortality, as well as a higher incidence of malformations and abnormal pigmentation in zebrafish larvae, compared to exposure to each compound individually.

An *in vitro* study on hemocytes of *Mytilus spp.* highlighted lysosomal membrane destabilization, extracellular lysozyme release, nitric oxide (NO) production, and reduced phagocytic activity

following exposure to environmental concentrations of a mixture of two fibrates (bezafibrate and gemfibrozil), demonstrating that, unlike exposure to single compounds, the mixture of hypolipidemic drugs can significantly affect mussels' immune function (Canesi et al., 2007).A 15-day exposure of *M. galloprovincialis* to a mixture of ibuprofen (250 ng/L), diclofenac (250 ng/L), and fluoxetine (75 ng/L) induced oxidative stress, as evidenced by an increased antioxidant response, and exhibited xenoestrogenic effects, such as the formation of vitellogenin-like proteins in females. These findings differed from those obtained in previous single-exposure studies, indicating the presence of interactions between the drugs within the mixture (Gonzalez-Rey et al., 2014). Dumas et al. (2024) observed potential additive effects in *M. galloprovincialis* exposed to a mixture of diclofenac, carbamazepine, and venlafaxine, including evidence of disrupted energy metabolism, altered fatty acid degradation, impaired protein synthesis and degradation, as well as induction of endoplasmic reticulum stress and oxidative stress. On the other hand, a mixture of the antidepressant fluoxetine (3 ng/L) and the beta-blocker propranolol (3 ng/L) caused antagonistic effects on mussels following a 7-day exposure, impacting gene expression related to serotonin receptors and oxidative stress responses (Franzellitti et al., 2013). Antagonistic interactions between carbamazepine and valsartan, with a reduced effect of the antiepileptic when combined with the cardiovascular drug, were also observed in *M. galloprovincialis* exposed to their mixture (0.5 µg/L for each drug) for 14 days (Mezzelani et al., 2023).

Pharmaceuticals are released in marine ecosystems that are facing deep physical and chemical changes, including ocean warming and acidification. Such factors that can alter the toxicity of chemical pollutants. Therefore, it is crucial to elucidate the impact of future projections of temperature and CO_2 - driven ocean changes will have on the environmental fate, bioavailability, and toxicity of emerging compounds (Delorenzo, 2015). From a biological and environmental perspective, even a limited disturbance caused by one stressor can indirectly impact the susceptibility of one organism toward a secondary stressor (Kroeker, Kordas, and Harley, 2017).

Changes in environmental conditions, such as temperature fluctuations and ocean acidification, not only affect the physical-chemical properties and biological responsiveness of contaminants (Cao et al., 2019; Cao et al., 2018; Giuliani et al., 2020; Götze et al., 2014; Hawkins and Sokolova, 2017; Munari et al., 2019; Nardi et al., 2018a; Nardi et al., 2018b) but also influence the physiological status of organisms (Nardi et al. 2018a). For instance, an increase in water temperature can enhance the metabolic activity of aquatic species, leading to increased feeding and adsorption of contaminants (Navarro et al., 2016). While the reduction of seawater pH has direct effects on several metabolic pathways in marine organisms, including immunocompetence, oxidative status, growth and

reproduction (Gazeau et al., 2013), and it also indirectly modulate bioaccumulation to other pollutants (Nardi et al., 2018a, Nardi et al., 2018b).

Several studies have investigated the interaction between ocean acidification or marine heatwaves and pharmaceuticals in non-target organisms, revealing that these contaminants exhibit increased toxicity under such conditions. The combination of the antiepileptic carbamazepine (3 µg/L) and reduced pH (7.1) scenario caused an increase of the drug toxicity in *Scrobicularia plana*, with reduced specimens' survival and enhanced oxidative stress (Freitas et al., 2016). At environmentally realistic concentration of carbamazepine (1 µg/L) and pH of 7.5, *R. philippinarum* exhibited changes in mRNA transcription of several genes related to neurotransmission, immune response and biomineralization (Almeida et al., 2018). Similarly, the combination of carbamazepine (1 µg/L) and an acidification scenario (pH 7.6) increased cellular hazards, including modulation of the immune and oxidative systems in *M. galloprovincialis* (Mezzelani et al., 2021). Furthermore, Nardi et al. (2022) observed that a marine heatwave scenario, with a peak temperature of 22.5 °C, increased the toxicity of carbamazepine (1 µg/L) in *M. galloprovincialis*, significantly altered its accumulation leading to weakened immunocompetence, oxidative stress, and consequent cellular damage and lipid metabolism disorders. On the other hand, it has also been demonstrated that the pharmaceuticals toxicity may decrease under climate change conditions, suggesting that the nature of the interaction is typically related to the specific type of drug and stressor considered. For instance, after a 21 days co-exposure of diclofenac (0.05 and 0.50 µg/L) and acidification condition (pH 7.4) in *M. galloprovincialis* and *R. philippinarum*, Munari et al. (2018) demonstrated that the biochemical parameters measured (activity of superoxide dismutase, catalase and cyclooxygenase, lipid peroxidation and DNA strand-break formation) in both bivalves were more influenced by the reduced pH than by the single drug or the combination of both stressors. Moreover, the impact of fluoxetine exposure (10 and 100 ng/L) on DNA damage in larval *Heliocidaris crassispina* was mitigated under reduced pH conditions (7.7), likely due to the decreased bioavailability of the antidepressant in acidified environments (Lo et al., 2021). Almeida et al. (2021) observed a beneficial impact of temperature (a gradual increase of 1 °C per day up to 21 °C) on the oxidative status of the clam *R. philippinarum*, including a higher reduced-to-oxidized glutathione ratio, and lower levels of lipid peroxidation and protein carbonylation, following a 28-day exposure to a mixture of carbamazepine (1 µg/L) and the antihistamine cetirizine (0.6 µg/L).

The majority of studies investigated the impacts of combining environmental contaminants with a single climate change-related stressor, whereas very few examined the interactions between pollutant-induced stress and multiple climate change factors. Among these, the research by Costa et al. (2020), aimed to assess the influence of pH (7.7) and temperature (20 °C) on diclofenac toxicity (1 µg/L) in

the clams *R. philippinarum* and *R. decussatus*. Obtained results revealed that climate change-related factors did not enhance the drug's impacts on metabolic capacity, oxidative responses, or neurotoxic responses, although both species exhibited specific mechanisms to cope with stress. Conversely, a 28-day exposure to diclofenac (from dietary sources, 500 ± 36 ng/kg d.w.), combined with warming (+5 °C) and acidification (ΔpCO_2 ~1000 µatm, equivalent to ΔpH = -0.4 units), exacerbated some of the drug's effects in juvenile seabass (*Dicentrarchus labrax*), including further reductions in erythrocyte viability and increases in brain GST activity and ubiquitin synthesis in muscle (Maulvault et al., 2018).

Given the wide range of potential dynamics and interactions among various types of multiple stressors affecting aquatic species, it is evident that the current knowledge on this topic is still limited. Consequently, the urgency to investigate the ecotoxicological potential of emerging contaminants, such as pharmaceuticals, under future projection of climate change scenarios is emphasized by the European Union through regulatory practices, including the 'Strategic Approach to Pharmaceuticals in the Environment' (COM/2019/128 final). This need is also highlighted by the 14th of the 17 Sustainable Development Goals (SDGs) of the 2030 Agenda for Sustainable Development, which aims to understand the long-term consequences of environmental pollution.

1.4 Existing regulatory guidelines on environmental pharmaceuticals

Despite their widespread use and potential environmental impacts, most pharmaceuticals are not included in current environmental regulations or monitoring programs (Mezzelani et al., 2020). The regulatory frameworks often lag behind the advancements in pharmaceutical science, resulting in a significant gap in addressing the ecological and health implications of these substances. Research emphasizes the need for better regulatory measures, including enhanced environmental risk assessments (ERAs) and more effective wastewater treatment processes. Since 2006, the European Medicines Agency (EMA) has mandated that pharmaceutical companies submit an environmental risk assessment as part of the drug approval process (OSMED, 2022). The current guidelines for the pharmaceuticals ERA primarily rely on estimating the Predicted Environmental Concentration (PEC) in surface water. This estimation assumes that WWTPs are the main source of these compounds and that no drug biodegradation or retention occurs within these facilities. The methodology also presumes an even distribution of the yearly consumed drug amounts over time and across regions, without considering metabolism (Mezzelani et al., 2020). The calculation of PEC involves variables such as the annual drug consumption in an EU country, the percentage removal by WWTPs, the number of inhabitants in the area, and the daily wastewater production per capita (Gaw et al., 2014; Kümmerer, 2009). If the PEC value is below 0.01 µg/L, the compound is considered not to pose an

environmental risk. Otherwise, a phase II analysis is conducted, assessing the PEC/PNEC ratio, where PNEC value (Predicted No Effect Concentration) is derived from acute toxicity bioassays. A PEC/PNEC ratio of less than 1 suggests that the compound is unlikely to pose a risk, whereas further evaluation is required if PEC exceeds PNEC (EMA, 2006).

However, this approach has conceptual limitations. Variables used in PEC calculation can significantly vary throughout the year, influenced by seasonal changes, population fluctuations (especially in touristic areas), and the type of pharmaceutical. Annual consumption data do not account for over the counter (OTC) drugs, illegal, or internet-marketed medicines (Mezzelani et al., 2020). Additionally, the PEC calculation does not consider specific treatments occurring in different WWTPs. Furthermore, the PNEC value, derived from acute toxicity tests, is often not environmentally representative since concentrations tested are usually orders of magnitude higher than those possibly found in aquatic ecosystems (Mezzelani et al., 2022). In the OsMed 2022 Report, the environmental risk of 90 pharmaceutical active principles was assessed based on three specific criteria: high consumption, high environmental toxicity, defined as a low PNEC value, and inclusion in or nomination to at least one version of the European monitoring list. Among these, the NSAIDs diclofenac and ibuprofen, as well as the antidepressants sertraline and venlafaxine showed highest environmental risk (Figures 1.2, 1.3).

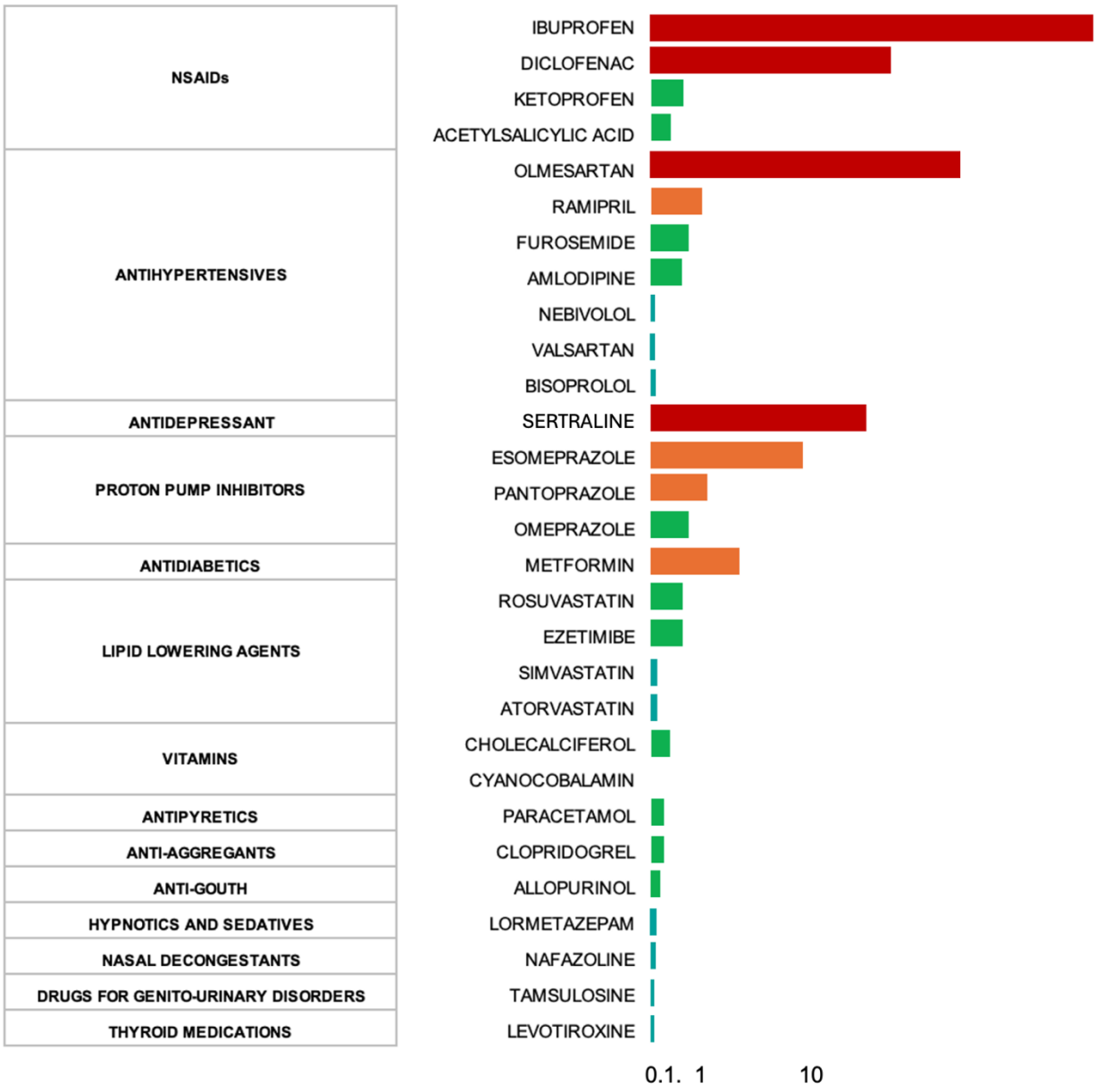


Figure 1.2. Environmental risk values for the 30 most widely consumed pharmaceuticals in Italy in 2022. The colors represent the risk category: red indicates high risk (PEC/PNEC ratio >10), orange moderate risk (PEC/PNEC ratio >1), green low risk (PEC/PNEC ratio >0.1), and blue negligible risk (PEC/PNEC ratio ≤0.1). (OSMED, 2022)

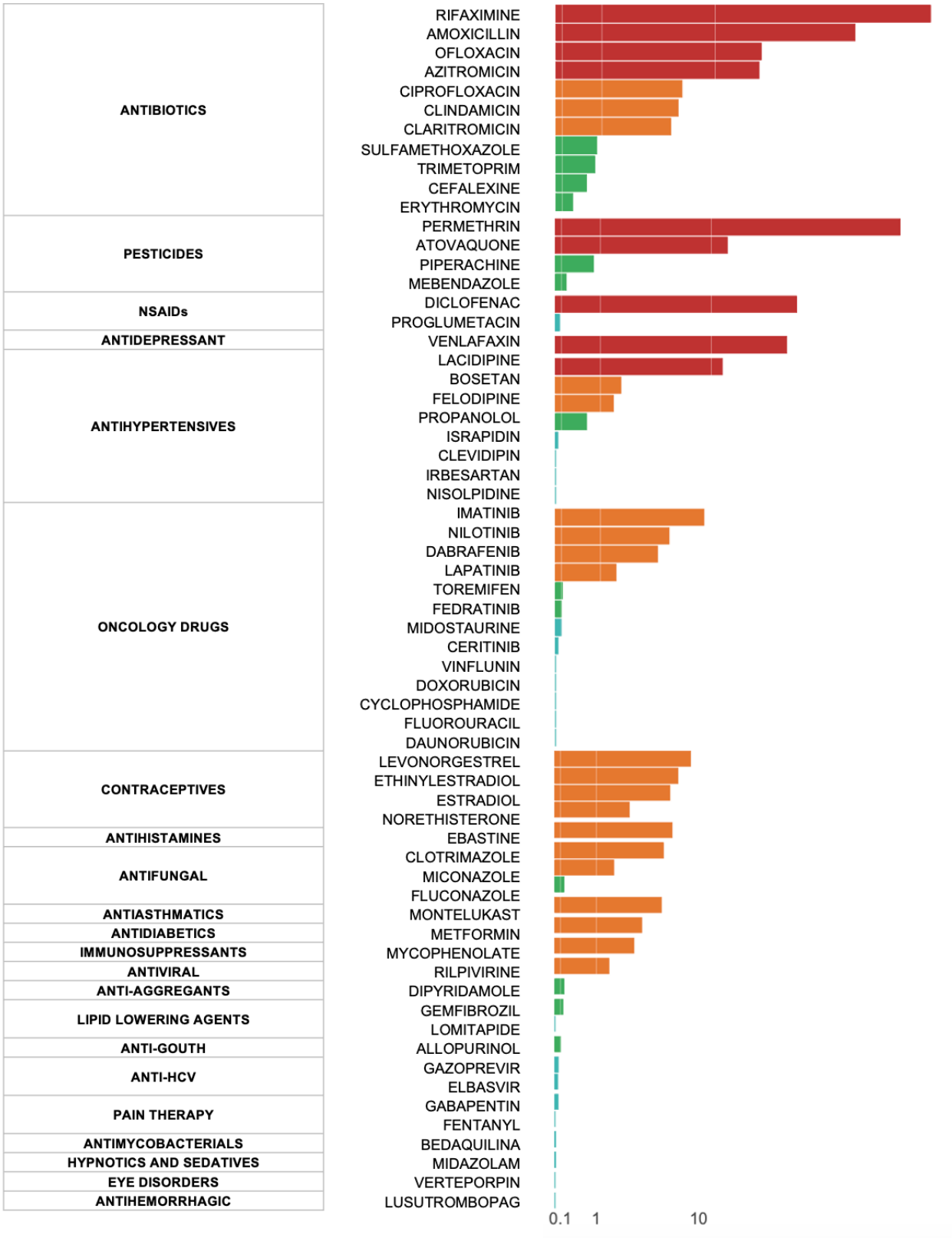


Figure 1.3. Environmental risk values for pharmaceuticals included in the European Watch List or with high environmental toxicity (low PNEC). The colors represent the risk category: red indicates high risk (PEC/PNEC ratio >10), orange moderate risk (PEC/PNEC ratio >1), green low risk (PEC/PNEC ratio >0.1), and blue negligible risk (PEC/PNEC ratio ≤0.1) (OSMED, 2022).

There is a consensus on the need for a more robust ERA methodology for pharmaceuticals, similar to those developed for traditional pollutants. A multidisciplinary approach combining chemical measurements of pharmaceuticals in the environment, their bioaccumulation in non-target species, and the adverse effects at various biological levels (from molecular to community level) would enhance the accuracy of environmental risk assessments for these emerging pollutants (Benedetti et al., 2012; Mezzelani et al., 2020). The European Union is actively funding numerous research projects aimed at addressing the environmental impact of pharmaceuticals, highlighting ongoing efforts to bridge knowledge gaps and promote sustainable pharmaceutical practices. Additionally, in recent years, guidelines for eco-pharmacovigilance have been developed, and regulatory measures have been implemented to mitigate the environmental risks associated with pharmaceutical residues (Mezzelani and Regoli, 2022). The Water Framework Directive (WFD) (2000/60/EC) is the primary European legislation aimed at protecting the water environment, encompassing freshwater, marine, and groundwater resources, making sure that they achieve good ecological and chemical status. Since the early 2000s, the WFD was the first European directive to focus on sustainability, with goals to enhance water quality, safeguard aquatic ecosystems, and promote the sustainable use of water resources. The WFD sets objectives on a six-year cycle, with the current goals to be achieved by 2027. In addition to the WFD, the Marine Strategy Framework Directive (MSFD) (2008/56/EC) has been established to specifically address the protection and improvement of marine ecosystems.

In this directive, the ecological status is assessed based on biological quality elements such as phytoplankton, phytobenthos, macrozoobenthos, macrophytes, and fish, as well as hydromorphological and physicochemical parameters (EEA Report No 7/2018). However, due to ecological variability across different ecosystems and communities, no absolute standard for this parameter can be established. Consequently, a waterbody is in good ecological status when there is minimal discrepancy between the measured values and the ideal expected values under conditions of minimal anthropogenic activity. If any biological quality element or parameter is not of good quality, the overall ecological status of the waterbody is determined by the lowest score among all the values, in accordance with the “one-out-all-out” principle (Carvalho et al., 2019).

Good chemical status is achieved when priority substances listed in Annex X of the WFD meet their environmental quality standards (EQS). Priority substances are chemicals identified by the Commission as posing a serious risk to, or via, the aquatic environment. These compounds include hazardous priority substances, which are defined as chemicals that are toxic, persistent, and capable of bioaccumulation (2000/60/EC). The rationale behind creating this "Watch list", and the ultimate goal of the Directive, is to progressively reduce the levels of naturally occurring priority substances to background levels and to eliminate, or reduce to near zero, hazardous priority substances

(2008/105/EC). According to Article 8b of the Environmental Quality Standards Directive (Directive 2008/105/EC, as amended by Directive 2013/39/EU), the watch list (WL) needs to be updated every two years. During each update, the Commission may remove substances from the WL if a risk-based assessment can be concluded without the need for additional monitoring data. Conversely, new substances or groups of substances may be added to the WL during each update. The maximum number of compounds or groups of substances that can be included on the WL increases by one with each update, up to a maximum of 14. Additionally, the continuous monitoring period for any individual substance on the WL may not exceed four years.

In the first WL, 4 pharmaceuticals were included, such as NSAID diclofenac, the hormones 17-beta-estradiol (E2) and 17-alpha- ethinylestradiol (EE2) and macrolide antibiotics. During the first update, the Commission determined the removal of 4 substances from the list, including diclofenac. This decision was based on the adequacy of the monitoring dataset, which allowed for a comprehensive risk assessment. Conversely, the antibiotics amoxicillin and ciprofloxacin were identified as appropriate candidates for inclusion and were subsequently added (EU 2018/840). In the second update of the WL, were included the antibiotics sulfamethoxazole and trimethoprim, the antidepressant venlafaxine and its metabolite o-desmethyl-venlafaxine, a group of ten azole compounds used as antifungal pharmaceuticals or food protection products, and the fungicides dimoxystrobin and famoxadone (EU 2020/1161). Finally, in the last update in 2022 9 new substances were added, including four pharmaceuticals such as antibiotics clindamycin and ofloxacin, and antidiabetic metformin and its transformation product guanylurea.

Although in the last years there have been efforts to include pharmaceuticals in regulatory assessments, these are not uniformly enforced or comprehensive enough to cover the vast array of pharmaceutical compounds detected in the environment.

To support policy-makers in mitigating the environmental impacts of pharmaceutical, it is crucial to further advance our understanding of their actual environmental distribution and biological effects in non-target organisms. For this reason, it is fundamental to expand environmental monitoring to encompass a broader range of pharmaceuticals and to enhance environmental risk assessments by including evaluations of bioaccumulation and sub-lethal biological effects across a diverse array of biological models representing different trophic levels and life stages.

2. Aims of the PhD study

The European Commission recently highlighted the importance of environmental pharmaceuticals with the 'Strategic Approach to Pharmaceuticals in the Environment' (COM/2019/128 final), emphasizing the need to gather data on their occurrence and ecotoxicological effects as crucial prerequisites for developing an appropriate environmental risk assessment (ERA) of these emerging contaminants. In this contest, the overall aim of my PhD thesis was to clarify the ecotoxicological impact of environmental pharmaceuticals in marine ecosystems evaluating the bioaccumulation and the onset of adverse effects of both single molecules and mixtures, contributing to the development of a comprehensive environmental risk assessment procedure for these pollutants in marine ecosystems. Several chronic and acute laboratory exposures were carried out, by adopting *in vivo*, *ex vivo* and *in vitro* approaches to evaluate the ecotoxicological effects on a selection of model species belonging to different trophic levels and life-stages. The application and integration of different methodologies aimed to deepen elucidate the intricate network of responses at different levels of biological organization which regulate the mechanisms of action of such molecules in non-target organisms. Tested active principles were selected based on their relevance in terms of volume of consumption and occurrence in coastal areas.

The research activities carried out for this PhD project will be presented in the following chapters.

Chapter 3 aimes to investigate pharmaceuticals' sub-lethal effects under chronic conditions, elucidating (1) the mechanism of actions of active principles, (2) the interactions among different therapeutic classes, and (3) the influence of climate change related stressors (namely, marine heatwaves and ocean acidification) in modulating the overall pharmaceuticals mixture toxicity. Three different *in vivo* experiments were conducted, exposing the mussel *Mytilus galloprovincialis* to environmentally realistic concentrations (ranging from 0.1 - 1.5 μg/L) of pharmaceuticals and their mixtures. *M. galloprovincialis* was selected as model organism due to its wide distribution, ecological relevance, commercial importance, and capacity to accumulate contaminants over relatively long lifespan. A multidisciplinary approach was applied integrating drugs bioaccumulation with a wide panel of biochemical, cellular and histological markers, including immunological and oxidative responses, genotoxic damage, neurotoxic effects, and alteration of lipid metabolism with the aim of clarifying the main metabolic pathways affected by pharmaceuticals exposure. Overall results were then elaborated by a quantitative Weight Of Evidence (WOE) model to clarify the environmental risk of these pollutants.

Chapter 4 reports the application of the *ex vivo* approach of Precision Cut Tissue Slices (PCTS) on mussel digestive gland to deepen knowledge on specific mechanisms of action of lipid lowering agents and its similarities to a natural active principle. Specifically, in this work the hypolipidemic

proprieties of the fenofibrate and of the of the natural alkaloid caulerpin were investigated in non-target aquatic organism, measuring, at molecular, catalytic and cellular levels, the responses reflecting the activation of lipolysis, modulation of the overall lipid metabolism and susceptibility of redox homeostasis.

Chapter 5 presents a study aimed at investigating the effects of three drugs (the antidiabetics metformin and rosiglitazone, and the lipid-lowering agent gemfibrozil), all known agonists of peroxisome proliferator-activated receptors (PPARs), on mussels' lipid metabolism integrating *in vivo* (14-day mussel exposure) and *ex vivo* (PCTS technique) approaches.

Chapter 6 aims to assess the ecotoxicological impact of environmental pharmaceuticals in two fish cell lines, PLHC-1 liver cells derived from topminnow *Poeciliopsis lucida*, and RT-gillW1 gill cells derived from the rainbow trout *Oncorhynchus mykiss*. These cell lines were selected for their well-characterized responses to environmental contaminants, as well as for the critical roles played by gills and liver tissues in xenobiotic uptake and metabolism in aquatic species. In this *in vitro* study, environmentally realistic concentrations (1µg/L - 10 µg/L) of several pharmaceuticals belonging to different therapeutic classes and their mixtures were tested for investigating their cytotoxicity, capability to induce oxidative stress and apoptosis measuring cell viability, production of reactive oxygen species and changes in mitochondrial membrane potential, respectively. For the first time, the responses obtained with this approach were elaborated and integrated through the WOE model to obtain synthetic hazard indices which reflect the severity of the disturbance caused by each tested drug and mixture in fish cell lines.

Finally, chapter 7 focuses on characterizing the ecotoxicological impact of the same panel of pharmaceuticals and their mixtures tested in fish cell lines, using a battery of bioassays. These included the algal growth inhibition on *Phaeodactylum tricornutum*, the embryo-development of the sea-urchin *Paracentrotus lividus* and the oyster *Crassostea gigas,* and the bioluminescence test on the bacterium *Aliivibrio fischeri*. The selection of species and endpoints was based on their sensitivity to contaminants, representation of different trophic levels and life-stages, and standardization / application in several international monitoring agencies and expert working groups. The findings of this work were elaborated through the WOE model, which provides a final cumulative level of hazard of each tested pharmaceutical and mixture related to the entire bioassays' battery.

3. Mussel exposures: ecotoxicological potential of single pharmaceuticals and their mixtures in model organism *Mytilus galloprovincialis*

3.1 Introduction

Active pharmaceutical ingredients (APIs) are increasingly being detected in all environmental matrices, including in seawater, sediments, and a wide range of aquatic species, from invertebrates to top predators (Oaks et al., 2004; Zenker et al., 2014; Álvarez-Muñoz et al., 2015; Mezzelani et al., 2018, 2020; Ali et al., 2018; Fernández-Rubio et al., 2019; Martínez-Morcillo et al., 2020), thereby becoming a significant class of pollutants of emerging concern for the scientific community.

Among the over 300 different types of active principles frequently detected in marine ecosystems there are nonsteroidal anti-inflammatory drugs (NSAIDs), psychiatric medications, antidiabetics, cardiovascular drugs, and lipid-lowering agents, each characterized by distinct physico-chemical properties and Mechanisms Of Action (MOA) (González-Alonso et al., 2017; Moreno-González et al., 2015; Alygizakis et al., 2016; Biel-Maeso et al., 2018; Fernandez Rubio, 2019; Mezzelani et al., 2020; Zhang et al., 2020; Ambrosio-Albuquerque et al., 2021). Due to their ability to permeate biological membranes, these compounds can interact with non-target marine species, potentially leading to adverse long-term effects on marine biodiversity and ecosystem health that are still poorly understood. These chemicals exert their biological effects by targeting metabolic, enzymatic, or cell signaling pathways through well-characterized mechanisms of action in humans. Notably, many molecular receptors are evolutionarily conserved across species, meaning that these pathways can also be modulated in aquatic organisms that possess identical or similar target organs, tissues, cells, or biomolecules (Fent et al., 2006; Mezzelani and Regoli, 2022). As a result, the continuous exposure of wild species to these pharmaceuticals can lead to significant physiological disturbances.

Current research has largely focused on elucidating the MOA of individual pharmaceuticals in non-target species. Studies have shown that many NSAIDs, psychiatric drugs, antidiabetics, cardiovascular drugs, and lipid-lowering agents target similar cellular components and metabolic pathways, such as those involved in lipid metabolism, oxidative stress response, and immune functions (Martin-Diaz et al., 2009; Almeida et al., 2014; Zenker et al., 2014; Ali et al., 2018; Elizalde-Velázquez and Gómez-Oliván, 2020; Mezzelani et al., 2020; Almeida et al., 2021; Sharma et al., 2021; Mezzelani and Regoli, 2022). Despite these findings, still remains a relative paucity of research on the complex interactions that may arise from pharmaceutical mixtures, which could result in additive, synergistic, or antagonistic effects, complicating the assessment of their full impact on marine ecosystems. The few studies available in the literature primarily examine interactions within mixtures composed of drugs from the same therapeutic class (Cleuvers et al., 2004, 2005; Rodrigues, 2020; Canesi et al., 2007) or, at most, involving two pharmaceutical compounds from different classes

(Franzellitti et al., 2013; Zuriaga et al., 2019; Mezzelani et al., 2023), an approach that can help elucidate the MOAs of the different active principles but fails to adequately reflect the complexity of real-world environmental scenarios.

Beside environmental pharmaceuticals, in natural conditions aquatic species are continuously exposed to a wide array of other stressors that can have an influence on the fate, bioavailability, and toxicity of these emerging compounds (Delorenzo, 2015; Kroeker et al., 2017; Martin et al., 2021). Changes in environmental conditions, such as temperature fluctuations and ocean acidification, can impact the physical-chemical properties and biological responsiveness of chemicals (Cao et al., 2019; Cao et al., 2018; Giuliani et al., 2020; Götze et al., 2014; Hawkins and Sokolova, 2017; Munari et al., 2019; Nardi et al., 2018a; Nardi et al., 2018b; Costa et al., 2020; Mezzelani et al., 2021; Almeida et al., 2021) and may also influence the physiological status of organisms (Nardi et al., 2018a). Although several studies have reported that interactions between ocean acidification and/or marine heatwaves with individual pharmaceuticals can enhance the toxicity of these contaminants in non-target organisms (Freitas et al., 2016; Almeida et al., 2018; Maulvault et al., 2018; Mezzelani et al., 2021; Nardi et al., 2022), there is limited information on how combined climate change stressors influence APIs toxicity in marine species. *In vivo* studies using model organisms, such as fish and aquatic invertebrates, provide an accurate representation of biological and ecological systems under natural conditions (Gunnarsson et al., 2019). This is crucial for a comprehensive understand of the long-term impacts of environmental stressors, including those related to climate change and pollutants such as pharmaceuticals. Additionally, this approach enables the detection of subtle sub-lethal effects that might not be evident in *in vitro* systems, such as changes in gene expression, immune responses, and metabolic pathways, which are critical for assessing the ecological risks posed by chronic exposure to low doses of contaminants (Johnson et al., 2020).

In this context, this chapter presents three distinct *in vivo* experiments based on the use of the model organism *Mytilus galloprovincialis*, which is recognized as an effective bioindicator in the Mediterranean Sea. This species was selected due to its ecological, economic, and ecotoxicological significance, as well as its relevance for studying the increasing impact of climate change-related stressors and pharmaceuticals in the region (Galli et al., 2017; Darmaraki et al., 2019; Mezzelani et al., 2020; Almeida et al., 2021; Musella et al., 2020). In these studies environmentally realistic concentrations (ranging from 0.1 - 1.5 μg/L) of the most common detected environmental pharmaceuticals and their mixtures were tested under chronic condition to (1) elucidate the MOA of poorly investigated active principles, (2) clarify interactions among different therapeutic classes of drugs, and (3) assess the toxicity of environmental mixtures in the context of climate change scenarios, including marine heatwaves and ocean acidification. The overall aim was to provide novel insights

into the mechanisms affecting bioaccumulation, excretion, and the onset of sublethal effects in this non-target species. A multidisciplinary approach was employed, integrating drug bioaccumulation with a comprehensive panel of biochemical, cellular, and histological biomarkers, such as immunological and oxidative responses, genotoxic damage, neurotoxic effects, and alterations in lipid metabolism. The overall results were elaborated though a quantitative Weight of Evidence model which integrates and summarizes complex heterogeneous data in synthetic hazard indices helpful for the pharmaceuticals environmental risk assessment.

3.2 Materials and methods

3.2.1 Animal collection

Mussels, *Mytilus galloprovincialis* (5.5 ± 1.00 cm shell length), were collected in March 2022 (experiment 1), November 2022 (experiment 2), and May 2023 (experiment 3) from a from a shellfish farm in Senigallia (Ancona, Adriatic Sea), and were acclimatized for 7 days with aerated artificial seawater (ASW; Istant Ocean®), at local seasonal environmental conditions of temperature (18 ± 1 °C), salinity (33 practical salinity units) and pH (8.20). Collection and experimental use of mussels is not subjected to ethical review permissions according to both European and Italian normative (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, 2010; Italian Legislative Decree n. 26, 2014), while monitoring guidelines recommend this species as appropriate bioindicator organism for assessing bioavailability of ecotoxicological effects of environmental pollutants in marine environments (Bocchetti and Regoli, 2006). The following section report a detailed description of the three different experimental plans that were carried out.

3.2.2 Chemicals and stock solutions

Selected pharmaceuticals were purchased from Sigma-Aldrich® (St. Louis, MO, USA): Ibuprofen (IBU, CAS 15687-27-1), Paroxetine (PAR, CAS 110429-35-1), Venlafaxine (VEN, CAS 99300-78-4), Gemfibrozil (GEM, , CAS 25812-30-0), Ramipril (RAM, CAS 87333-19-5), Metformin (MET, CAS 1115-70-4), Carbamazepine (CBZ, CAS 298-46-4), Diclofenac (DIC, CAS 15307-79-6), Valsartan (VAL, CAS 137862-53-4).

For experiment 1 (ibuprofen, paroxetine and their mixture), stock solutions of IBU and PAR were obtained by dissolving drug powder in methanol (MeOH) and stored at + 4°C, while working solutions were prepared daily by diluting the stock solution in ASW.

For experiment 2 (venlafaxine, gemfibrozil, ramipril, metformin and their mixture), stock solutions of VEN, GEM, RAM and MET were obtained by dissolving drug powder in dimethyl sulfoxide

(DMSO) and stored at + 4°C, while working solutions were prepared daily by diluting the stock solution in ASW.

For experiment 3 (Pharmaceuticals mixture under marine heatwave and/or reduced-pH scenario), stock solutions (5000 mg/L) were prepared for each pharmaceutical (IBU, CBZ, VEN, RAM, MET, GEM) in the mixture by dissolving drug powders in DMSO (for CBZ, VEN, IBU, RAM and GEM) or MEOH (for MET). All stock solutions were then diluted with DMSO to reach a concentration of 500 mg/L. From these intermediate stocks, a final stock solution of 5 mL was prepared with concentration of 90 mg/L for CBZ, IBU, and MET, 18 mg/L for VEN, 6 mg/L for RAM, and 24 mg/L for GEM. Finally, to reach the target concentrations for each pharmaceutical in the aquaria, 500 µL of the final stock were added to each aquarium.

3.2.3 Experimental plans

For the experiment 1, mussels were randomly distributed into 4 glass-tanks (n = 60 per tank) in a volume of 17 L of ASW, and exposed for 30 days to the four following treatments: CTRL, control condition (0.003 % of methanol); IBU, ibuprofen exposure (1 µg/L); PAR, paroxetine exposure (1 µg/L); IBU + PAR, mixture of ibuprofen (1 µg/L) and paroxetine (1 µg/L). After the exposure phase, mussels were maintained for additional 14 days in pharmaceuticals-free artificial seawater intended as a depuration phase.

For the experiment 2, organisms were randomly distributed into 6 glass-tanks (n = 60 per tank) in a volume of 17 L of ASW, and exposed for 30 days to the six following treatments: CTRL, control condition (0.0029 % of dimethyl sulfoxide, DMSO); VEN, venlafaxine exposure (1 µg/L); GEM, gemfibrozil exposure (1 µg/L); RAM, ramipril exposure (1 µg/L); MET, metformin exposure (1 µg/L); MIX, mixture exposure of VEN, GEM, RAM and MET (1 µg/L for each drug). After the exposure phase, mussels were maintained for additional 14 days in pharmaceuticals-free artificial seawater intended as a depuration phase. In both experiments, exposure doses of each pharmaceutical were selected within environmentally realistic concentrations for coastal areas (Togola and Budzinski, 2008; Seabra Pereira et al., 2016; Biel-Maeso et al., 2018; Seabra Pereira et al., 2016; Birch et al., 2015; Freitas et al., 2016; Gaw et al., 2014; Biel-Maeso et al., 2018; Mezzelani et al., 2018b).

For the experiment 3, 520 mussels were randomly distributed into 8 glass tanks (n = 65 per tank) in a volume of 17 L of ASW. The experimental setup consisted of eight treatments: 1) representing the solvent control condition, CTRL (0.0017 % of DMSO) with constant pH (8.1) and temperature (18 ± 1°C); 2) pH decrease, OA, simulating hypercapnic acidosis (pH 7.6, pCO_2 at 1700 µatm); 3) heatwaves scenario, HW, consisting of two consecutive dynamic heatwaves with peaks at 23 ± 1°C, where temperature increased daily by 1 °C until reaching the peak, maintained for 10 days, then

gradually decreased until returning to the climatological mean temperature (18 ± 1°C), and held constant for an additional 6 days before the second heatwave; 4) combination of OA and HW exposure, CC; 5) pharmaceuticals (APIs) mixture composed by environmentally realistic concentrations of each drug (Gonzalez-Rey et al., 2015; González-Alonso et al., 2017; Moreno-González et al., 2015; Alygizakis et al., 2016; Biel-Maeso et al., 2018; Fernandez Rubio, 2019; Zhang et al., 2020; Ambrosio-Albuquerque et al., 2021): IBU 1.5 µg/L, CBZ 1.5 µg/L, VEN 0.3 µg/L, RAM 0.2 µg/L, MET 1.5 µg/L and GEM 0.4 µg/L; 6) combination of OA and APIs mixture; 7) combination of HW and APIs mixture; and 8) combination of CC and APIs mixture combined (Figure 3.1).

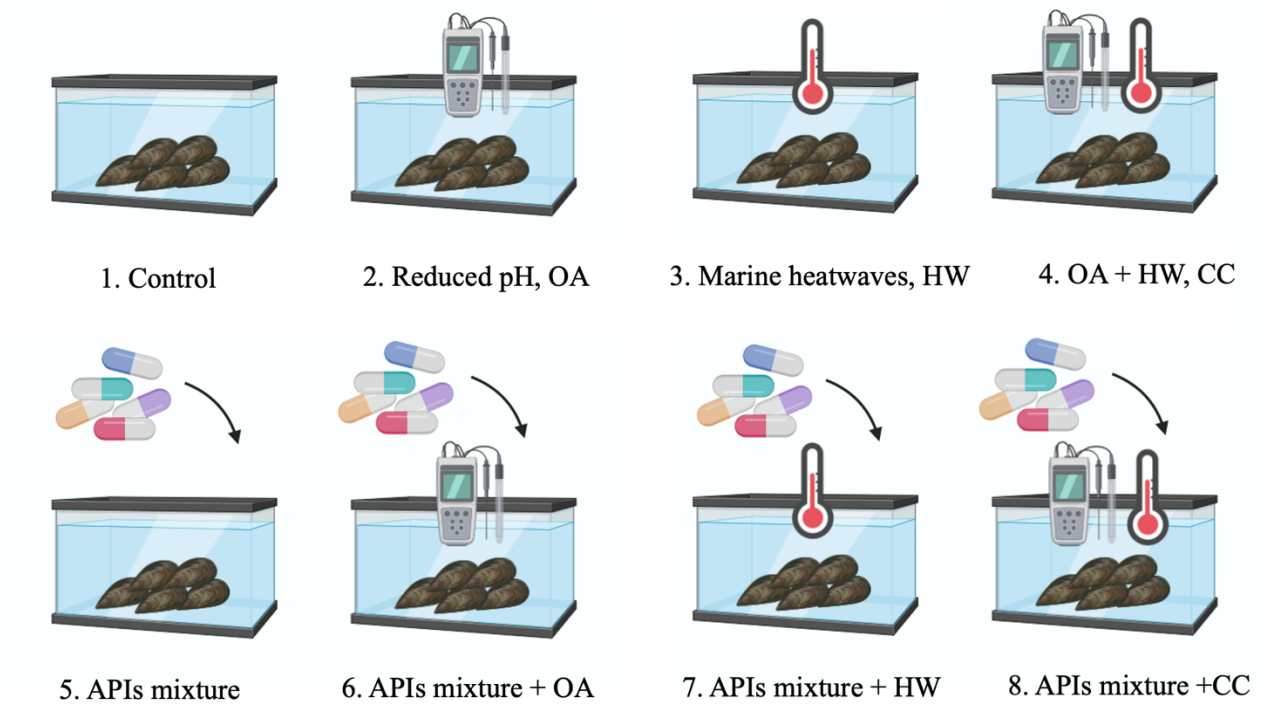


Figure 3.1 Experiment 3. The experimental setup consisted in mussels' exposure to 1) solvent (DMSO) control condition, with constant pH (8.1) and temperature (18 ± 1°C); 2) pH decrease, OA, simulating hypercapnic acidosis (pH 7.6, pCO_2 at 1700 µatm); 3) heatwaves scenario, HW, consisting of two consecutive dynamic heatwaves with peaks at 23 ± 1°C, where temperature increased daily by 1 °C until reaching the peak, maintained for 10 days, then gradually decreased until returning to the climatological mean temperature (18 ± 1°C), and maintained constant for an additional 6 days before the second heatwave; 4) combination of OA and HW exposure, CC; 5) pharmaceuticals (APIs) mixture composed by Ibuprofen, IBU 1.5 µg/L, Carbamazepine, CBZ 1.5 µg/L, Venlafaxine, VEN 0.3 µg/L, Ramipril, RAM 0.2 µg/L, Metformin, MET 1.5 µg/L and gemfibrozil, GEM 0.4 µg/L; 6) combination APIs mixture and OA; 7) combination APIs mixture and HW; and 8) combination of APIs mixture and CC.

Despite the effects related to climate changes are expected to occur over the course of decades, organisms were exposed to the tested environmental conditions of APIs mixture and acidification without gradual acclimation, thus simulating sudden changes. Lowered pH was adapted from scenario RCP 8.5 and IPCC CMIP6 model projections (IPCC 2023; Kwiatkowski et al., 2020) reporting a mean pH value of 7.6 for open oceans. Reduced pH/hypercapnia conditions were obtained by bubbling pure CO_2 in ASW until the desired pH was reached; this condition was monitored and maintained in exposure tanks through pH-stat system (Aquatronica®). For each experimental condition pH was measured continuously, temperature and salinity daily, while total alkalinity (A_T) twice per week according to Dickson et al. (2007). Seawater carbonate parameters (*p*CO2 and saturation state (Ω) for calcite and aragonite) were calculated in CO_2SYS (Pierrot et al., 2006) using barometric pressure values, as well as A_T, pH, temperature and salinity values of the respective samples. Details of constants used for calculations can be found in Nardi et al. (2017) and in Mezzelani et al. (2021).

In all experiments, water was changed every two days and, during the exposure phase, pharmaceuticals were re-dosed. Mussels were fed with 500 µL of a commercial mixture of marine plankton (EasySPS EVO, size range 0.2 - 400 mm) before the water change. At the end of the exposure (30 days for experiments 1 and 2; 42 days for the experiment 3) and depuration phases (44 days for experiments 1 and 2), 30 mussels for each treatment were sampled, dissected and pooled for bioaccumulation and biological analyses. For chemical analyses, 3 pools each constituted by the whole tissues of 5 organisms were collected and stored at - 20 °C. For biochemical and histochemical analyses, gills, digestive glands and hemolymph were collected from 15 individuals, pooled in 5 separate samples, each constituted by tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at - 80 °C. Aliquots of hemolymph were immediately processed for lysosomal membrane stability, phagocytosis activity, granulocytes-hyalinocytes ratio and DNA damage; while additional aliquots of hemolymph were fixed in Carnoy's solution (3:1 methanol and acetic acid solution) for the microscopy evaluation of micronuclei frequency.

3.2.4. Chemical analysis

Bioaccumulation of carbamazepine (CBZ), gemfibrozil (GEM), ibuprofen (IBU), metformin (MET), paroxetine (PAR), ramipril (RAM) and venlafaxine (VEN) was determined in the whole soft tissues of mussels on five replicates (n=5), each constituting of the tissues of 6 specimens. About 3 gr of wet tissue were homogenized in 5 mL of 100 % methanol (LC-MS grade) for 10 minutes using a dispersing, stirring, homogenizing and grinding system (ULTRA TURRAX Tube Drive – IKA) at a temperature of 4 °C in order to avoid oxidation or decomposition of the analytes; after centrifugation

at 5000 xg for 15 min, 1 mL of samples was purified using Phree Phospholipid Cartridges (Phenomenex, US), following the procedures described by the manufacturer, in order to remove part of the protein and lipidic material, reducing interferences and increasing stability of the samples. Finally, the purified extracts were analyzed using ultra-high performance liquid chromatography (UHPLC) with tandem triple quadrupole mass spectrometry (LC-MS/MS).

Chromatographic separation of CBZ, GEM, IBU, MET, PAR, RAM, and VEN were performed with PerkinElmer QSight® LX50 UHPLC system on Luna Omega® C18 column (1.6 µm, 50 mm length, 2.1 mm ID, Phenomenex, US) equipped with security guard column (C18, 5 µm, 4 mm length, 2.0 mm ID, Phenomenex, US). The chromatography column was maintained at controlled temperature of 30 °C and 10 µL of the sample was injected adopting the full loop filling technique to minimize injection variability and uncertainty. Chromatographic separation was carried out using a linear gradient of 0.1 % Formic Acid and 0.4 % Ammonium Acetate 1M in methanol (solvent A) and water (solvent B) as mobile phases at a constant flow rate of 0.250 mL/min, adopting a total run time of 12 min, with an equilibration time of 3 min between runs. The gradient specifications were: 0 min 20 % A; 6.5 min 65 % A; 7 min 100 % A, 8.5 min 100 % A; 9 min 20 % A.

Quantification and identification of target analytes, were performed employing a triple quadruple mass spectrometer PerkinElmer Qsight® 220 coupled at LX50 UHPLC system, using electrospray ionization (ESI) source in the positive-ion mode. A multiple reaction monitoring (MRM) mode was used to acquire the different analytes, with the exception of IBU were employed for each compound at least two selected MRM transitions: the one with the highest intensity for quantification (quantifier) while the other transition was used as a confirmation signal (qualifier). MRM transitions were obtained through direct infusions into a mass spectrometer of pure standard solutions of the individual analytes at a concentration of 10 µg/mL at a flow rate of 30 µL/sec and by testing the optimal operating conditions in order to obtain the formation of a parent ion and a series of subsequent products. These MRM experiments allowed the following operating conditions to be fixed: the electrospray voltage was set at 4850 V, the nebulizer and drying gas at 275 V and 120 V respectively. The ion source temperature was set at 400 °C, while the Hot-Surface Induced Desolvation (HSID) temperature was set at 250 °C. Additional key parameters such as collision energies (CE), entrance voltages (EV) and collision cell lens 2 (CCL2) were optimized for each analyte. The retention time (RT) of each compound was respectively: MET 0.62 min; VEN 4.98 min; CBZ 6.33 min; PAR 6.40 min; RAM 6.62 min; IBU 7.71 min and GEM 7.87 min. Concentrations of various analytes were quantified by comparison with signals of pure standard solutions obtained monitoring the signal of the quantifier transitions and verified using the qualifier transitions. Six different levels of the mixture of pure standard solutions: carbamazepine 1 mg/mL in methanol Cerilliant® (Supelco); gemfibrozil

100 mg/mL ⩾ 98.5 % GC (Sigma-Aldrich); ibuprofen 1mg/mL in methanol Cerilliant® (Supelco); metformin HCL (as free base) 1 mg/mL in methanol Cerilliant® (Supelco); paroxetine 10mg ⩾ 98 % HPLC, powder (Sigma-Aldrich); ramipril 100 mg/mL ⩾98 % HPLC (Sigma-Aldrich); venlafaxine hydrochloride 1 mg/mL in methanol Cerilliant® (Supelco) of the analytes of interest between a concentration range between 1.5 to 50 ng/mL were prepared in pure methanol (LC-MS), and each injected 3 times for each concentration level on the LC-MS system; calibration curves exhibited good linearity (R^2 > 0.98) for all the compounds. Due to the lack of appropriate Certified Standard Reference Materials (SRMs), recovery for each compound was estimated on samples of tissues of control mussels (n = 10) spiked with various concentrations of investigated molecules. The method was validated on control mussel samples fortified with pure standard solutions of target compounds at three concentration levels: 10 ng/g, 25 ng/g and 50 ng/g. To evaluate matrix effects mussel samples were prepared spiking known amount of the target analytes during the extraction procedure and the obtained signals were compared with those of the pure standard solution at the same concentrations. The mean recovery percentages obtained at various concentrations of CBZ, GEM, IBU, MET, PAR, RAM, and VEN were in the range of 90 % - 105 % (± 2 %, n = 10). Method detection limits (LOD) and method quantification limits (LOQ) were determined, using spiked matrix samples, as concentrations of the calibration curves with a signal-to-noise ratio of at least 10 and 20, respectively and a percentage variation coefficient (CV %) less than respectively 10 % and 5 % on the basin of at least five replicates (n = 5). The values obtained were the following: CBZ , 0.13 ng/g d.w.; GEM, 260 ng/g d.w.; IBU, 25 ng/g d.w.; MET, 0.46 ng/g d.w.; PAR 0.250 ng/gr d.w.; RAM 0.417 ng/g d.w. and VEN 0.623 ng/g d.w. for LOQs and CBZ, 0.013 ng/g; GEM, 36.75 ng/g; IBU, 2.54 ng/g; MET, 0.06 ng/g, PAR 0.028 ng/gr, RAM 0.033 ng/g and VEN, 0.07 ng/g for LODs respectively. All determinations were carried out after verifying the absence of signals in blank solutions (reagents treated in the same conditions as the analytical standards and samples) and in the working solutions, analysed repeatedly during each analytical session. All the working solutions, which include the blank solutions, the analytical standards, the mobile phases, etc., were prepared fresh during each analytical session, previously testing all the solvents adopted, selected among those of a grade suitable for the technique analytical used (LC-MS).

3.2.5 Biomarkers analysis

Standardized protocols (Bocchetti and Regoli, 2006; Mezzelani et al., 2021) were used to measure biomarkers in tissues. Lysosomal membrane stability (LMS) was evaluated collecting the hemolymph of 8 organisms and incubated on a glass slide with a neutral red (NR) working solution

(2 µl/mL filtered seawater). Hemocytes were microscopically examined every 20 minutes to define the time at which 50% of cells had lost into the cytosol the dye previously taken up by lysosomes.
Granulocyte/hyalinocyte ratio was analyzed by using aliquots of hemolymph dispersed on glass slides that, once dry, were fixed in Becker's fixative (2.5 NaCl). The slides were washed and stained with May Grunwald Giemsa before being mounted with Eukitt. Observations were carried out with a light microscope (1000x) and percentage of granulocytes was evaluated after counting at least 200 cells for each sample. Phagocytosis capacity assay was microscopically evaluated in hemolymph incubated for 2 hours at 15 °C in the dark with Fluorescein - labelled Zymosan A bioparticles (Invitrogen) at a 10:1 target:hemocyte ratio. Phagocytosis was expressed as percentage of cells that internalized at least 3 fluorescent particles. Genotoxicity was evaluated at chromosomal level by the micronucleus test and at molecular level, as single strand breaks (SB), by the Comet assay and Diffusion assay. Micronuclei (MN) frequency was measured in hemocytes, rapidly washed in a saline buffer, fixed in Carnoy's solution (3:1 methanol: acetic acid), dispersed on glass slides and stained with fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) at 100 ng/mL. 2000 cells with preserved cytoplasm were scored for each specimen for the presence of micronuclei. The criteria used to identify a micronucleus were: a round structure, smaller than 1/3 of the main nucleus diameter and on the same optical plan of it while still possessing distinguishable boundaries from it. Comet assay was carried out on mussels hemocytes included in 1 % normal-melting-point agarose on glass slides, followed by treatment in lysing solution, DNA denaturation, electrophoresis and staining with 1 µg/mL of DAPI. 100 randomly selected "nucleoids" per slide, with 2 replicates per sample, were examined under fluorescence microscopy (200x magnification on the Olympus BX-51) and the captured images (Image-Pro-Plus package) were analysed by Tritek CometScoreTM software. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.
DNA diffusion assay is a modified version of comet assay detecting low molecular weight DNA which is lost from agarose matrix during electrophoresis (Singh 2000). Slides were prepared following the comet protocol, but electrophoresis was omitted. Apoptotic cell nuclei have a hazy or undefined outline without any clear boundary due to nucleosomal-sized DNA diffusing into agarose and a diameter 3 times over the mean nuclear diameter. The degree of DNA diffusion pattern is subdivided in 5 classes: class 1, 1 – 5 % diffused DNA; class 2, 20 % diffused DNA around the nucleus; class 3, 50 % diffused DNA; class 4, 1 – 10 % diffused DNA; class 5, apoptotic cell (Cantafora et al. 2014). 100 randomly selected cells per slide were examined under fluorescence microscopy and a visual analysis was performed for evaluate the frequency of cells (%) belonging to the fifth class of damage (200x magnification; Olympus BX-51). Acetylcholinesterase (AChE) activity was spectrophotometrically assayed in mussels' gills (homogenized 1:3 w:v ratio in 0.1 M

Tris-HCl buffer pH 7.2, 0.25 M sucrose and centrifuged at 10,000 xg for 10 min) and haemolymph (centrifuged at 3000 xg for 5 min). Obtained supernatants were spectrophotometrically assayed by the Ellman's reaction at 18 ± 1 °C and λ = 412 nm. For antioxidant enzymes, samples were homogenized (1:5 w:v ratio) in 100 mM K- phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/mL bacitracin, 0.008 TIU/mL aprotinin, 1 g/mL leupeptin, 0.5 g/mL pepstatin and NaCl 2.5%, and were centrifuged at 110,000 xg for 1 hour at 4 °C. Measurements were carried out with a Varian (model Cary 3) spectrophotometer at a constant temperature of 18 °C (Regoli et al., 2004). Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, $\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$) due to the consumption of hydrogen peroxide, H_2O_2 [12 mM H_2O_2 in 100 mM K-phosphate buffer (pH 7.0)]. Glutathione S-tranferases (GST) were determined at 340 nm using 1-chloro2,4dinitrobenzene (CDNB) as substrate. They were assayed in 100 mM K-phosphate buffer (pH 6.5), 1.5 mM CDNB and 1 mM GSH ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of GSSG (λ= 340 nm, $\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$). The final assay conditions were 100 mM K- phosphate buffer (pH 7.0), 1 mM GSSG and 60 μM NADPH. Total glutathione was measured in samples homogenized (1:5 w:v ratio) in 5 % sulfosalicylic acid with 4 mM EDTA, kept for 45 minutes in ice and then centrifuged at 37,000 xg for 15 minutes. An enzymatic method was used to determine the rate of formation of TNB which is proportional (up to 2 μM) to the glutathione concentration. Glutathione peroxidases (GPx) were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to reconvert the formed GSSG into its reduced form (GSH). The decrease of absorbance was monitored at 340 nm ($\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$) in 100 mM K-phosphate buffer (pH 7.5), 1mM EDTA, 1 mM dithiothreitol, 1 mM NaN_3 (for hydrogen peroxide assay), 2 mM GSH, 1U GR, 0.24 mM NADPH and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates, respectively, for the Se-dependent and for the Se-dependent and Se-independent forms.

The Total Oxyradical Scavenging Capacity (TOSC) was measured in mussels' digestive glands homogenized as previously reported for enzymatic antioxidants, without PMSF in the homogenization buffer. The artificially generated radicals (ROO•, HO•) were obtained from the thermal homolysis of 20 mM 2'-azo-bis-(2- methylpropionamide)-dihydrochloride (ABAP) and the iron-EDTA (1.8 mM Fe^{3+}, 3.6 mM EDTA) plus 180 mM ascorbate, respectively. The absorption of artificially generated oxyradicals by cellular antioxidants was measured by the quantification of inhibited oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas. Determination of the ethylene formation was made by gas chromatographic analyses and TOSC values were quantified from the equation: $TOSC = 100 - (\int SA / \int CA \times 100)$, where $\int SA$ and $\int CA$ are the integrated areas calculated under the kinetic curve produced during the reaction course for respective sample

(SA) and control (CA) reactions. For all the samples, a specific TOSC was calculated by dividing the experimental TOSC values by the relative protein concentration, determined by the Lowry method with Bovine Serum Albumin (BSA) as standard (Regoli and Winston, 1999).

