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Impatto ecotossicologico di farmaci ad uso umano sulla specie modello *Mytilus galloprovincialis*: il caso studio di gemfibrozil, metformina e rosiglitazone.

Effects of human pharmaceuticals in *Mytilus galloprovincialis*: the case study of gemfibrozil, metformin and rosiglitazone.

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Abstract

Pharmaceuticals have become a significant threat for aquatic environments due to their widespread use and persistence. Since drugs are designed to be effective at very low concentrations, they have the potential to interfere with the biochemical and physiological processes of aquatic species. The aim of this thesis was to assess the ecotoxicological impact of the lipid-regulating agent gemfibrozil (GEM) and the antidiabetics metformin (MET) and rosiglitazone (ROS) on the bivalve *Mitylus galloprovincialis*. *In vivo* experiments were conducted by exposing mussels to single drugs at 2.5 μg/concentration for 14 days to final investigate the onset of sub-lethal effects on immune functions, genotoxicity, neurotoxicity, oxidative stress and lipid metabolism. In addition, an *ex vivo* approach was applied on Precision-Cut Tissue Slices of digestive gland (PCTS). PTCS were treated with higher drug doses (500-1000 μg/L) for 48h and 72h to stimulate clearer physiological responses, enabling a deeper investigation of the pharmaceutical's impact on lipid and oxidative metabolisms. Results obtained from both experiments were integrated to provide a comprehensive overview on the mechanisms of action (MOAs) and the main metabolic pathways modulated by these drugs in non-target organisms.

Obtained results revealed that all pharmaceuticals modulated the immune system, while no genotoxic and neurotoxic effects were observed. Furthermore, GEM, MET and ROS influenced the oxidative status and the lipid metabolism of mussels. Specifically, mussels treated with GEM and ROS showed overall similar responses: the *ex vivo* approach confirmed the hypolipidemic proprieties of these drugs also in non-target species at high exposure doses (500–1000 μg/L), while *in vivo* tests suggest that at lower concentrations (2.5 μg/L) the pronounced oxidative imbalance could be the cause of the higher level of neutral lipids. Similarly, the enhanced neutral lipids content detected under *ex vivo* conditions (500–1000 μg/L) following MET exposure appear to be associated with marked pro-oxidant effects rather than a stimulation of lipid synthesis. In conclusion, results obtained integrating *in vivo* and *ex vivo* tests allowed to increase our knowledge of the main metabolic pathways affected by the selected drugs in non-target organisms. Overall, the PCTS technique demonstrated to be a useful tool for elucidating the MOAs of pharmaceuticals, providing valuable insights into their environmental risk assessment.

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1 Italian summary

Impatto ecotossicologico di farmaci ad uso umano sulla specie modello Mytilus galloprovincialis: il caso studio di gemfibrozil, metformina e rosiglitazone.

I Contaminanti di Interesse Emergente (CECs) costituiscono un gruppo eterogeneo di sostanze caratterizzate da differenti applicazioni e proprietà chimico-fisiche, la cui presenza in ambiente minaccia gli ecosistemi a causa del loro potenziale ecotossicologico. Ne fanno parte prodotti per la cura personale (PPCPs), pesticidi, fragranze, ormoni, ritardanti di fiamma e farmaci.

Negli ultimi decenni, è aumentato l'interesse scientifico nei confronti dei composti farmaceutici a causa del loro ampio utilizzo in medicina umana e veterinaria, e della loro presenza ubiquitaria negli ambienti aquatici. Gli impianti di trattamento delle acque reflue (WWTPs) svolgono un ruolo cruciale nella diffusione di questi contaminanti, poiché le tecnologie attualmente in uso non sono efficaci nel rimuoverli completamente. Inoltre, le diverse proprietà chimico-fisiche di queste sostanze, come l'elevata polarità, la bassa volatilità e l'alta lipofilia, contribuiscono ulteriormente alla loro scarsa rimozione durante il trattamento. Di conseguenza, i farmaci rilasciati dai WWTPs possono accumularsi in vari compartimenti ambientali, nei quali vengono misurati livelli nell'ordine di ng – μg su litro o chilogrammo.

I composti farmaceutici, essendo progettati per avere effetti biologici a basse concentrazioni, possono interferire con i meccanismi fisiologici e biologici delle specie acquatiche mediante gli stessi meccanismi d'azione delle specie target (ad esempio i mammiferi).

Numerosi progressi normativi sono stati compiuti grazie alle evidenze scientifiche che hanno sottolineato il potenziale ecotossicologico di queste molecole per gli ecosistemi acquatici. Un esempio significativo è l'incremento del numero di composti farmaceutici presenti nella "Watch List" della Commissione Europea, passati da quattro nel 2015 a dieci nel 2022. Questa lista include gli inquinanti ambientali per cui è necessario un monitoraggio continuo, al fine di raccogliere ulteriori dati sul rischio ecologico e, in base a tali informazioni, adottare misure regolatorie adeguate. Tuttavia, nonostante questi progressi, ad oggi le legislazioni nazionali e internazionali non stabiliscono ancora limiti di concentrazione specifici per queste molecole nelle diverse matrici ambientali. Tra i farmaci maggiormente presenti negli ecosistemi marini, i regolatori lipidici e gli antidiabetici sono quelli meno studiati in termini

di tossicità su organismi non target.

I regolatori lipidici, come il gemfibrozil (GEM), riducono i trigliceridi e le lipoproteine a bassa densità (LDL) nel sangue, abbassando il rischio di malattie cardiovascolari. Il meccanismo d'azione di GEM coinvolge l'assorbimento degli acidi grassi, che vengono successivamente trasformati in derivati acil-CoA e degradati attraverso il processo di beta-ossidazione. Diversi studi hanno dimostrato la capacità di GEM di essere bioaccumulato da vertebrati e invertebrati marini, con conseguente capacità di indurre danni genotossici e citotossici, oltre a modulare del sistema antiossidante.

Gli antidiabetici, come la metformina (MET), utilizzati nel trattamento del diabete di tipo II, agiscono inibendo il complesso I della catena di trasporto degli elettroni mitocondriale, riducendo così la produzione di glucosio. Tra gli effetti collaterali riportati, è stato dimostrato che MET sopprime la lipogenesi e attiva l'ossidazione degli acidi grassi, attraverso un meccanismo che coinvolge l'attivazione della chinasi proteica attivata da AMP (AMPK). È stata osservata la potenzialità di questo farmaco di essere bioaccumulato, e di agire come distruttore endocrino, interagendo con il sistema riproduttivo, l'alimentazione e la locomozione di organismi non-target.

Il rosiglitazone (ROS), un antidiabetico utilizzato principalmente nel trattamento del diabete di tipo II, agisce per alleviare la resistenza insulinica periferica. È stato inoltre dimostrato che questo farmaco riduce le concentrazioni di trigliceridi nel sangue. Sebbene sia ampiamente rilevato negli ecosistemi acquatici, meno informazioni si hanno però sui suoi potenziali effetti avversi negli organismi non-target.

In questo contesto, l'obiettivo di questa tesi è stato quello di indagare gli effetti ecotossicologici di GEM, MET e ROS nell'organismo modello *Mitylus galloprovicinalis,* utilizzando un approccio integrato basato su esperimenti *in vivo* ed *ex vivo*. Nell'uomo questi composti sono tutti noti per interagire con il metabolismo lipidico, pertanto, questo studio ha avuto come ulteriore obiettivo quello di indagare se i loro meccanismi d'azione fossero conservati anche in specie non bersaglio.

L'esperimento *in vivo* ha previsto l'esposizione dei mitili a concentrazioni dei tre farmaci simili a quelle potenzialmente rilevate in ambienti marini costieri (2,5 μg/L) per 14 giorni, analizzando un panel di biomarkers biochimici e cellulari che comprendono risposte legate al sistema immunitario, la modulazione di percorsi ossidativi, effetti neuro e geno-tossici e cambiamenti del metabolismo lipidico.

Negli esperimenti *ex vivo*, le sezioni di tessuto della ghiandola digestiva (Precision-Cut Tissue Slices, PCTS) di mitili sono state esposte a concentrazioni più elevate (500 µg/L e 1000 µg/L) di GEM, ROS e MET per 48 e 72 ore, al fine di stimolare risposte fisiologiche più evidenti. I biomarcatori selezionati in questo caso si sono concentrati specificamente su parametri legati al metabolismo lipidico e alla pathway ossidativa, includendo l'accumulo di lipofuscina e lipidi neutri, nonché l'attività della catalasi.

I risultati hanno evidenziato la capacità dei farmaci testati di modulare il sistema immunitario nei mitili. Tutti i trattamenti *in vivo* hanno evidenziato una diminuzione nel tempo di ritenzione del rosso neutro, parametro indicatore della destabilizzazione della membrana lisosomiale, ed un contemporaneo aumento del rapporto tra le due popolazioni cellulari che caratterizzano gli emociti (granulociti e ialinociti). Tuttavia, solo MET ha causato una diminuzione della capacità fagocitaria degli emociti, suggerendo una riduzione dell'efficienza fagocitaria dei granulociti.

Gli effetti neurotossici sono stati valutati misurando l'attività dell'enzima acetilcolinesterasi nell'emolinfa e nelle branchie. Tutti i farmaci hanno determinato un'induzione significativa dell'attività dell'enzima nelle branchie, mentre ROS e GEM hanno causato un'induzione anche nell'emolinfa.

L'analisi della frequenza dei micronuclei ha mostrato un incremento significativo solo negli organismi esposti a MET. Tuttavia, il valore rilevato (1,5 ‰) è simile ai valori fisiologici di organismi di controllo (1‰), suggerendo che le concentrazioni di MET destate non causano effetti genotossici nei mitili.

I risultati ottenuti mediante entrambi gli approcci sperimentali, hanno dimostrato che tutti i farmaci influenzano il metabolismo ossidativo dei mitili. Negli esperimenti *in vivo*, tutti i trattamenti hanno indotto l'attività della catalasi (CAT), mentre negli esperimenti *ex vivo*, l'induzione della CAT è stata osservata solo per GEM e ROS. La MET ha, inoltre, indotto un amento significativo dell'enzima glutatione S-transferasi (GST) e delle glutatione perossidasi (GPx); la capacità antiossidante totale (TOSC) nei confronti dei radicali idrossilici e perossilici è diminuita solo a seguito dell'esposizione a GEM.

È interessante notare che l'antidiabetico MET sembri avere effetti dose-dipendenti sul sistema antiossidante: a basse dosi (2,5 µg/L, studi *in vivo*), MET induce la modulazione di parametri biochimici, come gli enzimi antiossidanti (CAT e GPx), mentre ad alte dosi (500-1000 µg/L, studi *ex vivo*) causa marcate alterazioni cellulari, come un forte accumulo di

lipofuscina. Tale alterazione dell'omeostasi ossidativa sembra essere la causa principale del significativo aumento dei livelli di lipidi neutri (NL) misurato a seguito dell'esposizione ad alte concentrazioni (500-1000 µg/L, studi *ex vivo*), piuttosto che un'azione diretta del farmaco nella sintesi lipidica.

A conferma di tale ipotesi, anche i marcati effetti pro-ossidati di GEM e ROS rilevati nell'esperimento *in vivo* (induzione enzimi antiossidanti, riduzione TOSC e aumento di prodotti di perossidazione lipidica) sono stati associati ad un aumento dei NL. Contrariamente, ad elevate dosi, quando non sono state misurate alterazioni nella pathway ossidativa, GEM e ROS hanno mostrato un effetto ipolipemizzante, confermando un meccanismo d'azione simile a quello misurato negli organismi target.

In conclusione, i risultati complessivi di questo lavoro mostrano che GEM, MET e ROS sono in grado di modulare diverse pathways metaboliche di *M. galloprovincialis*, con effetti significativi sul sistema immunitario, sul metabolismo lipidico e sull'omeostasi ossidativa.

Inoltre, la tecnica delle PCTS di ghiandola digestiva di mitilo si è rilevata un utile strumento per studiare e approfondire le conoscenze sui meccanismi d'azione dei farmaci in organismi non-target. In particolare, le proprietà ipolipemizzanti di GEM e ROS sono state osservate anche nei mitili, evidenziando un meccanismo d'azione simile a quello dimostrato per le specie target; mentre, nell'esposizione a MET, l'elevato accumulo di lipidi neutri sembra essere associato ad una marcata alterazione della pathway ossidativa. Tuttavia, ulteriori indagini saranno necessarie per confermare questi risultati. Infine, questo lavoro di tesi ha evidenziato l'efficacia di utilizzare un approccio integrato nella valutazione del rischio ambientale dei composti farmaceutici negli ecosistemi marini.

