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Effetti biologici dei composti farmaceutici in *Mytilus galloprovincialis*: modulazione del sistema immunitario e del metabolismo ossidativo

Evidence of biological effects of human pharmaceuticals and their mixtures in *Mytilus galloprovincialis*: modulation of immune system and oxidative metabolism

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Italian summary

Effetti biologici dei composti farmaceutici in *Mytilus galloprovincialis*: modulazione del sistema immunitario e del metabolismo ossidativo.

L'attenzione verso i contaminanti emergenti si è intensificata negli ultimi decenni. Il termine "contaminante emergente" è stato coniato per la prima volta dalla Agenzia di Protezione Ambientale degli Stati Uniti (US-EPA) nella metà degli anni Novanta, per identificare quelle sostanze che ancora non erano state regolamentate e che presentavano un alto potenziale ecotossicologico. Attualmente questa categoria è rappresentata da molecole eterogenee che hanno utilizzi e caratteristiche chimico-fisiche molto diverse, tra cui troviamo prodotti per la cura personale (PPCPs), pesticidi, profumi, ormoni, ritardanti di fiamma e farmaci.

Oggigiorno l'interesse verso questi ultimi è notevolmente aumentato a causa della loro presenza ubiquitaria negli ecosistemi acquatici e la poca conoscenza circa gli effetti che potrebbero avere a lungo termine sugli organismi non-target.

La scarsa capacità di rimozione da parte degli impianti di trattamento delle acque reflue insieme all'elevato consumo in medicina umana e veterinaria, hanno portato all'ampia diffusione dei farmaci in ambiente, dove sono state misurate concentrazioni nelle acque e nei sedimenti nell'ordine di ng/L-µg/L e ng/kg rispettivamente. Considerato che i farmaci sono molecole sintetizzate per essere bio-attive a dosi molto basse, tali livelli potrebbero essere sufficienti per andare ad interferire con i meccanismi fisiologici e biologici delle specie marine.

A livello normativo, grazie alle evidenze scientifiche raccolte negli ultimi anni sugli effetti e la distribuzione in ambiente di questi composti, sono stati fatti dei progressi. Il numero di farmaci presenti nella "Watch List" della Commissione Europea è infatti aumentato da quattro a tredici in sette anni. Tale lista indica quei contaminanti emergenti per cui è necessario un attento monitoraggio al fine di raccogliere maggiori informazioni sul potenziale rischio ambientale e, in base a ciò, applicare un'adeguata regolamentazione. Ad oggi, però, le legislazioni nazionali e internazionali, non prevedono ancora dei limiti di concentrazione per queste molecole nelle diverse matrici ambientali.

Tra le classi terapeutiche più rilevanti in ambiente marino troviamo gli antidepressivi, i farmaci cardiovascolari come i regolatori lipidici e gli antipertensivi, e gli antidiabetici.

Tra gli antidepressivi, gli inibitori della ricaptazione della serotonina e noradrenalina (SNRI) vengono comunemente utilizzati per il trattamento di diversi disturbi psichiatrici, come depressione, ansia, attacchi di panico e fobia sociale. La venlafaxina (VEN) è il principio attivo più utilizzato all'interno di questa classe terapeutica. Il suo meccanismo di azione prevede l'inibizione dei trasportatori responsabili per la ricaptazione della serotonina e, di conseguenza, un aumento della concertazione di questo neurotrasmettitore nelle sinapsi. Diversi studi hanno dimostrato la capacità

della venlafaxina di essere bioaccumulata e di modulare i processi neurofisiologici come riproduzione, locomozione e alimentazione di organismi marini non-target.

Il gemfibrozil (GEM), un regolatore lipidico appartenente alla classe dei fibrati, viene utilizzato per ridurre i livelli di trigliceridi e lipoproteine a bassa densità (LDL) nel sangue. GEM agisce aumentando l'assorbimento degli acidi grassi, che vengono poi convertiti in derivati acil-CoA e catabolizzati attraverso reazioni di beta-ossidazione. Anche per questo farmaco è stato ampiamente dimostrato il bioaccumulo, la capacità di modulare il sistema antiossidante, nonché di avere effetti citotossici in organismi marini vertebrati ed invertebrati.

L'antipertensivo ramipril (RAM) viene comunemente utilizzato per il trattamento della pressione alta, per le sue proprietà di promuovere la vasodilatazione. Tali proprietà derivano dalla capacità di questa molecola di inibire l'enzima che converte l'angiotensina I in angiotensina II, bloccando così l'interazione tra l'angiotensina II e il suo recettore (AT1R). Sebbene ci siano ancora poche informazioni in letteratura, alcuni studi hanno evidenziato la capacità di questo composto di interferire con il sistema immunitario ed antiossidante degli invertebrati marini.

Un'altra classe terapeutica ampiamente rilevata negli ecosistemi acquatici sono gli antidiabetici. Tra questi, la metformina, che viene utilizzata per la cura del diabete di tipo II, agisce inibendo il complesso I della catena di trasporto degli elettroni mitocondriale, con conseguente riduzione nella produzione di glucosio. Diversi studi hanno mostrato la potenzialità di questo farmaco di agire come distruttore endocrino nei vertebrati marini, interferendo con il sistema riproduttivo con effetti nella crescita, nel metabolismo, nell'alimentazione e nella locomozione delle specie non-target.

Nell'ambiente naturale gli organismi sono generalmente esposti a miscele complesse di composti chimici, inclusi i farmaci e molti altri tipi di inquinanti. Per questo motivo, oltre a studiare il meccanismo d'azione e, dunque, i potenziali effetti delle singole molecole nelle specie non-target, è estremamente importante comprendere le possibili interazioni additive, sinergiche o antagoniste che possono instaurarsi tra questi composti. Ad oggi gli studi che si occupano di indagare gli effetti delle miscele di farmaci negli organismi marini sono ancora pochi.

In questo contesto, l'obiettivo di questa tesi è quello di valutare il potenziale ecotossicologico dell'antidepressivo venlafaxina, del regolatore lipidico gemfibrozil, dell'antiipertensivo ramipril, dell'antidiabetico metformina e della loro miscela nell'organismo modello *Mytilus galloproincialis*. Il piano sperimentale ha previsto una fase di esposizione della durata di 30 giorni, durante la quale gli organismi sono stati esposti ad una concentrazione ambientalmente realistica di VEN (1µg/L), GEM (1µg/L), RAM (1µg/L), MET e VEN + GEM + RAM + MET (1µg/L per ciascun farmaco). Al termine della fase di esposizione i mitili sono stati lasciati per ulteriori 14 giorni in acqua priva di farmaci allo scopo di valutare la capacità di recupero dalle molecole testate.

Al termine dell'esperimento è stato applicato un approccio ecotossicologico che integra la misura di diverse risposte biologiche in grado di evidenziare l'insorgenza di alterazioni biochimiche e cellulari come la genotossicità e la modulazione del sistema immunitario ed ossidativo.

I risultati hanno evidenziato la capacità dei farmaci testati di modulare il sistema immunitario nei mitili. Nonostante sia stato già ampiamente documentato dalla letteratura per diverse classi terapeutiche, questi dati forniscono nuove informazioni sugli specifici meccanismi d'azione delle molecole testate. In particolare, il ramipril e la miscela hanno causato un'inibizione statisticamente significativa della stabilità delle membrane lisosomiali, effetto che si è mantenuto anche dopo il periodo di depurazione per entrambi i trattamenti. La venlafaxina ha causato una diminuzione della capacità di fagocitosi e del rapporto granulociti/ialinociti negli emociti, facendo ipotizzare un possibile effetto nella riduzione del numero di granulociti; ed infine per la miscela è stata documentata una diminuzione della capacità di fagocitosi suggerendo una possibile diminuzione dell'efficienza dei granulociti.

La genotossicità dei farmaci è stata testata analizzando la frequenza dei micronuclei nell'emolinfa dei mitili. I risultati ottenuti non hanno evidenziato nessuna variazione statisticamente significativa, confermando le informazioni già presenti in letteratura circa la non genotossicità dei composti testati.

L'aumento intracellulare di produzione di specie reattive dell'ossigeno, nonché gli squilibri tra forze pro-ossidanti e difese antiossidanti, sono state indagate integrando i risultati relativi alle attività dei singoli enzimi antiossidanti con la capacità antiossidante totale (TOSC) dei mitili di neutralizzare i radicali idrossilici (OH) e perossilici (ROO).

Le analisi dei singoli enzimi antiossidanti hanno mostrato una significativa induzione della catalasi, enzima coinvolto nella neutralizzazione del perossido di idrogeno (H_2O_2), a seguito dell'esposizione al gemfibrozil. Tali valori potrebbero essere giustificati dalla farmacocinetica di GEM, il quale, interagendo con il recettore attivato dal proliferatore del perossisoma α (PPAR α), va ad attivare i geni responsabili della β-ossidazione degli acidi grassi perossisomiali e a indurre la proliferazione dei perossisomi, con conseguente produzione di H_2O_2 che si traduce, dunque, in un'induzione della catalasi. Risultati simili sono stati confermati anche dalla letteratura, dove diversi studi evidenziano la capacità di GEM di indurre l'attività della catalasi nei pesci come *Sparus aurata.*

È stato ampiamente riconosciuto dalla letteratura che i farmaci vengono metabolizzati dagli enzimi appartenenti alla famiglia del citocromo P450 (fase I). Tuttavia, non sempre i prodotti del metabolismo di fase I sono sufficientemente solubili per essere escreti, ed in tal caso intervengono gli enzimi di fase II. Tra questi la glutatione-S- transferasi (GST) svolge un ruolo cruciale coniugando il glutatione (GSH) ai composti potenzialmente tossici, al fine di renderli più solubili in acqua e, dunque, renderli più facilmente eliminabili dalle cellule. I risultati ottenuti mostrano un'induzione

della GST e una conseguente diminuzione dei livelli di GSH totale per tutti i trattamenti al giorno 30 (fase di esposizione), evidenziando il processo di biotrasformazione di fase II come il percorso principale per la detossificazione dei mitili dai farmaci testati. Non sempre questi risultati sono stati confermati dalla letteratura. Ad esempio, la GST sembra non essere stata indotta nei pesci *Sparus aurata* e *Oryzias latipe*s a seguito dell'esposizione a GEM e MET rispettivamente, suggerendo che altri meccanismi di detossificazione possono essere coinvolti nel metabolismo di questi due farmaci nei vertebrati marini.

I risultati della glutatione reduttasi (GR), enzima coinvolto nella conversione del glutatione ossidato (GSSG) nella forma ridotta (GSH), hanno mostrato un'inibizione statisticamente significativa nella miscela al giorno 30, probabilmente correlata all'interazione additiva di VEN e MET che hanno mostrato un'attività inferiore rispetto agli organismi di controllo. Simili valori sono stati misurati in letteratura dopo una somministrazione a concentrazioni ambientalmente realistiche di fluoxetina (SSRIs) nei policheti *Hediste diversicolor*, corroborando l'ipotesi che gli antidepressivi possano avere un ruolo nel determinare l'effetto complessivo all'interno della miscela sull'attività della GR.

Inoltre, è stato osservato che l'effetto di VEN sulla attività della GR persiste anche dopo la fase di depurazione (44 giorni), suggerendo un effetto a lungo termine di questo farmaco.

L'azione bifasica e ritardata della VEN è stata sottolineata anche dai risultati delle glutatione perossidasi (GPx CHP e GPx H_2O_2), degli enzimi coinvolti nella detossificazione degli idroperossidi organici cellulari. GPx CHP agisce coniugando il GSH ai perossidi lipidici, neutralizzandoli e prevenendo il danno cellulare. Mentre, GPx H_2O_2 prevede la reazione di H_2O_2 nella neutralizzazione degli idroperossidi, formando ossigeno. Una leggera ma non significativa induzione è stata registrata per entrambi gli enzimi a 30 e 44 giorni nei mitili esposti alla VEN.

In ultimo, i risultati riguardanti la capacità antiossidante totale (TOSC) nei confronti dei radicali idrossilici (OH) e perossilici (ROO) non hanno evidenziato variazioni statisticamente significative per nessun trattamento.

In conclusione, i risultati complessivi di questo lavoro indicano che nell'organismo *M. galloprovincialis* le principali pathway modulate dai farmaci testati sono quelle coinvolte nel sistema immunitario ed ossidativo. Inoltre, le informazioni ottenute hanno migliorato la nostra comprensione sulle principali vie metaboliche dei prodotti farmaceutici, indicando effetti dipendenti dai meccanismi d'azione delle diverse classi terapeutiche indagate. I nostri risultati hanno anche fornito una nuova visione della potenziale interazione tra i farmaci all'interno della miscela, suggerendo l'importanza di studiare gli effetti combinati di diverse classi terapeutiche al fine di acquisire una maggiore comprensione del loro impatto sugli organismi non-target.