Lipofuscin and neutral lipids accumulation were evaluated through histological analysis on duplicate cryostat sections (8 μm thick) of digestive glands. Slides were fixed in Beker's fixative (+ 2.5 % NaCl) and stained by Schmrol reaction before mounting with Eukitt and by Oil red O (ORO) before mounting in glycerol gelatin for lipofuscin and neutral lipids content respectively. For both analysis, four measurements were made on digestive tubules of each section. Staining intensity was quantified with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of the digestive tubules.

Malondialdehyde (MDA) was quantified in digestive glands homogenized (1:3 w/v ratio) in 20 mM Tris–HCl (pH 7.4), centrifuged at 3,000 xg for 20 min. A conjugation reaction was performed in 1 mL reaction mixture (45 °C, 40 min), containing 10.3 mM 1- metyl-2-phenylindole (dissolved in acetonitrile/methanol, 3:1), 32% HCl, 100 μL water and 100 μL of sample or standard [standard range 0–6 μM 1,1,3,3-tetramethoxypropane, in 20 mM Tris-HCl (pH 7.4)]. Samples were cooled on ice, centrifuged at 15,000 xg for 10 min and spectrophotometrically analysed at 586 nm. MDA concentrations were determined as a function of the 1,1,3,3-tetramethoxypropane standard curve and expressed as nmol/g tissue. Acyl-CoA oxidase's (ACOX) activity was evaluated in digestive glands homogenized in 1 mM sodium bicarbonate buffer (pH 7.6), containing 1 mM EDTA, 0.1 % ethanol and 0.01 % Triton X-100 and centrifuged at 500 xg for 15 minutes at 4 °C. H_2O_2 production was measured in a coupled assay (Bocchetti and Regoli, 2006) by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF- DA, 40 μM sodium azide, 0.01 % Triton X-100, 1.2 U/mL HRP in a final volume of 1 mL. After a pre-incubation at 25 °C for 5 minutes in the dark with an appropriate amount of sample, reactions were started adding the substrate Palmitoyl-CoA at final concentration of 30 μM and 100 μM for Acyl-CoA oxidase (AOX); readings were carried out at λ = 502 nm against a blank without the substrate.

3.2.6 Statistical analysis and Weight Of Evidence (WOE) model

Statistical analysis for bioaccumulation and biomarkers data were performed using RStudio (version 1.2.5033). Data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test), with appropriate mathematical transformation if necessary.

For experiment 1 and 2 the analysis of variance was applied to test differences between treatments and exposure days (level of significance at $p < 0.001$), the Student Newman-Keuls test (SNK) was used for post-hoc comparison between means of values ($n = 5$).

For experiment 3, a two-way Analysis of Variance (ANOVA) was conducted to evaluate the differences between the treatment groups. This analysis aimed to determine the main effects of the treatment conditions (CTRL, OA, HW, and CC) and the presence of APIs, as well as their interaction effects on the response variables. The ANOVA provided insight into both the individual effects of the treatments and the exposure to APIs, as well as any interaction effects between these factors. Significant results from the ANOVA were further examined using *post-hoc* pairwise comparisons with appropriate adjustments (Tukey's HSD) to control for multiple testing. Statistical significance was set at ($p < 0.05$).

For experiments 1 and 2, for each experimental condition, the results on bioaccumulation and biomarkers analysis were further elaborated through a quantitative Weight Of Evidence model (WOE, Sediqualsoft) that provides synthetic hazard indices for each typology of data (or Line of Evidence, LOE) before the final integration (Regoli et al., 2019). Independent elaborations procedures were applied to LOE on bioaccumulation and biomarkers. For bioavailability hazard the model considered the fold increase and statistical significance of pharmaceuticals accumulation in exposed organisms (Piva et al., 2011); the elaboration of cellular responses is based on a specifically developed algorithm which, for every analyzed biomarker, evaluate the magnitude of observed variation in comparison to a specific threshold, the toxicological relevance (weight) of biological endpoint and the statistical significance of the difference in respect to controls. After normalization of indices to a common scale, individual hazard indices were integrated through a classical weight of evidence approach, and level of risk assigned to 1 of 5 classes, from "ABSENT" to "SEVERE". Whole calculations, detailed flow-charts, rationale for weights, thresholds and expert judgements have been previously described in detail (Regoli et al., 2019).

3.3 Results and discussions

3.3.1 Experiment 1. Ibuprofen, paroxetine and their mixture

Ibuprofen and Paroxetine are among the most widely consumed Non-Steroidal Anti-inflammatory Drugs (NSAIDs) and Selective Serotonin Re-uptake Inhibitors (SSRIs) in many European countries, including Italy (OsMed Report, 2022). Both drugs have being simultaneously detected in various wild marine organisms (Mezzelani et al., 2020; Blasco et al., 2023) and since they directly and indirectly target the oxidative metabolism (*e.g.* cytochrome P450 biotransformation pathway), this study aimed to investigate the potential additive mechanisms of interaction between these pharmaceuticals in the non-target species *Mytilus galloprovincialis*.

Table 3.1 presents the results of pharmaceutical accumulation in the whole soft tissues of mussels. After 30 days of exposure, a significant increase in the tissue concentrations of IBU and

PAR was observed in organisms treated with the individual drugs compared to the control group, confirming the species' ability to accumulate both tested compounds (Mezzelani et al., 2020; Fernandes et al., 2021; Blasco et al., 2023).A distinct bioaccumulation pattern was noted between organisms treated with the drugs individually and those exposed to the mixture. Notably, IBU accumulated at high concentrations in mussels exposed to the single compound, whereas levels were below the LOD (< 2.54 ng/g d.w.) in co-exposed organisms. In contrast, PAR exhibited similar concentrations in both mussels exposed to the mixture and those treated with PAR alone. Thus, co-exposure appeared to reduce IBU accumulation while leaving PAR accumulation unaffected. Although the mechanisms governing the uptake of pharmaceutical mixtures in mussels remain poorly understood, these results suggest a potential interaction between the two compounds, with a competitive mechanism favoring PAR over IBU when both are present. This pharmacodynamic interaction might be explained by the competition between IBU and PAR for the same cellular transporters, with PAR exhibiting a higher affinity, leading to increased uptake and inhibiting IBU bioaccumulation (Mezzelani and Regoli, 2022). After the depuration phase, bioaccumulation levels of both IBU and PAR returned below the LOD in all treatments, likely due to the mussels' ability to metabolize and excrete these compounds through their biotransformation pathways (Mezzelani and Regoli, 2022).

To achieve a deeper understanding of the potential interactive mechanisms between IBU and PAR, the chemical analysis were supported by a broad panel of responses measured at molecular, biochemical and cellular levels, reflecting the primary metabolic pathways in mussels. Notably, lysosomal membrane stability (LMS) was identified as a highly sensitive biomarker of IBU exposure (Figure 3.2A), corroborating previous findings that the immune system is a primary target of anti-inflammatory drugs (Parolini et al., 2011; Matozzo et al., 2012; Aguirre-Martínez et al., 2013; Mezzelani et al., 2016; 2018). Interestingly, no significant changes were observed in the granulocyte/hyalinocyte ratio (Figure 3.2B) or in phagocytosis capacity (Figure 3.2C), contrasting with the findings of Mezzelani et al. (2018), where a 30-day exposure to 2.5 µg/L of IBU resulted in a 70% and 50% reduction in these parameters, respectively. This discrepancy suggests that the lower IBU dose used in this study (1 µg/L) may not have been sufficient to elicit a detectable response on such parameters. Given its properties as a weakly basic drug with an amino group, PAR can be effectively sequestered by acidic subcellular compartments, such as mammalian lysosomes. Within these organelles, PAR becomes protonated and is unable to return to the cytosol, leading to its accumulation and subsequent loss of lysosomal function (Daniel and Wójcikowski, 1997). This sequestration mechanism is further facilitated by the single phospholipid layer of the lysosomal

membrane and the cation-π interactions between the aromatic rings of PAR and the ammonium group of glycerophospholipids, which are key components of eukaryotic membranes (Míguez et al., 2014). According to the biological read-across theory, which stated that a drug can induce similar effects in non-target organisms by acting via humans-conserved MOAs (Brooks et al., 2003a; Huggett et al., 2003; Gunnarsson et al., 2008), it is plausible to hypothesize that lysosomal sequestration of PAR also occurs in marine invertebrates. Supporting this hypothesis, the results of this work demonstrated a significant LMS reduction in mussel hemocytes following exposure to PAR (Figure 3.2A). The capacity of antidepressants to markedly alter lysosomal function in *Mytilus galloprovincialis* was also highlighted by Rafiq et al. (2023), where exposure to environmentally relevant concentrations (0.5 - 5 - 10 ng/L) of selective serotonin reuptake inhibitors (SSRIs) led to a reduction in LMS by up to 70 %. The functional efficiency of hemocyte lysosomes is crucial in invertebrates, as they are involved in the digestion of aged or damaged organelles and in the autophagy process, which is essential for cellular maintenance under environmental stress conditions (Moore et al., 2006). A loss of lysosomal function can, therefore, result in the inability of these organelles to degrade phospholipids and neutral lipids (Breiden and Sandhoff, 2020). Moreover, it can lead to the release of lysosomal proteolytic enzymes, such as phospholipases, which may increase the production of superoxide radicals and hydrogen peroxide, thereby inducing oxidative stress (Terman et al., 2006). Supporting this, the outcomes revealed an increase in neutral lipid content across all tested conditions, with a particularly pronounced effect observed in the IBU treatment group (Figure 3.3B). The notably stronger impact of IBU compared to other treatments may also be attributed to the drug's high affinity for peroxisome proliferator-activated receptors (PPARs), which can lead to the modulation of lipid metabolism (Chinetti et al., 2000).

The ability of pharmaceuticals in promoting reactive oxygen species (ROS) generation and related pro-oxidant mechanisms in marine organisms have been extensively documented in the literature (Ghosh et al., 2015; Mezzelani and Regoli, 2022). At the cellular functional level, such effects can lead to significant modulation of antioxidant enzymes and the accumulation of peroxidation products, such as lipofuscin (Viarengo et al., 2007). In fact, an increased lipofuscin content was observed in the digestive glands of mussels exposed to all tested conditions (Figure 3.3A), confirming the influence of these molecules on oxidative metabolism.

To assess potential changes in the oxidative status of mussels, data on the accumulation of lipid peroxidation products, such as lipofuscin content and malondialdehyde (Figure 3.3C), were integrated with measurements of individual antioxidant enzyme activities and the overall capacity to neutralize specific oxyradicals (Figure 3.4). Outcomes revealed an induction in catalase (CAT) and glutathione peroxidase (GP_x CHP) activity in all exposed organisms (Figure 3.4A, C). Both enzymes

play a crucial role in protecting the cell from oxidative damage: CAT counteracts the production of ROS hydrogen peroxide (H_2O_2), while GP_x CHP, as a peroxidase, reduces organic hydroperoxides. Some studies have reported the activation of antioxidant responses following short-term exposure to IBU (Gonzalez-Rey and Bebianno, 2011; 2012; Parolini et al., 2011; Trombini et al., 2019), while no changes were detected after mid- and long-term exposures (Aguirre-Martínez et al., 2015; Mezzelani et al., 2016). This suggests that over extended periods, the mussel *Mytilus galloprovincialis* may activate alternative defense mechanisms to counteract the pro-oxidant effects of IBU. Further analysis revealed an induction of glutathione S-transferase (GST) activity in all tested conditions (Figure 3.4B), highlighting the importance of phase II conjugation reactions in the biotransformation and excretion of pharmaceuticals in marine mussels (Mezzelani et al., 2021; 2023; Nardi et al., 2022). Although GST is the most extensively studied antioxidant enzyme in bivalves, other phase II enzymes may also play a crucial role in the biotransformation of the tested pharmaceuticals. For instance, in mammals, IBU undergoes phase I biotransformation via cytochrome P450 (CYP) enzymes, followed by phase II metabolism mediated by UDP-glucuronosyltransferases (UGT1A1, UGT2B7, UGT1A9, and UGT2B4) (Bindu et al., 2020; Mezzelani and Regoli, 2022). This suggests a need to investigate the activity of these enzymes in mussels to determine whether they are also involved in IBU metabolism. In terms of genotoxic damage, the study revealed a significant increase in DNA strand breaks and micronuclei (MN) frequency following IBU exposure, with these effects persisting even during the depuration phase (Figure 3.5A, B). This persistence suggests a lasting impact of IBU, although the observed DNA damage levels remained within the physiological range of variability (30 – 60 % and 26 – 50 %) reported in previous studies (Pisanelli et al., 2009; Bocchetti et al., 2008). The increase in MN frequency observed during both the IBU exposure and depuration phases is significant (Figure 3.7B), corroborating findings from previous studies on this drug (Mezzelani et al., 2016a, 2018a; 2018b; Bebianno and Gonzalez-Rey, 2015; Milan et al., 2013). Given that this drug has been previously documented as non-mutagenic (Philipose et al., 1997), these results support the hypothesis that IBU-induced genotoxic effects are not a result of direct DNA damage but may be linked to altered cellular turnover in hemocytes (Chang et al., 2009; Mezzelani et al., 2016a; 2016b). As regards PAR, the results showed an increase in DNA strand breaks after the depuration phase. However, these values are still within the range of seasonal variability (Pisanelli et al., 2009; Bocchetti et al., 2008), suggesting that the drug does not seem to exhibit genotoxic effects. Similar observations were reported by Lacaze et al. (2015), who found increased DNA strand breaks in *Mytilus edulis* following exposure to 1.5 µg/L of PAR.

Neurotoxic effects of the tested drugs were evaluated by measuring the activity of acetylcholinesterase (AChE), an enzyme which is responsible for terminating the nerve impulse

transmission through the hydrolysis of acetylcholine, a neurotransmitter. Given its essential role in the neuromuscular system, AChE activity serves as a valuable biomarker in aquatic organisms for detecting exposure to neurotoxic substances that may inhibit its function, potentially leading to the death of the organism (Lushchak, 2011). Figure 3.6 shows that the AChE activity was unaffected by any of the tested treatments. These results are consistent with the findings of Mezzelani et al. (2016), which showed that a 14-day exposure to 25 µg/L of IBU did not affect AChE activity, suggesting that this drug may not interact with the neurotransmission system of *Mytilus galloprovincialis*. Although there is limited information in the literature regarding the effects of PAR on AChE activity, substantial evidence indicates that environmental concentrations of fluoxetine (an SSRI) can inhibit AChE activity in mussels (Franzellitti et al., 2014) and down-regulate AChE mRNA expression in fish (Park et al., 2012). This suggests that, despite belonging to the same therapeutic class, paroxetine and fluoxetine may have different mechanisms of action, influencing the neurotransmission systems of non-target species in different way. Moreover, PAR has demonstrated a moderate to high affinity for mammalian muscarinic acetylcholine receptors (mAChRs) (Thomas et al., 1987; Owens et al., 1997), which have recently been identified in the oyster *Crassostrea gigas*. These receptors are integral to the cholinergic neuroendocrine-immune system, where they regulate tumor necrosis factor (TNF) expression and apoptotic processes (Liu et al., 2016). These observations suggest that PAR may indirectly influence the immune system in invertebrates through interactions with mAChR homologs. However, further molecular investigations are required to elucidate this potential interaction in more detail.

The hypothesis of an interaction mechanism between IBU and PAR which would lead to an additive effect in mixture-exposed organisms was denied by the obtained results on the responses measured at the molecular, biochemical and biological levels. The biological pathways altered in mussels exposed to the mixture were largely consistent with those observed in mussels exposed to PAR alone, which aligns with chemical analyses indicating a IBU bioaccumulation below of LOD in co-exposed organisms. Consequently, in MIX-exposed organisms the observed loss of lysosomal function, accumulation of lipofuscin, neutral lipids, and the variations in antioxidant responses, seems to reflect effects measured in PAR-exposed mussels, probably due to the lack of accumulation of IBU when in mixture. Nevertheless, further molecular investigations are required to validate these findings.

For an easier qualitative and quantitative comparison of pharmaceuticals accumulation and biomarkers responses in mussels exposed to different treatments, all obtained results were summarized in specific hazard indices. The Sediqualsoft model applies weighted criteria that consider the toxicological relevance (weight) of measured end points and both the number and magnitude of variations normalized to specific thresholds (Regoli et al., 2019).

The WOE approach confirmed the lack of significant interaction between IBU and PAR, with a risk level summarized as "MODERATE" for both individual pharmaceuticals and the mixture at day 30 (Figure 3.7A). After the depuration phase, the WOE risk level decreased to "SLIGHT" for all treatments, indicating that this filter-feeding species is capable of recovering from the harmful effects of pharmaceuticals within a relatively short period. However, it is noteworthy that the cellular effects of all treatments persisted longer, with a "MODERATE" hazard level, compared to the reduction in bioaccumulation hazard, which decreased from "MAJOR" to "ABSENT" (Figure 3.7B), thus suggesting that the depuration of the chemical burden is faster compared to the recovery of biological effects.

Table 3.1 Concentration at different sampling times of ibuprofen and paroxetine in the whole tissues of *M. galloprovincialis* exposed to experimental conditions. Data given in ng/g dry weight, d.w. (mean values ± standard deviations, n = 3). IBU Ibuprofen; PAR Paroxetine; IBU + PAR Ibuprofen + Paroxetine.

	IBU (ng/g d.w.)		PAR (ng/g d.w.)	
	Day 30	Day 44	Day 30	Day 44
CTRL	< 8.00	< 8.00	< 0.95	< 0.95
IBU	79.6 ± 24.33	< 8.00	< 0.95	< 0.95
PAR	< 8.00	< 8.00	12.82 ± 4.39	< 0.95
IBU + PAR	< 8.00	< 8.00	18.54 ± 10.75	< 0.95

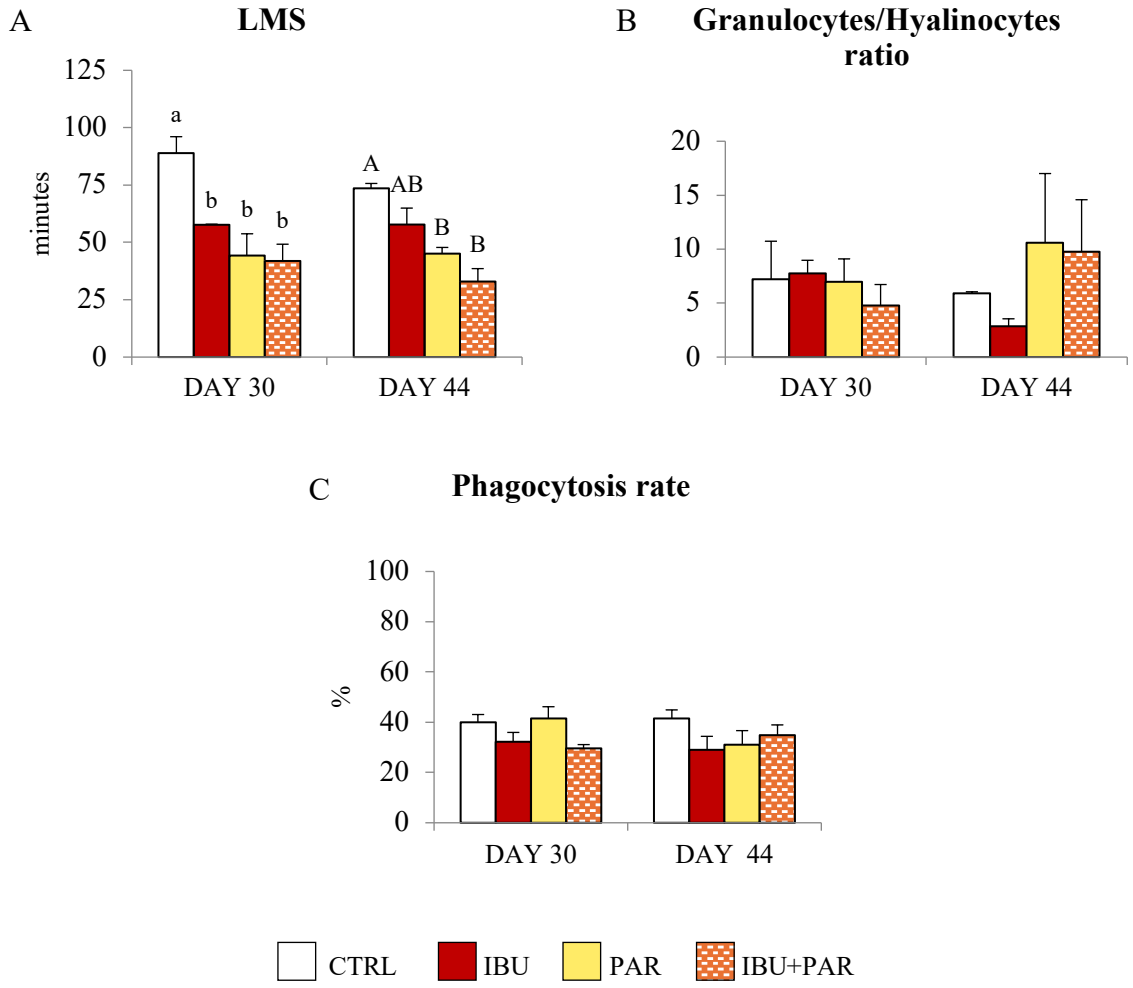


Figure 3.2 Immunological responses. A) Lysosomal membrane stability, LMS; B) Granulocytes/hyalinocytes ratio; C) Phagocytosis rate in hemocytes of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 4). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL Control; IBU Ibuprofen; PAR Paroxetine; IBU + PAR Ibuprofen + Paroxetine.

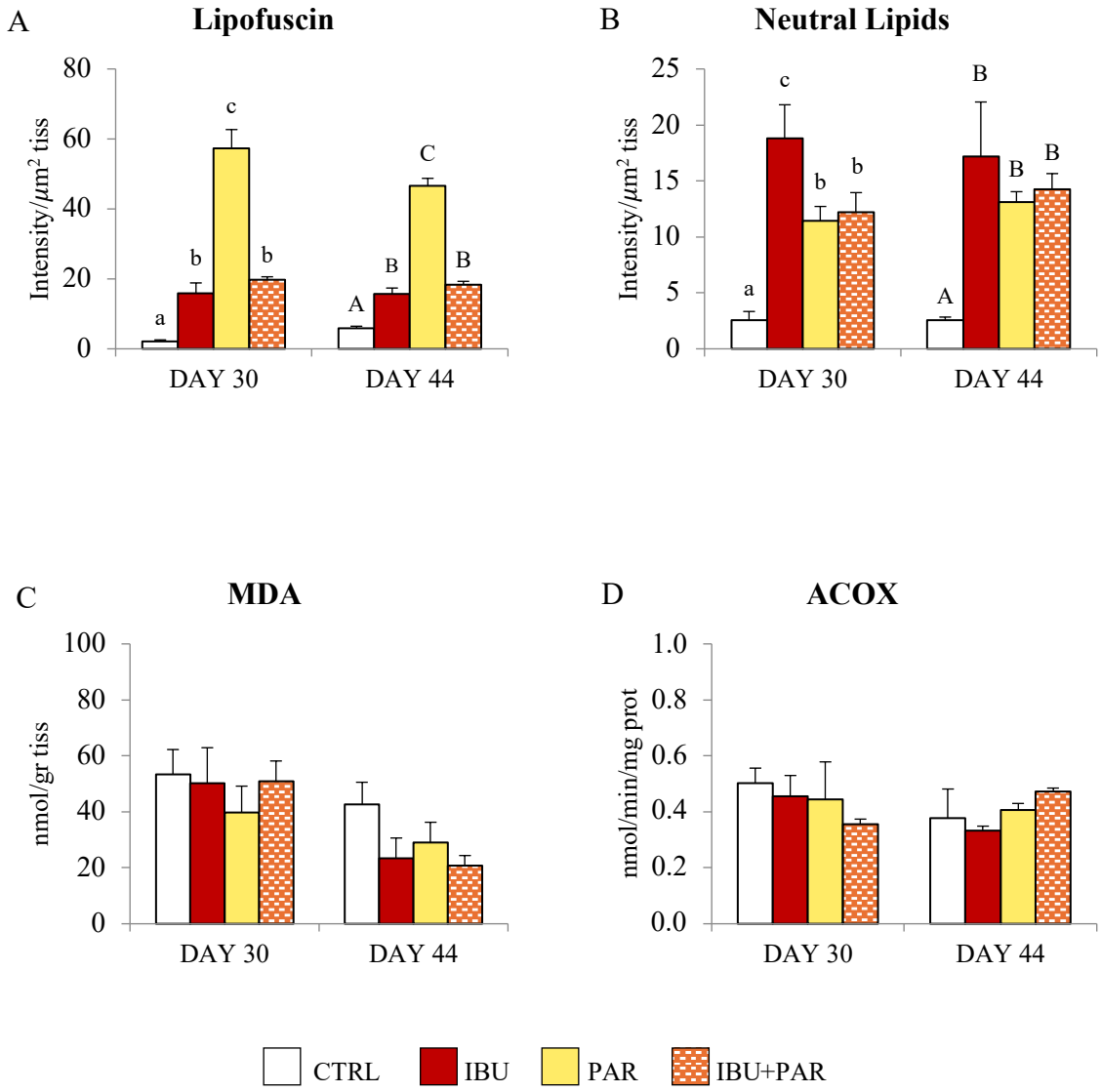
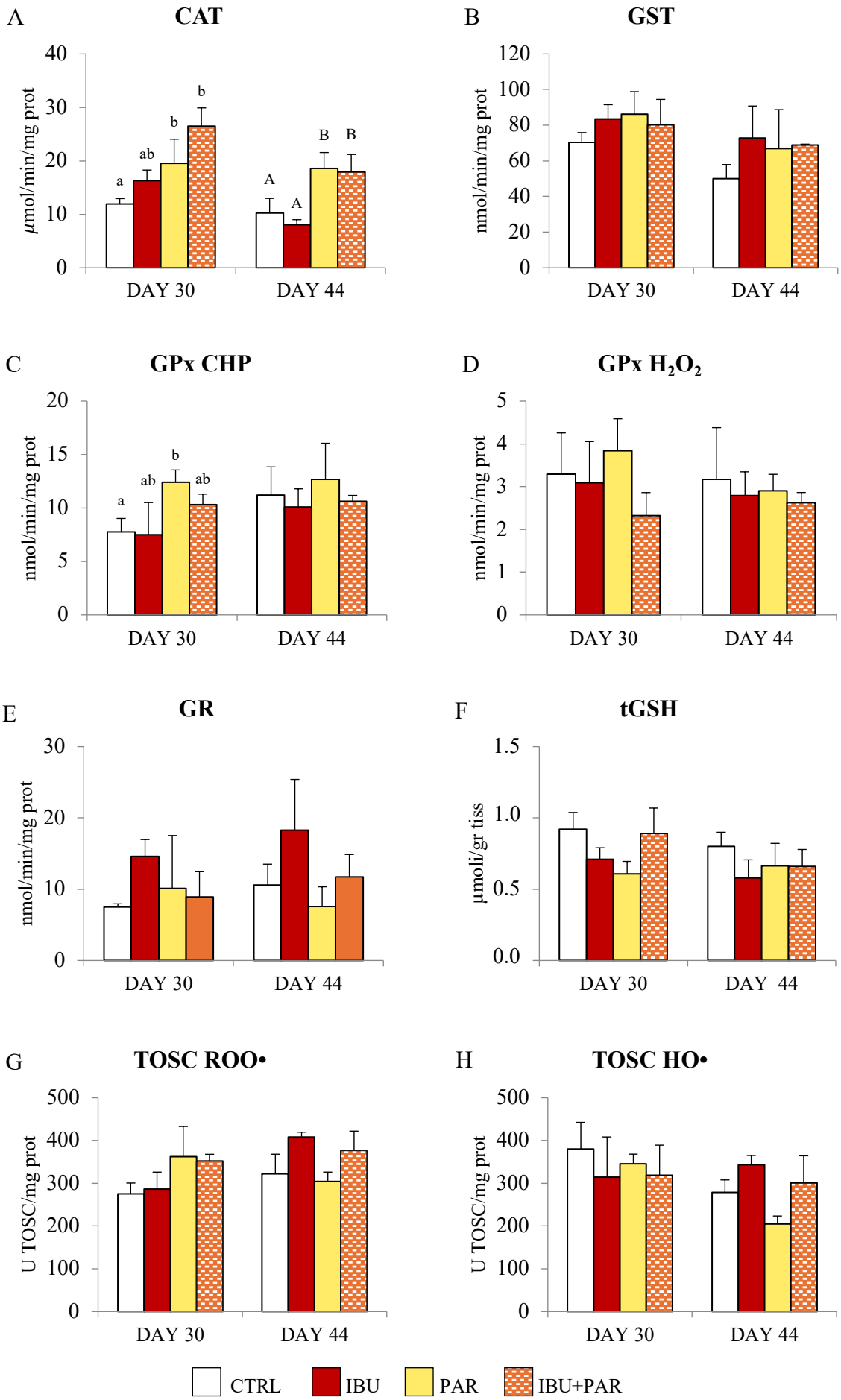


Figure 3.3 A) Lipofuscin and B) neutral lipids content; C) Levels of malondialdehyde, MDA; D) Acyl-CoA oxidase (ACOX) activity in mussels exposed to tested experimental conditions. Data are given as mean values ± Standard Error of the Mean (n = 4). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL Control; IBU Ibuprofen; PAR Paroxetine; IBU + PAR Ibuprofen + Paroxetine.


A
CAT
µmol/min/mg prot
40
30
20
10
0
a
ab
b
b
A
A
B
B
DAY 30
DAY 44
B
GST
nmol/min/mg prot
120
100
80
60
40
20
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DAY 30
DAY 44
C
GPx CHP
nmol/min/mg prot
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H
TOSC HO•
U TOSC/mg prot
500
400
300
200
100
0
DAY 30
DAY 44
CTRL
IBU
PAR
IBU+PAR

Figure 3.4 Antioxidant system responses in digestive glands of mussels exposed to different treatments. A) Catalase, CAT; B) Glutathione S-transferase, GST; C) Glutathione peroxidases CHP: sum of Se-dependent and Se-independent glutathione peroxidases; D) Glutathione peroxidases H_2O_2: Se-dependent glutathione peroxidases; E); Glutathione reductase, GR; F) Total glutathione, tGSH; G) TOSC ROO•: total oxyradical scavenging capacity toward peroxyl radical; H) TOSC HO•: total oxyradical scavenging capacity toward hydroxyl radical. Data are given as mean values ± Standard Error of the Mean (n = 3). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL Control; IBU Ibuprofen; PAR Paroxetine; IBU + PAR Ibuprofen + Paroxetine.

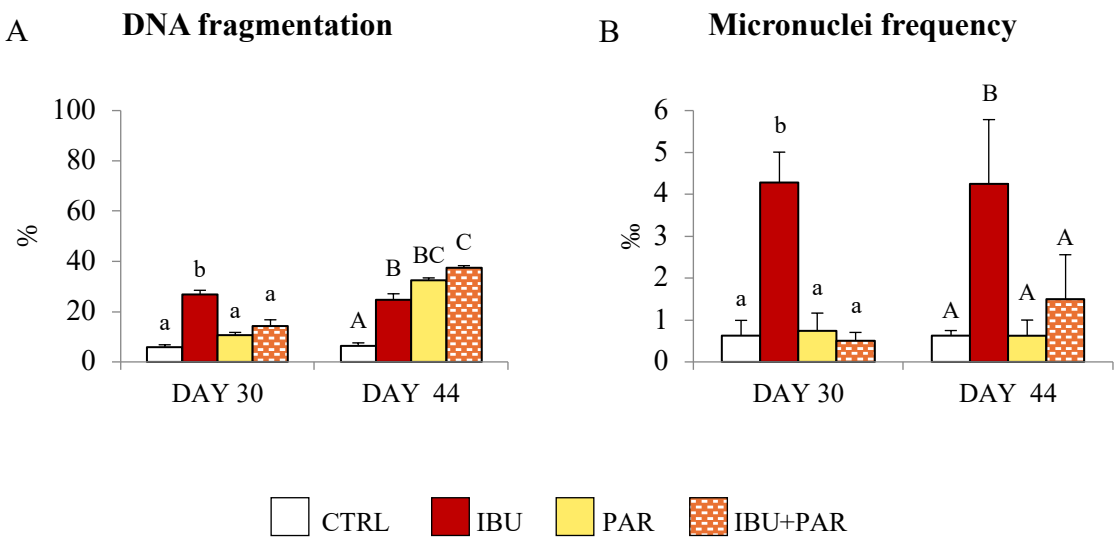


Figure 3.5 Genotoxic responses. A) Percentage of DNA in the comet tail; B) Micronuclei frequency (‰) in hemocytes of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 4). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL Control; IBU Ibuprofen; PAR Paroxetine; IBU + PAR Ibuprofen + Paroxetine.

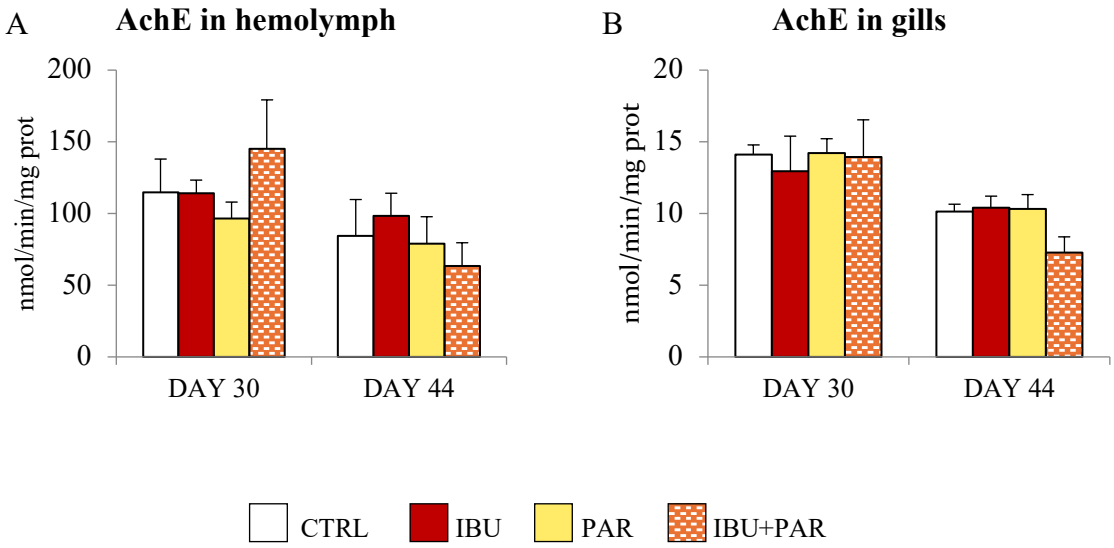


Figure 3.6 Neurotoxic damages. A) Acetylcholinesterase activity (AChE) in hemolymph and B) in gills of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 5). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL Control; IBU Ibuprofen; PAR Paroxetine; IBU + PAR Ibuprofen + Paroxetine.

A

TREATMENT	LOE 3 BIOAVAILABILITY	LOE 4 BIOMARKER	WOE INTEGRATION	LEVEL OF RISK
IBU	Major HQ: 7.96	Moderate HQ: 212.61	MODERATE	
PAR	Major HQ: 12.82	Moderate HQ: 163.68	MODERATE	
IBU + PAR	Major HQ: 7.3	Moderate HQ: 176.92	MODERATE	

B

TREATMENT	LOE 3 BIOAVAILABILITY	LOE 4 BIOMARKER	WOE INTEGRATION	LEVEL OF RISK
IBU	Absent HQ: 0	Moderate HQ: 203.21	SLIGHT	
PAR	Absent HQ: 0	Moderate HQ: 184.62	SLIGHT	
IBU + PAR	Absent HQ: 0	Moderate HQ: 192.31	SLIGHT	

Figure 3.7 Weighted elaboration of whole dataset for each treatment at A) exposure phase (day 30) and B) depuration phase (day 44).

3.3.2 Experiment 2. Venlafaxine, gemfibrozil, ramipril, metformin and their mixture

Despite significant research efforts over the past decades to understand the effects of individual pharmaceuticals, there is still a notable gap in knowledge regarding the toxicity of complex mixtures, especially those containing more than four pharmaceutical compounds.

In this context, the present study aimed to evaluate the potential interactive effects of four different therapeutic classes on the marine mussel *Mytilus galloprovincialis*. Selected compounds, the antidepressant venlafaxine, the lipid regulator gemfibrozil, the antihypertensive ramipril, and the antidiabetic metformin, are representative of the most frequently detected pharmaceuticals in marine ecosystems (Mezzelani et al., 2020; Fernandes et al., 2021; Blasco et al., 2023).

Table 3.2 shows the results on pharmaceuticals accumulation in the whole soft mussel tissues. The results highlighted specific bioaccumulation patterns for the different tested pharmaceuticals, each representing a distinct therapeutic class. Notably, the drugs exhibited similar behavior whether dosed individually or in a mixture. Outcomes highlighted that mussels can significantly bioaccumulate the antidepressant venlafaxine (VEN). After 30 days of exposure, higher levels of VEN compared to CTRL were detected in both mussels exposed to VEN alone and those exposed to a mixture, with similar concentrations observed in both scenarios. After the depuration phase, VEN levels decreased below the limit of detection in both treatments, thus suggesting a possible drug metabolism and excretion (Mezzelani and Regoli, 2022).

The presence of gemfibrozil (GEM) and metformin (MET) in control mussels indicates pre-existing environmental contamination. Even after 14 days in pharmaceutical-free water, mussels showed similar levels of GEM and MET, indicating an inability to effectively eliminate these compounds. This hypothesis is supported by baseline levels of GEM and MET detected in *Mytilus galloprovincialis* sampled from the Adriatic Sea (data not shown), with concentrations comparable to those in this study. GEM was approximately 2 μg/g d.w., while MET was around 4 ng/g d.w. The similarity in GEM and MET levels between exposed and control mussels suggests the low bioaccumulation potential of such drugs and/or a potential saturation point, beyond which further exposure does not increase tissues levels. Further research is needed to confirm this and to understand the mechanisms behind the persistence of these drugs in non-target marine organisms. At all exposure times, ramipril (RAM) levels were below the limit of detection (LOD) in both individually exposed and mixture-exposed mussels. Similar results were observed in a survey of wild mussels from the Adriatic Sea (data not shown), corroborating the limited capacity of *Mytilus galloprovincialis* to accumulate this drug.

Responses measured at the molecular, biochemical, and cellular levels enabled the identification of the primary metabolic pathways involved in the metabolism of individual drugs and their mixture.

For instance, immunological parameters confirmed that the immune system is one of the main targets of environmental pharmaceuticals (Mezzelani et al., 2018; Cortez et al., 2018; Mezzelani and Regoli, 2022; De Marco et al., 2022; Afsa et al., 2022; Franzellitti et al., 2011), particularly with respect to the reduction of lysosomal membrane stability (LMS) (Figure 3.8A).

Average lower levels of membrane stability were observed in mussels exposed to MET, while a significant decrease occurred in organisms treated with RAM and MIX. Notably, these effects persisted even after the depuration period, offering the first insights into the long-lasting impacts of the tested compounds on the immune system, particularly in response to RAM and the mixture. Although scientific knowledge on the effects of RAM is limited, there are case studies where antihypertensives, at similar exposure concentrations, have been shown to decrease LMS, suggesting a potential specific action of this class of pharmaceuticals. For instance, exposure to losartan (3000 ng/L) for 96 hours resulted in a decrease in LMS in the mussel *Perna perna* (Cortez et al., 2018). Additionally, the administration of increasing concentrations (0.3, 3, 30, 300, 30,000 ng/L) of the antihypertensive propranolol to *Mytilus galloprovincialis* led to a reduction in LMS (Franzellitti et al., 2011), consistent with the findings in this study. Notably, the LMS value observed in the mixture is lower than those in the individual treatments, indicating potential interactions among the tested pharmaceuticals. Given the greater effect of RAM compared to the other drugs, it is plausible to hypothesize that RAM plays a significant role in determining the overall impact of the mixture in this parameter. In the study by Minguez et al. (2014), lysosomal membrane damage caused by antidepressants was documented in abalone hemocytes and correlated with the hydrophobicity of the compounds. Molecular modeling suggested that the toxicity is significantly influenced by the distance between the ammonium groups of phosphatidylcholines (a major component of cell membranes) and the aromatic rings of the antidepressants, as well as by the angle between the aromatic rings in different drugs. This model explains the greater toxicity observed with paroxetine and the lower effect of VEN. These findings could explain the results of the previous experimental plan (Experiment 1. Ibuprofen, paroxetine and their mixture), where the SSRI paroxetine led to a significant decrease in LMS, and the current study, in which VEN did not exhibit any effect on this parameter.

Effects on the immune system were further evidenced by the granulocyte/hyalinocyte (G/I) ratio and phagocytosis capacity (Figure 3.8B, C). The G/I ratio reflects the relative abundance of the two primary immune cell subpopulations in mussels, with granulocytes, characterized by their higher phagocytic capacity, being the predominant population (Gorbi et al., 2012). Phagocytosis capacity measures variations in the ability of granulocytes to internalize external particles. Although not statistically significant, after 30 days of exposure, phagocytosis capacity decreased in mussels exposed to VEN and MIX. Notably, this decline was accompanied by a reduction in the ratio between

the two main hemolymph subpopulations in VEN-exposed organisms, but not in those treated with MIX. It is plausible to hypothesize that in VEN-exposed mussels, the lower phagocytosis rate may primarily be due to a reduction in granulocyte numbers rather than a decreased efficiency of granulocytes in phagocytosis. Conversely, in MIX-exposed organisms, where no significant effects on the ratio between the two hemocyte subpopulations were observed, the inhibition of phagocytosis may reflect a reduced efficiency of granulocytes in phagocytizing particles. Different effects were observed at higher VEN concentrations, where doses ranging from 0.15 mg/L to 75 mg/L decreased phagocytosis capacity in *M. galloprovincialis* (Lacaze et al., 2015), suggesting a concentration-dependent impact on immune function.

None of the tested treatments resulted in an increase in micronuclei (MN) frequency in the hemolymph of mussels (Figure 3.8D), indicating the lack of genotoxity of the selected pharmaceuticals in this model organism. Although there is no information in the literature regarding the genotoxicity of the other drugs, the study of Vijitkul et al. (2022) demonstrated that exposure to 1 mg/L of VEN for 96 hours resulted in an increased frequency of micronuclei in Nile tilapia (*Oreochromis niloticus*). These findings suggest potential genotoxic properties of this pharmaceutical in a different model species and at higher tested doses.

One of the primary responses to stress induced by xenobiotics is an imbalance between antioxidant defenses and pro-oxidant forces, leading to an increase in the generation of reactive oxygen species (ROS) (Regoli and Giuliani, 2014; Gorbi et al., 2013). To better elucidate the oxidative status of the mussels, this study investigated variations in individual antioxidant enzyme activities and assessed the total oxyradical scavenging capacity (TOSC) (Figure 3.9).

Results on the antioxidant system revealed a significant modulation of this pathway, identifying it as a primary target of all tested drugs (Strancova et al., 2017; Barreto et al., 2018; Cortez et al., 2018; Lee et al., 2019; Mezzelani et al., 2023). Catalase (CAT) is an enzyme that plays a critical role in decomposing hydrogen peroxide (H_2O_2) into water and oxygen, thereby reducing the potential for oxidative damage and maintaining cellular redox balance (Lores Arnaiz et al., 1995). After 30 days of exposure, a significant induction of CAT activity was measured in GEM exposed organisms, and higher average activity in the MIX treatment (Figure 3.9A). These findings align with those obtained vertebrates model species, which demonstrated that exposure of *Sparus aurata* to GEM concentrations of 1.5 µg/L and 15,000 µg/L resulted in a 50 % to 90 % increase in CAT activity in the gills, and up to 150 % in the liver, respectively (Barreto et al., 2018). These results can be explained by the pharmacodynamics of GEM, which acts as a Peroxisome Proliferator Agent (PPA). GEM interacts with the peroxisome proliferator-activated receptor α (PPARα), leading to the activation of genes involved in peroxisomal fatty acid β-oxidation and subsequent peroxisome proliferation

(Barreto et al., 2018). This process increases hydrogen peroxide (H_2O_2) levels, prompting an upregulation of CAT activity to counteract oxidative stress and potential damage.

Figure 3.9B highlights a significant induction of glutathione-S transferase (GST) activity in all treatments at day 30. GST is a phase II enzyme involved in the biotransformation process, which is a key process in the metabolism of exogenous compounds. Biotransformation comprises two phases: Phase I involves enzymes from the cytochrome P450 family, which are monooxygenases that enhance the solubility of compounds to facilitate their excretion. It is well-established in the literature that pharmaceuticals are metabolized by cytochrome P450 enzymes during phase I (Nichols and Sanders-Bush, 2003; Zurita et al., 2007; Mezzelani and Regoli, 2022; Godoy et al., 2018). However, many metabolites produced during phase I are not sufficiently soluble for excretion, necessitating their progression to phase II. In this phase, enzymes like GST play a pivotal role in excretion mechanisms by conjugating glutathione (GSH) to potentially toxic compounds, thereby increasing their water solubility and facilitating their elimination from cells. Consistent with this, the observed induction of GST activity was accompanied by a decrease in total GSH content across all treatments (Figure 3.9D), suggesting that phase II conjugation reactions are a key process in the metabolism of the tested pharmaceuticals in *M. galloprovincialis*. Our findings are further corroborated by Cortez et al. (2018), who reported that exposure to 3000 ng/L of the antihypertensive losartan in the mussel *Perna perna* resulted in significant GST induction, along with the activation of cytochrome P450 enzymes, specifically the CYP2C9 and CYP3A4 isoforms. In contrast, Barreto et al. (2018) did not observe any significant variation in GST activity in fish *Sparus aurata* exposed to GEM concentrations ranging from 1.5 to 15,000 µg/L. Similarly, Lee et al. (2019) reported that MET at concentrations of 40, 120, and 360 µg/L did not induce GST activity in fish *Oryzias latipes*. Additionally, Maranho et al. (2014) found that environmental concentrations of the antidepressant fluoxetine (ranging from 0.005 to 500 ng/L) did not modulate GST activity in the polychaete *Hediste diversicolor*. These studies highlight species-specific responses of the enzyme GST, suggesting that these pharmaceuticals may be metabolized through different pathways in other non-target organisms.

A statistically significant inhibition of glutathione reductase (GR), an enzyme responsible for the conversion of oxidized glutathione (GSSG) to its reduced form (GSH), was measured in mixture-exposed organisms (Figure 3.9C). An interesting biphasic and delayed response was observed in mussels treated with VEN, with lower average GR activity compared to control organisms after the exposure phase and a significant induction after the depuration phase (Figure 3.9C).

GP_x CHP and GP_x H_2O_2 are enzymes involved in the detoxification of cellular organic hydroperoxides. GP_x CHP (glutathione peroxidase conjugated to lipid hydroperoxides) acts by conjugating GSH to lipid peroxides, thereby neutralizing them and preventing cellular damage. In contrast, GP_x H_2O_2

(glutathione peroxidase reacting with hydrogen peroxide) facilitates the reaction of hydrogen peroxide to neutralize hydroperoxides, resulting in the formation of oxygen. An induction of both enzymes was observed at 30 and 44 days in VEN-exposed mussels, with a pronounced effect on GP_x CHP noted at the end of the depuration phase, further indicating a delayed response to this drug (Figure 3.9E, F). Supporting the suggestion that antidepressants can alter the oxidative status of bivalves, Magni et al. (2017) reported an induction of GP_x activity in the mussel *Dreissena polymorpha* following a 14-day exposure to the SSRI fluoxetine (500 ng/L).

The pro-oxidant effects of the tested drugs were further corroborated by the functional responses observed in the exposed mussels. Specifically, a significant increase in lipofuscin content, which is product of lipid peroxidation, was detected in organisms exposed to GEM, RAM, MET, and MIX (Figure 3.10A). Lipofuscin accumulation is indicative of cellular damage and impaired antioxidant defenses (Bocchetti et al., 2008), underscoring the extent of oxidative stress induced by these compounds. Notably, these oxidative effects persisted even after the depuration phase for all drugs except RAM. This observation suggests a differential capacity for recovery, where the absence of sustained RAM-induced stress allowed mussels to restore cellular homeostasis, highlighting the potential reversibility of oxidative damage when the source of contamination is removed.

The impact of the tested drugs on mussel lipid metabolism was evaluated at both functional and catalytic levels by measuring neutral lipid accumulation and the activity of acyl-CoA oxidase (ACOX), respectively. Treatments with VEN, GEM, MET, and their mixture led to significant alterations in lipid metabolism after a 30-day exposure, resulting in a statistically significant increase in neutral lipid content in the digestive gland. This effect persisted in all exposed mussels even after the depuration phase, with the exception of those treated with VEN (Figure 3.10B). Given GEM's mechanism of action in humans, a similar hypolipidemic effect was hypothesized for non-target organisms. Conversely to this expectation, however, an accumulation of lipids was observed at the functional level. Despite this unexpected outcome, GEM's mechanism of action was confirmed at the catalytic level, as indicated by the induction of ACOX activity in mussels at both exposure times (Figure 3.10C). ACOX, an enzyme essential to the β-oxidation pathway, catalyzes the initial step in the oxidation of very long-chain fatty acids by introducing a double bond into the acyl-CoA thioester. This process is critical for fatty acid degradation within peroxisomes (Ortiz-Zarragoitia and Cajaraville, 2006). The increased activity of ACOX in response to GEM exposure suggests a compensatory mechanism to manage excess fatty acids and maintain lipid homeostasis. Although there is still limited information on the effects of this drug in non-target organisms, analogous results have been observed with other fibrates. For example, Cancio et al. (1998) reported peroxisome

proliferation and increased peroxisomal acyl-CoA oxidase activity in *M. galloprovincialis* following the injection of 1.5 mg of clofibrate per mussel.

Figure 3.11 reported that the AChE activity was unaffected by any of the tested treatments. Although the trends were not statistically significant, drug-specific changes in AChE activity were observed in the hemolymph of mussels, indicating that this tissue exhibits greater sensitivity to pharmaceutical compounds compared to the gills. Opposite responses were observed for RAM and MET between the exposure and depuration phases. Mussels exposed to RAM exhibited an induction of AChE activity at day 30, followed by inhibition at day 44, suggesting a delayed neurotoxic effect associated with this drug. Conversely, MET treatment led to AChE inhibition at day 30, with a subsequent induction at day 44, indicating a potential recovery of the organisms after a period in drug-free water. An induction of AChE activity was also observed in *M. galloprovincialis* exposed for 14 days to the cardiovascular drug valsartan (0.5 μg/L), suggesting the potential of this pharmaceutical to interact with neuronal mechanisms and exhibit neuroprotective properties in non-target species (Mezzelani et al., 2023).

In this work, the biological significance of the observed responses was summarized in specific hazard indices, which allow an easier qualitative and quantitative comparison of pharmaceuticals accumulation and biomarkers responses in mussels exposed to various conditions (Regoli et al., 2019). The WOE approach highlighted a higher impact for the antidepressant VEN compared to all other single tested pharmaceuticals after the exposure phase (Figure 3.12A). The risk for mussels exposed to VEN alone was summarized as "MAJOR" based on “Severe” accumulation and “Moderate” cellular effects. While the "SLIGHT" risk for organisms treated with GEM, RAM and MET mainly depend on an "Absent" level of bioaccumulation of these drugs rather than their biological effects which resulted as "Moderate".

Although bioaccumulation levels being below the LOD for RAM or comparable to control organisms for GEM and MET, the observed cellular effects suggest that these drugs are absorbed and metabolized by the mussels, potentially impacting various metabolic pathways. MIX treatment showed a "MAJOR" level of risk, indicating a lack of a significant interaction between tested pharmaceuticals. In fact, this risk is based on the "Major" accumulation due mainly to the contribution of VEN levels in mussels, rather than the cellular effects which resulted "Moderate", similar to those observed for each individual drug. After the depuration phase, the WOE risk level was classified as "SLIGHT" for all treatments, with reductions observed for VEN and MIX, while the risk levels for GEM, RAM, and MET remained unchanged (Figure 3.12B). The decrease in risk for VEN and MIX is primarily attributed to a significant reduction in bioaccumulation hazard, shifting from "Severe" to "Absent" for VEN and from "Major" to "Absent" for MIX. However, the detrimental effects

associated with all treatments persisted even after a period in pharmaceutical-free water, with a "moderate" hazard level, highlighting the importance for further investigation into the long-term impacts of these pollutants.

Table 3.2 Concentration at different sampling times of venlafaxine, gemfibrozil, ramipril and metformin in the whole tissues of *M. galloprovincialis* exposed to experimental conditions. Data given in ng/g dry weight, d.w. (mean values ± standard deviations, n = 3), for venlafaxine, metformin and ramipril; data given in µg/g d.w. (mean values ± standard deviations, n = 3) for gemfibrozil. VEN venlafaxine; GEM gemfibrozil; RAM ramipril, MET metformin; MIX venlafaxine + gemfibrozil + ramipril + metformin.

	VEN (ng/g d.w.)		**GEM (µg/g d.w.*)**		**RAM (ng/g d.w.)**		**MET (ng/g d.w.)**	
	Day 30	Day 44	Day 30	Day 44	Day 30	Day 44	Day 30	Day 44
CTRL	0.847 ± 0.204	1.850 ± 1.509	1.730 ± 0.707	3.206 ± 0.938	< 0.417	0.696 ± 0.414	3.173 ± 2.327	5.285 ± 1.200
VEN	33.38 ± 5.136	< 0.623	2.164 ± 1.006	3.232 ± 0.745	< 0.417	< 0.417	1.934 ± 0.955	5.624 ±1.370
MET	< 0.623	< 0.623	2.910 ±1.553	2.117 ±1.341	< 0.417	< 0.417	5.430 ±1.322	2.573 ± 1.133
RAM	< 0.623	< 0.623	1.815 ± 0.781	2.878 ± 0.468	< 0.417	< 0.417	3.526 ± 0.057	3.597 ± 1.580
GEM	< 0.623	< 0.623	2.288 ± 0.186	4.124 ± 1.217	< 0.417	< 0.417	4.023 ± 2.892	1.417 ± 0.151
MIX	40.67 ± 4.544	< 0.623	2.266 ± 0.044	1.912 ± 0.893	< 0.417	< 0.417	2.924 ± 1.083	3.833 ± 2.313

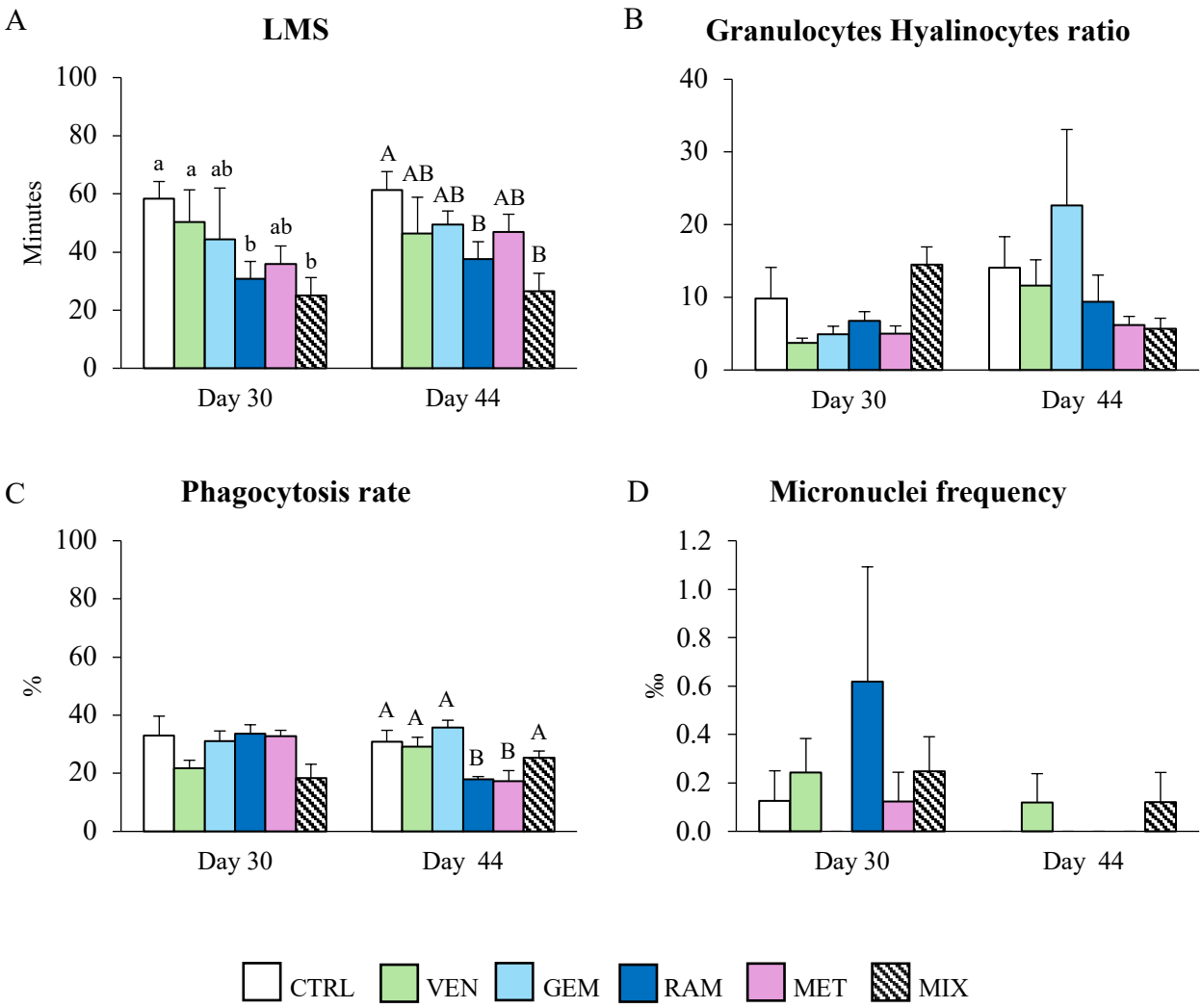


Figure 3.8. A) Lysosomal membrane stability, LMS; B) Granulocytes/hyalinocytes ratio; C) Phagocytosis rate; D) Micronuclei frequency in haemocytes of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 4). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL Control; VEN venlafaxine; GEM gemfibrozil; RAM ramipril, MET metformin; MIX venlafaxine + gemfibrozil + ramipril + metformin.