2 Introduction

2.1 Pharmaceuticals as emerging contaminants

Contaminants of Emerging Concern (CECs) are: “naturally occurring, manufactured or manmade chemicals or materials which have now been discovered or are suspected present in various environmental compartments and whose toxicity or persistence are likely to significantly alter the metabolism of a living being” (Sauvé & Desrosiers, 2014). The first interest for the so-called emerging contaminants was probably rise by the Rachel Carson's book, published in 1962, titled "Silent Spring". Such book highlighted a possible correlation between the widespread use of DDT in agriculture as pesticide and the deaths of many birds. The alarm raised by the environmental writer pushed the research to investigate the risks associated with DDT, bringing to its removal from the market. This can be seen as the first step toward considering that pesticides and chemicals can be harmful to human health. Since some compounds recognized as “emerging” twenty years ago are no longer considered as such, it turns out the concept of what is of “emerging interest” changes over time. Consequently, it can be useful to distinguish between “emerging contaminant”, which have only recently been identified, and “contaminants of emerging concerns”, which have been present in the environment for a long time but have only recently raised concerns (Sauvé & Desrosiers, 2014). CECs refer to a heterogeneous group of chemicals, which include personal care products and pharmaceuticals for everyday use (PPCPs), endocrine-disrupting chemicals (EDCs), hormones, artificial sweeteners (ASWs), flame retardants (FRs), herbicides, disinfection products (DPBs), plasticizers, musks and fragrances, laundry detergents and nanoparticles (NPs). They can be found in different compartments: surface water, sediments, aquatic organisms, drinking water and groundwater, effluent and influent of wastewater treatment plants, and atmosphere (Tang et al., 2020). These compounds have been present in the aquatic environment for decades, except for those recently formulated, but they were not detected previously due to a lack of analytical methods capable of identifying these molecules at low concentrations. However, recent improvements in analytical chemistry like the coupling of liquid chromatography with tandem mass spectrometry (HPLC/MS/MS), provides a more convenient means to quantifying polar compounds, such as pharmaceuticals, in complex matrixes. This has allowed the detection of many CECs in aquatic ecosystems, leading to a better understanding of their potential

hazard (Sedlak et al., 2000).

Among CECs, pharmaceutical products are of particular interest due to their widespread use in both human and veterinary medicine (Fent et al., 2006) and their continuous release into aquatic systems. Since 1990, the number of medicines sold in Europe has risen, as reported by the pharmaceutical industry. The document "Global Use of Medicines 2024- Outlook to 2028" published by IQVIA Institute for Human Data Science, provides an overview of the evolution of pharmaceutical market in the next five years. According to the last report, global annual medicine spending is projected to grow, form the 3-6% forecasted in 2023, to 5-8%, reaching $2,3 trillion by 2028 (OECD, 2023). In addition to the rise of pharmaceutical market, global pharmaceutical consumption is also growing, partly due to the increasing demand for drugs to treat ageing-related and chronic diseases, as well as changes in clinical practices (González Peña et al., 2021). For instance, the consumption of anti-hypertensive drugs increased by 65%, the use of lipid-modifying agents quadrupled, and the consumption of anti-diabetic and anti-depressant medications doubled in Organization for Economic Cooperation and Development (OECD) countries between 2000 and 2019 (OECD, 2021). As the pharmaceutical market grows and consumption increases, the release of pharmaceuticals and their metabolites into the environment also rises, mainly due to improper management, treatment, and disposal practice (González Peña et al., 2021).

Pharmaceuticals primarily enter aquatic ecosystems through domestic and hospital discharges, mainly due to their not fully metabolization by the human body. In fact, these substances are often excreted either partially transformed or unchanged, but typically conjugated with polar molecules, and are subsequently released into the environment through effluents from sewage treatment plants (Heberer, 2002). Another significant, albeit more localized, source of pharmaceuticals in the aquatic environment comes from wastewater generated by intensive livestock farming and aquaculture facilities, where veterinary medicines are commonly used (Bottoni et al., 2010). The role of wastewater treatment plants (WWTPs) in the persistence of pharmaceutical contamination is particularly notable, as their current technological design makes them inefficient at removing these compounds.

WWTPs are efficient to remove suspended solids, organic and pathogens, partial removal of nitrogen and phosphorus and heavy metals (Hamza et al., 2016). Since this technology is not specifically designed to eliminate active pharmaceutical ingredients some of these may

be transformed into by-products and released directly into the environment becoming a threat for the wildlife (Luo et al., 2014; Fernández-López et al., 2016; Mezzelani et al., 2018). It was estimated that the percentage of removal rates of WWTPs for pharmaceuticals is from 10 to almost 100% and it depends on the physicochemical characteristics of the pharmaceutical and the type of treatment technology (Gaw et al., 2014). The wastewater treatment process is typically based on two phases: absorption to suspended solids (sewage sludge) and biodegradation (Fent et al., 2006). The primary wastewater treatment process consists in a series of physical separations such as coagulation, flocculation and sedimentation (Mezzelani et al., 2018). The objective of this preliminary treatment is to remove and reduce the size of large, suspended or floating inorganic solids such as wood, paper, plastics, garbage and fecal matter. Instead, the secondary process consists in a biological treatment through a battery of microorganisms in a controlled environment, such as aerobic microbes that consume the organic matter (Sonune & Ghate, 2004). Some WWTPs also provide a tertiary treatment system, which includes several stages, such as membrane processes (microfiltration, ultrafiltration, nanofiltration, and reverse osmosis), UVC photolysis, and advanced oxidation processes (ozonation, H_2O_2/UVC, photo-Fenton/UVC) (Moreira et al., 2016). Unfortunately, this tertiary treatment is expensive and consequently poorly used at global scale (Mezzelani, Gorbi, & Regoli, 2018a). The assessment of the efficiency of WWTPs in the removal process is essential for optimizing treatment procedures. With this purpose, Behera (2011) investigates the occurrence and removal efficiencies of 20 pharmaceuticals and personal care products in five different WWTPs in Korea. The results clearly show the difference in removal efficiency between the primary and secondary treatment processes. In the primary treatment, the removal rate was minimal, reaching a maximum of 28% for the Non-Steroidal Antinflammatory drug (NSAID) diclofenac and the hormone estriol. In contrast, the secondary treatment, which uses biological processes, was the main mechanism for pharmaceutical removal, as evidenced by the amounts of diclofenac and estriol removed, which corresponded to 68% and 93% respectively. Another study investigates the fate of 13 PPCPs, eight of which are pharmaceuticals, in the municipal sewage treatment plant in Galicia (Spain). Even here, the primary treatment had very low efficiency, with no significant reduction in NSAIDs ibuprofen and naproxen, the antibiotic sulfamethoxazole, and the hormone estrone. In the secondary treatment, during the biological step, it took place removal efficiencies of 60-70%

for ibuprofen, 40-55% for naproxen, and of 67% for sulfamethoxazole (Carballa et al 2004). Moreover, certain physicochemical properties of pharmaceuticals can reduce their removal efficiency during treatment processes, including the high polarity, low volatility, high lipophilicity and persistence (Khasawneh & Palaniandy, 2021). The octanol-water partition coefficient (K_{ow}) is one of the most important parameters that influence the removal rate of organic contaminants, such pharmaceuticals, from wastewater treatment by adsorption. K_{ow} is a physicochemical property of compound that is defined as the ratio of the concentration of a solute in a water-saturated octanolic phase to its concentration in an octanol-saturated aqueous phase (Erdem, 2024). Rogers (1996) identified three levels of sorption of compound based on the value of the K_{ow}: molecules with log K_{ow} < 2.5 have low sorption potential, 2.5 to 4 have medium sorption potential, and K_{ow} > 4.0 high sorption potential.

Despite the susceptibility of pharmaceuticals to degradation, their continuous input into the environment results in a pseudo-persistent behaviour: once released by WWTPs, such molecules can accumulate in various environmental matrices, including seawater, sediment, and biota (Fabbri & Franzellitti., 2016a). Pharmaceuticals can be distributed in different compartments thanks to physicochemical properties, interaction with different matrices and the local hydrodynamic conditions (Bayen et al., 2013).

Along with the octanol-water partition coefficient (K_{ow}), the dissociation constant (pK_a) is an important property that can influence the sorption of organic compounds. The pK_a of a substance describes the pH at which half of the molecules are in the ionized (charged) form and the other half in the non-ionized (neutral) form. The ionized form is water-soluble and cannot easily cross cell membrane, whereas the neutral form is lipid-soluble and can be readily adsorbed by cells and tissues (Manjooran, 2020). Generally, pharmaceuticals with a pK_a lower than 8 are predominantly in their ionized form, making them more water-soluble and less permeable through membranes. A study comparing four active pharmaceuticals compounds -acetaminophen (APAP), caffeine (CAF), carbamazepine (CBZ), and diclofenac (DCF)- evaluated their bioavailability and bioaccumulation in clams *Ruditapes decussatus* based on pK_a. CAF, APAP and CBZ are neutral at the pH of receiving water, with water solubility ranked in the order: CBZ<<APAP<=CAF. This explains why CAF and APAP are more bioavailable and bioaccumulated by exposed organisms, whereas CBZ, being almost water insoluble, has limited bioaccumulation. On the other hand, DCF, with his negative charge, is hindered in the permeation of mollusc tissues (Cravo et al 2022).

Furthermore, it is important to consider that the active principle may have different fates also depending on the ionic strength and temperature of surrounding environment. This is demonstrated by a study that aimed to develop devices for passive integrative sampling of both polar and nonpolar compounds, with a focus on their applicability in pharmaceutical monitoring. The results show that higher temperature modifies the compound solubility and the octanol-water partition coefficient, enhancing the sampling rate of pharmaceuticals. The salinity enhances water ionic strength and consequently the lipophilicity of compounds decrease (Togola & Budzinski, 2007).

Pharmaceuticals have been documented to be accumulated in various environmental matrices, including seawater, sediment, and biota (Fabbri & Franzellitti., 2016). The main therapeutic classes detected in marine ecosystems include NSAIDs, psychiatric drugs, cardiovascular drugs, antidiabetics, steroids and antibiotics (Liu et al., 2015).

The concentrations in coastal areas range from a few ng/L up to hundreds of μg/L (Mezzelani & Regoli, 2022; barr et al., 2021). The most significant concentration levels, on ranges, of pharmaceuticals detected in surface water are up to 92 ng/L for the NSAID ibuprofen, 134-203 ng/L for the lipid regulator benzafibrate and 170- 242 ng/L for the beta-blocker atenolol. While the antibiotics lincomycin, erythromycin, and spiramycin are characterized by levels up to 14, 17, 68 ng/L, respectively. On the other hand, in the sediments the highest detected concentrations were 130 ng/Kg for benzafibrate and lincomycin and 220, 410, 630 and 2900 ng/Kg for ibuprofen, ranitidine, erythromycin, and spiramycin, respectively (Zuccato et al 2000). The antidiabetic metformin was found in WWTP effluent at concentration of 1 μg/L to 47 μg/L and in surface waters at concentrations from 0.06 μg/L to 3 μg/L (MacLaren et al 2018).

Pharmaceuticals, designed to permeate biological membranes, have been identified in various wild freshwater and marine species, from primary producers to top predators (Swiacka et al., 2019; Mezzelani and Regoli, 2022). For instance, a study which investigated different species of macroalgae, bivalves, and fish from Portugal, Sapin, Italy, Netherlands and Norway, detected the concentrations up to 36.1 ng/g dry weight (*dw*) of velanfaxine and up to 13.3 ng/g *dw* of the antibiotic azithromycin in bivalves from the Po delta (Italy). Besides, dimetridazole was found in bivalves in Tagus estuary with a concentration of 7.7 ng/g *dw* (Alvarez-Munoz et al., 2015). Furthermore, considering the distribution of various pharmaceuticals in the Mar Menor lagoon, it was found the highest concentrations of 2.3

ng/g of citalopram in bivalve *Cerastoderma glaucum*, 3.2 ng/g of hydrochlorothiazide in bivalve *Pinna nobilis* and 2.3 ng/g of carbamazepine in mollusk *Murex trunculus*. Lastly, it was detected concentration of 6.3 ng/g of carbamazepine in teleost *Liza aurata* and 3.89 ng/g of hydrochlorothiazide in teleost *Gobius niger* (Moreno-González et al., 2016).

2.2 Ecotoxicological approaches: *in vivo* and *ex vivo* models

Ecotoxicology not only aims to understand the fate of chemicals when they are introduced, voluntary or accidentally, into the environment and how they interact with biota, but also focuses on integrating the effects of contaminants from genetic and cellular level to organisms, populations, and entire ecosystems. To achieve these objectives, ecotoxicological studies require an interdisciplinary approach, considering chemical, biological and molecular processes, while also accounting for mechanisms of toxicity and ecological dynamics (Fent, 1996). By integrating chemical and biological effects measurements, it is possible to achieve a higher level of interpretation of the individual data and assess environmental status more accurately. These two types of measurements, when considered in a complementary and integrated approach, can lead to the identification of substances present in the environment that are responsible for the observed effects (Vethaak et al., 2017).