1 Introduction

1.1 Pharmaceuticals as emerging contaminants: sources, distribution and occurrence in aquatic environments

The term "contaminants of emerging concern" (CECs) includes a wide group of molecules classified into multiple categories based on their functional and chemical properties. CECs have recently garnered significant attention from environmental toxicologists, government regulators, and the general public. The emergence of awareness regarding emerging contaminants can be attributed to Rachel Carson and her book "Silent Spring" (Sauvé & Desrosiers., 2014), in which she demonstrated the toxicological potential of DDT. The term "emerging contaminant" was first used by the US Environmental Protection Agency in the mid-1990s, identifying them as contaminants with no regulatory standards associated with them but with suspected high ecotoxicological potential. The US Geological Survey (USGS, 2016) defines CECs as: "Any synthetic or naturally occurring chemical or any microorganism that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects" (Poynton & Robinson., 2018). Nowadays, thousands of molecules have been measured in seawater belonging to different categories such as pharmaceuticals, personal care products (PPCPs), pesticides, fragrances and hormones (Dulio et al., 2018). Althought the majority of CECs consist of previously unused molecules, there are notable cases where certain compounds have been used for decades, and their presence in water, soil, and tissues has only recently been quantified using modern technologies. Pharmaceuticals are considered a class of emerging contaminants due to their increasing detection in aquatic ecosystems (Mezzelani et al., 2018); this is primarily attributed to their widespread use and the lack of effective controls and regulations for their disposal and treatment. Pharmaceuticals are extensively used worldwide in both human and veterinary domains, and their consumption is expected to grow: according to current projections, global medicine spending, which refers to the amount spent on purchasing medicines, is expected to increase and reach $1.9 trillion by 2027, growing at a rate of 3–6% per year (QuintilesIMS health, 2023). This increase is driven by the demand for medications to prevent and cure diseases and address age-related medical issues. Among all countries, China, the world's second-largest country for pharmaceutical spending, is projected to experience an 8% increase in volume over five years, while spending is expected to increase by 19% (QuintilesIMS health, 2023).

Globally, antidepressants, cardiovascular drugs and antidiabetics are among the most commonly used pharmaceuticals, and it was estimated that 264 million people suffered from depression (our world in data, 2017) with Europe stands as the largest consumer of antidepressants worldwide, according to the Organization for Economic Cooperation and Development, OECD (Castillo-Zacaris

et al., 2021). Regarding cardiovascular disease, they remain the major cause of death for the European Society of Cardiology, ESC (Timmis et al., 2020, Figure 1) and Europe has one of the highest death rates per 100,000 people due to cardiovascular diseases (CVDs), with 3.9 million deaths in 2017 (our world in data, 2017). Obesity and hypercholesterolemia, increasing in Europe in the last years, are the major causes which contribute to the onset of CVDs by affecting the concentration of low-density lipoprotein (LDL), leading to arterial obstruction. To manage CVDs, lipid-regulating agents, which help to control cholesterol, are commonly prescribed together with hypertensives, which act by decreasing the vasoconstriction substances, thereby improving blood flow and increasing the efficiency of the heart pump. In the United States, the use of beta blockers increased by 57% from 2001 to 2015 while in Brazil, for instance, enalapril (an antihypertensive drug) represented 2.45% of total generic medicines sold in 2013, and the fourth most commonly sold drug was atenolol, a beta blocker (Godoy et al., 2015). Relating to antidiabetics drugs, they are used for the treatment of diabetes type II, which is a significant health concern globally, and in 2019, it was the eighth leading cause of mortality, resulting in 1.55 million deaths (our world in data, 2019). The prevalence of diabetes type II is particularly high in America, with Mexico being heavily affected. While there are multiple drugs used for the treatment of diabetes type II, a large percentage of patients, specifically 73% among 280,000 patients under treatment from 2000 to 2017, use metformin. Sulfonylurea is used by 15% of patients, and the remaining 7% choose for other options (Wilkinson, 2018).

The occurrence of pharmaceuticals in the water is attributed to the inappropriate disposal of domestic or hospital drugs into sewage systems, the potential discharge by industries, aquaculture and animal farming practices and the incorrect functioning of wastewater treatment plants (WWTPs) against these molecules. In fact, many drugs have been found in the effluents of WWTPs because these treatment facilities are not specifically designed to remove micropollutants (Luo et al., 2014). The removal efficiency of pharmaceuticals in WWTPs can vary significantly depending on the specific properties of the pharmaceuticals. For instance, in the case of diclofenac (a Non-Steroidal Anti-Inflammatory Drugs, NSAIDs), the removal efficiency has been reported to range between 20% and 50% (Thiebault et al., 2017). WWTPs typically employ primary, secondary, and potentially tertiary or advanced treatment processes. Primary treatment involves physical procedures (flocculation, coagulation, sedimentation) that reduce suspended particles such as sand, fats, and oil. Secondary treatment, which involves biological processes such as activated sludge and trickling filters, removes pharmaceuticals through absorption, biodegradation, and biotransformation. Lastly, tertiary treatment employs processes such as chlorination, UV decontamination, ozonation, membrane filtration, and activated carbon to enhance the treatment process and potentially discharge higher-quality effluents. Unluckily the advanced treatment is rarely used for its high cost (Khasawneh & Palaniandy., 2021).

Usually, WWTPs are not effective for the removal of a wide variety of pharmaceuticals, some authors hypothesise microbial activities or hydrolysis in WWTPs could restore pharmaceuticals metabolites or their conjugates into the parent compound (Martinez-Alcalà et al., 2021), other studies hypothesise that compounds can be absorbed by faecal particles in sewage and released later. Lastly, some research suggest that could be a mismatch between influent and effluent flow, resulting in uncertain calculation of removal efficiency (Khasawneh and Palaniandy., 2021). For instance, for carbamazepine and atenolol, efficiency of removal has found to be less than 20% (Desbiolles et al., 2018). Nonetheless, the effectiveness of the treatment can be hindered by factors such as technical properties of pharmaceuticals. Despite the susceptibility of pharmaceuticals to degradation, their continuous input into the environment results in a pseudo-persistent behaviour: once released by WWTPs, pharmaceuticals can accumulate in various environmental matrices, including seawater, sediment, and biota (Fabbri & Franzellitti., 2016a). The concentrations in coastal areas range from a few ng/L up to hundreds of μg/L (Mezzelani & Regoli, 2022). Out of all the pharmaceuticals available, the presence of antidepressants in WWTPs has widely been reported, for example, in the Douro and Leça rivers of Portugal, common antidepressants such as fluoxetine, citalopram, and sertraline have been detected at concentrations of up to 2.0 ng/L. Additionally, in the UK, citalopram and fluoxetine have been found in drinking water at concentrations of approximately 2.26 ng/L and 0.27 ng/L, respectively (Castillo-Zacaris et al., 2021). Venlafaxine (VEN) is one of the most widely prescribed antidepressants worldwide, is commonly found in water samples, with concentrations ranging from ng/L to μg/L (Castillo-Zacaris et al., 2021), the higher concentrations are observed in the areas surrounding the Black Sea and the North Sea, as shown in Figure 2. The map was created using a Geographical Information System software (GIS) using data of four drugs usually prescribed in Europe (venlafaxine, gemfibrozil, ramipril, metformin) downloaded by the EMPODAT (Environmental monitoring data module) dataset from the Information Platform for chemical monitoring. The data for single pharmaceuticals and their metabolites are downloaded and successively uploaded on the software, in order to create complete description of the distribution of compounds. Among the pharmaceuticals detected in WWTPs that are utilized for the treatment of cardiovascular disease, lipid regulators are the most commonly found, with a detection rate of 70-85% (Debiolles et al., 2018) and their concentration in water samplers range from ng/L to μg/L (Hernando et al., 2005). In particular, gemfibrozil (GEM) is commonly detected in water at levels ranging from ng/L to μg/L (Korkmaz et al., 2022), with higher levels found in the rivers of northern Europe (Figure 2). Furthermore, for the antihypertensive drugs ramipril (RAM), an ACE inhibitor widely used worldwide, has been found to have higher concentrations in the Black Sea and rivers in Eastern Europe (Figure 2). Antidiabetic medications represent a commonly class of pharmaceuticals

frequently detected in water samples, among them, metformin stands out as the most widely used antidiabetic drug globally, estimates suggest that over 100 million patients are treated with this drug annually (Godoy et al., 2018), its presence has been documented across Europe, with significant records in Eastern Europe and the Black Sea region. Interestingly, the human body does not biotransform metformin (MET), meaning that it is excreted from the body in its natural form (Ambrosio-Albuquerque et al., 2021). This, coupled with its widespread use, contributes to the high concentrations found in the effluents of WWTPs, often exceeding the concentrations of other pharmaceuticals by approximately two orders of magnitude (Oosterhuis et al., 2013). It is noteworthy that sediment serves as a reservoir for contaminants, which can be released during multiple events including storms, tides, and dredging activities. Sorption of pharmaceuticals depends on their chemical properties, the more hydrophilic ones occur in surface water and the more lipophilic ones in sediment or biota (Mezzelani et al., 2018). Usually, the lipophilicity of a chemical is evaluated using the Octanol/Water partition coefficient (log Kow). This coefficient compares the concentration of the chemical in n-octanol (a surrogate for lipids) to the concentration of the chemical in water, expressing the tendency of an organic compound to partition into lipids (Kümmerer, 2008). Organic compounds are categorized based on their log Kow values: compounds with log Kow < 2.5 are the more hydrophilic ones, those with values between 2.5 and 4, and those with log Kow > 4, the more lipophilic ones (Korkmaz et al., 2022). Furthermore, the dissociation constant (pKa) has a minor influence on the sorption of organic compounds. Pharmaceuticals with low pKa (acidic tendency) and high log Kow usually show affinity for sediment (Hernando et al., 2006). Among all the different classes of drugs, antidepressants usually have a log Kow > 3, although venlafaxine is the only exception with a log Kow of 2.8 (Minguez et al., 2014). It is noteworthy that antidepressants have been found in aquatic species. In 40% of wild mussels, lormetazepam and paroxetine have been detected, and 64% of bivalves collected in California bioaccumulate sertraline (Mezzelani and Regoli, 2022). Cardiovascular drugs exhibit different values depending on their categories. Ramipril, for instance, has a low log Kow value of 0.70, indicating a higher probability of being found in the water column (Tong et al., 2015). On the other hand, lipid regulators generally have higher values. Gemfibrozil, one of the most commonly used lipid regulators, has a log Kow value of 4.39, suggesting its tendency to accumulate in sediment (Capolupo et al., 2018). Among the antidiabetic drugs, metformin has a low log Kow value of -4.90, indicating its tendency to be found in the water column (Briones et al., 2016). Pharmaceuticals pose a potential risk due to their ability to bioaccumulate in marine organisms, crossing biological membranes and causing adverse effects on in crucial homeostatic mechanisms. Bioaccumulation refers to the process by which the concentration of a chemical in a living organism exceeds its concentration in surrounding environment (Puckowski et

al., 2016). It is evaluated according to the criteria of OECD, for which the potential bioaccumulation of chemicals is provided by the evaluation of the Octanol/Water partition coefficient discussed before. A log (Kow) value greater than 3 indicates the propensity of substances to accumulate in the lipid-rich tissues of organisms (Puckowski et al., 2016). The bioaccumulation rate is influenced by the bioavailability of the chemical in the aquatic system, the physicochemical nature of pharmaceuticals, biotic factors relating to the exposure of aquatic organisms, and the pH and temperature of seawater. Analysing previous data, is evident that there is a relatively low number of publications discussing the bioaccumulation of pharmaceuticals in animals compared to the extensive research on analysis in water matrices, this because of the easer analyses procedures, in addition to the fact that the amount of marine biota mass collected in sampling activity is typically low. Additionally, pharmaceuticals in marine organisms can undergo metabolism and exist in forms different from those found in humans and this makes the analysis tougher to conduct (Świacka et al., 2022). The accumulation of molecules among marine organisms has been established in various research, in which the bivalves have the highest rates of bioaccumulation due to their filter-feeding behaviour. Among all pharmaceuticals, NSAIDs have been found to be the class that bioaccumulates the most, followed by psychoactive drugs (Świacka et al., 2022). The primary classes identified in marine environments include antibiotics, steroid hormones, NSAIDs, antidepressants, lipid-regulating agents, cardiovascular drugs, and anti-diabetic drugs (Mezzelani and Regoli., 2022). According to previous investigations, hotspots of pharmaceutical occurrence have been identified in coastal areas subjected to anthropogenic pressure (Fabbri & Franzellitti, 2016a). In this scenario exposure to pharmaceuticals can become an additional stressor in an environment where multiple factors occur, such as climate change related stressors such as acidification and heatwaves, due to the capability of these factors to influence the chemical properties of most molecules (Fabbri & Franzellitti, 2016b). Furthermore, it is worth mentioning that in the environment, organisms are exposed to complex mixtures of compounds that can interact with each other, often in ways that differ from their intended human uses (Mezzelani and Regoli, 2022). To enhance our understanding, it is mandatory to integrate studies on released loads with the evaluation of the bioavailability, uptake, and biological effects of pharmaceuticals in marine organisms. This comprehensive approach will provide valuable insights into the potential risks and impacts of contamination in marine ecosystems.