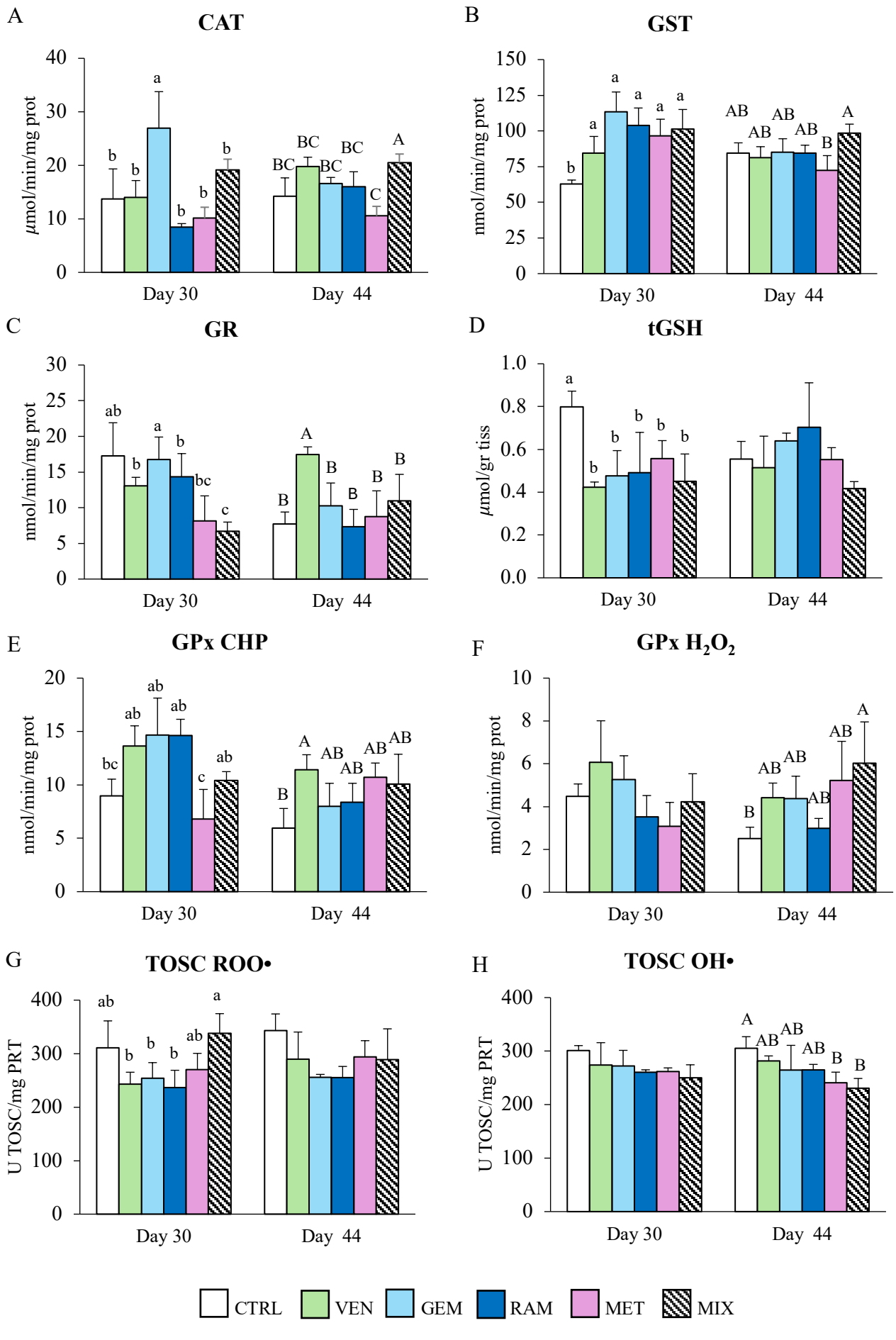

A
CAT
µmol/min/mg prot
Day 30
Day 44
B
GST
nmol/min/mg prot
C
GR
nmol/min/mg prot
D
tGSH
µmol/gr tiss
E
GPx CHP
nmol/min/mg prot
F
GPx H2O2
nmol/min/mg prot
G
TOSC ROO•
U TOSC/mg PRT
H
TOSC OH•
U TOSC/mg PRT
CTRL
VEN
GEM
RAM
MET
MIX

Figure 3.9 Antioxidant system responses in digestive glands of mussels exposed to different treatments. A) Catalase, CAT; B) Glutathione S-transferase, GST; C) Glutathione reductase, GR; D) Total glutathione, tGSH; E) Glutathione peroxidases CHP: sum of Se-dependent and Se-independent glutathione peroxidases; F) Glutathione peroxidases H_2O_2: Se-dependent glutathione peroxidases; G) TOSC ROO•: total oxyradical scavenging capacity toward peroxyl radical; H) TOSC HO•: total oxyradical scavenging capacity toward hydroxyl radical. Data are given as mean values ± Standard Error of the Mean (n = 3). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL Control; VEN venlafaxine; GEM gemfibrozil; RAM ramipril, MET metformin; MIX venlafaxine + gemfibrozil + ramipril + metformin.

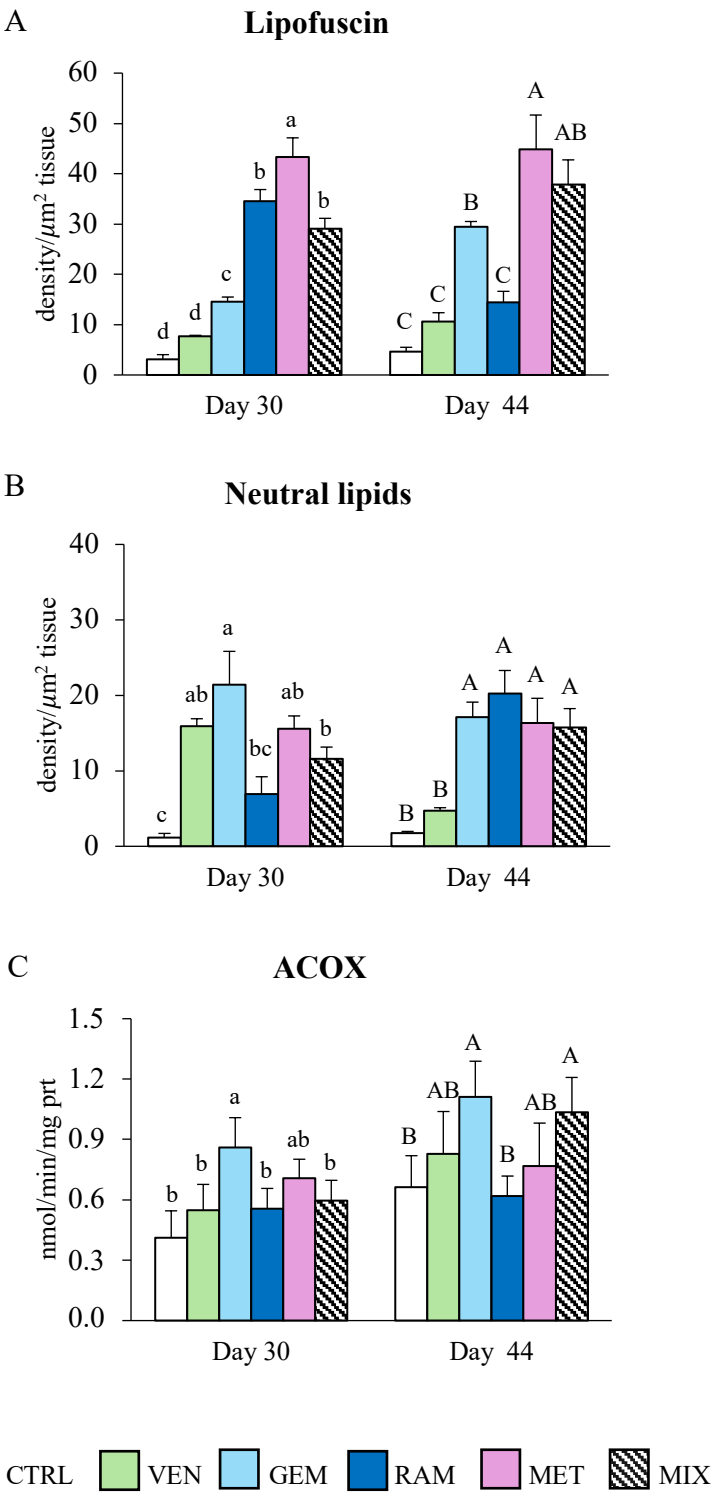


Figure 3.10 A) Lipofuscin and B) neutral lipids content; C) Acyl-CoA oxidase (ACOX) activity in mussels exposed to tested experimental conditions. Data are given as mean values ± Standard Error of the Mean (n = 4). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL Control; VEN venlafaxine; GEM gemfibrozil; RAM ramipril, MET metformin; MIX venlafaxine + gemfibrozil + ramipril + metformin.

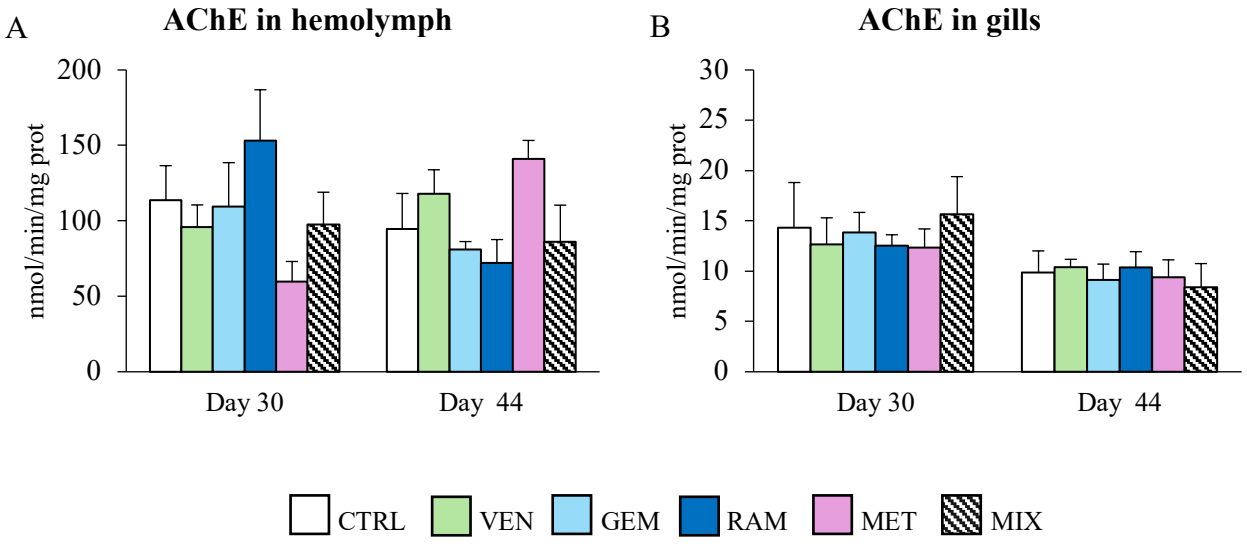


Figure 3.11 Neurotoxic damages. A) Acetylcholinesterase activity (AChE) in hemolymph and B) in gills of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 5). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL Control; VEN venlafaxine; GEM gemfibrozil; RAM ramipril, MET metformin; MIX venlafaxine + gemfibrozil + ramipril + metformin.

A

TREATMENT	LOE 3 BIOAVAILABILITY	LOE 4 BIOMARKERS	WOE INTEGRATION	LEVEL OF RISK
VEN	Severe HQ: 8100	Moderate HQ: 189.9	MAJOR	
GEM	Absent HQ: 100	Moderate HQ: 209.2	SLIGHT	
RAM	Absent HQ: 100	Moderate HQ: 215.9	SLIGHT	
MET	Absent HQ: 100	Moderate HQ: 198.3	SLIGHT	
MIX	Major HQ: 2100	Moderate HQ: 195.7	MAJOR	

B

TREATMENT	LOE 3 BIOAVAILABILITY	LOE 4 BIOMARKERS	WOE INTEGRATION	LEVEL OF RISK
VEN	Absent HQ: 100	Moderate HQ: 206.8	SLIGHT	
GEM	Absent HQ: 100	Moderate HQ: 176.6	SLIGHT	
RAM	Absent HQ: 100	Moderate HQ: 158.5	SLIGHT	
MET	Absent HQ: 100	Moderate HQ: 218.6	SLIGHT	
MIX	Absent HQ: 100	Moderate HQ: 238.2	SLIGHT	

Figure 3.12 Weighted elaboration of whole dataset for each treatment at A) exposure phase (day 30) and B) depuration phase (day 44). VEN venlafaxine; GEM gemfibrozil; RAM ramipril, MET metformin; MIX venlafaxine + gemfibrozil + ramipril + metformin.

3.3.3 Experiment 3. Effects of climate change-related stressors on pharmaceuticals toxicity

Pharmaceuticals, particularly mixtures of such compounds, are considered as a major environmental challenge in aquatic ecosystems. While recent efforts have emphasized the need of monitoring and mitigation strategies for these emerging contaminants, research on the combined effects of pharmaceutical mixtures remains limited (Mezzelani and Regoli, 2022; Kidd et al., 2024). Concurrently, climate change exacerbates the vulnerability of marine ecosystems, inducing critical alterations such as ocean warming, marine heatwaves, and acidification (Oliver et al., 2019; IPCC, 2023). Marine heatwaves (MHWs) are defined as a short-term extreme event characterized by seawater temperatures exceeding a seasonally varying threshold (usually the 90th percentile) for at least five consecutive days (Nardi et al., 2022). These phenomena have become increasingly prevalent and severe. The Mediterranean Sea is considered one of the most vulnerable regions, with projections indicating that the frequency and intensity of MHWs will continue to increase, leading to severe consequences for the structure, functioning, and services of marine ecosystems (Oliver et al., 2019). Another direct consequence of anthropogenic CO_2 emissions is ocean acidification (OA), a process in which the absorption of carbon dioxide by the oceans leads to a decrease in seawater pH, resulting in increased acidity. These environmental changes can modify the toxicity of pharmaceuticals by altering their chemical composition due to increased temperatures or acidification processes, and by influencing various aspects of aquatic species' health, including metabolic and feeding rates, which in turn affect the bioaccumulation of chemicals in their tissues (Kroeker et al., 2017; Serra-Compte et al., 2018; Nardi et al., 2018). In the present study, the influence of ocean acidification and marine heatwaves was investigated, both separately and in combination, on the bioaccumulation and toxicity of a selected pharmaceutical mixture in the Mediterranean mussel *Mytilus galloprovincialis*.

Figure 3.13 show the levels of VEN, CBZ, GEM and MET in the whole tissue of mussels.

The obtained results offered both novel insights and corroborated previously observed bioaccumulation patterns for the tested pharmaceuticals, each representing different therapeutic class. Additionally, the findings allowed to elucidate their behavior within mixtures and their responses to one or more climate change-related stressors. Outcomes revealed that *M. galloprovincialis* can bioaccumulate VEN and CBZ (Figure 3.13A, B), as indicated by the statistically significant increase in their levels in all organisms exposed to the APIs mixture, compared to those with no-APIs (< LOD). These results are consistent with those of the previous experiment (Exp. 2. Venlafaxine, gemfibrozil, ramipril, metformin and their mixture), where chemical analyses demonstrated the ability of mussels to bioaccumulate the antidepressant VEN when exposed either alone or in a mixture. Levels of VEN and CBZ were modulated by climate change stressors. The general assumption was that organisms subjected to thermal stress, such as the dynamic heatwaves (HW) in this study, would exhibit

enhanced metabolism, ventilation, and higher feeding rates in response to increased metabolic demands. These physiological changes often result in a greater uptake of contaminants dissolved in the water column (Maulvault, Barbosa, et al. 2018). A similar trend was observed for the anticonvulsant CBZ; although not statistically significant, higher average levels of this drug were found in the tissues of mussels exposed to the HW scenario (Figure 3.13B). This increasing trend of bioaccumulation under HW conditions was also reported by Nardi et al. (2022), who observed similar results in *M. galloprovincialis* exposed to 1 μg/L of the same pharmaceutical.

On the other hand, VEN displayed a distinct pattern, with lower average levels in mussel tissues exposed to the HW scenario (Figure 3.13A). This finding aligns with observations by Maulvault et al. (2018), who reported that co-exposure to increased temperatures (ΔT °C = +5 °C) and higher concentrations (20 μg/L) reduced VEN uptake in the liver of the fish *Argyrosomus regius.* The measured decrease in contaminant levels at higher temperatures may be attributed to accelerated metabolic rates, which can enhance the metabolism and excretion of contaminants (Maulvault et al. 2018). Moreover, ocean acidification (OA) has been shown to impact the physiology of aquatic organisms. Calcifying and sessile species, such as mussels, are particularly sensitive to this stressor: increased energy demands for homeostatic processes can lead to reduced growth, fitness, and metabolic rates (Kroeker et al. 2013, 2017). Consequently, it can be hypothesized that the decreased biological activity of mussels under acidification may result in reduced contaminant uptake. The findings of this work support this hypothesis, indicating a slight decrease in the bioaccumulation levels of VEN and CBZ under OA conditions. Similar findings were reported by Almeida et al. (2018), who observed reduced CBZ bioaccumulation in *V. philippinarum* exposed to decreased pH (7.5) and 1.0 μg/L of the anticonvulsant, and by Maulvault et al. (2018), who noted decreased VEN levels in *A. regius* exposed to elevated pCO_2 levels (~1,000 μatm) and 20 μg/L of the antidepressant.

Levels of GEM and MET were measured in all organisms, including those not treated with the APIs mixture (Figure 3.13C, D), suggesting pre-existing environmental contamination for both drugs. These findings are consistent with those observed in Experiment 2 (Exp. 2. Venlafaxine, gemfibrozil, ramipril, metformin and their mixture) and discussed in the previous paragraph.

A statistically significant increase in MET levels was observed in mussels exposed to either the HW or OA scenarios (HW scenario, no APIs; OA scenario, no APIs) compared to the control group (no APIs) (Figure 3.13D). These findings are likely due to a decrease in pre-existing MET levels after 42 days of pharmaceutical-free water exposure in the control mussels. Notably, MET excretion was not observed in organisms subjected to individual stressors related to climate change (HW and OA scenarios), but it occurred when these stressors were combined (CC scenario).

IBU and RAM were the only pharmaceuticals in the mixture detected below the limit of detection (< LOD of 25 ng/g d.w. and 0.33 ng/g d.w., respectively) in all treatments (data not shown). In Experiment 1 of this chapter (Exp. 1. Paroxetine, ibuprofen and their mixture), IBU was also not bioaccumulated (levels < LOD) when co-exposed with other pharmaceutical, indicating consistent behavior of this drug in mixtures.

To provide a more comprehensive assessment of how climate change stressors influence the toxicity of the API mixture, findings on its bioaccumulation behavior are integrated with a broad panel of biochemical and cellular responses reflecting the main metabolic pathways in *M. galloprovincialis*.

The results of immune parameters measured in mussel hemolymph are shown in Figure 3.14. A decreasing trend in LMS was observed in all organisms compared to their controls (CTRL no-APIs and CTRL treated with APIs-MIX), with a statistically significant reduction in mussels treated with HW and CC (Figure 3.14A). These findings highlight LMS impairment in all treatments involving thermal stress, without a clear influence of the APIs mixture, confirming results reported in the hemolymph of other invertebrate species (Beesley et al., 2008; Matozzo et al., 2012). Supporting these results, literature studies have reported lysosomal membrane destabilization due to co-exposure to climate change related stressors. For instance, Matozzo et al. (2012) observed decreased LMS in *M. galloprovincialis* exposed to low pH (7.4) and high temperatures (28 °C) over 7 days. Likewise, Nardi et al. (2017) found significant lysosomal membrane instability in mussels exposed to acidification (pH 7.4) and thermal stress (15 °C during winter) over a 28-day period.

Outcomes of this work showed no change in LMS in OA treatments compared to the control, consistent with findings by Mezzelani et al. (2021), where mussels exposed to OA (pH 7.6) exhibited unaltered LMS values. However, LMS was significantly altered in organisms co-exposed to the mixture and OA, indicating the deleterious effects of hypercapnic conditions in the presence of the APIs mixture. This is supported by Mezzelani et al. (2021), who also observed lysosomal membrane destabilization in mussels co-exposed to OA and CBZ (1 μg/L) for 28 days, further corroborating our findings that OA combined with the API mixture significantly alter this immune parameter. The ability of OA and HW to influence the mussels' immune system is further highlighted by the significant decrease in the granulocyte-to-hyalinocyte ratio and the simoultaneous inhibition of phagocytosis capacity observed in all treatments involving these climate change-related stressors (Figure 3.14B, C). In mussels, granulocytes are essential for cell-mediated immunity, contributing to efficient phagocytosis and cytotoxic reactions, while hyalinocytes are specialized in coagulation and encapsulation. The observed decrease in phagocytosis may therefore be associated with the reduced number of granulocytes, as suggested by the decreased G/H ratio (Gorbi et al., 2013). Finding of this study are in line with those of Mezzelani et al. (2021) and Nardi et al. (2022), which observed

decreases in the granulocyte-to-hyalinocyte ratio and phagocytic capacity in mussel hemocytes exposed to OA, HW and CC scenarios.

Climate change stressors appear to be the primary factor influencing immune system modulation, regardless of the presence of the APIs mixture. Notably, treatments exposed solely to environmentally realistic concentrations of the pharmaceutical's mixture showed no significant effects on these immune parameters, suggesting that the tested mixture had limited impact on immune function. However, investigations on individual drugs such as VEN (Rafiq et al., 2023), IBU (Exp. 1; Mezzelani et al., 2016), CBZ (Mezzelani et al., 2021), and RAM (Exp. 2) have demonstrated that these pharmaceuticals, when exposed alone, can affect the immune system and lead to a decrease in LMS. This observation suggests the possibility of antagonistic interactions within the tested mixture. Although not statistically significant, a trend of increased MN frequency was highlighted in hemolymph of mussels co-exposed to the APIs mixture and both climate change stressors. (Figure 3.15A). Previous research has shown that individual pharmaceuticals, such as the NSAID IBU (Exp. 1; Mezzelani et al., 2016) and the cardiovascular drug valsartan (Mezzelani et al., 2021), can increase MN frequency in non-target species, affecting cellular turnover. In contrast, this study revealed that the mixture, despite containing the same APIs, did not affect MN frequency, suggesting potential antagonistic interactions among its components. These interactions appear to be less pronounced when the mixture is combined to climate change stressors, highlighting the complexity involved in evaluating such effects.

Conversely, the increase in DNA damage observed in hemocytes following exposure to the APIs mixture, may be attributed to the cumulative effects of each component within the mixture (Figure 3.15B). For instance, have been reported that exposures to single VEN, GEM, and IBU enhanced DNA strand breaks in marine organisms (Exp. 1; Lacaze et al., 2015; Mezzelani et al., 2016; Nardi et al., 2022). In the present study, is however important to highlight that levels of DNA fragmentation measured in mussels in all treatments (ranging from 15.9 % to 37.3 %) fall within the physiological variability observed in natural, uncontaminated organisms (20 % to 50 %) as reported by Bocchetti et al. (2008) and Pisanelli et al. (2009), indicating that these values are not alarming but rather typical for this parameter. Interestingly, DNA damage significantly decreased under co-exposure to the HW scenario, and the APIs mixture compared to both stressors tested alone, suggesting also in this case a potential antagonistic interaction between these stressors. The DNA diffusion assay, employed as a genotoxic endpoint, was conducted on mussel hemocytes to quantify the percentage of apoptotic cells in tested treatments (Figure 3.15C). Apoptosis, a crucial mechanism of programmed cell death, plays a fundamental role in maintaining cellular homeostasis and mediating responses to environmental stressors. By measuring the number of apoptotic cells, it is possible to assess the extent of cellular

damage and the stress responses elicited by environmental conditions and/or pollutants (Singh, 2005; Cantafora et al., 2014; Mottola et al., 2022). The results revealed a significant increase in the percentage of apoptotic cells in mussels co-exposed to both climate change stressors and APIs mixture. In contrast, treatments involving both climate change stressors alone and the APIs mixture alone exhibited lower levels of apoptosis. These findings suggest that simultaneous exposure to multiple stressors intensifies the stress response in mussel hemocytes, potentially overwhelming the cellular defense mechanisms.

AChE in the hemolymph and gills of bivalves is widely recognized as a sensitive biomarker for assessing the neurotoxicity of xenobiotics (Viarengo et al., 2007; Gorbi et al., 2013).As demonstrated in literature studies, certain pharmaceuticals, such as the antidepressant fluoxetine (Franzellitti et al., 2014) and the NSAIDs IBU and paracetamol (Mezzelani et al., 2016), have been shown to inhibit AChE activity in mussel gills, thereby exhibiting neurotoxic properties. Conversely, other drugs within the tested mixture, including the antiepileptic CBZ (Siebel et al., 2010; Mezzelani et al., 2021) and the cardiovascular drug RAM (Exp. 2), have demonstrated the ability to enhance AChE activity, likely through interactions with ion channels, neurotransmitter systems, and receptors essential for its function (Mezzelani et al., 2023). The outcomes of this study, in fact, revealed a significant induction of AChE activity in the gills of mussels treated with the APIs mixture compared to the control condition without the mixture, as well as in the hemolymph of mussels exposed to the APIs mixture under HW scenario compared to HW condition alone (Figure 3.16).

The impact of the APIs mixture and climate change-related stressors on the oxidative system of mussels was assessed by integrating changes in the activity of key antioxidant enzymes with the overall capacity to neutralize specific oxyradicals (Figure 3.17). Additionally, the study evaluated the accumulation of lipid peroxidation products, such as lipofuscin and malondialdehyde (Figure 3.18A, C), as biomarkers of oxidative damage. The marked accumulation of malondialdehyde (MDA) observed in the organisms exposed to APIs mixture compared to the control group (Figure 3.18C), along with the significant induction of catalase, CAT, activity (Figure 3.17A) and the concurrent suppression of the total oxyradical scavenging capacity (TOSC) to neutralize the ROO• and HO• radicals (Figure 3.17G, H) and of the GST activity (Figure 3.17B), provides strong evidence that the exposure to the tested pharmaceuticals mixture induces a pronounced state of oxidative stress. This oxidative imbalance is characterized by enhanced lipid peroxidation, as indicated by elevated MDA levels, and a disrupted antioxidant defense mechanism, reflected in the altered CAT activity and reduced ability to counteract ROS, thereby suggesting a potential compromission in the redox cellular homeostasis. Supporting these outcomes, several studies have previously documented the capacity of some of these individual drugs to alter the oxidative status of bivalves, including the lipid-lowering

agent GEM (Exp. 2; Barreto et al., 2018), the antidepressant VEN (Exp. 2), the anticonvulsant CBZ (Mezzelani et al., 2023), and the NSAID IBU (Exp. 1; Gonzalez-Rey and Bebianno, 2011; 2012; Parolini et al., 2011; Trombini et al., 2019), suggesting that these compounds play a key role in the pro-oxidant effects observed in the mixture. A statistically significant increase in TOSC values toward ROO• and HO• radicals was observed in mussels treated with the APIs mixture in combination with one or both climate change-related stressors, compared to those exposed to the APIs mixture alone (Figure 3.17G, H). The induction of such antioxidant defenses, coupled with the concomitant increase of lipid peroxidation products (Figure 3.18A, C), likely represents an adaptive response by the organisms to counteract the intensified pro-oxidant challenge induced by the simultaneous exposure to multiple stressors.

Interestingly, the results of this study indicate that hypercapnic conditions can exacerbate oxidative stress in cells induced by the pharmaceutical mixture, as evidenced by a significant increase in lipofuscin content in mussels treated with the APIs mixture under combined OA and CC scenarios, compared to those exposed to the APIs mixture alone or in the presence of HW conditions (Figure 3.18A). Similar findings were reported by Mezzelani et al. (2021), who observed that co-exposure to lower pH and CBZ enhanced lipofuscin levels in mussel digestive glands.

An opposite pattern of variation was observed in terms of lipofuscin and MDA content among APIs-exposed organisms: specifically, an increase in lipofuscin and a decrease in MDA levels was measured in OA and CC-treatments; conversely higher MDA levels were associated to a reduced lipofuscin content in HW-treatment. This peculiar pattern of variation is related to the specific role of MDA and Lipofuscin in lipid peroxidation process: MDA represent as an intermediate product and, as a reactive toxic metabolite, is rapidly degraded and converted into lipofuscin (Viarengo et al., 2007), thus an opposite trend in content of these molecules has been well-documented in Literature (Franzellitti et al. 2014; Rafiq et al. 2023). Specifically, the results demonstrated an increase in lipofuscin content accompanied by a decrease in MDA levels in mussels exposed to the APIs mixture in combination with OA and CC scenarios. Conversely, in treatments involving the APIs mixture alone and the APIs mixture combined with HW conditions, low lipofuscin levels were correlated with high MDA levels (Figure 3.18A, C).

Neutral lipids (NL) in the digestive gland are a primary energy reserve in bivalves, and fluctuations in their quantity are considered a key indicator of metabolic disturbance (Bocchetti & Regoli, 2006). Changes in NL levels can be associated with either the inhibition or induction of peroxisomal enzyme activity, such as acyl-CoA oxidase (ACOX), often revealing opposite trends between these parameters. The tested APIs mixture exhibited a notable impact on lipid metabolism in mussels, as indicated by a significant increase in NL levels, accompanied by a corresponding inhibition of ACOX activity

(Figure 3.18B, D). Similar effects were observed for most of the individual drugs tested, including IBU (Mezzelani et al., 2018; Exp. 1), CBZ (Nardi et al., 2022), VEN, GEM, and MET (Exp. 2), with the exception of RAM, which likely does not contribute to the overall mixture effect on this pathway. On the other hand, the statistically significant induction of ACOX activity observed in mussels treated with the APIs mixture and individual stressors (HW and OA) was not associated to a decrease in NL content. This discrepancy can be likely due to a temporal difference between the catalytic and functional responses. Nevertheless, the results indicated that both individually climate change-related stressor modified the impact of the drug mixture on mussel lipid metabolism, leading to an acceleration of fatty acid β-oxidation as evidenced by increased ACOX activity. The faster β-oxidation could be a compensatory response to manage increased lipid load or oxidative stress induced by the combined exposure to pharmaceuticals and environmental stressors. This interaction underscores the complexity of metabolic responses in marine organisms under multifaceted environmental pressures, where climate change effects can modulate the biochemical impact of contaminants.

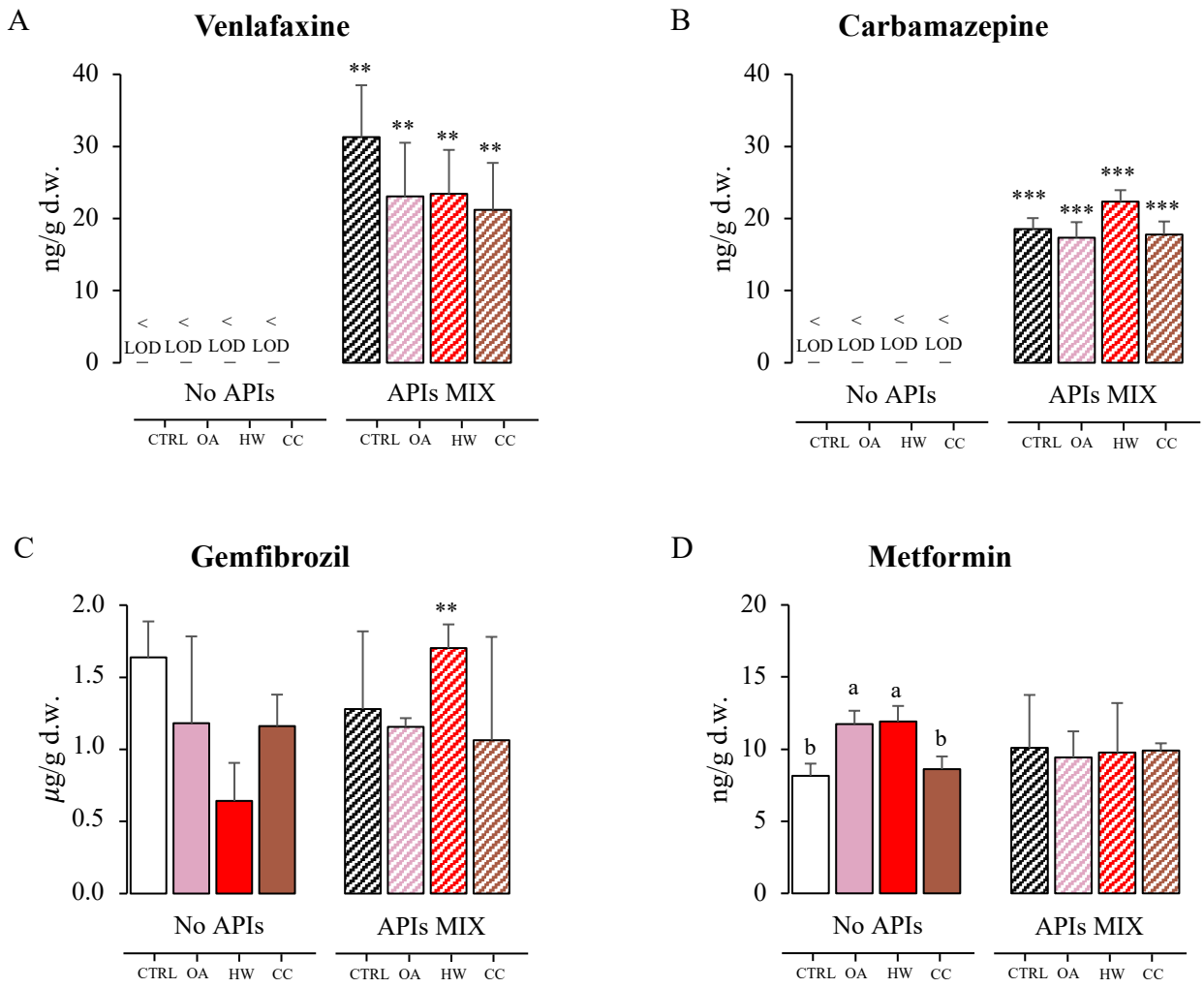


Figure 3.13 Levels of A) Venlafaxine (ng/g d.w.); B) carbamazepine (ng/g d.w.); C) gemfibrozil (µg/g d.w.); and D) metformin (ng/g d.w.) in whole tissues of *M. galloprovincialis*. Data are given as mean ± standard deviation. Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

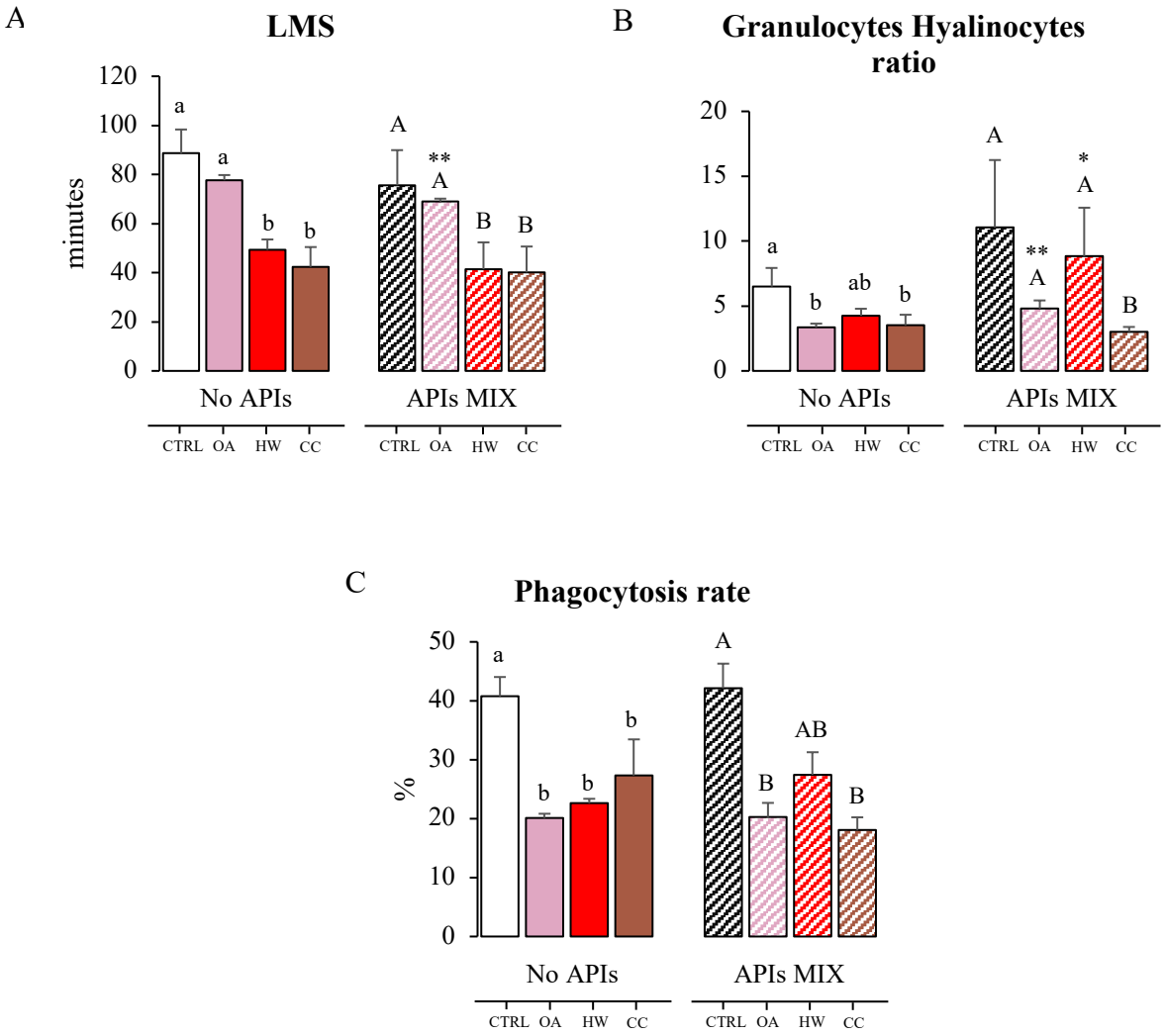


Figure 3.14 Immunological responses. A) Lysosomal membrane stability, LMS; B) Granulocytes/hyalinocytes ratio; and C) Phagocytosis rate in haemocytes of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 4). Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (* p <0.05; ** p <0.01; *** p <0.001). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

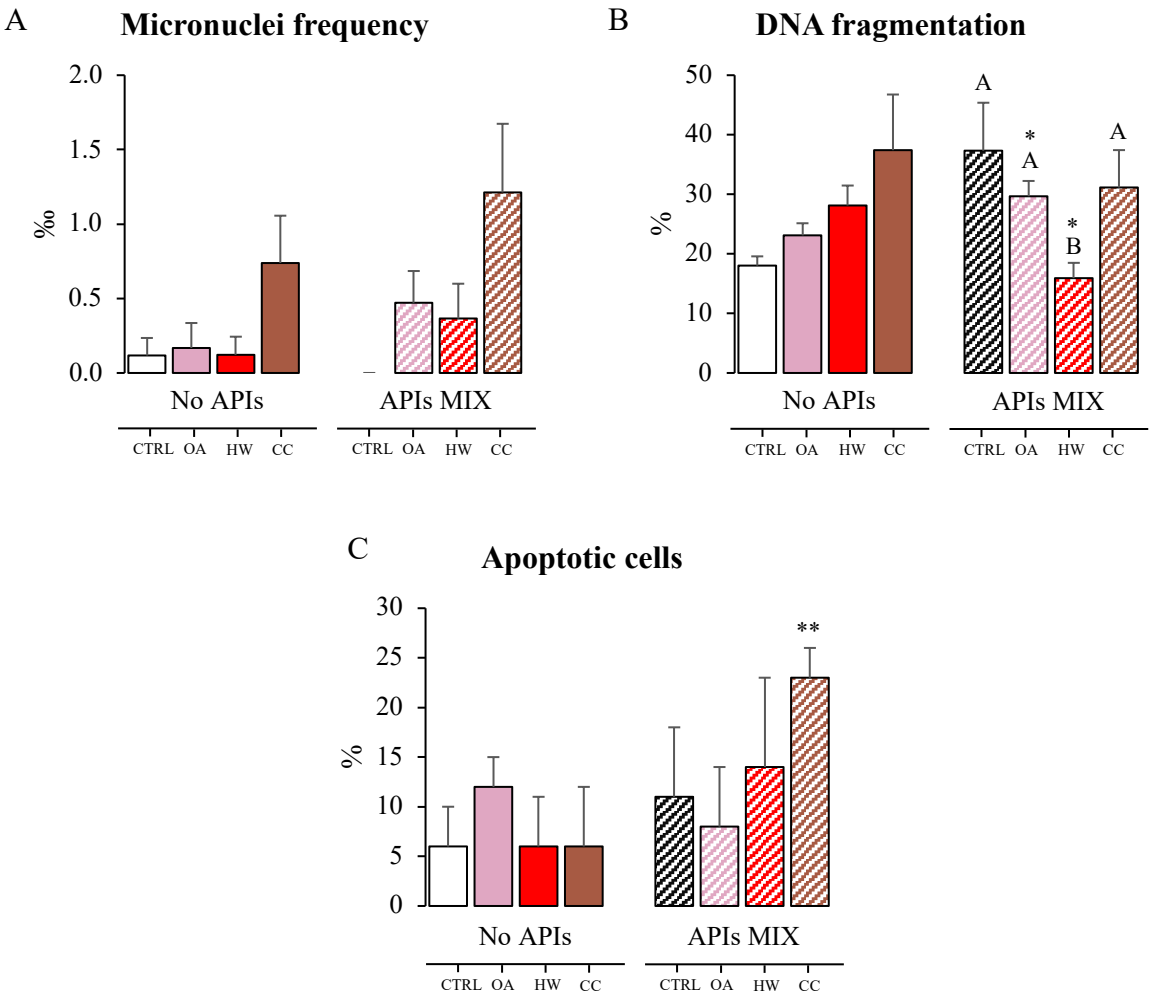


Figure 3.15 Genotoxic responses. A) Micronuclei frequency (‰); B) Percentage of DNA in the comet tail; and C) percentage of apoptotic cells in hemocytes of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 4). Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (* p <0.05; ** p <0.01; *** p <0.001). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

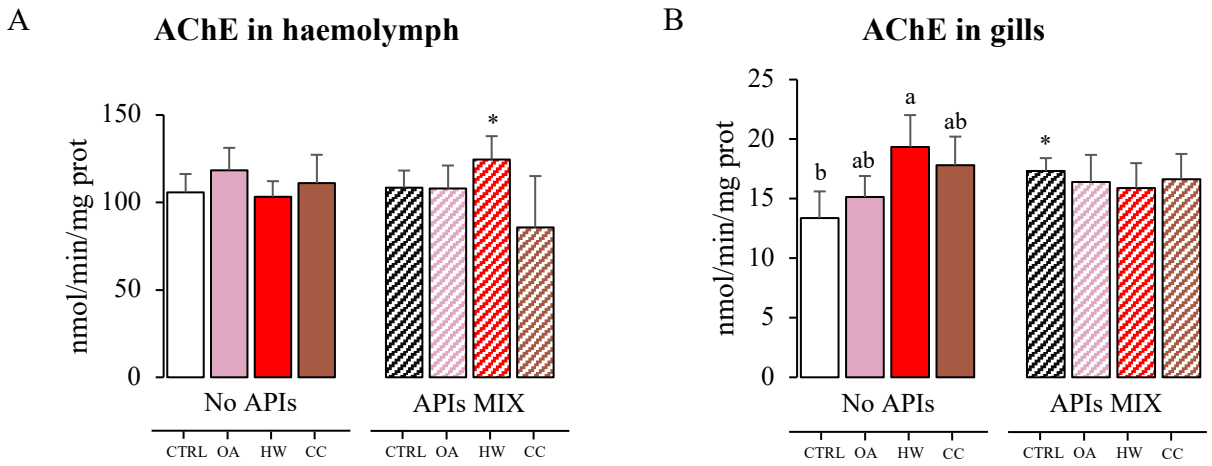
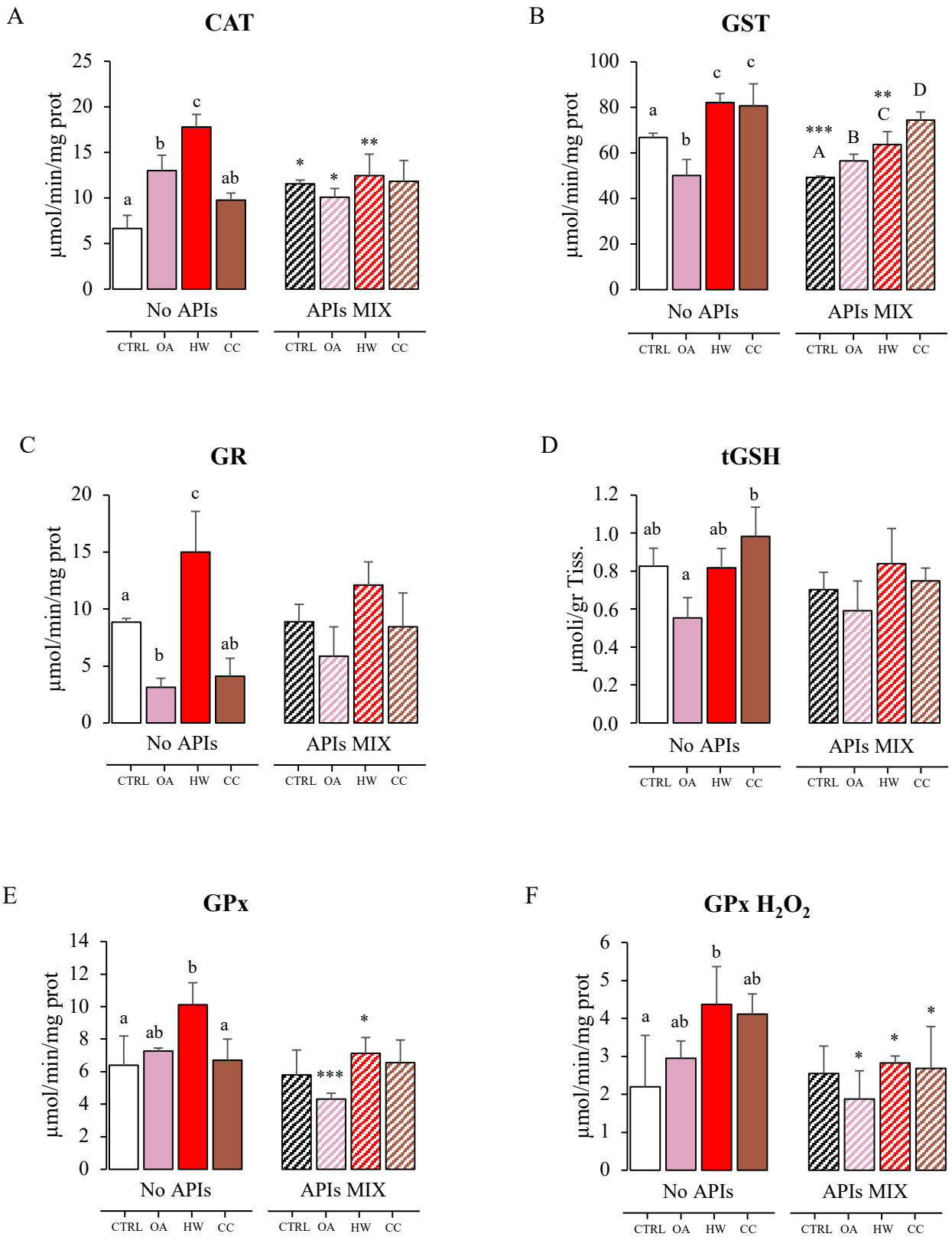


Figure 3.16 Neurotoxic damages. A) Acetylcholinesterase activity (AChE) in hemolymph and B) in gills of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 5). Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (* p <0.05; ** p <0.01; *** p <0.001). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.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CAT
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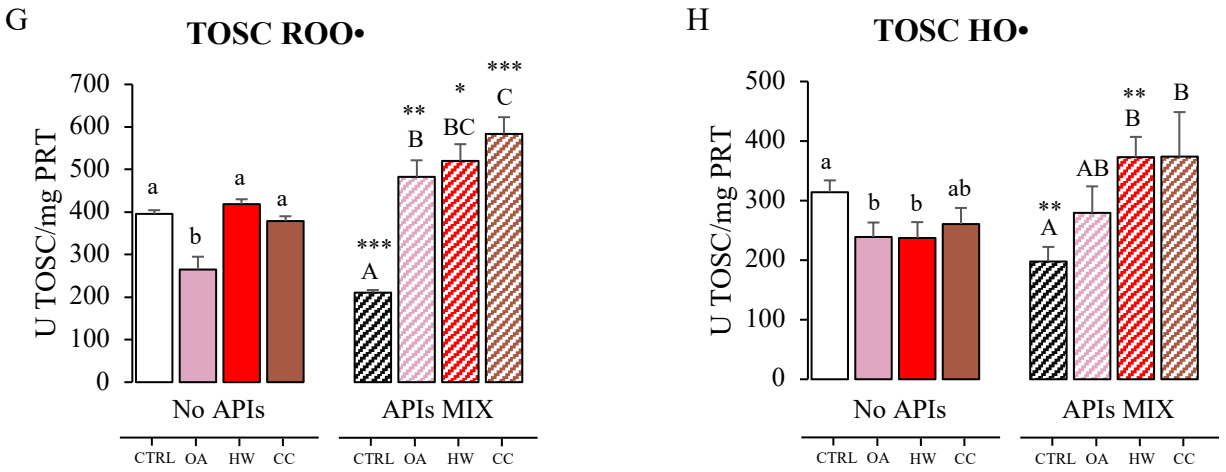


Figure 3.17 Antioxidant system responses in digestive glands of mussels exposed to different treatments. A) Catalase, CAT; B) Glutathione S-transferase, GST; C) Glutathione reductase, GR; D) Total glutathione, tGSH; E) Glutathione peroxidases CHP: sum of Se-dependent and Se-independent glutathione peroxidases; F) Glutathione peroxidases H_2O_2: Se-dependent glutathione peroxidases; G) TOSC ROO•: total oxyradical scavenging capacity toward peroxyl radical; H) TOSC HO•: total oxyradical scavenging capacity toward hydroxyl radical. Data are given as mean values ± Standard Error of the Mean (n = 3). Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (* p <0.05; ** p <0.01; *** p <0.001). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

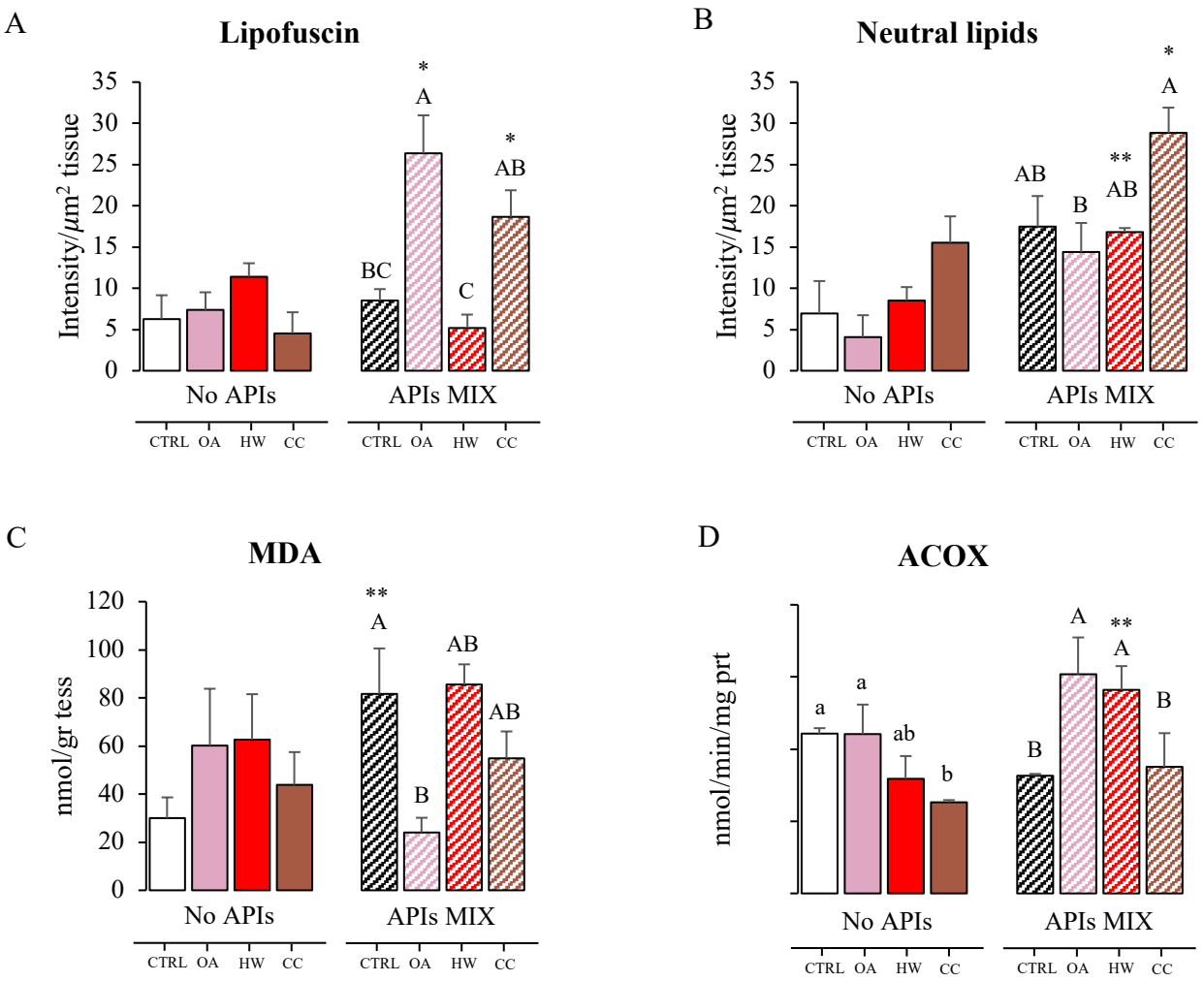


Figure 3.18 A) Lipofuscin and B) neutral lipids content; C) Levels of malondialdehyde, MDA; and D) Acyl-CoA oxidase (ACOX) activity in mussels exposed to tested experimental conditions. Data are given as mean ± standard deviation for MDA and ACOX while mean ± SEM for lipofuscin and neutral lipids (n = 4). Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (* p <0.05; **p <0.01; ***p <0.001). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

3.4 Conclusions

In conclusion, the presented studies have demonstrated significant biological reactivity to all tested pharmaceuticals, providing novel insights into their mechanisms of action in non-target species.

Notably, each individual compound was observed to modulate the oxidative system, albeit through distinct pathways. Immune parameters were most significantly affected by the NSAID IBU, the antidepressant PAR, and the cardiovascular drug RAM. Furthermore, lipid metabolism was notably influenced by IBU, the lipid-lowering agent GEM, the antidiabetic MET, while the effects of the antidepressants PAR and VEN were less pronounced. Although additive or antagonistic interactions were observed in specific metabolic pathways, the overall severity of biochemical and cellular responses was similar to that induced by each individual drug ("Moderate" cellular hazard levels).

While the severity of these effects varied based on the pharmaceutical treatment and specific endpoint, most were not mitigated during the depuration phase, as indicated by the persistent “Moderate” hazard level measured for cellular effects, raising concerns about the resilience of mussels to sustained pharmaceutical exposure.

The bioaccumulation patterns highlighted for individual drugs, were consistent across the three experimental plans and showed similarities among compounds belonging to the same therapeutic class (*e.g.*, the antidepressants VEN and PAR). Furthermore, while most drugs exhibited similar behavior regardless of whether they were administered individually or in a mixture, others, such as IBU, did not show bioaccumulation in mussels when co-exposed with other pharmaceuticals. In this case, the lack of IBU uptake was attributed to a potential competitive mechanism with other drugs, as supported by the absence of interactive cellular effects observed in the mixtures. Conversely, cellular effects ("Moderate" hazard level) observed for GEM, RAM, and MET, suggest that these substances undergo metabolism and excretion, despite the lack on their bioaccumulation in exposed mussels. These findings suggest that environmental monitoring alone, which focuses on the detection of pharmaceutical compounds in marine ecosystems, may be insufficient to fully assess the overall environmental risk of these substances.

Finally, the third study provided evidence on the complex interactions between environmental pharmaceuticals and climate change-related stressors. Such findings highlighted that hypercapnia and dynamic heatwaves significantly impact the bioaccumulation of drugs such as CBZ and VEN by modulating mussels’ metabolic activity: OA reducing and HW accelerating it. Additionally, cellular and biochemical responses indicated that OA exacerbates oxidative stress caused by pharmaceuticals mixture, whereas thermal stress seems to amplify the impact of these mixtures on mussels’ immune systems. These results underscore potential risks to the health of *Mytilus galloprovincialis* in coastal regions where pharmaceutical presence is simultaneous to climate change stressors. Given the

species' ecological importance as a key filter-feeder and its economic significance in the seafood market, it is crucial to conduct further long-term studies. Such research will be essential to comprehensively understand the ecological consequences of pharmaceutical mixtures under future oceanic conditions, thereby informing effective management strategies to mitigate risks to marine biodiversity and human health.