For these reasons is important in ecotoxicological studies the use of bioindicator organisms in the assessment of environmental quality. A bioindicator is any plant or animal that reflects exposure to and the toxicity of xenobiotics (Li et al., 2010). These organisms are valuable for assessing the impact on human and environmental health due to their sensitivity, ecological role, likelihood of exposure, and their geographic and ecological distribution or abundance (McCarthy, 2018). Bioindicators help compensate for the lack of data on pollutant concentrations in water and sediments, as only organisms can incorporate the bioavailable fraction of xenobiotics present in the aquatic environment, often accumulating at levels significantly higher than the environmental background (Chahouri et al., 2023). A good bioindicator should have several qualities, including the capacity to accumulate high levels of pollutants without facing death, being suitably distributed and easily accessible both for sampling and for culturing in the laboratory, long-lived enough to allow comparisons between various ages, and possessing a clear and observable dose-effect

relationship (Hamza-Chaffai, 2014). Bivalves, particularly mussels, are excellent bioindicators due to their filter-feeding behaviour, which enables them to reflect the environmental quality of surrounding waters. As sessile organisms, mussels provide information about specific locations. They form mussel beds in shallow waters, making them easy to access in the field and simple to maintain in culture. Mussels filter large volumes of water to feed on phytoplankton, a process that leads to the accumulation of pollutants. Furthermore, they provide essential ecological services, such as food and habitat for many species (Beyer et al., 2017). The mussel species most commonly used in European biomonitoring programs are *Mytilus edulis*, *Mytilus galloprovincialis* and *Mytilus trossulus*, which are employed to assess the chemical quality of coastal and estuarine waters (Martínez-Gómez et al., 2017). Bioindicators are organisms that provide useful information about the variation of pollutants present in the environment over time and space. Indeed, these species not only provide information about the bioaccumulation rate of pollutants but are also widely used in ecotoxicology studies to assess their effects through the use of biomarkers measurable parameters across different levels of biological organization (molecular, cellular, and physiological). Biomarkers reflect the effects of anthropogenic stressors on organisms, which is translated in changes in metabolic regulatory processes (Hamza-Chaffai, 2014). Measuring biomarkers at molecular or cellular levels can be considered a sensitive "early warning" tool for detecting biological effects in environmental quality assessments. Based on the use of "exposure biomarkers" or "effect biomarkers", it is possible to determine whether the organism has been exposed to pollutants or to assess the magnitude of the organism's response to the pollutant, respectively. These early warning biomarkers can be used to implement bioremediation strategies, serving as predictive indicators (Hamza-Chaffai, 2014). Depledge (1994) defined a biomarker as "a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of chemical pollutants". Among the biomarkers used, some are activated at molecular and cellular levels, while others are activated at tissue or organism level, with potential consequences for the entire population. The most investigated biomarkers in tissues of exposed mussels include immunological responses as lysosomal membrane stability (LMS), granulocytes/hyalinocytes ratio (G/H) and phagocytosis activity; antioxidant responses as the susceptibility of single antioxidant enzymes and total antioxidant scavenging capacity

(TOSC); accumulation on lipid peroxidation products as lipofuscin (LIPO) and malondialdehyde (MDA); responses of lipid metabolism as accumulation of neutral lipids (NL) in digestive gland and Acyl-CoA oxidase activity (ACOX); neurotoxic responses as AChE activity; and genotoxic damages in hemolymph as DNA strand breaks and micronuclei frequency (MN) (Mezzelani et al., 2018).

In ecotoxicology, biological responses can be evaluated on different model organisms through different experimental approaches such as *in vivo*, *in vitro* and *ex vivo* tests. The *in vivo* approach involves exposing whole, living organisms to specific concentrations of contaminants under controlled laboratory conditions. This allows for the assessment of the toxicological potential of selected substances without the influence of environmental variability of natural ecosystems, both with short- and long-term exposures that reflect acute and chronic effects. This approach provides the opportunity to explore the full effects of a toxic compound without excluding any biochemical pathways. In several studies, the concentrations of the substances investigated are much higher than the actual environmental levels (Franzellitti et al., 2014; Matozzo et al., 2014; Solé et al., 2010). While this helps to immediately reveal the effects and the mechanism of action exerted on the selected biological model, the high concentrations do not reflect the real risk present in the aquatic environment. On the other hand, studies that use concentrations found in the aquatic environment are more realistic in environmental risk assessments (Binelli et al., 2009). Thus, *in vivo* studies play a crucial role in ecotoxicological research by enhancing our understanding of the impact of toxic compounds on living organisms within their natural habitats, using biomarkers such as survival and growth rates, reproductive success, and biochemical indicators of stress to assess the ecological impact (Pastorino et al., 2024).

Ex vivo and *in vitro* studies are alternative methods frequently used in ecotoxicological research for the different investigation model that propose. Such models are preferred for several reasons, including cost-effectiveness, rapid reproduction and easy adoption to automated high- throughput screening technologies. From a social and ethical perspective, their use is also supported by the replacement and reduction of use of whole animals (Taju et al., 2012). This aligns with the principles of the 3Rs (Replacement, Reduction, and Refinement), developed by Russel and Burch in 1960s in their book “The Principles of Humane Experimental Technique”. The authors highlighted the ethical issue concerning animal testing, particularly in relation to vertebrate species, with an emphasis on humane

treatment of animals and the improvement of research methods (Pastorino et al., 2024).

The *in vitro* techniques, such as cell lines which are cultures of animal cells capable of being propagated repeatedly and sometime indefinitely (Bols et al., 2011), are employed to explore the mechanisms of action, molecular pathways and potential targets of contaminants. These approaches offer the advantage of assessing specific biological responses in controlled experimental conditions (Giuliani et al., 2019). However, these systems are not able predict the physiological response of an organ or tissue because they lack cell-cell and cell-extracellular matrix interactions (Cho & Yoon, 2017).

Ex vivo methods involve the use of organs, tissues or fragments of tissues instead of isolated cells, with the aim to preserve cellular interactions among various cell types and to provide a more realistic model (El Haj et al., 2019). Specifically, the model of the Precision-Cut Tissue Slices (PCTS) is viable tissue explants that are maintained and grown under controlled conditions to preserve their viability. Tissue slices contain different types of cells, with intracellular and cell-matrix interactions remaining intact. This technique has been used in studies of biochemical functions, including 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). Despite the increasing applications of PCTS technique on a wide range of organisms, from rats to fish, there are very few examples of its use on invertebrates, with the notable exception of the work of Giuliani (2019), which propose the use of mussels' digestive gland to study molecular and cellular responses. The slicing technique is applicable to non-solid organs such as intestine, liver, spleen, brain and heart, as well as to carcinomas of varying solidity (De Graaf et al., 2010). To facilitate the cutting process, the organ of interest is included in an agarose gel, which helps during slicing with an automated vibratome. This machine, set with specific values of speed, amplitude and blade angle, ensures precise and consistent slices. Embedding the organ in agarose is crucial, not only to achieve high quality slices but also to preserve the three-dimensional structure of soft tissues (Giuliani et al., 2019). The thickness of the slices is another important factor because it should be thin enough to allow the diffusion of oxygen and molecules from the culture medium, but thick enough to preserve the original histological architecture. For instance, a thickness of 400 µm for mussel's digestive gland seems to meet these requirements (De Graaf et al., 2010; Giuliani et al., 2019).

As demonstrated by several studies (Vitale et al., 2019 ; Pan et al., 2016; Yu et al., 2019), in

the field of ecotoxicology, the integration of different types of techniques is fundamental to gaining a deeper understanding of the environmental impact of chemical pollutants. For this reason, this thesis aims to combine both *in vivo* and *ex vivo* approaches to investigate the main metabolic pathways involved in mechanisms of action of pharmaceutical classes that have been poorly studied.

2.3 Ecotoxicological potential of Gemfibrozil, Metformin and Rosiglitazone in aquatic ecosystems

Pharmaceuticals are designed to exert their biological effects at low concentrations by interacting with metabolic, enzymatic, or cell signalling pathways through well-established mechanisms of action in humans and animals. As certain molecular receptors are evolutionarily conserved across various aquatic species, these same pathways may also be modulated in organisms with similar target organs, tissues, cells, or biomolecules (Fent et al., 2006; Mezzelani and Regoli, 2022). However, in other species, these molecular targets might differ or even be completely absent, leading to different and potentially unknown mechanisms of action (Fent et al., 2006; Daughton & Ternes, 1999).

Among all class of pharmaceuticals occurring in marine ecosystems and accumulated by aquatic organisms, antibiotics, antiepileptics, and painkillers are the most extensively studied, primarily due to their high global consumption (Hughes et al., 2013). However, other therapeutic classes have received less attention in toxicity assessments. Notably, there remains a significant knowledge gap concerning the impact of certain drug classes, particularly lipid regulators and antidiabetics, on aquatic non-target species and ecosystems.

Lipid regulators, typically prescribed combined with cardiovascular drugs, are used to treat dyslipidemias reduce the risk of severe cardiovascular diseases, and can be divided into two major subclasses, the statins and fibrates (Mezzelani & Regoli, 2022).

Among fibrates, gemfibrozil (GEM) lowers levels of fatty acids, triglycerides, and low-density lipoproteins (LDL), primarily by enhancing peroxisomal β-oxidation and, to a lesser extent, mitochondrial β-oxidation (Pahan, 2006; Barradell, 1996). This action occurs through the activation of peroxisome proliferator-activated receptor alpha (PPARα) (Schmidt et al., 2014), which enhances peroxisomal β-oxidation, increases the number and size of peroxisomes, and induces oxidative stress, thereby facilitating the breakdown of lipids

(Barreto et al., 2017).

Although the International Agency for Research on Cancer has classified GEM as non-carcinogenic to humans (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1987), this pharmaceutical should be further investigated for its potential harm to aquatic ecosystem (Zurita et al., 2007). In fact, while its pharmacological mechanisms in the human body have been well described, there is still a lack of information regarding its potential adverse effects on non-target species (Zurita et al., 2007). The few studies carried out on lipid regulators indicate that they can affect lipid metabolism, immune system and redox homeostasis, and induce genotoxicity in aquatic organisms.

For instance, a significant reduction of triglycerides and an increase in fatty acids were observed in mussels exposed to 0.2 μg/L of the fibrate clofibrate (Lazzara et al. 2012). While variations of plasma triglycerides, cholesterol levels, and changes of genes involved in lipid metabolism were observed in fish fathead minnow (*Pimephales promelas*) exposed to 15 and 600 μg/L of GEM for 2-8 days (Skolness et al., 2012).

Zebra mussels (*Dreissena polymorpha*) exposed to 1000 μg/L of GEM (96h) showed an induction of glutathione-S- transferase (GST) and an increase in DNA damage (Quinn et al., 2011). In addition, Canesi et al. (2002) reported a destabilization of lysosomal membranes, a release of extracellular lysozyme and a decrease of phagocytic activity in mussels' hemocytes treated with the fibrates GEM and benzafibrate for 30 min (from 100 mM stock solutions). The same drugs were also tested in *in vivo* condition, highlighting an induction of GST activity and total glutathione (GSH) content (Canesi et al., 2007).

The teleost *Sparus aurata* exposed to increasing sublethal concentrations of GEM, ranging from 1.5 to 15,000 μg/L (96h), showed a reduction in its ability to swim against a water flow (in terms of time of swimming), which could be an indicator of damage to neurotransmission. An induction of enzymes involved in antioxidant defences (catalase, glutathione peroxidase and glutathione reductase) occurs at higher concentrations of GEM (15- 15,000 μg/L) (Barreto et al., 2018a). In addition, to evaluate the genotoxic effects of GEM, the teleost *Danio rerio* was exposed to the average concentrations detected in some Italian treatment plants (380 ng/L) for 5 and 35 days. The levels of DNA damage are a sensitive indicator of genotoxicity. *D. rerio* showed a significant loss of DNA integrity (60,67%) after one week of exposure, which was partially repaired after 14 days (Rocco et al., 2012).

Another class of drugs that remains underexplored in the field of ecotoxicology is

antidiabetics. Antidiabetics are used to treat the hyperglycaemia and include sulfonylurea derivates, biguanides, thiazolidinediones, meglitinides, and α-glucosidase inhibitors. Among biguanides, metformin (MET) is widespread use to treat various common human disorders, such as type-2 diabetes, polycystic ovary syndrome, and cancers where insulin resistance is a contributing factor. The prevalence of diabetes is steadily increasing, with the International Diabetes Federation estimating 537 million cases in adults aged 20 to 79 in 2021. If no effective measures are taken to address this trend, the number of people affected by diabetes is expected to rise to 643 million by 2030 (Magliano & Boyko, 2021). Because millions of people consume MET annually, is one of the most detected drugs in sewage effluents (Niemuth et al., 2015). Despite MET was introduced in clinical treatment from 1950s, the exact mechanism of action has not been fully discovered. The biological activity of this drug primarily involves the inhibition of complex I in the electron transport chain, which alters hepatic energy status. This inhibition increases the adenosine monophosphate to adenosine triphosphate (AMP:ATP) ratio, thereby activating AMP-activated protein kinase (AMPK), a key metabolic sensor. Upon activation, AMPK inhibits gluconeogenesis and stimulates glycolysis (Viollet et al., 2012). Furthermore, AMPK suppresses sterol regulatory element binding protein 1 (SREBP1), a key transcription factor involved in lipid and cholesterol synthesis.

MET is commonly detected in aquatic environments (Niemuth et al., 2015; Raja & Kathiravan, 2021), primarily due to its widespread consumption and its low rate of biotransformation in the human body. About 70% of the ingested dose is excreted unchanged in urine and feces (Ussery et al., 2018). Due to its ubiquitous presence in aquatic ecosystems, MET has become a subject of growing interest, particularly about its potential adverse effects on aquatic biota.