1.2 Ecotoxicological potential of pharmaceuticals

In order to properly address the adverse outcomes of pharmaceuticals in aquatic systems, information on bioaccumulation should be integrated with the analysis of specific biological indicators or measurable parameters that provide information about the biological effects of pollutants, known as biomarkers. They can be physiological, molecular, or genetic responses to the exposure contaminants. Among the various procedures used in ecotoxicological studies, acute toxicity tests are an essential component, they involve exposing organisms to varying concentrations of pollutants for a short period of time to evaluate their toxic effects. These tests measure multiple endpoints such as lethality, growth rates, and the embryotoxicity.

One of the most widely consumed pharmaceuticals therapeutic classes in the world are the antidepressants. They have the ability to modulate the central nervous system and they are classified based on their mechanism of action in serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs). Among the SNRIs, venlafaxine is commonly prescribed worldwide (Figure 2) for the treatment of conditions such as agitation, depression, insomnia, borderline syndrome, hypertension, and weight loss (Gutierrez et al., 2003), and it has been classified as an environmental pollutant since 2008 when it was identified as one of the major compounds detected in various water sources (Zhu et al., 2022). VEN is rapidly absorbed through oral administration, especially when taken with food. Upon administration, VEN undergoes extensive first-pass metabolism, resulting in the formation of a major metabolite, which is O-desmethylvenlafaxine (O-VEN) and two minor metabolites, which are N,O-didesmethylvenlafaxine and N-desmethylvenlafaxine. The major forms detected in WWTPs are VEN and O-VEN (Mehdi et al., 2019). The formation of these metabolites is primarily mediated by the cytochrome P450 enzymes, which are a family of enzymes located in the smooth endoplasmic reticulum of hepatocytes and other cells. They play a crucial role in various transformations, including demethylation and oxidation, to render toxic compound more water-soluble and facilitate their excretion. Venlafaxine acts by increasing the concentration of extracellular serotonin (5-hydroxytryptamine), which is a small molecule that serves as a neurotransmitter in the central nervous system. Serotonin is synthesized from tryptophan and stored in synaptic vesicles to prevent its degradation, and under normal conditions, these vesicles fuse with the presynaptic membrane and release serotonin into the extracellular space, where it binds to 5HT receptors, triggering signalling processes (Nichols and Nichols, 2008). The excess serotonin can be degraded by monoamine oxidase or be reuptaken by the serotonin transporter. In this pathway, SNRIs act as inhibitors of the serotonin transporter, leading to an accumulation of serotonin in the synaptic cleft and enhancing its binding to receptors (Figure 3).

The consequentially permanence of serotonin in extracellular space cause an activation of 5HT1 and 5HT5 receptors which inhibits adenylate cyclase, resulting in a decrease in cyclic adenosine monophosphate (cAMP) and cAMP-dependent enzymes. On the other hand, 5HT3, 5HT6, and 5HT7 receptors have an opposite role, increasing cAMP levels and activating downstream signalling pathways, including the mitogen-activated protein kinase (MAPK) pathway, extracellular signal-regulated kinase 1 and 2 (ERK1/2), and the cAMP response element-binding protein (CREB) cascade, which induces the expression of brain-derived neurotrophic factor (BDNF), which is a neurotrophins involved in neurogenesis (the generation of new neurons), synaptic plasticity and neuroimmune regulation (Mezzelani and Regoli, 2022). At higher doses (>150 mg/day), venlafaxine also inhibits the reuptake of norepinephrine, resulting in increased extracellular norepinephrine levels. Furthermore, it has a minimal role in the inactivation of dopamine reuptake, possibly due to partial absorption of dopamine by norepinephrine transporters. At least, venlafaxine has a low potential for anticholinergic (inhibition of parasympathetic nervous system) and orthostatic hypotensive effects (alteration of blood pressure), and it is unlikely to cause sedation or weight gain due to its lack of affinity for muscarinic, alpha1-adrenergic, and histaminergic receptors (Gutierrez et al., 2003). Numerous studies have examined the biological effects of antidepressant on non-target species and some of them demonstrated that fluoxetine (SSRIs) has genotoxic and potential immunotoxic effects on marine invertebrates, causing mortality in worms (*Schmidtea mediterranea*) (Wang et al., 2021). Additionally, fluoxetine has a negative impact on phytoplankton, affecting as a consequence the primary productivity. Similarly, other antidepressants like sertraline have been shown to induce physiological damage in marine vertebrates, resulting in reduced respiration and impaired motor coordination in rainbow trout (*Oncorhynchus mykiss*) and acute toxicity tests have indicated that the lethal concentration for 50% of *Oncorhynchus mykiss* (LC50 96h) is 0.38 mg/L (Wang et al., 2021). Although venlafaxine has been primarily studied for its effects on human health, recent research has highlighted its impact on the serotonergic system in marine organisms, including molluscs and fish. In mussels, serotonin plays a crucial role in various neurophysiological processes, such as the sensitization and facilitation of withdrawal reflexes, feeding behaviour, locomotion, and reproduction (Fabbri and Franzellitti, 2016). Studies have shown that venlafaxine can bioaccumulate in the liver of marine vertebrates (Maulvault et al., 2019; Lin et al., 2022), enhancing antioxidant mechanisms in fish (*Argyrosomus regius*), leading to modulation of several antioxidant enzymes, including catalase (which catalyse the degradation of peroxide of hydrogen in water), glutathione S-trasferase (which catalyse the conjugation of glutathione to toxic molecules) and superoxide dismutase (which dismutase the superoxide anion, dangerous for all the cells, in oxygen and peroxide of hydrogen). Furthermore, in marine vertebrates, exposure to VEN has been found to cause inhibition of the

expression of the DNMT1 gene, which codify for the DNA methyltransferases involved in DNA methylation, for about 37% in zebrafish (*Danio rerio*) after 30 days of exposure to concentration between 0.1 and 100 µg/L (Lin et al., 2022).

Other commonly detected compounds are cardiovascular drugs. Among them, lipid-regulating agents are commonly prescribed for the treatment of CVDs. These drugs are able to decrease serum triglyceride and very low-density lipoprotein levels, while increasing high-density lipoprotein levels. GEM, a frequent drug approved by the Food and Drug Administration in 1976, is a widely prescribed lipid-regulating agent usually detected in WWTPs. GEM, a fibric acid derivative, is absorbed by the gastrointestinal tract and metabolized in the liver by cytochrome P450, with approximately 70% excreted in the urine, mostly as a glucuronide conjugate (Zurita et al., 2007). This medication is prescribed to patients at high risk of coronary heart disease to decrease cholesterol concentration (Schmidt et al., 2014). Fibrates, including gemfibrozil, generally work in the liver by increasing the uptake of fatty acids, which are then converted to acyl-CoA derivatives and catabolized through beta-oxidation, the degradation pathway for fatty acids (Figure 4). During this process, acyl-CoA oxidase, an enzyme that oxidizes very long-chain fatty acids, is activated, leading to the release of peroxide of hydrogen (H_2O_2), resulting in modulation of cell oxidative homeostasis and formation of hydroxyl radicals (Gonzalez, 1998). In response to oxidative stress, cells activate various antioxidant enzymes to maintain redox homeostasis. These enzymes include catalase (CAT), which is present in peroxisomes, as well as cytosolic enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST). Additionally, cells can activate non-enzymatic antioxidants such as glutathione (GSH), which is oxidized to glutathione disulfide (GSSG). These antioxidant defences systems play a crucial role in preventing damage such as lipid peroxidation, DNA damage, and protein degradation. Gemfibrozil is classified as a peroxisome proliferator because it induces the activation of the nuclear peroxisomal proliferator-activated receptor, which plays a role in enhancing lipid catabolism and increasing the size and number of peroxisomes (Mimeault et al., 2006). Previous research on acute toxicity test on non-target species investigating the effects of lipid regulators bezafibrate and fenofibric acid, highlighted instances of damage in various organisms at concentration higher than those typically found in marine environment. For example, the EC50 values for bezafibrate were 172.73 mg/L in *V. fisheri,* 7.62 mg/L in *Anabaena*, and 240.4 mg/L in *Daphna magna*, while values for fenofibric acid were determined to be 1.72 mg/L in *V. fisheri*, 10.85 mg/L in *Anabaena*, and 4.90 mg/L in *Daphna magna* (Rosal et al., 2010). In studies on marine invertebrates, clofibrate at a concentration of 0.2 µg/L caused a significant reduction in triglycerides and an increase in fatty acids in zebra mussels (Mezzelani and Regoli, 2022). In particular, the impact of gemfibrozil has also been extensively evaluated. Among the several studies on invertebrates, particularly on

mussels, an investigation focused on *Dreissena polymorpha* has shown modulation of antioxidant pathway. Exposure to high and not environmental real concentration of gemfibrozil (1000 μg/L) for 24 hours resulted in a significant induction in glutathione s-transferase and, simultaneously, an increase of DNA damage and lipid peroxidation was observed (Quinn et al., 2011). Concerning marine vertebrates, in experiments involving *Sparus aurata*, exposure to varying concentrations of gemfibrozil ranging from 1.5 to 15 000 μg/L, has been found to result in a decreased ability of the fish to swim against water flow (between 50 and 65%), indicating potential issues in neurotransmission. This decrease of swimming performance has potential dangerous environmental consequences, leading to a reduction in species' fitness. In the same study a modulation of antioxidant pathways was observed, with induction in catalase, glutathione peroxidase, and glutathione reductase in the liver of fish *Sparus autata* exposed to GEM (Barreto, 2018). Furthermore, a loss of DNA integrity was assessed in zebrafish (*Danio rerio*) exposed to environmentally relevant concentrations ranging from 13 to 380 ng/L for 7 days (Mezzelani and Regoli, 2022).

Antihypertensives are another class of drugs used for the treatment of CVDs. These pharmaceuticals act by inhibiting vasoconstriction substances, promoting vasodilation, resulting in enhanced blood circulation and improved cardiac function. They belong to different categories, including beta blockers, angiotensin II converter enzyme (ACE) inhibitors, calcium antagonists, and angiotensin II receptor blockers (ARBs) and, despite the different mechanisms of action, they all treat hypertension. Antihypertensives are commonly detected in WWTPs worldwide, and their ecotoxicological effects have been evaluated several times. Ramipril, a widely used ACE inhibitor worldwide, inhibits the enzyme that converts Angiotensin I to Angiotensin II (renin) resulting in a final decreasing of angiotensin II (Regoli and Mezzelani, 2022). Blocking the production of Angiotensin II has downstream effects through both the angiotensin II type 1 (AT1) receptor and angiotensin II type 2 (AT2) receptors. In particular, the effect of blocking the interaction between angiotensin II and its receptor (AT1R) is primarily related to vasodilation and the reduction of blood pressure (Messerli et al., 2018; Figure 5). This drug is metabolized by cytochrome P450 enzymes and undergoes phase II glucuronidation reactions to facilitate the formation of water-soluble metabolites (Regoli and Mezzelani, 2022). Although the ecotoxicological impacts of cardiovascular drugs on marine organisms are not extensively explored, pivotal investigation revealed the presence of beta-adrenergic receptors in mussels, and proteins such as PKA have been purified in marine invertebrates like *Mytilus galloprovincialis* (Cao et al., 1995a, b), suggesting the potential for effect in these organisms. There is limited research on the effects of ramipril specifically on marine organisms. Notwithstanding, previous studies have assessed the effects of other antihypertensives drugs on various non-target species. For instance, in case of mussels *Mytilus galloprovincialis*, prolonged

exposure to propranolol (beta blocker) at concentrations ranging from 0.3 to 30 000 ng/L resulted in modulation of immune system measured through lysosomal membrane damage in haemocytes (Franzellitti et al., 2011). Similar effects were observed in the case of *Perna perna* exposed to losartan, an angiotensin II receptor blocker (ARB), at concentration ranging from 30 to 300 ng/L (Cortez et al., 2018). Additionally, exposure to antihypertensive propanolol caused a modulation of antioxidant system, as demonstrated in Franzellitti et al., 2010, in which *Mytilus galloprovincialis* showed an inhibition of catalase and an induction glutathione transferase, and in Sanzi Cortez et al., 2018, where *Perna perna* exposed to losartan resulted in induction in glutathione transferase and glutathione peroxidases. Concerning other invertebrates, adverse effects were observed in *Daphnia magna*, where exposure to propranolol led to alterations in heart function and metabolic rate (Mezzelani and Regoli, 2022). Furthermore, data provided anomalies in the embryo development of sea urchin *Paracentrotus lividus* exposed to Propranolol (5 µg/L) (Mezzelani and Regoli, 2022).