4. Mechanisms of cellular responses of caulerpin as a target of the peroxisome proliferator-activated receptor α (PPARα) and its similarities with the lipid lowering agent fenofibrate

4.1. Introduction

During the last century the progress of medical science allowed to treat several pathologies, determining a general improvement of health quality, an increment of life expectancy finally leading to the current increase of the demographic trend towards an aging population (Mezzelani et al., 2022). In this respect, the need to find new active principles to improve the health of elderly people can be accomplished by marine-derived compounds. Indeed, several marine natural products, recently proved their efficacy against a wide array of diseases due to antibacterial, antifungal, antiprotozoal, antituberculosis, antiviral, antidiabetic, anti-inflammatory, and anticancer properties (Montaser et al., 2011; Giuliani et al., 2024).

Aging represents the most important risk factor for aging-related pathologies, which include cardiovascular, metabolic and neurodegenerative diseases (Guo et al., 2022). Cardiovascular diseases, CVDs, are one of the greatest health burdens of modern era and elevated lipid plasma levels, were shown to be the most predictive risk factor for development and progression of CVDs (Mourikis et al., 2020); for this reason beta blockers, ACE inhibitors, angiotensin II receptor and calcium channel blockers are commonly prescribed with lipid-lowering agents, such as statins and fibrates, which indirectly reduce the risk of severe CVDs by treating dyslipidemias. Among lipid lowering agents, fenofibrate (FFB) is an amphipathic carboxylic fibrate, with many blood lipid-modifying properties, including the decrease of triglycerides and increase of the high-density lipoprotein (HDL) cholesterol levels. These effects are mediated by activation of the peroxisome proliferator-activated receptor, PPARα, which enhances peroxisomal β-oxidation and activate lipoprotein lipase (Chen et al., 2012). Interestingly, recent studies discovered that FFB has anti-aging properties since it reduces the number of senescent cells via apoptosis, increases autophagic flux, and protects against cartilage degradation. FFB was shown to reduce senescence and inflammation and to increase autophagy in both ageing human and osteoarthritis chondrocytes whereas PPARα knockdown elicited the opposite effect (Nogueira-Recalde et al., 2019).

In marine environment, the bisindole alkaloid caulerpin (CAU) is a secondary metabolite of several green algae of the genus *Caulerpa* (Shah et al., 2020) and other seaweeds (Abdelrheem et al., 2020; Ara et al., 2019). Diverse bioactive properties have been attributed to caulerpin, including anti-viral Macedo et al., 2012; Esteves et al., 2019; Ahmed et al., 2020), anti-tubercolosis (Canché et al., 2014), anti-nociceptive, anti-inflammatory (De Souza et al., 2009) and anti-cancer (Li et al., 2018; Mehra et al., 2019; Ferramosca et al., 2016) activities. Besides that, several evidence have driven the hypothesis

of potential caulerpin-mediated effects on lipid metabolism. The first observations on fish feeding on *Caulerpa cylindracea* highlighted that hepatic accumulation of caulerpin was associated with the changes in responses involved in fatty acid β-oxidation, *e.g.* activity of acyl CoA oxidase (ACOX) and modulation of PPARα transcription (Gorbi et al., 2014). Moreover, in the same fish a reduction of polyunsaturated fatty acids was observed in the muscle (Felline et al., 2019). CAU was then identified as the direct cause of alterations in the lipid composition of fish meat (Del Coco et al., 2018). Additional *in silico*, *in vitro* and *ex vivo* experiments demonstrated this active principle as agonist of PPARs (Vitale et al., 2018).

Under this perspective, CAU shows several similarities to FFB (Russo et al., 2023; 2024) and many literature studies demonstrated the FFB capability to induce lipid metabolism abnormalities and decrease long-chain omega-3s content, as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), when administered to freshwater fish (Du et al., 2004; 2008); notably, the same effects has been noted in wild populations of *D. sargus* feeding on *C. cylindracea* (Krawczyk et al., 2012). As abovementioned, hypolipidemic agents like FFB activate lipolysis through the modulation of peroxisomal genes/enzymes: reactive oxygen species generated by peroxisomal reactions also participate in lipolysis (Krawczyk et al., 2012; Mezzelani et al., 2024), for this reason the activation of lipid metabolism is typically associated to an unbalance of the cellular redox homeostasis that in turn increase can increase the oxidative stress (Liu et al., 2019; Giuliani et al., 2019).

In this respect, the present study, was aimed to investigate if CAU can represent a possible marine-derived candidate hypolipidemic agent. An *ex vivo* approach was applied using Precision Cut-Tissue Slices (PCTS) of *Mytilus galloprovincialis* digestive glands, selected as invertebrate marine model species. PCTS offers the main advantages of cell cultures while preserving the physiological architecture of the tissue: it has already been useful to demonstrate the activation of genes of lipid metabolism by CAU in the marine fish *Diplodus sargus* (Vitale et al., 2019) and has been already validated also for the development of ecotoxicological tests in invertebrates marine species like the Mediterranean mussel *Mytilus galloprovincialis* (Giuliani et al., 2019). This *ex vivo* approach fulfill the 3R principle (Refinement, Reduction and Replacement) and the animal welfare regulation for promoting responsible scientific experimentation (Directive 2010/63/EU).

The potential hypolipidemic *FFB-like* effect of CAU, was investigated through an integrated approach which combined measurements at both molecular and cellular levels of responses reflecting the activation of lipolysis, modulation of the overall lipid metabolism and susceptibility of redox homeostasis. The same endpoints were also assessed for FFB exposed-PCTS for a comparison.

The present study was expected to provide novel insights on the possible use of marine natural products as a new wave of drugs.

4.2. Materials and methods

4.2.1 Solutions and culture medium

The composition of buffers and culture medium was based on standardized protocols (Giuliani et al, 2019). Physiological solution (PS) (NaCl 436 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgSO_4$ 53 mM, pH 7.3) and Leibovitz's medium (L-15) (Gibco; adjusted to NaCl 436 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgSO_4$ 53 mM and supplemented with L-glutamine 2 mM) were sterilized and supplemented with 1 % penicillin/streptomycin antibiotic mix.

4.2.2 Animal maintenance

Mussels *Mytilus galloprovincialis* (5.5 ± 1 cm) were obtained from a local aquaculture farm (Ancona, Adriatic Sea) and acclimatized for two weeks with recirculating Artificial Sea Water (ASW), at 35 ‰ salinity and 20 °C (2 mussels/L). The animals were fed daily with a commercial mixture of marine plankton (EasySPS EVO, size range 0.2 - 400 µm) and the water changed three times a week.

4.2.3 Exposure of Precision Cut-Tissue Slices (PCTS)

PCTS were prepared from digestive glands (DG) according to standardized method (Giuliani et al., 2019). Briefly, each DG was excised carefully, the gastro-intestinal tract was washed with cold sterilized ASW and DG were maintained in cold (4 °C) sterilized ASW until slicing. DG were included in 2.5 % low melting point agarose and sectioned with a vibratome (VT1200S, Leica, Wetzlar, Germany) set at the following parameters: thickness 400 µm, amplitude 0.1 mm, speed 0.18 mm/s. Immediately after slicing, the PCTS were placed in cold PS supplemented with 10 mM D-glucose and then pre-incubated in 12-wells plate (4 PCTS/well) in L-15 with 10 % fetal bovine serum (FBS) (1 mL/well), at 18 °C and mild shaking (50 rpm) for 1:30 h. 24 PCTS were obtained from each DG. The experimental dose (100 µM for both CAU and FFB) were selected based on the results obtained from preliminary tests with different concentrations of FFB (data not shown).

After pre-incubation, PCTS were exposed to FFB 100 µM (36.08 mg/L) and to CAU 100 µM (39.84 mg/L) in L-15 medium (both dissolved in DMSO at 1 % final concentration) for 24 h, 48 h and 72 h. A control (PCTS in L-15 alone) and a vehicle control (PCTS in L-15 with 1 % DMSO) were included. For both FFB and CAU exposure, the PCTS from each individual DG (24 PCTS/DG) were distributed in 6 wells of a 12-wells plate (4 PCTS/well) and incubated in 1 mL of control, vehicle control or exposure medium (8 PCTS/condition). Every 24 h, the culture medium was renewed and FFB or CAU were re-dosed. The experimental plan was replicated many times to obtain, at the end of the exposure, for each treatment 2 PCTS to include in cryostat embedding medium (Killik, Bio Optica)

and frozen at -80 °C for histological analysis, 6 PCTS to pool and frozen at -80 °C for each gene expression analysis, and 16 PCTS (pooled from 2 different DG) to frozen at -80°C for the evaluation of the Acyl-CoA oxidase's (ACOX) activity.

4.2.4 Histological analysis of neutral lipids and lipofuscin

Neutral lipids and Lipofuscin staining was performed on 4 cryostat sections (10 µm) obtained from 2 PCTS (400 µm) from the 3 DG used for each treatment and experimental time. Cryosections were fixed in Beker's fixative (2.5 % NaCl) for 15 min and stained by Oil red O (ORO) before mounting in glycerol gelatin and by Schmrol reaction before mounting with Eukitt for neutral lipids and lipofuscin content, respectively. Slides were observed under light microscope. For both analysis, four measurements were made on digestive tubules of each section (one sections from each of the 2 PCTS of the 3 DG for each treatment). Quantification of staining intensity was performed with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of digestive tubules. Results were expressed as fold change of control and averaged (n = 3).

4.2.5 Quantitative analysis of mRNA levels

mRNA levels of acyl CoA oxidase 1 (*acox1*), catalase (*cat*), glutathione S-transferase pi-isoform (*gst-pi*), 70 kDa heat shock proteins (*hsp70*), Cu, Zn superoxide dismutase (*Cu/Zn-sod*), cytochrome P450 3A1 (*cyp3A1*) and ATP binding cassette (ABC) transporter (*abcb1*) were quantified through absolute real time PCR (qPCR).

Total RNAs were extracted from 4 pool of PCTS from 4 mussels (each pool composed of 6 PCTS from single digestive gland) for each treatment and experimental time, using the Hybrid-R kit (GeneAll) according to manufacturer's protocol. Total RNA concentrations and purity were measured with spectrophotometric methods (NanoDrop, ND1000); 260/280 and 260/230 ratios were >1.8. cDNA samples were synthesized from 1 µg of total RNA through retrotranscription reactions using oligo-dT and random examers (iScript cDNA synthesis, BioRad). qPCRs were performed with the Sybr Green method in a 96-well thermocycler (StepOnePlus, Applied Biosystems). Reactions were set up with 7.5 µL of SYBR Select Master Mix (Life Technologies), 5 µL of total cDNA (diluted 1:5) and 0.2 mM of forward and reverse primers (Table S3, Supplementary Material). In Table S3 were indicated the annealing temperatures for all genes. A non-template control was included. For each gene, standard curve was built with 8 serial dilutions of a plasmid at known copy numbers containing the amplicon of interest. Sample and standards were amplified in duplicate in the same run. A calibration curve was built by plotting cycle threshold (Ct) values of standards versus $\log_{10}$ copy numbers. Ct values resulting from each sample run were interpolated on the standard curve and

converted in copy number/µg of total RNA. Results were expressed as fold change of control and averaged (n = 4).

4.2.6 Activity of Acyl-CoA oxidase (ACOX)

For the evaluation of the Acyl-CoA oxidase's (ACOX) activity 3 pool of PCTS from 6 mussels (each pool composed of 14/16 PCTS from two digestive gland) for each treatment and experimental time were homogenized in 1 mM sodium bicarbonate buffer (pH 7.6), containing 1 mM EDTA, 0.1 % ethanol and 0.01 % Triton X-100 and centrifuged at 500 xg for 15 minutes at 4 °C. H_2O_2 production was measured in a coupled assay (Mezzelani et al., 2023) by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF- DA, 40 µM sodium azide, 0.01 % Triton X-100, 1.2 U/mL HRP in a final volume of 1 mL. After a pre-incubation at 25°C for 5 minutes in the dark with an appropriate amount of sample, reactions were started adding the substrate Palmitoyl-CoA at final concentration of 30 µM and 100 µM for Acyl-CoA oxidase (ACOX); readings were carried out at λ = 502 nm against a blank without the substrate. Results were expressed as fold change of control (n = 3).

4.2.7 Statistical analysis

Statistical analyses were performed using RStudio (version 2023.12.0 Build 369). Data were assessed for normal distribution using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. For each exposure time, the comparison between the treatment and its solvent control was evaluated using one-way analysis of variance (ANOVA), level of significance at the 95 % of confidence interval, α = 0.05. Post-hoc analysis was performed using the Student Newman Keuls Test to compare each experimental group.

4.3 Results

Figure 4.1 shows the results of lipid metabolism related parameters in PCTS exposed for 24, 48, 72 h to fenofibrate (FFB) and caulerpin (CAU). A decreasing trend in neutral lipids content compared to the control is shown for both FFB and CAU exposed-PCTS, with significant differences after 48 and 72 h (Fig. 4.1A, B; Fig. S1, S2). No variation of ACOX activity is observed for both FFB and CAU at 24 and 48 h (Fig. 4.1.C, D); while a significant induction was measured for CAU after 72 h of exposure (Fig. 4.1D). At molecular level, a statistically significant increase of ACOX transcript levels compared to the control was observed in PCTS treated with FFB for 48 h (Fig. 4.1E), while no variations were detected for CAU and for the other FFB exposure times (Fig. 4.1E, F).

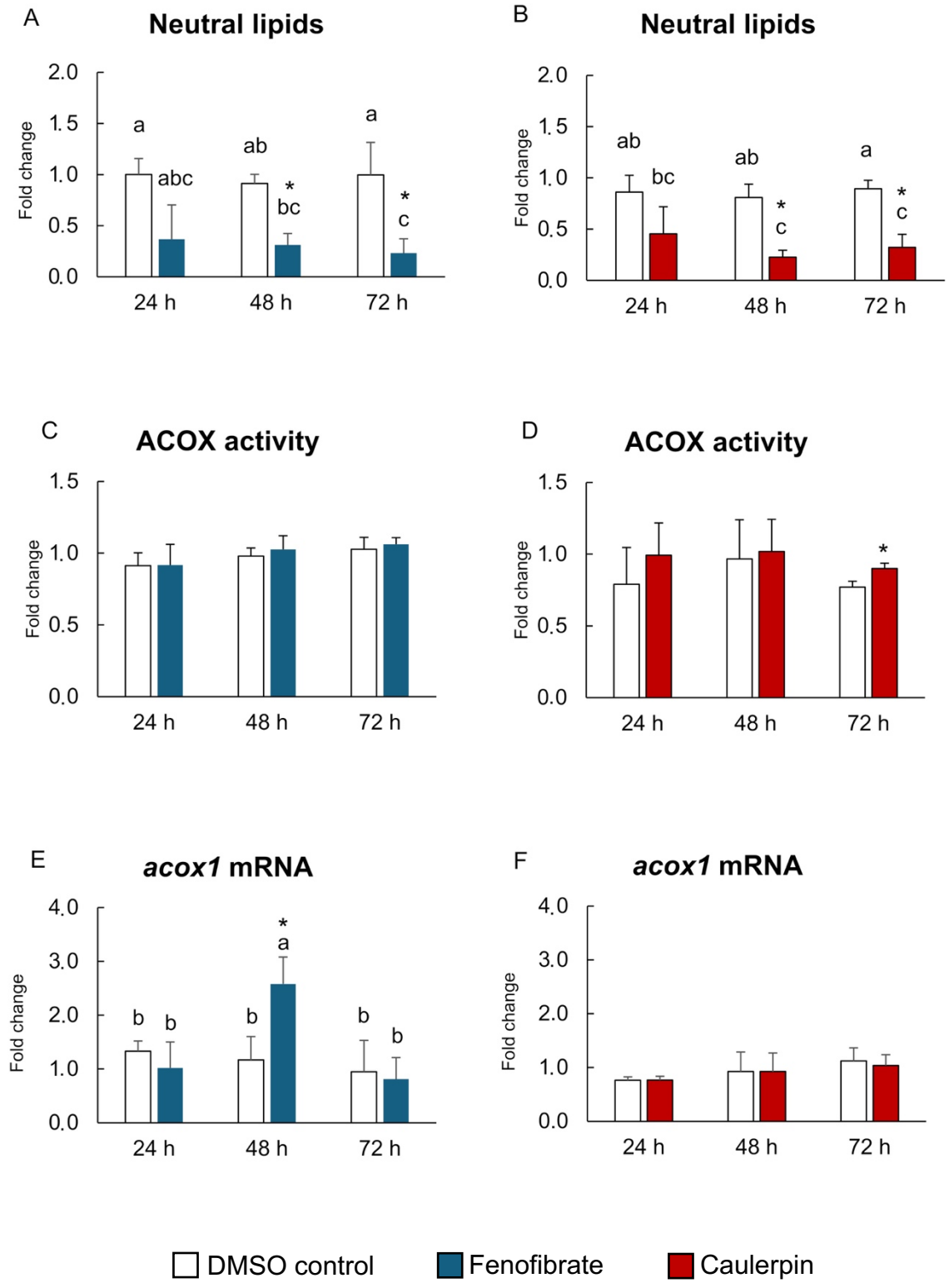
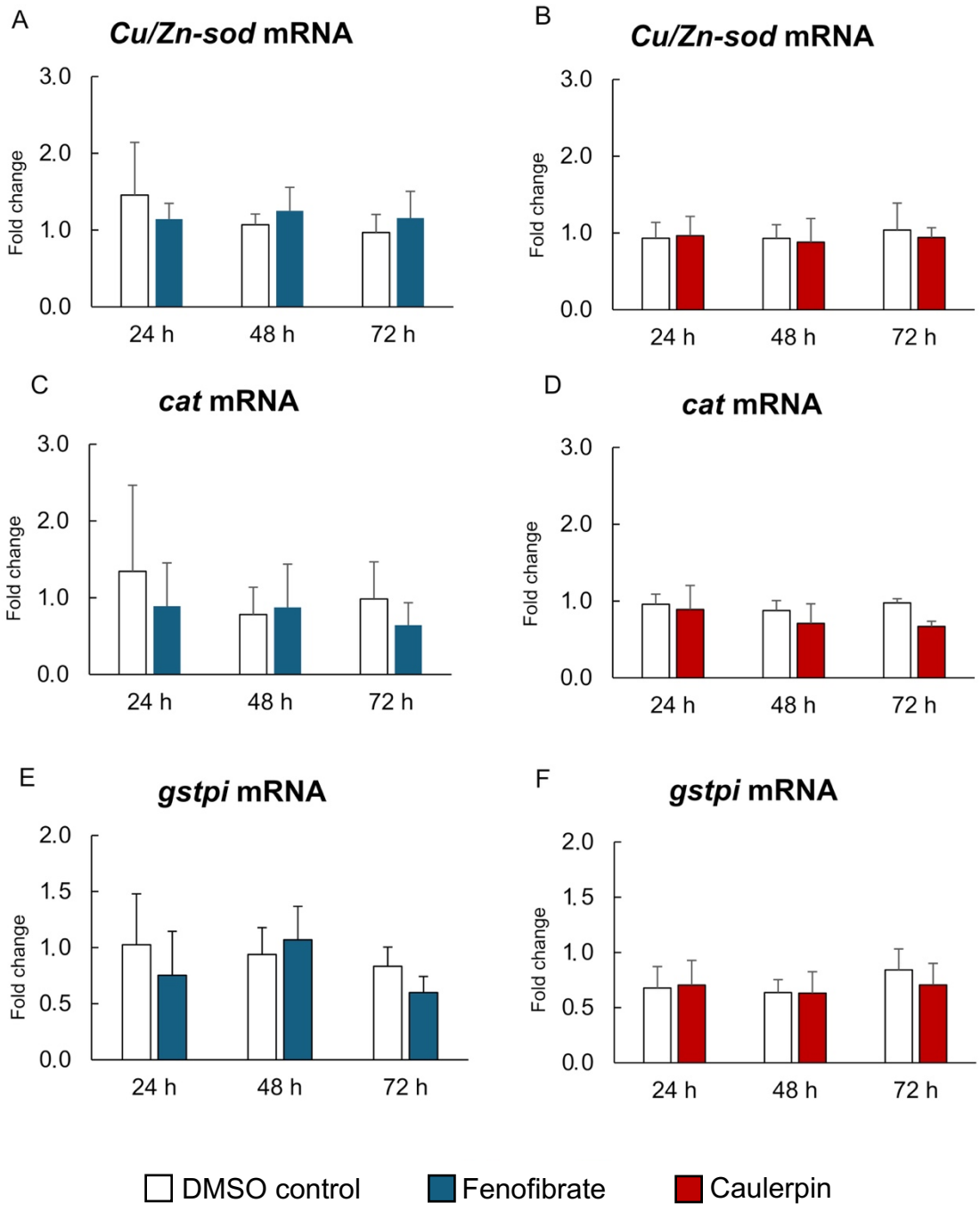


Figure 4.1. Lipid metabolism related parameters. Content of neutral lipids (A, B), catalytic activities (C, D) and mRNA levels (E, F) of acyl CoA oxidase (ACOX) in PCTS exposed, for 24, 48, 72 h, to Control in DMSO (white), Fenofibrate (blue) and Caulerpin (red). Data are expressed as mean values ± standard errors (n = 3). * (p-value <0.05) represents statistical significance compared to solvent control PCTS, determined using one-way ANOVA; while letters indicate differences between groups (results of post-hoc Student Newman Keuls Test).

Figure 4.2 shows the results of oxidative stress related responses in PCTS exposed for 24, 48, 72 h to FFB and CAU. At all exposure times, no significant differences in mRNA levels of genes related to oxidative stress (*Cu, Zn superoxide dismutase*, *catalase*, *glutathione S-transferase* and *70 kDa heat shock proteins*) were observed in PCTS exposed to FFB and CAU compared to their control (Fig. 7.3.2A - H). While, at histological level a markedly increase in lipofuscin content were measured in PCTS treated with both molecules at all exposure times (Fig. 4.2I, L; Fig. S1, S2).



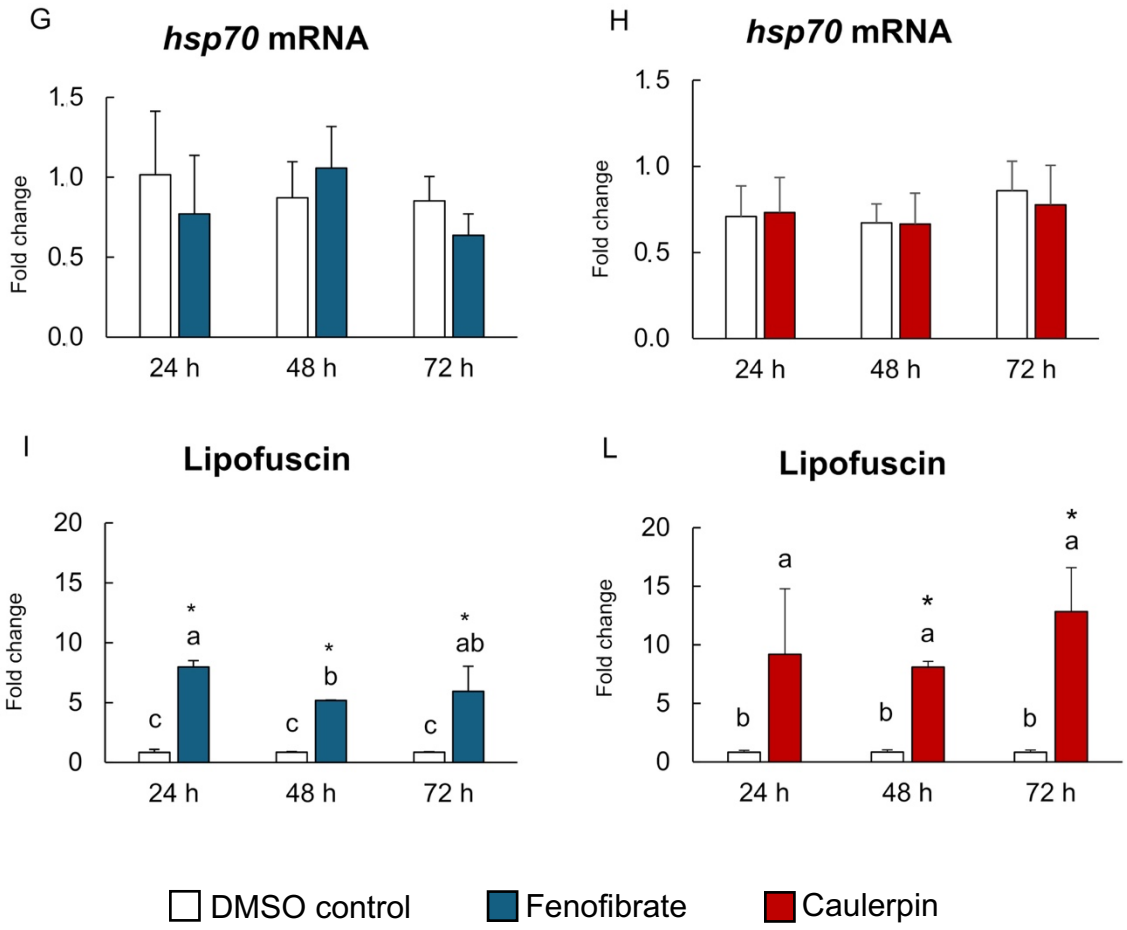


Figure 4.2. Oxidative stress related responses. mRNA levels of *Cu/Zn-sod* (A, B), *cat* (C, D), *gst-pi* (E, F) and *hsp70* (G, H); and content of lipofuscin (I, L) in PCTS exposed, for 24, 48, 72h, to Control in DMSO (white), Fenofibrate (blue) and Caulerpin (red). Data are expressed as mean values ± standard errors (n = 3). * (p-value <0.05) represents statistical significance compared to solvent control PCTS, determined using one-way ANOVA; while letters indicate differences between groups (results of post-hoc Student Newman Keuls Test).

Figure 4.3 reports the transcript levels of Cytochrome P450 3A1 (*cyp3a1*) and ABCB1/P-glycoprotein (*abcb1*) measured in PCTS exposed for 24, 48, 72 h FFB and CAU. A significant increase of levels of *cyp3a1* was observed after 48 h in PCTS exposed to FFB compared to the control (Fig. 4.3A), while no statistical variations were detected in in CAU-exposed PCTS (Fig. 4.3B). Transcript levels of *abcb1* were significant higher in PCTS exposed for 48 h to FFB, while similar values to control PCTS were observed at 24 and 72 h (Fig. 4.3C). CAU did not cause any significant modulation in *abcb1* gene at all exposure times (Fig. 4.3D).

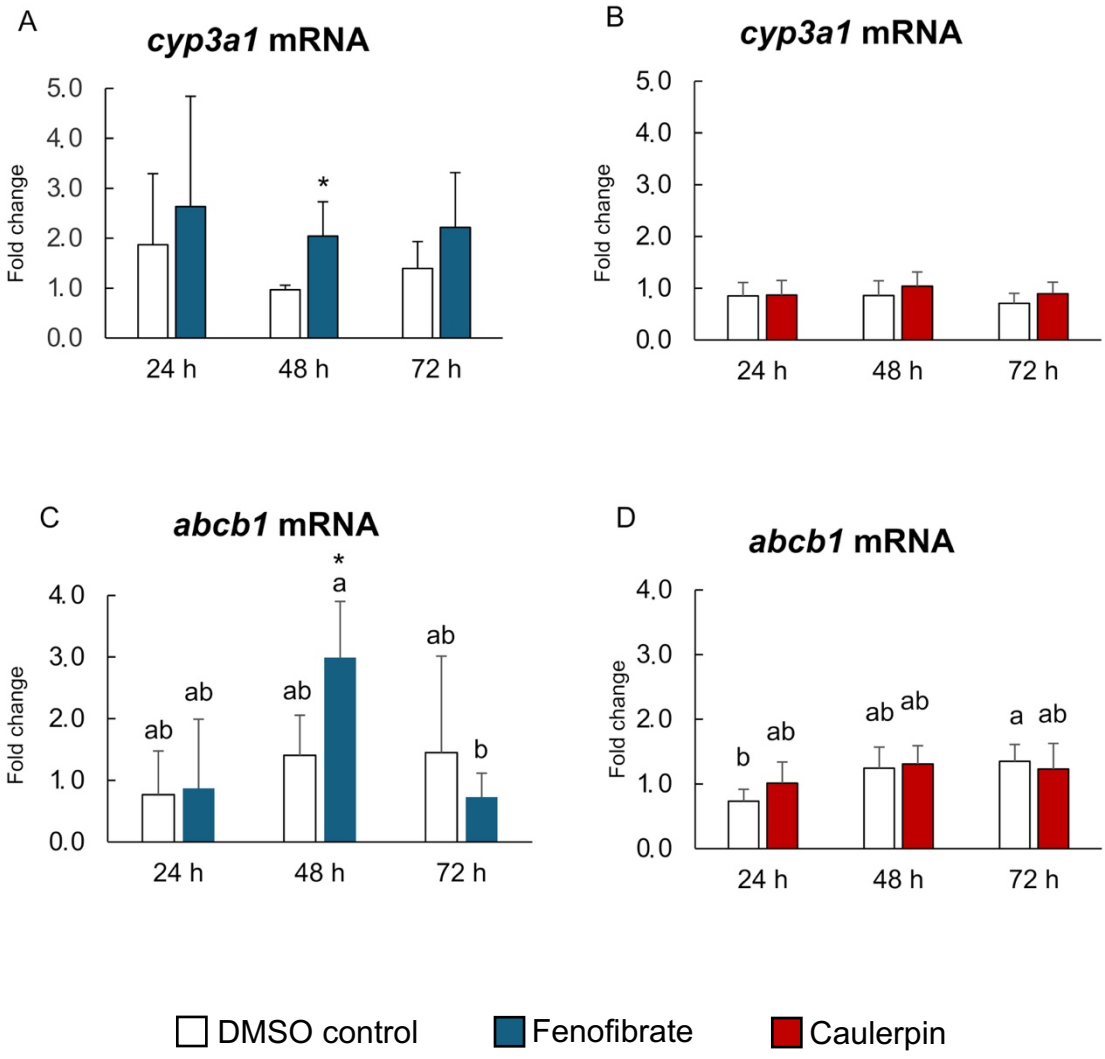


Figure 4.3. Metabolism/excretion related genes. mRNA levels of *cyp3a1* (A, B) and *abcb1* (C, D) in PCTS exposed, for 24, 48, 72h, to Control in DMSO (white), Fenofibrate (blue) and Caulerpin (red). Data are expressed as mean values ± standard errors (n = 3). * (p-value <0.05) represents statistical significance compared to solvent control PCTS, determined using one-way ANOVA; while letters indicate differences between groups (results of post-hoc Student Newman Keuls Test).

4.4 Discussion

Cardiovascular diseases and dyslipidemia are among the most widespread chronic pathologies in elderly individuals and often required long-term pharmacological therapies. Although many challenges affect aging and aging-related diseases, the discovery of new effective active principles characterized by low side effects represent one of the main objectives of the drug development research (Montaser et al., 2011; Giuliani et al., 2024). In this context, there is a growing interest on properties of natural molecules from marine environments since many studies have already proved their efficacy against a wide array of diseases. Among marine-derived compounds, the natural alkaloid from the invasive green algae *Caulerpa cylindracea* called caulerpin was investigated in the present study as possible compound with hypolipidemic properties, given some evidence on its potential role as agonist of peroxisome proliferator-activated receptors PPARα like the synthetic drug fenofibrate. Homologs of such nuclear receptor proteins were previous defined in marine bivalves (Ran et al., 2019), and they act as transcription factors for genes involved in cellular differentiation, development, inflammation, reproduction and lipid metabolism (Vitale et al., 2019; Vázquez-Carrera et al., 2023). To deepen knowledge on possible CAU hypolipidemic properties, an *ex vivo* approach based on *M. galloprovincialis* PCTS exposure, was applied in the present study. The application of this methodology agrees with the 3R principle (Refinement, Reduction and Replacement) for the adoption of measures to improve the welfare of animals used in research; it requires a reduced number of organisms and lower amounts of exposure compounds for experimental settings with advantages on both ethical issues and laboratory costs, thus significantly enhancing the socio-economic and environmental sustainability. PCTS from mussels digestive gland are considered a suitable and valid model for investigating molecular and cellular responses of different xenobiotics, since is preserved the tissue original architecture which is more similar to *in vivo* conditions than isolated cells, which resulted often more sensitive (Giuliani et al., 2019). Moreover, the use of PCTS has a wide range of applications in biological sciences: beside the study of toxicological effects, mechanisms of action of chemical substances can be clarified, as well as metabolism of pharmaceutical compounds in both target and non-target species (Giuliani et al., 2019).

In this respect, results obtained from mussel's PTCS exposure revealed a decrease in neutral lipids content in response to both CAU and FFB, with marked effects after 48 h and 72 h of exposure, thus highlighting, for the first time, the CAU lipid-lowering effect in mussels. In model species, the FFB hypolipidemic function relies on its role as PPARs agonist and the known mechanism of action consist in the activation of lipolysis processes through the β-oxidation of long-chain fatty acids and lipid derivatives mediated by Acyl-CoA oxidase (Srivastava et al., 2011; Gureev et al., 2016). In the present study, the decrease of neutral lipids was associated to enhanced mRNA levels of ACOX in

mussels treated with FFB (48 h) and to the induction of ACOX enzymatic activity in CAU treatments (72 h): beside suggesting similarities in FFB and CAU mechanisms of action, such findings revealed for the first time that CAU-hypolipidemic properties relies on the activation of lipid catabolism in invertebrate marine species, an aspect so far seldom investigated. In agreement with data obtained in the present study, the CAU-mediated activation of lipid β-oxidation pathway was also demonstrated in both *ex vivo* and *in vivo* exposures of the white seabream *D. saragus* treated with caulerpin (Vitale et al., 2019). The onset of pro-inflammatory processes is strictly related to the enhancement of ROS production and oxidative stress, considered as crucial factors in promoting CVDs and aging (Li et al., 2023). Lipid lowering agents are typically prescribed with cardiovascular compounds based on the evidence of their anti-inflammatory properties (Thomas et al., 2010). In the present study, for a more comprehensive overview on CAU properties, further characterizing the potential benefits and harms, the CAU capability to alter the redox homeostasis was assessed through the measurements of the accumulation of lipid peroxidation products and the expression of oxidative stress-related genes. At histological level, CAU and FFB treatments highlighted at all exposure times the enhancement of the lipofuscin content: lipofuscin is heterogenous amalgam mainly composed of oxidized proteins (30 to 70 %) and lipids such as triglycerides, free fatty acids, cholesterol, and lipoproteins (20 to 50 %), while carbohydrates represent a small contribution that proportionally may increase with age (4 to 7 %) (Snyder et al., 2020). Although, increased lipofuscin is typically associated to the onset of oxidative stress (Giuliani et al., 2014) our results on gene expression highlighted that both CAU and FFB exposure did not cause variation in oxidative stress-related genes, thus allow to hypothesize that the increase in lipofuscin levels may be related to the enhanced lipid catabolism and degradation rather than the impairment of oxidative homeostasis. A similar result reporting a link between lipofuscin formation and the oxidation of polyunsaturated fatty acids has already been found in the housefly *Musca domestica* (Sohal et al., 1984).

Moreover, cellular metabolism and peroxisomal reactions induced by FFB and CAU can promote the generation of reactive oxygen species (Laville et al., 2004; Krawczyk et al., 2012; Dutta et al., 2021), but in physiological conditions, ROS production is neutralized by antioxidant defenses; this mechanism possibly explain the lack of variation of oxidative-stress related responses observed in the present study at molecular level (Krawczyk et al., 2012; Schieber et al., 2014; Shin et al., 2016). In agreement with our results, previous investigation on white seabream *D. sargus* and *M. galloprovincialis* confirmed the low prooxidant challenge of CAU (Gorbi et al., 2014; Russo et al., 2023). This evidence is consistent with results on human cell lines suggesting caulerpin to be also characterized by anti-inflammatory and anti-atherogenic properties (Gorbi et al., 2014). On the other hand, the 28-days *in vivo* mussels exposure to CAU and FFB revealed the latter to markedly cause

oxidative stress-related morphological alterations in the mussels' gills and digestive tubules, compared to CAU, which instead did not produce any significant alterations in the mussels (Russo et al., 2023). In the present study, limited differences between CAU and FFB were observed for responses of the oxidative pathways. Is important to consider that oxidative stress responses may not necessarily be associated with transcriptional variations of genes, but rather with post-translational modifications of proteins (Regoli et al., 2011). Furthermore, several processes can mask the links between effects occurring at various intracellular levels, including different timing for transcriptional and translational mechanisms, metabolic capability of tissues, post-transcriptional modifications of proteins and bi-phasic responses of antioxidant enzymes (Regoli et al., 2014). For example, a significant induction of catalase activity was observed in brown trout (*Salmo trutta*) exposed to heavy metals, but without variations on mRNA expression (Hansen et al., 2007), suggesting a different time-course of these responses and/or a shorter half-life of mRNA (Regoli et al., 2011). Therefore, since not always modulation of genes reflects functional responses to oxidative challenge, to avoid possible underestimation of the pro-oxidative challenge caused by CAU and FFB, further investigation can provide the integration of these responses with variation of catalytic activities.

In this work, changes in the mRNA levels of *cyp3a1* and *abcb1*, which are genes related to metabolism and excretion of xenobiotic compounds, respectively, was evaluated. The results showed an increase in the expression of both genes after 48 h exposure to FFB, while no variations were observed in CAU treatments, suggesting a different time of action between these two molecules.

Considering that the half-life of the synthetic drug FFB in humans is estimated to be 19 - 27 hours (Miller et al., 1998), it is possible to explain the onset of its effects in metabolism (*cyp3a1*) and excretion (*abcb1*) only after 48 h of treatment. Interesting, although not significant, the induction of *cyp3a1* in FFB-exposed PCTS seems to be unchanged even after 72 h of exposure, indicating the progress of biotransformation processes which however was parallel to the unexpected decrease of *abcb1* mRNA levels, suggesting a reduction of FFB excretion.

Although our results showed no differences in levels of metabolism and excretion-related genes in response to CAU exposures, other studies demonstrated that CAU accumulation can cause effects on important cellular processes in fish, primarily those related to the CYP450 biotransformation pathway. Increased enzymatic activities and mRNA levels of *cytochrome P450* were measured in *D. saragus* sampled in sites characterized by abundant levels of *C. racemosa* proliferation (Gorbi et al., 2014), suggesting that CAU could be metabolized in longer times than those used in this experimental plan.

Overall, the present study highlighted the CAU capability to decrease neutral lipids content in PCTS of mussels digestive glands. The comparison between FFB and CAU obtained responses, provide

first insights on similarities in mechanisms of action of tested active principles, thus suggesting CAU as agonist of PPARα. A slower metabolism of the natural alkaloid CAU was observed compared to the synthetic FFB, while the possible hypolipidemic action of CAU appeared to not be associated to the onset of detrimental oxidative stress, even if additional investigation is needed to clarify possible adverse outcomes of CAU in marine invertebrates. Overall results suggest the importance to further deepen knowledge on caulerpin in order to support its possible valorization in pharmaceutical and nutraceutical applications.

5. Biological reactivity of pharmaceuticals targeting lipid metabolism: the case study of gemfibrozil, metformin and rosiglitazone

5.1 Introduction

Pharmaceuticals are considered contaminants of emerging concern, with lipid regulators and antidiabetic agents being among the most detected therapeutic classes in marine ecosystems and accumulated by aquatic non-target species. Among lipid regulators, the fibrate gemfibrozil reduces levels of fatty acids, triglycerides, and low-density lipoproteins (LDL), mainly by stimulating the peroxisomal, and partly the mitochondrial, β-oxidation pathways (Pahan, 2006). These effects are mediated by the activation of peroxisome proliferator-activated receptor alpha (PPARα), which enhances peroxisomal β-oxidation, increases both the number and size of peroxisomes, and induces oxidative stress, thereby promoting lipid catabolism (Barreto et al., 2017). The antidiabetic metformin, one of the most commonly prescribed treatments for type 2 diabetes, is well known for its potent glucose-lowering effects (Wu et al., 1990; Stumvoll et al., 1995). Among its reported side effects, metformin has been shown to suppress lipogenesis and activate catabolic processes, such as fatty acid oxidation, through a mechanism involving the activation of AMP-activated protein kinase (AMPK) and the downregulation of sterol regulatory element-binding protein 1c (SREBP1c), a key transcription factor involved in lipid synthesis (Fryer and Carling, 2005). Similarly, the thiazolidinedione rosiglitazone, primarily used in the treatment of type 2 diabetes to alleviate peripheral insulin resistance, acts as a PPAR-γ agonist. This nuclear receptor regulates the expression of genes involved in glucose metabolism, lipid metabolism, and inflammation. In humans, rosiglitazone has been shown to reduce postprandial triglyceride concentrations (Mayerson et al., 2002; Tan et al., 2004). Interestingly, these pharmaceuticals' effects on lipid metabolism have also been reported in non-target aquatic species. For example, gemfibrozil has been shown to induce variations in plasma triglyceride and cholesterol levels, along with alterations in the expression of genes involved in lipid metabolism, such as *FABP*, *APOA1*, *APOB*, *FASN*, *SREBP2*, *HMGCR1*, *PPARA*, and *SREBP1*, in fish (Skolness et al., 2012; Al-Habsi et al., 2016). Moreover, Zhou et al. (2019) reported that acute exposure to metformin induced AMPK activation, enhanced lipid metabolism, and reduced lipid accumulation in fish hepatocytes. In contrast, such hypolipidemic effects of rosiglitazone in non-target organisms remain insufficiently investigated.

In this respect, in the present study, an integrated approach was applied to investigate the biological reactivity and the involvement of lipid metabolisms in response to gemfibrozil, metformin and rosiglitazone in the Mediterranean mussel *Mytilus galloprovincialis*. The three selected APIs were singularly tested though and *in vivo* (2.5µg/L) and *ex vivo* (500 and 1000µg/L) exposure to assess their potential to alter lipid metabolism and redox homeostasis in mussels. Sublethal effects were

evaluated following *in vivo* exposure, where a comprehensive panel of biochemical, cellular, and histological markers was assessed, including immunological and oxidative responses, genotoxic damage, neurotoxic effects, and changes in lipid metabolism. The *ex vivo* model of Precision-Cut Tissue Slices (PCTS) of mussels' digestive glands, previously validated for investigating the acute toxicological effects and mechanisms of action of chemical substances (Giuliani et al., 2019), was used as a supplementary methodology to further elucidate histological alterations induced by the selected pharmaceuticals in non-target marine species. The PCTS approach is considered a good compromise between the *in vivo* and *in vitro* studies, as it preserves the organ's physiology and natural architecture while maintaining tissue heterogeneity and cell-cell, as well as cell-matrix, interactions. This allows the PCTS model to more closely mimic the *in vivo* organization of the organ compared to traditional *in vitro* models (de Kanter et al., 2002; Cho & Yoon, 2017).

Overall, this work aims elucidate the impact of drugs targeting peroxisome proliferator-activated receptors (PPARs) on mussels' lipid metabolism, thereby enhancing our knowledge on mechanisms of action and potential risks of such emerging pollutants in non-target aquatic species.

5.2 Materials and methods

5.2.1 In vivo study

5.2.1.1 Animal collection and experimental plan

Mussels, *Mytilus galloprovincialis* (5.5 ± 1.00 cm shell length), were collected in May 2023 from a from a shellfish farm in Senigallia (Ancona, Adriatic Sea), and were acclimatized for 7 days with aerated artificial seawater (ASW; Istant Ocean®), at local seasonal environmental conditions of temperature (18 ± 1 °C), salinity (33 practical salinity units) and pH (8.20). Collection and experimental use of mussels is not subjected to ethical review permissions according to both European and Italian normative (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, 2010; Italian Legislative Decree n. 26, 2014), while monitoring guidelines recommend this species as appropriate bioindicator organism for assessing bioavailability of ecotoxicological effects of environmental pollutants in marine environments (Bocchetti & Regoli, 2006). Mussels were randomly distributed into 4 glass-tanks (n = 60 per tank) in a volume of 17 L of ASW, and exposed for 14 days to the four following treatments: CTRL, control condition (0.0029 % of dimethyl sulfoxide, DMSO); GEM, gemfibrozil exposure (2.5 µg/L); MET, metformin exposure (2.5 µg/L); ROS, rosiglitazone exposure (2.5 µg/L).

Stock solutions of GEM (Sigma Aldrich, CAS 25812-30-0), MET (Sigma Aldrich, CAS 1115-70-4), and ROS (Sigma Aldrich, CAS 122320-73-4) were obtained by dissolving drug powder in DMSO

and stored at +4 °C, while working solutions were prepared daily by diluting the stock solution in ASW. Water was changed every two days and pharmaceuticals were re-dosed. Mussels were fed with 500 µL of a commercial mixture of marine plankton (EasySPS EVO, size range 0.2 - 400 µm) before the water change. At the end of the experiment, for biochemical and histochemical analyses, hemolymph, gills and digestive glands were collected from 60 individuals, pooled in 20 separate samples, each constituted by tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at −80 °C. Aliquots of hemolymph were immediately processed: one for lysosomal membrane stability, phagocytosis activity, granulocytes-hyalinocytes ratio and DNA damage, while additional aliquots of hemolymph were fixed in Carnoy's solution (3:1 methanol and acetic acid solution) for the microscopy evaluation of micronuclei frequency.

5.2.1.2. Biomarkers analysis

Standardized protocols (Bocchetti and Regoli, 2006; Mezzelani et al., 2021) were used to measure biomarkers in tissues. Lysosomal membrane stability (LMS) was evaluated collecting the hemolymph of 8 organisms and incubated on a glass slide with a neutral red (NR) working solution (2 µl/mL filtered seawater). Hemocytes were microscopically examined every 20 minutes to define the time at which 50% of cells had lost into the cytosol the dye previously taken up by lysosomes.

Granulocyte/hyalinocyte ratio was analyzed by using aliquots of hemolymph dispersed on glass slides that, once dry, were fixed in Becker's fixative (2.5 NaCl). The slides were washed and stained with May Grunwald Giemsa before being mounted with Eukitt. Observations were carried out with a light microscope (1000x) and percentage of granulocytes was evaluated after counting at least 200 cells for each sample. Phagocytosis capacity assay was microscopically evaluated in hemolymph incubated for 2 hours at 15 °C in the dark with Fluorescein- labelled Zymosan A bioparticles (Invitrogen) at a 10:1 target:hemocyte ratio. Phagocytosis was expressed as percentage of cells that internalized at least 3 fluorescent particles.

Genotoxicity was evaluated at chromosomal level by the micronucleus test and at molecular level, as single strand breaks (SB), by the Comet assay and Diffusion assay. Micronuclei (MN) frequency was measured in hemocytes, rapidly washed in a saline buffer, fixed in Carnoy's solution (3:1 methanol: acetic acid), dispersed on glass slides and stained with fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) at 100 ng/mL. 2000 cells with preserved cytoplasm were scored for each specimen for the presence of micronuclei. The criteria used to identify a micronucleus were: a round structure, smaller than 1/3 of the main nucleus diameter and on the same optical plan of it while still possessing distinguishable boundaries from it.

Acetylcholinesterase (AChE) activity was spectrophotometrically assayed in mussels' gills (homogenized 1:3 w:v ratio in 0.1 M Tris-HCl buffer pH 7.2, 0.25 M sucrose and centrifuged at 10,000 ×g for 10 min) and haemolymph (centrifuged at 3000 ×g for 5 min). Obtained supernatants were spectrophotometrically assayed by the Ellman's reaction at 18 ± 1 °C and λ = 412 nm. For antioxidant enzymes, samples were homogenized (1:5 w:v ratio) in 100 mM K- phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/mL bacitracin, 0.008 TIU/mL aprotinin, 1 g/mL leupeptin, 0.5 g/mL pepstatin and NaCl 2.5 %, and were centrifuged at 110,000 x g for 1 hour at 4 °C. Measurements were carried out with a Varian (model Cary 3) spectrophotometer at a constant temperature of 18 °C (Regoli et al., 2004).

Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, ε = 0.04 mM^{-1} cm^{-1}) due to the consumption of hydrogen peroxide, H_2O_2 [12 mM H_2O_2 in 100 mM K-phosphate buffer (pH 7.0)]. Glutathione S-tranferases (GST) were determined at 340 nm using 1-chloro2,4dinitrobenzene (CDNB) as substrate. They were assayed in 100 mM K-phosphate buffer (pH 6.5), 1.5 mM CDNB and 1 mM GSH (ε = 9.6 mM^{-1} cm^{-1}). Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of GSSG (λ= 340 nm, ε = −6.22 mM^{-1} cm^{-1}). The final assay conditions were 100 mM K- phosphate buffer (pH 7.0), 1 mM GSSG and 60 μM NADPH. Total glutathione was measured in samples homogenized (1:5 w:v ratio) in 5 % sulfosalicylic acid with 4 mM EDTA, kept for 45 minutes in ice and then centrifuged at 37,000 xg for 15 minutes. An enzymatic method was used to determine the rate of formation of TNB which is proportional (up to 2 μM) to the glutathione concentration. Glutathione peroxidases (GP_x) were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to reconvert the formed GSSG into its reduced form (GSH). The decrease of absorbance was monitored at 340 nm (ε = -6.22 mM^{-1} cm^{-1}) in 100 mM K-phosphate buffer (pH 7.5), 1mM EDTA, 1 mM dithiothreitol, 1 mM NaN_3 (for hydrogen peroxide assay), 2 mM GSH, 1U GR, 0.24 mM NADPH and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates, respectively, for the Se-dependent and for the Se-dependent and Se-independent forms.

The Total Oxyradical Scavenging Capacity (TOSC) was measured in mussels' digestive glands homogenized as previously reported for enzymatic antioxidants, without PMSF in the homogenization buffer. The artificially generated radicals (ROO•, HO•) were obtained from the thermal homolysis of 20 mM 2'-azo-bis-(2- methylpropionamide)-dihydrochloride (ABAP) and the iron-EDTA (1.8 mM Fe^{3+}, 3.6 mM EDTA) plus 180 mM ascorbate, respectively. The absorption of artificially generated oxyradicals by cellular antioxidants was measured by the quantification of inhibited oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas. Determination of the ethylene formation was made by gas chromatographic analyses and TOSC values were

quantified from the equation: TOSC = 100 − (∫SA / ∫CA × 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curve produced during the reaction course for respective sample (SA) and control (CA) reactions. For all the samples, a specific TOSC was calculated by dividing the experimental TOSC values by the relative protein concentration, determined by the Lowry method with Bovine Serum Albumin (BSA) as standard (Regoli and Winston, 1999).

Lipofuscin and neutral lipids accumulation were evaluated through histological analysis on duplicate cryostat sections (8 μm thick) of digestive glands. Slides were fixed in Beker's fixative (+ 2.5 % NaCl) and stained by Schmrol reaction before mounting with Eukitt and by Oil red O (ORO) before mounting in glycerol gelatin for lipofuscin and neutral lipids content respectively. For both analysis, four measurements were made on digestive tubules of each section. Staining intensity was quantified with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of the digestive tubules.

Malondialdehyde (MDA) was quantified in digestive glands homogenized (1:3 w/v ratio) in 20 mM Tris–HCl (pH 7.4), centrifuged at 3,000 xg for 20 min. A conjugation reaction was performed in 1 mL reaction mixture (45 °C, 40 min), containing 10.3 mM 1- metyl-2-phenylindole (dissolved in acetonitrile/methanol, 3:1), 32 % HCl, 100 μL water and 100 μL of sample or standard [standard range 0–6 μM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl (pH 7.4)]. Samples were cooled on ice, centrifuged at 15,000 xg for 10 min and spectrophotometrically analysed at 586 nm. MDA concentrations were determined as a function of the 1,1,3,3-tetramethoxypropane standard curve and expressed as nmol/g tissue. Acyl-CoA oxidase's (ACOX) activity was evaluated in digestive glands homogenized in 1 mM sodium bicarbonate buffer (pH 7.6), containing 1 mM EDTA, 0.1 % ethanol and 0.01 % Triton X-100 and centrifuged at 500 xg for 15 minutes at 4 °C. H_2O_2 production was measured in a coupled assay (Bocchetti and Regoli, 2006) by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF- DA, 40 μM sodium azide, 0.01 % Triton X-100, 1.2 U/mL HRP in a final volume of 1 mL. After a pre-incubation at 25 °C for 5 minutes in the dark with an appropriate amount of sample, reactions were started adding the substrate Palmitoyl-CoA at final concentration of 30 μM and 100 μM for Acyl-CoA oxidase (AOX); readings were carried out at λ = 502 nm against a blank without the substrate.

5.2.2 Ex vivo study

5.2.2.1 Solutions and culture medium

The composition of buffers and culture medium was based on standardized protocols (Giuliani et al., 2019). Physiological solution (PS) (NaCl 436 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgSO_4$ 53 mM, pH 7.3) and Leibovitz's medium (L-15) (Gibco; adjusted to NaCl 436 mM, KCl 10 mM, $CaCl_2$ 10 mM,

$MgSO_4$ 53 mM and supplemented with L-glutamine 2 mM) were sterilized and supplemented with 1 % penicillin/streptomycin antibiotic mix.

5.2.2.2 Animal maintenance

Mussels *Mytilus galloprovincialis* (5.5 ± 1 cm) were obtained from a local aquaculture farm (Ancona, Adriatic Sea) and acclimatized for two weeks with recirculating Artificial Sea Water (ASW), at 33 ‰ salinity and 18 ± 1 °C (2 mussels/L). The animals were fed daily with a commercial mixture of marine plankton (EasySPS EVO, size range 0.2 - 400 µm) and the water changed three times a week.

5.2.2.3 Exposure of Precision Cut-Tissue Slices (PCTS)

PCTS were prepared from digestive glands (DG) according to standardized method (Giuliani et al., 2019). Briefly, each DG was excised carefully, the gastro-intestinal tract was washed with cold sterilized ASW and DG were maintained in cold (4 °C) sterilized ASW until slicing. DG were included in 2.5 % low melting point agarose and sectioned with a vibratome (VT1200S, Leica, Wetzlar, Germany) set at the following parameters: thickness 400 µm, amplitude 0.1 mm, speed 0.18 mm/s. Immediately after slicing, the PCTS were placed in cold PS supplemented with 10 mM D-glucose and then pre-incubated in 12-wells plate (3 PCTS/well) in L-15 with 10 % fetal bovine serum (FBS) (1 mL/well), at 18 °C and mild shaking (50 rpm) for 1:30 h. 24 PCTS were obtained from each DG. After pre-incubation, PCTS were exposed to ROS, GEM and MET (500 and 1000µg/L) in L-15 medium (both dissolved in DMSO at 0.5 % final concentration) for 48 h and 72 h. A control (PCTS in L-15 alone) and a vehicle control (PCTS in L-15 with 0.5 % DMSO) were included. For all treatments, the PCTS from each individual DG (24 PCTS/DG) were distributed in 8 wells (3 PCTS/well) of two 12-wells plate (each plate corresponded at an exposure time) and incubated in 1 mL of control, vehicle control or exposure medium at two doses. Every 24 h, the culture medium was renewed, and pharmaceuticals were re-dosed. The experimental plan was replicated many times to obtain, at the end of the exposure, for each treatment and experimental time, 2 PCTS from 3 mussels to include in cryostat embedding medium (Killik, Bio Optica) and frozen at -80 °C for histological analysis.

5.2.2.4 Histological analysis of neutral lipids and lipofuscin

Neutral lipids and Lipofuscin staining was performed on 4 cryostat sections (10 µm) obtained from 2 PCTS (400 µm) from the 3 DG used for each treatment and experimental time. Cryosections were fixed in Beker's fixative (2.5 % NaCl) for 15 min and stained by Oil red O (ORO) before mounting

in glycerol gelatin and by Schmrol reaction before mounting with Eukitt for neutral lipids and lipofuscin content, respectively. Slides were observed under light microscope. For both analysis, four measurements were made on digestive tubules of each section (one sections from each of the 2 PCTS of the 3 DG for each treatment). Quantification of staining intensity was performed with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of digestive tubules.

5.2.3 Statistical analysis

Statistical analyses were performed using RStudio (version 2023.12.0 Build 369). Data were assessed for normal distribution using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. For the *in vivo* study, the ANOVA was applied to test differences between treatments (level of significance at $p < 0.001$), and the Student Newman-Keuls test (SNK) was used for post-hoc comparison between means of values ($n = 5$). For the *ex vivo* study, at each exposure time, the comparison between the treatment and its solvent control was evaluated using one-way analysis of variance (ANOVA), level of significance at the 95 % of confidence interval, $\alpha = 0.05$. Post-hoc analysis was performed using TuckeyHSD to compare each experimental group.

5.3 Results

5.3.1 In vivo study

Results on immunological biomarkers revealed a significant reduction of the lysosomal membrane stability in mussels exposed to ROS, GEM and MET compared to control organisms (Fig. 5.1A), while a significant reduction of phagocytosis capacity was reported only in MET treatment (Fig. 5.1B). A significant increase in granulocyte/hyalinocyte ratio was observed in all treatments compared to the CTRL (Fig. 5.1C). Levels of micronuclei exhibited a marked enhancement in organisms exposed to MET, while no significant effect was observed in all other treatments (Fig. 5.1D).