The exposure of fathead minnows (*Pimephales promelas*) at 40 µg/L of MET for 365 days demonstrated its potential as endocrine disruptor and highlighted its impact on the reproductive system of the organism, causing the development of intersex gonads in males, reducing the size of male fish, and decreasing the fecundity of treated pairs (Niemuth & Klaper, 2015). In addition, Koagouw and Coiocan (2018) reported an increase on vitellogenin mRNA expression levels, an induction of pathological conditions in the gonad, and destabilization of lysosomal membrane in hemocytes of mussels (*Mytilus edulis*) treated with MET (40 µg/L, 7 days), suggesting such drug as a nontraditional endocrine disruptor

in the environment. A significant increase in mRNA expression of vitellogenin, estrogen receptor α, cytochrome P450 3A4-like isoform, and gonadotropin-releasing hormone in juvenile *P. promelas* exposed to MET (1, 10 and 100 µg/L,) represent a further example of disruption of endocrine system (Godoy et al., 2018). Moreover, two generations of *Oryzias latipes* were exposed to MET (40 µg/L, 120 µg/L, and 360 µg/L) for 133 days. The results showed in females a decrease in gene expression of vitellogenin and ERβ1. In addition, male fish exhibited a reduced glutathione (GSH) content, while female fish showed increased catalase an increase in the gene expression of cytochrome P450 19a and estrogen receptor α (ERα) in males, while activity, highlighting MET's potential as endocrine disruptor (Lee et al., 2019). In contrast, a lack of neurotoxic effects was observed in Zebrafish (*Danio rerio*) exposed to MET concentrations ranging from 0 to 2000 mg/L (24h). The results showed no significant changes in swimming behaviour and survival rate was unaffected (Godoy et al., 2018). Negative behavioural effects caused from MET exposure (40 µg/L and 80 µg/L, 40 weeks) was also reported for *Betta spendens*. Normally, males of these species exhibit discrete aggressive behaviours. However, following exposure to MET, individuals showed a reduction in aggressive behaviours, which could potentially affect male fitness (MacLaren et al., 2018).

Moreover, Zhou et al. (2019) reported that 48h exposure to 200 µM MET induced AMPK activation, enhanced lipid metabolism, and reduced lipid accumulation in hepatocytes of fish blunt snout bream.

Among antidiabetic drugs, the thiazolidinedione rosiglitazone (ROS) is commonly used to treat type 2 diabetes mellitus by alleviating peripheral insulin resistance. Although its precise mechanism of action remains unclear, ROS aims to increase insulin sensitivity and can modulate various processes, including insulin receptor kinase activity, receptor phosphorylation, and hepatic glucose metabolism (Balfour and Plosker, 1999). ROS acts as a PPAR-γ agonist, reducing plasma glucose levels and an increasing the production of endogenous glucose (Wagstaff & Goa, 2002). In addition to the expression of genes involved in glucose metabolism, PPAR-γ regulates also genes involved in lipid metabolism and inflammation. Indeed, in humans, ROS has been reported to reduce postprandial triglyceride concentrations (Mayerson et al., 2002; Tan et al., 2005).

ROS is extensively metabolized in the human body and excreted as metabolites in the urine. The primary metabolites are produced through N-demethylation and hydroxylation,

followed by conjugation with sulphate and glucuronic acid. All these metabolites are less potent than the original compound (Khetan & Collins, 2007).

Despite the large consumption by human of this pharmaceutical, there are a severe lack of information about its possible impact on aquatic organisms.

Guan et al. (2021) reported an increase of growth rates, feed efficiency and muscle glycogen accumulation, and an improved insulin sensitivity and levels in farmed tilapia (GIFT) fed with high-starch diets supplemented with ROS (10, 20, 30 mg/kg), suggesting that such drug could be useful to improve the aquaculture productivity. In addition, dietary supplementation with ROS (15 mg/kg, 4 weeks), as a *pparg* activator to increase the esterification of free fatty acid into triglycerides, could reduce oxidative stress and significantly improve stress resistance in zebrafish (Wang et al., 2022). On the other hand, a study evaluated the effects of exposure to pioglitazone (9 mg/kg, 18 mg/kg, 9 days), a member of the thiazolidinedione family, on cognitive impairment induced by scopolamine in mice. Pioglitazone reduced scopolamine-induced memory deficits by the enhancing the cholinergic system, as indicated by the increase of Ach levels, demonstrating significant neuroprotective effects (Xiang et al., 2012). Considering the limited knowledge on the effects of ROS on non-target aquatic species, further research is crucial to better understand the potential ecological consequences these pharmaceuticals may have on marine ecosystems. Expanding studies in this area will provide valuable insights into the long-term impacts and help the implementation of strategies for mitigating risks associated with pharmaceutical occurrences in aquatic environments.

2.4 Current regulatory frameworks

Pharmaceuticals are increasingly recognized as ubiquitous environmental contaminants due to their presence in trace amounts and their characteristic to be biologically active, which may negatively impact non-target organisms. For these reasons, the potential risks associated with the release of drugs into the environment have attracted the attention of both the pharmaceutical industries and environmental regulators (Silva et al., 2011). The urgency to protect and monitor the health status of the environment, increased the need to develop guidelines for ecopharmacovigilance in Europe, United States, Japan and Australia (Mezzelani & Regoli, 2022). The concept of Ecopharmacovigilance (EPV) involves the

collaboration among diverse stakeholders, including pharmaceutical industry, healthcare providers, environmental regulators, and scientific researchers (Silva et al., 2011). The World Health Organization (WHO) defines EPV as "the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other possible drug related problems'' (Weltgesundheitsorganisation & Collaborating Centre for International Drug Monitoring, 2002). EPV includes the development of environmentally friendly pharmaceuticals, the application of green chemistry in process design, the creation of biodegradable products, the reduction of manufacturing emissions, education on responsible use of medicines, improvement in prescribing practices, and the proper management of unused pharmaceuticals (Gómez-Oliván & Arizmendi-Cotero, 2019).

Over the last four decades, the European Community has issued a series of directives addressing to various areas of environmental policy. These directives have marked a shift for the EU Member States, moving from sectoral environmental protection to a more integrated approach, establishing environmental objectives that aim to achieve a good environmental status (Petersen et al., 2009). Some of the most relevant EU directives focused on environmental protection, with major emphasis on waters, starts with the Nitrate Directive (ND) (91/676/ECC). Issued in 1991, the ND aimed to "protect water quality across Europe by preventing nitrates from agricultural sources polluting ground and surface waters and by promoting the use of good farming practices". In the same year, the EU's Urban Wastewater Treatment Directive (91/271/EEC) was adopted, aiming to protect the environment from adverse effects of untreated urban wastewater and ensuring that domestic and industrial wastewater was efficiently collected, treated and discharged. Some years later, in 1998, the Drinking Water Directive (DWD) (98/83/EC) was established to regulate the water quality in EU, setting a series of quality standards and health parameters.

In the early 2000s, in this context of water-related EU directives, was introduced the Water Framework Directive (WFD) (2000/60/EC), bringing a significant level of innovation to water policy, being the first European Directive aimed at protecting the water environment, encompassing freshwater, marine, and groundwater, focused on the environmental sustainability. The WFD sets goals on a six-year cycle, with the current set of objectives to be achieved by 2027. In addition, the Marine Strategy Framework Directive (MSFD) (2008/56/EC), which specifically focuses on marine ecosystems, complements the WFD by addressing environmental protection in marine waters.

In contrast with the old water-related directives, which considered isolated pressures and reduced environmental systems to their basic components, the WFD introduced a holistic approach that considers all parts of the environmental systems in relation to pressures (Voulvoulis et al., 2017). This paradigm shift aims to assess the overall environmental status with the goal of achieving Good Environmental Status (GES), which is defined by a set of characteristics that represent the desired high quality of both water and ecosystems.

In the GES assessment process, reference conditions are established. These include both pristine states, which are rarely found and free from human impact, as well as slightly disturbed conditions that do not negatively affect the ecosystem (Borja et al., 2010). Establishing these reference conditions is crucial as they provide a baseline for assessing the health and quality of natural systems. Once the reference conditions are determined, monitoring programs are designed to assess any divergence from them. For the classification of ecological status, Annex V of the WFD outlines three groups of "quality elements": biological, hydro morphological, and physico-chemical (Voulvoulis et al., 2016). Data collected from these indicators are used to calculate environmental parameters that are then compared against metrics tailored to specific water body types as part of the GES evaluation. The difference between the reference and estimated values is used to calculate the Ecological Quality Ratio (EQR), which ranges from 0 to 1 and classifies the environment into five categories: high, good, moderate, poor, and bad. These categories inform subsequent management decisions (Hering et al., 2018).

It is important to note that even a single significant deviation from the reference value for any individual metric is sufficient to define an environmental status as poor, following the "one-out-all" principle. This principle emphasizes that the environmental status of a water body is determined by its worst-performing element, ensuring that no single metric can mask broader environmental degradation.

Zacharias (2020) conducted a comparison of the condition of water basins from the first years of 2000s to 2020. This analysis estimates the overall improvement in water body over the past twenty years. The results show that 20% of water bodies have improved their status, and a significant number of those with an unknown status now have their chemical status identified. Furthermore, lakes and coastal waters have reached higher quality levels than rivers and transitional waters. These findings prove that significant progress has been made towards improving the environmental status.

Despite the environmental threat posed by pharmaceuticals, their disposal remains poorly regulated.

The watch-list (WL) is designed to identify priority substances-chemicals selected by the European Commission (Decision 495/2015/EU) due to their significant environmental risks. The main goal is to reduce these substances to natural background levels or to eliminate those that do not occur naturally. This List aims to monitor EU water basins, assess the concentrations in the aquatic environments, and evaluate the potential risks for the ecosystems of those pollutants that currently lack adequate or sufficient monitoring information (Serrano et al., 2019).

The criteria for selection include data collected from scientific literature, member states, and stakeholders, as well as an assessment of substances from previous watch-lists. To effectively prioritize these chemicals, continuous monitoring is essential to detect their presence in the environment. Furthermore, obtaining information on the biological impacts of these substances is vital for understanding their distribution patterns and evaluating their potential effects on the environment. Additionally, the availability of analytical techniques capable of quantifying these contaminants in various environmental matrices plays a crucial role in ensuring accurate monitoring (Cortes & Cuenca, 2022).

Moreover, a crucial aspect is the Predicted No Effect Concentration (PNEC) which defines the safety threshold. The PNEC is derived from ecotoxicity data and hazard characteristics such as persistence, bioaccumulation, carcinogenicity, mutagenicity, reproductive toxicity, endocrine disruptor, and the contribution to antimicrobial resistance. Using these criteria, the Joint Research Centre divided the selected substances into three groups: Priority 1, substances suitable for inclusion in the WL; Priority 2, substances suitable for inclusion but lacking a reliable PNEC or analytical method; and Priority 3, substances that are good candidate for the future WL updates (Cortes & Cuenca, 2022).

The first Watch list included 4 pharmaceuticals: the NSAID diclofenac, the hormons 17-beta-estradiol (E2), and 17-alpha-ethinylestradiol (EE2) and macrolide antibiotics. However, in the latest update in 2022, the number of pharmaceuticals increased at 10, included the antibiotics sulfamethoxazole, clindamycin, ofloxacina, and trimethoprim; the antidepressant venlafaxine and its metabolite O-desmethylvenlafaxine; the antimicotics clotrimazole, fluconazole, miconazole; and the antidiabetic metformin and its metabolite guanylurea (Commission Implementing Decision (EU) 2022/1307), emphasising the rising of awareness

concerning the presence of pharmaceuticals in water and their effects on non-target organisms (Cortes et al., 2022).

Given the urgency, one of the primary objectives is to adopt an integrated, multidisciplinary approach to optimize environmental risk assessment. While precise chemical quantification is essential for monitoring, it offers limited insight into ecosystem health without biological data. It is therefore crucial to compare analytical chemical data with biological assessments, in alignment with European Directives, which advocate for the use of multiple quality indicators. Biological evaluations are vital not only for detecting the presence of chemicals but also for assessing their effects on organisms and the broader ecological consequences. Consequently, new scientific data are fundamental to supporting policymakers in developing robust regulatory guidelines and strategies for assessing environmental risks (Mezzelani et al., 2018).

In this context, through both *in vivo* and *ex vivo* laboratory approaches, this thesis seeks to enhance our understanding of the mechanisms of action and potential risks associated with emerging pollutants on non-target organisms.

3 Aim of the study

In recent decades, pharmaceuticals have been widely produced and consumed by humans and consequently detected in marine environments. Their presence in marine ecosystems is reflected not only in the water column and sediments but also in non-target organisms, which may accumulate these substances and be subjected to adverse effects, even at low concentrations. Despite extensive research on pharmaceutical pollution, significant knowledge gaps remain, particularly regarding the impact of certain therapeutic classes on aquatic ecosystems. Among these, antidiabetic drugs and lipid regulators have received relatively limited attention. In this context, this thesis aims to investigate the potential harmful effects of three poorly studied pharmaceuticals: the lipid-regulating agent gemfibrozil, and the antidiabetic drugs metformin and rosiglitazone. These compounds are all known to interact with lipid metabolism in humans; therefore, this study explores whether their mechanisms of action are also conserved in non-target species.

The model organism chosen for the exposure tests was *Mytilus galloprovincialis*, a bivalve widely used as a bioindicator due to its filter-feeding capability, high tolerance to various environmental conditions, broad distribution, and easy breeding. The potential effects of tested pharmaceuticals were assessed by applying an integrated approach that combines both *in vivo* and *ex vivo* studies. The *in vivo* tests involved a mussels exposure to concentrations of the three pharmaceuticals potentially close to those measured in coastal environments, for 14 days (2.5 µg/L), while in the *ex vivo* technique, tissue slices of digestive gland (PCTS) were exposed to higher drug concentrations (500 µg/L; 1000 µg/L) for 48h and 72h to stimulate clearer physiological responses. The biomarkers analysed after the *in vivo* exposure included those related to the immune system modulation, such as the granulocytes/hyalinocytes ratio, phagocytosis activity, and lysosomal membrane stability; oxidative pathway responses through the single antioxidant defences, including the activity of catalase, glutathione S-transferases, glutathione peroxidases, Se-dependent glutathione peroxidases (GPx H_2O_2), glutathione reductase, total glutathione, and the total oxyradical scavenging capacity toward hydroxyl (HO•) and peroxyl (ROO•) radicals; genotoxic and neurotoxic effects measuring the frequency of micronuclei and the activity of acetylcholinesterase in haemolymph and gills; and the modulation of lipid metabolism

through the accumulation of lipid peroxidation products (lipofuscin and malondialdehyde), neutral lipids content and activity of the Acyl-COA oxidase in the digestive gland. In the *ex vivo* technique selected biomarkers were specifically focussed on parameters related to the lipid metabolism and the oxidative pathway, including the accumulation of lipofuscin and neutral lipids, as well as catalase activity. The overall results of this study will provide novel insights into the main metabolic pathways modulated by the tested drugs, helping to clarify their mechanisms of action on non-target organisms. Such findings will be helpful to fill the knowledge gap regarding the ecological consequences of the tested therapeutic class in aquatic environments.