Antidiabetic drugs are another class of medications widely used worldwide. Metformin is undoubtedly the primary antidiabetic drug used worldwide and one of the most frequently prescribed medications globally. Chemically, metformin is a biguanide compound composed of two guanidine groups joined together. Regarding its mechanism, the liver is the primary site of action for metformin, in fact, it enters hepatocytes preferentially through the organic cation transporter 1 (OCT1), which transports metformin into the liver cells. Once inside the cell, metformin acts as an inhibitor of complex I of the mitochondrial electron transport chain, leading to the suppression of glucose output and this inhibition results in an insufficient ATP flux, which is necessary for driving hepatic gluconeogenesis, an energy-consuming process. Metformin act increasing the levels of AMP (adenosine monophosphate) by activating 5' AMP-activated protein kinase (AMPK) (Figure 6), resulting in the inhibition of Target of Rapamycin complex1 (TORC1) with downstream effect on the cell growth, proliferation, and metabolism. AMPK inhibits Fru-1,6-bisphosphatase (FBPase), a key enzyme involved in gluconeogenesis which convert fructosio-1,6- bisphosphate in fructosio -6-bisphosphate, resulting in an inhibition of the pathway. Furthermore, AMPK suppresses sterol regulatory element binding protein 1 (SREBP1), a key transcription factor involved in lipid and cholesterol synthesis. Eventually, inhibition of mitochondrial activity leads to the accumulation of lactate due to an increase in glycolysis. From the liver cells, it is further transported to the bile unchanged, through the multidrug and toxin extrusion protein (MATE). Once in the bile, metformin is transported to the renal tubular epithelial and excreted without being metabolized (Rena et al., 2013). Regarding its biological effects on non-target species, acute toxicity tests have shown toxicity of metformin to various organisms. For example, it has been found to be toxic to *Daphnia similis* (EC50 48h 14.3 mg/L), *Hydra attenuata* (EC50 96h 3918 mg/L) and *Danio rerio* (LC50 96h 1315.5

mg/L) (Godoy et al., 2018). Noteworthy research has highlighted interesting findings related to the effects of metformin on various organisms. In the case of vertebrates, acute test was performed on newly fertilized embryos (3 h after fertilization) of *Danio rerio*. Exposure to concentration less than 600 mg/L of metformin did not modify the number of heartbeats, number of malformation or the swimming behaviour of larvae. Malformations such as scoliosis and abnormal pigmentation in zebrafish larvae exposed only to higher concentration of MET (1100 mg/L) as well as an increased oxidative stress in organisms. Exposure to MET on medaka fish (*Oryzias latipes*) (Ambrosio-Albuquerque et al., 2021) and fathead minnows (*Pimephales promelas*) (Godoy et al., 2018) has been shown to induce an increase in cytochrome P45019a and estrogen receptor (ER) expression in males, resulting in feminization, while females exhibited a decrease in vitellogenin (VGT2), a protein typically associated with female reproduction, and ER expression. Histological studies of female gonads have revealed intersex phenomena with abnormal spermatogonium cell stages (Ambrosio-Albuquerque et al., 2021). Another interesting observation involves *Betta splendens*, where exposure to metformin at concentrations of 40 and 80 μg/L for 20 weeks resulted in reduced aggressive behaviour. This has potential evolutionary implications for species in which aggression is vital for territorial defence and mate acquisition (Ambrosio-Albuquerque et al., 2021). These findings highlight the potential impacts of metformin on various aspects of animal physiology and behaviour (Godoy et al., 2018).

It is noteworthy that nature marine organisms are exposed to mixtures of different pollutants in their environment. Even if a single compound has a concentration too low to cause an effect on its own, it can still contribute to toxicity within the framework of multiple stressors. Recent research focuses on understanding the possible synergistic, antagonistic, and additive effects that a mixture of pharmaceuticals could have on non-target organisms. Regarding effect of mixture of drugs on marine organisms, some authors found an enhancement of growth inhibition in *Vibrio fischeri* when exposed to a combination of ibuprofen (NSAIDs) and omeprazole (proton pump inhibitor). Additionally, ibuprofen has been found to have a toxic effect on the green neon shrimp (*Neocaridina denticulate*), even when mixed with acetaminophen. In relation to antihypertensives, it was observed that the effect of a mixture of atenolol, metoprolol, and propranolol in immobilization tests using *Daphnia magna* and growth inhibition tests using *D. subspicatus* was higher than the sum of the effects of the individual compounds in both bioassays (Godoy et al., 2015). However, when conducting a growth inhibition test using *Lemna minor*, it was found that the effect of the mixture of propranolol and losartan was lower than the effect observed when the pharmaceuticals were tested individually (Godoy et al., 2015). Regarding antidepressants, it was discovered that the combination of fluoxetine and propranolol, both at 0.3 ng/L, had an antagonistic effect on *Mytillus galloprovincialis*.

Interestingly, the authors found that propranolol inhibited the expression of the gene for the serotonin receptor (5-HT1), which was induced by fluoxetine (Godoy et al., 2015). Other studies have characterized the effects of a mixture of venlafaxine, 2000 ng/L, and norfluoxetine, 3.2 ng/L, both antidepressants, on zebrafish larvae, resulting in an increase in mortality (13% after 8h) and in percentage of malformations and abnormal pigmentation (Rodrigues et al., 2020). While in another study, zebrafish embryos exposed to a mixture of pharmaceuticals (carbamazepine, diltiazem, fluoxetine, gemfibrozil, and metformin) showed a significant correlation between exposure and the expression of genes involved in the metabolism of steroid intermediates (Ambrosio-Albuquerque et al., 2021). Similarly, exposure of *Limnodynastes peronii* larvae (amphibians) to a mixture of metformin, atorvastatin, and bezafibrate resulted in effects on growth and development, energy reserves (negative impact on triglyceride and cholesterol metabolism), and hepatic metabolite profiles (increased levels of fatty acids and amino acids due to liver stress) (Ambrosio-Albuquerque et al., 2021). These findings highlight the importance of evaluating and focusing on the mixtures of pharmaceuticals, as there is currently limited knowledge in this area. Prioritize further research in this field should be mandatory.

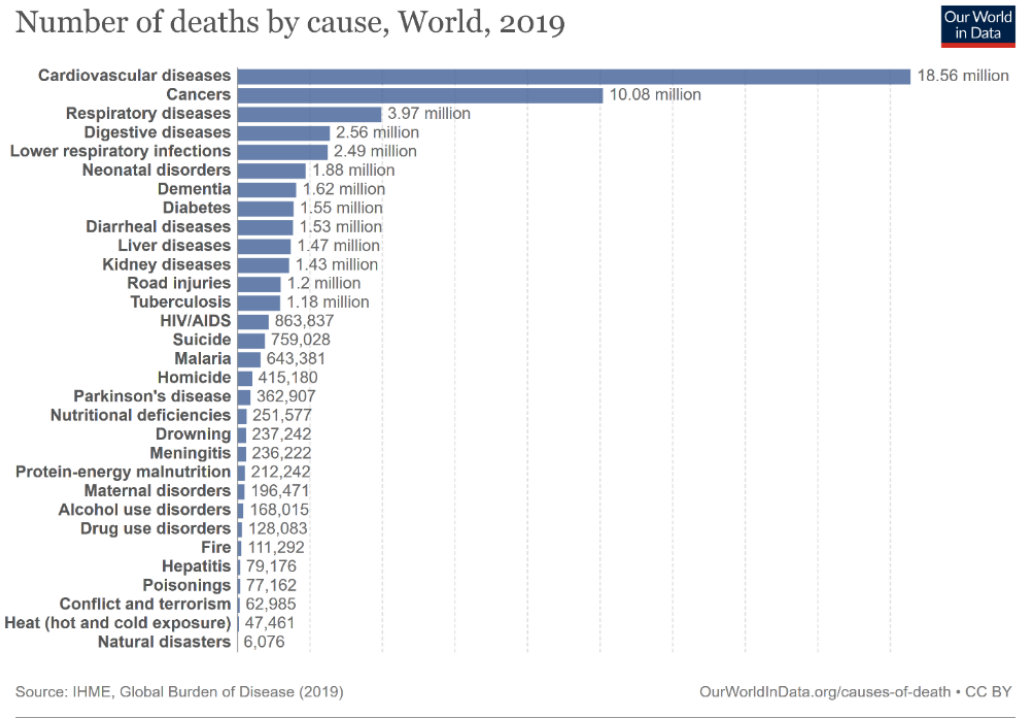

Figure 1. Number of deaths by cause in Europe (https://ourworldindata.org/)

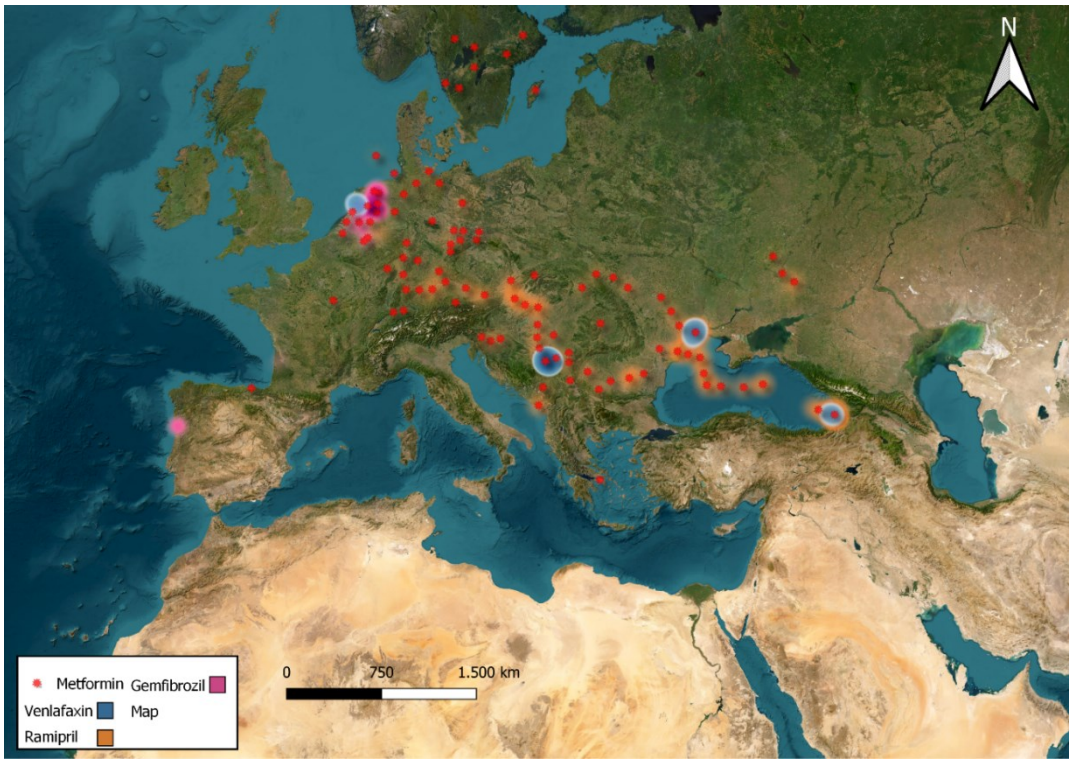

Figure 2. GIS map describing the distribution of venlafaxine, gemfibrozil, ramipril and metformin in Europe. Data are downloaded from database EMPODAT and uploaded on software qGIS.

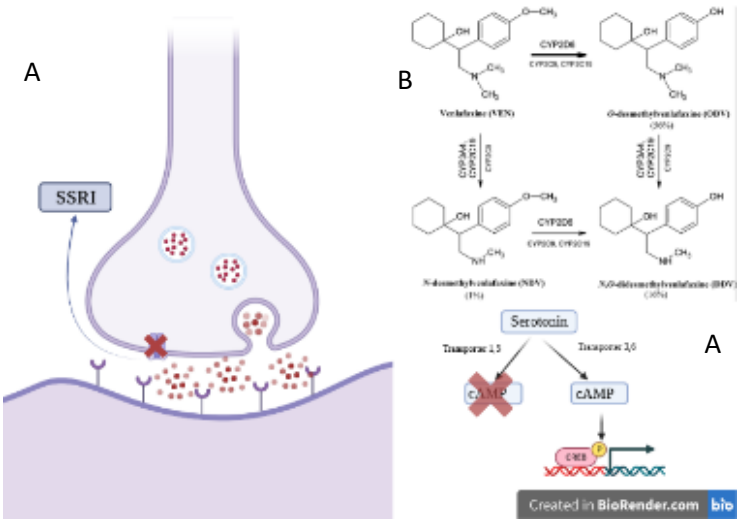

Figure 3. Mechanism of action of SSRIs (A) and mechanism of degradation of venlafaxine (B)