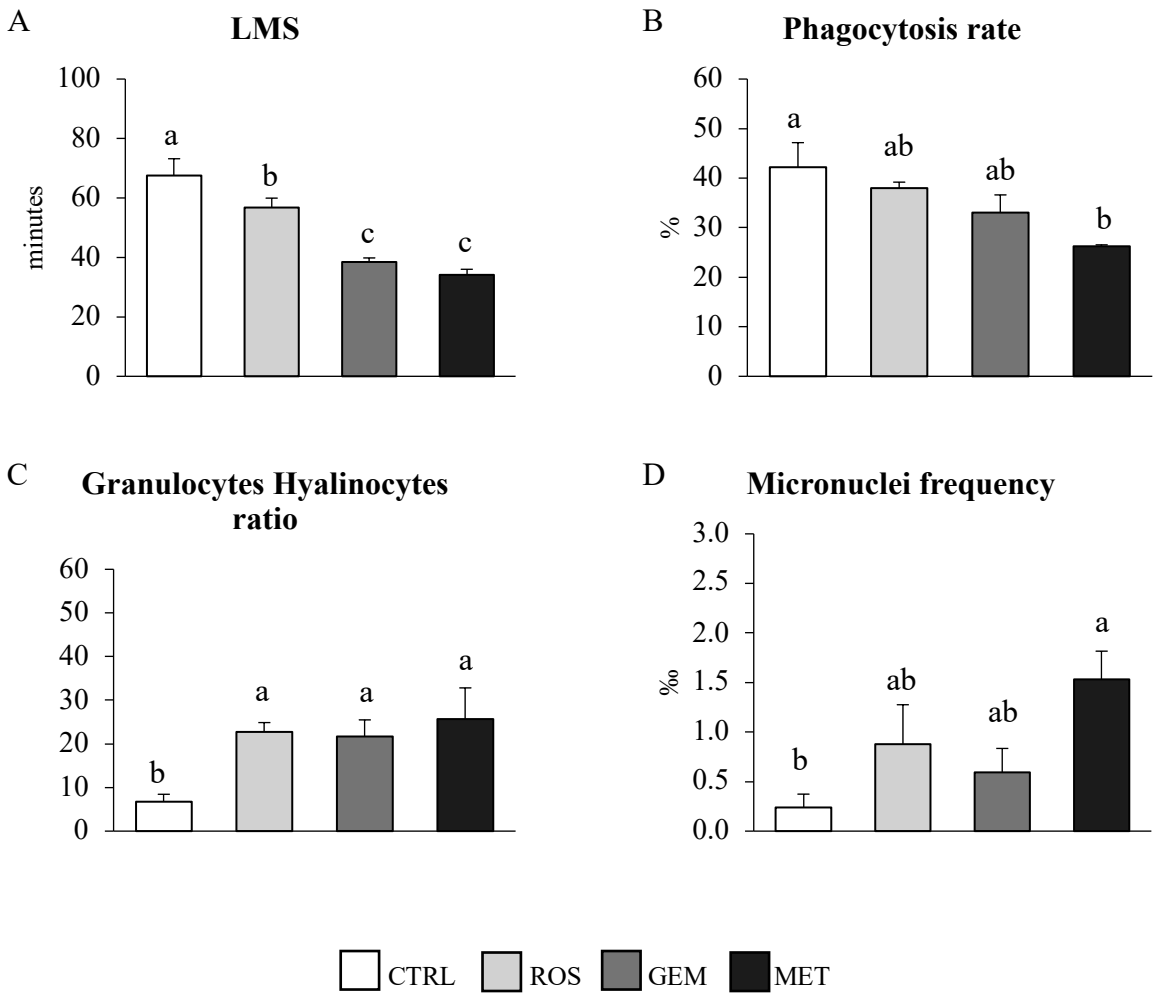


Figure 5.1 A) Lysosomal membrane stability, LMS; B) Granulocytes/hyalinocytes ratio; C) Phagocytosis rate; D) Micronuclei frequency in haemocytes of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 4). Different letters indicate significant differences between treatments. CTRL Control; ROS rosiglitazone; GEM gemfibrozil; MET metformin.

Acetylcholinesterase activity in hemolymph was significantly induced in ROS and GEM-exposed organisms, while in gills was significantly induced in organisms treated with ROS, GEM and MET (Fig. 5.2).

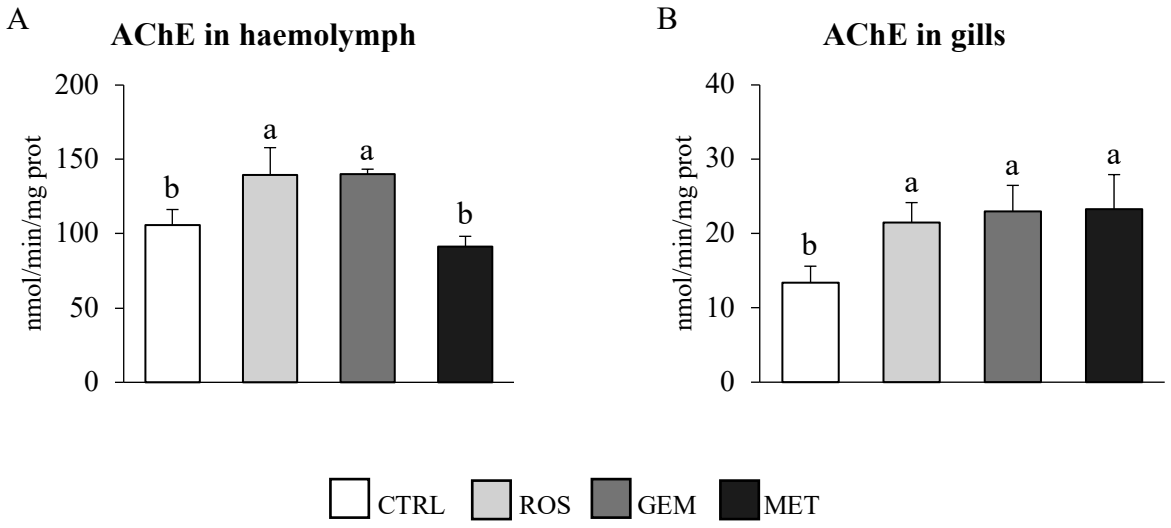
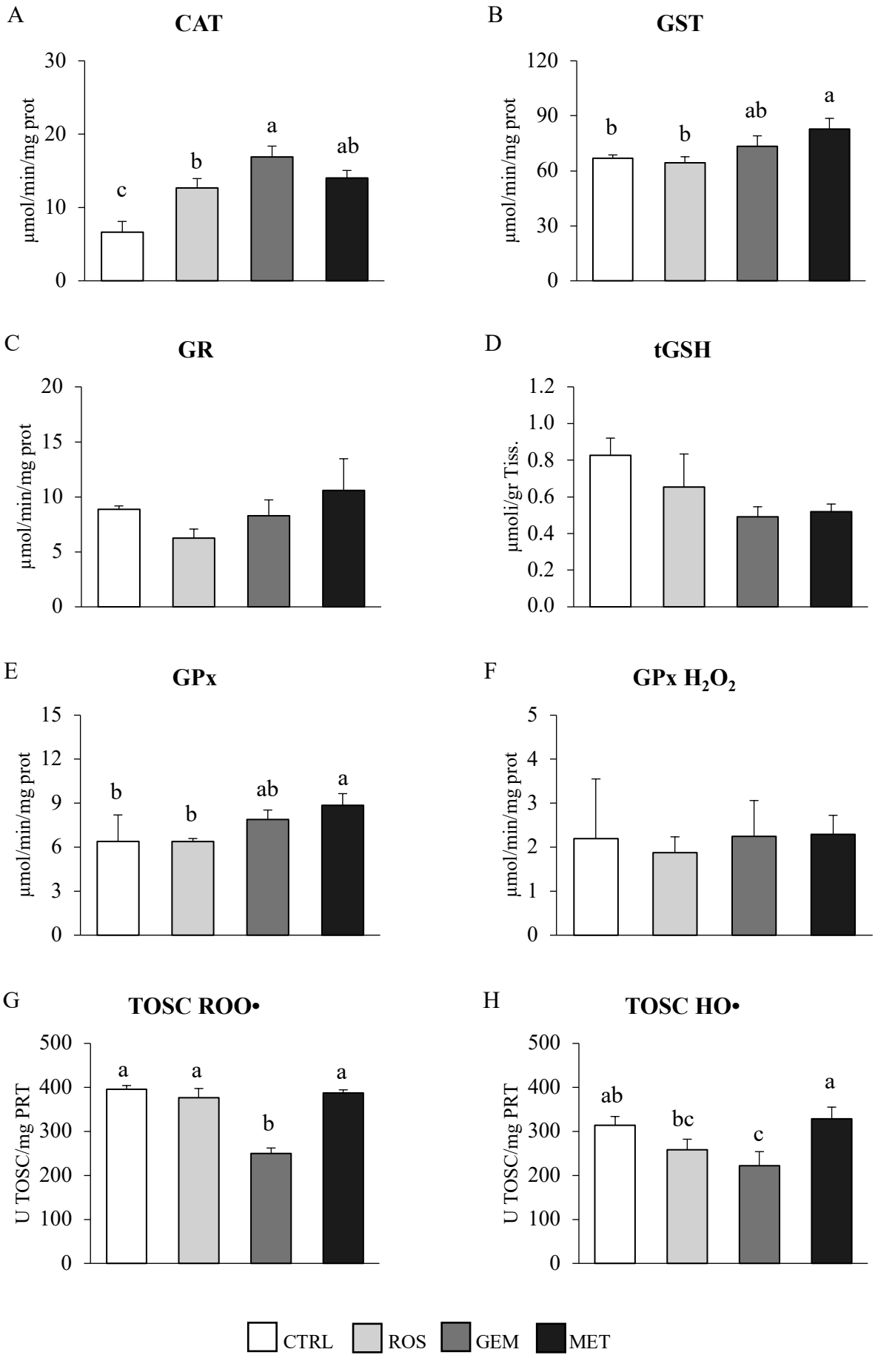


Figure 5.2 Neurotoxic damages. A) Acetylcholinesterase activity (AChE) in hemolymph and B) in gills of mussels exposed to different treatments. Data are given as mean values ± Standard Error of the Mean (n = 5). Different letters indicate significant differences between treatments. CTRL Control; ROS rosiglitazone; GEM gemfibrozil; MET metformin.

Results of single antioxidant enzymes and of the Total Oxyradical Scavenging Capacity are shown in Figure 5.3. Catalase activity statistically increased in all treatments (Fig. 5.3A), while glutathione-S-transferase and Sum of Se-dependent and Se-independent glutathione peroxidases activity was statistically induced only in MET exposed mussels (Fig. 5.3B, E).A lack of significant variation was reported for Se-dependent glutathione peroxidases, glutathione reductase and levels of total glutathione (Fig. 5.3C, D, F). The Total Oxyradical Scavenging Capacity toward peroxyl (ROO•) and hydroxyl (HO•) radicals highlighted significant lower values in organisms exposed to GEM, while limited variations were reported for all other treatments (Fig. 5.3G, H).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0
a
a
b
a
H
TOSC HO•
U TOSC/mg PRT
500
400
300
200
100
0
ab
bc
c
a
CTRL
ROS
GEM
MET

Figure 5.3 Antioxidant system responses in digestive glands of mussels exposed to different treatments. A) Catalase, CAT; B) Glutathione S-transferase, GST; C) Glutathione reductase, GR; D) Total glutathione, tGSH; E) Glutathione peroxidases CHP: sum of Se-dependent and Se-independent glutathione peroxidases; F) Glutathione peroxidases H_2O_2: Se-dependent glutathione peroxidases; G) TOSC ROO•: total oxyradical scavenging capacity toward peroxyl radical; H) TOSC HO•: total oxyradical scavenging capacity toward hydroxyl radical. Data are given as mean values ± Standard Error of the Mean (n = 3). Different letters indicate significant differences between treatments. CTRL Control; ROS rosiglitazone; GEM gemfibrozil; MET metformin.

A significant increase of lipofuscin and neutral lipids content was measured in mussels treated with ROS and GEM compared to CTRL, while no significant effects were highlighted for MET treatment (Fig. 5.4A, B). At cellular level, average higher values of malondialdehyde were measured in all treatments with a significant increased only in GEM exposed organisms (Fig. 5.4C), while a significant induction of acyl-CoA oxidase activity was reported only in mussels treated with MET (Fig. 5.4D).

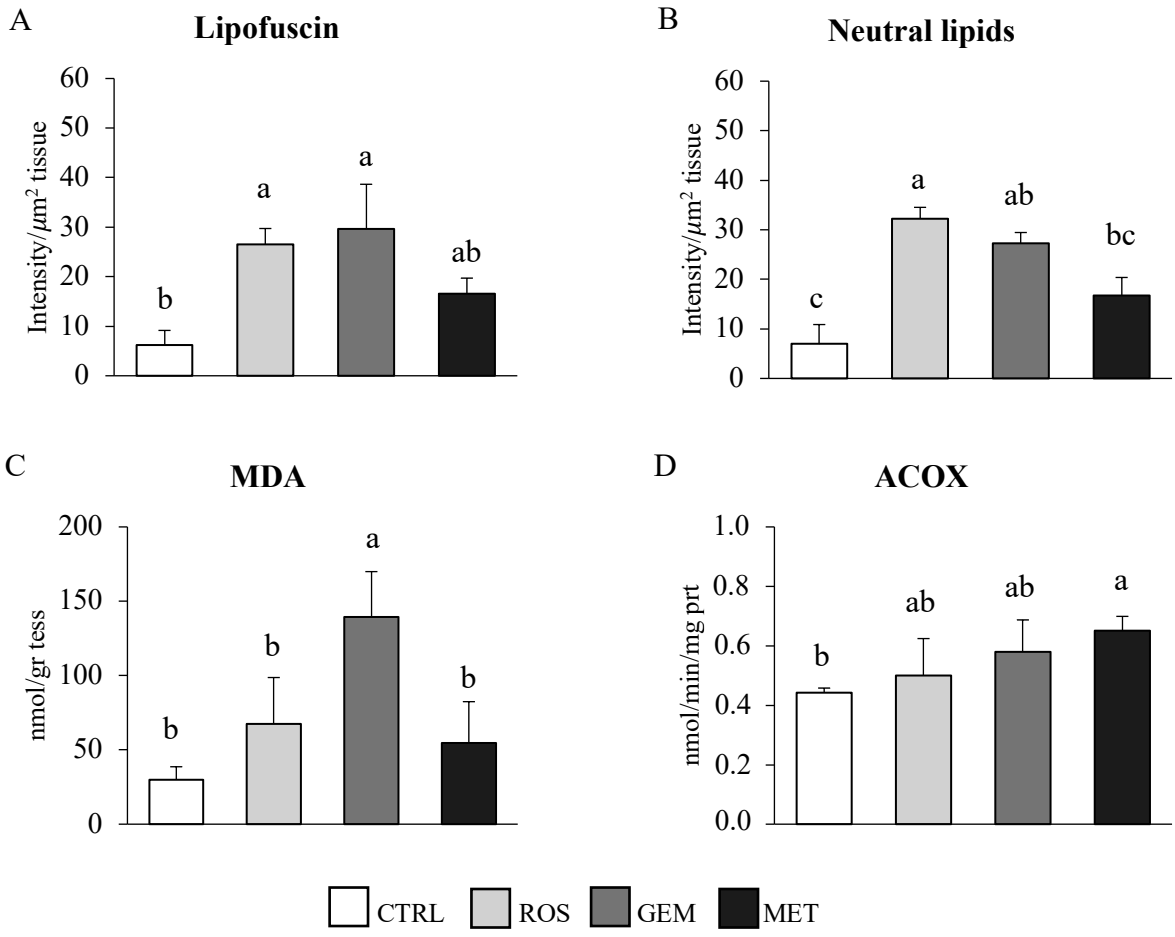
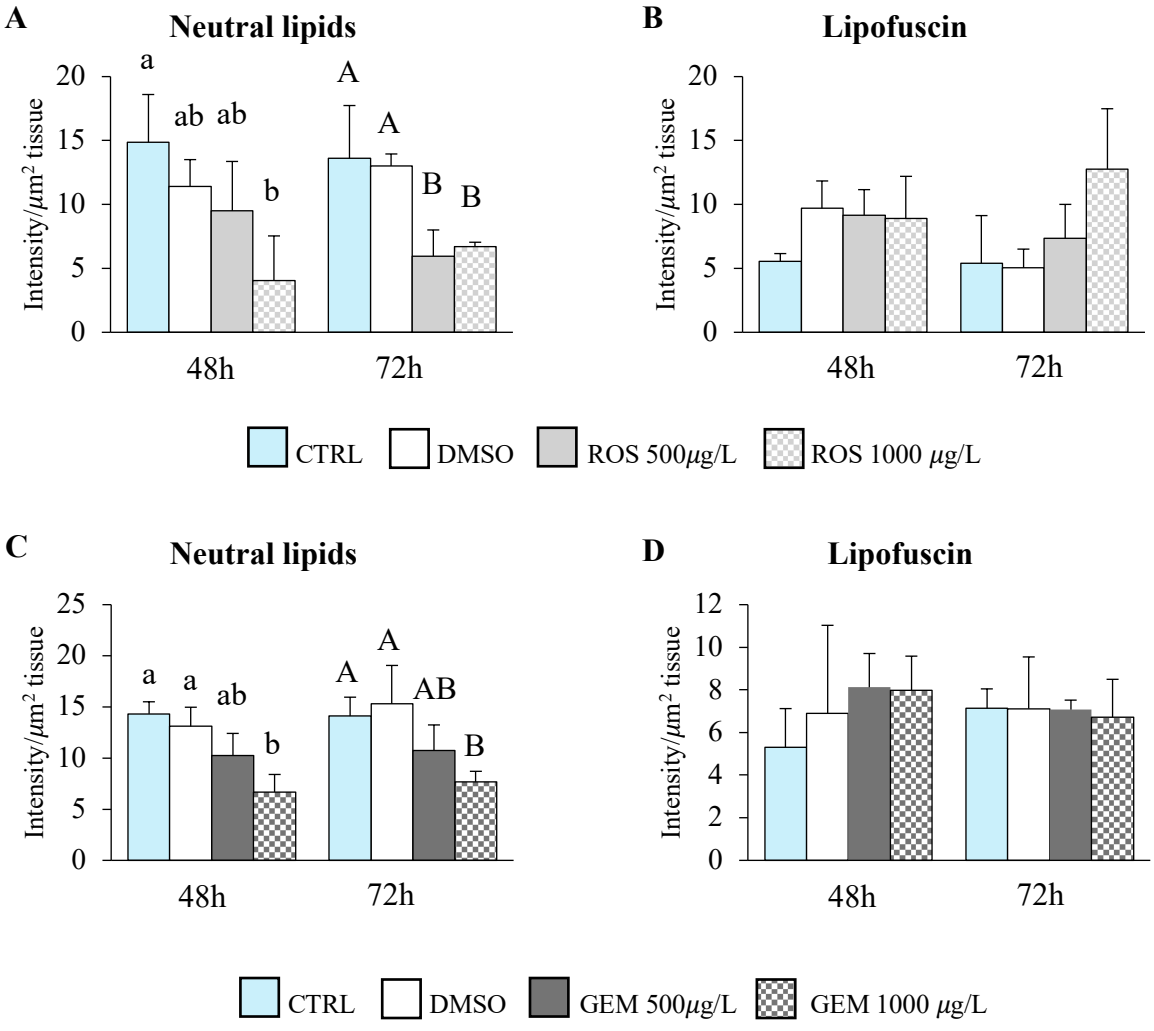


Figure 5.4 A) Lipofuscin and B) neutral lipids content; C) Levels of malondialdehyde, MDA; D) Acyl-CoA oxidase (ACOX) activity in mussels exposed to tested experimental conditions. Data are given as mean values ± Standard Error of the Mean (n = 4). Different letters indicate significant differences between treatments. CTRL Control; ROS rosiglitazone; GEM gemfibrozil; MET metformin.

5.3.2 Ex vivo study

Figure 5.5 shows the results of neutral lipids and lipofuscin content in PCTS exposed for 48 and 72 h to ROS, GEM and MET. A decreasing trend in neutral lipids content compared to the DMSO control was shown for ROS and GEM exposed-PCTS a both 48 and 72 h. A significant decrease was measured for ROS 500 µg/L after 72h and for ROS 1000 µg/L at both exposure times. GEM caused a significant depletion of NL levels only at the higher dose at both 48 and 72h (Fig 5.5A, C). An opposite trend was highlighted for MET treatment, which showed for both doses a significant increase after 48h of exposure, and average higher levels at 72h (Fig 5.5E). No variation of lipofuscin levels was measured for ROS and GEM at both doses and exposure times, while a significant enhance was reported for both doses of MET at 48h, and for 500 µg/L at 72h (Fig 5.5B, D, F).



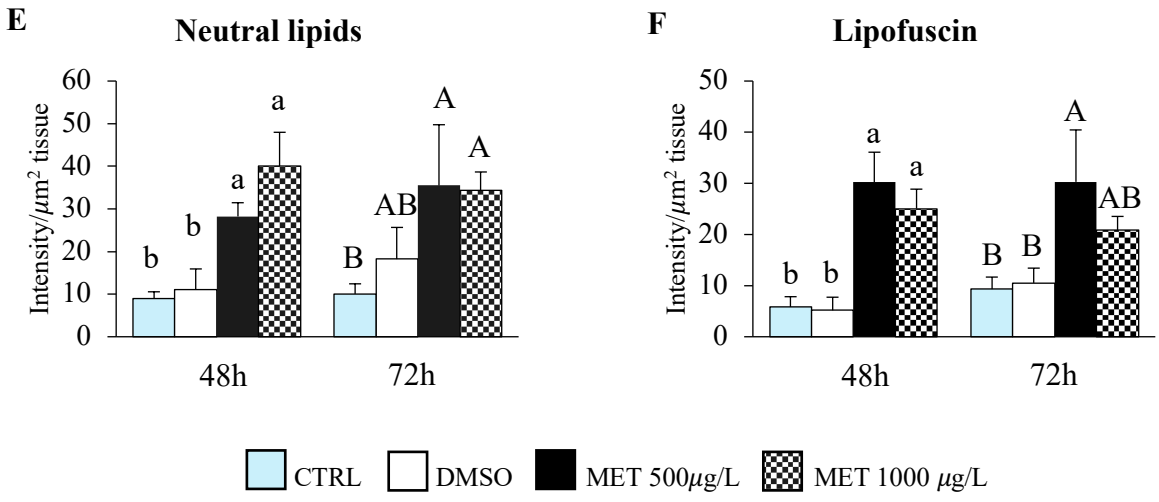


Figure 5.5 Content of neutral lipids (A, C, E), and content of lipofuscin (B, D, F) in PCTS exposed, for 48 and 72 h, to CTRL, CTRL in DMSO, ROS (500 μg/L – 1000 μg/L), GEM (500 μg/L – 1000 μg/L), and MET (500μ g/L – 1000 μg/L). Data are expressed as mean values ± standard errors (n = 3). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at 48h; capital letters are used to highlight significant differences between treatments at 72h. CTRL control; DMSO Dymethilsulfoxide; ROS rosiglitazone; GEM gemfibrozil; MET metformin.

5.4 Discussion

The environmental consequences of pharmaceuticals have recently emerged as a significant threat to aquatic ecosystems. Despite extensive research efforts over the past few decades to understand the effects of these emerging contaminants on non-target species, there remains a notable knowledge gap regarding the impact of certain therapeutic classes, particularly antidiabetic drugs and lipid regulators. Specifically, the present study investigated gemfibrozil, rosiglitazone, and metformin, all known agonists of peroxisome proliferator-activated receptors (PPARs), as potential pharmaceuticals with hypolipidemic properties in non-target species, by applying an integrated approach that combines both *in vivo* and *ex vivo* studies. The Mediterranean mussel (*Mytilus galloprovincialis*) was chosen as the model species due to its ecological significance, its sensitivity to molecular and cellular responses to environmental stressors, and the presence of PPAR homologs (Ran et al., 2019; Mezzelani et al., 2024). *In vivo* exposure allowed the detection of a wide panel of molecular, biochemical, and cellular responses, providing insights into the main metabolic pathways modulated by the tested drugs on this non target species.

The obtained results revealed that immune parameters are sensitive to these classes of pharmaceuticals, supporting previous studies that identified the immune system as one of the primary targets of these pollutants (Chapter 3; Mezzelani and Regoli, 2022; Afsa et al., 2022; Franzellitti et al., 2011). While all tested drugs caused a significant reduction of lysosomal membrane stability

(LMS) and increase of granulocyte/hyalinocyte ratio, only MET statistically reduced the hemocytes phagocytosis capacity. Although an increase in the granulocytes/hyalinocytes ratio was observed in all treatments, the phagocytosis capacity, primarily attributed to granulocytes, remained unchanged or even decreased (MET-exposed mussels), suggesting a reduction in the efficiency of granulocytes to phagocytize particles. In contrast to these findings, under different experimental conditions (lower dose of 1 µg/L and longer exposure time of 30 days), MET and GEM did not affect any immune parameters, suggesting a likely dose-dependent response (Chapter 3 - Experiment 2: Venlafaxine, gemfibrozil, ramipril, metformin, and their mixture). Confirming this hypothesis, the study of Koagouw and Ciocan (2018) reported severe pathologies of the gonadal tissues and the destabilization of the lysosomal membrane in hemocytes in the mussel *Mytilus edulis* after exposure to high MET concentrations (40 µg/L). Similarly, high doses of GEM (250 ng/g) injected in mussels' hemocytes caused lysosomal membrane destabilization (Canesi et al., 2007).

A significant increase in micronuclei frequency was observed in mussels treated with MET compared to the control organisms. Potential genotoxic effects of MET were also reported by Sibiya et al. (2023), who documented substantial DNA damage in *Labeo rohita* after 28 days of exposure to 80 µg/L of MET. In the same study, a lower exposure dose of 40 µg/L did not induce any detectable effects, highlighting a higher sensitivity of aquatic invertebrates to this drug compared to vertebrates. The neurotoxic properties of the tested drugs were investigated by measuring the activity of the enzyme acetylcholinesterase (AChE) in the hemolymph and gills. The results revealed a significant induction after exposure to ROS and GEM in both hemolymph and gills, whereas an enhancement of AChE activity was measured only in the gills following MET treatment. Slight AChE induction was also observed by Zurita et al. (2007) in the PLHC-1 cell line following acute exposure to high doses (100 µM) of GEM. Although limited information is available in the literature regarding the neurotoxic effects of the antidiabetics ROS and MET in aquatic non-target organisms, a previous study on mice reported that the PPAR agonist pioglitazone protected against cognitive impairment induced by scopolamine, a muscarinic antagonist, by reducing the increased AChE activity (Xiang et al., 2012). Such findings revealed a mechanism of action in mammals that differs from the one observed in our study on aquatic invertebrate species.

Pro-oxidant effects of tested pharmaceuticals were assessed integrating measurements of individual antioxidant enzyme activities and the total oxyradical scavenging capacity (TOSC) toward peroxyl (ROO•) and hydroxyl (HO•) radicals with data on the accumulation of lipid peroxidation products, such as lipofuscin and malondialdehyde content. Outcomes revealed the capability of all PPARs agonists to influence oxidative status of mussels. A significant induction in catalase (CAT) activity was observed in all exposed organisms, while a marked decrease in TOSC toward both radicals was

detected in response to GEM, further supporting previous findings of its pro-oxidant effects in aquatic non-target species (Chapter 3- Experiment 2; Barreto et al., 2018). Interestingly, a significant induction of glutathione S-transferase (GST) activity was observed in MET treatment, confirming the involvement of phase II conjugation reactions in the biotransformation and excretion of this drug in marine mussels (Chapter 3- Experiment 2). It is noteworthy that the effects of MET on the antioxidant system were dose-dependent, with low doses (1 µg/L, Chapter 3, Experiment 2) not inducing pro-oxidant effects, slightly higher concentrations (2.5 µg/L, *in vivo* study) modulating catalytic responses through the induction of antioxidant enzymes such as CAT and glutathione peroxidase (GP_x), and higher doses (500 - 1000 µg/L, *in vitro* study) causing cellular alterations, including a marked increase in lipofuscin accumulation.

Results obtained from mussel's PTCS exposure revealed an increase in neutral lipids content in response to MET. Such findings would appear to be in contrast with the hypolipidemic properties previously demonstrated for this drug in target organisms (Fryer and Carling, 2005). Considering the significant increment in lipofuscin levels, a marker typically associated with the onset of oxidative stress (Giuliani et al., 2014), it can be hypothesized that the observed enhancement of neutral lipids may be linked to a strong impairment of oxidative homeostasis, rather than a direct induction of lipid synthesis. Similarly, results from the *in vivo* study, which demonstrated pronounced pro-oxidant effects of GEM and ROS at both catalytic (induction of antioxidant enzymes) and cellular level (increase in lipofuscin and malondialdehyde), also showed a significant enhance in neutral lipid content. Furthermore, MET exposure in the same experiment did not cause significant variations in lipofuscin content, suggesting a lower pro-oxidant effect compared to the other tested drugs, and consequently, no increase in neutral lipids content was detected. Different behavior was observed for GEM and ROS: mussels' PCTS treated with these drugs showed no changes in lipofuscin content but exhibited a decrease in neutral lipid levels, with significant variations observed at the higher dose (1000 µg/L) at both 48 and 72 hours, confirming their lipid-lowering effects also in non-target organisms.

In conclusion, the present work highlighted the PPARs agonists capability to modulate mussels' lipid and oxidative metabolisms under both *in vivo* and *ex vivo* conditions. The hypolipidemic proprieties of GEM and ROS were confirmed for higher exposure dose (1000 µg/L), while the increased neutral lipids levels observed at lower exposure dose (2.5 µg/L) and in MET treatment (500 - 1000 µg/L) appear to be associated to a significant imbalance of redox homeostasis rather than a lipogenesis induction. Overall, combination of such approaches allowed to deepen knowledge on mechanism of actions of tested drugs in non-target organisms, demonstrating that the PCTS model could represent an added value within integrated strategies for the toxicity assessment of these pollutants.

6. Cellular effects of environmental pharmaceuticals: mechanisms of toxicity on fish hepatoma (PLHC-1) and gill (RTgill-W1) cell lines

6.1 Introduction

Active pharmaceutical ingredients (APIs) represent emerging contaminants of particular concern for their potential impact on the environment. Although their environmental persistence is influenced by several factors (such as their physical and chemical properties, pH, temperature, and photolysis by solar irradiation (Couto et al., 2019), these substances can behave as pseudo persistent pollutants due to their continuous release and the inefficient removal by wastewater treatments plants (Bahlmann et al., 2014; Peake et al., 2016; Mo et al., 2024). Psychiatric compounds, non-steroidal anti-inflammatory drugs (NSAIDs), antidiabetics, cardiovascular drugs and lipid lowering agents are widely documented in aquatic ecosystems and organisms worldwide (Mezzelani et al., 2020; Mezzelani and Regoli, 2022; Swiacka et al., 2022).

The exposure of non-target species to complex mixtures of pharmaceuticals with similar, different, or even opposing mode of actions can determine unexpected effects on several metabolic pathways (Mezzelani and Regoli, 2022). A better knowledge on early molecular and cellular responses is thus useful for both elucidating mechanisms of action of environmental pharmaceuticals and also to predict the onset of long-term toxicological effects or changes at higher levels of biological organization (Mezzelani and Regoli, 2022).

Fish cell cultures represent an appropriate model for such investigations (Franco et al., 2018), fulfilling the 3R principle (Refinement, Reduction and Replacement) and the animal welfare regulation (*e.g.* Directive 2010/63/ EU on the protection of animals used for scientific purposes). The hepatocellular carcinoma cell line derived from the desert topminnow *Poeciliopsis lucida* (PLHC-1) has been used to evaluate metabolic activity and cytotoxicity of a wide panel of chemical substances, including pharmaceuticals (Thibaut and Porte, 2008; Traven et al., 2008; Pérez-Albaladejo et al., 2016), due to its capacity to express the cytochrome P4501A system through the aryl hydrocarbon receptor (AHR): such biotransformation pathway is often involved in hepatic drug metabolization of vertebrates (Schlenk et al., 2008). Similarly, the RTgill-W1 cell line, derived from *Oncorhynchus mykiss*, has become widely used *in vitro* model for evaluating the toxicity of environmental pollutants (OECD, 2014). Beyond its role in environmental monitoring, RTgill-W1 is increasingly employed in ecotoxicological studies to assess the cytotoxicity, bioaccumulation, and sub-lethal effects of pharmaceutical compounds on aquatic non-target organisms (Zeng et al., 2016; Franco et al., 2018). Several studies also demonstrated that metabolism of APIs is associated to an increase in reactive oxygen species (ROS) production (Thibaut et al., 2008), and such enhanced oxidative conditions could promote the pharmaceuticals capability to exert cytotoxic effects (Ghosh et al., 2015;

Mezzelani and Regoli, 2022). Some drugs, including NSAIDs and psychiatric compounds, further affect the ROS-mediated induction of apoptosis through the inhibition of protein kinase B (Akt) signaling pathways, the modulation of the transcription factor nuclear factor κB (NF-κB), and the alteration of the protein 53 (p53) pathway (Djordjevic et al., 2011; Mezzelani and Regoli, 2022).

The aim of this work was to provide novel insights on mechanisms of action and toxicity of a wide number of pharmaceuticals dosed alone and as mixtures on fish hepatic (PLHC-1) and gill (RTgill-W1) cell lines, selected for their critical roles in xenobiotic uptake and metabolism in aquatic species. Cell viability, ROS production and mitochondrial membrane potential were assessed to evaluate respectively the cytotoxicity, the capability to induce oxidative stress and cell apoptosis of 15 pharmaceuticals belonging to psychiatric drugs (venlafaxine, O-Desmethylvenlafaxine, paroxetine, fluoxetine, carbamazepine, carbamazepine-10,11 epoxide), NSAIDs (ibuprofen, naproxen, diclofenac, nimesulide), cardiovascular compounds (valsartan, atenolol, ramipril), lipid lowering agents (gemfibrozil) and antidiabetics (metformin). The obtained results were summarized within a weight of evidence approach allowing to better compare the biological significance of the experimental conditions. The findings of this work were expected to add further evidence on toxicological proprieties of environmental pharmaceuticals and to improve future environmental risk assessments in aquatic ecosystems.

6.2 Materials and methods

6.2.1 Chemicals

Selected pharmaceuticals including both parent compounds and metabolites were purchased from Sigma-Aldrich® (St. Louis, MO, USA): Venlafaxine (VEN, CAS 99300-78-4), O-Desmethylvenlafaxine (O-DESVEN, CAS 93413-62-8), Paroxetine (PAR, CAS 110429-35-1), Fluoxetine (FLU, CAS 56296-78-7), Carbamazepine (CBZ, CAS 298-46-4), Carbamazepine-10,11 epoxide (CBZ-EP, CAS 36507-30-9), Ibuprofen (IBU, CAS 15687-27-1), Naproxen (NAP, CAS 22204-53-1), Diclofenac (DIC, CAS 15307-79-6), Nimesulide (NIM, CAS 51803-78-2), Valsartan (VAL, CAS 137862-53-4), Atenolol (ATE, CAS 29122-68-7), Ramipril (RAM, CAS 87333-19-5), Gemfibrozil (GEM, , CAS 25812-30-0) and Metformin (MET, CAS 1115-70-4). Stock solutions (1 mg/mL) were prepared in methanol (MeOH) as solvent and subsequently diluted in culture medium the day of the experiment. The MeOH concentration in the experimental solutions was 0.001 %. The toxicological effects of the 15 pharmaceuticals were assessed on single compounds (at two environmental realistic doses of 1 and 10 μg/L) and in 6 different mixtures (MIX). MIX were selected combining drugs based on the mechanism of action of single molecules, their frequency of detection in the environment, and the typical combination in human therapy. In this respect, the tested MIX

were: IBU + PAR (MIX A); CBZ + VAL (MIX B); IBU + PAR + CBZ + VAL (MIX C); VEN + RAM + GEM + MET (MIX D); VEN + NAP (MIX E); VAL + ATE + GEM + RAM (MIX F). Also in the MIX treatments, individual compounds were tested at both 1 and 10 μg/L, as carried out for single molecules.

6.2.2 Cell cultures: PLHC-1 and RTgill-W1

PLHC-1 liver cells, derived from topminnow *Poeciliopsis lucida* (Ryan and Hightower, 1994), were cultured using a Eagles's minimum essential medium supplemented with 5 % fetal bovine serum (FBS) (Biowest) and 1 % penicillin-streptomycin (5000 U/mL) (Biowest) in a 5 % carbon dioxide (CO_2) humidified incubator at 30 °C (Blanco et al., 2018). Rainbow trout RTgill-W1 gill cells were cultured using the Leibovitz's L-15 medium supplemented with 10% FBS and 1 % penicillin and streptomycin (Biowest) and maintained at 18 ◦C. Both cell lines were grown in 75 cm^2 culture flasks and, when 90 % of confluence was reached, they were detached using trypsin (0.25 % in ethylenediamine tetraacetic acid, EDTA) in subculture. Cells were seeded in 96 well plates with a cell density of 30,000 cells/mL, determined using the Muse® Cell Analyzer, from a culture flask with 90 % monolayer confluency and > 90 % of cell viability. The methods for cell count and viability measurements of the cell lines are provided in the Supplementary Materials. After 24 h, the culture medium was replaced with the exposure solution. The present experiment was carried out in accordance with European normative (Directive 2010/63/EU).

6.2.3 Cell viability

Cell viability was measured as a marker of cytotoxicity by the fluorescent dye resazurin (AlmarBlue™) monitoring cell metabolic activity (Dayeh et al., 2003) after 24h of exposure to single pharmaceuticals and MIXs. The fluorescence at 530/590 nm was recorded using a SpectraMax Paradigm Multi-Mode plate reader. Results were expressed as percentage viability (mean ± standard deviation) compared to the solvent control (0.001 % MeOH). Seven replicates were prepared for each tested concentration of the single pharmaceutical, MIXs, control, solvent control and 0.1 μM H_2O_2 (positive control).

6.2.4 Generation of reactive oxygen species (ROS)

The fluorescence of the oxidized probe (2′7′-dichlorodihydrofluorescein diacetate, Invitrogen) at 485/528 nm (LeBel et al., 1992; Blanco et al., 2018) was measured to assess the generation of ROS after 1h of exposure to single pharmaceuticals and MIXs. Results were expressed as percentage

change in fluorescence (mean ± standard deviation) compared to the solvent control. Seven replicates were prepared for each tested concentration of the single pharmaceutical and MIXs, control, solvent control and positive control.

6.2.5 Mitochondrial membrane potential (Δψ)

The change in mitochondrial inner membrane electrochemical potential was detected after 1h of exposure to single pharmaceuticals and MIXs using the cationic, lipophilic dye JC-1 (5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimi-dazoylcarbocyanine iodide), applying the Mitochondria Staining Kit protocol (CS0390 Sigma-Aldrich). The principle of the method is the comparison between the red fluorescence (490 nm excitation/ 530 nm emission) of the aggregated JC-1, representing intact mitochondria, to the green fluorescence (525 nm excitation/ 590 nm emission) of the monomeric JC-1, which represents disrupted mitochondria. Results were expressed as percentage change in fluorescence (mean ± standard deviation) compared to the solvent control. Seven replicates were prepared for each concentration of the single pharmaceutical and mixtures, control, solvent control and Valinomycin (0.2 μg/mL, used as a positive control for this assay).

6.2.6 Statistical analyses

Statistical analyses were performed using RStudio (version 2023.12.0 Build 369). Data were assessed for normal distribution using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. Comparisons between each treatment and control groups were analyzed using one-way analysis of variance (ANOVA), level of significance at the 95 % of confidence interval, $\alpha = 0.05$. The ANOVA was used to test differences between treatments within the same therapeutic class of APIs. Post-hoc analysis was performed using the Tukey HSD to compare the means of values ($n = 7$).

6.2.7 Weight of Evidence (WOE) elaboration of all biological endpoints in PLHC-1 and RTgill-W1 exposed to single pharmaceuticals and mixtures

Weighted criteria were applied to elaborate and integrate the results on cell viability, ROS production and mitochondrial membrane potential summarizing a qualitative and quantitative hazard index, which reflected the severity of effects caused by different pharmaceuticals and MIXs. The conceptual framework was adapted from the mathematical structure developed for elaborating ecotoxicological bioassays within the Sediqualsoft Weight of Evidence (WOE) model (Piva et al., 2011; Regoli et al., 2019). Briefly, each typology of response had a "weight" based on toxicological relevance of the measured endpoint, and a "threshold" reflecting its sensitivity and the percentage of variation

considered of biological relevance. The assigned "weights" were 1.0 for ROS production, 1.2 for mitochondrial membrane potential, and 1.5 for cell viability. The selected "thresholds" were 20 % for the inhibition for all the endpoints, and 20 % for induction for both cell viability and ROS production. For each experimental condition the cumulative Hazard Quotient (HQ) was calculated from the magnitude of variations observed for various responses normalized to their thresholds and toxicological relevance (weights). The HQ obtained for each tested pharmaceutical and MIX was finally assigned to one of five classes of hazard, which were Absent, Slight, Moderate, Major, and Severe. A detailed description of whole calculations, detailed flow-chart, rationale for weights, thresholds and expert judgements used in this work are given in Supplementary Materials.

6.3. Results

6.3.1 PLHC-1

6.3.1.1 Cell viability

For psychiatric drugs (Fig. 6.1A), a significant and dose-dependent decrease in the cell viability was observed in response to VEN, PAR, FLU and CBZ-EP; particularly marked was the effect measured for PAR and CBZ-EP which, at the highest concentration (10 μg/L), showed similar viability to cells exposed to the positive control (0.1 μM H_2O_2). More limited or not significant effects in viability were observed for the cells exposed to O-DES and CBZ (1 - 10 μg/L). Pharmaceuticals belonging to therapeutic class of NSAIDs (Fig. 6.1B) did not cause any variation in the cell viability of PLHC-1 cells, with the only exception of NAP that showed a significant and dose-dependent effect in exposed cells. Among cardiovascular drugs (Fig. 6.1C), a major reduction in viability was observed for ATE at both concentrations, with values similar to cells exposed to the positive control. On the other hand, an increased cell viability was highlighted for RAM at both tested doses, and not significant variations were measured for cells exposed to VAL. The lipid lowering GEM caused a dose-dependent decrease in PLHC-1 cell viability (Fig. 6.1D), while the antidiabetic MET significantly increased this response at both tested doses (Fig. 6.1E).

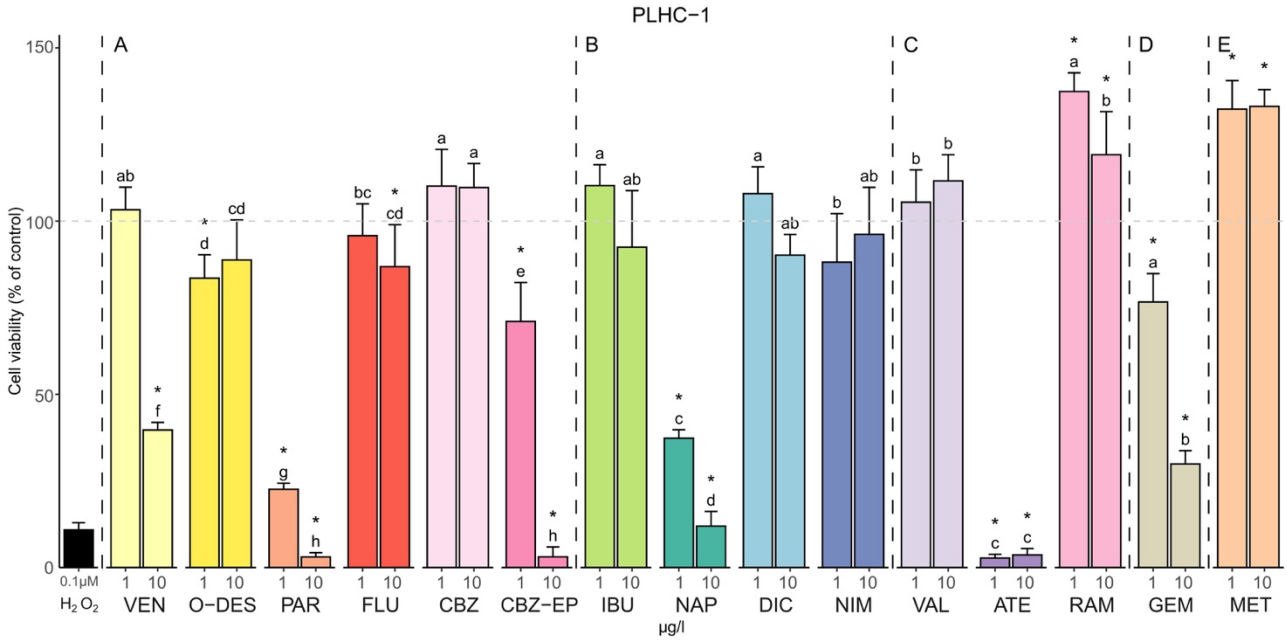


Figure 6.1. The effect of (A) psychiatric drugs, (B) Non-Steroidal Antinflammatory Drugs, (C) cardiovascular drugs, (D) the lipid lowering agent and (E) the antidiabetic on cell viability in fish liver cell cultures (PLHC-1). The graph describes the cell viability after 24h exposure to pharmaceuticals (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.1 µM H_2O_2 was used as a positive control of reduced viability. Results are presented as mean ± SD (n = 7). * (*p*-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA.
Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). VEN Venlafaxine; O-DESVEN O-Desmethylvenlafaxine; PAR Paroxetine; FLU Fluoxetine; CBZ Carbamazepine; CBZ-EP Carbamazepine-10,11 epoxide; IBU Ibuprofen; NAP Naproxen; DIC Diclofenac; NIM Nimesulide; VAL Valsartan; ATE Atenolol; RAM Ramipril; GEM Gemfibrozil; MET Metformin.

The exposure to pharmaceuticals MIXs (Fig. 6.2) highlighted dose-dependent effects for all treatments with a statistically significant decrease in cell viability in MIXs B and F at both exposure doses, and only at the higher concentration (10 µg/L) for all the other MIXs. Cytotoxic effects comparable to positive control were observed for MIXs B and D at the higher exposure dose.

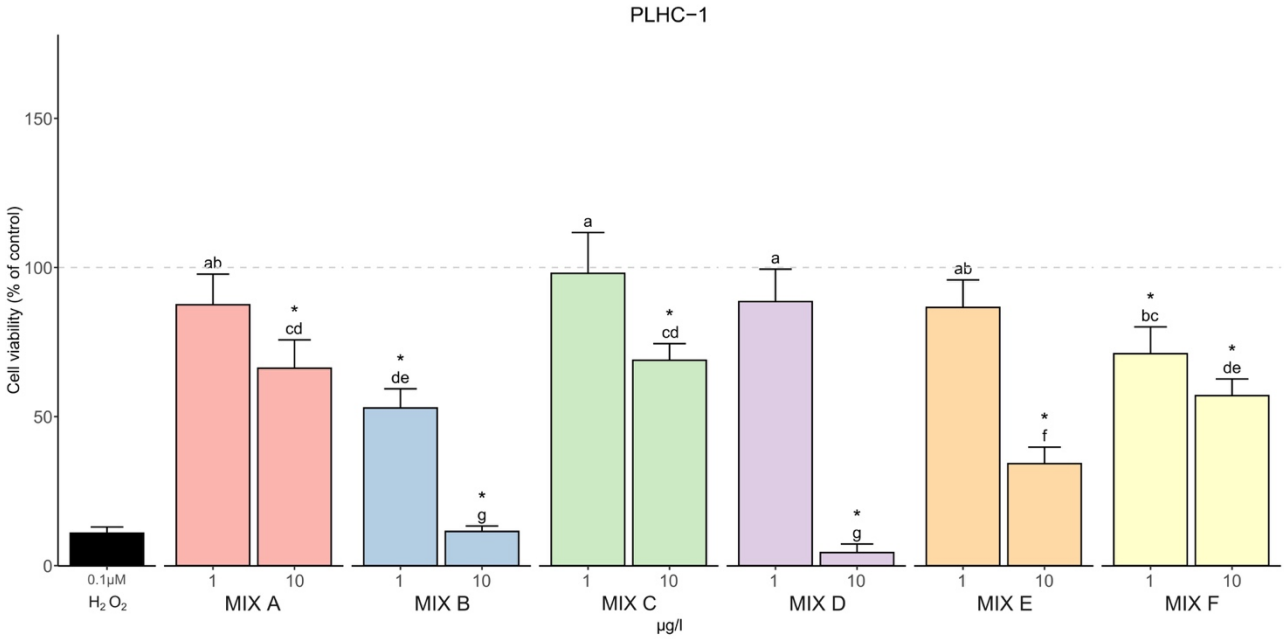


Figure 6.2. The effect of pharmaceuticals mixtures on cell viability in fish liver cell cultures (PLHC-1). The graph describes the cell viability after 24h exposure to pharmaceuticals mixtures (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.1 µM H_2O_2 was used as a positive control of reduced viability. Results are presented as mean ± SD (n = 7). * (p-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX C: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Ramipril + Gemfibrozil + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril.

6.3.1.2 Reactive oxygen species production

A statistically significant and generally dose-dependent induction of ROS was observed after exposure to the psychiatric drugs VEN, PAR and CBZ, while a significant decrease was highlighted for O-DES, FLU and CBZ-EP (Fig. 6.3A). Among NSAIDs, no variations were measured for IBU and DIC, a lowered ROS formation was observed in cells exposed to NIM, a statistically significant and dose-dependent production of ROS was highlighted in response to NAP (Fig. 6.3B). Cardiovascular drugs (Fig. 6.3C) showed increased levels of ROS for VAL (1 µg/L), ATE (1 µg/L) and RAM (10 µg/L), no effects were measured for the lipid lowering GEM at both tested concentrations (Fig. 3D), and a significant enhancement of ROS was highlighted after the exposure of PLHC-1 cells to the lower dose of the antidiabetic MET (Fig. 6.3E).

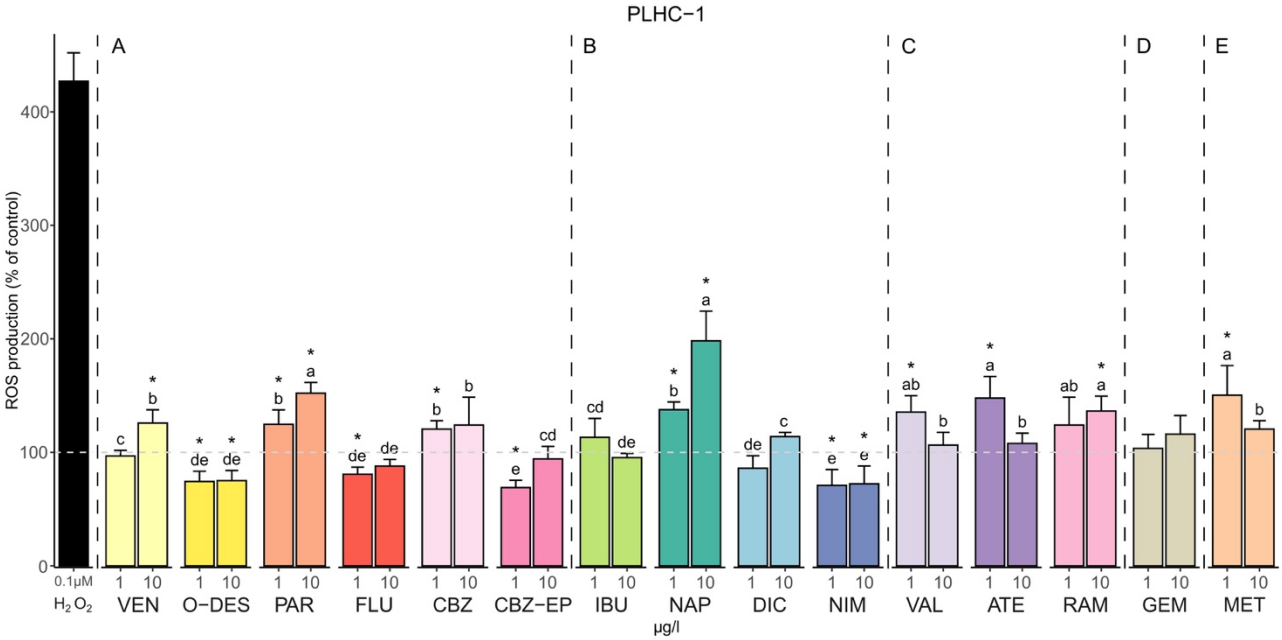


Figure 6.3. The effect of (A) psychiatric drugs, (B) Non-Steroidal Antinflammatory Drugs, (C) cardiovascular drugs, (D) the lipid lowering agent and (E) the antidiabetic on reactive oxygen species (ROS) production in fish liver cell cultures (PLHC-1). The graph describes ROS generation measured in PLHC-1 after 60 minutes of exposure to pharmaceuticals (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.1 µM H_2O_2 was used as a positive control of cellular ROS production. Results are presented as mean ± SD (n = 7). * (*p*-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). VEN Venlafaxine; O-DESVEN O-Desmethylvenlafaxine; PAR Paroxetine; FLU Fluoxetine; CBZ Carbamazepine; CBZ-EP Carbamazepine-10,11 epoxide; IBU Ibuprofen; NAP Naproxen; DIC Diclofenac; NIM Nimesulide; VAL Valsartan; ATE Atenolol; RAM Ramipril; GEM Gemfibrozil; MET Metformin.

A statistically significant increase in ROS formation was caused by the exposure to MIXs A, D, E at the higher dose, and MIX B at both doses, while a decrease was measured in cells treated with MIXs A (1 μg/L) and C (10 μg/L) (Fig. 6.4).

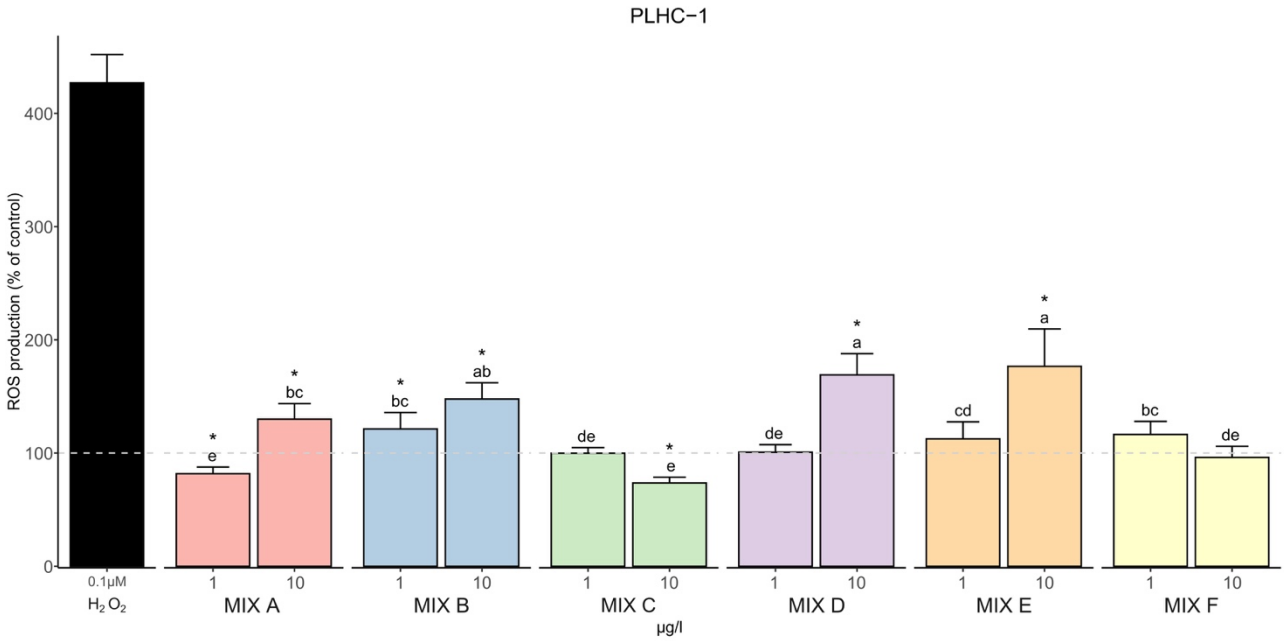


Figure 6.4. The effect of pharmaceutical mixtures on reactive oxygen species (ROS) production in fish liver cell cultures (PLHC-1). The graph describes ROS generation measured in PLHC-1 after 60 minutes of exposure to pharmaceuticals mixtures (1 - 10 μg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.1 μM H_2O_2 was used as a positive control of cellular ROS production. Results are presented as mean ± SD (n = 7). * (*p*-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX C: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Ramipril + Gemfibrozil + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril.

6.3.1.3 Mitochondrial membrane electrochemical potential change

Results of psychiatric drugs highlighted a dose-dependent loss of $\Delta\psi_m$ in cells exposed to VEN, PAR, FLU, CBZ, CBZ-EP, with significant decrease for VEN (1 - 10 µg/L), PAR (10 µg/L), FLU (10 µg/L) and CBZ-EP (10 µg/L). No effect was observed for O-DES at both exposure doses (Fig. 6.5A). All the NSAIDs caused a significant loss in mitochondrial membrane potential at the higher tested concentration, and a similar effect was obtained also at the lower exposure dose for NAP and DIC (Fig. 6.5B). No variations were observed for the cardiovascular drugs VAL and RAM, while a significant and dose-dependent decrease of $\Delta\psi$ was highlighted for ATE (Fig. 6.5C). The lipid lowering GEM caused a decrease in mitochondrial membrane potential at the higher exposure dose (Fig. 6.5D), and a significant effect was observed for the antidiabetic MET at both tested concentrations (Fig. 6.5E).