4 Material and methods

4.1 Animals collection

Mediterranean mussels, *M. galloprovincialis*, (5.5 ± 1.00 cm shell length), were collected from a local farm in Senigallia (Ancona, Adriatic Sea). Organisms were acclimated for 7 days with aerated artificial sea water (ASW), at local seasonal environmental conditions of salinity (33‰), temperature (18 ± 1°C), and pH (8.0). Collection and experimental use of mussels do not require ethical review permission according to both European (European Directive 2010/63/EU) and Italian normative (Italian Legislative Decree No. 26, 2014).

4.2 *In vivo* experiment

4.2.1 Experimental design

240 organisms were randomly distributed into 4 glass-tanks (n=60 per tank) in a volume of 17 L of ASW. The experimental setup consisted of four treatment groups: CTRL, control condition (0.0029% of dimethyl sulfoxide, DMSO); ROS, rosiglitazone exposure (2.5 µg/L); GEM, gemfibrozil exposure (2.5 µg/L); MET, metformin exposure (2.5 µg/L) (Figure 1).
Stock solutions of GEM, MET, and ROS 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 zooplankton for filter-feeding organisms (Easy-sps EVO) every 12 hours. 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, 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.

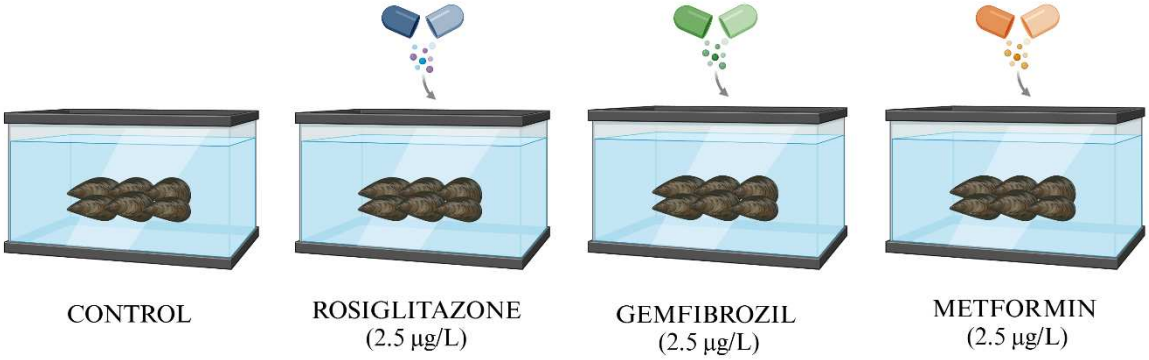


Figure 1. *in vivo* experiments: tanks with mussels (n=60) exposed to rosiglitazone (2.5 µg/L), gemfibrozil (2.5 µg/L), and metformin (2.5 µg/L).

4.2.2 Biomarkers analysis

Biomarkers in tissues were assessed using standardized protocols (Bocchetti and Regoli, 2006; Mezzelani et al., 2021). 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.

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 $\lambda = 412$ 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).

Genotoxicity was evaluated at chromosomal level by the micronucleus test. 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.

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 x g 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 x g 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 x g 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 $\lambda = 502$ nm against a blank without the substrate.

4.3 *Ex vivo* experiment

4.3.1 *Experimental solutions*

Culture medium and buffers were prepared according to standardized protocols (Giuliani et al., 2019). 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) and HEPES physiological solution (NaCl 436 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgSO_4$ 53 mM, pH 7.3) were sterilized and supplemented with 1% antibiotic mix (penicillin 10000 units/mL, streptomycin 10 mg/mL, amphotericin-B 0.025 mg/mL).

4.3.2 *Experimental design*

Precision Cut Tissue Slices (PCTS) were prepared from mussels' digestive glands (DG) followed Giuliani et al. (2019) method.

Before DG dissection, the shell surface of mussels was cleaned with 75% ethanol, then the digestive glands were excised with sterile blade, tweezers, and scissors. The digestive canal was washed through a syringe four times by cold HEPES physiological solution. Dissected DG were immediately placed in cold (4 °C) physiological solution until the cutting phase. Each DG was cut into 2 parts along the dorsoventral axis, using a sterile blade, and embedded in an agarose gel cylinder (2.5%), to facilitate slicing procedure. Slices with thickness of 300 μm were cut using a motorized vibrating blade vibratome (VT1200, Leica, Wetzlar, Germany) with a razor blade, setting speed of 0.18 mm/s, amplitude of 1.0 mm, knife angle

of 18°. The slicing procedure was carried out in cold deionized water (4 °C) with the antibiotic mix. After the cutting, the agarose was removed with small brushes and the PCTS were maintained in 12-wells culture plates (2 PCTS/wells) (Figure 2) with cold physiological solution with 1% of D-glucose 180 mg/mL until incubation. To reactivate the cellular functions after the stress imposed by the cutting and the cold exposure, a pre-incubation phase was carried out in Leibovitz's medium L-15 supplemented with 10% of fetal bovine serum (FBS), at 18 °C, for 1 h, in absences of light, with gentle shaking. After the pre-incubation, the medium was removed and the PCTS were rinsed three times with physiological solution to remove any residual of FBS.

After this phase, PCTS were exposed to GEM, MET and ROS (500 and 1000µg/L) 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. Every 24 h, the culture medium was renewed, and pharmaceuticals were re-dosed. Incubation occurs under static conditions to avoid mechanical stress, at 18 °C, normal atmosphere and in the darkness. 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, 9 PCTS to pool and frozen at –80 °C for the evaluation of the catalase activity.

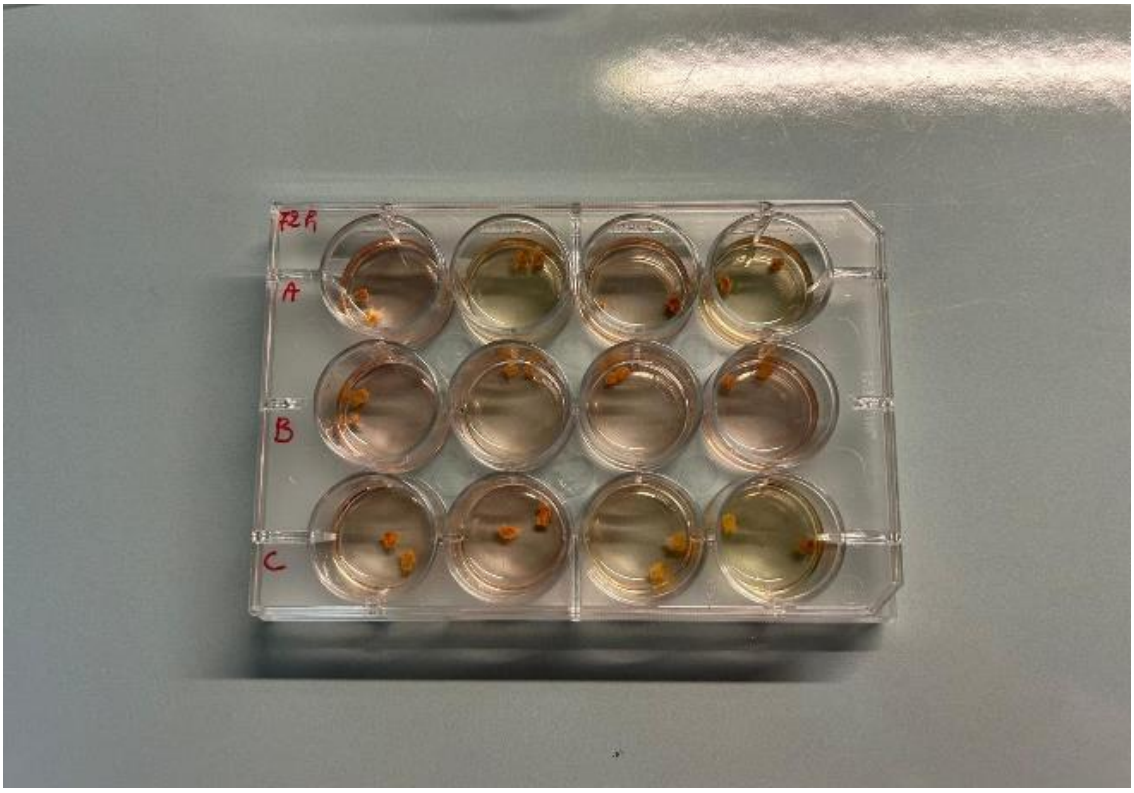


Figure 2. 12-well culture plate with PCTS exposed to pharmaceuticals

4.3.3 *Biomarkers analysis*

Biomarkers analysis on PCTS were assessed by using standardized protocols (Bocchetti and Regoli, 2006; Mezzelani et al., 2021).

Lipofuscin and Neutral lipids staining was performed on 4 cryostat sections (8 µm) obtained from 2 PCTS (300 µm) from the 3 DG used for each treatment and experimental time (Figure 3). After cutting, cryosections were fixed in Beker's fixative (2.5 % NaCl) for 15 min 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. Slides were observed under light microscope. For both analysis, four measurements were made on digestive tubules of each section (one section 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 DMSO control and averaged (n = 3).

Catalase activity was assessed in 3 replicates (each replicate consisted in a pool of 9 PCTS from one DG) measuring 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)]. Results were expressed as fold change of DMSO control and averaged (n = 3).

Figure 3. Inclusion of mussels' PCTS in Killik medium for cryostat cutting.

4.4 Statistical analysis

Statistical analyses were carried out using RStudio (version 2023.12.0 Build 369). Normal distribution of data was assessed using the Shapiro-Wilk test and the homogeneity of variances using Levene's test. For the *in vivo* experiment, the ANOVA was used to test differences between treatments (level of significance at $p < 0.001$), and the Student Newman-Keuls test (SNK) was applied for post-hoc comparison between means of values (n = 5). For the *ex vivo* experiment, the comparison 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 and exposure times. Post-hoc analysis was performed using TuckeyHSD to compare each experimental group.

5 Results

5.1 *In vivo* experiment

Results of the immune system biomarkers are showed in Figure 4. A statistically significant reduction in neutral red retention time was observed for all treatments compared to the CTRL (Fig. 4A). The granulocytes-hyalinocytes ratio exhibited a statistically increased compared to the CTRL, without significative differences among the treatments (Fig. 4B). Additionally, average lower values were measured for the percentage of phagocytosis activity, with a statistically significant decrease observed only in mussels exposed to MET (Fig. 4C).

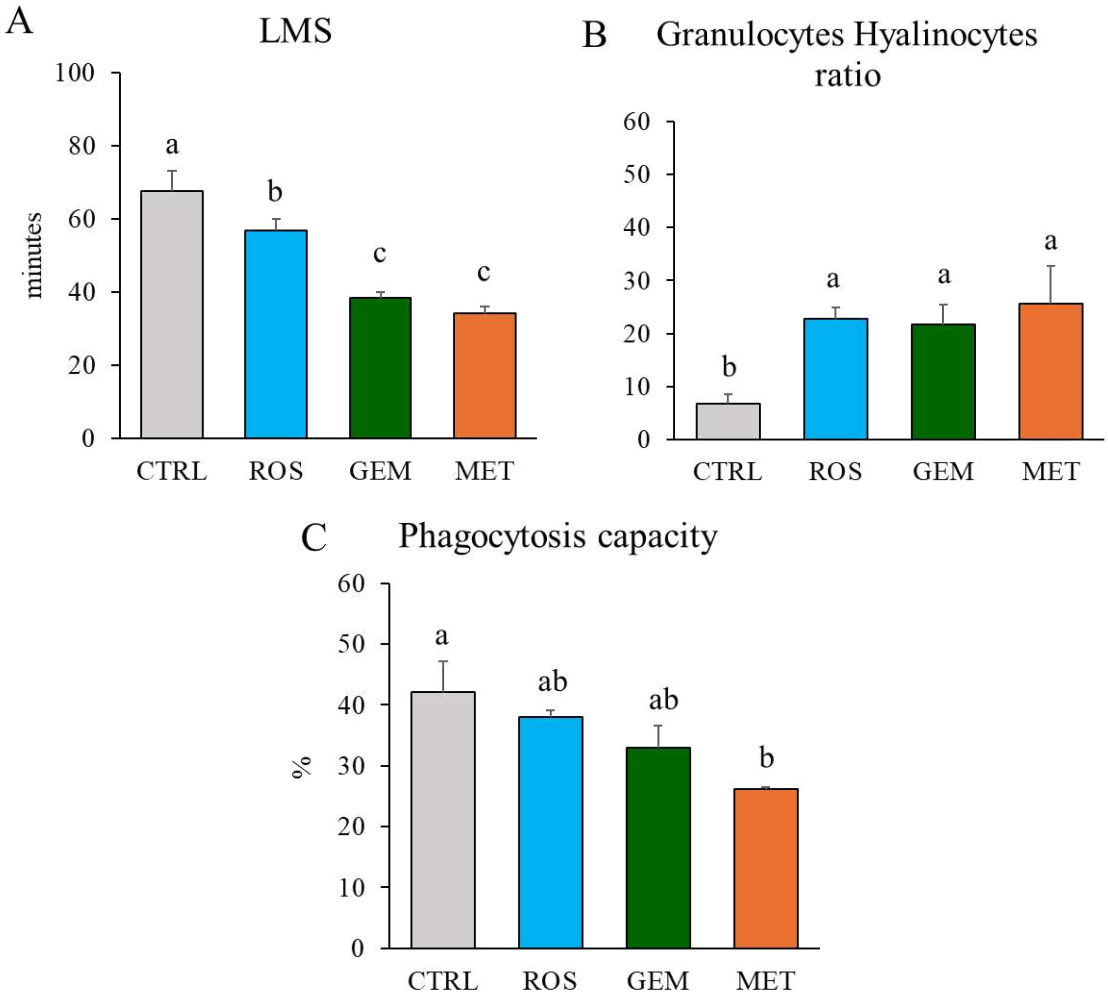


Figure 4. Lysosomal Membrane Stability (LMS) (A), granulocytes hyalinocytes ratio (B), phagocytosis (C) in haemocytes of mussels exposed to different pharmaceuticals. Data are given as mean values±Standard Error of the Mean. Different letters indicate significant differences between group of means. CTRL, control; ROS, rosiglitazone; GEM, gemfibrozil; MET, metformin.