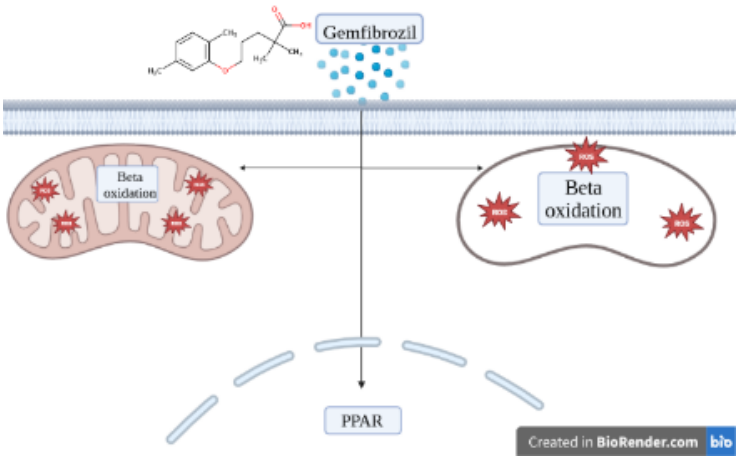

Figure 4. Mechanism of action of lipid regulating agent

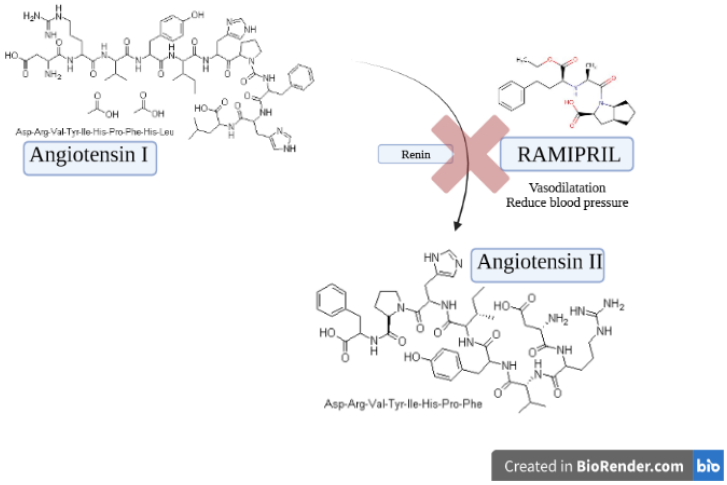

Figure 5 Mechanism of action of ACE inhibitor

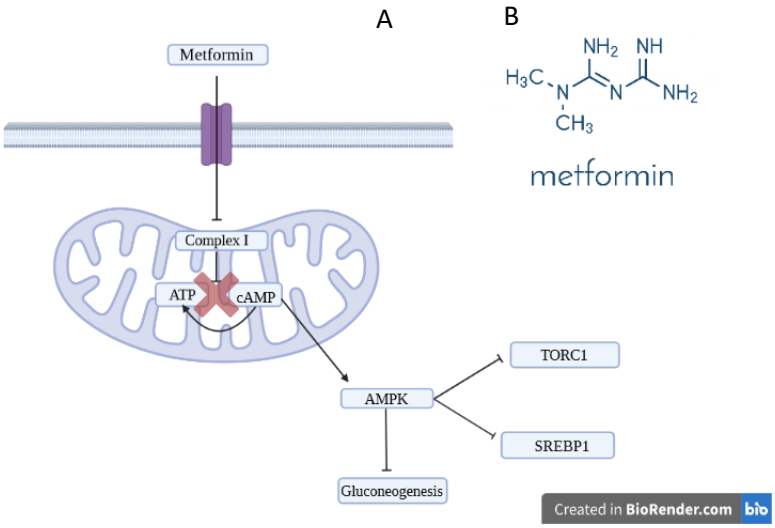

Figure 6 Mechanism of action of metformin (A) and structure of metformin (B)

1.3 Pharmaceuticals as emerging contaminants: a normative perspective

Awareness on the presence of pharmaceuticals in the marine environment is relatively recent and, in most cases, poorly regulated. However, there is now a growing focus on the distribution of drugs in the marine environment due to numerous research studies providing toxicological data that demonstrate the adverse effects of these substances in marine non-target species. Several countries, including Japan (Pharmaceutical Affairs Law), Australia (Environmental Protection Authority), the United States (Environmental Protection Agency) and Europe (Water Framework Directive) have recognized this concern and responded by developing guidelines for eco-pharmacovigilance and implementing regulatory measures (Mezzelani & Regoli, 2022). These initiatives align with the European Strategic Approach to Pharmaceuticals in the Environment, which promotes innovative and multidisciplinary ecosystem-oriented approaches to identify the long-term consequences of pharmaceutical compounds (Eur. Comm., 2019). Eco pharmacovigilance involves the science and activities related to detecting, evaluate, and prevent the adverse effects of molecules products in the environment. Its scope includes studying the toxicological effects and accumulation of pharmaceuticals in the environment and biota, as well as educating on the correct use of medicines, proper handling of unused compounds, and the design and development of environmentally friendly chemistry and medicines. (Gómez-Oliván et al., 2019). The Water Framework Directive (WFD) (2008/105/EC) is the primary legislation for protecting the water environment, covering freshwater, marine, and groundwater, and has been in effect since the early 2000s. It represents the first European directive to focus on sustainability and aims to enhance water quality, safeguard aquatic ecosystems, and promote the sustainable use of water resources (Voulvoulis et al., 2017). The goals of the WFD are set on a six-year cycle, with the current objects to be achieved by 2027, more recently, this aspect has been also considered by Marine Strategy Framework Directive (MSFD) (2008/56/EC) which specially focus on marine ecosystem. However, despite monitoring and investigation efforts involved over 100,000 rivers, lakes, transitional waters, and coastal waters by 2018 (Hering et al., 2018), 47% of EU surface water did not reach a good environmental status by the end of the first cycle (2009-2015), with only a 10% improvement in water quality (Voulvoulis et al., 2016). The WDF promotes a comprehensive and integrated approach to evaluate environmental quality and unlike the previous approach that considered ecosystems as a collection of individual elements and examined single environmental stressor in isolation, such as Drinking Water Directive, the Urban Wastewater Directive or the Nitrates Directive (Petersen et al., 2009), the WFD adopts a holistic perspective, recognizing the complexity of ecosystems and the interactions between different elements and pressures (Voulvoulis et al., 2017). This new approach aims to assess the environmental status in terms of achieving Good Environmental Status (GES), which comprises a set of characteristics

defining the desired high quality for water and ecosystems. In the assessment of GES, reference conditions are established, involving both pristine states without anthropic disturbance, which are relatively rare, and states in which there is a slight disturbance which does not have negative impact on the ecosystem (Borjia et al., 2010). The purpose of establish standard conditions is to provide a baseline for assessing the health and quality of natural system. Once reference conditions are created, monitoring programs are used to assess any divergence from them, using standardized methodologies to evaluate indicators, defined as "subjective mental construction aiming to capture one or several aspects of reality considered of importance when it comes to a specific subject". For the classification of ecological status Annex V of WFD provides three groups of "quality elements": biological, hydro morphological and physico-chemical (Voulvoulis et al., 2016). Data collected are used to calculate parameters, which are compared with metrics in baselines specific to each type of water body as part of GES evaluation. The difference between the reference and estimated values is used to calculate the Ecological Quality Ratio, which ranges between 0 and 1 and categorizes the environment into five classes (high, good, moderate, poor, and bad), on which management decisions are taken (Hering et al., 2018). It is important to note that even a significant deviation of a single metric, from the reference value considered, is sufficient to define a poor-quality environmental status, following the principle known as the "one-out-all" principle. Despite the significant attention given to pharmaceuticals use and prescription in human healthcare, there are very few rules regarding their proper disposal (Mezzelani et al., 2018), and out of the list of 4,000 substances classified as emerging substances, only 13 of them are included in the dynamic watch-list of the European Union Water Framework Directive. The watch-list is established to identify priority substances, which are chemicals selected by the Commission due to their significant risk to the environment. The objective is to reduce these substances to their natural background levels or eliminate those that are not naturally occurring. The criteria for the selection include information gathered from literature, member states and stakeholders as well as reviewed substances for the previous watch list. To prioritize the chemicals, it is necessary to conduct monitoring activities to track their presence in the environment. Additionally, gathering information about the biological effects of these substances is crucial in order to understand the distribution patterns of these pollutants assessing their potential impact on the environment; and it is important to consider the availability of analytical methodologies that allow the quantification of these contaminants in various matrices. Among the different categories comprised in the list, there are pesticides, medicines, UV filters, endocrine disruptors, cosmetics, antibiotics (Cortes et al., 2022). In the first Watch list 4 pharmaceuticals were included (diclofenac,17-beta-estradiol (E2), 17-alpha-ethinylestradiol (EE2) and macrolide antibiotics) while the last update in 2022 includes 9 new pharmaceuticals (antibiotics including clindamycin, cefalexin, ofloxacin; allopurinol, gabapentin,

gemfibrozil, irbesartan, propranolol, metformin with its transformation product guanylurea) emphasising the rising of awareness concerning the presence of pharmaceuticals in water and their effects on non-target organisms (Cortes et al., 2022).

Considering the urgency, one of the main objectives is to employ an integrate multidisciplinary approach to optimize environmental risk assessment. Indeed, evaluation such as sterile quantification of chemicals, essential in monitoring, provide no meaningful information on the status of ecosystems without biological data. It's mandatory compare analytical data of chemicals to biological evaluation, in alignment with the European Directives which recommend the use of multiple quality indicators**.** Biological evaluations are effective for quantifying not only the presence of chemicals in the environment but also, more importantly, their effects on living organisms and the resulting consequences for the ecosystem. These evaluations provide insights into the impact of organic compounds on the organisms and help assess consequently ecological implications. Furthermore, laboratory studies are equally important, as they allow researchers to assess the effects of pollutants in a controlled environment. Laboratory analysis provide insight into how molecules impact on the biology of organisms and how different molecules can interact with each other. These analyses are functional to understand the mechanism of action, physiological responses of the compound on living organisms. Besides, laboratory studies provide information on acute pollution using specific test which evaluate biological endpoints in selected organisms. To achieve an effective evaluation of the hazard induced by chemicals, an integrate holistic approach is the better choice for a better understanding of the hazard of the molecules and its potential consequences on the environment and human health. In reference to this, the Weight of Evidence is an example of an integrated approach, used for the evaluation of sediments, which integrate different results coming from different type of analysis providing an accurate and impactful result of Risk Assessment (Regoli et al., 2019).

This thesis places particular emphasis on laboratory studies that investigate the effects of pharmaceuticals and their mixture on non-target organisms, evaluating the impacts of drugs not extensively studied so far. By conducting an experiment for over 30 days this study aims to simulate the chronic effects of exposure to these compounds. Through laboratory experiments, this research aims to enhance our understanding of the potential effects and risks associated with these emerging contaminants on non-target organisms.

2. Aim of the study

Pharmaceuticals and their mixtures are extensively detected on a global scale and have emerged as a significant contemporary challenge owing to their potential to be bioaccumulated and cause negative effects in biological and physiological mechanisms of non-target organisms, even at low concentrations. This study aimed to assess the effects of four different therapeutic classes representative of the most frequently detected compounds in marine ecosystem, namely the antidepressant venlafaxine, the lipid-regulating agent gemfibrozil, the antihypertensive ramipril and antidiabetic drug metformin. Since in nature many pharmaceuticals are simultaneously detected, possible synergistic, antagonistic or additive effects on marine non-target organisms can occur and are still poorly known, in this thesis the effect of single compound were integrated with investigations of pharmaceuticals mixture. The model species chosen for this evaluation was *Mytilus galloprovincialis*, a typical bioindicator organism widely distributed, highly accessible for research purposes, with high tolerance to various environmental conditions. The experimental plan provided mussels' exposure to single and combined pharmaceuticals for 30 days, to simulate chronic exposure scenario and 14 days depuration period, in order to evaluate mussels' capability to recover possible adverse effect caused by pharmaceuticals. The exposure dose was selected considering concentrations typically found in coastal areas. An integrated ecotoxicological approach was applied measuring a wide panel of biological responses that typically reflect impact on several pathways. These biomarkers assessed of the modulation of the immune system, including phagocytosis activity, granulocytes and hyalinocytes ratio and lysosomal membrane stability. Genotoxic effects were evaluated measuring the frequency of micronuclei and lastly alterations in oxidative metabolism, through measurement of single antioxidant defences activity and total oxyradical scavenging capacity (TOSC) toward hydroxyl (HO •) and peroxyl (ROO•) radicals. The overall results of this study will be helpful to clarify the ecotoxicological effects of both single drug and their mixtures in non-target marine organisms, contributing to fill the gap of knowledge regarding the ecological consequences of these emerging pollutants in aquatic system.

3. Materials and methods

3.1 Experimental design

Mussels, *Mytillus galloprovincialis*, were collected from a shellfish farm in a reference area of central Adriatic Sea (Ancona, Adriatic coast) in October 2022. Organisms were acclimatized for 7 days with aerated artificial sea water, at local seasonal environmental condition of salinity (33 ‰) and temperature (18± 1 °C). Collection and experimental use of mussels is not subjected to ethical review permission according to both European and Italian normative (Directive 2010/63/EU, of the European Parliament and of the Council of 22 September while monitoring guidelines indicate this species as an adequate bioindicator organisms for assessing bioavailability of ecotoxicological effects on the protection of animals used for scientific purpose, 2010; Italian Legislative Degree n. 26, 2014) of environmental pollutants in marine environment. 360 mussels were randomly distributed into 6 glass tanks (n=60 per tank) in a volume of 17L artificial seawater ensuring that no death animals were included (Figure 7). Individuals within each tank were intentionally distributed together to promote cohesion among the animals. Out of the six glass tanks, four tanks contained organisms exposed to following treatments: CTRL control condition (DMSO), VEN, venlafaxine exposure (1 µg/L); GEM, gemfibrozil exposure (1 µg/L); RAM, ramipril exposure (1 µg/L); MET, metformin exposure (1 µg/L); MIX, mixture exposure of VEN, GEM, RAM and MET (1 µg/L for each drug). The mussels were initially exposed to the pharmaceuticals for a period of 30 days and then they were kept in pharmaceutical-free artificial water for an additional 14 days. The rationale for selecting 14 days for the depuration phase was based on the previous research on physiology and responsiveness of this bioindicator specie; 14 days, in fact, is the shortest time enabling *M. galloprovincialis* to recover or highlight the persistence of stressful conditions. This phase, eventually, was functional for the evaluation of the depurative capacity of mussels. The stock solutions of pharmaceuticals were prepared dissolving the drug powders in 1 ml of DMSO and diluted to a final concentration of 34 mg/L. From this final stock, 500 µL were added in each tank in order to obtain a single APIs and mixture concentration of 1 µg/L and 1 µg/L for each pharmaceutical respectively. The stock solutions were stored at 4°C for the duration of the experiment, and working solution were prepared daily by diluting the stock solution in water. Additionally, a water change and pharmaceuticals redosing were carried out every two days in order to maintains the desired concentration in each tank. The day before the exposure, organisms were fed with 500 µL of algae Easy sps EVO. For each experimental condition (CTRL, VEN, GEM, RAM, MET and MIX) at each experimental phase (exposure 30 and recovery 44), 25 individuals were sampled from each tank and dissected for biological analysis. For each organism haemolymph, digestive glands and gills were dissected, frozen in liquid nitrogen and stored at -80°C for histochemical and biochemical analysis. Aliquot of haemolymph for treatments

were processed immediately for *in vivo* analysis of lysosomal membrane stability in haemocytes, phagocytosis activity and granulocytes-hyalinocytes ratio, while additional aliquots of haemolymph were fixed in Carnoy's solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency.