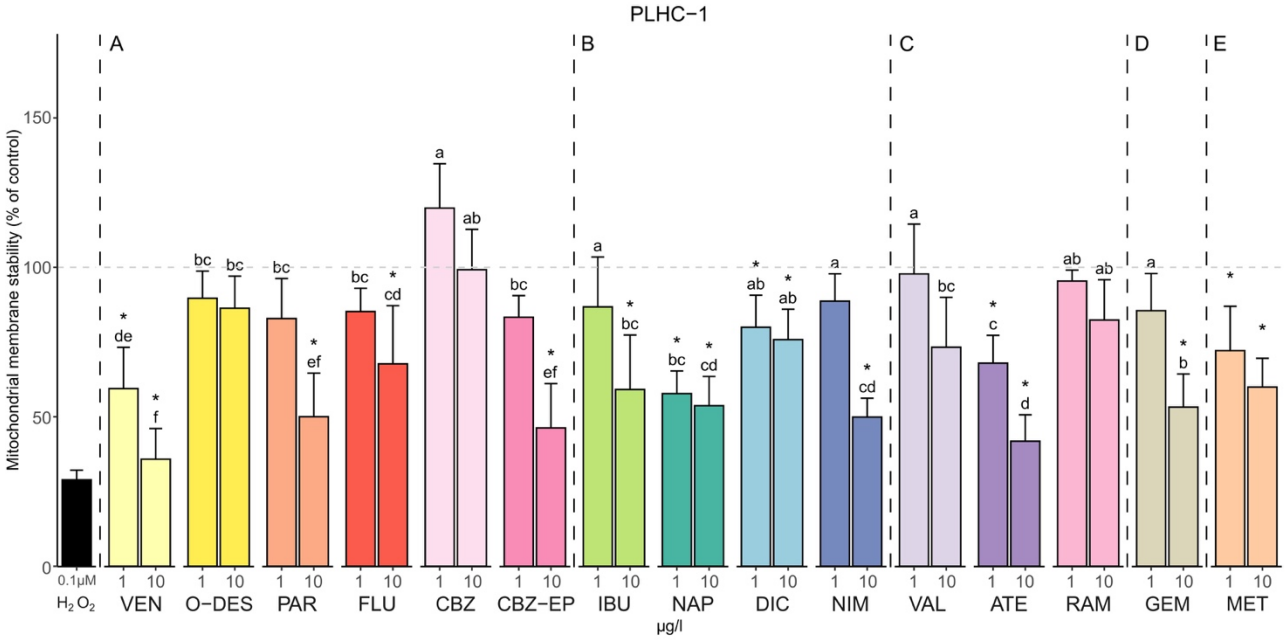


Figure 6.5. The effect of (A) psychiatric drugs, (B) Non-Steroidal Antinflammatory Drugs, (C) cardiovascular drugs, (D) the lipid lowering agent and (E) the antidiabetic on mitochondrial membrane electrochemical potential damage ($\Delta\psi$) in fish liver cell cultures (PLHC-1). The graph describes $\Delta\psi_m$ measured in PLHC-1 after 60 minutes of exposure to pharmaceuticals (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.2 µg/mL of Valynomicin was used as a positive control. Results are presented as mean ± SD (n = 7). * (p-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). VEN Venlafaxine; O-DESVEN O-Desmethylvenlafaxine; PAR Paroxetine; FLU Fluoxetine; CBZ Carbamazepine; CBZ-EP Carbamazepine-10,11 epoxide; IBU Ibuprofen; NAP Naproxen; DIC Diclofenac; NIM Nimesulide; VAL Valsartan; ATE Atenolol; RAM Ramipril; GEM Gemfibrozil; MET Metformin.

A statistically significant loss in $\Delta\psi_m$ was observed in PLHC-1 cells treated with MIXs B, C, D and E at higher exposure dose, and mixture A at both doses (Fig. 6.6).

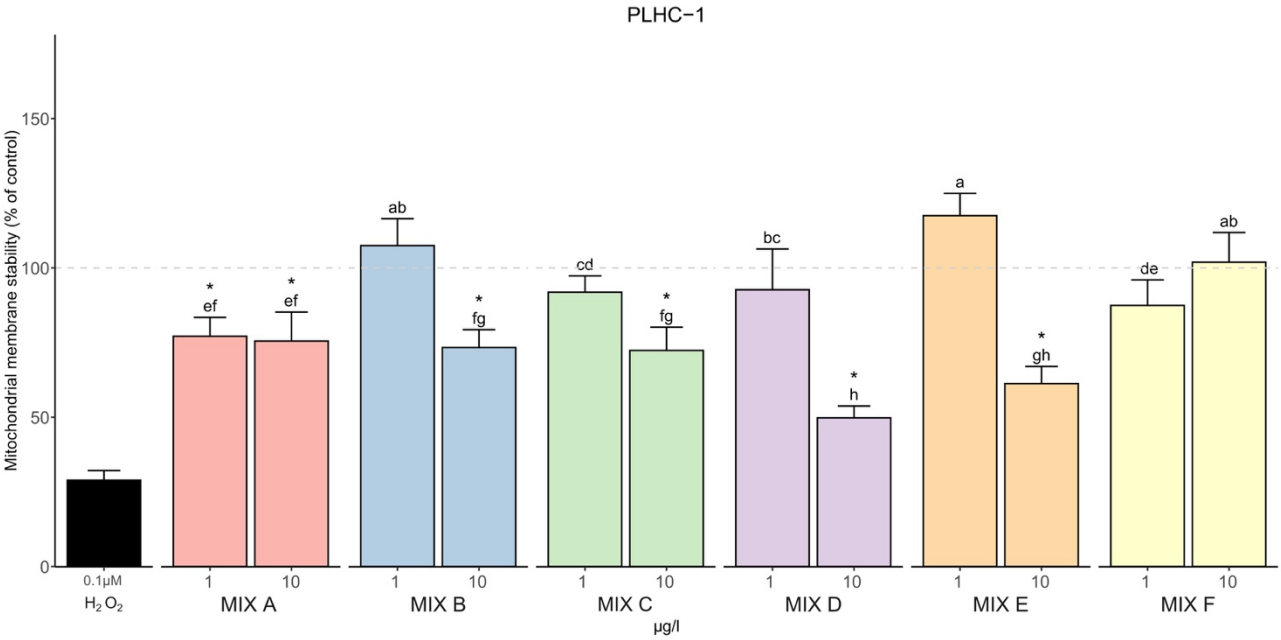


Figure 6.6. The effect of pharmaceutical mixtures on mitochondrial membrane electrochemical potential damage ($\Delta\psi$) in fish liver cell cultures (PLHC-1). The graph describes $\Delta\psi_m$ measured in PLHC-1 after 60 minutes of exposure to pharmaceuticals (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.2 µg/mL of Valynomicin was used as a positive control. Results are presented as mean ± SD (n = 7). * (p-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX C: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Ramipril + Gemfibrozil + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril.

6.3.1.4 Weight of Evidence (WOE) elaboration

For psychiatric drugs, the WOE model summarized a level of hazard "Severe" for PAR (10 µg/L), "Major" for PAR (1 µg/L), VEN (10 µg/L) and CBZ-EP (10 µg/L), "Moderate" for CBZ-EP (1 µg/L) and "Absent" for all the other treatments and doses. The NSAIDs always showed an "Absent" level of hazard, with the exception of NAP ("Major" at 1 µg/L and "Severe" at 10 µg/L) and NIM ("Slight" at 10 µg/L). Among cardiovascular drugs, an "Absent" level of hazard was elaborated for VAL at both doses, "Slight" for RAM and "Major" for ATE. For the lipid lowering GEM the level of hazard increased from "Absent" to "Major" at 1 and 10 µg/L, respectively, while it was "Moderate" at both doses of the antidiabetic MET (Fig. 6.7).

Treatment	Endpoint	% of effect (1µg/L)	HQ BATTERY	% of effect (10µg/L)	HQ BATTERY	Class of hazard (1µg/L)	Class of hazard (10µg/L)
Psychiatric drugs							
Venlafaxine	Cell V ROS MMS	0.61 0.81 42.61	0.71	61.01 25.87 61.57	4.57	Absent 	Major 
O-Desmethyl venlafaxine	Cell V ROS MMS	16.46 25.35 12.29	0.88	5.22 24.92 15.62	0.70	Absent 	Absent 
Paroxetine	Cell V ROS MMS	77.4 25.87 17.14	3.69	96.93 52.15 50.37	6.59	Major 	Severe 
Fluoxetine	Cell V ROS MMS	1.23 16.74 16.72	0.52	6.11 4.82 35.31	0.76	Absent 	Absent 
Carbamazepine	Cell V ROS MMS	10.11 20.46 20.79	0.48	9.68 9.48 0.35	0.32	Absent 	Absent 
Carbamazepine-10,11 epoxide	Cell V ROS MMS	28.95 32.19 16.23	1.64	96.91 1.74 55.35	5.24	Moderate 	Major 
NSAIDs							
Ibuprofen	Cell V ROS MMS	10.24 16.07 4.62	0.50	2.03 0.81 44.03	0.77	Absent 	Absent 
Naproxen	Cell V ROS MMS	62.66 43.77 43.1	4.51	89.83 98.28 48.05	7.59	Major 	Severe 
Diclofenac	Cell V ROS MMS	7.91 14.01 21.9	0.70	9.83 16.59 23.46	0.80	Absent 	Absent 
Nimesulide	Cell V ROS MMS	5.02 29.06 13.76	0.72	0.93 27.74 50.09	1.46	Absent 	Slight 
Cardiovascular drugs							
Valsartan	Cell V ROS MMS	1.63 36.42 0.48	0.53	10.13 1.92 23.9	0.62	Absent 	Absent 
Atenolol	Cell V ROS MMS	97.41 47.85 34.53	5.91	96.45 3.01 59.06	5.4	Major 	Major 
Ramipril	Cell V ROS MMS	37.4 9.48 7.57	1.02	19.17 36.42 17.61	1.37	Slight 	Slight 
Lipid lowering agents							
Gemfibrozil	Cell V ROS MMS	23.16 0.86 18.24	0.78	70.62 16.07 46.77	4.17	Absent 	Major 
Antidiabetics							
Metformin	Cell V ROS MMS	30.73 50.39 26.62	2.65	31.36 20.46 41.15	2.30	Moderate 	Moderate 

Figure 6.7. Weighted elaboration of results on cell viability, ROS production and mitochondrial membrane electrochemical potential damage in in PLHC-1. The % of effect measured for each endpoint (*Ew(i)*, calculation detail is reported in Fig. S5, Supplementary Material), the Hazard Quotient (HQ) elaborated for the battery of responses and the overall Class of hazard are given for each dose (1 - 10 µg/L) and pharmaceutical of the various therapeutic classes. Colors are used to highlight hazard classes as follow: white-Absent, blue-Slight, yellow-Moderate, red-Major, black-Severe.

The overall elaboration of results on MIXs effects summarized a level of hazard "Absent" for all treatments at 1 µg/L with the exception of mixture B (hazard "Moderate"). At the higher exposure dose of 10 µg/L the elaborated hazard level was "Absent" for MIX F, "Moderate" for MIXs A and C, "Major" for MIXs B and E, and "Severe" for MIX D (Fig. 6.8).

Treatment	Endpoint	% of effect (1µg/L)	$HQ_{BATTERY}$ (1µg/L)	% of effect (10µg/L)	$HQ_{BATTERY}$ (10µg/L)	Class of hazard (1µg/L)	Class of hazard (10µg/L)
MIX A	Cell V ROS MMS	12.49 17.63 21.7	0.84	33.78 29.92 24.51	2.09	Absent	Moderate
MIX B	Cell V ROS MMS	47.12 30.00 0.95	1.81	88.76 47.71 29.52	5.33	Moderate	Major
MIX C	Cell V ROS MMS	1.01 0.14 8.13	0.15	31.11 24.21 27.08	1.89	Absent	Moderate
MIX D	Cell V ROS MMS	11.79 0.15 3.11	0.29	96.1 69.01 50.22	7.06	Absent	Severe
MIX E	Cell V ROS MMS	15.86 18.75 17.5	0.57	65.8 81.1 38.06	5.61	Absent	Major
MIX F	Cell V ROS MMS	29.86 16.45 13.36	0.82	42.99 0.99 0.26	0.88	Absent	Absent

Figure 6.8. Weighted elaboration of results on cell viability, ROS production and mitochondrial membrane electrochemical potential damage in in PLHC-1. The % of effect measured for each endpoint (*Ew(i)*, calculation detail is reported in Fig. S5, Supplementary Material), the Hazard Quotient (HQ) elaborated for the battery of responses and the overall Class of hazard are given for each dose (1 - 10 µg/L) and pharmaceutical mixtures. Colors are used to highlight hazard classes as follow: white-Absent, blue-Slight, yellow-Moderate, red-Major, black-Severe. MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX C: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Ramipril + Gemfibrozil + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril.

6.3.2 RTgill-W1

6.3.2.1 Cell viability

For all psychiatric drugs a dose-dependent decrease in the cell viability was measured; particularly marked effect was observed for the highest dose of VEN and PAR (10 µg/L), showed similar viability to cells exposed to the positive control (0.1 µM H_2O_2). Lack of significant effects in terms of viability were observed for the cells exposed to the lower dose (1 µg/L) of VEN, O-DES, FLU and CBZ-EP, and for both concentrations of CBZ (Fig. 6.9A). More limited effects were observed for the therapeutic class of NSAIDs, with a significant decrease detected in cells exposed to NAP, DIC (1 µg/L) and NIM (10 µg/L) (Fig. 6.9B). Among cardiovascular drugs, a statistically significant reduction in viability was observed at the higher dose of ATE (10 µg/L), with values similar to cells exposed to the positive control; conversely, an increased cell viability was measured for the lower concentration of RAM (10 µg/L) and no significant variations were highlighted for all other treatments (Fig. 6.9C). Lack of effects were shown in cells exposed to both doses of the lipid lowering agent GEM (Fig. 6.9D), while the antidiabetic MET significantly increase the cell viability at the higher concentration (Fig. 6.9E).

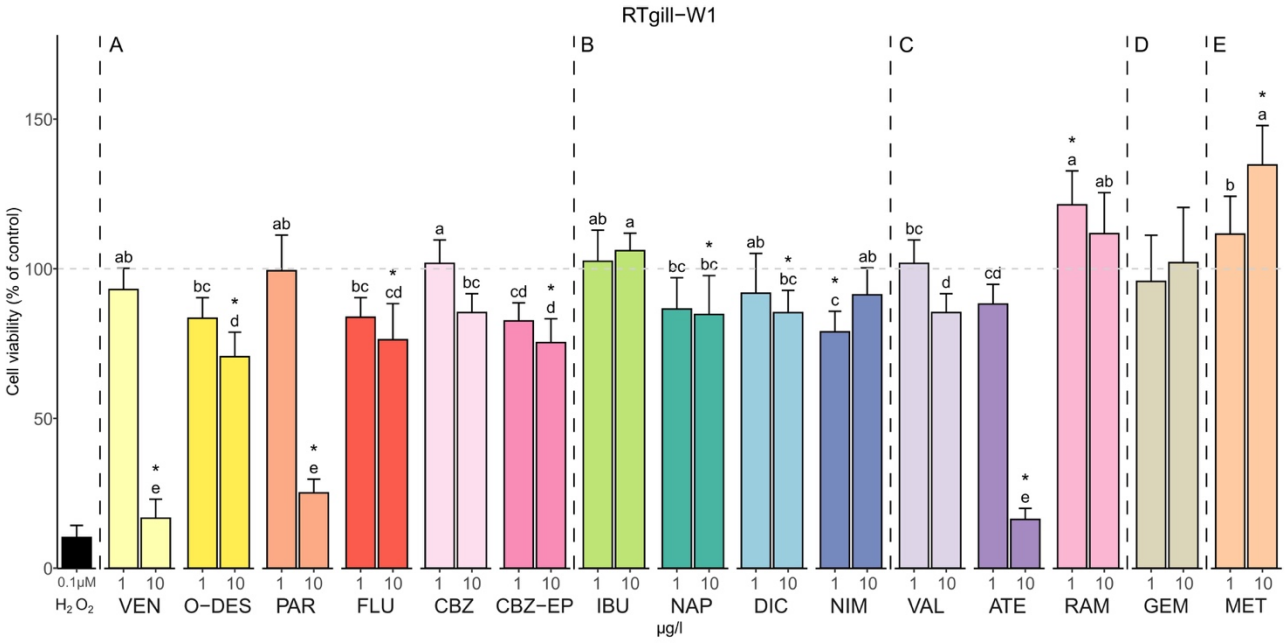


Figure 6.9. The effect of (A) psychiatric drugs, (B) Non-Steroidal Antinflammatory Drugs, (C) cardiovascular drugs, (D) the lipid lowering agent and (E) the antidiabetic on cell viability in fish gill cell cultures (RTgill-W1). The graph describes the cell viability after 24h exposure to pharmaceuticals (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.1 µM H_2O_2 was used as a positive control of reduced viability. Results are presented as mean ± SD (n = 7). * (p-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). VEN Venlafaxine; O-DESVEN O-Desmethylvenlafaxine; PAR Paroxetine; FLU Fluoxetine; CBZ Carbamazepine; CBZ-EP Carbamazepine-10,11 epoxide; IBU Ibuprofen; NAP Naproxen; DIC Diclofenac; NIM Nimesulide; VAL Valsartan; ATE Atenolol; RAM Ramipril; GEM Gemfibrozil; MET Metformin.

No cytotoxicity was observed for MIX A, while a dose-dependent effects was highlighted for MIXs B, C, D, E and F, with a statistically significant decrease in viability for the higher concertation (10 µg/L) (Fig. 6.10).

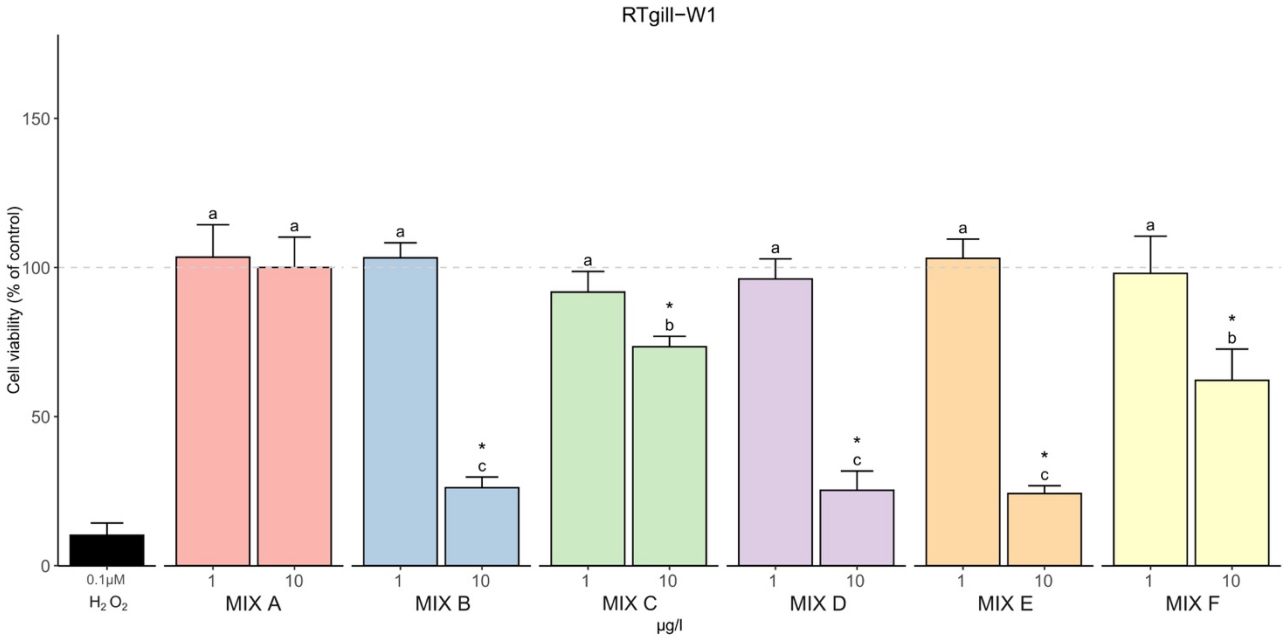


Figure 6.10. The effect of pharmaceuticals mixtures on cell viability in fish gill cell cultures (Rtgill-W1). The graph describes the cell viability after 24h exposure to pharmaceuticals mixtures (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.1 µM H_2O_2 was used as a positive control of reduced viability. Results are presented as mean ± SD (n = 7). * (*p*-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical mixture, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX C: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Ramipril + Gemfibrozil + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril.

6.3.2.2 Reactive oxygen species production

A statistically significant decrease in ROS production was observed after exposure to all psychiatric drugs, with the only exception of the highest dose (10 µg/L) of PAR that showed a significant induction (Figure 6.11A). Similarly, lowered ROS formation was detected in cells exposed to NSAIDs, while no variations were highlighted for the highest concentration (10 µg/L) of IBU (Figure 6.11B). Among cardiovascular drugs, limited effects were observed for the highest dose of VAL and ATE, while all the other treatments caused a statistically significant reduction in ROS production (Figure 6.11C). Gills cell lines exposed to both the lipid lowering agent GEM (Figure 6.11D) and the antidiabetic MET (Figure 6.11E) showed a statistically decrease in ROS formation at all tested doses.

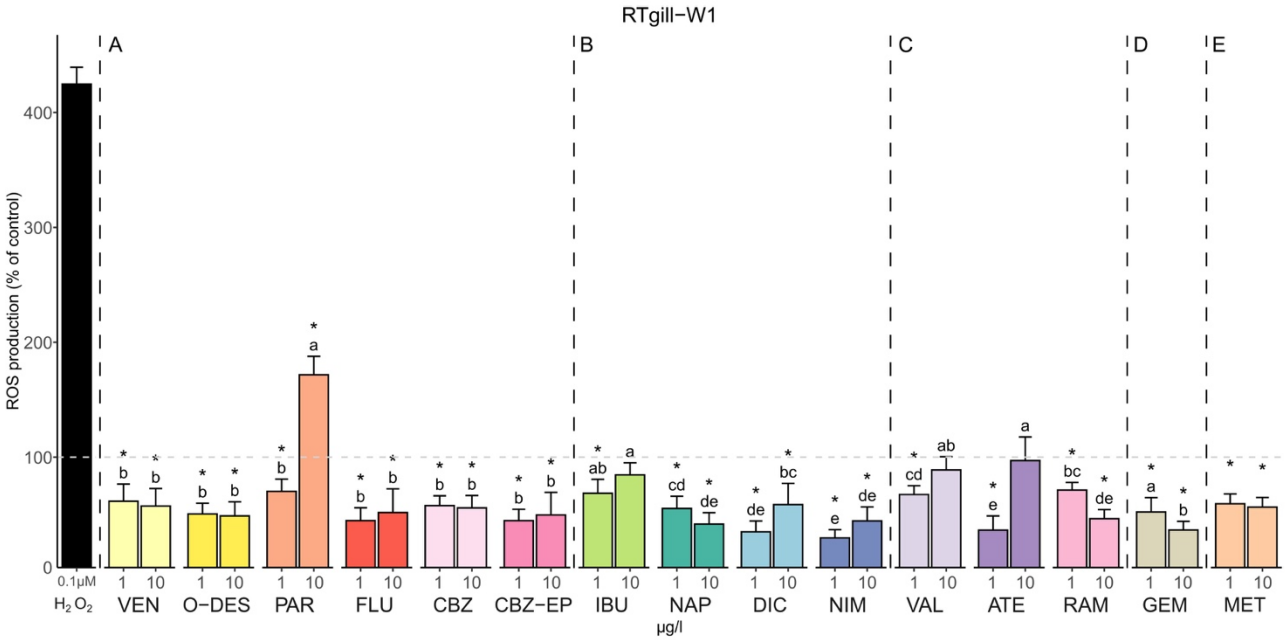


Figure 6.11. The effect of (A) psychiatric drugs, (B) Non-Steroidal Antinflammatory Drugs, (C) cardiovascular drugs, (D) the lipid lowering agent and (E) the antidiabetic on reactive oxygen species (ROS) production in fish liver gill cultures (RTgill-W1). The graph describes ROS generation measured in PLHC-1 after 60 minutes of exposure to pharmaceuticals (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.1 µM H_2O_2 was used as a positive control of cellular ROS production. Results are presented as mean ± SD (n = 7). * (*p*-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). VEN Venlafaxine; O-DESVEN O-Desmethylvenlafaxine; PAR Paroxetine; FLU Fluoxetine; CBZ Carbamazepine; CBZ-EP Carbamazepine-10,11 epoxide; IBU Ibuprofen; NAP Naproxen; DIC Diclofenac; NIM Nimesulide; VAL Valsartan; ATE Atenolol; RAM Ramipril; GEM Gemfibrozil; MET Metformin.

Lack of variations were observed for both doses of MIXs A, B and for the lower concentration of MIX F, while a statistically significant ROS reduction was highlighted for all other MIXs (Figure 6.12).

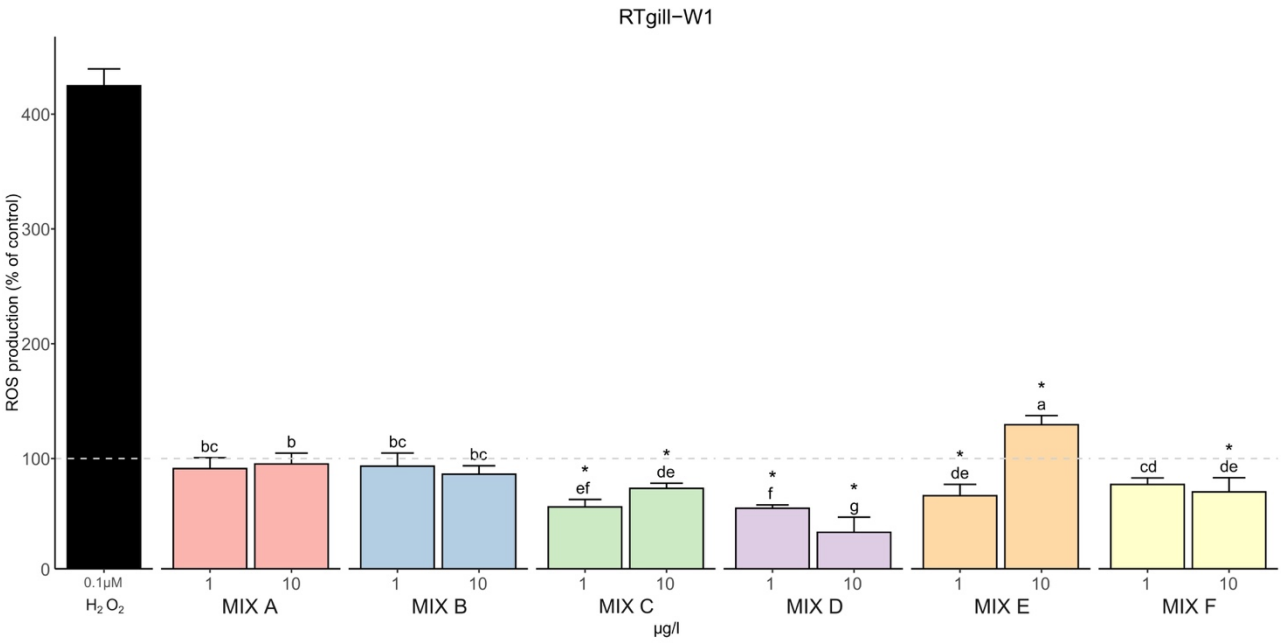


Figure 6.12. The effect of pharmaceutical mixtures on reactive oxygen species (ROS) production in fish gill cell cultures (RTgill-W1). The graph describes ROS generation measured in PLHC-1 after 60 minutes of exposure to pharmaceuticals mixtures (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.1 µM H_2O_2 was used as a positive control of cellular ROS production. Results are presented as mean ± SD (n = 7). * (*p*-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical mixture, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX C: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Ramipril + Gemfibrozil + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril.

6.3.2.3 Mitochondrial membrane electrochemical potential change

A dose-dependent loss of Δψ was measured in all gill cells exposed to psychiatric drugs, except for the lower dose (1 µg/L) of CBZ and CBZ-EP, which did not exhibit significant variations (Figure 6.13A). A lack of effect was detected for the lower concentration of NIM, while a significant dose-dependent loss in mitochondrial membrane potential was observed in all other NSAIDs at both tested doses (Figure 6.13B).Among cardiovascular drugs, no variations were measured in cells exposed to both concentrations of RAM and to the lower dose of VAL; while ATE and VAL (10 µg/L) showed a significant reduction in mitochondrial membrane potential (Figure 6.13C). A significant loss of Δψ was measured for the lipid lowering agent GEM (Figure 6.13D), while a significant increase in mitochondrial membrane potential was detected for the antidiabetic MET (Figure 6.13E).

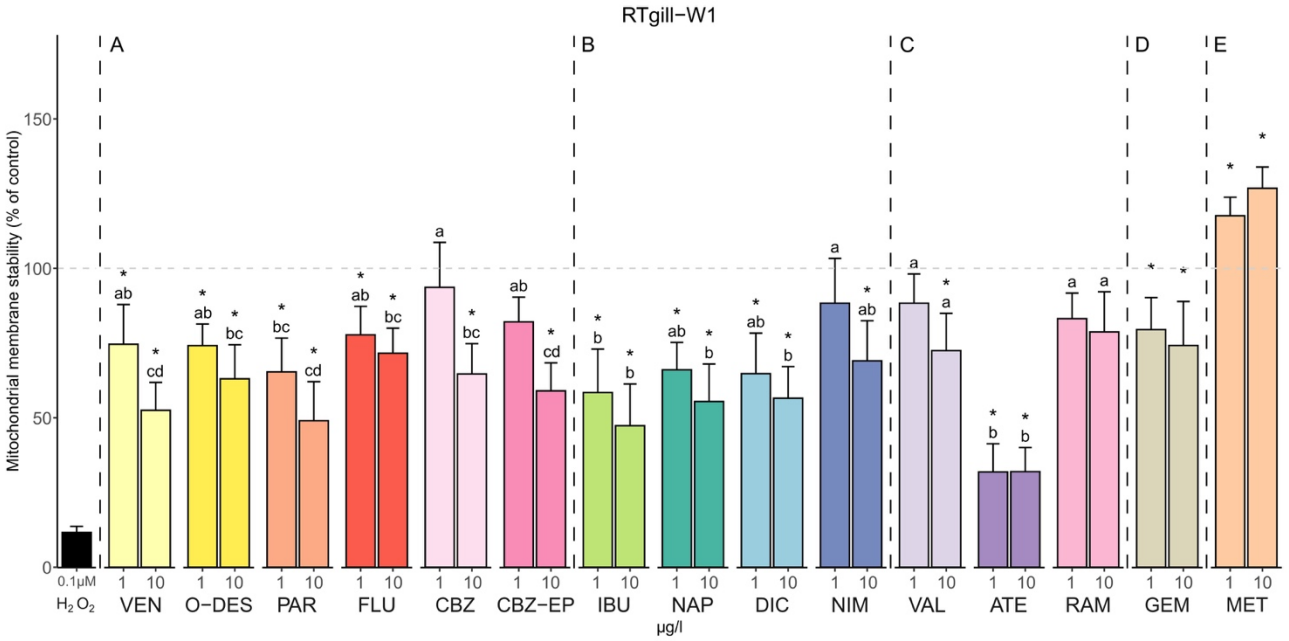


Figure 6.13. The effect of (A) psychiatric drugs, (B) Non-Steroidal Antinflammatory Drugs, (C) cardiovascular drugs, (D) the lipid lowering agent and (E) the antidiabetic on mitochondrial membrane electrochemical potential damage (Δψ) in fish gill cell cultures (RTgill-W1). The graph describes $\Delta\psi_m$ measured in PLHC-1 after 60 minutes of exposure to pharmaceuticals (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.2 µg/mL of Valynomicin was used as a positive control. Results are presented as mean ± SD (n = 7). * (*p*-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). VEN Venlafaxine; O-DESVEN O-Desmethylvenlafaxine; PAR Paroxetine; FLU Fluoxetine; CBZ Carbamazepine; CBZ-EP Carbamazepine-10,11 epoxide; IBU Ibuprofen; NAP Naproxen; DIC Diclofenac; NIM Nimesulide; VAL Valsartan; ATE Atenolol; RAM Ramipril; GEM Gemfibrozil; MET Metformin.

A dose-dependent decrease in mitochondrial membrane potential was observed in MIXs A, B, C and D, with statistically significant effect for the highest exposure dose. Cells exposed to both doses of MIX E exhibited a statistically significant loss of $\Delta\psi$, whereas treatment with MIX F did not reveal any significant variations (Figure 6.14).

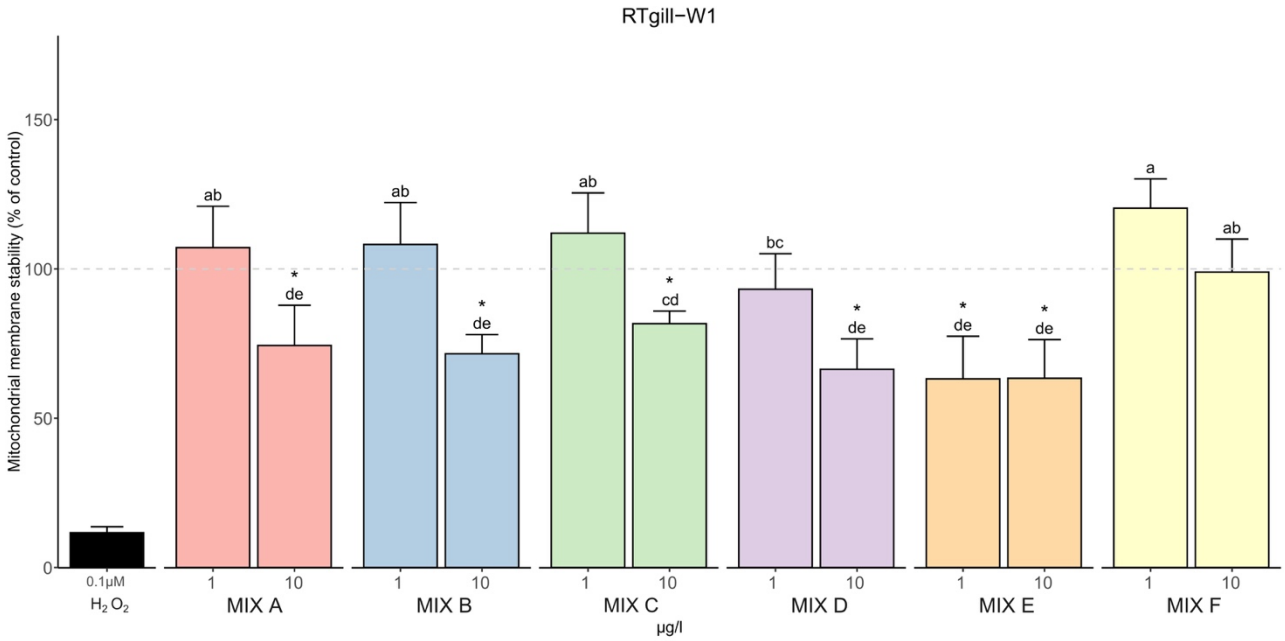


Figure 6.14. The effect of pharmaceutical mixtures on mitochondrial membrane electrochemical potential damage ($\Delta\psi$) in fish liver gill cultures (RTgill-W1). The graph describes $\Delta\psi_m$ measured in PLHC-1 after 60 minutes of exposure to pharmaceuticals (1 - 10 µg/L) as the percentage of fluorescent response from each treatment compared to cells exposed to solvent control (0.001 % MeOH = 100 %). 0.2 µg/mL of Valynomicin was used as a positive control. Results are presented as mean ± SD (n = 7). * (p-value < 0.05) represents statistical significance compared to solvent control cells (0.001 % MeOH = 100 %), determined using one-way ANOVA. Different letters indicate significant differences between each pharmaceutical mixture, the Tukey test (HSD) was used for post-hoc comparison between means of values (n = 7). MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX C: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Ramipril + Gemfibrozil + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril.

6.3.2.4 Weight of Evidence (WOE) elaboration

For psychiatric drugs, the overall elaboration of results summarized a level of hazard "Severe" for PAR (10 µg/L), "Major" for VEN and O-DES (10 µg/L), "Moderate" for O-DES (1 µg/L), FLU, CBZ and CBZ-EP at both doses, and "Absent" for VEN and PAR at the lower concentration.

IBU showed a "Slight" level of hazard for both exposure doses, while all other NSAIDs revealed a "Moderate" level of hazard. Among cardiovascular drugs, it was elaborated a level of hazard "Absent" for VAL, "Slight" for RAM and "Major" for ATE. A dose-dependent hazard level increase was elaborated for the lipid lowering agent GEM and the antidiabetic MET passing from "Slight" to "Moderate" and from "Absent" to "Moderate", respectively (Figure 6.15).

Treatment	Endpoint	% of effect (1µg/L)	HQ BATTERY (1µg/L)	% of effect (10µg/L)	HQ BATTERY (10µg/L)	Class of hazard (1µg/L)	Class of hazard (10µg/L)
Psychiatric drugs							
Venlafaxine	Cell V ROS MMS	7.03 41.08 51.89	1.00	55.50 19.54 24.95	5.38	Absent 	Major 
O-Desmethyl venlafaxine	Cell V ROS MMS	23.26 46.34 30.39	1.99	31.12 36.78 32.10	3.05	Moderate 	Major 
Proxetine	Cell V ROS MMS	1.97 39.44 58.59	0.96	45.84 29.20 24.96	6.21	Absent 	Severe 
Fluoxetine	Cell V ROS MMS	23.05 51.62 25.33	1.95	31.50 38.41 30.09	2.19	Moderate 	Moderate 
Carbamazepine	Cell V ROS MMS	2.62 93.01 4.37	0.62	19.86 40.25 39.89	2.12	Absent 	Moderate 
Carbamazepine-10,11 epoxide	Cell V ROS MMS	25.79 53.15 21.06	1.84	26.44 37.22 36.34	2.86	Moderate 	Moderate 
NSAIDs							
Ibuprofen	Cell V ROS MMS	1.19 37.38 61.43	1.3	5.39 18.87 75.73	1.23	Slight 	Slight 
Naproxen	Cell V ROS MMS	18.84 43.10 38.06	2.01	8.67 47.14 44.19	2.47	Moderate 	Moderate 
Diclofenac	Cell V ROS MMS	3.41 59.60 36.98	2.14	18.84 41.78 39.38	2.3	Moderate 	Moderate 
Nimesulide	Cell V ROS MMS	29.48 65.49 5.03	2.01	12.38 52.76 34.86	1.95	Moderate 	Moderate 
Cardiovascular drugs							
Valsartan	Cell V ROS MMS	2.59 67.24 30.17	0.63	32.54 18.64 48.82	0.91	Absent 	Absent 
Atenolol	Cell V ROS MMS	10.81 38.94 50.25	3.7	60.67 0.29 39.04	5.1	Major 	Major 
Ramipril	Cell V ROS MMS	39.60 35.40 25.00	1.21	8.55 61.89 29.56	1.38	Slight 	Slight 
Lipid lowering agents							
Gemfibrozil	Cell V ROS MMS	1.79 62.40 35.81	1.13	0.00 64.43 35.57	1.74	Slight 	Moderate 
Antidiabetics							
Metformin	Cell V ROS MMS	10.96 89.04 0.00	0.59	50.22 49.78 0.00	1.53	Absent 	Moderate 

Figure 6.15. Weighted elaboration of results on cell viability, ROS production and mitochondrial membrane electrochemical potential damage in RTgill-W1. The % of effect measured for each endpoint (*Ew(i)*, calculation detail is reported in Fig. S5, Supplementary Material), the Hazard Quotient (HQ) elaborated for the battery of responses and the overall Class of hazard are given for each dose (1 - 10 µg/L) and pharmaceutical of the various therapeutic classes. Colors are used to highlight hazard classes as follow: white-Absent, blue-Slight, yellow-Moderate, red-Major, black-Severe. VEN, Venlafaxine; O-DES, O-Desmethylvenlafaxine; PAR, Paroxetine; FLU, Fluoxetine; CBZ, Carbamazepine; CBZ-EP, Carbamazepine-10,11 epoxide; IBU, Ibuprofen; NAP, Naproxen; DIC, Diclofenac; NIM, Nimesulide; VAL, Valsartan; ATE, Atenolol; RAM, Ramipril; GEM, Gemfibrozil; MET, Metformin.

The WOE model summarized a level of hazard "Absent" for the lower dose of MIXs B, C, D and F, and for both doses of MIX A; "Slight" for MIXs E (1 µg/L) and F (10 µg/L); "Moderate" for MIX C (10 µg/L); and "Major" for the highest concentration of MIXs B, D and E (Figure 6.16).

Treatment	Endpoint	% of effect (1µg/L)	$HQ_{BATTERY}$ (1µg/L)	% of effect (10µg/L)	$HQ_{BATTERY}$ (10µg/L)	Class of hazard (1µg/L)	Class of hazard (10µg/L)
MIX A	Cell V ROS MMS	45.45 54.55 0.00	0.06	0.00 4.35 95.65	0.43	Absent	Absent
MIX B	Cell V ROS MMS	42.11 57.89 0.00	0.05	69.65 8.46 21.89	3.64	Absent	Major
MIX C	Cell V ROS MMS	22.71 77.29 0.00	0.74	44.03 28.76 27.21	1.50	Absent	Moderate
MIX D	Cell V ROS MMS	3.75 90.42 5.83	0.65	51.71 29.64 18.65	5.34	Absent	Major
MIX E	Cell V ROS MMS	1.79 41.54 56.67	1.12	60.79 15.70 23.50	4.44	Slight	Major
MIX F	Cell V ROS MMS	2.59 97.41 0.00	0.31	66.05 33.72 0.23	1.37	Absent	Slight

Figure 6.16. Weighted elaboration of results on cell viability, ROS production and mitochondrial membrane electrochemical potential damage in RTgill-W1. The % of effect measured for each endpoint (*Ew(i)*, calculation detail is reported in Fig. S5, Supplementary Material), the Hazard Quotient (HQ) elaborated for the battery of responses and the overall Class of hazard are given for each dose (1 - 10 µg/L) and pharmaceutical mixtures. Colors are used to highlight hazard classes as follow: white-Absent, blue-Slight, yellow-Moderate, red-Major, black-Severe. MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX C: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Ramipril + Gemfibrozil + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril.

6.4 Discussions

6.4.1 PLHC-1

The ecotoxicological prioritization of emerging pollutants as pharmaceuticals, requires the analysis of multiple biological endpoints. This study was aimed to evaluate specific mechanism of toxicity caused by 15 pharmaceuticals commonly detected in aquatic environments, and to validate the use of weighted criteria for summarizing severity of effects and hazard indices of various drugs and their mixtures. Among all tested drugs, the therapeutic class of antidepressants showed the greater effects on PLHC-1 cell line. The reduction of cell viability observed by the AlamarBlue assay reflects a decrease in metabolic activity that affects mainly mitochondria, while the strong increase in ROS levels can be due to the ability of pharmaceuticals to disrupt the mitochondrial electron transport chain (Bermejo-Nogales et al., 2016). Deleterious effects of ROS include oxidation of the cell constituents (proteins, lipids, DNA) and the permeabilization of lysosomes located in the proximity of uncoupled mitochondria, which initiate apoptosis or necrotic pathways (Boya and Kroemer, 2008; Bermejo-Nogales et al., 2016). Furthermore, acidic subcellular compartments such as lysosomes can easily sequester weakly basic drugs as antidepressants which, once protonated, cannot return to the cytosol thus causing lysosomal destabilization (Daniel and Wójcikowski, 1997; Miguez et al., 2014). This mechanism, often referred as lysosomotropism, can be associated to compounds with a Log P > 2 and a basic pKa between 6.5 and 11 (Lemieux et al., 2004; Nadanaciva et al., 2011); and, despite their structural differences, most antidepressants share this physicochemical property. In this respect, the potential damage to lysosomal function induced by these pharmaceuticals could lead to the release of lysosomal proteolytic enzymes, such as phospholipases, which may attack mitochondrial membranes, initializing apoptotic processes. The release of lysosomal contents can further enhance the production of superoxide radicals and hydrogen peroxide, increasing level of oxidative stress (Terman et al., 2006). The data elaboration through weighted criteria revealed a "Major" and “Severe” level of hazard for PAR-exposure at 1 and 10 μg/L respectively, highlighting an elevated toxicity of this molecule. Despite PAR and FLU belong to the same therapeutic class (SSRIs), our results did not highlight similar toxicity, suggesting the role of oxidative metabolism in modulating biological effects of these drugs. Indeed, the significant increase in ROS might have contributed to the higher cytotoxicity of PAR, while the limited response of redox homeostasis and ROS production after FLU-exposure was paralleled by a lower cytotoxicity, and an “Absent” level of hazard.

Among psychiatric drugs, the biological effects of parent compounds CBZ and VEN could be compared with those of their metabolites, CBZ-EP and O-DES respectively. Considering the analyzed endpoints, this study showed a higher toxicity of CBZ-EP compared to CBZ, while VEN appeared to have greater effects than its metabolite O-DES. CBZ metabolism is catalyzed by CYP

450 biotransformation pathway, mainly by CYP3A4, CYP2C8 and CYP3A5 enzymes (Kerr et al., 1994). A typical side effect in the metabolism of CBZ is the enhancement of ROS production (Mezzelani and Regoli, 2022), confirmed by our results after 1h of CBZ-exposure at both doses; exposed cells, however, were able to counteract the prooxidant challenge, showing a lack of cytotoxicity after 24 h of exposure and no effects on the stability of mitochondrial membranes; unlike other psychiatric drugs, CBZ does not show lysosomotropism (Matsumoto et al., 1998), possibly explaining the lower magnitude of the oxidative damage and the ability of cells to restore the general homeostasis. Limited toxicity of CBZ in PLHC-1 cell lines resulted in a level of hazard elaborated as "Absent" by the weighted criteria. On the other hand, CBZ-EP, the main product of CBZ metabolism, is considered an active metabolite in mammals (Bourgeois et al., 1984), and the present study results revealed a different mechanism of toxicity compared to its parent compound. CBZ-EP caused higher cytotoxicity and loss of the mitochondrial membranes integrity in a dose-dependent manner, effects which did not appear directly linked to increased ROS production. The greater biological reactivity of CBZ-EP compared to CBZ was highlighted by the "Moderate" (1 μg/L) and "Major" (10 μg/L) level of hazard elaborated by the weighted criteria.

Considering the similarities in VEN and O-DES chemical structure and pharmacodynamics, analogous effects could have been expected. However, obtained results confirmed this hypothesis only at the lower exposure dose while at 10 μg/L a much greater toxicity of VEN compared to O-DES was observed in PLHC-1, with a level of hazard decreasing from "Major" to "Absent" in the parent compound and its metabolite, respectively. VEN caused cytotoxicity, the enhancement of ROS formation and a decrease of mitochondrial membrane potential, confirming previous literature data. Exposure to 550.8 mg/L of VEN promoted ROS production and subsequent toxic effects including collapse of mitochondrial membrane potential and cell death in rat hepatocytes (Ahmadian et al., 2016). In the same study, mitochondrial functional damage was detected with inhibition of mitochondrial respiratory complexes II and IV in response to increased doses of VEN (27.7 – 55.5 – 138.7 mg/L). On the other hand, the more limited effects of O-DES could be related to the decreased production of ROS at both exposure doses, suggesting the possible activation of antioxidant defenses that prevent the onset of cytotoxicity and decrease in mitochondrial membrane potential.

The therapeutic class of NSAIDs generally provoked limited effects on the PLHC-1 cell line, as also highlighted by the "Absent" level of hazard elaborated by the weighted criteria for most of tested drugs. A notable exception was obtained for NAP, whose marked effects in terms of cytotoxicity, enhancement of ROS and decrease in mitochondrial membrane potential resulted in a "Major" and "Severe" level of hazard elaborated for 1 and 10 μg/L exposure doses, respectively. The lower cytotoxicity of NSAIDs compared to other therapeutic classes also agrees with the limited effects

measured in the rainbow trout *Oncorhynchus mykiss* (RTL-W1) cell line (Schnell et al., 2009). The majority of data on fish cell lines did not show relevant effects of NAP compared to other NSAIDs (Caminada et al., 2006; Schnell et al., 2009; Fernández et al., 2012), despite the oxidative potential of this molecule was highlighted in liver of the rohu fish (*Labeo rohita*) after chronic exposures to environmentally relevant concentrations of NAP (1 µg/L and 50 µg/L) (Priyadarshinee et al., 2023). Interestingly, in mammalian cell lines (UM-UC-5 and UM-UC-14) the decreased viability was associated to NAP capability to suppress PI3K activity and downstream signaling, resulting in cell-cycle arrest and apoptosis induction (Kim et al., 2014). Since biological targets of pharmaceuticals are evolutionarily conserved among vertebrates and invertebrates, the greater toxicity of NAP highlighted in the present study could be mechanistically related to its higher binding affinity toward PI3K and the related inhibition of cell cycle. This hypothesis is further corroborated by the evidence of immunosuppression and genotoxic effects on the wolf fish (*Hoplias malabaricus*) cell lines and adult marine mussel *Mytilus galloprovincialis* exposed to NSAIDs (Coelho Ribas et al., 2014; Mezzelani et al., 2016).

Among cardiovascular drugs, the beta blocker ATE was the only active ingredient showing an elevated toxicity in the PLHC-1 cell line, with a "Major" level of hazard at both exposure doses. Conversely, the limited effects caused by the antihypertensive VAL and the ACE inhibitor RAM were summarized in an "Absent" and "Slight" hazard level, respectively. The higher ATE toxicity is not in line with previous data, showing only a slight dose-dependent decrease in mitochondrial activity (measured by the MTT (3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay) in gill, digestive gland and hemolymph cells of zebra mussel (*Dreissena polymorpha*) exposed to increasing concentrations of atenolol (1 – 10 – 100 – 1000 – 10000 µg/L) (Parolini et al., 2011). Similarly, a low ATE cytotoxicity was observed in PLHC-1 and RTG-2 fish cell lines exposed to doses ranging from 2.66 to 2663.36 mg/L (Caminada et al., 2006). On the other hand, a stronger oxidative stress was observed in zebrafish exposed for 7 days to ATE (1 mg/L) compared to the other drugs (DIC and ketoprofen), demonstrating a greater prooxidant potential of this molecule (Diniz et al., 2015). In addition, zebrafish larvae exposed to environmentally relevant concentrations of ATE showed an overall decrease in lipid content, more specifically of C16 ceramide, phosphatidylethanolamine, and phosphatidylcholine (Ivantsova et al., 2022): such lipids are important for lipid bilayer integrity and their decrease may compromise plasma membranes, potentially explaining the reduced mitochondrial membrane stability observed in our study.

The lipid regulator GEM showed a dose-dependent cytotoxicity after 24 h with an overall increase in hazard level from "Absent" to "Major" at 1 and 10 µg/L exposures, respectively. Fibrates, as GEM, are characterized by the pronounced induction of hepatic peroxisome proliferation, mediated via

peroxisome proliferator-activated receptor alpha (PPARα), leading to an increase of the number and size of peroxisomes and potential oxidative stress (O' Brien et al., 2000; Barreto et al., 2017). From our study, GEM cytotoxicity does not seem to be a direct consequence of enhanced ROS production. However, other oxidative mechanisms might be responsible for the observed reduction of mitochondrial membrane potential, and a more prolonged prooxidant challenge could explain the recorded GEM cytotoxicity after 24 h of exposure.

The exposure of PLHC-1 cell line to the antidiabetic MET determined significant effects summarized in a "Moderate" level of hazard at both tested concentrations. The mechanism of action of MET includes the inhibition of the complex I in the mitochondrial electron transport chain, leading to adenosine triphosphate (ATP) depletion and increase in adenosine monophosphate (AMP) levels (Foretz et al., 2014; Godoy et al., 2018). A genetic and functional homology between mammalian and zebrafish mitochondria has already been reported (Pinho et al., 2013), suggesting the potential involvement of these organelles in the MET metabolism also for aquatic vertebrates. Other studies also indicated that environmentally relevant concentrations of MET cause oxidative stress and cell apoptosis in fish and in mammalian cell lines (Anedda et al., 2008; Queiroz et al., 2014; Lee et al., 2019), in line with results of this work, which showed a slight increase in ROS production and a decrease in mitochondrial membranes stability at both exposure doses. Interestingly, the increase in cell viability measured after 24h, indicates the acceleration of cell metabolism caused by longer exposure to this drug.

In natural field conditions, organisms are commonly exposed to mixtures of several co-occurring drugs, with potentially unexpected effects in terms of toxicity or metabolic changes. In our work, interactions between different pharmaceuticals were investigated exposing PLHC-1 cell line to 3 binary (A, B, E) and 3 quaternaries MIXs (C, D, F).

Binary MIXs A and E were both composed of an antidepressant and a NSAID (PAR and IBU, VEN and NAP, respectively). These MIXs showed a significant effect in all measured endpoints only at the higher exposure dose (10 μg/L), while more variable results were observed at 1 μg/L. The overall effect of PAR on cell viability, ROS production and mitochondrial membrane potential appeared to be mitigated by co-exposure with IBU with a hazard index of MIX A classified as "Absent" or "Moderate" at the two exposure doses. A similar trend was highlighted in MIX E, where the magnitude of NAP toxicity was decreased when co-exposed with the antidepressant VEN, and an overall hazard index ranging from "Absent" to "Major". These results suggest the presence of antagonistic mechanisms when NSAIDs are co-exposed with antidepressants, since the magnitude of the MIX effects were typically in between compared to those measured for the single compounds. Such antagonistic interactions were not previously observed after *in vivo* long-term exposure (30-

days) of Mediterranean mussels (*M. galloprovincialis)* to 1 μg/L of IBU, PAR and their mixture. In this case, bivalves highlighted a similar magnitude of cellular effects in PAR and mixture-treated organisms, possibly related to metabolism and excretion of IBU, which appeared below detection limit in co-exposed individuals (exp. 1 *in vivo* mussels).

The binary MIX B was composed by the antiepileptic CBZ and the antihypertensive VAL. In this case, the co-exposure caused a synergistic and dose-dependent effect on cytotoxicity, with an overall hazard index increasing from "Moderate" to "Major". Both individual drugs and the MIX showed a similar enhancement of ROS production, while the decrease of mitochondrial membrane potential caused by the MIX appeared mostly related to VAL. An *in vivo* exposure conducted using the Mediterranean mussel at environmental concentrations of CBZ, VAL and their mixture for 14 days revealed greater biological effects due to CBZ compared to VAL, and clear evidence of antagonistic interactions between these compounds, at least partly modulated by a lower accumulation of CBZ in co-exposed mussels (Mezzelani et al., 2023; Mezzelani et al., 2024). The evidence that the same molecules can interact in a different way depending on biological models, exposure conditions and measured endpoints, highlights the importance of an integrated approach, combining acute and chronic exposures, species of different trophic levels, and the measurement of several pathways and responses to evaluate the mechanisms of action and risk of environmental pharmaceuticals on non-target organisms.

The quaternary MIX C was the combination of the MIXs A and B. This treatment showed an overall effect on the analyzed endpoints which, at the higher exposure dose, appeared intermediate (hazard "Moderate") to those of the MIXs A and B (hazard "Moderate" and "Major", respectively). Interestingly, the ROS production was decreased after exposure to MIX C, suggesting the possible activation of antioxidant defenses following the interaction among IBU, PAR, CBZ and VAL. Despite the requirement of additional studies to prove such hypothesis, our study findings are supported by molecular and cellular effects highlighted on the redox metabolism of the Mediterranean mussels exposed to the same APIs (Mezzelani et al., 2023; Mezzelani et al., 2024).

The MIX D included the antidepressant VEN, the ACE inhibitor RAM, the lipid regulator GEM and the antidiabetic MET. At the higher dose a marked reduction of cell viability was observed along with increased ROS production and destabilization of mitochondrial membranes, which determined a "Severe" class of hazard. As mentioned above, all the pharmaceuticals are prooxidant chemicals, and no synergistic or additive effects were detected in terms of ROS formation compared to single molecule exposures. Additionally, for the potential of mitochondrial membranes, the effect of the MIX probably reflects the contribution of VEN, GEM and MET, but not of RAM due to the lack of

effect measured in cells exposed to the single drug. For cytotoxicity, the effect of the mixture appeared mostly influenced by those of VEN and GEM.

The combination of the antihypertensive VAL, the beta blocker ATE, the ACE inhibitor RAM and the lipid regulator GEM (MIX F), was the less toxic active compound, with a hazard level "Absent" at both exposure doses. This result allowed to exclude any evidence of synergistic effects, and rather a lower toxicity of the mixture compared to ATE and GEM dosed alone.

6.4.2 RTgill-W1

The RTgill-W1 cell line, derived from the gill cells of *Oncorhynchus mykiss* (rainbow trout), is widely recognized as a suitable model for ecotoxicological screening of both acute and chronic toxicity, as well as for assessing cytotoxicity, oxidative stress, and specific mechanisms of action at environmentally relevant concentrations of pollutants, including pharmaceuticals (Lee et al., 2009; Tanneberger et al., 2013). Since the gills represent the primary interface between fish and their aquatic environment, RTgill-W1 cells are highly representative of the organ's role in mediating the uptake and bioaccumulation of waterborne contaminants (Evans et al., 2005). This study evaluated the toxicity of a panel of 15 pharmaceuticals commonly found in aquatic environments and their mixtures using the RTgill-W1 cell line, and to compare these toxic effects with those observed in the liver (PLHC-1 cell line), a key organ responsible for the metabolism and detoxification of drugs. This work aimed to elucidate an organ-specific responses and differences in susceptibility between gill and liver tissues to pharmaceutical exposure in fish, thereby providing a more comprehensive understanding of the toxicological impact of these compounds in non-target species.

Among all tested therapeutic classes in RTgill-W1 cells, antidepressants such as VEN, O-DES, PAR, and FLU demonstrated the greatest toxicity. At the highest exposure dose (10 μg/L), these compounds exhibited marked effects in terms of variation in cell viability, ROS production and mitochondrial membrane potential, with elaborated hazard indices ranging from "Moderate" to "Severe", corroborating and reinforcing the toxicity profiles observed in the previous experiment with the PLHC-1 cell line. In line with our findings, the study by Best et al. (2014) demonstrated that environmentally realistic concentrations (1 μg/L) of VEN impair the metabolic capacity of the gills in *O. mykiss* by enhancing glycolytic capacity, as well as decreased Na^+–K^+ ATPase activity and increasing the protein expression of this enzyme. On the other hand, in contrast with our outcomes, the study by Mittal et al. (2022) reported the absence of cytotoxicity for the antidepressant FLU at concentrations below 1 mg/L in RTgill-W1 cells. However, there is a lack of studies investigating the effects of other pharmaceutical compounds on fish gill cells, making it difficult to draw comparisons with existing literature.

The comparison between the parent compounds CBZ and VEN with their metabolites (CBZ-EP and O-DES, respectively) highlighted higher toxicity of CBZ-EP and O-DES ("Moderate" hazard level) compared to CBZ and VEN ("Absent" hazard level) at the lower exposure dose, while similar effects were observed at the higher exposure dose ("Moderate" hazard level).

