UNIVERSITÀ POLITECNICA DELLE MARCHE

FACOLTÀ DI SCIENZE DELLA VITA E DELL'AMBIENTE

Corso di Laurea Magistrale in Biologia Marina

Effetti ecotossicologici di farmaci umani in *Mytilus galloprovincialis*: prime evidenze su ibuprofene, paroxetina e la loro miscela

Ecotoxicological effects of human pharmaceuticals in *Mytilus galloprovincialis*: first insights on ibuprofen, paroxetine and their mixture

Relatore: Prof.
Stefania Gorbi

Tesi di Laurea di:
Alessia Saccardi

Correlatore: Dr.
Marica Mezzelani

Sessione Straordinaria
A.A. 2021/2022

Italian Summary

Effetti ecotossicologici di farmaci umani in *Mytilus galloprovincialis*: prime evidenze su ibuprofene, paroxetina e la loro miscela

Negli ultimi decenni l'interesse da parte della comunità scientifica nei confronti dei contaminanti emergenti è notevolmente aumentato. Fanno parte di questa categoria prodotti per la cura personale, pesticidi, profumi, plastificanti, ormoni e ritardanti di fiamma. Tra questi, particolare attenzione è stata prestata ai composti farmaceutici. Nonostante gli innumerevoli benefici che hanno apportato alla società, il loro elevato consumo sta recentemente suscitando un crescente livello di preoccupazione a causa della loro presenza ubiquitaria e degli sconosciuti effetti biologici ed ecotossicologici che potrebbero avere nel lungo termine sugli organismi non-target. La principale fonte di diffusione in ambiente è rappresentata dagli effluenti degli impianti di trattamento delle acque reflue, in quanto non essendo stati progettati per trattenere queste molecole la loro efficienza di rimozione non risulta sempre essere ottimale. I farmaci sono stati misurati nelle acque superficiali con concentrazioni comprese tra i ng/L e μg/L, questi livelli sono sufficienti per rappresentare un potenziale rischio per le specie aquatiche non-target ed interferire a livello biologico e fisiologico, dato che vengono sintetizzati per essere bioattivi a dosi molto basse.

Da un punto di vista legislativo, sulla base delle evidenze scientifiche, alcuni composti farmaceutici sono stati inseriti dalla Commissione Europea a partire dal 2015 in una "Watch List", una lista di inquinanti emergenti che rappresentano un potenziale rischio ambientale e che quindi necessitano di un attento monitoraggio per la raccolta di dati al fine di trarre conclusioni definitive sulla loro regolamentazione. Nonostante questo, le legislazioni nazionali e internazionali, ad oggi non prevedono dei limiti di concentrazione di queste molecole nelle matrici ambientali. La situazione però potrebbe cambiare a breve, nel caso in cui venisse adottata una proposta di revisione della lista delle "priority substances" della Water Framework Directive, avanzata ad ottobre 2022, che prevede l'inserimento di 9 composti farmaceutici.

Tra le classi terapeutiche più rilevante in ambiente marino troviamo i Farmaci Antinfiammatori Non-Steroidei (FANS) e gli Inibitori Selettivi della Ricaptazione della Serotonina (SSRIs).

I FANS vengono prescritti per le loro proprietà analgesiche, antipiretiche e antinfiammatorie dato che il loro meccanismo d'azione consiste nell'inibizione della conversione di acido arachidonico in prostanoidi, i quali giocano un ruolo importante nei processi omeostatici e patologici e nell'attivazione delle risposte immunitarie. Studi passati hanno già dimostrato la capacità del mitilo Mediterraneo *Mytilus galloprovincialis* di bioaccumulare concentrazioni ambientalmente realistiche di diversi FANS e provocare effetti ecotossicologici dose-dipendenti a livello biochimico e cellulare, interferendo con il sistema immunitario e il metabolismo ossidativo e lipidico.

Gli SSRIs, invece, sono farmaci prescritti per trattare depressione, disturbo ossessivo-compulsivo, attacchi di panico, ansia sociale e disturbo dell'attenzione. Sono farmaci che hanno come bersaglio il sistema nervoso centrale e vanno ad interferire con il sistema serotoninergico aumentando la concentrazione extracellulare di serotonina nelle fessure sinaptiche, incentivandone l'interazione con i suoi recettori. È stato dimostrato che i SSRIs possono agire da distruttori endocrini, interferire con il sistema riproduttivo e causare danni alla crescita, metabolismo, immunità, alimentazione e locomozione di pesci e molluschi a concentrazioni ambientalmente realistiche.

In ambiente marino, tuttavia, è essenziale considerare il fatto che i farmaci non sono presenti come composti singoli ma come miscele complesse costituite da più medicinali e inquinanti di diversa natura. Diventa dunque estremamente importante comprendere come queste sostanze chimiche interagiscono tra loro in ambiente naturale al fine di comprendere l'insorgenza di potenziali effetti sinergici, additivi o antagonisti. Ad oggi, pochi studi sono stati pubblicati sulla tossicità di miscele di farmaci in ambienti acquatici.

In quest'ottica, questa tesi ha avuto come obiettivo quello di valutare il potenziale ecotossicologico del FANS ibuprofene (IBU) e dell'SSRI paroxetina (PAR) e della loro miscela, nell'organismo modello *M. galloprovincialis*. Il piano sperimentale ha previsto una fase di esposizione della durata di 30 giorni in cui gli organismi sono stati esposti ad una concentrazione ambientalmente realistica di IBU (1µg/L), PAR (1µg/L) e IBU + PAR (1 µg/L + 1 µg/L). Al termine della fase di esposizione gli organismi sono stati lasciati per ulteriori 14 giorni in acqua priva di farmaci con l'obiettivo di valutare la capacità di recupero

dalle molecole testate. Al termine dell'esperimento è stato applicato un approccio ecotossicologico integrando le analisi chimiche del bioaccumulo con un ampio numero di risposte biologiche in grado di evidenziare l'insorgenza di alterazioni biochimiche e cellulari come, ad esempio, la modulazione del sistema immunitario, l'insorgenza di effetti neurotossici, cambiamenti del metabolismo ossidativo e lipidico, l'accumulo di prodotti della perossidazione e l'insorgenza di genotossicità. I risultati ottenuti sono stati infine integrati ed elaborati con un modello di analisi di rischio basato su un approccio "Weight of Evidence".

I risultati delle analisi chimiche hanno confermato la capacità dei mitili di bioaccumulare IBU e PAR quando presenti come composti singoli. L'esposizione alla miscela, invece, ha rivelato concentrazioni di IBU al di sotto dei limiti di rilevamento strumentali. Dunque, è stato osservato che in presenza di PAR, l'accumulo di IBU è ridotto. Questi risultati suggeriscono la presenza di una possibile interazione tra le due molecole con un meccanismo di competizione che vede PAR avere la meglio su IBU. Questa dinamica potrebbe essere spiegata dal fatto che entrambe i farmaci competono per gli stessi trasportatori di membrana e PAR, essendo più affine ad essi, impedisce il bioaccumulo all'interno della cellula di IBU.

Per quanto riguarda i risultati relativi ai biomarker, lo studio del tempo di ritenzione del rosso neutro ha evidenziato una diminuzione della stabilità delle membrane lisosomiali, che si è mantenuta anche dopo il periodo di depurazione, in tutti gli organismi esposti ai trattamenti. Effetti simili erano già stati documentati in letteratura per entrambe le molecole. Nessun effetto sinergico è stato osservato negli organismi esposti alla miscela. La modulazione del sistema immunitario, però, non è stata confermata dagli altri parametri coinvolti, quali capacità di fagocitosi e rapporto granulociti-ialinociti, i quali non hanno mostrato variazioni significative in nessun trattamento rispetto al controllo.

La genotossicità è stata testata analizzando l'integrità strutturale del DNA e la frequenza di micronuclei. Un aumento significativo nella percentuale di frammenti di DNA è stato verificato negli organismi esposti ad IBU al giorno 30 e in tutti gli altri trattamenti dopo la fase di depurazione, a riprova del fatto che gli effetti dell'esposizione ai farmaci persistono anche dopo la loro escrezione. Nonostante i livelli di frammentazione del DNA (da 10,7% a 37,5%) dei mitili esposti ai trattamenti si sono rivelati essere sempre più alti rispetto ai controlli (6,4% di valore massimo), questi valori rientrano comunque nel naturale range di

variazioni fisiologiche misurate in natura. Anche nel caso della frequenza dei micronuclei, livelli significativamente più alti del controllo sono emersi in tutti gli esposti, e in maniera statisticamente significativa per IBU, sia durante la fase di esposizione che dopo la depurazione. Dati analoghi erano già stati documentati per IBU e, dato che non risulta essere un composto mutageno, è probabile tale risposta sia legata ad una modulazione del ciclo cellulare da parte dei FANS che va a promuovere il turnover cellulare, e non ad un effettivo danno genotossico.

L'attività dell'acetilcolinesterasi analizzata nell'emolinfa e branchie dei mitili come biomarker di neurotossicità non ha mostrato differenze statisticamente significative in nessun trattamento rispetto agli organismi di controllo. Nel caso degli organismi esposti a PAR era ipotizzabile una modulazione dell'attività dell'acetilcolinesterasi data l'interazione di questa classe terapeutica di farmaci con il sistema nervoso, tenendo conto anche del fatto che effetti neurotossici erano già stati documentati in studi precedenti in seguito all'esposizione di organismi ad altre tipologie di SSRIs.