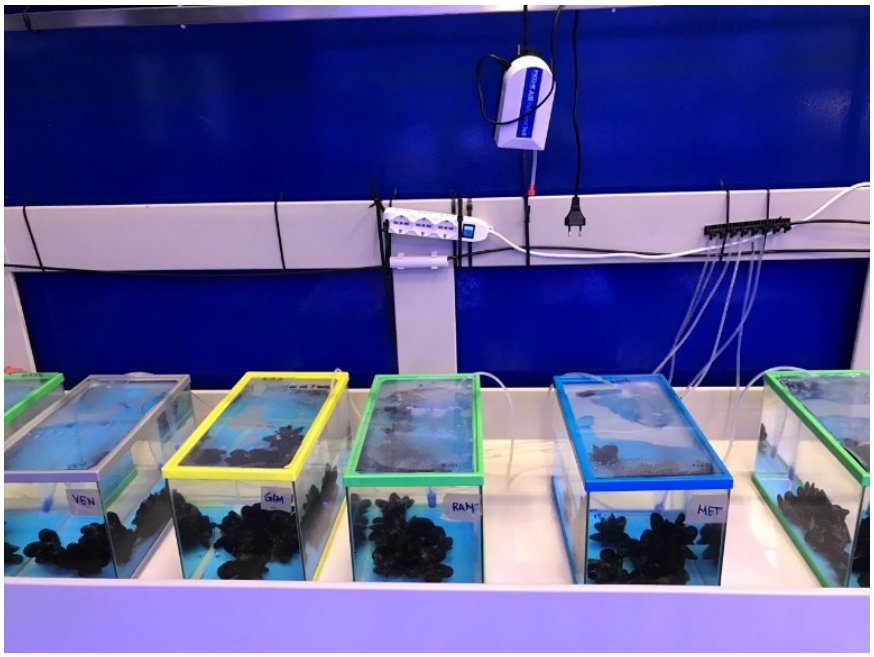

Figure 7. Tanks with mussels (n=60) exposed to single pharmaceuticals and mixture.

3.2 Biomarker analysis

Biomarkers were assessed by using standardized protocols (Bocchetti and Regoli., 2006; Mezzelani et al., 2022). Immunological system is activated under exposure to multiple stressor and immunological biomarker were evaluated in terms of lysosomal membranes stability (LMS), granulocytes-hyalinocytes ratio (G/I) and phagocytosis capacity in haemocytes. For the evaluation of lysosomal membrane stability (NRRT) haemocytes were incubated on glass slides with a freshly prepared Neutral Red (NR) working solution, then were examined microscopically at 20 min intervals in order to determine the time at which 50% of cells had lost into the cytosol the dye previously assimilated by lysosomes. Granulocyte/Hyalocytes ratio was analysed by using aliquots of haemolymph dispersed on glass side and fixed in Becker's fixative (2.5 NaCl). Following fixation, the slides were washed and stained with Mary Grunwald Giemsa before being mounted with Eukitt. Successively observations were evaluated with a light microscope (1000x) and percentage of granulocytes was quantified counting 100 cells for each sample. The cells were coloured using pH sensitive dye which change colour between rose and violet based on pH. The nucleus, bigger in granulocyte, appears violet. For the Phagocytosis assay, 50µL of haemolymph was spread on a glass slide and incubated for 2h in the dark at 15 °C with fluorescein labelled Zymosan. A bioparticles (Invitrogen) at a 10:1 target:hemocyte ratio. After the incubation period, uninternalized particles were washed away with physiological solution and slides were fixed in Beker's fixative and mounted in Eukitt. Phagocytosis was evaluated under a fluorescence microscope counting 100 granulocytes and calculating the percentage of cells that had internalized at least 3 fluorescent particles. For the genotoxic damage DNA micronuclei were evaluated. 50 µL of haemolymph were placed on the glass slides and stained with 100 ng/mL of fluorescent agent 4',6-diamidino-2-phenylindole (DAPI). Micronuclei (MN) frequency was determined at the fluorescent microscope (40x) by counting 2000 cells for each slide. The criteria used to identify a micronucleus were: dimension between 1/12 and ¼ of the nucleus diameter and it must be totally detached and located within the same optical plan as the nucleus, indicating that they belong to the same cytoplasm. Xenobiotics as pharmaceuticals, typically, have the ability to enhance pro-oxidant effects on marine organisms by interacting with the antioxidant system and inducing the formation of Reactive Oxygen Species (ROS). To evaluate antioxidant defences and the onset of oxidative damage, both the variation of single antioxidant defences and the total oxyradical scavenging capacity (TOSC) were evaluated. Catalase (CAT) enzyme was quantified by the decrease in absorbance at 240 nm due to the consumption of 12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0. Glutathione reductase (GR) was determined at 340 nm, from NADPH oxidation during reduction of 1 mM GSSG in 100 mM k-phosphate buffer pH 7.0 and 60mM NADPH. Glutathione peroxidases (GPx) activities were assessed in a coupled enzyme system

where NADPH is used by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was observed at 340 nm in 100 mM K-phosphate buffer pH 7.5 1mM EDTA, 1 mM dithiothreitol, 1mM sodium azide (NaN3) (for hydrogen peroxide assay), 2mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.5mM hydrogen peroxide or 0.8mM cumene hydroperoxide as substrates respectively for the Se-dependent and for the sum of Se-dependent and Se-independent forms. Glutathione S-transferases (GST) were measured at 340 nm using 1.5 mM 1-chloro-2,4-dinitrobenzene as substrates (CNDB) and 1mM GSH, in 100 mM K-phosphate buffer pH 6.5. Total glutathione was enzymatically determined in supernatant samples obtained after the homogenization in 5% sulfosalicylic acid with 4 mM EDTA, stored for 45 min on ice and centrifuged at 37,000 x g for 15 min. Total oxyradical scavenging capacity assesses the organism's antioxidant ability to cope with increase in ROS production. The assay utilizes substrates which generate artificial ROS, inducing the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas. Thermal homolysis of 20 mM of 2'-azo-bis-(2-methylpropionamide)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer (pH 7.4) generates Peroxyl radical, while Hydroxyl radicals (•OH) are generated by Fenton reaction of iron-EDTA (1.8 mM Fe3+,3.6 mM EDTA) with 180 mM ascorbate in 100 mM K-phosphate buffer. Ethylene is measures at 37°C at 15 minutes time intervals by gas chromatographic analysis. Total analysis involved 108 minutes and TOSC values were quantified from the equation: TOSC = 100 – (∫SA/∫CA x 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. Each experimental TOSC value is divided by the sample protein concentration determined using the Lowry method, with Bovine Serum Albumin (BSA) as the standard (Regoli and Winston, 1999).

4. Results

Results on immune system and genotoxicity biomarkers analysed in mussels' haemolymph are shown in the Figure 8. Average lower values of neutral red retention time were observed in all exposed mussels compared to control, both at the end of the exposure and depuration phase. A statistically significant reduction of the lysosomal membrane stability was measured in mussels exposed to RAM and MIX after 30 days of exposure, with major effects for mixture compared to the single compound. In both treatments these significant effects were maintained even after the end of depuration phase (DAY 44) (Fig. 8A). Fluctuating results were obtained for granulocytes-hyalinocytes ratio with average lower values for single molecules compared to CTRL and MIX at day 30. Although not significant, at the end of depuration phase average lower values were measured for most of tested compounds, with the only exception of VEN which showed a ratio almost doubled compared to the CTRL. Pharmaceuticals exposure (DAY 30) caused a slight inhibition of phagocytosis capacity in VEN and MIX-exposed mussels, while after the end of depuration phase (DAY44), a statistically significant decrease of such parameter was measured in RAM and MET-exposed organisms compared to the CTRL (Fig. 8C).

Regarding genotoxic damage, the micronuclei frequency (MN) showed a lack of statistically significant variations between control and exposed organisms after the end of both exposure and depuration phases (Fig. 8D).

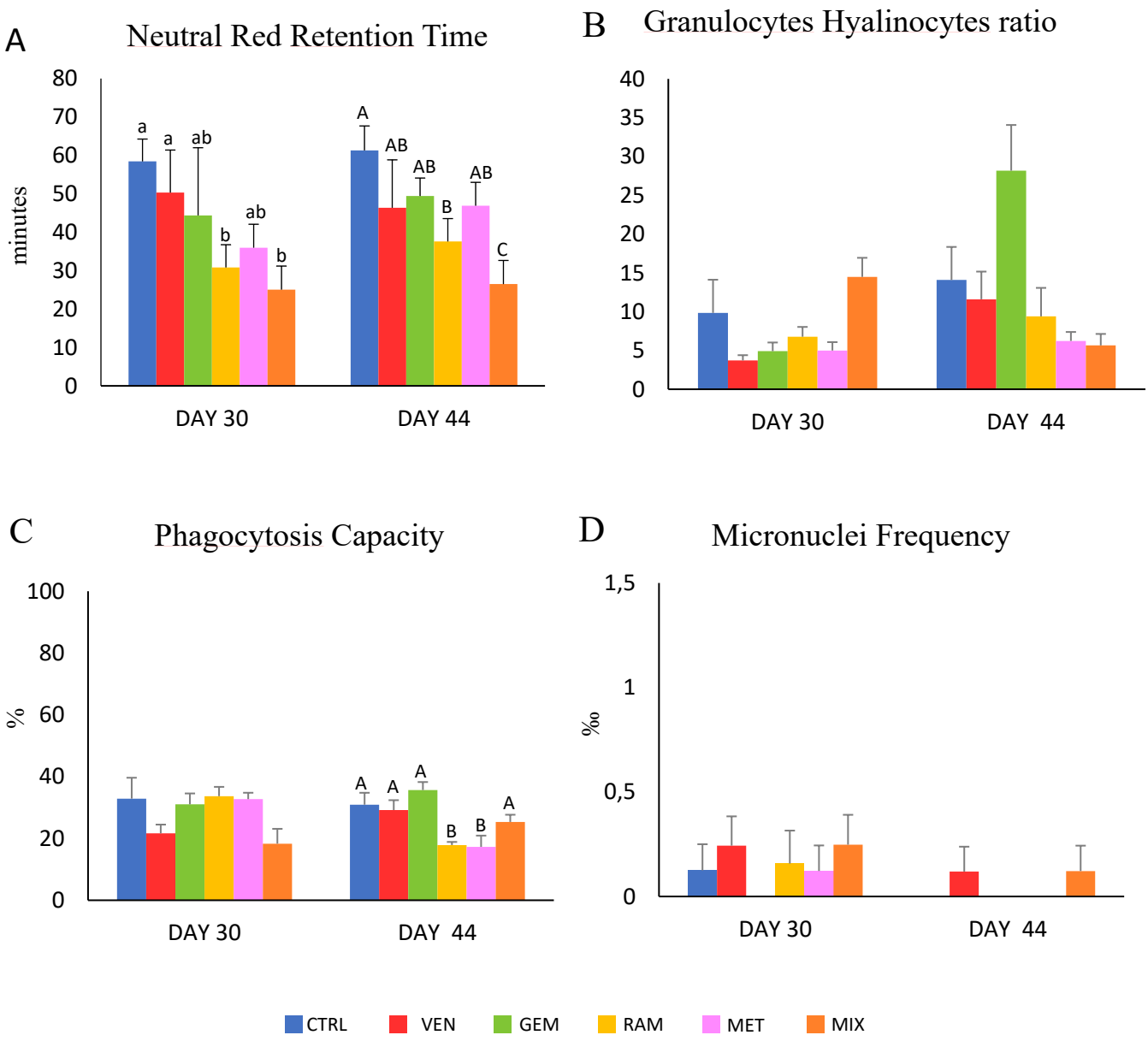

Figure 8. Lysosomal membrane stability, LMS (A), granulocytes/hyalinocytes ratio (B), phagocytosis capacity (C), micronuclei frequency (D) in haemocytes of mussels exposed to various treatments. Data are given as mean values±Standard Error of the Mean (n=4). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL, control; VEN, venlafaxine; GEM, gemfibrozil; RAM, ramipril; MET, metformin; MIX, mixture.