A study investigating the uptake and tissue distribution of pharmaceuticals demonstrated the metabolization of CBZ through the CBZ-EP pathway in the gills of *Jenynsia multidentata* (Valdés et al., 2016). This finding could suggest that the comparable hazard levels observed in both treatments (HQ values of 2.12 for CBZ and 2.86 for CBZ-EP) at the higher exposure dose may be attributed to the conversion of CBZ into its metabolite in RTgill-W1 cells, leading to similar concentrations of CBZ-EP under both conditions. On the other hand, this effect resulted less pronounced at concentration of (1 μg/L) of CBZ, likely due to the lower levels of CBZ-EP produced at this dose.

Unlike the PLHC-1 cell line, where significant effects were observed only for NAP, the RTgill-W1 cell line exhibited comparable levels of toxicity in all tested NSAIDs, regardless of the exposure dose. Specifically, IBU showed a "Slight" hazard level, whereas DIC, NAP, and NIM were categorized as having a "Moderate" hazard level. These findings suggest a non-specific response of this tissue to the different active principles within this therapeutic class, indicating that the toxicological effects may be driven by a generalized mechanism of action rather than compound-specific interactions.

A study on *O. mykiss* reported that a 28-day exposure to environmentally relevant concentrations (1-500 μg/L) of DIC induced gill toxicity, characterized by the proliferation of interlamellar and chloride cells, along with an increased frequency of necrosis in respiratory epithelial cells (Schwaiger et al., 2004), which, together with our findings, confirms the susceptibility of this tissue to this class of pharmaceuticals under both acute and chronic exposure conditions.

In this work, interactions between different pharmaceuticals were investigated testing 3 binary (A, B, E) and 3 quaternaries MIXs (C, D, F) in RTgill-W1 cell line.

The results highlighted no toxic effects at the lower exposure dose (1 μg/L), while variable responses were observed at the higher concentration (10 μg/L). In particular, the most harmful combinations for RTgill-W1 cell line were MIXs B, E (binary) and D (quaternary), suggesting that the increased detrimental impact was driven not by the number of drugs, but by potential interactions between them.

In the previous experimental plan, antagonistic effects were detected in the PLHC-1 cell line between the therapeutic classes of antidepressants and NSAIDs (MIXs A and E). These interactions, however, were not observed in this cell line. On the contrary, MIX A exhibited lower overall toxicity (hazard level "Absent") compared to individual exposures to ibuprofen (hazard level "Slight") and paroxetine (hazard level "Severe"), further suggesting a tissue-specific response to these compounds.

Interestingly, similar effect to that previously observed in the PLHC-1 cell line for mixture B (CBZ + VAL) at the higher exposure dose (10 μg/L) was identified in the present work. Both studies demonstrated a synergistic interaction between the antiepileptic CBZ and the antihypertensive VAL, resulting in higher overall toxicity for the combination (hazard level "Major" in both cell lines) compared to the individual compounds (hazard levels "Absent" for both in PLHC-1; "Moderate" for CBZ and "Absent" for VAL in RTgill-W1). As reported by Mezzelani et al. (2023; 2024), the effects caused by the same mixture on a different species (*M. galloprovincialis*) and under distinct experimental conditions (14-day *in vivo* exposure), revealed antagonistic interactions, further supporting the importance of adopting an integrated approach to assess the mechanisms of action and environmental risks associated with these compounds.

Overall results of this study highlight a distinct tissue-specific response to pharmaceuticals, demonstrating a generally higher level of hazard in RTgill-W1 compared to PLHC-1, which may be attributed to the enhanced capacity of hepatic cells to mitigate chemical stress owing to their critical role in detoxification processes (Schlenk et al., 2008). RTgill-W1 cell line exposed to most treatments exhibited lower levels of ROS compared to the control, whereas an opposite trend was observed in the PLHC-1 cell line, which showed increased ROS production. This heightened ROS production in PLHC-1 cells is consistent with their role in drug detoxification and metabolism, processes that are inherently associated with increased oxidative stress due to the activation of cytochrome P450 enzymes and other metabolic pathways involved in xenobiotic processing (Finkel & Holbrook, 2000). Pharmaceuticals may have been easily transported inside the gill cells, where they can exhibit toxicity due to limited biotransformation capabilities, likely due to the low or absent activity of CYP3A-like enzymes. This reduced metabolic capacity is further supported by the study of Franco et al. (2018), which suggests an increased susceptibility of these cells to cytotoxicity. The decrease in ROS levels observed in RTgill-W1 cell line could be due to a cellular metabolic slowdown in response to the drugs. This can occur as a protective mechanism to conserve energy or due to impaired cellular functions (Finkel & Holbrook, 2000; Sies 2015). Normally, ROS serve as signaling molecules that help activate oxidative response pathways. Their reduction might mean that the cells are unable to effectively engage in adaptive stress responses or repair mechanisms, leading to increased vulnerability to damage and higher cytotoxicity in the long run (Foyer & Noctor, 2005). Confirming this hypothesis, the study results indicated that, at the highest exposure dose (10 μg/L), most of the tested pharmaceuticals and mixtures (VEN, O-DES, FLU, CBZ-EP, NAP, DIC, MIX C, MIX D, and MIX F) that showed statistically significant decreases in ROS levels after 1 hour also exhibited significant cytotoxicity after 24 hours. The different responses observed for other treatments may be attributed to the gill cells' ability to mitigate chemical insults over longer periods at lower exposure

doses (1 μg/L) and to the specific cellular adaptative response to certain active principles (*e.g.*, CBZ, RAM, GEM, and MET). A reduced metabolic rate in gills due to toxic conditions was also hypothesized by Malarvizhi et al. (2012), who linked this condition to decreased lactate dehydrogenase activity measured after an acute exposure to CBZ (5.97 mg/L) in fish *Cyprinus carpio*. Interestingly, a biphasic response to the antidiabetic MET was observed. After one hour of exposure at both doses, there appears to be a slowdown in cellular metabolism, as indicated by a decrease in ROS levels. However, prolonged exposure (24 hours) seems to result in an acceleration of cellular metabolism, as evidenced by the increased cell viability.

The precise mechanisms underlying the behavior of PAR and MIX E (VEN + NAP) at the highest exposure dose remain unclear and require further investigation using molecular techniques. Notably, these two treatments, unlike all other tested conditions, induced a significant increase in ROS production, which was accompanied by a marked rise in cytotoxicity after 24 hours of exposure, suggesting a similar cellular response between this tissue and hepatic tissue under high-dose conditions.

6.5 Conclusions

Overall, the presented studies demonstrated that both the PLHC-1 and RTgill-W1 cell lines are suitable and sensitive models for assessing toxicity of pharmaceuticals and their MIXs at concentration ranges at least one order of magnitude lower than those reflecting EC_{50} obtained for environmental drugs in other cell lines (Caminada et al., 2006; Schnell et al., 2009). The selected endpoints revealed significant effects on toxicity-related pathways by a large panel of drugs, identifying the therapeutic class of antidepressants as the most reactive and PAR as the most toxic molecule among psychiatric drugs for both tissues, while a great toxicity was found also for the NSAID NAP in PLHC-1 cell line. Future investigations would be necessary to deepen the knowledge on molecular mechanisms of action of these compounds in non-target aquatic species. Results obtained in MIX treatments demonstrated that the toxicity cannot be related to the number of the combined drugs but rather to their mechanistic interactions, it varies depending on the tissue, with a greater effect of single pharmaceuticals compared to their MIXs. For the first time, weighted criteria were applied to elaborate and integrate the overall biological effects in cell cultures, summarizing synthetic hazard indices which reflect the severity of disturbance in *in vitro* models. This innovative approach provided quantitative insights on toxicity of several pharmaceuticals and their MIXs, allowing an easier comparison between different molecules, tissues and exposure conditions. This is of particular importance for future prioritization and risk assessment of environmental pharmaceuticals on non-target aquatic species.

7. Application of a battery of bioassays within the assessment procedure for the ecotoxicological impact of environmental pharmaceuticals

7.1 Introduction

The ubiquitous occurrence of pharmaceuticals in aquatic ecosystems is raising interest at both scientific and normative level. Due to their massive use in human and veterinary medicine and the limited removal by wastewater treatment plants (WWTPs), several active principles from different therapeutic classes (Bayer et al., 2014; Boxall et al., 2012; Mezzelani et al., 2018a) are commonly detected in freshwater, seawater sediments and organisms worldwide, with variable levels ranging from ng/L to μg/L and ng/g, respectively (Mezzelani et al. 2018a; Moreno-González et al. 2015). Pharmaceuticals are specifically designed for being effective at low doses and since the molecular targets of these bioactive compounds are evolutionarily conserved, their interaction with non-target aquatic species has been shown to interfere with several physiological and biochemical processes (Álvarez-Muñoz et al., 2015; Cerveny et al., 2021; Trombini et al., 2019). Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently detected therapeutic classes in tissues of marine organisms (Martínez-Morcillo et al., 2020; Mezzelani et al., 2016a; Wolecki et al., 2019), particularly diclofenac, ibuprofen, nimesulide and naproxen. Several studies have evaluated the sublethal effects induced by NSAIDs on non-target organisms, highlighting increased oxidative stress, changes on lipid metabolism, immune responses and genotoxicity (Ericson et al., 2010; Mezzelani at al., 2016a; Shmidt et al., 2012). Largely prescribed at global level, also psychiatric pharmaceuticals, can be bioaccumulated in aquatic organisms, as already documented for the antidepressants paroxetine, sertraline, fluoxetine, venlafaxine and citalopram, the anxiolytic lormetazepam and the antiepileptic carbamazepine (Martínez-Morcillo et al., 2020; Mezzelani et al., 2016a, 2018a, 2020): demonstrated adverse effects of these compounds include both physiological and behavioral alterations caused at ecologically relevant concentrations (Castillo-Zacarías et al., 2021; Ramírez-Morales et al., 2020; Sehonova et al., 2018). Cardiovascular drugs and lipid lowering agents are the therapeutic classes typically combined in human medicine and extensively consumed worldwide (Bayer et al., 2014; Cleuvers, 2005). Due to their widespread use and incomplete metabolism (Zhang et al., 2020), compounds as atenolol, propanol, gemfibrozil, ramipril and valsartan, are often detected in aquatic ecosystems (Ali et al., 2018; Álvarez-Muñoz et al., 2015; Godoy et al., 2015; Mezzelani et al., 2020; Moreno-González et al., 2016), but only a few data are available on non-target species. The antidiabetic drug metformin, owing to its widespread use, is frequently detected in marine environments (Meador et al., 2016; Ambrosio-Albuquerque et al., 2021), where it can significantly affect locomotor activity, exert estrogenic and neurotoxic effects, and impair the immune system function of non-target species (Niemuth et al., 2015a; Koagouw and Ciocan, 2018).

All the above-mentioned pharmaceuticals typically co-occur in marine ecosystems as complex mixtures containing several compounds with different chemical structures and modes of action, potentially leading to still unexplored additive, synergistic or antagonistic interactions. Various model species can be used for the toxicological assessment of pharmaceuticals on aquatic organisms and to evaluate the environmental risk of such compounds. However, the majority of the studies performed so far, were focused on individual compounds, after dosed at acute toxicity test dosing concentrations orders of magnitude higher (mg/L) than those realistic for aquatic ecosystems (Ferrari et al., 2003; Nunes et al., 2006; Riberio et al., 2015). In addition, comparison between different studies and molecules is often hampered by the use of single species with different sensitivity and the measurement of biological endpoints with different toxicological relevance.

In this respect, the application of a battery of model organisms to different trophic levels, the measurements of various biological endpoints and the overall elaboration of results through weighted criteria has been proposed as a reliable procedure to summarize complex datasets of toxicological effects and to better compare the ecotoxicological risk of pollutants in various environmental scenarios (Regoli et al., 2019). Weighted criteria are based on specific weights and thresholds assigned to each bioassay and derived from the biological relevance of measured endpoint and sensitivity of the tested species (Piva et al., 2011) providing a cumulative level of hazard, and a more robust and quantitative assessment of the adverse outcomes of tested compounds or mixtures.

In this respect, this work aimed to investigate the toxicological potential of several pharmaceuticals' representative of psychiatric, NSAIDs, cardiovascular drugs, lipid lowering agents and antidiabetics acting as individual compounds and complex mixtures. Their effects were evaluated on the growth of the unicellular algae *Phaeodactylum tricornutum*, on the embryo development of sea urchin *Paracentrotus lividus* and of the oyster *Crassostea gigas,* and on the bioluminescence of the bacterium *Aliivibrio fischeri*. These species have been largely characterized in terms of sensitivity of their biological responses, allowing their use as a battery and the weighted elaboration of overall results in an ecotoxicological hazard quotient. This standardized procedure is now included in Italian Law on dredged marine sediments to define quality and management actions for these materials (DM 173/2016). In the present study, this battery was applied to 16 widespread pharmaceuticals characterized by different mode of action to provide novel insights into their potential interactions toward the development of a more comprehensive ecotoxicological risk assessment procedures.

7.2 Materials and methods

7.2.1 Tested pharmaceuticals and experimental conditions

Selected pharmaceuticals including both parent compounds and metabolites were purchased from Sigma-Aldrich® (St. Louis, MO, USA): Venlafaxine (VEN, CAS 99300-78-4), O-Desmethylvenlafaxine (O-DESVEN, CAS 93413-62-8), Paroxetine (PAR, CAS 110429-35-1), Fluoxetine (FLU, CAS 56296-78-7), Lormetazepam (LORM, CAS 848-75-9), Carbamazepine (CBZ, CAS 298-46-4), Carbamazepine-10,11 epoxide (CBZ-EP, CAS 36507-30-9), Ibuprofen (IBU, CAS 15687-27-1), Naproxen (NAP, CAS 22204-53-1), Diclofenac (DIC, CAS 15307-79-6), Nimesulide (NIM, CAS 51803-78-2), Valsartan (VAL, CAS 137862-53-4), Atenolol (ATE, CAS 29122-68-7), Ramipril (RAM, CAS 87333-19-5), Gemfibrozil (GEM, , CAS 25812-30-0) and Metformin (MET, CAS 1115-70-4).

Stock solutions (1 mg/mL) of VEN, PAR, IBU, DIC, NAP, GEM, CBZ, CBZ-EP, VAL, RAM and MET were prepared in methanol (MeOH), while for the other molecules (O-DESVEN, FLU, NIM, LORM, ATE and GEM) the MeOH stock solution (1 mg/mL) was already supplied by Sigma-Aldrich®. Stock solutions were diluted in DMSO to obtain the selected exposure concentrations in the medium. DMSO in the experimental solutions was 0.1 %.

The toxicological effects of the 15 pharmaceuticals were assessed on individual compounds (at two environmental realistic doses of 1 and 10 μg/L) and in six different mixtures. The latter were selected combining drugs based on the mechanism of action of single molecules, frequency of detection in the environment, and their typical combination in human therapy. In this respect, the tested mixtures were MIX A: IBU + PAR; MIX B: CBZ+ VAL; MIX D: IBU + PAR + CBZ + VAL; MIX D: VEN + GEM + RAM + MET; MIX E: VEN + NAP; MIX F: VAL + ATE + GEM + RAM; MIX G: IBU + PAR + CBZ + VAL + LORM + DIC + NIM. In the mixtures, indicated doses (1 - 10 μg/L) represent the concentrations added for each single compound.

7.2.2 Algal growth of Phaeodactylum tricornutum

The algal growth was measured in unicellular algae *Phaeodactylum tricornutum*, according to standardized procedure (ISO, 2016). After exponential growth of the algal cultures, the cell density was adjusted to 10^4 cells/mL. At this time, 0.5 mL of algal cultures and 0.5 mL of nutrient solutions were added to 4 mL of tested solution (3.95 mL of ISO water + 0.05 mL of pharmaceuticals). For each condition, an experimental control (ISO water, salinity 33 ‰), a solvent control (ISO water and 0.1 % DMSO) and two concentrations (1 and 10 μg/L) of each pharmaceutical and mixtures were setup in triplicate using sterile 12-wells culture plates.

After 72 h of incubation, under 10.000 lux of continuous illumination, at 20 ± 2 °C, algal cells were fixed by adding two drops of Lugol and ethanol (1:3) and were counted using an optical microscope (OLYMPUS BX51) and Burker's counter chamber (Broccoli et al., 2021). Results were expressed as percentage of inhibition.

7.2.3 Embryo development of sea urchin Paracentrotus lividus

Spawning of adult of sea urchins was induced by osmotic shock, and the gametes were collected separately in male and female specimens: eggs were recovered in 100 ml glass beakers filled with sea water, placing the female with the genital pore facing the water, while the sperm was aspirated directly from the gonopores with a glass Pasteur pipette and stored at 4 °C in a 1.5 ml tube (Eppendorf® type). The quality of eggs was checked discarding female animals with eggs not round, immature forms or debris. Suspensions with density eggs of 10^3 cells/mL were prepared and fertilization with sperm/egg ratio 10:1 was induced (ISPRA, 2017).

After 20 min of incubation at 18 ± 1 °C, 1 mL of fertilized suspensions was mixed with 9 mL of tested solution (8,9 mL of ISO water + 0,1 mL of pharmaceuticals) (Broccoli et al., 2021). For each condition, an experimental control (ISO water, salinity 33 ‰), a solvent control (ISO water and 0.1 % DMSO) and two concentrations (1 and 10 μg/L) of each pharmaceutical and mixtures were setup in triplicate.

After 72 h at 18 ± 1 °C in a dark room, the larvae were fixed by adding formalin and were observed in an inverted microscope (MEIJI TECHNO). The morphological evaluation was performed, and the results were expressed as percentage of abnormal embryos, using the Abbott's correction (Abbott, 1975). To obtain a more accurate estimate of the embryotoxic effects different categories of developmental anomalies have been identified according to Carballeira et al. (2012).

7.2.4 Embryo development of oyster Crassostrea gigas

The embryotoxicity test was conducted using the sea oyster *Crassostrea gigas*, following a standardized method (ICES, 2013). After the collection of the sperm and eggs of male and female specimen, the quality of gametes and the correct density (eggs suspension of $3*10^3$ cells/mL) was checked. Gametes were put together for a fertilization period of 2 hours in the dark. The suspension should be stirred at least every 10 - 15 minutes to prevent excessive settling of embryos and potential oxygen starvation. An aliquot of this suspension was collected to control, microscopically, if there was fertilization and embryos development (*i.e.* zygotes have reached the 16 - 32 cell stage), then, 1 mL of fertilized suspensions was mixed in 9 mL of tested solution (8.9 mL of ISO water + 0.1 mL of pharmaceuticals). For each tested condition an experimental control (ISO water, salinity 33 ‰), a

solvent control (ISO water and 0.1 % DMSO) and two concentrations (1 and 10 µg/L) of each pharmaceutical and mixture were setup in triplicate, using sterile 6-wells culture plates. After 24h at 24 ± 2 °C in the dark room, a solution of Lugol and ethanol was added to each well to fix and preserve larvae. Estimation of percentage of normal and abnormal larvae was done counting 100 larvae for every sample using an inverted microscope (MEIJI TECHNO). Larvae were considered malformed when presented extruded and granulated tissues (Beiras & His, 1994), pre-D larvae stage, protruded mantle and indented shell as described by His et al. (1997). The toxicity of the treatments was determined by calculating the percentage of malformed larvae.

7.2.5 Bioluminescence in the bacterium Aliivibrio fischeri

The bioluminescence was measured in the luminescent bacterium *Aliivibrio fischeri* following the basic test procedure for Microtox (ISO, 2019). All necessary reagents (freeze-dried bacteria, diluents, osmotic adjusting and reconstituting solutions) were supplied by AQUA SCIENCE.

Pharmaceuticals and mixtures treatments were incubated with *A. fisheri* for 15 minutes at 15 °C and variation in light intensity were measured and automatically converted in bioluminescence inhibition (%) by Microtox OmniTM v. 1.16 software. The assays were performed in 5 replicates for each experimental solution.

7.2.6 Statistical analysis

Statistical analyses were performed using RStudio (version 2023.12.0 Build 369). Data were assessed for normal distribution using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. Comparisons between treatments within the same therapeutic class of pharmaceuticals were analyzed using one-way analysis of variance (ANOVA), level of significance at the 95 % of confidence interval, $\alpha = 0.05$. Post-hoc analysis was performed using the Student Newman Keuls Test to compare the means of values ($n = 3$).

7.2.7 Weight of Evidence (WOE) elaboration

Overall results of the algal growth in *P. tricornutum*, of the embryo development in *P. lividus* and of the bioluminescence in *A. fisheri* were elaborated through weighted criteria, which assign to each bioassay specific weights and thresholds derived from the biological relevance of measured endpoint and sensitivity of the tested species respectively (Piva et al., 2011), to provide a cumulative level of hazard related to the entire bioassays battery which reflected the severity of effects caused by different pharmaceuticals and mixtures.

For each experimental condition the cumulative Hazard Quotient ($HQ_{BATTERY}$) was calculated by the summation of the weighted effects (E_w), i.e., the variations measured for each test normalized to its specific thresholds, corrected for the statistical significance of the difference (w), biological importance of the endpoint and exposure conditions (w_2):

$$HQ_{Battery}=\sum_{k=1}^{N} Effectw(k) \cdot w_2$$

The $HQ_{Battery}$ was normalized to a scale ranging from 0 to 10, where 1 is the Battery Threshold when all the measured bioassays exhibit an effect equal to the threshold, 10 when all the assays exhibit 100 % of effect (Regoli et al., 2019). Based on these criteria, each tested pharmaceutical and mixture were assigned to one of five classes of hazard, which are Absent, Slight, Moderate, Major and Severe.

7.3 Results

7.3.1 Algal growth on Phaeodactylum tricornutum

In this bioassay, a 15 % inhibition of algal growth represents the threshold for identifying a biologically relevant effect in the unicellular algae *P. tricornutum*.

For all the antidepressants (VEN, O-DES, PAR, and FLU) was observed a dose-dependent effect, with a growth stimulation at the lower exposure dose of 1 µg/L, and inhibition at the concentration of 10 µg/L. Particularly marked effects were highlighted for 10 µg/L of PAR (Figure 7.1A). Significant decrease in algal growth was highlighted for the antiepileptic CBZ (1 and 10 µg/L) and its metabolite CBZ-EP (10 µg/L); while no significant variations were showed for LORM (1 and 10 µg/L) and the lower dose of CBZ-EP (Figure 7.1A).

An algal growth stimulation was observed at the lower concentration of IBU, while a significant inhibition was reported at the higher concentration of 10 µg/L. No variations were measured for NAP, DIC at both 1 and 10 µg/L, and NIM at 1 µg/L; however, NAP caused a decrease in *P. tricornutum* growth at 10 µg/L (Figure 7.1B).

Among cardiovascular drugs (Figure 7.1C), a significant inhibition of algal growth was observed for the higher dose of VAL, ATE and RAM, whereas no significant effects were showed at the lower tested concentration. The highest dose of the lipid-lowering agent GEM caused a significant decrease in *P. tricornutum* growth (Figure 7.1D), while no effects were observed for both doses of the antidiabetic MET (Figure 7.1 E).

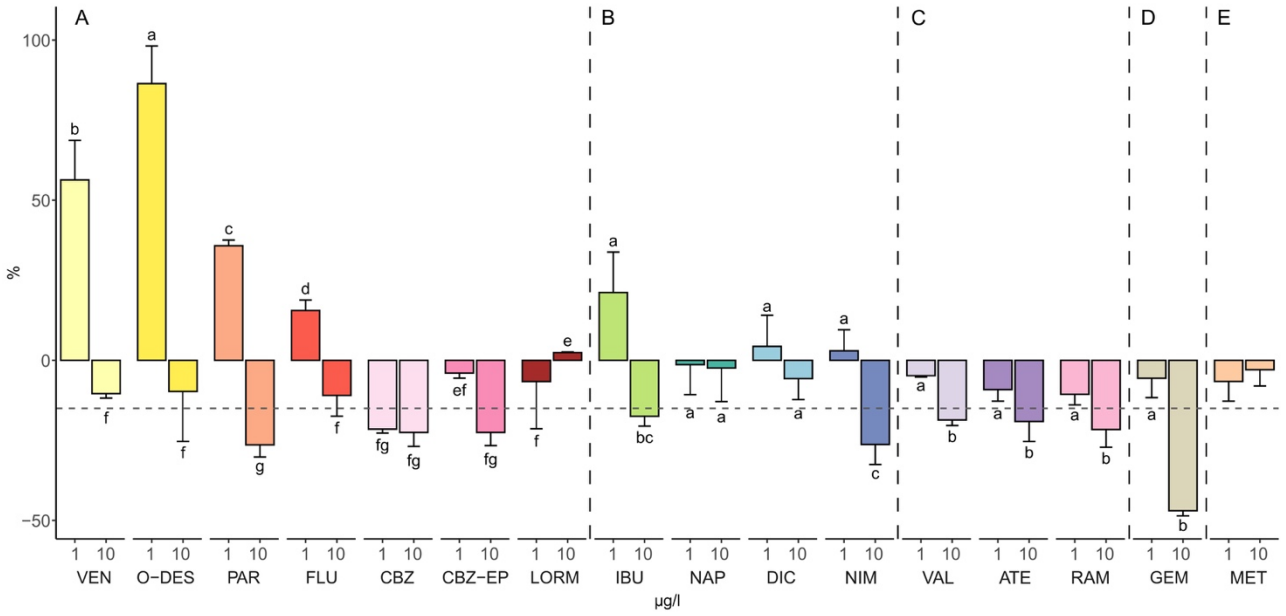


Figure 7.1. The effect of (A) psychiatric drugs, (B) Non-Steroidal Antinflammatory Drugs, (C) cardiovascular drugs, (D) the lipid lowering agent and (E) the antidiabetic on the algal growth of *Phaeodactylum tricornutum.* The graph describes the percentage of growth measured in *P. tricornutum* after 72 hours of exposure to individual pharmaceuticals (1 - 10 µg/L) compared to those detected in algae exposed to solvent control (0.1 % DMSO). The gray dashed line (15 % of inhibition) indicates the threshold above which the effect is considered biologically relevant. Results are presented as mean ± SD (n = 3). Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Student Newman Keuls Test was used for post-hoc comparison between means of values (n = 3). VEN Venlafaxine; O-DESVEN O-Desmethylvenlafaxine; PAR Paroxetine; FLU Fluoxetine; CBZ Carbamazepine; CBZ-EP Carbamazepine-10,11 epoxide; LORM Lormetazepam; IBU Ibuprofen; NAP Naproxen; DIC Diclofenac; NIM Nimesulide; VAL Valsartan; ATE Atenolol; RAM Ramipril; GEM Gemfibrozil; MET Metformin.

The exposure to both doses of mixtures A, B, C, D, E and G highlighted a decrease in *P. tricornutum* growth. Lower toxicity was observed for mixture F, showing significant effects only for the concentration of 10 µg/L (Figure 7.2).

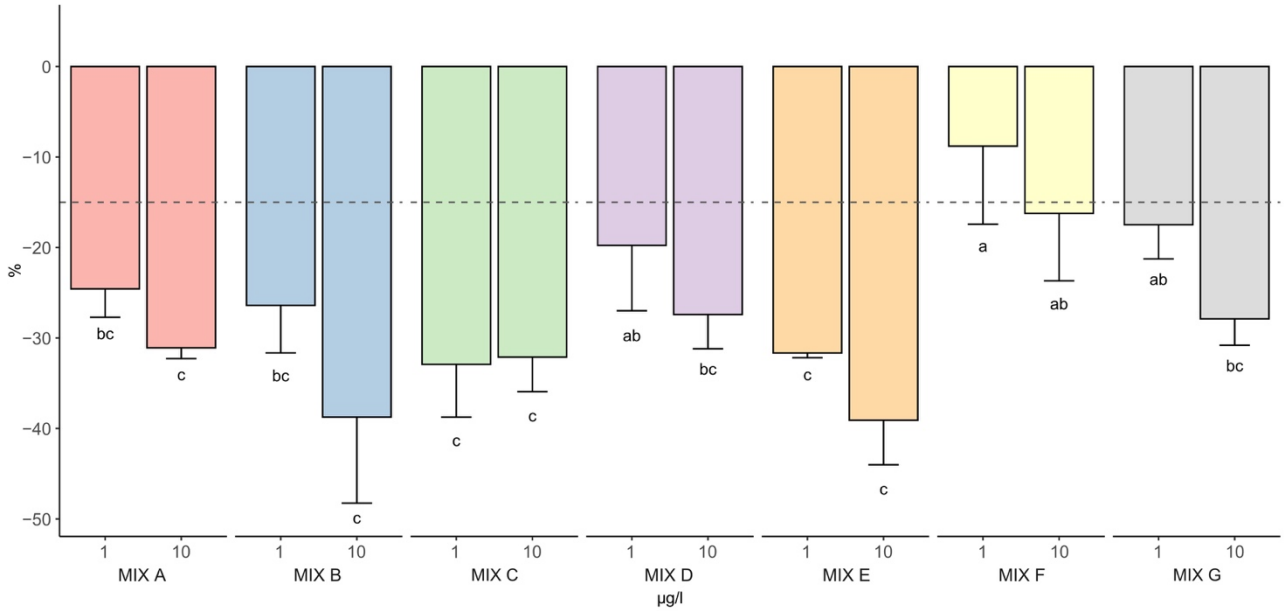


Figure 7.2. The effect of pharmaceutical mixtures on the algal growth of *Phaeodactylum tricornutum.* The graph describes the percentage of growth measured in *P. tricornutum* after 72 hours of exposure to pharmaceutical mixtures (1 - 10 µg/L) compared to those detected in algae exposed to solvent control (0.1 % DMSO). The gray dashed line (15 % of inhibition) indicates the threshold above which the effect is considered biologically relevant. Results are presented as mean ± SD (n = 3). Different letters indicate significant differences between each pharmaceutical mixture, the Student Newman Keuls Test was used for post-hoc comparison between means of values (n = 3). MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine+ Valsartan; MIX D: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Gemfibrozil + Ramipril + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril; MIX G: Ibuprofen + Paroxetine + Carbamazepine + Valsartan + Lormetazepam + Diclofenac + Nimesulide.

7.3.2 Embryo development of sea urchin Paracentrotus lividus

In this test, a 15 % of abnormal larvae indicates the threshold for identifying a biologically relevant effect in the sea urchin *P. lividus*.

Due to the limited availability of sexually mature specimens of *P. lividus* throughout the year, it was not possible to conduct this bioassay on two treatments: the antidiabetic metformin and the mixture D (VEN + GEM + MET + RAM).

A lack of significant variations was observed for 1 and 10 µg/L of the psychiatric drug O-DES and for 1 µg/L of PAR (Figure 7.3A). A dose-dependent effect was highlighted for all other treatments (Figure 7.3, 7.4). Considering the individually exposed pharmaceuticals, a significant increase of abnormal larvae was measured for both doses of the psychiatric drug CBZ-EP (Figure 7.3A) and cardiovascular drug VAL (Figure 7.3C), and for the highest concentration (10 µg/L) of the psychiatric drugs VEN, PAR, FLU, CBZ and LORM (Figure 7.3A), the NSAIDs IBU, NAP and DIC (Figure 7.3B) and the lipid lowering agent GEM (Figure 7.3D).

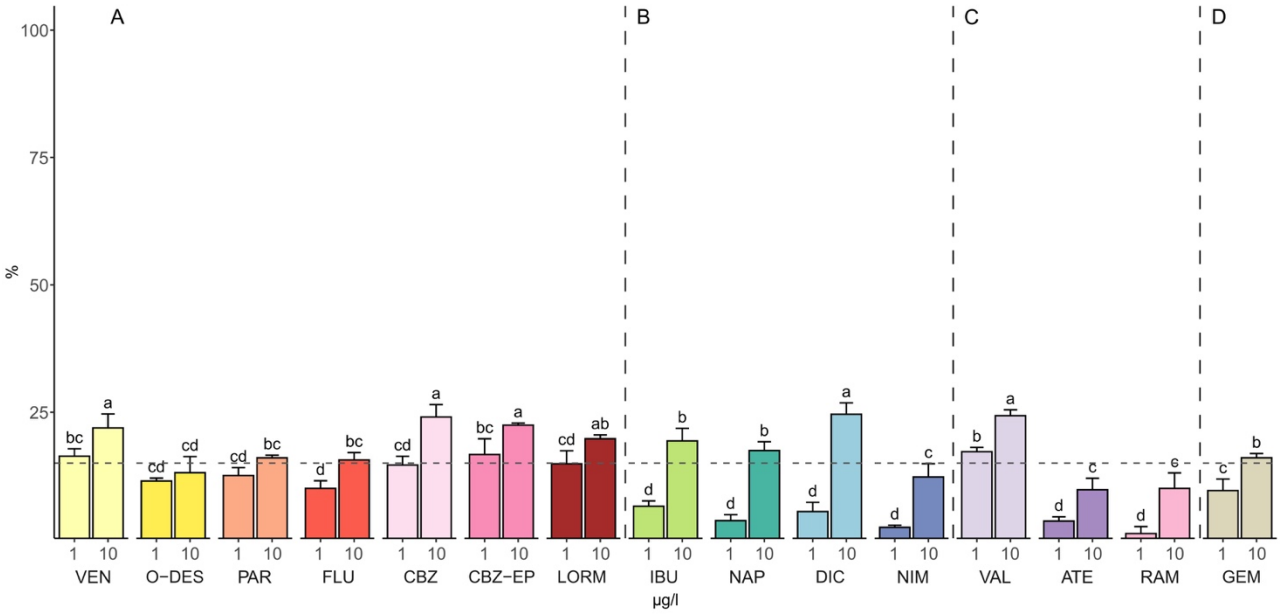


Figure 7.3. The effect of (A) psychiatric drugs, (B) Non-Steroidal Antinflammatory Drugs, (C) cardiovascular drugs, (D) and the lipid lowering agent on the embryo development of *Paracentrotus lividus.* The graph describes the percentage of abnormal larvae measured in *P. lividus* after 72 hours of exposure to individual pharmaceuticals (1 - 10 µg/L) compared to those detected in sea urchins exposed to solvent control (0.1 % DMSO). The gray dashed line (15 % of of abnormal larvae) indicates the threshold above which the effect is considered biologically relevant. Results are presented as mean ± SD (n = 3). Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Student Newman Keuls Test was used for post-hoc comparison between means of values (n = 3). VEN Venlafaxine; O-DESVEN O-Desmethylvenlafaxine; PAR Paroxetine; FLU Fluoxetine; CBZ Carbamazepine; CBZ-EP Carbamazepine-10,11 epoxide; LORM Lormetazepam; IBU Ibuprofen; NAP Naproxen; DIC Diclofenac; NIM Nimesulide; VAL Valsartan; ATE Atenolol; RAM Ramipril; GEM Gemfibrozil.

Biological relevant effects on sea urchin embryo development were observed for 1 and 10 µg/L of mixtures C, E and G; and for the highest concentration of mixtures A and B (Figure 7.4).

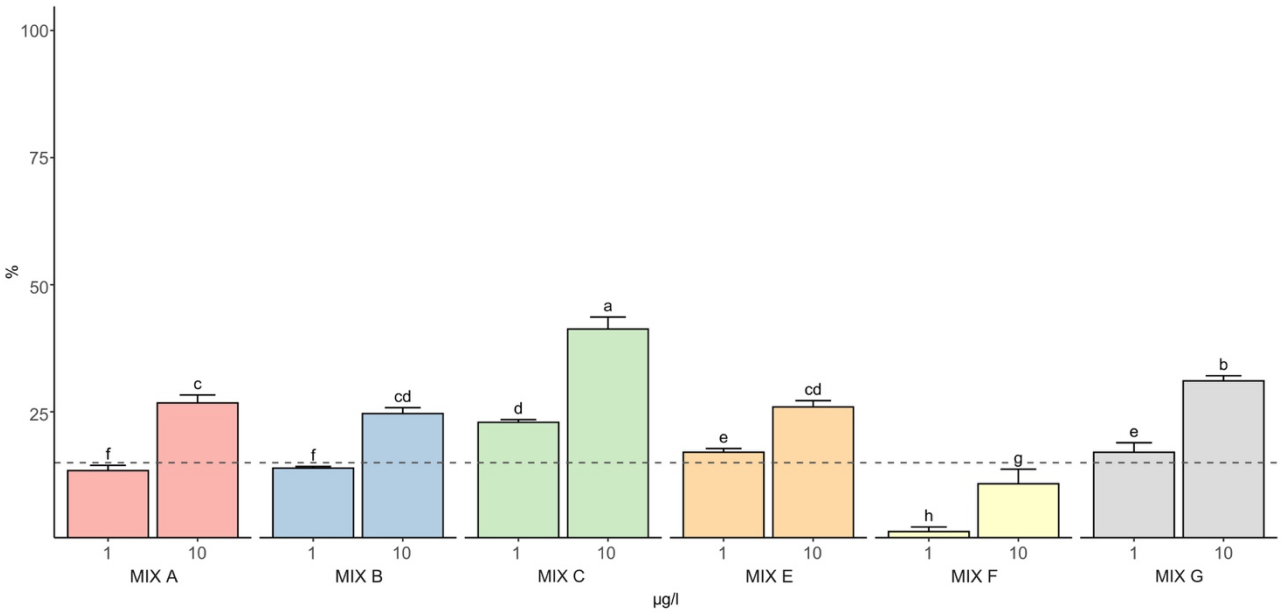


Figure 7.4. The effect of pharmaceutical mixtures on the embryo development of *Paracentrotus lividus.* The graph describes the percentage of abnormal larvae measured in *P. lividus* after 72 hours of exposure to pharmaceutical mixtures (1 - 10 µg/L) compared to those detected in sea urchins exposed to solvent control (0.1 % DMSO). The gray dashed line (15 % of abnormal larvae) indicates the threshold above which the effect is considered biologically relevant. Results are presented as mean ± SD (n = 3). Different letters indicate significant differences between each pharmaceutical mixture, the Student Newman Keuls Test was used for post-hoc comparison between means of values (n = 3). MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine+ Valsartan; MIX D: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril; MIX G: Ibuprofen + Paroxetine + Carbamazepine + Valsartan + Lormetazepam + Diclofenac + Nimesulide.

7.3.3 Embryo development of oyster Crassostrea gigas

In this bioassay, a 15 % of abnormal larvae represents the threshold for identifying a biologically relevant effect in the sea oyster *C. gigas*.

No significant variation was observed in embryo development of *C. gigas* exposed to psychiatric drugs (Figure 7.5A) and NSAIDs (Figure 7.5B).

Among cardiovascular drugs, significant increase in oyster abnormal larvae was measured for the highest dose of ATE, while no significant effects were reported for all other treatments (Figure 7.5C).

No relevant biological effects were highlighted for the lipid lowering agent GEM (Figure 7.5D) and for the antidiabetic MET (Figure 7.5E).

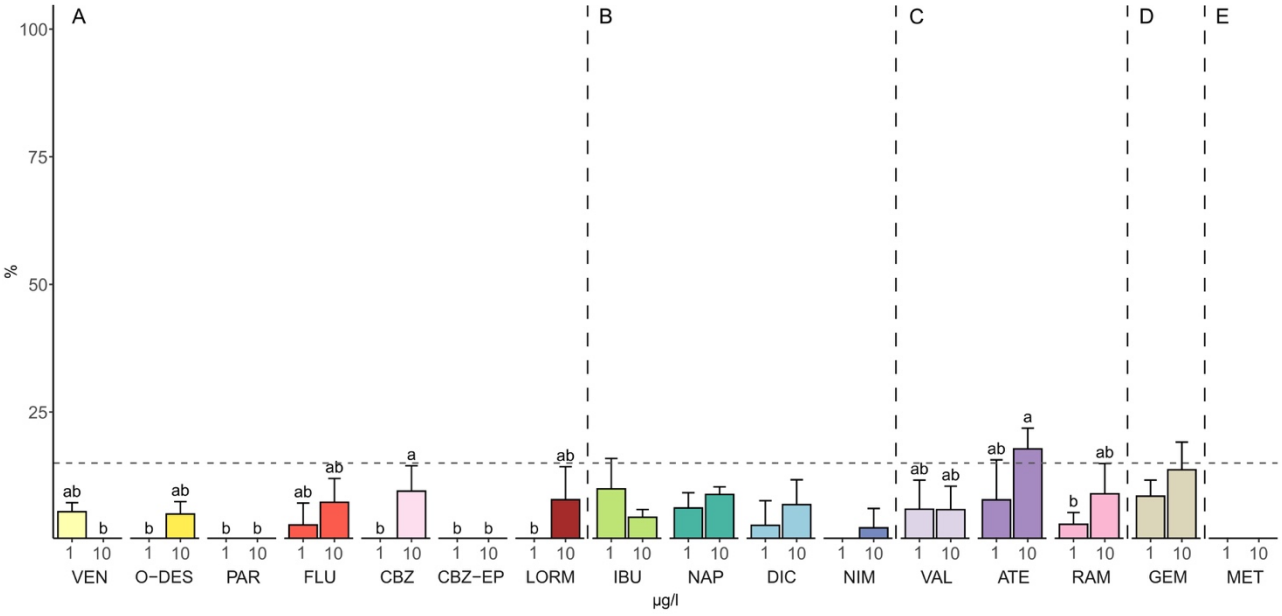


Figure 7.5. The effect of (A) psychiatric drugs, (B) Non-Steroidal Antinflammatory Drugs, (C) cardiovascular drugs, (D) the lipid lowering agent and (E) the antidiabetic on the embryo development of *Crassostrea gigas.* The graph describes the percentage of abnormal larvae measured in *C. gigas* after 72 hours of exposure to individual pharmaceuticals (1 - 10 µg/L) compared to those detected in oysters exposed to solvent control (0.1 % DMSO). The gray dashed line (15 % of abnormal larvae) indicates the threshold above which the effect is considered biologically relevant. Results are presented as mean ± SD (n = 3). Different letters indicate significant differences between each pharmaceutical inside the same therapeutic class, the Student Newman Keuls Test was used for post-hoc comparison between means of values (n = 3). VEN Venlafaxine; O-DESVEN O-Desmethylvenlafaxine; PAR Paroxetine; FLU Fluoxetine; CBZ Carbamazepine; CBZ-EP Carbamazepine-10,11 epoxide; LORM Lormetazepam; IBU Ibuprofen; NAP Naproxen; DIC Diclofenac; NIM Nimesulide; VAL Valsartan; ATE Atenolol; RAM Ramipril; GEM Gemfibrozil; MET Metformin.

A significant increase in *C. gigas* abnormal larvae was observed for the higher concentration of the mixture G, whereas no variations were showed in both doses of all other mixtures (Figure 7.6).

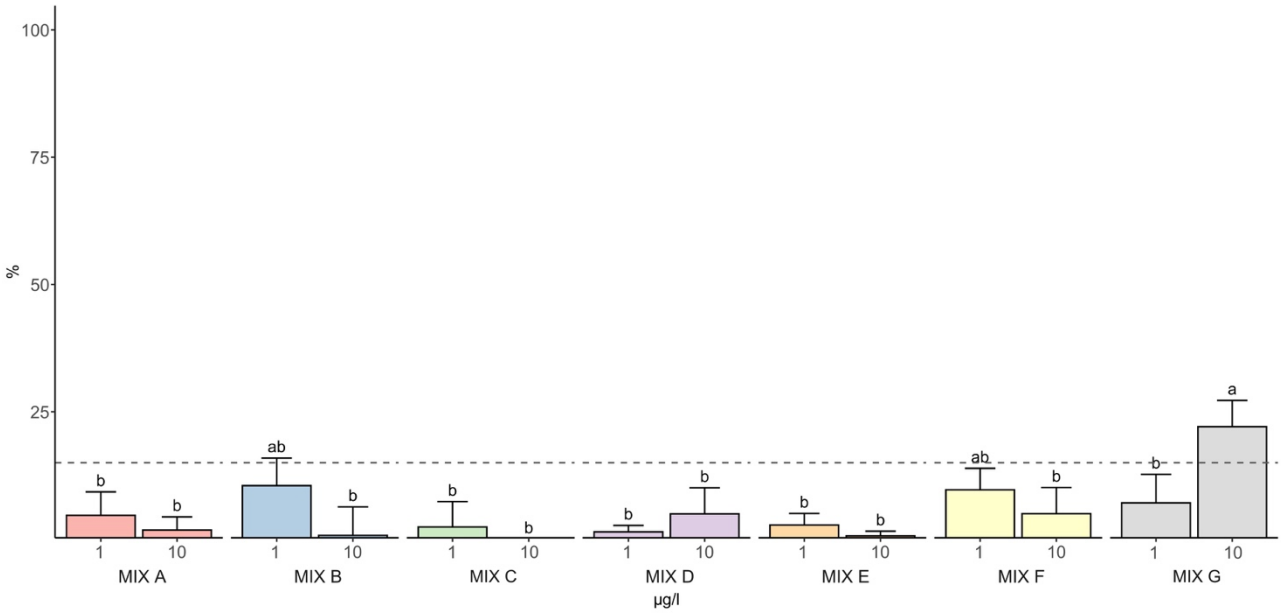


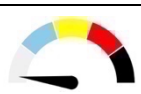
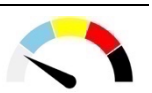
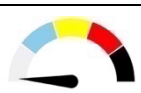
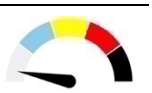
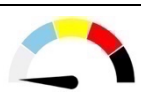
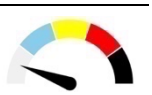
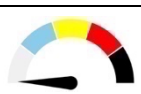
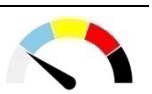
Figure 7.6. The effect of pharmaceutical mixtures on the embryo development of *Crassostrea gigas.* The graph describes the percentage of abnormal larvae measured in *C. gigas* after 72 hours of exposure to pharmaceutical mixtures (1-10 µg/L) compared to those detected in oysters exposed to solvent control (0.1 % DMSO). The gray dashed line (15% of abnormal larvae) indicates the threshold above which the effect is considered biologically relevant. Results are presented as mean ± SD (n = 3). Different letters indicate significant differences between each pharmaceutical mixture, the Student Newman Keuls Test was used for post-hoc comparison between means of values (n = 3). MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX D: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Gemfibrozil + Ramipril + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril; MIX G: Ibuprofen + Paroxetine + Carbamazepine + Valsartan + Lormetazepam + Diclofenac + Nimesulide.

7.3.4 Bioluminescence in the bacterium Alivibrio fischeri

In this test, a 15 % of bioluminescence inhibition indicates the threshold for identifying a biologically relevant effect in the bacterium *A. fischeri*. No biological effects were observed for both the individually exposed pharmaceuticals and the mixtures (data not shown).

7.3.5 Weight of evidence (WOE) elaboration

The figures 5.7 and 5.8 show the weighted elaboration of results of the algal growth in *P. tricornutum*, the embryo development in *P. lividus* and the bioluminescence in *A. fisheri* for individually exposed pharmaceuticals and mixtures, respectively.For psychiatric drugs, the WOE model summarized a level of hazard "Slight" for the higher concentration (10 µg/L) of PAR, CBZ and CBZ-EP, and "Absent" for all other treatments and doses. The NSAIDs and cardiovascular drugs always showed an "Absent" level of hazard, while the level of hazard of the lipid lowering agent GEM increased from "Absent" to "Moderate" at 1 and 10 µg/L, respectively (Figure 7.7).

Treatment	Species	$HQ_{BIOASSAY}$ (1µg/L)	$HQ_{BATTERY}$ (1µg/L)	$HQ_{BIOASSAY}$ (10µg/L)	$HQ_{BATTERY}$ (10µg/L)	Class of hazard (1µg/L)	Class of hazard (10µg/L)
Psychiatric drugs							
Venlafaxine	*P.tricornutum*	1.29		1.07		Absent 	Absent 
	P. lividus	1.02	0.65	1.36	0.68		
	A. fisheri	0.08		0.04			
O-Desmethyl venlafaxine	*P.tricornutum*	1.29		0.26		Absent 	Absent 
	P. lividus	0.71	0.55	0.81	0.30		
	A. fisheri	0.01		0.02			
Paroxetine	*P.tricornutum*	0.01		2.72		Absent 	Slight 
	P. lividus	0.78	0.22	1.00	1.05		
	A. fisheri	0.00		0.07			
Fluoxetine	*P.tricornutum*	0.03		1.13		Absent 	Absent 
	P. lividus	0.62	0.18	0.97	0.6		
	A. fisheri	0.00		0.09			
Carbamazepine	*P.tricornutum*	2.21		2.32		Absent 	Slight 
	P. lividus	0.91	0.89	1.49	1.07		
	A. fisheri	0.11		0.03			
Carbamazepine -10,11 epoxide	*P.tricornutum*	0.14		2.32		Absent 	Slight 
	P. lividus	1.04	0.36	1.39	1.07		
	A. fisheri	0.14		0.11			
Lormetazepam	*P.tricornutum*	0.17		0.00		Absent 	Absent 
	P. lividus	0.92	0.31	1.23	0.35		
	A. fisheri	0.04		0.06			
NSAIDs							
Ibuprofen	*P.tricornutum*	0.00		1.8		Absent 	Absent 
	P. lividus	0.41	0.15	1.2	0.82		
	A. fisheri	0.13		0.00			
Naproxen	*P.tricornutum*	0.03		0.06		Absent 	Absent 
	P. lividus	0.23	0.08	1.08	0.32		
	A. fisheri	0.05		0.03			
Diclofenac	*P.tricornutum*	0.00		0.2		Absent 	Absent 
	P. lividus	0.34	0.1	1.53	0.49		
	A. fisheri	0.04		0.05			
Nimesulide	*P.tricornutum*	0.00		2.7		Absent 	Absent 
	P. lividus	0.15	0.04	0.76	0.99		
	A. fisheri	0.00		0.13			
Cardiovascular drugs							
Valsartan	*P.tricornutum*	0.23		1.92		Absent 	Absent 
	P. lividus	1.07	0.36	1.51	0.96		
	A. fisheri	0.01		0.08			
Atenolol	*P.tricornutum*	0.94		1.96		Absent 	Absent 
	P. lividus	0.23	0.36	0.61	0.72		
	A. fisheri	0.15		0.06			
Ramipril	*P.tricornutum*	1.09		2.22		Absent 	Absent 
	P. lividus	0.02	0.34	0.62	0.78		
	A. fisheri	0.13		0.01			
Lipid lowering agents							
Gemfibrozil	*P.tricornutum*	0.2		4.83		Absent 	Moderate 
	P. lividus	0.6	0.23	1.00	1.86		
	A. fisheri	0.04		0.10			
Antidiabetic							
Metformin *	*P.tricornutum*	0.12		0.23		Absent 	Absent 
	C. gigas	0.00	0.31	0.00	0.45		
	A. fisheri	0.00		0.00			

*Due to the lack of data on embryotoxicity in sea urchin *P. lividus*, the weighted elaboration for this treatment was calculated using the results obtained from the species *C. gigas*.

Figure 7.7. Weighted elaboration of results on the algal growth in *P. tricornutum*, the embryo development in *P. lividus* and the bioluminescence in *A. fisheri* for individual pharmaceuticals. The Hazard Quotient (HQ) elaborated for each bioassay, the HQ elaborated for the whole battery and the overall Class of hazard are given for each dose (1 - 10 µg/L) and pharmaceutical of the various therapeutic classes. Colors are used to highlight hazard classes as follow: white-Absent, blue-Slight, yellow-Moderate, red-Major, black-Severe.

The overall elaboration of results on mixtures effects summarized a level of hazard "Moderate" for 10 µg/L of mixtures B, C and E, "Slight" for 10 µg/L of mixtures A and G, and for 1 µg/L of mixtures B, C and E, and "Absent" for all other treatments and doses (Figure 7.8).

Given the lack of data regarding the effects of the antidiabetic MET and mixture D on the sea urchin *P. lividus*, the weighted elaboration for these treatments was conducted using the results of the embryo development in oyster *C. gigas*. The WOE elaboration summarized an "Absent" and "Slight" hazard level for both doses of MET (Figure 7.7) and mixture D (Figure 7.8), respectively.

Treatment	Species	$HQ_{BIOASSAY}$ (1µg/L)	$HQ_{BATTERY}$ (1µg/L)	$HQ_{BIOASSAY}$ (10µg/L)	$HQ_{BATTERY}$ (10µg/L)	Class of hazard (1µg/L)	Class of hazard (10µg/L)
MIX A	*P.tricornutum*	2.53	0.94	3.2	1.48	Absent	Slight
	P. lividus	0.83		1.66			
	A. fisheri	0.07		0.007			
MIX B	*P.tricornutum*	2.72	1.00	3.99	1.74	Slight	Moderate
	P. lividus	0.86		1.53			
	A. fisheri	0.05		0.09			
MIX C	*P.tricornutum*	3.39	1.46	3.30	1.84	Slight	Moderate
	P. lividus	1.42		2.56			
	A. fisheri	0.06		0.03			
MIX D *	*P.tricornutum*	1.75	1.15	2.65	1.35	Slight	Slight
	C. gigas	1.12		1.74			
	A. fisheri	0.00		0.00			
MIX E	*P.tricornutum*	3.26	1.25	4.02	1.76	Slight	Moderate
	P. lividus	1.06		1.61			
	A. fisheri	0.00		0.04			
MIX F	*P.tricornutum*	0.04	0.16	0.67	0.67	Absent	Absent
	P. lividus	0.40		1.67			
	A. fisheri	0.14		0.08			
MIX G	*P.tricornutum*	1.80	0.8	2.87	1.48	Absent	Slight
	P. lividus	1.06		1.93			
	A. fisheri	0.06		0.13			

*Due to the lack of data on embryotoxicity in sea urchin *P. lividus*, the weighted elaboration for this treatment was calculated using the results obtained from the species *C. gigas*.

Figure 7.8. Weighted elaboration of results on the algal growth in *P. tricornutum*, the embryo development in *P. lividus* and the bioluminescence in *A. fisheri* for pharmaceutical mixtures. The Hazard Quotient (HQ) elaborated for each bioassay, the HQ elaborated for the whole battery and the overall Class of hazard are given for each dose (1 - 10 µg/L) and pharmaceutical mixtures. Colors are used to highlight hazard classes as follow: white-Absent, blue-Slight, yellow-Moderate, red-Major, black-Severe. MIX A: Ibuprofen + Paroxetine; MIX B: Carbamazepine + Valsartan; MIX C: Ibuprofen + Paroxetine + Carbamazepine + Valsartan; MIX D: Venlafaxine + Ramipril + Gemfibrozil + Metformin; MIX E: Venlafaxine + Naproxen; MIX F: Valsartan + Atenolol + Gemfibrozil + Ramipril; MIX G: Ibuprofen + Paroxetine + Carbamazepine + Valsartan + Lormetazepam + Diclofenac + Nimesulide.

7.4 Discussion

Considering the increasing concern about environmental pharmaceuticals, scientific community and regulatory authorities are actively working to establish appropriate procedures for the ecotoxicological assessment of their impacts on aquatic ecosystems. Currently, Italian legislation regarding dredged marine sediments (DM 173/2016) employs standardized approaches to summarize extensive datasets into integrative indices, thereby making the findings more accessible and interpretable for stakeholders and decision-makers. This approach involves applying a battery of bioassays using model species from different trophic levels, measuring various biological endpoints, and elaborating the overall results through weighted criteria (Regoli et al., 2019). In this context, this work aimed to assess the ecotoxicological hazards of environmental pharmaceuticals and their mixtures by applying a standardized battery of bioassays, which included the growth of the unicellular algae *Phaeodactylum tricornutum*, the embryo development of the sea urchin *Paracentrotus lividus*, and the bioluminescence of the bacterium *Aliivibrio fischeri*. Additionally, the embryo development of the oyster *Crassostrea gigas* was assessed to identify an alternative species suitable for the risk assessment of these emerging pollutants, serving as a potential replacement for the sea urchin, which is often limited by the scarcity of sexually mature individuals throughout the year.

The outcomes of this study highlighted a lack of overall toxicity for both doses of most individual pharmaceuticals, as supported by the "Absent" hazard level determined using weighted criteria. However, greater effects were observed for the highest concentration (10 μg/L) of the antidepressant PAR, the antiepileptic CBZ and its metabolite (CBZ-EP) and the lipid regulator GEM, with hazard levels ranging from "Slight" to "Moderate".

Although the overall toxicity assessed through the battery of ecotoxicological bioassays was found to be absent for most of the tested compounds, it was possible to hypothesize and discuss the mechanisms of toxicity for certain pharmaceuticals based on the results of individual bioassays.

The observed decrease in growth of *Phaeodactylum tricornutum* exposed to CBZ may be attributed to its capacity to alter cell membrane integrity and ion dynamics through interactions with the phospholipid bilayer (Xin et al., 2017). Specifically, phospholipids have been shown to be particularly sensitive to CBZ exposure, likely due to the drug's molecular structure and lipophilicity (log K_{ow} = 2.5) (Loftsson et al., 2004). CBZ has the ability to expand the bilayer by disrupting polar head groups and acyclic phospholipid chains (Suwalsky et al., 2006), leading to a reduction in ion permeation, particularly for Na^+ and K^+ (Lamichhane et al., 2013). Consequently, CBZ may induce hydrophobic interactions that compromise the integrity of the phospholipid bilayer, further impairing membrane functionality. Similarly, the observed toxicity of PAR in *P. tricornutum* can be correlated with its molecular structure, particularly the presence of both the aromatic ring and the amino group,

which may interact with biological membranes (Villain et al., 2016). Despite the limited data on algal responses to PAR, results of this study align with those of Johnson et al. (2007), who confirmed through the computerized predictive system ECOSAR (Ecological Structure–Activity Relationship) that selective serotonin reuptake inhibitors (SSRIs), including PAR, can have significant toxic effects on microalgae. Furthermore, as discussed in the previous chapter (Experiment 2: Exposure to venlafaxine, gemfibrozil, ramipril, metformin, and their mixture), the model proposed by Minguez et al. (2014) may elucidate why PAR causes greater membrane damage compared to other antidepressants due to its specific chemical structure, thereby potentially justifying our findings.

Additionally, Antonopoulou et al. (2022) reported algal growth inhibition ranging from 20 % to 40 % in three different microalgae (*Scenedesmus rubescens, Chlorococcum sp.,* and *Dunaliella tertiolecta*) exposed to 10 µg/L of PAR for 72 hours.