Squilibri tra forze pro-ossidanti e difese antiossidanti, così come un aumento intracellulare della produzione di specie reattive dell'ossigeno, sono stati analizzati integrando i risultati delle attività di singoli enzimi antiossidanti con la capacità antiossidante totale (TOSC) degli organismi di neutralizzare i radicali idrossilici e perossilici. Per i singoli enzimi antiossidanti è stata misurata un'induzione statisticamente significativa della catalasi in tutti i trattamenti sia al giorno 30 che al giorno 44, e per glutatione perossidasi, nei trattamenti al giorno 30. Fluttuazioni non statisticamente significative sono state osservate per le attività degli altri enzimi antiossidanti, livelli di glutatione totale e capacità antiossidante totale.

È stato interessante osservare come a livello cellulare si sia osservato un aumento dell'accumulo di prodotti della perossidazione lipidica, in particolare lipofuscina e lipidi neutri. La lipofuscina ha mostrato livelli statisticamente più alti rispetto al controllo ad entrambi i tempi di esposizione in tutti i trattamenti, ma soprattutto in organismi esposti a PAR. I mitili trattati con la miscela hanno evidenziato risultati comparabili a quelli esposti a IBU, suggerendo un'influenza dovuta solamente al FANS. Per quanto riguarda i lipidi neutri, tutti i trattamenti hanno mostrato un significativo aumento rispetto al controllo, anche al termine della fase di depurazione, specialmente negli organismi esposti a IBU. Nessun cambiamento significativo è stato evidenziato per i livelli di malondialdeide e acyl-CoA ossidasi.

Per fornire un'indicazione del potenziale rischio ambientale derivante dalla presenza di questi composti nei mitili, i risultati ottenuti sono stati elaborati con un modello di analisi di rischio basato sull'approccio Weight of Evidence (WOE). Questo modello integra dati eterogenei in un indice sintetico di rischio sulla base della rilevanza biologica della risposta analizzata, l'entità della variazione rispetto al controllo ed a specifici valori soglia. I risultati di questa elaborazione hanno evidenziato un livello di rischio "Moderato" per tutti i trattamenti al giorno 30 e una diminuzione del livello di rischio a "Lieve" al termine della fase di depurazione, confermando la capacità di questa specie di recuperare le alterazioni subite in un lasso di tempo relativamente breve.

In conclusione, i risultati complessivi suggeriscono la potenziale presenza di meccanismi di competizione tra le molecole testate nel momento in cui vengono combinate in miscela. Tale interazione negli organismi esposti alla miscela causa un minore accumulo di IBU e di conseguenza una modulazione delle risposte biochimiche e cellulari prevalentemente legata alla presenza di PAR.

Alla luce di questi risultati si evince la necessità di investigare in studi futuri il meccanismo di bioaccumulo di queste molecole e di studiare in maniera più approfondita i meccanismi di azione e interazione.

La rielaborazione dei dati tramite il modello WOE ha permesso di fornire un indice di rischio complessivo per le molecole testate, rappresentando un importante valore aggiunto per facilitare la disseminazione e divulgazione di queste informazioni di rilevanza ambientale al di fuori della comunità scientifica.

1. Introduction

1.1 Pharmaceuticals as contaminants of emerging concern

Contaminants of emerging concern (CECs) are “naturally occurring, manufactured or manmade chemicals or materials which have now been discovered or are suspected present in various environmental compartments and whose toxicity or persistence are likely to significantly alter the metabolism of a living being" (Sauvé et al., 2014).

The scarcity of information in the scientific literature and the poorly documented issues about the associated potential problems they could cause, are the reason why CECs are defined as "emerging". However, an official unified definition of CECs has not yet been reached by the scientific community making it more difficult for the different regulatory agencies to monitor and regulate these chemicals, leading to potentially underestimate the risk posed to the marine environment. As of today, several thousands of CECs compounds have been identified (Dulio et al., 2018) belonging to different categories of organic chemicals such as pharmaceuticals, personal care products (PPCPs), pesticides, fragrances, plasticizers, hormones and flame retardants.

Among all of them, pharmaceuticals represent one of the most interesting economies to follow, being the driver for medical progress by researching, developing and delivering every year new medicines. The world’s population increasing by 1 billion people since 2010, together with the increase in life expectancy levels (UN World Population Prospects, 2022), caused a growing demand of drugs to prevent and cure diseases and age-related medical issues. In fact, the consumption in OECD countries of anti-hypertensive drugs, lipid-modifying agents, anti-diabetic and anti-depressants has risen by 65%, 300% and 100% respectively between 2000 and 2019 (OECD Health at a Glance, 2021). Consequently, the value of the world pharmaceutical market has steadily increased with an annual rate of 5.8% since 2017 and in 2020 it was estimated at around 1 billion euros, with the American’s being the world’s largest market, followed by Europe, China and Japan (EFPIA The Pharmaceutical Industry in Figures, 2021).

Despite the innumerous benefits that pharmaceutical consumption has produced in human society, their massive and unregulated release into the environment has, rather recently, sparked an increasing level of concern regarding their ubiquitous distribution and the unknown biological and ecotoxicological long-term effects they might have on non-target

organisms. Detected for the first time in the early 1990s when traces of anticancer drugs were found in sewage effluents, rivers and drinking water, pharmaceuticals belonging to a variety of therapeutic classes have since been identified in the marine environment (Aherne et al. 1990; Mezzelani et al., 2018a). Nowadays, the most frequently detected therapeutic classes of drugs in surface waters are anti-inflammatory drugs, analgesics, antibiotics, lipid regulators, steroids and related hormones, beta-blockers, psychiatric and cytostatic drugs (Fatta-Kassinos et al., 2010).

Medicine residues and their metabolites enter the marine environment through different pathways including hospital effluents, industrial wastewaters, landfill leachate, runoffs from concentrated animal feeding operations and aquaculture, but the main one is represented by wastewater treatment plants (WWTPs) effluents (Kasprzyk-Horderna et al., 2009). These facilities receive and treat sewage waters from various sources including domestic households. Many individuals improperly discard unutilized or expired prescription medications into their sinks and toilets unaware of their fate (Petrovic et al., 2004). Moreover, ingested drugs that aren't completely absorbed by the organism are metabolized and then excreted through urine and faeces. The amount of drugs released into the environment after human and/or veterinary usage depends on metabolism and excretion processes, which vary according to the type of ingested chemical, and the posology.

Once medicines arrive to WWTPs, unfortunately, these systems aren't often efficient in the removal of pharmaceuticals since they weren't originally designed to decompose these compounds but rather to control substances such as particulates, carbonaceous substances, nutrients and pathogens (Luo et al., 2014). The stability of the molecules, their non-volatility properties and the polar functional groups in the chemical structure frequently determine the transfer of pharmaceuticals from WWTPs to surface waters (Madikizela et al., 2020; Ojemaye et al., 2018). Removal rates can range from less than 10% to 100% depending on the physio-chemical properties of the compound and the type of treatment applied (Gaw et al., 2014).

Conventional WWTPs usually offer a primary and a secondary treatment process while technologically advanced WWTPs also have a tertiary treatment which increases the efficiency and consistency of the system in the removal of selected compounds up to 100% (Balabanič et al., 2012). Primary treatment processes have the objective of removing suspended solids mainly through sorption. Removal efficiency at this stage for

pharmaceuticals ranged up to only 28% for some and had no significant reduction for others (Behera et al., 2011; Carballa et al., 2004). In secondary treatment chemicals are subjected to a biological degradation resulting in mineralization or production of by-products (Luo et al., 2014). Pharmaceutical compounds exhibit substantial variability in their biodegradability. For instance, among Non-Steroidal Anti-Inflammatory-Drugs (NSAIDs), ibuprofen showed high tendency to biodegradability (> 75%) compared to diclofenac (<25%) (Salgado et al., 2012). Lastly, tertiary treatment processes are based on more advanced technologies like finer filtration techniques and absorption by activated carbon, however their implementation is still relegated to higher quality water purposes since it's associated to higher costs (Mezzelani and Regoli, 2022). The outcome of these transformation processes is the mineralization of pharmaceuticals and their metabolites to carbon dioxide and water or, in the case of lipophilic compounds, adsorption on suspended solids or discharge through WWTPs effluents as deteriorated products. From these effluent sources, the fate for these chemicals is to be transported and accumulated in different environmental matrices like surface water, groundwater, sediments and biota (Parra-Saldivar et al., 2021).

In 2018, at least 80 different pharmaceuticals were found at significant concentrations in all the environmental matrices (Ojemaye et al., 2018) with levels ranging from a few nanograms per liter, ng/L, to hundreds of micrograms per liter, µg/L (Huerta et al., 2012).

More precisely, concentrations ranging from 0.01 ng/L to 2.4 mg/L have been detected in surface seawater. Such a big discrepancy between the lowest and the highest values can be attributed to different factors. The geographical location and population density of the area influence the usage and therefore the discharge of these chemicals which are found in higher levels close to largely populated coastal areas that can be considered as hotspots of pharmaceutical release (Fabbri and Franzellitti, 2016). Physical and chemical characteristics of the active principles affect their dispersion in the environment. For instance, the more hydrophilic a molecule is the more likely it's going to persist in the water column, differently from lipophilic molecules that are more easily available for organisms or can sink into sediments (Mezzelani et al., 2018a). Physiochemical properties of seawater such as temperature, salinity and water pH can also control the behavior of pharmaceuticals. Even atmospheric conditions can impact pharmaceutical concentration in the marine environment. Solar irradiation can trigger photodegradation processes while rainfall can act both as natural

attenuation, causing river water dilution, but also as intensifier of the emission of pharmaceuticals on surface waters because of leaching and sewer overflows resulting in the direct discharge in the marine environment (Luo et al., 2014).