Results of antioxidants biomarker evaluated both in terms of single antioxidant enzymes and the total oxyradical scavenging capacity are shown in Figures 9 and 10. Average higher values were measured for catalase activity at day 30 in mussels exposed to GEM and MIX, with a statistically significant induction only for GEM, while no variation was assessed for the other treatments. After the end of depuration phase (DAY 44) a statistically significant induction of CAT was measured in MIX compared to the CTRL (Fig. 9A).

A significant induction of the glutathione s- transferase (GST) was measured at the end of exposure phase (DAY 30) in all treatments. After the depuration phase (DAY 44), all values were similar to the CTRL (Fig. 9B).

Glutathione reductase (GR) showed a statistically significant inhibition in the mixture at 30 days, and a statistically significant induction in VEN after depuration phase (Fig. 9C).

Levels of total glutathione displayed a statistically significant decrease in all treatments at day 30, while a lack of significant variations were measured at DAY 44 (Fig. 9D). Although not statistically significant, higher average values were measured at day 30 for the activity of GPx CHP and GPX H_2O_2 in VEN-exposed mussels. This trend of induction was confirmed for all treatments compared to CTRL at the end of the depuration phase, with statistically significant higher values in mussels exposed to MIX (Fig. 9E-F). Results on Total Oxyradical Scavenging Capacity (TOSC) toward peroxyl and hydroxyl radicals are shown in Figure 10. Although not significant, after 30 days of exposure average lower values of the total oxyradical scavenging capacity toward peroxyl radicals (Fig. 10 B) were measured for VEN, GEM, RAM and MET compared to the CTRL-organisms, while such changes were not observed at the end of the depuration period (Fig. 10A). Mussels' capability to neutralize hydroxyl radicals were significantly reduced in MIX treatments at the end of the recovery phase (Fig. 10B).

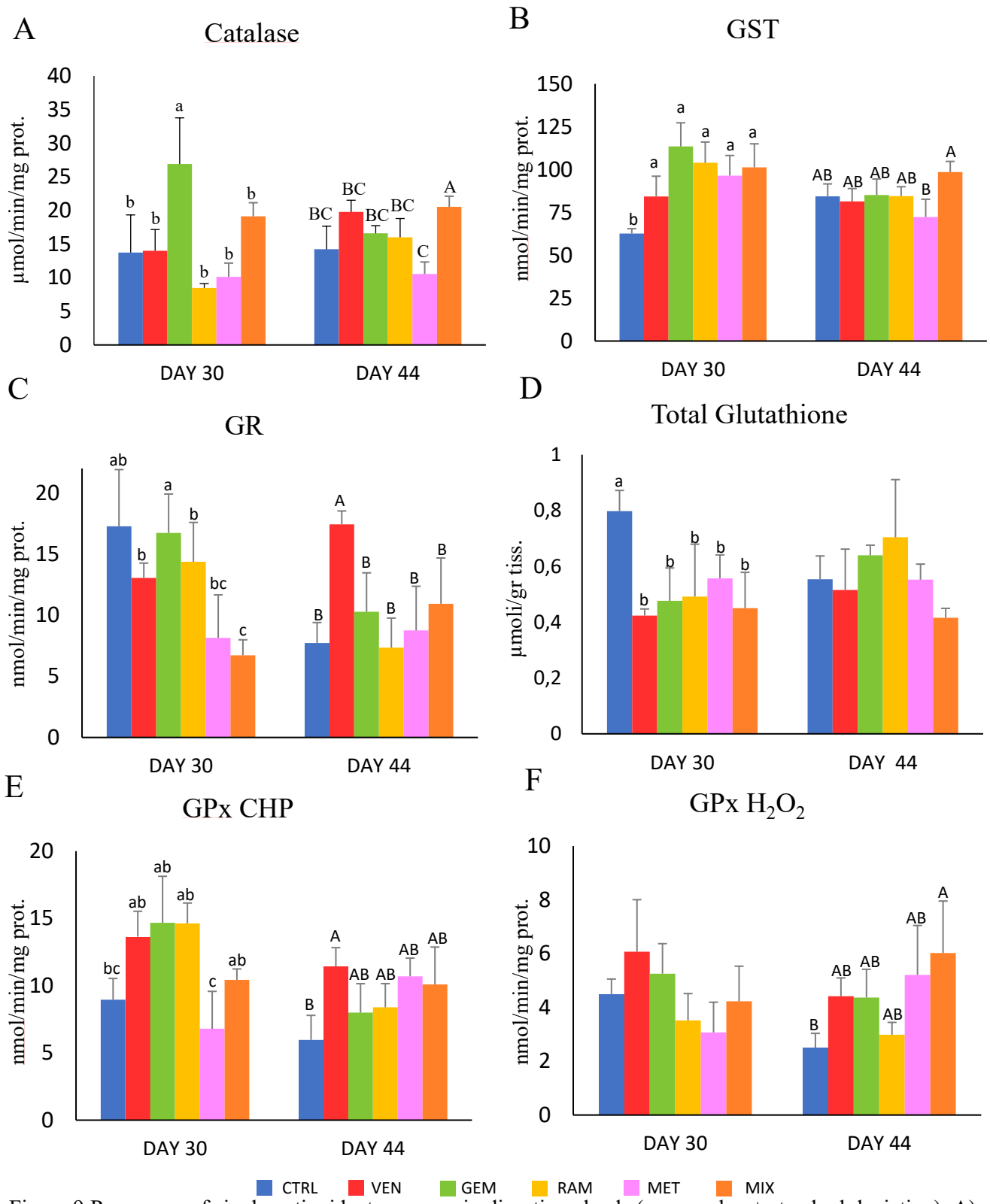

Figure 9 Responses of single antioxidant enzymes in digestive glands (mean value ± standard deviation). A) Catalase activity (CAT), B) glutathione S-transferase activity (GST), C) glutathione reductase activity (GR), D) total glutathione, E) Sum of Se-dependent and Se- independent glutathione peroxidases (GPx CHP), F) Se-dependent glutathione peroxidases (GPx H2O2). Data are given as mean values±Standard Error of the Mean (n=4). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL, control; VEN, venlafaxine; GEM, gemfibrozil; RAM, ramipril; MET, metformin; MIX, mixture.

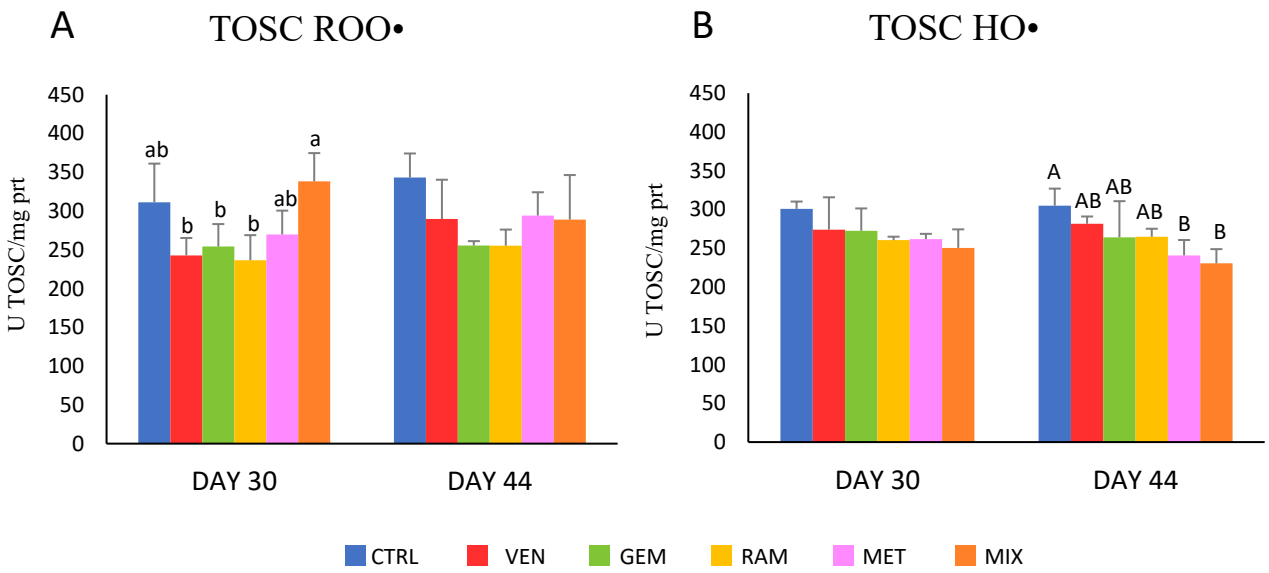

Figure 10 Total Oxyradical Scavenging Capacity for A) peroxyl radicals (ROO·) and B) hydroxyl radicals (HO·). Data are given as mean values±Standard Error of the Mean (n=4). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 30; capital letters are used to highlight significant differences between treatments at day 44. CTRL, control; VEN, venlafaxine; GEM, gemfibrozil; RAM, ramipril; MET, metformin; MIX, mixture.

5. Discussion

In recent years, interest on the potential consequences of pharmaceuticals in aquatic ecosystem is increased. For a considerable period, their risk was underestimated, due to the belief that seawater had an unlimited dilution capacity (Mezzelani and Regoli, 2022). Although these compounds in natural environment are present at low concentrations, it is crucial the evaluation of the potential effects in long-term period in non-target organisms. Wild organisms are continuously exposed to a cocktail of chemical substances, which, based on their chemical-physical properties and modes of action, can interact, and modify their toxicity causing potential additive, synergistic or antagonistic biological effects. For this reason, in this study was investigated the ecotoxicological effects of both single pharmaceuticals belonging to different therapeutic classes and their mixtures, by the assessment of the main metabolic pathway in the Mediterranean mussels *M. galloprovincialis*. Considering the mechanism of action of drugs, a panel of biomarker has been evaluated: the modulation of immune system was assessed by analysing lysosomal membrane stability (LMS), granulocytes/hyalinocytes ratio and phagocytosis capacity in haemocytes. Genotoxicity was studied through the measurement of Micronuclei frequency (MN) and the modulation of antioxidant pathway was investigated by assessing variation in single antioxidant enzymes and in total oxyradical scavenging capacity (TOSC) toward two of the main ROS generated by cellular metabolism: the peroxyl and the hydroxyl radicals. The mussels' immune system is a particularly sensitive parameter in response to environmental drugs (Mezzelani et al., 2018; Cortez et al., 2018, Mezzelani and Regoli, 2022, De Marco et al., 2022, Afsa et al., 2022, Franzellitti et al., 2011), and overall results of our study further confirm this evidence, especially in terms of reduction of Lysosomal Membrane Stability (LMS). LMS, is a sensitive biomarker frequently used in ecotoxicological investigations (Gorbi et al., 2012). Lysosomes are integral organelles involved in the degradation and recycling of molecules; they play a crucial role in cell immune responses. In mussels, the principal immune responses reside in haemolymph. Based on this, membrane stability was evaluated on mussels' haemocytes using Neutral Red Retention Time Assay (NRRT). Average lower levels of membrane stability were measured in MET exposed mussels, while a significant decreased occurred for organisms treated with RAM and MIX. Interestingly, the measured effects persisted even after the depuration period, providing the first insights of long-lasting effects of tested compounds in immune system, especially in response to RAM and the mixture. Although the scientific knowledge on the effect of RAM is limited there are case studies where antihypertensives, at similar exposure concentrations, induce decrease in NRRT, suggesting a possible specific action of these therapeutic class of pharmaceuticals. Noteworthy in literature other antihypertensives drugs were demonstrated to modulate immune system. For instance, exposure for 96 hours to losartan, at concentration of 3000