In accordance with our findings, which indicated a 10 % growth inhibition in algae treated with 10 µg/L of the antidepressant FLU, IC50 values of 47.3 µg/L, 44.99 µg/L, and 48.2 µg/L for FLU were reported for the marine diatoms *P. tricornutum* and *Skeletonema marinoi*, as well as for the freshwater microalga *Raphidocelis subcapitata* (Johnson et al., 2007; Minguez et al., 2018; Feijão et al., 2020). Specifically, exposure to 80 µg/L FLU in *P. tricornutum* resulted in an 83 % reduction in cell density and induced stress response mechanisms, including increased production of β-carotene and antioxidant enzymes, as well as the accumulation of lipid peroxidation products (Feijão et al., 2020).

An hormetic effect was observed for all antidepressants (VEN, O-DES, PAR, and FLU) and the NSAID IBU, showing stimulatory effects at lower tested doses and growth inhibition at higher concentrations. Literature reports a similar phenomenon for the NSAID DIC, which at low concentrations (< 10 mg/L, an order of magnitude greater than that used in this study) stimulated algal growth of *Chlorella pyrenoidosa*, promoting chlorophyll and lipid accumulation, as well as increasing antioxidant enzyme activities (Zhang et al., 2019). The study by Zhou et al. (2024) suggested that hormesis in algae could function as a defensive mechanism against chemicals, such as antibiotics, leading to increased secretion of extracellular organic matter that can accelerate their indirect degradation. This hypothesis may also apply to other pharmaceuticals; however, further analysis is necessary to confirm it.

In line with our results, exposure to environmentally relevant concentrations of IBU (100 and 300 µg/L) for 24 hours negatively impacted the growth of *P. tricornutum*, inhibiting chloroplastic energy transduction within the electron transport chain and resulting in reduced energy transfer to Photosystem I (Silva et al., 2020). This growth inhibition may be further explained by IBU's ability to alter the microalgae's cytoplasmic membrane, leading to its depolarization and, in some cases, inducing chlorosis (Prado et al., 2015).

Although there is limited information on the effects of ATE in algae, we can hypothesize a mechanism of toxicity based on studies of other β-blockers. For instance, exposure to propranolol (0.3 - 300 µg/L) significantly inhibited the growth of *P. tricornutum*, likely due to a decrease in energy transduction from Photosystem II to the electron transport chain, which elevated oxidative stress levels. Specifically, the impaired energy production at the PSII donor side forced diatoms to utilize stored lipids as an energy backup, resulting in increased respiratory activity (Duarte et al., 2020).

The marked toxicity of the lipid regulator GEM in *P. tricornutum* may be linked to its ability to bind to the sulfhydryl groups of proteins (Zurita et al., 2007), leading to increased oxidative stress and impaired growth. In contrast to our findings, several studies have reported adverse effects on the proliferation of other algal species, such as *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*, at GEM concentrations an order of magnitude higher than those used in this study (Isidori et al., 2007; Calvo et al., 2012; Sanderson et al., 2003), suggesting a greater sensitivity of *P. tricornutum* to this drug.

The results on the embryo development of the sea urchin highlighted a high sensitivity of this species to most of the individually tested pharmaceuticals: relevant biological effects were observed for the highest exposure dose of the antidepressants PAR and FLU; the NSAIDs IBU, NAP and DIC; the lipid lowering agent GEM; and both concentrations of the antidepressant VEN; the benzodiazepine LORM; the antiepileptic CBZ and its metabolite CBZ-EP; and antihypertensive VAL.

In accordance with our findings, Aguirre-Martínez et al. (2015) reported that exposure of environmental concentrations (ranging from 0.01 to 10 µg/L) of IBU and CBZ significantly reduced the embryo-larval development in sea urchins, suggesting high sensitivity of this organism to selected drugs. Furthermore, Ribeiro et al. (2015) identified a lowest observed effect concentration (LOEC) of 12.5 µg/L for DIC in *P. lividus*, posing concern on its long-term effects for aquatic organisms. The same study also highlighted significant impacts on sea urchin embryo development caused by environmental concentrations (0.64 - 10 µg/L) of the antidepressant sertraline, further corroborating the high sensitivity of this species to this class of pharmaceuticals.

Exposure to the benzodiazepine delorazepam (1, 5, 10 µg/L) resulted in anatomical anomalies in plutei, accelerated or delayed development, and alterations in the presence and distribution of glycoconjugates such as N-acetyl-glucosamine, α-linked fucose, and α-linked mannose in both morulae and plutei, thereby reducing the fertilization capacity of male and female gametes and interfering with fertilization and embryonic development in sea urchins (Fogliano et al., 2024).

The presence of one of the primary targets of benzodiazepines, TSPO (translocator protein, formerly known as the peripheral benzodiazepine receptor, PBR) receptors, in *P. lividus* may explain the significant effects caused by both delorazepam (Fogliano et al., 2024) and lormetazepam (our

findings) on this species. Specifically, it has been reported that this class of drugs, by binding the TSPO receptors can generate oxidative stress (Krishna et al., 2013) which consequently induces microtubule disassembly in sperm (Chakrabarti et al., 1998), leading to harmful effects on gametogenesis.

The toxicity of the antihypertensive VAL on the embryo development of sea urchins was particularly evident at both tested doses (1 and 10 µg/L). Given the limited information available in the literature regarding the effects of this drug on non-target species, it is challenging to hypothesize a specific mechanism of toxicity. However, observations by Mezzelani et al. (2023) indicate that chronic exposure to VAL (0.5 µg/L) adversely affects lipid metabolism and lysosomal membranes integrity in *Mytilus galloprovincialis*, suggesting that the toxicity of this pharmaceutical may be linked to its interaction with biological membrane components.

Short-term exposure to the beta-blocker propranolol (0.1 - 1 µg/L) resulted in extensive lysosomal damage in the Eastern oyster *Crassostrea virginica*, accompanied by the release of lysosomal contents. This release was linked to interactions between the drug and the membrane Mg^{2+}ATPase-dependent proton pump, leading to its dysfunction and subsequent membrane damage (Khan et al., 2018). Such a mechanism of toxicity could also explain the significant effects observed at the highest dose (10 µg/L) of the beta-blocker ATE in the species *Crassostrea gigas*.

In general, the results of the embryonic development assessment in the oyster *Crassostrea gigas* are comparable to those obtained in *P. lividus*, even if some exceptions indicated a slight lower sensitivity of the oyster compared to the sea urchin. Hence, considering the greater availability of mature adults for gamete collection provided by oysters, it can be considered a reliable organism to be included in the battery of bioassays for the assessment of environmental risk of pharmaceuticals, when gametes of sea urchin are not available.

Wild organisms are often exposed to mixtures of various pharmaceuticals, which may interact and lead to unpredictable additive, synergistic, or antagonistic effects in terms of toxicity and alterations in metabolic pathways. In this contest, this work aimed to investigate interactions between different drugs exposing the battery of bioassays to three binary mixtures (A, B, E), three quaternary mixtures (C, D, F) and one seven-drug mixture (G).Overall results indicated that mixtures exhibited greater toxicity compared to individual pharmaceuticals, with hazard levels ranging from "Slight" to "Moderate" at the highest exposure dose. The only exception was mixture F (VAL + ATE + GEM + RAM), composed by active ingredients commonly used together in human therapy, which showed an "Absent" hazard level elaborated with weighted criteria.

In accordance with our findings, several studies in the literature have reported that pharmaceutical mixtures are more toxic to non-target species (such as algae, crustaceans, oysters, and bacteria) than

the active ingredients tested individually (Geiger et al., 2016; Godoy, 2019; Di Poi et al., 2018; Tominaga et al., 2022). Interestingly, the highest overall toxicity ("Moderate" hazard level) was observed in binary mixtures B and E and in quaternary mixture C, suggesting that this increased toxicity is not necessarily related to the number of combined drugs, but rather to specific interactions among individual compounds.

The binary mixture B was composed of the antiepileptic CBZ and the antihypertensive VAL. An additive effect on the growth of *P. tricornutum* was observed for both doses of this treatment, where the magnitude of the mixture's effect on algal growth (26 % and 39 % inhibition at 1 and 10 µg/L, respectively) corresponds to the sum of the effects measured for the individual drugs (CBZ showing 21 % and 22 % inhibition, and VAL showing 5 % and 18 % inhibition at 1 and 10 µg/L, respectively). To support the importance of using an approach that integrates multiple biological species for assessing the ecotoxicological impacts of these pollutants, it is significant that these interactions were not reported in the sea urchin *P. lividus*, where the effects of the mixture were similar to those measured for the individual drugs CBZ and VAL.

A 14-day exposure to environmentally realistic concentrations of CBZ, VAL, and their mixture on the mussel *M. galloprovincialis* revealed greater toxicity for CBZ compared to VAL (Mezzelani et al., 2023; 2024), corroborating our findings that indicate an overall higher hazard level for the antiepileptic ("Slight") compared to the antihypertensive ("Absent"). However, the same study reported antagonistic effects between these compounds, suggesting that their interactions can vary depending on biological models, exposure conditions, and measured endpoints.

The quaternary mixture C consisted of the antidepressant PAR, the antiepileptic CBZ, the NSAID IBU, and the antihypertensive VAL. Both doses of this treatment exhibited significant toxicity in the species *P. tricornutum* and *P. lividus*, with a dose-dependent effect observed in the embryo development of the sea urchin.

A synergistic interaction between the antidepressant VEN and the NSAID NAP (mixture E) was highlighted in species *P. tricornutum*, such that, for the higher exposure dose (10 µg/L), the magnitude of the mixture effect on the algal growth is greater than the sum of the effects measured for the individual drugs. Noteworthy, the phenomenon of hormesis previously reported for lower dose (1 µg/L) of VEN was not observed in the mixture, suggesting that such effects of the antidepressant may be counteracted by the presence of NAP. Once again, this interaction was not detected in the sea urchin *P. lividus*, where the effects of the mixture on embryo development were similar to those reported for the individual drugs VEN and NAP.

Overall, our findings indicate that the bioluminescence of the bacterium *Alivibrio fischeri* is not affected by any of the tested pharmaceuticals or their mixtures, suggesting that this test is not sensitive

enough to detect adverse effects at environmental realistic doses. Several studies have corroborated these results, reporting acute toxicity levels for various NSAIDs and psychiatric drugs that exceed environmental concentrations by several orders of magnitude (mg/L) in relation to bacterial bioluminescence (La Farré et al., 2001; Aguirre-Martínez et al., 2015; Zhang et al., 2008; Ionescu et al., 2020; Minagh et al., 2009).

7.5 Conclusions

In conclusion, this study demonstrated the importance of employing an approach that integrates the use of various species from different trophic levels and the measurement of different biological responses to assess the environmental impact of pharmaceuticals and their mixtures. The antidepressant PAR, the antiepileptic CBZ and its metabolite CBZ-EP, and the lipid-lowering agent GEM exhibited the highest toxicity in the ecotoxicological bioassays, with elaborated hazard level ranging from "Slight" to "Moderate". An overall lower level of hazard was observed for individual drugs compared to their mixtures, where the greater toxicity was not associated with the number of combined drugs but rather with interactions detected among individual molecules.

Obtained results highlighted different sensitivity of the tested species to these pollutants, with *P. tricornutum* and *P. lividus* > *C. gigas* > *A. fisheri*. Considering their role as the base of aquatic food webs and their importance in energy and nutrient transfer to higher trophic level (Bi et al., 2018), the observed reduction in diatom biomass due to exposure to potentially environmentally realistic concentrations (10 µg/L) of certain pharmaceuticals (PAR, CBZ, CBZ-EP, IBU, NIM, VAL, ATE, RAM and GEM) and their mixtures, may have significant long-term repercussions for aquatic ecosystems. Furthermore, endpoints applied in this work highlighted the need of using more sensitive responses when assessing risk of pharmaceuticals in aquatic environments, since those applied in current guidelines may not be suitable, as the bioluminescence test on *A. fisheri*. In fact, the hazard levels elaborated and summarized by the overall responses of the battery of ecotoxicological bioassays could underestimate the impact of the tested pharmaceuticals and mixtures, due to the low sensitivity of the used tests in detecting potential adverse effects resulting from exposure to low environmental concentrations of these pollutants.

8. General conclusions

The main objective of this PhD thesis was to clarify the ecotoxicological impact of environmental pharmaceuticals in marine ecosystems, evaluating the bioaccumulation and the onset of sublethal adverse effects through different approaches under laboratory conditions, including *in vivo*, *ex vivo* and *in vitro* tests, using a selection of model species belonging to different trophic levels, thereby contributing to the development of a comprehensive environmental risk assessment procedure for these pollutants.

Chronic *in vivo* exposures revealed the mussel's capability to bioaccumulate most of tested pharmaceuticals. The bioaccumulation patterns highlighted for individual drugs were consistent across all experimental plans and showed similarities among compounds belonging to the same therapeutic class (*e.g.* the antidepressants VEN and PAR). Although no clear bioaccumulation of lipid lowering agent GEM, cardiovascular drug RAM, and antidiabetic MET was observed, the biological alterations measured in exposed organisms suggest a possible metabolism and excretion of these drugs. Such finding indicates that environmental monitoring alone, focusing on the detection of pharmaceuticals in aquatic matrices, may be insufficient to fully assess the overall impacts of these pollutants.

Significant biological reactivity was highlighted for all tested drugs, with marked effects on oxidative system. Immune parameters were most significantly affected by the therapeutic classes of NSAIDs (IBU), antidepressants (PAR), and cardiovascular drug (RAM); while lipid metabolism was most influenced by NSAIDs (IBU), lipid-lowering agents (GEM) and antidiabetics (MET).

No additive or synergistic interactions were highlighted in tested mixtures, which showed similar toxicity compared to single compounds ("Moderate" cellular hazard levels). Furthermore, it was demonstrated that drugs elimination was faster compared to the recovery of the adverse effects, highlighting the importance to adopt integrated effect-based procedures in future study on long-term toxicity. Climate change-related stressors have been shown to modulate the toxicity of pharmaceuticals mixture under chronic exposure scenario, with acidification exacerbating oxidative stress and thermal stress amplifying alterations in the immune system of mussels, highlighting the need for further research to elucidate the ecological consequences of pharmaceutical mixtures under future oceanic conditions, toward the development of effective management strategies aimed at mitigating risks to marine biodiversity and human health.

Responses obtained with the *ex vivo* model of PCTS of *M. galloprovincialis* digestive glands confirmed the PPARs agonists (GEM, ROS, MET, and FFB) hypolipidemic proprieties, providing novel insights into the mechanisms that modulate lipolysis activation, redox homeostasis imbalance, and biotransformation processes in non-target species. Such findings demonstrated the sensitivity and

suitability of this technique in investigating mechanisms of action of environmental pharmaceuticals, representing an added value within integrated strategies for the toxicity assessment of these compounds.

Outcomes of the acute *in vitro* test revealed that psychiatric drugs were the most reactive therapeutic class and, among single active principles, PAR, NAP and ATE were the most toxic molecules in terms of cell viability, production of reactive oxygen species and changes in mitochondrial membrane potential. Insights on their specific mechanism of action were highlighted including apoptosis through cascade reactions affecting lysosomes, mitochondria and oxidative pathway, inhibition of cell cycle and depletion of lipid metabolism. Single pharmaceuticals showed a greater effect compared to their mixtures, in which the toxicity was not related to the number of the combined drugs, but rather to their interactions, with evidence of antagonistic mechanisms between the class of non-steroidal anti-inflammatory drugs and antidepressants in PLHC-1 cell line.

A higher biological reactivity of psychiatric drugs (PAR, CBZ, and CBZ-EP) was also observed in acute *in vivo* tests. In this context, the results of the ecotoxicological bioassays highlighted lower toxicity for the individual compounds compared to their mixtures. However, it was confirmed that the greater effects observed in mixtures were not related to the number of combined drugs, but rather to potential interactions between their components, underscoring the importance of deepen knowledge on mixtures effects to clarify the role of possible additive, synergic or antagonistic interactions in modulating the overall toxicity in non-target species.

Finally, the application of weighted criteria to elaborate and integrate the overall biological effects in *in vivo* and *in vitro* systems, summarized hazard indices which for the first time provided quantitative insights on toxicity of several pharmaceuticals and their mixtures, allowing to communicate the risk in a simple way without reducing the scientific soundness of the overall investigation. At the same time, the elaboration through weighted criteria allowed an easier comparison between different molecules, exposure conditions, tissues, species, and level of biological organization, which is fundamental for the prioritization and risk assessment of these environmental pollutants.

The comparison between the responses measured at sub-lethal (mussels' *in vivo* exposure), cellular (*in vitro* test on fish cell lines) and organism (*in vivo* test with a battery of ecotoxicological bioassays) levels for those pharmaceuticals tested with all these approaches (psychiatric drugs VEN and PAR, lipid lowering agent GEM, cardiovascular drug RAM, antidiabetic MET, and NSAID IBU), highlighted an higher sensitivity of sub-chronic responses compared to acute toxicity, thus confirming the importance to use an integrated approach which consider the alteration at different level of biological organization for a more compressive evaluation of ecotoxicological impacts of

environmental pharmaceuticals. In conclusion, the integration of results obtained from these laboratory investigations with those obtained from field studies will allow to clarify a list of priority pharmaceuticals and to provide a scalable science-based and flexible effect-based risk assessment procedure useful to support the implementation of relevant European policies.

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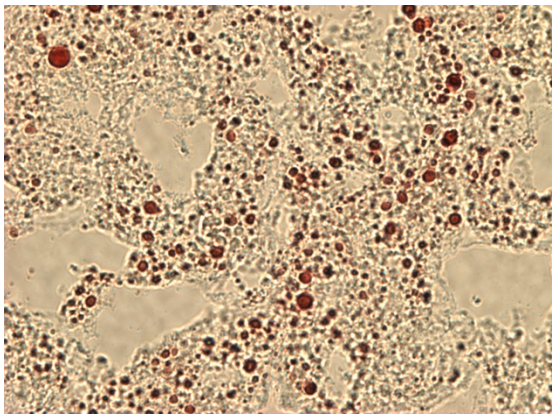
11. Supplementary materials

Mechanisms of cellular responses of caulerpin as a target of the peroxisome proliferator-activated receptor α (PPARα) and its similarities with the lipid lowering agent fenofibrate

A)



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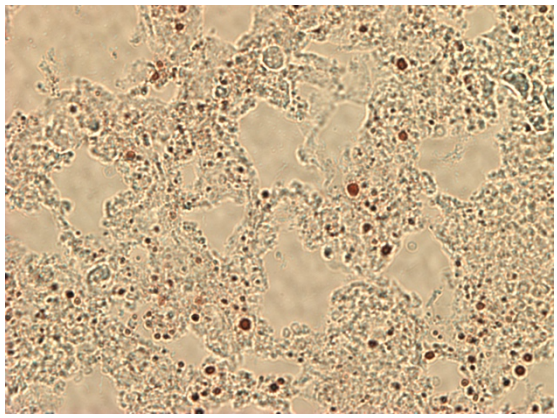

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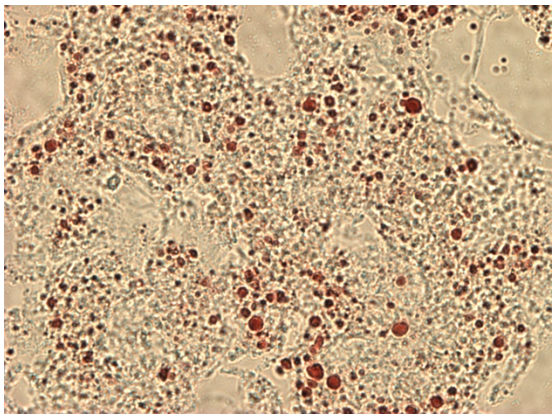

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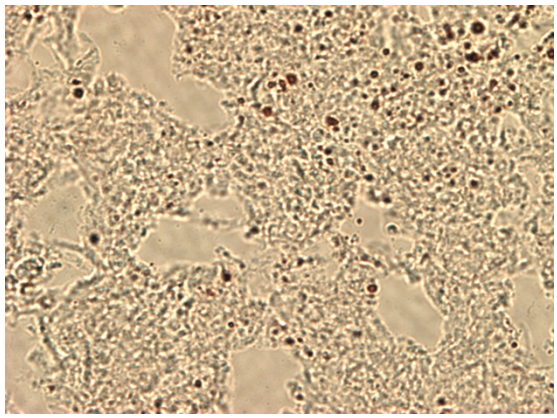

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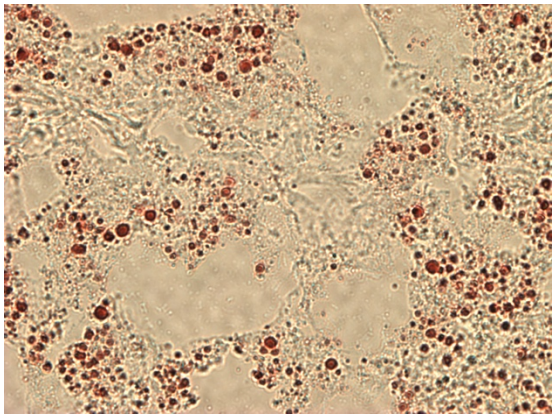

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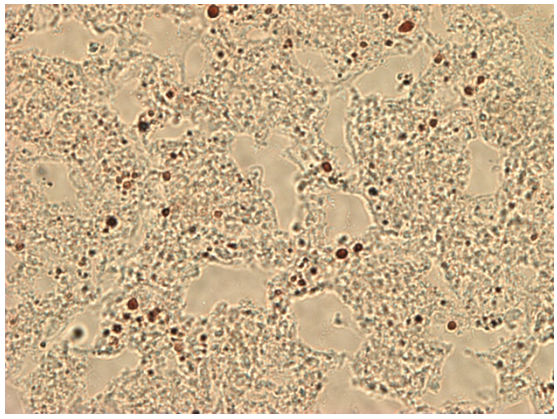


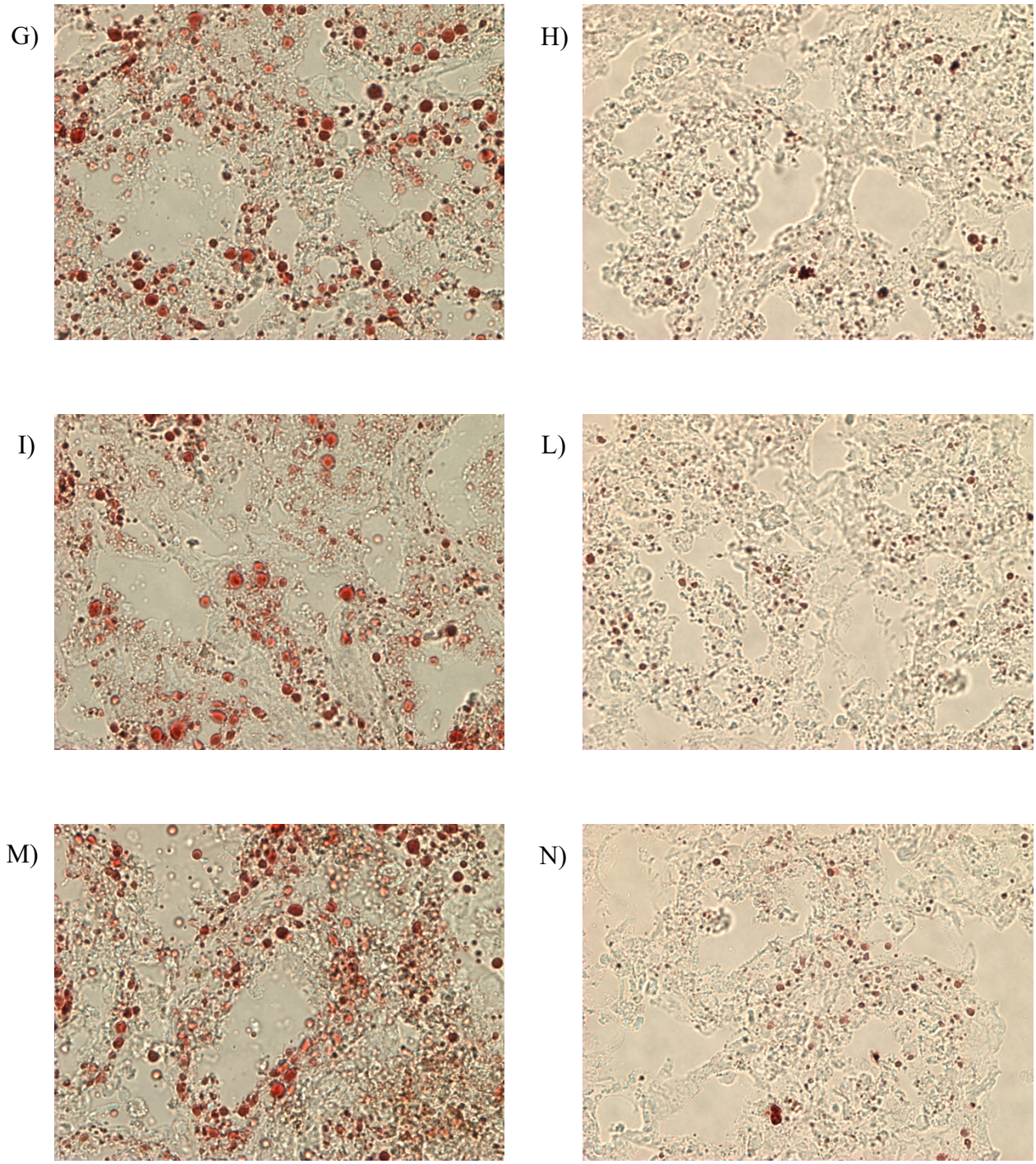
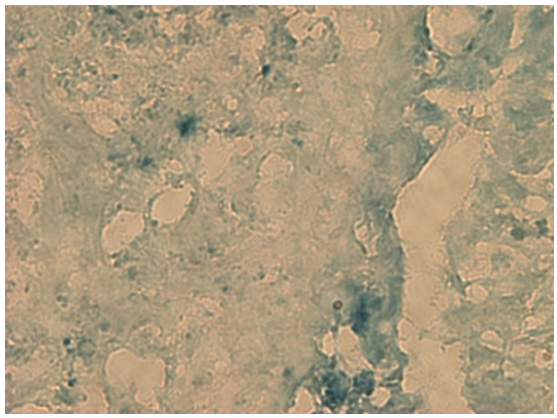


Figure S1. Oil Red O (ORO) staining on 10 µm longitudinal cryostatic sections of *M. galloprovincialis* digestive gland. The pictures represent the content of neutral lipids in PCTS exposed for 72 h to CTRL (A, C, E) and FFB (B, D, F); and to CTRL (G, I, M) and CAU (H, L, N). CTRL, control; FFB, fenofibrate; CAU, caulerpin.

A)



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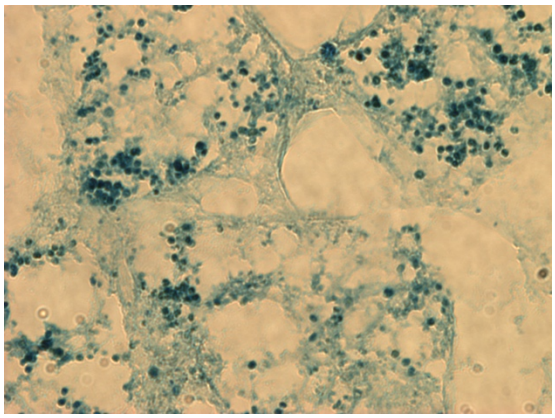

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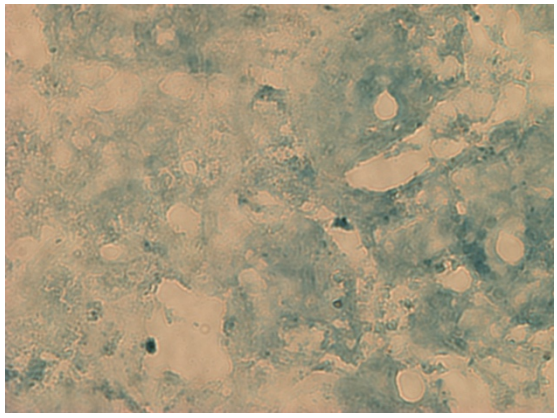

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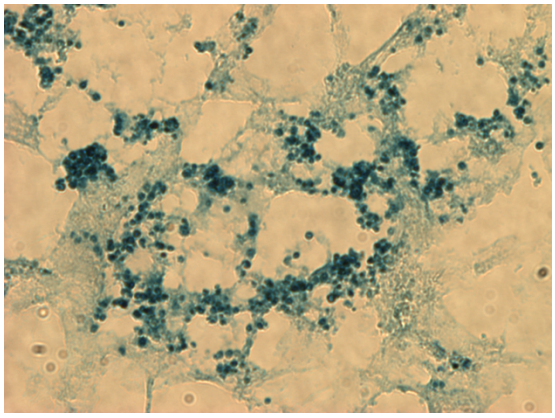

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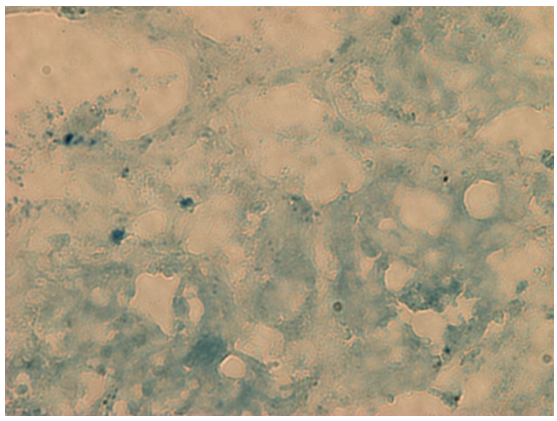

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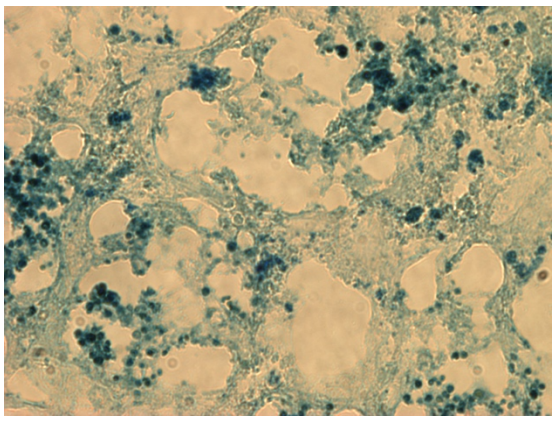

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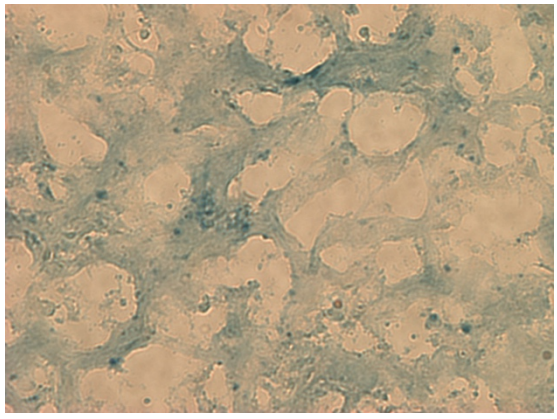

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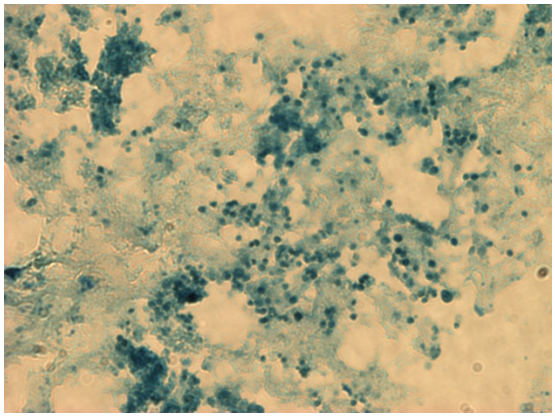

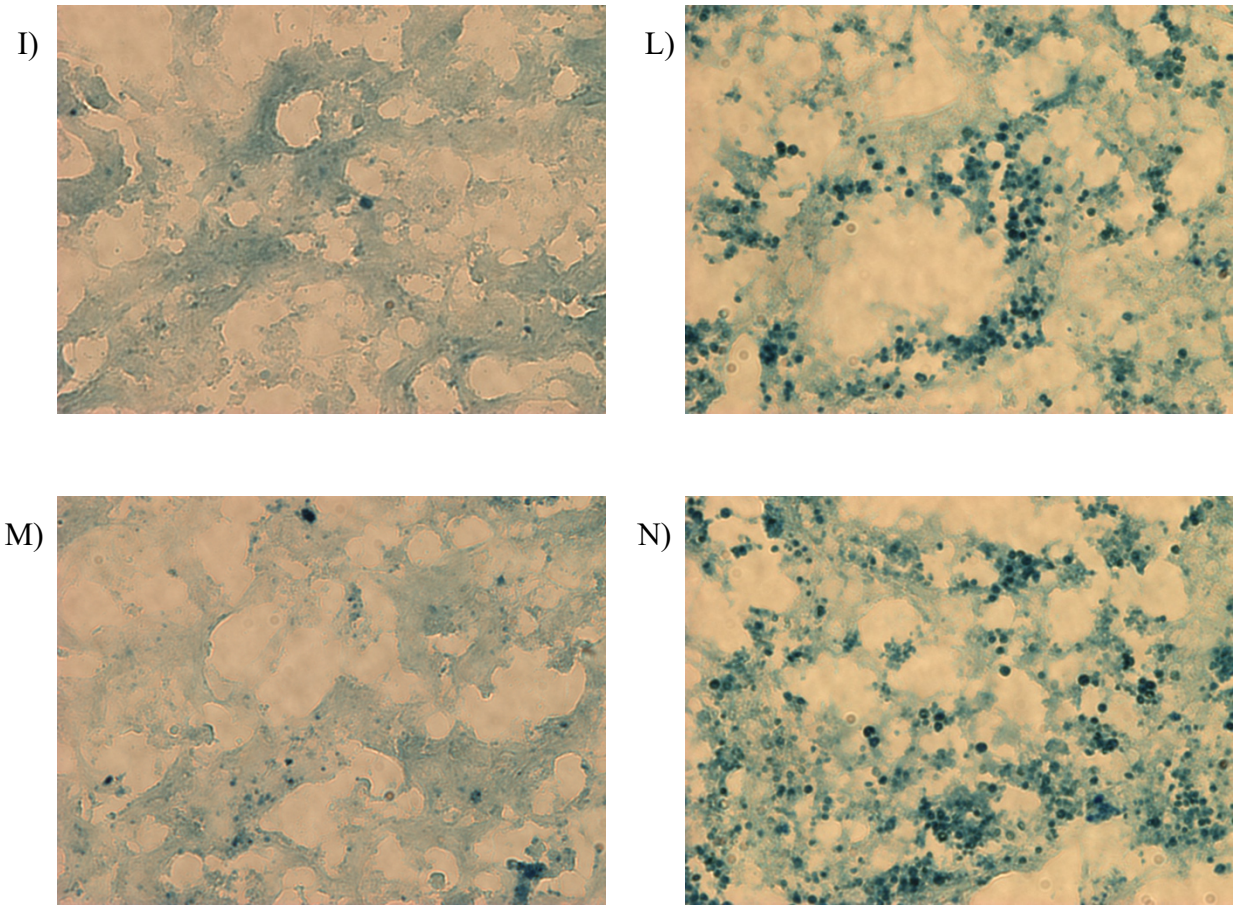

Figure S2. Schmrol reaction staining on 10 µm longitudinal cryostatic sections of *M. galloprovincialis* digestive gland. The pictures represent the content of lipofuscin in PCTS exposed for 72 h to CTRL (A, C, E) and FFB (B, D, F); and to CTRL (G, I, M) and CAU (H, L, N). CTRL, control; FFB, fenofibrate; CAU, caulerpin.

Table S3. Sequences of forward and reverse primers and annealing temperatures used for qPCR analysis, amplicon sizes and accession numbers of genes.

Gene	Primer sequences	Annealing T	Amplicon size	Accession n.
cat	F: CGACCAGAGACAACCCACC[1] R: GCAGTAGTATGCCTGTCCATCC[1]	55 °C	132 bp	AY743716
gst-pi	F: TCCAGTTAGAGGCCGAGCTGA[2] R: CTGCACCAGTTGGAAACCGTC[2]	55 °C	172 bp	AF527010
hsp70	F: GGTGGTGAAGACTTTGACAACAG [3] R: CTAGTTTGGCATCGCGTAGAGC[3]	62 °C	295 bp	AY861684
Cu/Zn-sod	F:AGCCAATGCAGAGGGAAAAGCAGA [4] R: CCACAAGCCAGACGACCCCC[4]	65 °C	177 bp	FM177867
acox1	F: ACAGTCGTGCAAAACAGGGAC[5] R: CTGCTGCTTCAACCAACCTGG[5]	62 °C	153 bp	EF525542
abcb1	F: CACCATAGCCGAGAACATCC[6] R: CTCCACGCTCTCCAACTAG[6]	56 °C	140 bp	FM999809
cyp3A1	F: TGAACTCGCAAAAAGAACCA[7] R: GGAACACTGGAGCCTTGAAC[7]	55 °C	80 bp	AY566247

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Cellular effects of environmental pharmaceuticals: mechanisms of toxicity on fish hepatoma (PLHC-1) and gill (RTgill-W1) cell lines

Counts and viability measurements of PLHC-1 and RTgill-W1 cell lines using the Muse® Cell Analyzer

The Muse™ Count & Viability Kit was used to determine both the viability and total cell count of PLHC-1 and RTgill-W1 cell lines. Briefly, 50 µL of the cell suspension was incubated in the dark for 5 minutes with 450 µL of Muse® Count & Viability reagent. The assay uses a combination of two DNA-intercalating fluorescent dyes in a single reagent. One dye is membrane-permeant, staining all nucleated cells, while the second dye only stains cells with compromised membranes, indicating dead or dying cells. This dual-staining approach allows for the discrimination between nucleated cells and non-nucleated debris, as well as the differentiation between live and dead/dying cells. As a result, the assay provides both accurate cell concentration and viability measurements (Millipore, 2012).

Cell health was assessed by measuring the population profile, viability profile, and viability percentage (%) using the Muse® Cell Analyzer. The total cell count (cells/mL) was used to calculate the required volume of cell suspension to seed each well at a final density of 30,000 cells/mL (Figure S4). Achieving a uniform cell density across all wells is critical for normalizing data obtained from each assay. For each experiment, only cell cultures with > 90 % viability were used.

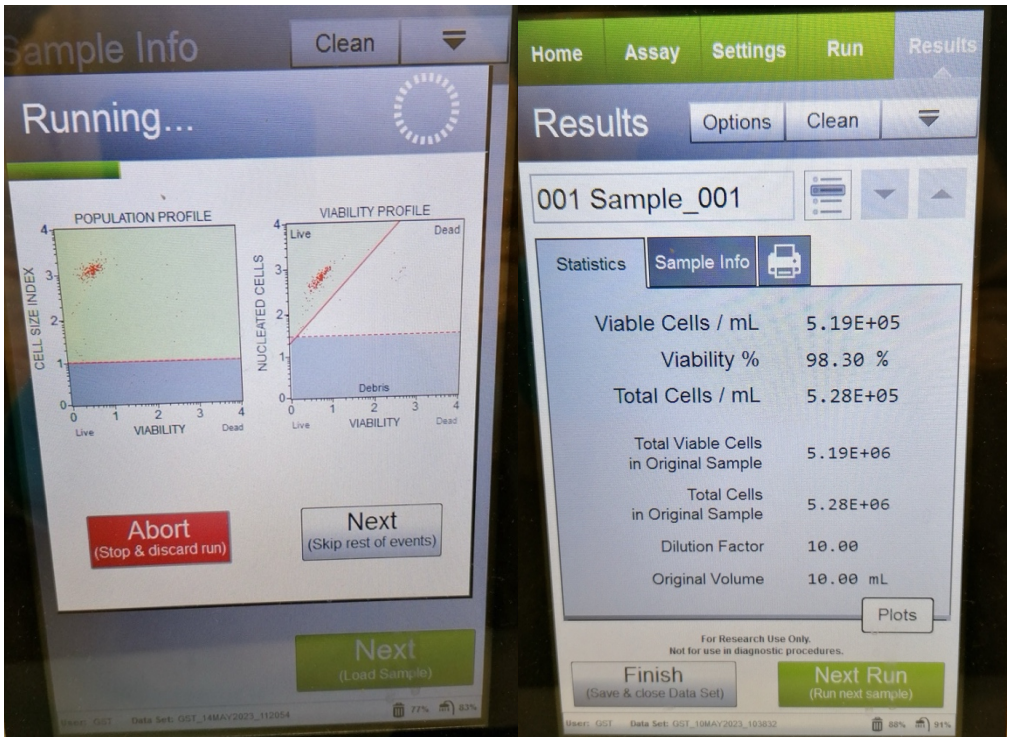

FIGURE S4. Example of count and viability results screen.

Weighted criteria for elaboration and integration of all biological endpoints measured in PLHC-1 and RTgill-W1 exposed to single pharmaceuticals and their mixtures

The weighted criteria applied in the present study allowed to elaborate and integrate the overall results obtained on cell viability, ROS production and mitochondrial membrane electrochemical potential, summarizing a qualitative and quantitative hazard index which reflect the severity of effects. The conceptual framework was adapted from the mathematical structure developed for elaborating ecotoxicological bioassays and biomarkers data within the quantitative Weight of Evidence (WOE) Sediqualsoft model (Piva et al., 2011; Regoli et al., 2019). Specific "weights" and "thresholds" are assigned to each in vitro response depending on toxicological relevance of analyzed endpoint and the magnitude of variation considered of biological relevance in terms of inhibition and/or induction. The "weights" were assigned following criteria developed within the framework of the WOE Sediqualsoft model (*e.g.* Lines of Evidence 3 and 4, Piva et al., 2011). With some variations given by the considered levels of biological organization (molecular/ biochemical/ cellular/ organism), weights range between 1 and 3, with the weighting of 1.0 generally associated to biological variations that do not necessarily imply the onset of a toxicity state, and the weighting of 3.0 assigned to mortality at the organism level (Piva et al., 2011). In the present study, the assigned "weights" were 1.0 for ROS production (variation that do not necessarily induce the onset of a toxicity state), 1.2 for mitochondrial membrane potential (variation that could be prognostic of impairment at higher levels of biological organization), and 1.5 for cell viability (mortality at cellular level). The selected "thresholds", extrapolated from the scientific literature, were 20 % for the inhibition for all the endpoints, and 20 % for induction for both cell viability and ROS production (Table S5). As indicated in the flow-chart (Figure S6), for each biological endpoint, the percentage of variation compared to the control conditions was calculated, and then corrected for the statistical significance according to function z; the Ew (i) was then obtained from the ratio between the percentage of variation and the "threshold" of each endpoint, and this value was further weighted for the specific "weight" of the endpoint ($Ew(k)^* w_2$). The HQBATTERY was given by the summation (Σ) of weighted effects measured for various endpoints. The HQBATTERY obtained for each tested pharmaceutical and mixture was normalized to a scale ranging from 0 to 10, where 1 is the Battery threshold when all the measured endpoints exhibit an effect equal to their threshold, and 10 when all endpoints exhibit 100 % of effect. The HQBATTERY is then assigned to one of five classes of hazard, which are Absent (< 1), Slight (from 1 to 1.5), Moderate (from 1.5 and 3), Major (from 3 and 6), and Severe (from 6 to 10).

TABLE S5. Specific weights and thresholds (%) of the analyzed endpoints, ROS = reactive oxygen species.

Endpoint	Weight	Threshold of induction (%)	Threshold of inhibition (%)
Cell viability	1.5	20	20
Mithocondrial membrane potential	1.2	-	20
ROS production	1.0	20	20

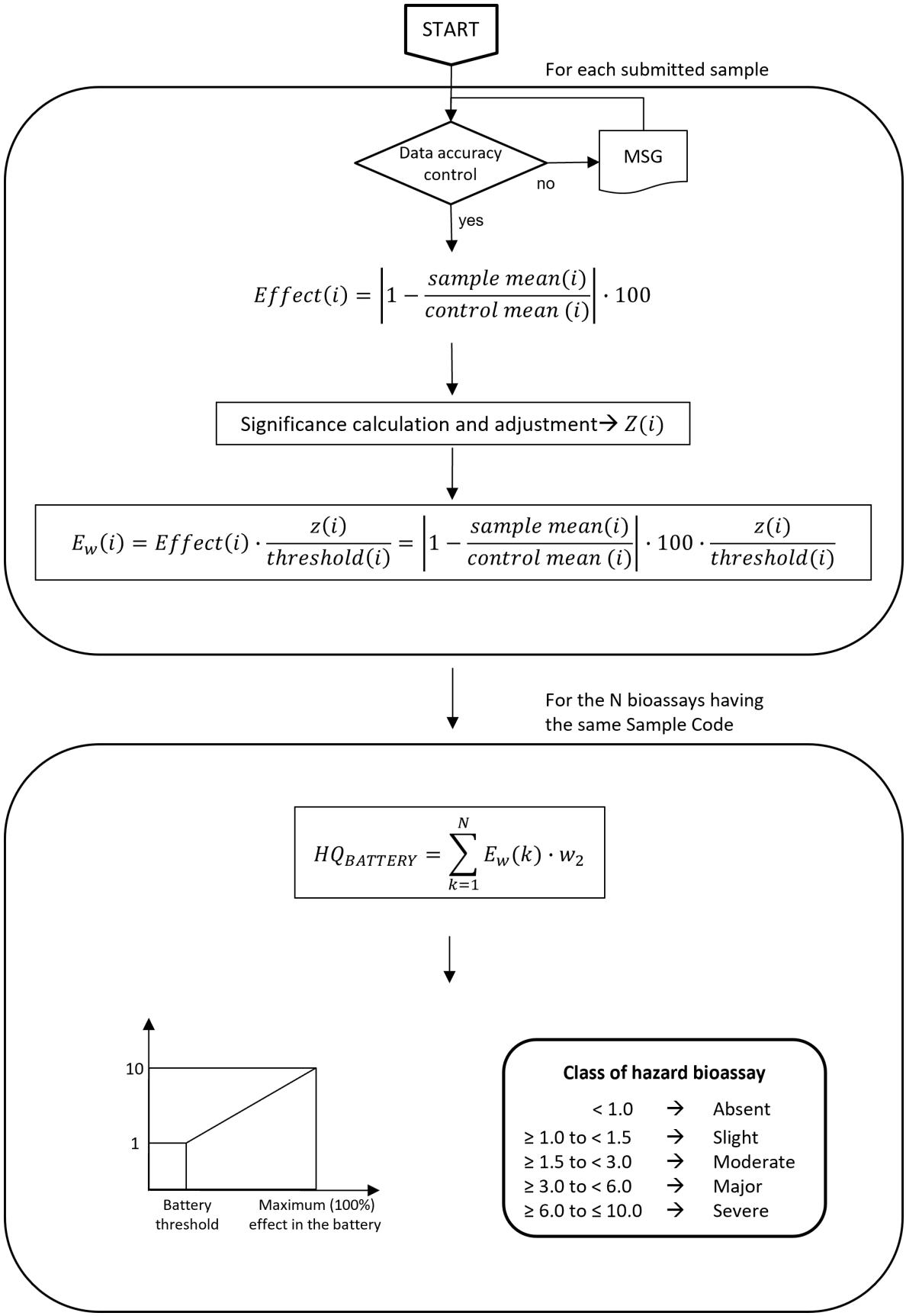

FIGURE S6. Flow-chart of cell line Line of Evidence, MSG = alert Message, %VAR = percentage of Variation, HQ = Hazard Quotient.

Appendix

List of Publications in International Journals

1. M. Mezzelani, V. Notarstefano, **M. Panni**, E. Giorgini, S. Gorbi and F. Regoli (2024). Exposure to environmental pharmaceuticals affects the macromolecular composition of mussels digestive glands. *Scientific Reports*, 14(1), 9369. https://doi.org/10.1038/s41598-024-59663-7

2. Bernardini, I., Mezzelani, M., **Panni, M.,** Dalla Rovere, G., Nardi, A., El Idrissi, O., et al., (2025). Transcriptional modulation in Mediterranean Mussel *Mytilus galloprovincialis* following exposure to four pharmaceuticals widely distributed in coastal areas. *Aquatic Toxicology*, 107255. https://doi.org/10.1016/j.aquatox.2025.107255

3. **Panni, M**., Mezzelani, M., d'Errico, G., Vitale, M., Gorbi, S., Pampanin, D.M., Regoli, F. (2024) Cellular effects of environmental pharmaceuticals: mechanisms of action on *Poeciliopsis lucida* fish hepatoma cell line. *(under revision)*

4. Mezzelani, M., **Panni, M**., Giuliani, M.E., N. Cerioni, P., Nerdi, A., Benedetti, M., Mollo, E., Regoli, F., Gorbi, S. (2024) Mechanisms of cellular responses of caulerpin as a target of the peroxisome proliferator-activated receptor α (PPAR α) and its similarities with the lipid lowering agent fenofibrate. *(under revision)*

5. **Panni, M.,** Mezzelani, M., Gorbi, S., d'Errico, G., Nardi, A., Benedetti, M., Regoli, F. Application of a battery of bioassays within the assessment procedure for the ecotoxicological impact of environmental pharmaceuticals. *(in preparation)*

6. **Panni, M.,** Mezzelani, M., d'Errico, G., Vitale, M., Gorbi, S., Pampanin D.M., Regoli, F. Cellular effects on *Oncorhynchus mykiss* fish gill cell line by environmental pharmaceuticals and their mixtures. *(in preparation)*

7. Mezzelani, M., **Panni, M.**, El Idrissi, O., d'Errico, Milan, M., Ferraresso, S., Dalla Rovere, G., Nardi, A., Benedetti, M., Gorbi, S., Regoli, F. Evidence on modulation of *M. galloprovincialis* metabolic pathways by environmental pharmaceuticals: first insights on ibuprofen, paroxetine and their mixture. *(in preparation)*

8. Mezzelani, M., **Panni, M.,** d'Errico, G., Di Carlo, M., Fattorini, D., Nardi, A., Gorbi, S., Benedetti, M., Regoli, F. Interactive effects modulate toxicity of gemfibrozil, venlafaxine, ramipril, and metformin APIs mixture. *(in preparation)*

9. Mezzelani, M., **Panni, M**., d'Errico, G., Di Carlo, M., Fattorini, D., Nardi, A., Gorbi, S., Benedetti, M., Regoli, F. Spatial and temporal distribution of active pharmaceutical ingredients in in biotic and abiotic matrices of Adriatic Sea. *(in preparation)*

Contributions to national and international workshops and congresses

Oral presentations

Panni, M., Mezzelani, M., Vitale, M., d'Errico, G., Gorbi, S., Regoli F., Pampanin, D.M. (An *in vitro* approach to assess the cellular effects of several therapeutic classes of pharmaceuticals as single compounds and mixtures. 22° International Symposium on Pollutants Responses in Marine Organisms, PRIMO, Nantes, 26-29 May 2024.

Panni, M., Mezzelani, M., d'Errico, G., Benedetti, M., Gorbi, S., Regoli, F. Application of a battery of bioassays within the assessment procedure for the ecotoxicological impact of environmental pharmaceuticals. First international workshop of the Society of Environmental Toxicology and Chemistry (SETAC), Italian Branch. Siena, 15 September 2022.

Poster presentations

Panni, M., Mezzelani, M., d'Errico, G., Vitale, M., Gorbi, S., Benedetti, M., Pampanin, D.M., Regoli, F. A multidisciplinary approach to assess the impacts of environmental pharmaceuticals and their mixtures in non-target model species. AquaticPollutants Final Conference, 22-23 october 2024, Frankfurt.

Panni, M. Presence, behavior and risk assessment of pharmaceutical products in aquatic ecosystems. Polytechnic University of Marche PhD week, June 2022, 2023, 2024.

Panni, M. Mezzelani, M., d'Errico, G., Benedetti, M., Gorbi, S., Regoli F. Application of a battery of bioassays within the assessment procedure for the ecotoxicological impact of environmental pharmaceuticals. SETAC Europe, Dublin, 30 April - 4 May 2023.

Other contributions

Mezzelani, M., **Panni, M.,** d'Errico, G., Regoli, F., Pampanin, D.M., Gorbi, S. Mechanisms of molecular and cellular responses of human pharmaceuticals mixtures in non-target model species. SETAC Italian Language Branch 8-9 october 2024, Milan *(Oral presentation)*

Mezzelani, M., **Panni, M.,** d'Errico, G., Di Carlo, M., Fattorini, D., Nardi, A., Benedetti, M., Gorbi, S., Regoli, F. Interactive biological responses modulate the toxicity of pharmaceuticals mixtures. 22° International Symposium on Pollutants Responses in Marine Organisms, PRIMO, Nantes, 26-29 May 2024. *(Oral presentation)*

Nardi, A., Bernardini I., Mezzelani, M., Vivani, V., **Panni, M.,** Peruzza, L., Dalla Rovere, G., El-Idrissi, O., d'Errico, G., Di Carlo, M., Pittura, L., Benedetti, M., Gorbi, S., Milan, M., Regoli, F. Mode of action of stimulant alkaloids: cellular metabolism and effects of cocaine, caffeine and their mixture in *Mytilus galloprovincialis.* 22° International Symposium on Pollutants Responses in Marine Organisms, PRIMO, Nantes, 26-29 May 2024. *(Oral presentation)*

García-Pimentel, M.M., **Panni, M.,** Vitale, M., Campillo, J.A., León, V.M., Pampanin, D.M. *In vitro* effects of pharmaceuticals at environmental relevant concentrations on liver fish cell line. SETAC Europe, Seville, 5 - 9 May 2024 *(Poster)*

García-Pimentel, M.M., **Panni, M.,** Vitale, M., Campillo, J.A., León, V.M., Pampanin, D.M. *In vitro* effects of plastic additives and their mixtures with pharmaceuticals on fish liver cell line. SETAC Europe, Seville, 5 - 9 May 2024 *(Poster)*

Mezzelani, M., **Panni, M.,** d'Errico, G., Benedetti, M., Gorbi, S., Regoli F. Molecular, biochemical and cellular effects of single and combined pharmaceuticals in *Mytilus galloprovincialis*. SETAC Europe, Dublin, 30 April - 4 May 2023. *(Oral presentation)*

Garcìa Pimentel, M.M., Campillo, J.A., Valdés, J., Castano-Ortiz, J.M., Rodriguez-Mozaz, S., Mezzelani, M., Gorbi, S., **Panni, M.,** Regoli, F., Leòn, V.M. Biological effects of simultaneous and separated exposure of mussels to citalopram/bezafibrate and polyethylene microplastics in seawater. SETAC Europe, Dublin, 30 April - 4 May 2023. *(Poster)*

List of periods spent abroad

Exchanging period (17th March- 23rd June 2023) at the University of Stavanger (UiS), Norway.
Activities: *In vitro* test in fish cell lines (PLHC-1 liver cells; RTgill-W1 gill cells): exposure to single pharmaceuticals and mixtures to evaluate cell viability, ROS production and mitochondrial membrane stability.

List of awards

Best Student Poster Presentation Award, PhD week organized by Department of Life and Environmental Sciences, Polytechnic University of Marche (Ancona, Italy). 3 – 7 June 2024.

List of attended courses/seminars/schools

1. Scientific Summer School in "Global Change in Transitional Environments". The advanced course was organized by the University of Aveiro (Professor Rosa Freitas and Professor Eduarda Pereira) and the activities were held at Department of Biology (dbio), Department of Chemistry (dqua) and Laboratory Central of Analysis (lca), from 15 to 26 of July 2024.
2. "The resolution revolution in Cryo-electron-microscopy, in Structural Biology and in Life Sciences", Martino Bolognesi, Department of Biosciences, University of Milan, 7 June 2022.
3. "Current threats to research ethics and how to cope with them", Marco Seeber, Department of Political Science and Management, University of Agder, Norway, 9 June 2022.

4. “Cambiamenti climatici, comunità e Sindaci resilienti: contributi e riflessioni con il mondo universitario”, Università Politecnica Marche, Facoltà Di Ingegneria, 12 October 2022.
5. “Ecotoxicology under global changes: impacts in marine wildlife”, Rosa Freitas, Departamento de Biologia, Universidade de Aveiro. 27 April 2023.
6. 2nd Workshop of SETAC Italian Language Branch. Consiglio Nazionale delle Ricerche, Roma 11 October 2023.

Educational/ Teaching and Scientific Outreach Activities

1. Co-supervisor of Master's Thesis in Marine Biology “Effects of pharmaceuticals in *Mytilus galloprovincialis*: the case study of gemfibrozil, metformin and rosiglitazone”, Dr. Elena Alfero
2. Teaching support in the “Biological Bases and Methods of Biotechnology” course (SSD BIO/13), Prof. Alessandro Nardi, Bechelor’s Degree in Biomedical Engineering, Faculty of Engineering – Polytechnic University of Marche. Lesson on *in vitro* and *ex vivo* approach in ecotoxicological field
3. Co-supervisor of Master's Thesis in Marine Biology “Evidence of biological effects of human pharmaceuticals and their mixtures in *Mytilus galloprovincialis*: modulation of immune system and oxidative metabolism”, Dr. Gaia Flori
4. Teaching support in the Applied Biology Laboratory course (SSD BIO/13), Prof. Stefania Gorbi and Prof. Maura Benedetti, Bachelor's Degree in Biological Sciences, Department of Life and Environmental Sciences - Polytechnic University of Marche. Practical exercises on the main biological investigation techniques.
7. Sharper 2021-2022-2023-2024 with several activities related to contaminants of emerging concern and sustainable development goals of Agenda 2030
8. Participation to Moby Litter 2022-2023-2024. Event organized the Department of Life and Environmental Sciences – DISVA, Polytechnic University of Marche, about marine plastic pollution and activities proposed to reduce plastic dispersion, recycle and recover marine plastic debris
9. Participation at SostenibilMente 2021. Event on sustainability organized by Ancona Municipality
10. Participation to Climbing for Climate 2021, 2022, 2023. Event organized by the four University of Marche Region, annually, for promoting responsible and sustainable action against climate change and environmental pollution