Lower concentrations, compared to surface water, have been found in groundwater going from 10 ng/L to 1 μg/L (Lapworth et al., 2012). Sediments represent an environmental matrix that can act as a sink and secondary pollution source from which these chemicals can be released back into seawater following environmental physiochemical changes, storm events or tidal changes. Concentrations found in this matrix are rather variable (e.g. 0.1 ng/g of dry weight, d.w.; 820 mg/g of wet weight, w.w. Fabbri and Franzellitti, 2016).

Much less documented is the bioaccumulation of pharmaceuticals in biological tissues from both wild populations and under controlled laboratory conditions mainly because it's more complex to extract, separate, purify and analyze these compounds in biota compared to abiotic matrices. As a rule, it is assumed that chemicals with an octanol-water partition coefficient (log K_{OW}) value equal to 3 or higher can be bioaccumulated in biota. However, accumulation in biological tissues depends also on other factors such as uptake and depuration kinetics that represent a key concept to understand the biodegradation and biotransformation pathway of these chemicals, especially in non-target species where there is the need for more in depth investigation (Huerta et al., 2012). Studies have detected the presence of pharmaceuticals in several marine organisms including macroalgae, bivalves, fish, cephalopods and arthropods (Álvarez-Muñoz et al., 2015a; Ali et al., 2018; Martínez-Morcillo et al. 2020).

Świacka et al. in a recent review in 2022 found that the most frequently detected classes of pharmaceuticals in marine organisms greatly varied among countries with psychoactive drugs and antibiotics being at the top of the list in Europe, antihistamines and antibiotics in the United States and antibiotics and NSAIDs in Asia. This could be related to the different health problems and drugs' prescriptions that are prevalent in these geographical areas.

Regarding concentration levels, the highest values in wild non-target species belonged to antibiotics and NSAIDs with concentrations exceeding 500 ng/g, followed by psychoactive drugs and hormones reaching significant levels of 100-500 ng/g (Świacka et al., 2022).

Active Pharmaceutical Ingredients (APIs) pose a potential risk to non-target aquatic species since they are designed to be bioactive at very low concentrations and, because of this, can potentially interfere at the biological and physiological level. This has been confirmed by

recent investigations that found particular targets of human pharmaceuticals conserved in the evolution process in marine animals (Fabbri and Franzellitti, 2016).

In order to evaluate possible adverse outcomes caused by environmental pharmaceuticals to no-target marine species, a series of ecotoxicology studies have been performed to characterize both acute and sublethal effects.

The acute toxicity is typically evaluated through a battery of toxicity and biological tests with whom different biological endpoints can be evaluated: death, development anomalies, behavioral modifications, changes in reproductive success, growth alterations and bioluminescence. These tests are performed under controlled laboratory conditions where standard organisms (e.g. *Vibrio fischeri*, *Paracentrotus lividus*, *Crassostrea gigas*, *Mytilus galloprovincialis*) are exposed to increasing doses, including lethal doses, of contaminants, or mixtures of contaminants contained in a matrix. The aim for the use of toxicity tests is to define a relationship between severity of an effect and doses of the corresponding chemical that causes the effect. Regarding pharmaceuticals, large number of laboratory studies demonstrated as highly impossible the onset of acute toxicity at environmentally realistic concentrations and much more plausible the emergence of sub-lethal effects due to chronic exposure (Adeleye et al., 2022).

The most widespread approach used by the scientific community to investigate the capability of contaminants to cause chronic toxicity, rely on investigation of specific bioindicator organisms in order to measure a wide panel of molecular, biochemical and cellular alterations, defined as biomarkers. Bioindicator organisms are used in the ecotoxicological approach because they can give us an information about the bioavailability of a contaminant and because they allow us to analyze integrated biological effects.

An organism to be considered as a bioindicator should present specific characteristics such as: resistance to environmental stress, tolerance to pollutants, reactivity to a wide spectrum of environmental changes, contaminants baseline levels known but, most importantly, inability to regulate tissue concentrations of contaminants in order to show in tissues higher concentrations that in the abiotic matrices. Mussels, in particular the Mytilidae family, check all these requirements and because of that have now been used for decades as sentinel organisms for marine monitoring programs and laboratory experiments.

Many xenobiotics can act as stressors and cause direct or indirect alterations on different biological pathways leading to an imbalance between prooxidant forces and antioxidant

defenses, an activation of the immune system, modification of the lipidic metabolism and ultimately provoke toxic effects. In this context, biomarkers have been used both in laboratory conditions and in field studies to detect early warning signals of chemical toxic effects (Monserrat et al., 2007). Their investigation is performed in target organs and tissues (e.g. liver, gills, hepatopancreas, hemolymph and blood).

At the cellular and sub-cellular level, biomarker analysis has shown that NSAIDs can cause an early activation of the immune system in *M. galloprovincialis* and *Ruditapes philippinarum* with a significant impairment of hemocytes responsiveness and destabilization of lysosomal membranes (Almeida et al., 2020). They can also promote ROS generation and prooxidant mechanisms by modulating antioxidant enzymes, causing accumulation of peroxidation products and the onset of genotoxicity, as observed in *Mytilus spp.*, *R. philippinarum*, *Gibbula umbicalis* and *Hediste diversicolor* (Bebianno and Gonzalez-Rey, 2015; Mezzelani et al., 2018a, b; Milan et al., 2013). Similarly, also psychiatric drugs have shown the ability to modulate intracellular ROS formation and antioxidant levels with the peculiar characteristic of having either antioxidant or prooxidant activity depending on the tissues, dose and presence of a preexisting oxidative imbalance (Stefan et al., 2020). For example, *M. galloprovincialis*, *Perna perna* and *R. philippinarum* exposed to fluoxetine (0.003-5 μg/L) showed induction of glutathione S-tranferase activity coupled with impairment of immune parameters, decreased lysosomal stability, increased levels of malondialdehyde (a typical peroxidation product) and variations of antioxidant enzymes, indicating activation of xenobiotic metabolism and an antioxidant response (Franzellitti et al., 2014; Munari et al., 2014). Concerning steroidal hormones, one of the most frequently measured effects in fish is an increase in vitellogenin (Mezzelani and Regoli, 2022), precursor of the egg-yolk proteins in oviparous organisms. Normally this protein is produced only in females in response to endogenous estrogens, however, it has been shown that it can be found also in males exposed to xenoestrogens, making it an excellent biomarker to detect if an organism has come into contact with these chemicals. Even though knowledge on its mechanism of action in invertebrates is more limited, a significant increase in the expression of vitellogenin and the estrogen receptor ER2 has been detected in female and male mussels (*Mytilus edulis*) exposed to environmentally realistic concentrations (5 or 50 ng/L) of ethynilestradiol, a synthetic steroidal estrogen (Almeida et al., 2020a).

More complex and still more difficult to predict are long-term effects at higher levels of biological organization from the organismal level to ecosystem functioning. Nevertheless, studies have found that NSAIDs impact early mussel embryos by modulating genes involved in the shell formation and mineralization while in adult specimens they can reduce byssus abundance and strength causing a decrease in their growth (Balbi et al., 2018).

Selective serotonin reuptake inhibitors' (SSRIs) chronical exposure demonstrated that they play a role in the regulation of feeding and metabolism in bivalves (Gonzalez-Rey and Bebianno, 2013) as well as on locomotion in various marine invertebrates (Estévez-Calvar et al., 2017; Fong and Ford, 2014), phototactic responses in amphipods (*Gammarus pulex*) and inhibition of striking prey efficiency in newborn cuttlefish (*Sepia officinalis*) (Bidel et al., 2016). Reproduction effects in fish caused by estrogen exposure include feminization of males, with the presence of oocytes in the male gonads, compromised spermatogenesis, decrease in sperm counts and motility (Almeida et al., 2020; Aris et al., 2014; Notch et al., 2007) and altered reproductive behavior (Saaristo et al., 2010). Marine invertebrates (polychaete worms, molluscs and crustaceans) show similar effects such as developmental delays and female-biased sex ratios (Roark, 2020), but also changes in energy metabolism, particularly energy depletion of spermatozoa, which in *M. galloprovincialis* led to low percentage of fertilized eggs (Almeida et al., 2020).

1.2 The Water Framework Directive

Despite the massive consumption and release into the environment and the widespread awareness about biologically active compounds, the majority of pharmaceuticals have not been included neither in environmental regulation nor in routine monitoring programs (Mezzelani et al., 2020).

Regulation of water quality started in 1975 with the European water legislation to protect human and environmental health from potential adverse effects of toxic and persistent pollutants and finally in October 2000 the EU Water Framework Directive (2000/60/EC) was adopted as a pioneering piece of legislation to satisfy the need for a more comprehensive and global approach to water policy. The main objective of EU Water Framework Directive, which covers surface waters including inland, transitional, coastal waters and groundwaters, is to protect aquatic ecosystems and make sure that they achieve good ecological and chemical status. However, there is growing concern that this goal will be far from being achieved in many countries (Carvalho et al., 2019). To date, in fact, only 38% of EU surface waters are considered in good status by the European Environment Agency (EEA Report No 7/2018). Ecological status is evaluated by looking at biological quality elements, such as phytoplankton, phytobenthos, macrozoobenthos, macrophytes and fish, but also hydromorphological and physiochemical parameters (EEA Report No 7/2018), however, because of ecological variability across different ecosystems and communities, no absolute standard for biological quality can be set. Therefore, a good ecological status is considered when there is a minimal discrepancy between the measured values and the ideal expected values in conditions of minimal anthropogenic activity; even if one biological quality element or other parameter isn't of good quality, then the overall ecological status of the waterbody will be dictated by the lowest score of all the values, following the so called "one-out-all-out" principle (Carvalho et al., 2019). Good chemical status, on the other hand, is achieved when priority substances listed in Annex X of the WFD meet their environmental quality standards (EQS).