ng/L, caused a decrease of the NRRT in the mussels *Perna perna* (Cortez et al., 2018). Other research highlighted that the administration of increasing concentrations (0.3, 3, 30, 300, 30 000 ng/l) of the antihypertensive propranolol on *Mytilus galloprovincialis* determined a decrease in NRRT (Franzellitti et al., 2011), as suggested in our case. Notably, in the mixture, NRRT value is lower than those in the individual treatment, indicating potential interaction among the tested pharmaceuticals. It is possible to hypothesize that RAM plays a significant role in determining the outcome of the mixture. Many other studies previously demonstrated effects of the other therapeutic classes on NRRT (Aguirre- Martinez et al., 2013), including, for instance, gemfibrozil. GEM, shown to influence LMS of *M. galloprovincialis* at concentration of 1 μM, with the most significant effect at concentration of 100 μM (Canesi et al., 2007), but it is important to highlight that these concentrations were higher than the ones used in our research. Effects on immune system were also evidenced by the results of granulocytes/hyalinocytes ratio (G/I) and phagocytosis capacity. These two distinct sub-populations of immune cells in mussels play a crucial role in immune responses. Granulocytes, characterized by higher phagocytosis capacity, represent the dominant population (Gorbi et al., 2012); while the phagocytosis capacity is a parameter that evaluates the variation in the ability of granulocytes to embed external particles. Although not significant, after 30 days of exposure, phagocytosis capacity declined in VEN and MIX-exposed mussels, and notably this was parallel to the decrease in ratio between the two main haemolymph sub population in mussel in VEN but not in MIX treatment. It is possible to hypothesize that in VEN exposed mussels the lower phagocytosis rate might be related mainly to a decrease of granulocytes rather than to a lower efficiency of granulocytes to phagocytize; while for the MIX, considering the lack of effects on the two typologies of haemocyte's sub-population, the inhibition of phagocytosis analyses might reflect a lower efficiency of granulocytes to phagocytize. Different studies demonstrated that drugs belonging to different therapeutic classes, as NSAIDs, could modify both population of granulocytes and hyalinocytes. Exposure to sialic acid (NSAIDs), for instance, induced an increase in both populations, the same was observed in the co exposure of caffeine and sialic acid (Afsa et al., 2022; De Marco et al., 2022), conversely previous investigation of Mezzelani et al., 2023 reported a lack of significant changes in G/I ratio after 14 days of exposure to the antihypertensive valsartan (VAL) at concentration of 0.5 μg/L. In our study, after 14 days of depuration the lower G/I ratio for RAM, MET was also parallel to the inhibition of phagocytosis in RAM and MET, confirming both how these two parameters are strictly related and interconnected, but also the long-lasting modulation of immune system. The results of RAM treatment differed from those of *M. galloprovincialis* exposed to VAL at concentration of 0.5 μg/L for 14 days, where no variation occurred in phagocytosis capacity (Mezzelani et al., 2023). This different behaviour can be attributed to the specific mechanism of action

of these two pharmaceuticals, despite both are antihypertensive agents. Different results were found in studies of Lacaze at al., 2015 and Canesi et al 2007, which assessed a decrease in phagocytosis capacity in *M. galloprovincialis* exposed to VEN at concentration between 0.15 mg/L and 75 mg/L (Lacaze et al., 2015) and a 41% decrease in phagocytosis capacity at concentration 100 µM of GEM (Canesi et al., 2007) respectively. This may be because the concentrations used in these works are an order of magnitude higher than those used in this study.

Regarding genotoxicity, the frequency of micronuclei (MN) was quantified in haemolymph of mussels. It is noteworthy that no significative presence of MN was registered for all the treatments, in contrast with other studies. For instance, in *Mytilus galloprovincialis,* 14 days of exposure to VAL (0.5 µg/L) resulted in a significant increase in micronuclei frequency, with values two-fold exceeding the control. Specifically, the value was greater than 1 ‰, which was more than twice the value of 0.5 ‰ observed in the control (Mezzelani et al., 2023). Furthermore, the antidepressant fluoxetine (FLX) led to an increase in micronuclei frequency in Nile tilapia *Oreochromis niloticus* following exposure to 1000 µg/L for 96 hours, where in the control the frequency was 0.04 ‰, whereas in the exposed individuals was 0.26 ‰ (Vijitkul et al., 2022). It is noteworthy that the concentrations used in this works was significantly higher than the ones used in our study. The diversity of outcomes of this research demonstrate how significant are factors such as the selection tested pharmaceuticals, the duration of exposure and the selection of the model species.

One of the main responses to stress induced by xenobiotics is an imbalance between antioxidant defences and pro-oxidant forces and an increase in generation of reactive oxygen species (ROS) (Regoli and Giuliani, 2014; Gorbi et al., 2013). To better understand the oxidative status of mussels, this study evaluated the variation in single antioxidant enzymes, which are catalase, glutathione S-transferase, glutathione reductase, glutathione peroxidases, and the variation in the total oxyradical scavenging capacity (TOSC) of mussels. The results regarding the antioxidant system demonstrate a significant modulation of this pathway, indicating it as one of the principal targets of drug action (Strancova et al., 2017; Barreto et al., 2018; Cortez et al., 2018; Lee et al., 2019; Mezzelani et al., 2023). During the exposure time, it is evident that all the pharmaceuticals activated the biotransformation pattern (Nichols and Sanders-Bush, 2003; Zurita et al., 2007; Mezzelani and Regoli, 2021; Godoy et al., 2018), with a particular pronounced effect observed in the case of the mixture, suggesting a potential interaction among the tested drugs. Catalase is an enzyme which has a role in neutralizing hydrogen peroxide. After exposure phase, among all the treatments, GEM stimulated a significant induction of CAT and higher average levels were reported for MIX. Obtained results are supported by previous literature, where *Sparus aurata* exposed to 1.5 µg/L GEM determined and induction of CAT activity in gills from 50% to 90% compared to control, and up to

150% at 15 000 µg/L in liver (Barreto et al., 2018). These results can be explained by the pharmacodynamics of GEM, which is a Peroxisome Proliferator Agent (PPA). It interacts with the peroxisome proliferator activated receptor α (PPAR α), thereby activating genes responsible for peroxisomal fatty acid β oxidation system and inducing proliferation of peroxisomes, resulting in possible occurring of oxidative damage (Barreto et al., 2018). In fact, the induction of this pathway leads to an increase of H_2O_2 levels, which results in an induction of CAT used to cope with the ROS (Lores Arnaiz et al., 1995). Although the use of a different species and significantly higher concentrations, similar results were obtained from Lee et al. in 2019, where exposure of fish *Oryzias latipes* to different concentrations of MET 40 µg/L, 120 µg/L, 360 µg/L resulted in induction of CAT. After the depuration phase, however, the results showed an induction in CAT only in organisms exposed to the mixture, suggesting a delayed effect for this treatment. In a study with concentrations similar to those tested in the present investigation, Mezzelani et al reported an induction in CAT for *M. galloprovincialis* after administration of 0.5 µg/L of VAL at the end of depuration phase (Mezzelani et al. 2023). This suggest that in our evaluation, the antihypertensive may have played a crucial role in influencing the outcome of the mixture at 44 days. Concerning GST a significant induction was observed for all the treatments at day 30. GST is phase II enzyme involved in the biotransformation process. Biotransformation is a key process in the metabolism of exogenous compounds. Phase I enzyme belong to the cytochrome family, they are monooxygenase which aim to make compound more soluble to facilitate the excretion. It is widely recognized in literature that exposed pharmaceuticals are metabolized by cytochrome P450 enzymes (phase I) (Nichols and Sanders-Bush, 2003; Zurita et al., 2007; Mezzelani and Regoli, 2021; Godoy et al., 2018). However, many of the metabolite produced during phase I were not sufficiently soluble to be excreted and thus proceeded to phase II. Phase II enzymes play a pivotal role in the excretion mechanisms and in this phase, in fact, enzymes like GST play a crucial role by conjugating glutathione (GSH) to potentially toxic compounds, making them more water-soluble and easily eliminated from cells. In fact, results showed both the induction of the GST and the decrease of total GSH content in all treatments, highlighting the phase II biotransformation process as the main pathway for detoxification of tested pharmaceuticals (Burkina et al., 2015; Cortez et al., 2018). In addition to its primary function, GST also play a minor role in handling peroxidative products of DNA and lipids (Luis et al., 2018). The decrease of GSH level can be attributed to its use in phase II reactions and its role as a direct scavenger of ROS neutralization. Supporting our result Cortez et al. found that exposure to 3000 ng/L of LOS, in *Perna perna,* for 48 hours resulted in an induction of GST (Cortez et al. 2018). The article highlighted the involvement of the cytochrome family, specifically CYP2C9 and CYP3A4 families, which are activated by pharmaceuticals. On the contrary, Barreto et al. (2018), did not observed

induction of GST in *Sparus aurata* exposed to a range from 1.5 to 15000 µg/L of GEM. Regarding other drugs, MET at different concentrations of 40 µg/L, 120 µg/L, 360 µg/L did not induce GST in *Oryzias latipe*s, while in the same study variation of GSH was detected at 120 µg/L and 360 µg/L (Lee et al., 2019), such differences may be attributed to species-specific responses. Previous investigations suggested the absence of induction of GST by antidepressants. Based on this, research on FLX (ranging from 0.005 to 500 ng/g) showed that in polychaetes *Hediste diversicolor* the compound did not induced GST (Maranho et al., 2014). Furthermore, limited studies were conducted on modulation of GST in response to VEN, and some suggest that no differences were detected in super oxide dismutase (SOD), a key enzyme of antioxidant pathways, following exposure to a range from 1 to 1000 µg/L of VEN of Big Ramshorn Snail (*Planorbarius corneus)* (Ziegler et al., 2021). Concerning the results of Glutathione reductase (GR), involved in the conversion of oxidized glutathione (GSSG) in the reduced form (GSH), this enzyme during the exposure phase, showed a statistically significant inhibition in MIX, probably related to the additive interaction of VEN and MET which displayed lower activity compared to CTRL. Conversely to our results, Barreto et al, 2018 reported the induction of GR ranging from 46% to 72 % in gills of *Sparus aurata* at concentrations of 15, 150 and 1500 µg/L of GEM, and in liver an induction ranging from 42 to 75% at concentrations of GEM higher than 1.5 µg/L, this discrepancy with our results may be attributed to the use of different and higher doses employed. Other cases confirmed the induction of GR after administration of FLX at environmental concentration of 1 ng/g for polychaetes *Hediste diversicolor* (Maranho et al., 2014). Again, Mezzelani et al., 2023 observed an induction of GR after 14 days exposure to VAL at concentration of 0.5 µg/L in *M. galloprovincialis*, when comparing these results to our findings, a dominant role of antihypertensive in determining the effect on GR within the mixture can be hypothesized. At depuration phase, GR activity was induced in VEN treatment, suggesting a biphasic and delayed response. GPx CHP and GPx H_2O_2 are enzymes involved in the detoxification of cellular organic hydroperoxides. GPx CHP functions by conjugating GSH to lipid peroxides, thereby neutralizing them and preventing cellular damage. On the other hand, GPx H_2O_2 involves the reaction of H_2O_2in neutralizing hydroperoxides, forming oxygen. A slight but not significant induction was registered for both enzymes at 30 and 44 days in VEN-exposed mussels, This trend is consistent with previous data found in literature, documenting the induction of GPx in *Perna perna* exposed for 48 hours to losartan at concentration of 3000 ng/L (Cortez et al., 2018) and for *Sparus Aurata* exposed to concentration higher than 1.5 µg/L of GEM, where GPx was significantly induced by 156% to 243 % (Barreto et al, 2018). In the present study, the magnitude of variations was not compared to those measured by Barreto et al., (2018) probably due to the higher concentrations and the different used species. At depuration time, the mixture has value higher than

all the treatment, because of interplay among drugs and this suggests that mixture may have a delayed action. Results related the total scavenger capacity (TOSC) of mussels did not reveal significant variation for any treatment after 30 days. Similar results were evidenced by Mezzelani et al., 2023, where the capability to neutralize peroxyl and hydroxyl radicals were not affected by the exposure to VAL (0.5 μg/L) in *M. galloprovincialis*. An inhibition of TOSC toward hydroxyl radical was highlighted after the depuration phase for MET and mixtures, suggesting that these treatments could have a delayed action.

Notably, our results highlighted that all tested therapeutic classes have an influence on antioxidant processes after the exposure phase, inducing the activation of detoxification pathway. The majority of these effects resolved during the depuration phase, as demonstration of the depurative capacity of mussels, as illustrated from previous literature (Mezzelani et al., 2021). The remarkable ability of our model species *Mytilus galloprovincialis*, to return to stable condition and detoxify following exposure to toxic compound, is noteworthy. This resilience highlights the species' capacity to adapt and recover from environmental stressors, it is important from both an ecological point of view and economic perspective. Ecologically, it means the species' ability to recover following anthropogenic stress situation, such as presence of pharmaceuticals in sea water, which is vital for maintaining biodiversity. Additionally, from an economic point of view, mussels' ability to depurate themselves after stress period is crucial for the commercial industries.

6. Conclusions

This thesis evaluated for the first time the biological effects of mixture of four therapeutic classes of drugs, the antidepressant venlafaxine, the lipid regulating agent gemfibrozil, the antihypertensives ramipril and the antidiabetic metformin, in mussels' organisms *Mytilus galloprovincialis*. The overall results on biological parameters highlighted the immune and the antioxidant systems as those mainly modulated by all the tested pharmaceuticals, with evidence of interactive effects for the mixture.

Outcomes of the immune system indicated the lysosomal membrane stability and the phagocytosis capacity of haemocytes as the most sensitive endpoints for the tested drugs, highlighting the lipid regulating agents and the antihypertensives as the most effective among the used therapeutic classes. Such effects were measured both after the exposure and depuration phases, suggesting a long-term effect on the immune system modulation by tested pharmaceuticals.

Results on antioxidant system highlighted an induction of glutathione-S-transferase (GST) and a consequent decrease in level of total glutathione (GSH) for all treatments, suggesting phase II conjugation reactions as one of the key processes for tested pharmaceuticals metabolism in *M. galloprovingialis.*

In conclusion, results of this thesis enhanced our comprehension of the main pathways involved in pharmaceuticals metabolism, indicating dependent effects by the MOAs of different investigated therapeutic class. Furthermore, our results provided a novel insight into potential additive interaction among drugs within the mixture, suggesting the importance of studying the combined effects of different therapeutic classes in order to acquire a more comprehensive understanding of their impact on non-target organisms.

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