Biomarkers of neurotoxicity and genotoxicity were showed in Figure 5. Considering neurotoxicity, exposures to ROS and GEM caused statistically significant induction of Acetylcholinesterase (AChE) activity in both haemolymph and gills (Fig. 5A), while MET-treated mussels highlighted a significant induction only in gills (Fig. 5B).

Regarding genotoxic damage, the micronuclei frequency exhibited average increased values in all treatments, with statistically significant variations only in organisms exposed to MET, (Fig. 5C).

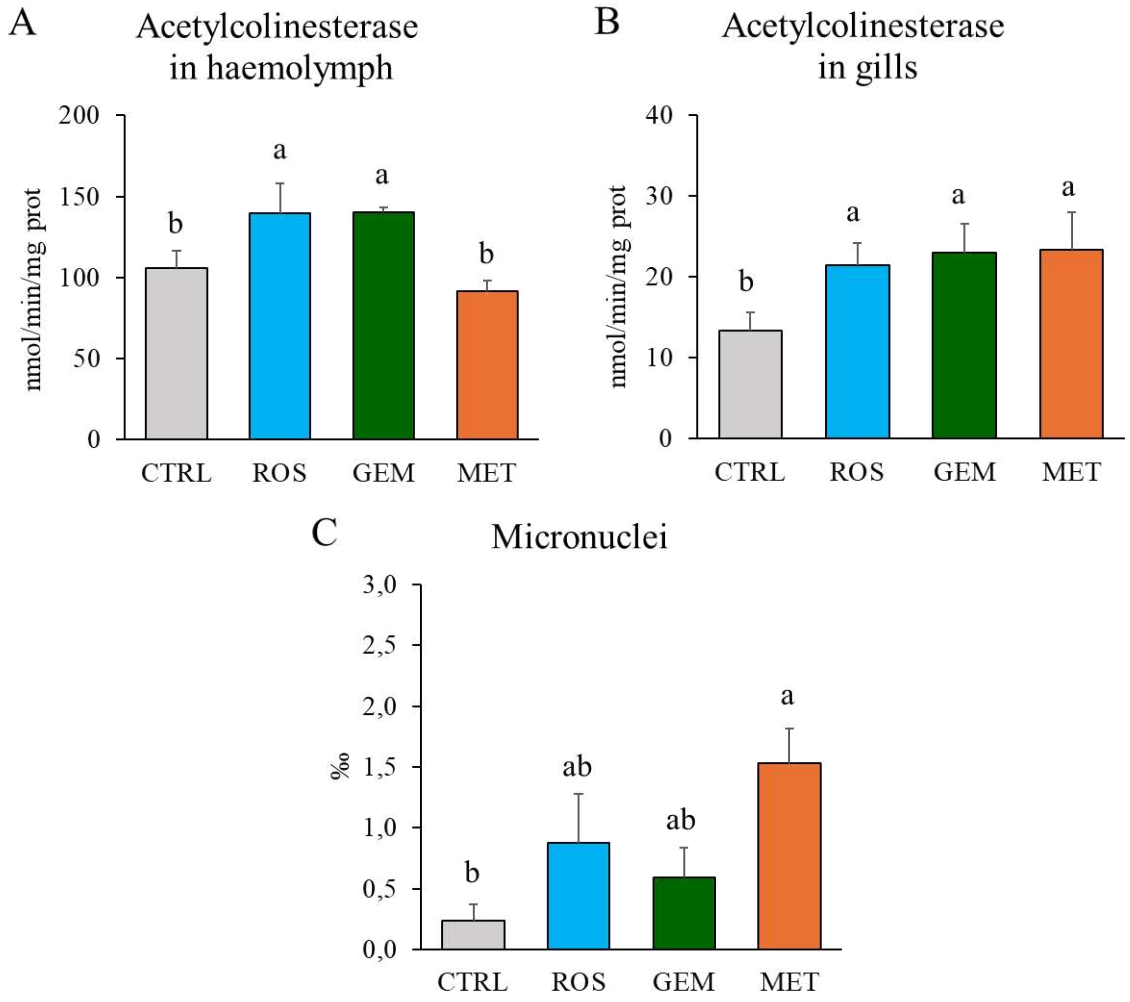
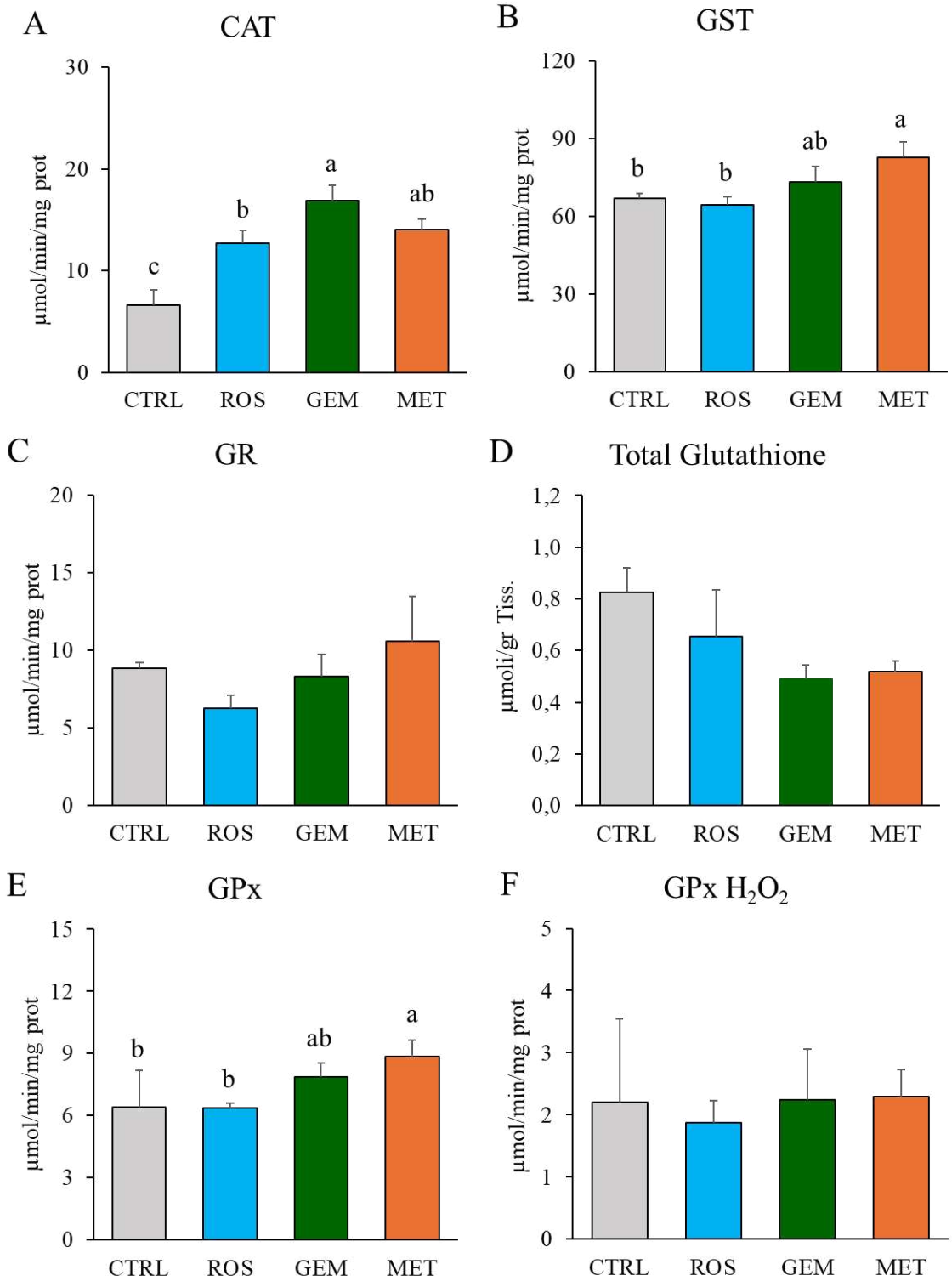


Figure 5. Neurotoxicity responses: Acetylclineserase activity (AChE) in hemolymph (A) and gills (B). Genotoxic effects: Micronuclei frequency (MN) (C). Data are given as mean values±Standard Error of the Mean. Different letters indicate significant differences between group of means. CTRL, control; ROS, rosiglitazone; GEM, gemfibrozil; MET, metformin.

Responses of antioxidant responses, evaluated both in terms of single antioxidant enzyme activity and the total oxyradical scavenging capacity (TOSC) toward hydroxyl (HO·) and peroxyl (ROO·) radicals, are shown in Figures 6. Among the antioxidant enzymes, Catalase (CAT) activity showed a statistically significant induction in all treatments (Fig. 6A), while average enhanced activity was observed for Glutathione S-transferase (GST) activity, with significant differences only in MET-exposed organisms (Fig 6B). No significant differences were measured in the activity of glutathione reductase (GR) (Fig. 6C), while lower average levels of total glutathione were observed in all treatments compared to CTRL (Fig. 6D).

Statistically significant induction of Glutathione Peroxidase (GPx) was highlighted in mussels exposed to MET (Fig. 6E), while no variations were observed in Se-dependent Glutathione Peroxidases (GPx H_2O_2) activity (Fig. 6F).

The TOSC values toward both radicals showed a statistically significant decrease in GEM-exposed mussels, while no significant variations were measured for the other treatments (Fig.6G, H).


A
CAT
µmol/min/mg prot
30
20
10
0
c
b
a
ab
CTRL
ROS
GEM
MET
B
GST
µmol/min/mg prot
120
90
60
30
0
b
b
ab
a
CTRL
ROS
GEM
MET
C
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1,2
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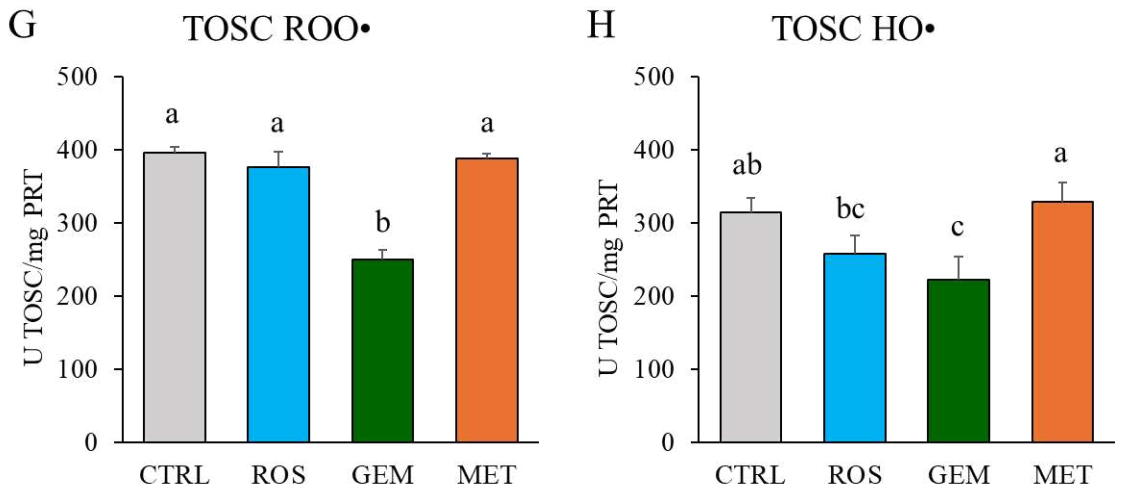

Figure 6. Responses of single antioxidant enzymes in mussels' digestive glands: Catalase activity (A); Glutathione S-transferase activity (GST) (B); Glutathione reductase activity (GR) (C); Total glutathione (D); Glutathione peroxidase (GPx) (E); Se-dependent glutathione peroxidases (GPx H_2O_2) (F); Total Oxyradical Scavenging Capacity for peroxyl radicals (ROO·) (G) and hydroxyl radicals (HO·) (H). Data are given as mean values±Standard Error of the Mean. Different letters indicate significant differences between group of means. CTRL, control; ROS, rosiglitazone; GEM, gemfibrozil; MET, metformin.