Priority substances are chemicals selected by the Commission because they pose a serious risk to or via the aquatic environment. Among them we can distinguish hazardous priority substances, which can be defined as chemicals that are toxic, persistent and that are able to bioaccumulate (2000/60/EC).

The reason behind the creation of this list, and the ultimate goal of this Directive, is to progressively reduce back to background levels those naturally occurring priority substances and to eliminate, or get values really close to zero, for hazardous priority substances (2008/105/EC).

The list of priority substances has been introduced by the Environmental Quality Standards Directive in 2008 (2008/105/EC) with 33 compounds and has been updated in 2013 (2013/39/EU) with the total number increasing to 45.

No pharmaceutical compound has ever been part of this list, yet this could change in the near future. As a matter of fact, in October 2022 the European Commission has adopted a proposal for the revision of the priority substances list. This would entail the addition of 24 substances among which we find 9 pharmaceuticals: the sexual hormones 17-beta estradiol, Estrone (E1) and EE2; the antibiotics azithromycin, clarithromycin and erythromycin; the anticonvulsant carbamazepine; and the NSAIDs diclofenac and ibuprofen.

If this proposal gets to be accepted by the Council and the European Parliament, Member States will be required to meet the quality standards for all the substances added making it the first time ever in the European Union that the emissions and release into the environment of pharmaceutical compounds get to be regulated.

Having said that, it isn't the first time that human drugs' concentrations in surface waters have been monitored. A Watch List was established in 2015 under the EQSD (2013/39/EU) to improve the available monitoring knowledge on emerging pollutants and substances of greater concern that could pose a risk at Community level but for which there is insufficient data to draw definitive conclusions (Cortes et al., 2022). The Watch List has since been updated every two years in 2018, 2020 and more recently in 2022.

Member States should monitor at least once a year, up to four years, all the substances reported on the list since each substance can't be continuously monitored for more than four years. After that period, the substance must be taken out of the list and other substances or group of substances can be added to it, to a maximum of 14.

The first Watch List included 4 pharmaceuticals [diclofenac,17-beta-estradiol (E2), 17-alpha-ethinylestradiol (EE2) and macrolide antibiotics] while in the latest update in 2022 we find 6 of them (5 antibiotics including sulfamethoxazole, trimethoprim, venlafaxine and o-desmethylvenlafaxine, clindamycin, ofloxacin and the anti-diabetic drug metformin with its transformation product guanylurea).

1.3 Ibuprofen and paroxetine and their ecotoxicological potential

Over the last few decades, a growing scientific interest has been shown towards the presence and bioaccumulation of pharmaceuticals in the aquatic environment but more recently a pending task has been to identify the ecotoxicological potential of these molecules that are persistent at low concentrations in the environment and could produce sub-lethal effects correlated to a chronic exposure (Mezzelani et al., 2018a).

Among the most relevant therapeutic classes of pharmaceuticals that represent contaminants of emerging concern in the marine environment there are NSAIDs and psychiatric drugs including SSRIs (Parolini, 2020; Mezzelani and Regoli, 2022).

NSAIDs are some of the most commonly used pharmaceutically active compounds worldwide and are prescribed for their analgesic, antipyretic and anti-inflammatory properties to both humans and animals for veterinary use (Parolini, 2020). In Italy, during 2021, about 15 out of 100 citizens received at least one prescription of NSAIDs (OsMed, 2021).

The onset of pain in the body causes increased levels of arachidonic acid which is transformed by the cyclooxygenase (COX) pathway into prostanoids – prostaglandin and thromboxane (Mazaleuskaya et al., 2015). These prostanoids differently modulate intracellular levels of cAMP and calcium playing an important role in a series of homeostatic and pathological processes and in the activation of immune responses (Mezzelani and Regoli, 2022). NSAIDs inhibit the conversion of arachidonic acid into prostanoids by inhibiting one or both isoforms of the COX enzyme. Ibuprofen is classified as a non-selective NSAID because it acts on both COX-1 and COX-2. Once in the organism, it's partially metabolized and the remaining residue is then found in the urine (Mazaleuskaya et al., 2015).

The mechanism of action of NSAIDs displays similarities between vertebrates and invertebrates which were confirmed by a differential expression of genes involved in arachidonic acid metabolism after the exposure of *Mytilus spp.* to different concentrations of ibuprofen (Almeida et al., 2020b).

Because of their massive use, NSAIDs account for 15% of drugs detected in aquatic ecosystems worldwide (Santos et al., 2010) and among them ibuprofen is one of the most commonly identified (He et al., 2017).

Numerous studies under laboratory conditions showed not only the ability of *M. galloprovincialis* to accumulate ibuprofen and other NSAIDs at environmentally realistic concentrations but also the onset of dose-dependent ecotoxicological effects with biochemical and cellular alterations including transitory induction of antioxidant enzymes, increase of lipid peroxidation, decrease of lysosomal membrane stability and inhibition of acyl-CoA oxidase activity, an enzyme of the peroxisomal ß-oxidation pathway, typical biomarker for peroxisome proliferation (Gonzalez-Rey and Bebianno, 2011, 2012, 2014; Mezzelani et al., 2018b).

Among antidepressants, SSRIs are the most commonly prescribed to treat clinical depression, obsessive-compulsive disorder (OCD), panic disorders, social anxiety and attention deficit disorder (ADHD) (Mezzelani et al., 2018a). Consumption of antidepressant drugs in OECD countries more than doubled from 2000 to 2019 (OECD, 2021) creating a concerning picture at environmental level since their high biological activity can pose risks to non-target species (Schulze et al., 2010).

Paroxetine is one of the most used SSRIs and ranked second in terms of popularity index (index representing the proportion of articles published on paroxetine compared to all the articles on the topic of major depressive disorder in the period 1988-2017) (Tran et al., 2019). It's a pharmaceutical that targets the central nervous system and interferes with the serotonergic system. It modulates the concentration of serotonin [or 5-hydroxytryptamine (5-HT)] by increasing its extracellular level in the synapsis cleft. Serotonin is a molecule that functions both as a neurotransmitter and a hormone. In natural conditions, 5-HT is stored in synaptic vesicles and, only following an action potential, it's released allowing it to bind with 5-HT receptors and modulate the associated pathway. Paroxetine is designed to inhibit 5-HT re-uptake by binding with the 5-HT transporter protein and distorting its tertiary structure causing it to be blocked and accumulate in the synaptic cleft, enhancing its interaction with 5-HT receptors (Mezzelani and Regoli, 2022). This serotonergic signaling pathway is conserved in marine organisms, both vertebrates and invertebrates, even if its sensitivity can greatly vary among different organisms (Sumpter et al., 2014). Because of this, paroxetine could cause adverse effect in many marine species acting as an endocrine disruptor and interfering at the reproductive level, but it has also been showed that it can cause impairment to the growth, metabolism, immunity, feeding and locomotion of fish and molluscs even at environmentally realistic concentrations (Mezzelani et al., 2018a).

Paroxetine was detected in more than 40% of wild mussels in the Mediterranean (Mezzelani and Regoli, 2022) nonetheless, scientific knowledge on its potential negative effects on non-target organisms is still scarce.

In marine ecosystems it's important to consider that pharmaceuticals aren't present as single compounds but rather as complex mixtures with other drugs and pollutants. Understanding whether interactions between the single compounds can have additive or antagonistic effects is becoming a priority, to get an idea of how these chemicals really interact in the natural environment and what could be the actual effects on marine organisms. Very few studies have been published on the toxicity of mixtures of pharmaceuticals in aquatic ecosystems.

One by Gonzalez-Rey et al. (2014) exposed *M. galloprovincialis* for 7 days to a mixture of two NSAIDs (diclofenac and ibuprofen) and one SSRI (fluoxetine) and found out that the observed effects were significantly different in mixture-exposed organisms compared to those treated with single molecules. Another study, by Franzellitti et al. (2015), tested the toxicity of a mixture of two SSRIs (propranolol and fluoxetine) in the same model organism, *M. galloprovincialis*. In this case it was observed that co-exposure induced antagonistic effects to the cAMP content of the organisms.

Although these studies have started the discussion around the effects of pharmaceutical mixtures on marine organisms, no one yet has investigated specifically the interactions between ibuprofen and paroxetine and their ecotoxicological potential in marine invertebrates after chronic exposure and, in this context, this thesis aims to fill this gap of knowledge.

1.4 Environmental Risk Assessment and WOE model

One of the big challenges related to the presence of APIs in marine environment is represented by the need to develop an Environmental Risk Assessment (ERA) procedure that can give a characterization of the environmental quality of the ecosystem investigated.

With that in mind, recently the scientific community has agreed on the importance to combine information on the presence of contaminants in abiotic matrices with data on bioaccumulation and effects on biota to obtain a multidisciplinary integrative approach that has also been strongly encouraged in the context of the Water Framework Directive (Directive 2000/60/EC).