Results on the lipid metabolism and the accumulation of lipid peroxidation products are reported in Figure 7. Average higher levels of lipofuscin (Fig. 7A) and neutral lipids (Fig. 7B) content were observed in all treated mussels compared to the CTRL organisms, with statistically significant variations in ROS and GEM treatments. Similarly, the accumulation of Malondialdehyde (MDA) showed average enhanced values in all treated organisms, with statistical differences only in mussels exposed to GEM (Fig. 7C), while a statistically significant induction of acyl-CoA oxidase (ACOX) was observed in only in MET treatment (Fig. 7D).

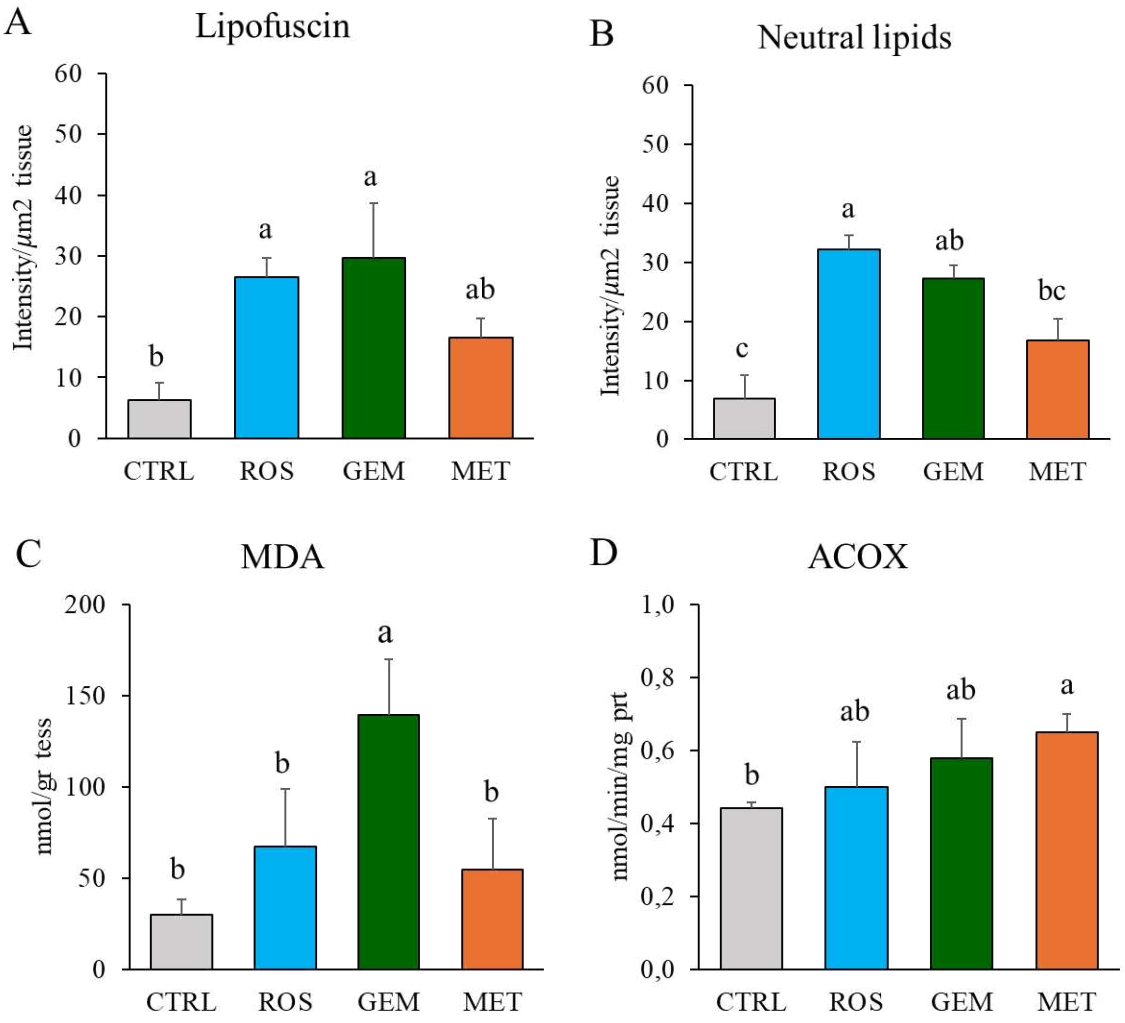

Figure 7. Lipid peroxidation products and lipid metabolism analysed in digestive gland. Lipofuscin (A), Neutral lipids (B), Malondialdehyde (MDA) (C), acyl-CoA oxidase activity (ACOX) (D). Data are given as mean values±Standard Error of the Mean. Different letters indicate significant differences between group of means. CTRL, control; ROS, rosiglitazone; GEM, gemfibrozil; MET, metformin.

5.2 *Ex vivo* experiment

Figure 8 shows results on neutral lipid and lipofuscin content in mussels' PCTS exposed to selected pharmaceuticals. In this case, results are expressed as percentage variations compared to relative control organisms.

PCTS treated with ROS showed a statistically significant reduction of neutral lipids content for both concentrations after 72h of exposure and for 1000 µg/L after 48h (Fig. 8A). Lipofuscin levels highlighted a significant increase in PCTS exposed to 1000 µg/L of ROS for 72h compared to the control PCTS, while no variations were observed for other exposure dose and experimental time (Fig. 8B)

Exposure to GEM caused a reduction in neutral lipids content, which was statistically significant for both tested concentrations after 72 hours, and for 500 µg/L at 48h (Fig.8C). No significant variations were observed in lipofuscin levels in PCTS exposed to GEM (Fig. 8D).

A statistically significant increase in neutral lipids and lipofuscin content was measured in MET-treated PCTS for all exposure doses and times (Fig. 8E, F).

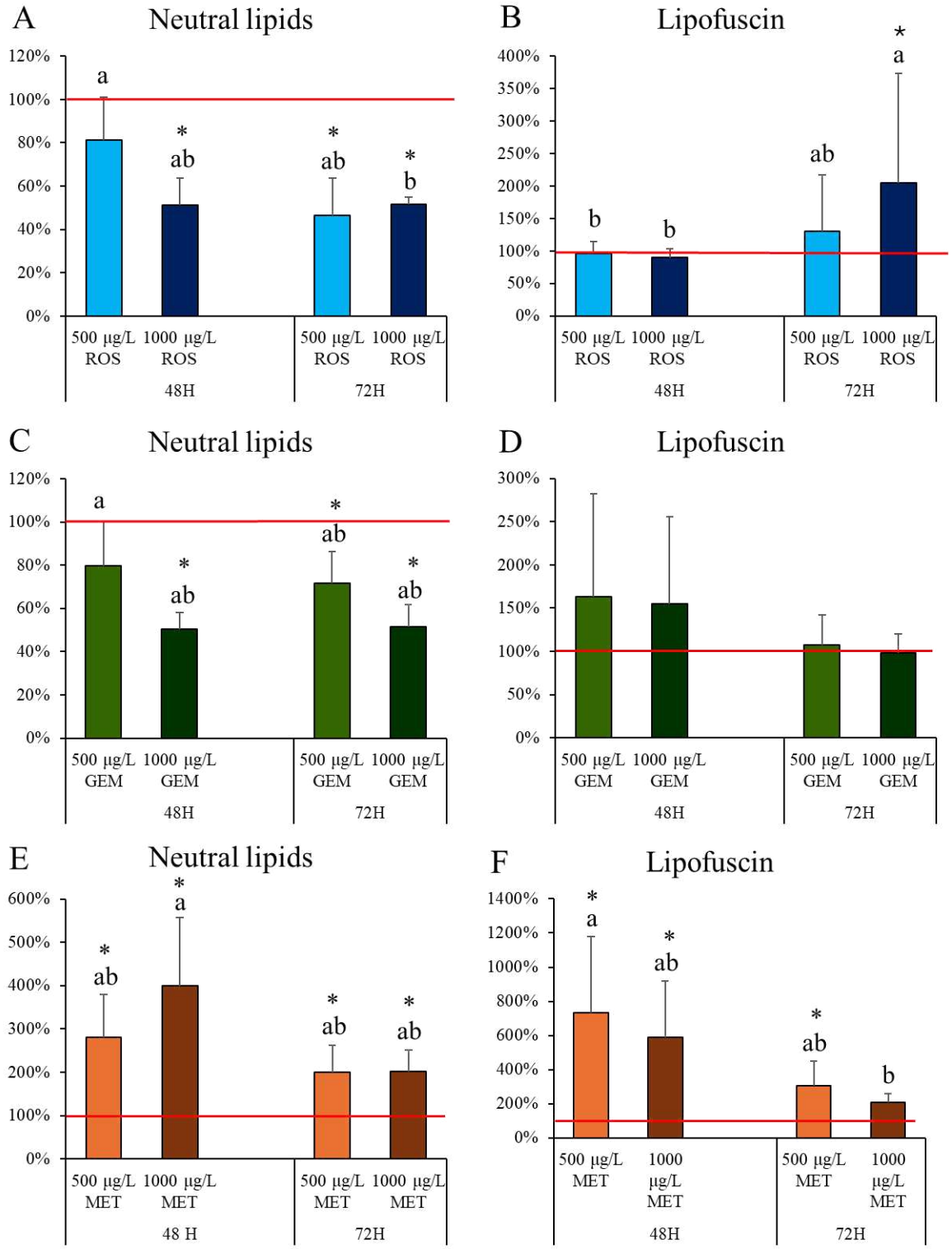

Figure 8. Neutral lipids and lipofuscin values in mussels' PCTS exposed to 500 and 1000 µg/L of ROSe (A, B), GEM (C, D), MET (E, F) for 48 and 72 h. Data are expressed as percentage compared to relative controls. * represents statistical significance compared to control PCTS; while letters indicate differences between groups. ROS, rosiglitazone (blue); GEM, gemfibrozil (green); MET, metformin (orange).

Results of the antioxidant enzyme CAT are showed in Figure 9. Once more, results are expressed as percentage variations compared to relative control organisms. Increased CAT induction was highlighted in PCTS treated with both concentrations of ROS, with statistically significant variations only for the higher dose (Fig. 9A).

Conversely, exposure to GEM caused a statistically significant inhibition of CAT activity at both tested concentrations (Fig. 9B). A lack of effects was observed for both 500 and 100 µg/L of MET (Fig. 9C).

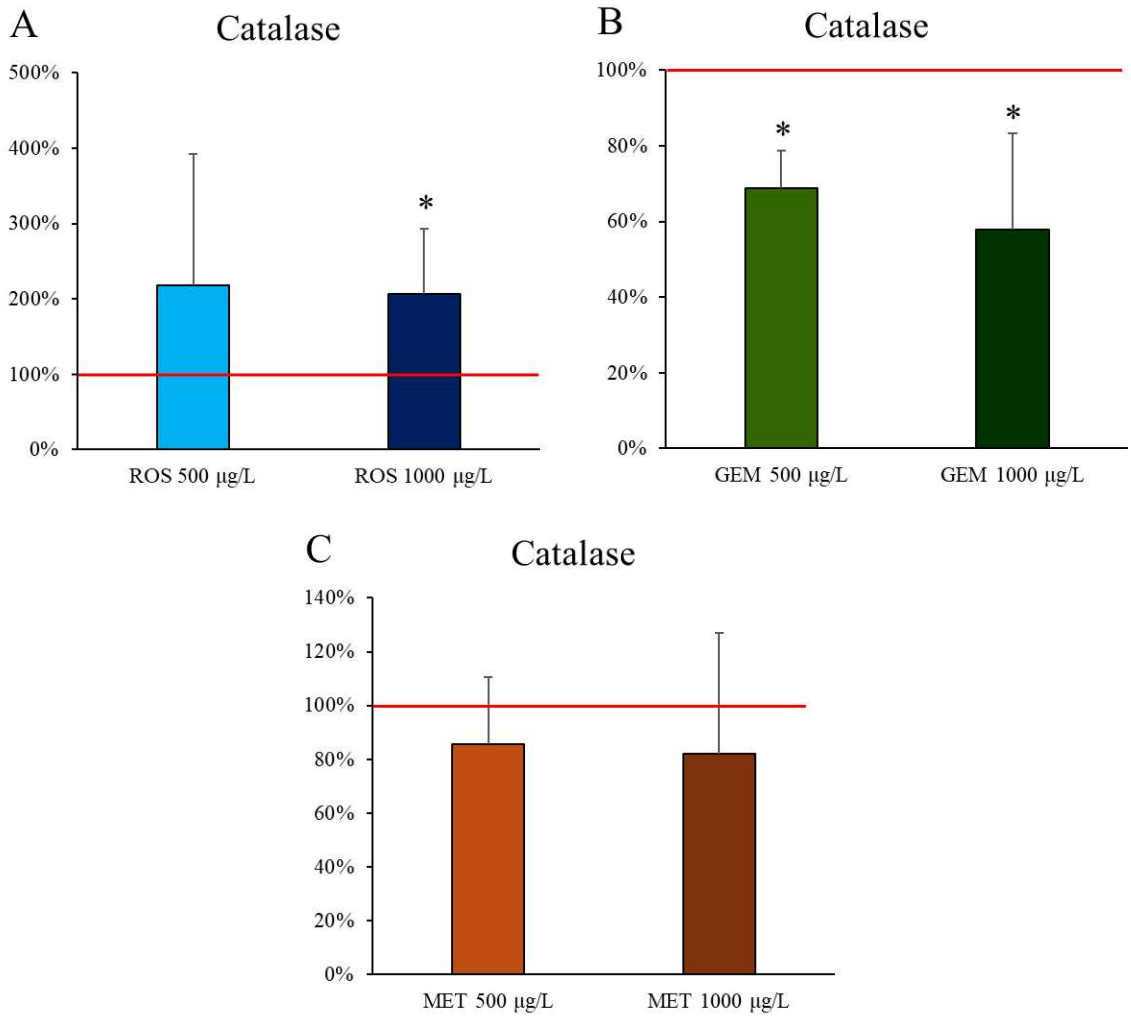

Figure 9. Catalase activity in mussels' PCTS exposed to 500 and 1000 µg/L of ROS (A), GEM (B), MET (C) for 48 h. Data are expressed as percentage compared to relative controls. * represents statistical significance compared to control PCTS; while letters indicate differences between groups. ROS, rosiglitazone (blue); GEM, gemfibrozil (green); MET, metformin (orange).

6 Discussion

The extensive use of pharmaceuticals in recent years has raised significant environmental concerns due to their persistence in wastewater and their consequent accumulation in aquatic ecosystems. These substances, designed to be biologically active at very low concentrations, can interfere with biochemical and physiological processes of marine organisms, posing potential risks on non-target species (Mezzelani et al., 2018). Although extensive research in recent decades has aimed at understanding the effects of pharmaceuticals on non-target organisms, a significant knowledge gap remains regarding the impact of certain therapeutic classes, particularly antidiabetic drugs and lipid-lowering agents. In this contest, this thesis aimed to investigate the ecotoxicological effects of three widely prescribed and consumed pharmaceuticals, the antidiabetics rosiglitazone (ROS) and metformin (MET) and the lipid-regulating agent gemfibrozil (GEM), using the Mediterranean mussel *Mytilus galloprovincialis* as a model organism. *In vivo* and *ex vivo* studies were combined to provide a more complete overview on the mechanisms of action and the main metabolic pathways modulated by these drugs. The *in vivo* experiment involved chronic exposure of mussels to environmentally relevant concentrations of selected pharmaceuticals to assess their sub-lethal effects in real coastal contamination scenarios. While, in the *ex vivo* study, the innovative approach of Precision Cut Tissue Slices (PCTS) of mussels digestive gland was employed to deepen knowledge on potential hypolipidemic effects of tested drugs, measuring endpoints related to lipid metabolism and oxidative homeostasis following high-dose exposures.

In *in vivo* exposure a wide panel of molecular, biochemical, and cellular biomarkers were investigated, including responses related to immune system, genotoxic and neurotoxic effects, the antioxidant pathway, and changes in lipid metabolism and accumulation of lipid peroxidation products.

The immune system was identified as one of the main pathways modulated by all the tested drugs, as reported for other therapeutic classes, including nonsteroidal anti-inflammatory drugs (NSAIDs), selective serotonin reuptake inhibitors (SSRIs), and antiepileptics (Afsa et al., 2022; Mezzelani, Gorbi, & Regoli, 2018b; Mezzelani & Regoli, 2022). Lysosomal membrane stability (LMS) in mussels' haemocytes is a well-established indicator of cellular stress caused by chemical pollution. It is commonly evaluated using the neutral red retention

time (NRRT) assay (Pantea et al., 2023) which is based on principle that lysosomes in healthy cells retain the neutral red dye for a longer duration compared to compromised cells (Aguirre-Martínez et al., 2013). All tested organism highlighted a significant decrease in LMS, indicating that all selected pharmaceuticals caused lysosomal destabilization. In line with these results, the study by Canesi et al. (2007) demonstrated that exposure of *Mytilus spp.* to increasing concentrations of GEM (2.50, 25.03, and 250.35 ng/g *d.w.* for 24 hours) resulted in a reduction of LMS, with a clear concentration-dependent lysosomal destabilization. Similarly, other research examining the biological effects of MET (100 ng/L, 80 µg/L, and 150 µg/L; 7 and 21 days) demonstrated that exposure to higher concentrations drastically reduced the retention time of haemocytes (Koagouw et al., 2024). Although no specific information is available on the effects of ROS on lysosomal stability, the observed decrease in LMS in exposed organisms suggest its potential immunotoxic effects. Haemocytes are also responsible of cell-mediated immune mechanisms, such as phagocytosis, the most important cell-mediated immune response process in mussels, by which phagocytic non-self-components are internally degraded through the release of lysosomal enzymes and reactive oxygen species (Hinzmann, 2016; Mezzelani et al., 2021). Haemocytes involved in phagocytosis are classified into granulocytes, which have abundant granules containing hydrolytic enzymes, and hyalinocytes, with few or no granules in their cytoplasm. Although both cell types are capable of phagocytosis, it has been hypothesized that granulocytes, having a greater number of granules, have higher phagocytic activity than hyalinocytes. In contrast, hyalinocytes appear to have a more important role in haemocyte aggregation processes (Prado-Alvarez et al., 2012). Results revealed that all tested drugs caused an increase of granulocyte/hyalinocyte (G/H) ratio, while only MET statistically reduced the haemocytes phagocytosis capacity. Although an increase in the G/H ratio was observed in MET treatment, the phagocytosis capacity, mainly attributed to granulocytes, decreased, suggesting a reduction in the ability of granulocytes to engulf particles.

The neurotoxic effects of tested pharmaceuticals were assessed by evaluating the alterations in the activity of the enzyme Acetylcholinesterase (AChE) in both haemolymph and gills tissues. AChE is crucial for the deactivation of acetylcholine at nerve endings, preventing continuous nerve signalling. This process is essential for the normal functioning of sensory and neuromuscular systems (Van Der Oost et al., 2003). The results showed a statistically significant induction in AChE activity in gills for all tested compounds, while in

haemolymph, only ROS and GEM caused such induction. Although limited information is available in the literature regarding the neurotoxic effects of antidiabetic and lipid-regulating drugs in aquatic non-target species, a slight induction of AChE was also reported in the PLHC-1 cell line treated with 100 μM of GEM (Zurita et al., 2007), suggesting similar effects of this drug in both vertebrate and invertebrate species. The genotoxic potential of pharmaceuticals was evaluated by analysing micronuclei frequency (MN), which exhibited a statistically significant increase only in organisms exposed to MET. MN are small, intracytoplasmatic masses composed of chromatin, resulting from chromosomal breakage, or errors in chromosome segregation during cell division (Nigro et al., 2006). Based on the baseline MN frequency of approximately 1‰ in bivalve haemocytes (Binelli et al., 2008), it's possible that the frequency of 1.5‰ observed after MET exposure remains within physiological ranges. On the other hand, DNA damage in *Labeo rohita* was measured after 28 days of exposure to 80 μg/L of MET, suggesting a potential genotoxic effect of this drug at higher exposure doses (Sibiya et al., 2023).

This study revealed the capability of all tested drugs to influence oxidative status of mussels. In particular, results of *in vivo* experiment showed a significant induction of catalase (CAT) activity in all treatments, while a modulation of CAT was observed only in PCTS exposed to GEM and ROS. CAT catalyses the reduction of hydrogen peroxide (H_2O_2) into H_2O and O_2, mitigating oxidative damage (Giuliani & Regoli, 2014). Similar findings have been reported in other species, for instance *Sparus aurata* exposed to increasing concentrations of GEM (1.5, 15, 150, 1500, 15000 μg/L) for 96h, exhibited a 50 and 93% increase in CAT activity in the gills at concentrations above 1.5 μg/L. Furthermore, CAT activity in the liver increased by 150% at the higher concentrations (15000 μg/L) (Barreto et al., 2018b). A notable increase in glutathione S-transferase (GST) activity was observed following MET treatment, suggesting a kind of metabolism, given the crucial role played by this phase II conjugation enzyme in biotransformation of xenobiotics in *M. galloprovincialis*. The role of phase II enzymes is to catalyse conjugation reactions that facilitate the excretion of chemicals by adding polar groups to the molecules (Van Der Oost et al., 2003). GST deactivates hydrophobic xenobiotic compounds, which can exert toxic effects at cytosolic and genetic levels. The deactivation occurs trough the conjugation of glutathione (GSH) with the substrate, forming a GS-hydrophobic conjugate that is excreted from the cells via an ATP-dependent membrane pump (Blanchette et al., 2007). Similarly, juveniles of Atlantic

salmon exposed to different concentrations of MET (5, 50, 500 µg/L) for 3, 7, 10 days showed increased GST activity, particular at day 3 and 10 at the highest concentrations of (500 µg/L) (Doujet, 2016).

Concerning the Glutathione peroxidase (GPx), results showed a statistically significant induction following MET treatment. GPx is an enzyme that catalyses the reduction of H_2O_2 to water, while oxidizing reduced glutathione (GSH) to its oxidized form (GSSG). This enzyme plays a crucial role in protecting cells membranes from damage due to lipid peroxidation (Van Der Oost et al., 2003). These findings are aligned with those of Doujet (2016), who reported a significant GPx induction on juveniles of Atlantic salmon treated with 500 µg/L of MET for 7 days, confirming the pro-oxidant effect of this pharmaceutical.

Total oxyradical scavenging capacity (TOSC) toward both radicals showed a statistically significant decrease in mussels exposed to GEM. The TOSC assay provides an index of biological resistance to oxidative stress. A reduced TOSC capacity indicates a compromised ability to neutralize oxidative radicals, which may anticipate the oxidative damages at cellular level (Gorbi et al., 2008). This can lead to a higher incidence of lysosomal alterations (Gorbi et al., 2008), including an increased accumulation of lipid peroxidation products, as indicated by the higher levels of lipofuscin and malondialdehyde (MDA) measured in mussels exposed to GEM.

It is interesting that the antidiabetic MET seem to have a dose-dependent effect in antioxidant system, with low doses (2.5 µg/L, *in vivo* study) modulating catalytic responses through the induction of antioxidant enzymes such as CAT and GPx, while higher doses (500–1000 µg/L, *in vitro* study) causing cellular alterations, including a marked increase in lipofuscin accumulation.

Results obtained from *ex vivo* exposure showed an increase of neutral lipids (NL) level in PCTS treated with MET. NL accumulation in digestive tubules represents an energy reserve for the cells and is considered as marker of altered metabolism (Bocchetti & Regoli, 2006). These findings seem to contradict the hypolipidemic effects previously reported for this pharmaceutical in the target species (Fryer & Carling, 2005). Given the marked enhance of lipofuscin levels, a well-known marker of oxidative stress, it can be hypothesized that the measured increase in NL is more likely associated with a significant alteration of oxidative pathway, rather than a direct effect on lipid synthesis.

In support of this, the results of *in vivo* study reported a significant enhance in NL content

following GEM and ROS treatments, both of which also exhibited marked pro-oxidant effects at the catalytic level (induction of antioxidant enzymes) and the cellular level (increase in lipofuscin and MDA). Furthermore, MET exposure in the same experiment did not cause significant changes in lipofuscin content, indicating a weaker pro-oxidant effect in comparison to GEM and ROS, and consequently, no increase in neutral lipid content was observed. Otherwise, the hypolipidemic effects of GEM and ROS was confirmed also in non-target organisms: mussels' PCTS exposed to these pharmaceuticals exhibited no variations in lipofuscin levels but showed a decrease in neutral lipid content.

7 Conclusions

This thesis evaluated the biological effects of three pharmaceuticals from to two therapeutic classes, the lipid-regulating agent GEM, and the antidiabetics MET and ROS, using the mediterranean mussel (*Mytilus galloprovincialis*) as a model organism. The overall findings demonstrated that the tested drugs modulated various biological pathways, including immune system, lipid metabolism, and oxidative homeostasis.

The exposure to GEM and ROS showed overall similar responses: the *ex vivo* approach confirmed the hypolipidemic effects of these pharmaceuticals also in non-target species at high exposure doses (500–1000 μg/L), while *in vivo* tests suggest that at lower concentrations (2.5 μg/L) the pronounced oxidative imbalance could be responsible for the increased neutral lipid content. Similarly, the high levels of neutral lipids measured under *ex vivo* conditions (500–1000 μg/L) following MET exposure appear to be associated with marked pro-oxidant effects rather than actual lipid synthesis, revealing a different mechanism of action for this pharmaceutical compared to those known in target organisms. However, further investigations would be necessary to validate this hypothesis.

In conclusion, results obtained integrating *in vivo* and *ex vivo* approaches allowed to enhance our knowledge of the main metabolic pathways affected by these drugs in non-target organisms, such as *Mytilus spp*. The PCTS technique provides tissue samples that retain the original three-dimensional architecture of the selected organ, allowing the study of complex pathways in a context similar to that of the whole organism. Although in the present thesis only a limited number of parameters were analyzed using this technique, the obtained results clearly demonstrate its potential application in the field of ecotoxicology. Overall, PCTS proved to be a valuable tool for investigating the mechanisms of action of pharmaceuticals and assessing their environmental risks, while also offering an experimental system that reduces the animal use in line with the 3R principles.

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