In this respect, the WOE (Weight of Evidence) model represents a fundamental tool for summarizing and interpreting complex heterogeneous datasets and deliver qualitative and quantitative evaluations with the development of indices and scales. The model is innovative since it has abandoned the pass-to-fail approach, which was based on comparison of individual thresholds or the worst result for ecotoxicological risk and has instead introduced a weighted criteria system (d'Errico et al., 2021), enhancing the capability to discriminate different environmental conditions. Moreover, the WOE model is versatile, easy to adapt to different case studies and its user-friendly format allows to deliver an easy-to-understand communication of the environmental risks to policy makers and non-expert stakeholders, while maintaining scientific robustness (Linkov et al., 2009).

In this context, this study aimed to use the WOE model as a practical tool to better communicate the results obtained and integrate them in environmental impact assessment.

2. Aim of the Study

Widely detected in aquatic ecosystems, pharmaceuticals can bioaccumulate and potentially interfere with physiological and biochemical processes of marine species. Since this class of emerging pollutants is characterized by compounds with different chemical structures and modes of action, the adverse effects of APIs mixtures on non-target organisms are still little unknown.

In this respect, this thesis aims to assess the ecotoxicological effects produced in the model organism *Mytilus galloprovincialis* due to long-term exposure at environmentally realistic concentrations to the NSAID ibuprofen, the SSRI paroxetine, and their mixture, compounds belonging to two of the main therapeutic classes detected in marine ecosystems.

An ecotoxicological approach was applied integrating chemical analyses on drug accumulation with a large panel of biomarkers reflecting the impairment of various biochemical and cellular districts including variations of the immune system, the onset of neurotoxic effects, changes in oxidative and lipid metabolism, accumulation of peroxidation products and the onset of genotoxicity.

The overall results were elaborated to summarize a cellular hazard index based on toxicological relevance, magnitude of variations and responsiveness of analysed endpoints.

The present study was expected to advance the scientific knowledge on the potential interactions (e.g. additive, synergistic or antagonistic) among different pharmaceuticals to better clarify the environmentally realistic conditions in which organisms live and fill the gap of information on the ecological consequences of these molecules in aquatic ecosystems.

3. Materials and Methods

3.1 Experimental Design

Mussels (*Mytilus galloprovincialis*) were collected in March 2022 from a local farm in Senigallia (Ancona, Adriatic Sea) and acclimatized for 7 days with aerated artificial seawater, at temperature 18 ± 1 °C and salinity 33 ‰.

The 240 specimens (average valves length size: 5,5 ± 1,00 cm) were randomly distributed into 4 glass-tanks (n=60 per tank) in a volume of 17 L of artificial seawater.

The experimental design included two tanks with organisms exposed to 1 μg/L of a single pharmaceutical compound, namely ibuprofen IBU (NSAID) and paroxetine PAR (SSRI), one tank with organisms exposed to a mixture of IBU + PAR (1 μg/L + 1 μg/L) and one solvent control tank (CTRL) where methanol was added at the same concentration used in the treatments.

Due to their low solubility in water, stock solutions (1 mg/mL) of IBU and PAR were obtained by dissolving each drug powder in methanol (MeOH) and stored at + 4°C. The final experimental solutions (34 mg/L) were prepared daily by diluting the stock solutions in MeOH and in each tank were added 500 μL in order to obtain the single APIs and mixtures concentration of 1 μg/L and 1μg/L + 1μg/L respectively. Water changes in the tanks and pharmaceutical administration were done every 2 days. Food (Easy sps EVO) was administered every 2 days, before the water change.

Mussels were exposed for 30 days and then were maintained for additional 14 days in pharmaceuticals-free artificial seawater intended as a depuration phase.

At days 30 and 44, mussels were dissected and the whole tissues collected for chemical analysis and stored at -20°C. Hemolymph, digestive glands and gills were frozen in liquid nitrogen and stored at -80°C for histochemical and biochemical analysis.

Two aliquots of hemolymph were immediately processed: one for lysosomal membrane stability, phagocytosis activity, granulocytes-hyalinocytes ratio and DNA damage; the other was fixed in Carnoy's solution (3:1 methanol and acetic acid solution) for the microscopy evaluation of micronuclei frequency.

Biomarkers in tissues were measured through standardized protocols which included: microscopy analysis of hemocytes for lysosomal membrane stability through neutral red retention time (NRRT), phagocytosis activity and granulocytes-hyalinocytes ratio (G/H);

histochemical quantification of lipofuscin (LIPO) and neutral lipids (NL) on digestive gland cryostat sections; spectrophotometric determination of acyl-CoA oxidase (ACOX), acetylcholinesterase (AChE) activity in hemolymph and gills, single antioxidant defenses activity in digestive glands [catalase (CAT), glutathione S-transferases (GST), Se-dependent glutathione peroxidases (GPx H_2O_2), sum of Se-dependent and Se-independent glutathione peroxidases (GPx CHP), glutathione reductase (GR) and total glutathione (GSH) content]; gas-chromatographic assay of the total oxyradical scavenging capacity (TOSC) toward peroxyl (ROO•) and hydroxyl radicals (HO•) in digestive glands; chromatographic determination of malondialdehyde (MDA); electrophoretic and cytogenetic analysis of DNA integrity in hemocytes through DNA fragmentation (COMET assay) and micronuclei frequency (MN).

3.2 Chemical analysis

Analytical standards (purity > 97%) were purchased from Sigma Aldrich (Milan, Italy) for ibuprofen IBU (CAS 15687-27-1) and paroxetine PAR (CAS 110429-35-1). Stock solutions (1 mg/mL) were prepared in methanol acidified at 0.1% with acetic acid in amber vials and stored at +4°C in the dark to reduce possible degradation.

Samples were prepared by using 3 g of wet tissue homogenized in 5 mL of buffer (acetic acid 0.1%) at room temperature for 10 minutes using a dispersing, stirring, homogenizing and grinding system (IKA ULTRA® TURRAX® Tube Drive). After centrifugation at 4500 xg for 30 minutes, samples were purified by Solid Phase Extraction (SPE). SPE tubes were conditioned with 1 mL of methanol, followed by 3 mL of ultra-pure water. Samples were diluted (1:1) with ultra-pure water and loaded onto the SPE cartridges, washed with 2 volumes (6mL) of potassium bicarbonate $KHCO_3$ and 1 volume (6 mL) of ultra-pure water. Analytes were eluted and recovered using 2 mL of a solution made by methanol acidified at 0.1% with acetic acid (HPLC, gradient grade, Carlo Erba). Samples obtained were filtered using Phenex™-RC membrane (Regenerated Cellulose/Polypropylene 0.45 μm, 15 mm syringe filters, Phenomenex, US). Analytical detection of extracted APIs was executed by High Performance Liquid Chromatography with fluorometric and diode array detectors DAD by using an Agilent Infinity 1260 series.

Chromatographic separations of IBU, PAR and their mixture were performed on a Kinetex column (C18,5 μm, 150 mm length, 4.6 mmID, Phenomenex, US), equipped with a security guard column (C18,5 μm, 4 mm length, 2.0 mmID, Phenomenex, US).

Analysis was performed using ultra-pure water, acetonitrile and acetic acid 0.1% gradient (from 35%:30%:35% to 0%:65%:35% linearly for 23 minutes) and obtained by fluorometric detector with excitation/emission wavelengths at 230/290 nm for IBU and 296/338 nm for PAR. Concentrations of various APIs were quantified by comparison with signals of pure standard solutions.

Because of the unavailability of appropriate Certified Standard Reference Materials (SRMs) for this kind of compounds and environmental matrices, recovery for each compound was estimated by testing control mussels' samples spiked with various concentrations of the investigated molecules. According to EU standards (2002/657/EC), retention time of all analytes are comparable with those obtained from the calibration standard in spike mussels,

with a margin of ± 2,5%. For the detection of each analyte the following identification criteria were applied: retention time (RT); at least two qualifying signals; a main signal and qualifiers easily distinguishable from the background; cross checking of signals obtained by fluorescence with those of the DAD detector; comparison of the UV/VIS spectrum obtained by the DAD with that of the corresponding standard solutions and with that of mussels tested with known aliquots of pure standards; evaluation of peak purity detected with DAD by comparing the spectra of standard and sample solutions with the use of the software provided by Agilent Technologies (ChemStation Edition-OpenLAB CDS, Rev.C.01.03[37]).

Considering the analytical conditions and the described preparation procedures, the minimum measurable amounts (Limit of Quantification, LOQ) in mussels' tissues were 8 ng/g d.w. for IBU and 0.95 ng/g d.w. for PAR.

3.3 Biomarker Analysis

Immunological responses were evaluated in terms of lysosomal membrane stability, granulocytes-hyalinocytes ratio and phagocytosis capacity in hemocytes.

Lysosomes are cellular organelles that contain a wide range of enzymes that have the ability to degrade cellular components. Lysosomal membrane stability is a sensitive marker for the evaluation of cell damage since lysosomal membranes can be destabilized by lipid peroxidation caused by reactive oxygen species (ROS) (Broeg and Gorbi, 2011). This biomarker was analyzed through neutral red retention time (NRRT). Hemolymph was collected from the adductor muscle of 8 mussel's specimens and incubated on a glass slide with a neutral red (NR) working solution (2 µl/mL filtered seawater). Hemocytes were microscopically examined every 20 minutes to define the time at which 50% of cells had lost into the cytosol the dye previously taken up by lysosomes.

Granulocyte/hyalinocyte ratio was analyzed by using aliquots of hemolymph dispersed on glass slides that, once dry, were fixed in Becker's fixative (2.5 NaCl). The slides were washed and stained with May Grunwald Giemsa before being mounted with Eukitt. Observations were carried out with a light microscope (1000x) and percentage of granulocytes was evaluated after counting at least 200 cells for each sample.

Phagocytosis capacity assay was microscopically evaluated in 100 mL of hemolymph dispersed on a glass slide and incubated for 2 hours at 15°C in the dark with Fluorescein-labelled Zymosan A bioparticles (Invitrogen) at a 10:1 target:hemocyte ratio. Uninternalized particles were washed away with physiological solution and slides were fixed in Beker's fixative and mounted in with Eukitt. Phagocytosis was expressed as percentage of cells that internalized at least 3 fluorescent particles.

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

Acyl-CoA oxidase's (ACOX) activity was measured in samples homogenized in 1 mM sodium bicarbonate buffer (pH 7.6), containing 1 mM EDTA, 0.1% ethanol and 0.01% Triton X-100. They were centrifuged at 500 xg for 15 minutes at 4°C. H_2O_2 production was detected in a coupled assay (Bocchetti and Regoli, 2006) by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 μM sodium azide, 0.01% Triton X-100, 1.2 U/mL HRP in a final volume of 1 mL. After a pre-incubation at 25°C for 5 minutes in the dark with an appropriate amount of sample, reactions were started adding the substrate Palmitoyl-CoA at final concentration of 30 μM and 100 μM for Acyl-CoA oxidase (AOX); readings were carried out at $\lambda = 502$ nm against a blank without the substrate.

Acetylcholinesterase (AChE) activity was spectrophotometrically assayed in hemolymph and gills. Hemolymph samples were centrifuged at 3000 xg for 5 minutes and obtained supernatants were analyzed by Ellman's reaction at 18 ± 1°C and $\lambda = 412$ nm. Gills were homogenized (1:3 w:v ratio) in a 0.1 M Tris-HCl buffer (pH 7.2), 0.25 M sucrose and centrifuged at 10,000 xg for 10 minutes; obtained supernatants were assayed as stated above.

Antioxidant defenses and onset of oxidative damage can be used as effective biomarkers to determine pro-oxidant effects of xenobiotics in marine organisms since they can interfere with the normal functioning of the antioxidant system and can contribute to the formation of ROS (Monserrat et al., 2011). Both variations of single antioxidant defenses and total oxyradical scavenging capacity (TOSC) were analyzed.

For antioxidant enzymes, samples were homogenized (1:5 w:v ratio) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/mL bacitracin, 0.008 TIU/mL aprotinin, 1 g/mL leupeptin, 0.5 g/mL pepstatin and NaCl 2.5%. Then, they were centrifuged at 110,000 xg for 1 hour at 4°C. Measurements were carried out with a Varian (model Cary 3) spectrophotometer at a constant temperature of 18°C (Regoli et al., 2004).

Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, $\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$) due to the consumption of hydrogen peroxide, H_2O_2 [12 mM H_2O_2 in 100 mM K-phosphate buffer (pH 7.0)].

Glutathione S-tranferases (GST) were determined at 340 nm using 1-chloro2,4dinitrobenzene (CDNB) as substrate. They were assayed in 100 mM K-phosphate buffer (pH 6.5), 1.5 mM CDNB and 1 mM GSH
($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

Glutathione peroxidases (GPx) were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to reconvert the formed GSSG into its reduced form (GSH). The decrease of absorbance was monitored at 340 nm ($\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$) in 100 mM K-phosphate buffer (pH 7.5), 1mM EDTA, 1 mM dithiothreitol, 1 mM NaN3 (for hydrogen peroxide assay), 2 mM GSH, 1U GR, 0.24 mM NADPH and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates, respectively, for the Se-dependent and for the Se-dependent and Se-independent forms.

Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of GSSG (λ= 340 nm, $\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$). The final assay conditions were 100 mM K-phosphate buffer (pH 7.0), 1 mM GSSG and 60 μM NADPH.

Total glutathione was analyzed in samples homogenized (1:5 w:v ratio) in 5% sulfosalicylic acid with 4 mM EDTA, kept for 45 minutes in ice and then centrifuged at 37,000 xg for 15 minutes. The Akerboo and Sies enzymatic method was used to determine the rate of formation of TNB which is proportional (up to 2 μM) to the glutathione concentration. Final assay conditions were 100 mM K-phosphate (pH 7.0), 1 mM EDTA, 20 mM DTNB in MetOH, 4 mg/mL NADPH solution and 1U GR. Spectrophotometric reading was done at λ= 412 nm.

Total oxyradical scavenging capacity assay (TOSCA) measures the overall capability of tissues to neutralize different forms of artificially generated oxyradicals, thus inhibiting the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas. Peroxyl radicals (ROO•) were generated by thermal homolysis of 20 mM 2'-azo-bis-(2-methylpropionamide)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer (pH 7.4). Hydroxyl radicals (•OH) were produced by the Fenton reaction of iron-EDTA (1.8 mM Fe^{3+}, 3.6 mM EDTA) with 180 mM ascorbate in 100 mM K-phosphate buffer. Under these conditions the different oxyradicals produced quantitatively similar yields of ethylene in control reactions, allowing to compare the relative efficiency of cellular antioxidants toward a quantitatively similar radical flux. Ethylene formation was analysed at 15 minutes time

intervals by gas chromatographic analysis and the 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. For all the samples, a specific TOSC was calculated dividing the experimental TOSC values by the sample protein concentration determined with the Lowry method with Bovine Serum Albumin (BSA) as standard (Regoli and Winston, 1999).

Malondialdehyde (MDA) is a product of lipid peroxidation and it's used as a biomarker of oxidative stress. MDA was quantified in digestive glands homogenized (1:3 w/v ratio) in 20 mM Tris–HCl (pH 7.4), centrifuged at 3,000 xg for 20 min. A conjugation reaction was performed in 1 mL reaction mixture (45 °C, 40 min), containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol, 3:1), 32% HCl, 100 µL water and 100 µL of sample or standard [standard range 0–6 µM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl (pH 7.4)]. Samples were finally cooled on ice, centrifuged at 15,000 xg for 10 min and spectrophotometrically analysed at 586 nm. MDA concentrations were determined as a function of the 1,1,3,3-tetramethoxypropane standard curve and expressed as nmol/g tissue.

Genotoxicity was evaluated at chromosomal level by looking at the frequency of micronuclei (MN) and at molecular level, as single strand breaks (SB), by the Comet assay. Samples for micronuclei frequency evaluation were made with haemocytes, rapidly washed in a saline buffer, fixed in Carnoy's solution (3:1 methanol:acetic acid), dispersed on glass slides and stained with fluorescent dye 4',6-diamidino-2-phenylindole at 100 ng/mL. 2,000 cells with preserved cytoplasm were scored for each specimen for the presence of micronuclei. The criteria used to identify a micronucleus were: a round structure, smaller than 1/3 of the main nucleus diameter and on the same optical plan of it while still possessing distinguishable boundaries from it.

Comet assay was carried out on mussels' haemocytes included in 1% normal-melting-point agarose on glass slides, followed by treatment in lysing solution, DNA denaturation, electrophoresis and staining with 1µg/mL 4',6-diamidino-2-phenylindole. 100 randomly selected "nucleoids" per slide, with 2 replicates per sample, were examined under fluorescence microscopy (200x magnification on the Olympus BX-51) and the captured images (Image-Pro-Plus package) were analysed by Tritek CometScore™ software. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation.

3.4 Statistical Analysis and Weight of Evidence approach

Statistical analysis for bioaccumulation and biomarkers data were performed using RStudio (version 1.2.5033). Data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test), with appropriate mathematical transformation if necessary. Analysis of variance was applied to test differences between treatments and exposure days (level of significance at $p < 0.001$), the Student Newman-Keuls test (SNK) was used for post-hoc comparison between means of values ($n = 5$).

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

4. Results

4.1 Chemical Analysis

Figure 1 shows the levels of IBU and PAR in tissues of control and exposed mussels for both exposure (day 30) and depuration (day 44) phases.

Control organisms at day 30 and day 44 showed bioaccumulation rates below the Limit of Detection (LOD) which corresponds to 8 ng/g d.w. for IBU and 0.95 ng/g d.w. for PAR.

Mussels exposed to IBU alone (Figure 1A) during the exposure phase showed statistically significant higher concentrations than in the organisms exposed to the mixture, where IBU levels were below the LOD. After the depuration phase, IBU levels decreased back below the LOD.

During the exposure phase, organisms exposed to PAR (Figure 1B) showed higher concentrations than those found in control organisms, without significant differences between single and mixture's treatments. After the depuration phase, levels of PAR in both treatments were below the LOD.

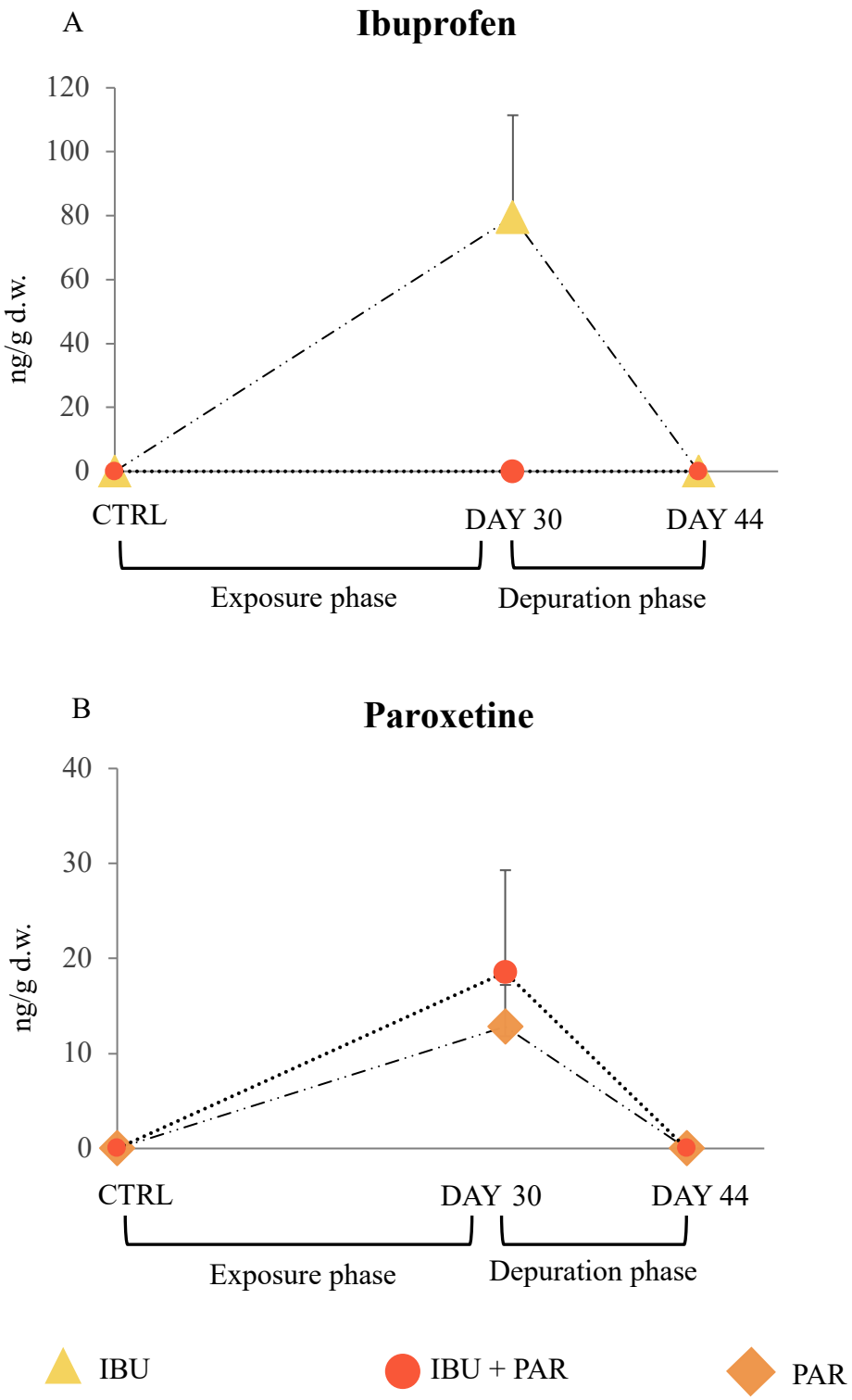


Figure 1. Concentration (ng/g d.w.) of A) IBU and B) PAR in whole tissues of *M. galloprovincialis*. Data are given as mean value ± standard deviation.

4.2 Biomarker Analysis

The results of immune parameters measured in mussels' hemolymph are shown in Figure 2. Lysosomal membrane stability significantly decreased in all exposed organisms compared to the controls both at the end of the exposure and depuration phase (Figure 2A). At day 30 all the treatments exhibited a significant decrease in NRRT as well as organisms exposed to PAR and IBU+PAR at day 44, while organisms exposed to IBU after the depuration phase were statistically comparable with both the control and the other treatments.

The phagocytosis capacity (Figure 2B) and granulocytes-hyalinocytes ratio (Figure 2C) didn't show any statistically significant variations between the control and the exposed organisms in both exposure and depuration phases.

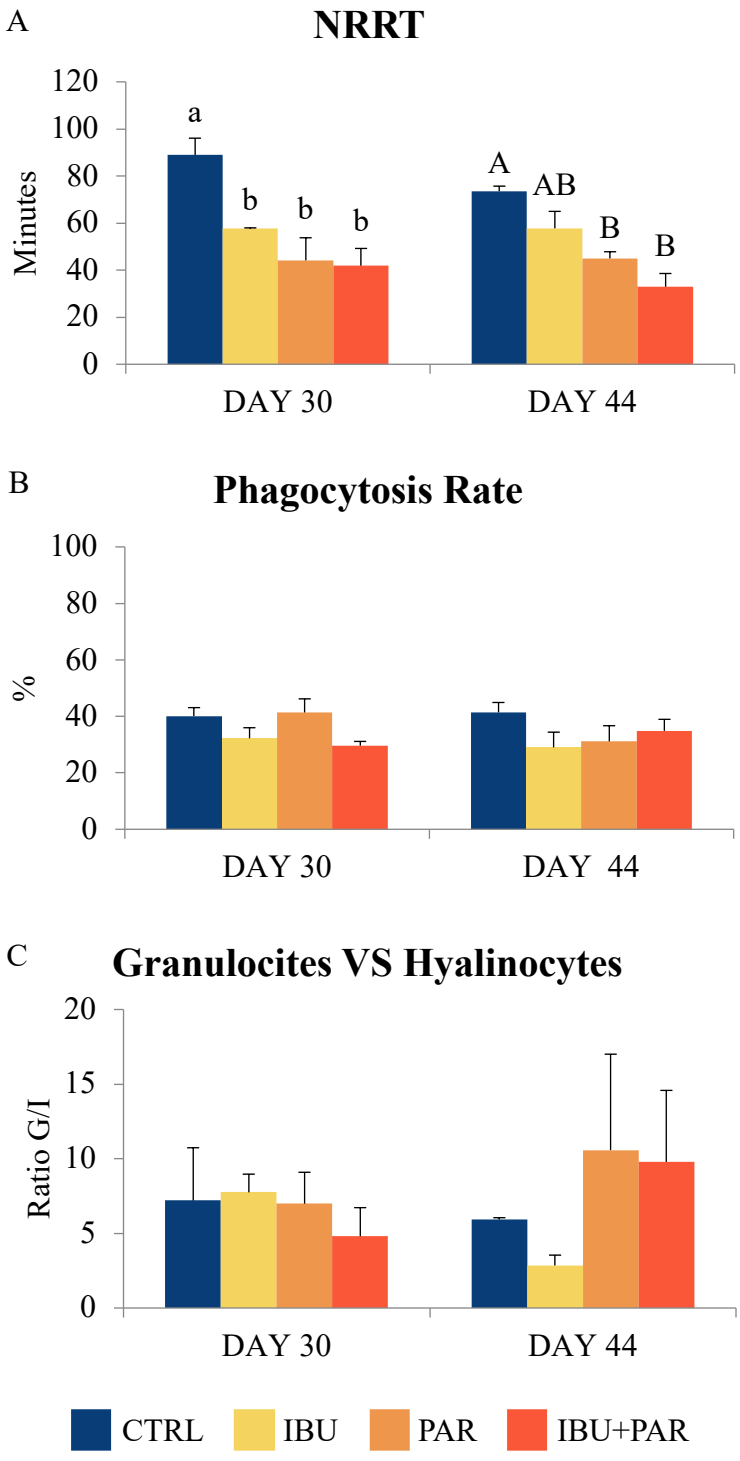


Figure 2. Immunological responses. A) Neutral Red Retention Time (Minutes), B) phagocytosis capacity (%), C) granulocyte/hyalinocyte ratio in hemocytes of *M. galloprovincialis.* Data are given as mean value ± standard deviation. Different letters indicate significant differences between groups of mean.

Considering biomarkers of genotoxicity, a significant increase of DNA fragmentation was measured in organisms exposed to IBU at day 30 and in all the treatments at day 44 (Figure 3A). During the depuration phase mussels exposed to PAR and IBU+PAR showed the highest percentage of DNA in the comet tail with IBU and IBU+PAR exhibiting a statistically significant difference.

A statistically significant increase of micronuclei frequency was measured in organisms exposed to IBU for both exposure and depuration phases, while no significant effect was observed in all other treatments (Figure 3B).

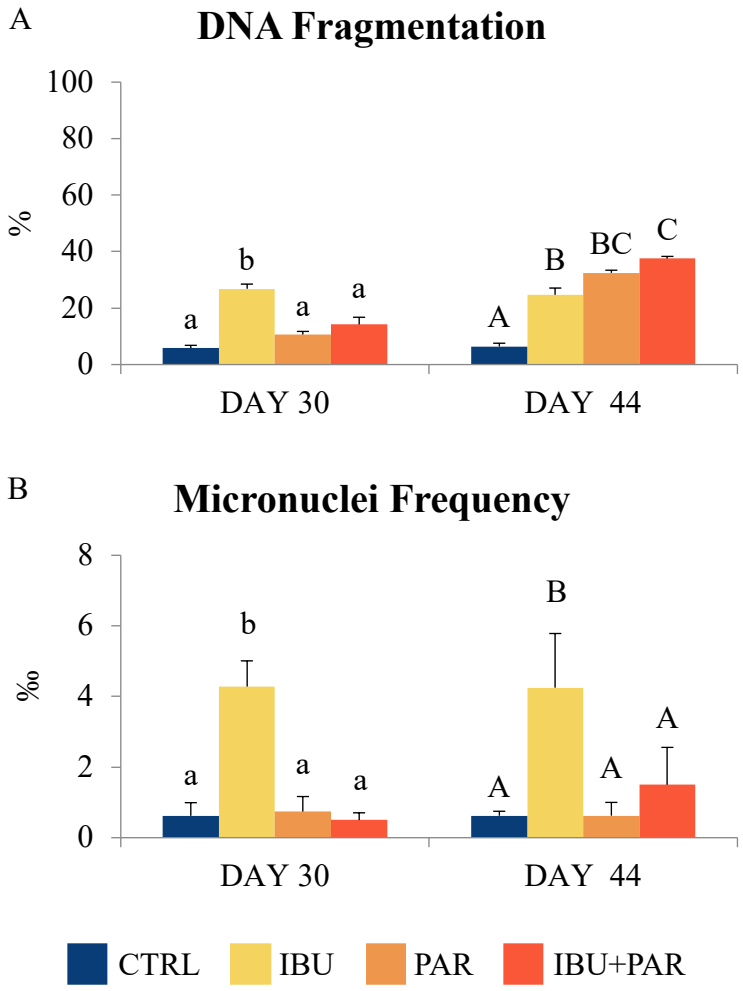


Figure 3. Genotoxic effects. A) Percentage of DNA in the comet tail, B) Micronuclei frequency (‰) in hemocytes. Data are given as mean value ± standard deviation. Different letters indicate significant differences between groups of mean.

No significant variations were measured in acetylcholinesterase activity in both hemolymph and gills for all treatments at both exposure and depuration phases (Figure 4A, B).

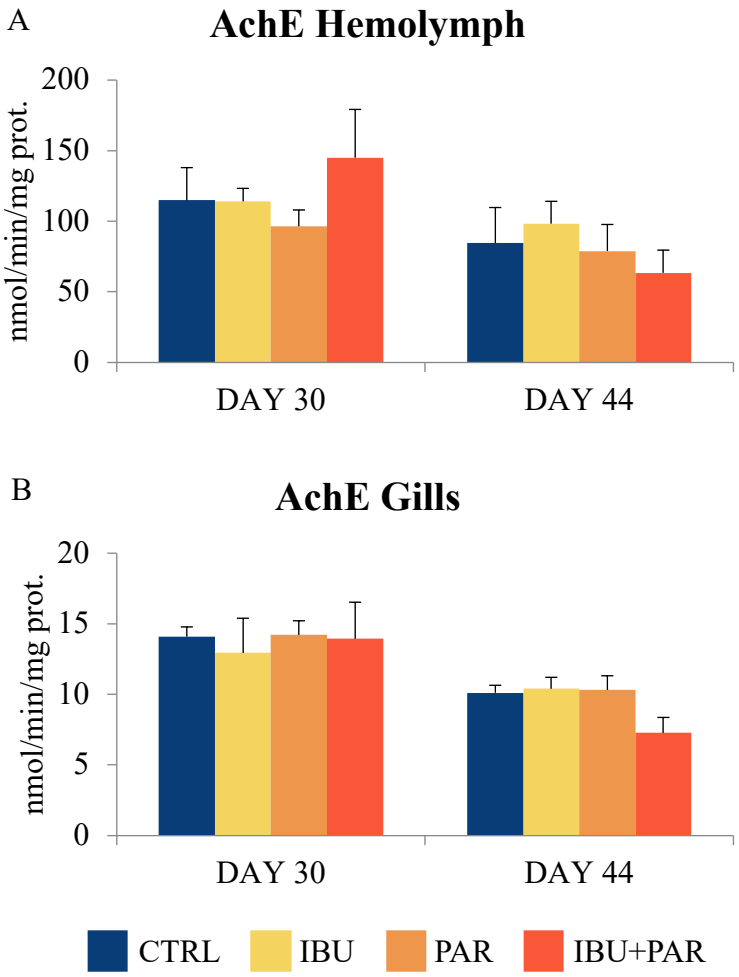


Figure 4. Neurotoxicity responses. A) Acetylcholinesterase activity (AChE) in hemolymph (nmol/min/mg prot.), B) acetylcholinesterase activity (AChE) in gills (nmol/min/mg prot.). Data are given as mean value ± standard deviation.

Results of single antioxidant enzymes and of the total oxyradical scavenging capacity are shown in Figure 5.

Catalase activity increased during the exposure phase in all three treatments, with a statistically significant effect in organisms exposed to PAR and IBU+PAR (Figure 5A) even after the depuration phase.

Sum of Se-dependent and Se-independent glutathione peroxidases at day 30 exhibited statistically significant induction in organisms exposed to PAR (Figure 5C). No variations were detected during the depuration phase in all treatments.

Glutathione reductase, Se-dependent glutathione peroxidases, glutathione S-transferase and levels of the total glutathione did not show any variations in all treatments for both exposure and depuration phases (Figure 5B, D, E, F).

Total oxyradical scavenging capacity toward peroxyl and hydroxyl radicals showed a steady profile at the end of both the exposure and depuration phases (Figure 5G, H). Although not significant, a reduction of total antioxidant capacity towards hydroxyl radicals was highlighted in organisms exposed to PAR after the depuration phase.

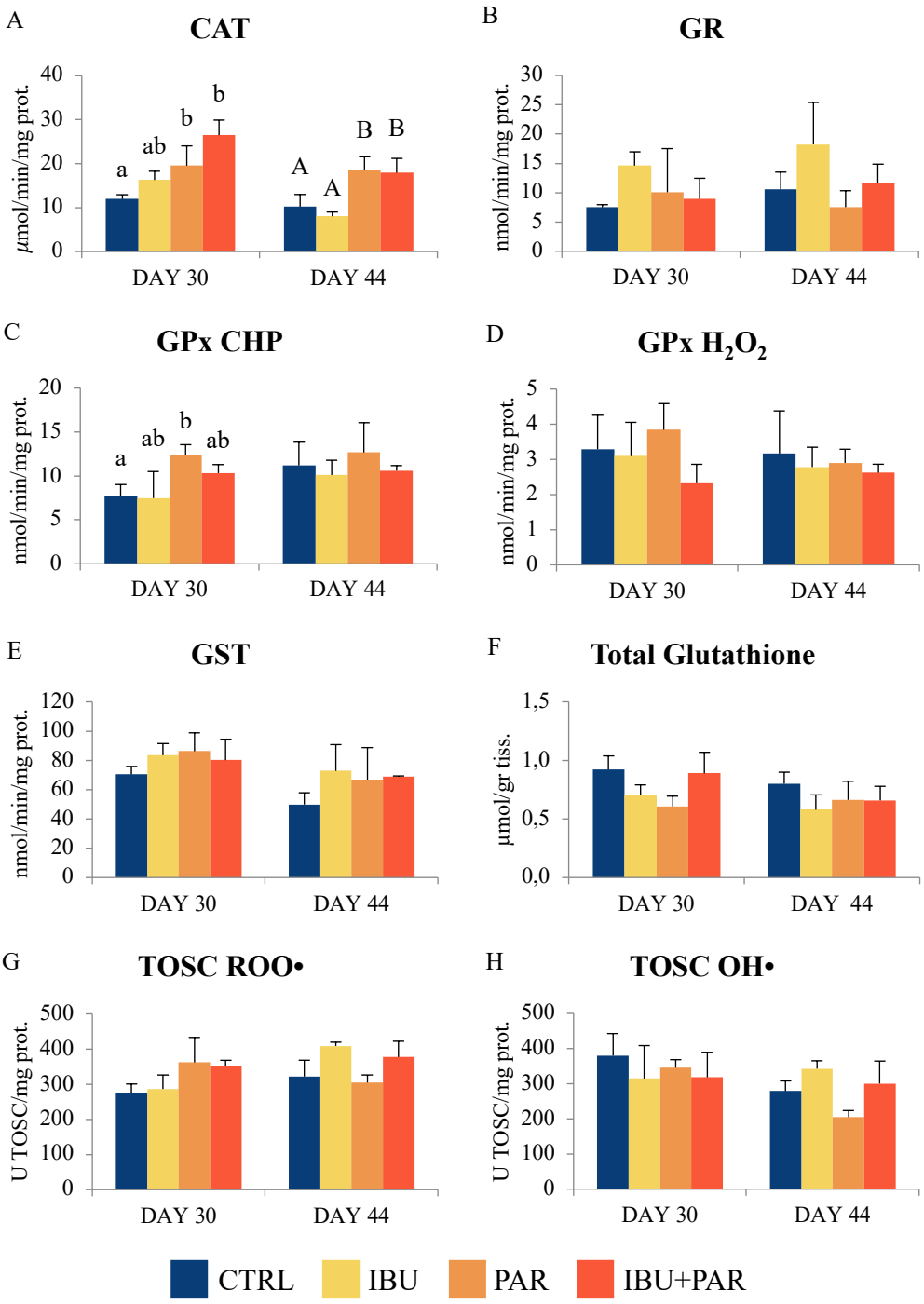


Figure 5. Antioxidant system responses in digestive glands (mean value ± standard deviation). A) Catalase activity (CAT), B) glutathione reductase activity (GR), C) Sum of Se-dependent and Se-independent glutathione peroxidases (GPx CHP), D) Se-dependent glutathione peroxidases (GPx H_2O_2), E) glutathione S-tranferase activity (GST), F) total glutathione, G) TOSC for peroxyl radicals (ROO·), H) TOSC for hydroxyl radicals (OH·). Different letters indicate significant differences between groups of mean.

Results on accumulation of peroxidation products and the lipid metabolism are shown in Figure 6.

A significant accumulation of lipofuscin was measured at day 30 and day 44 in all treatments, with major levels in organisms exposed to PAR (Figure 6A).

Neutral lipids content increased in all treatments even after the depuration phase, with higher levels in organisms exposed to IBU (Figure 6B).

No significant variations were highlighted at the cellular level with malondialdehyde levels not being statistically different from the controls (Figure 6C).

Similarly, the activity of acyl-CoA oxidase remained constant in all treatments at both experimental phases (Figure 6D)

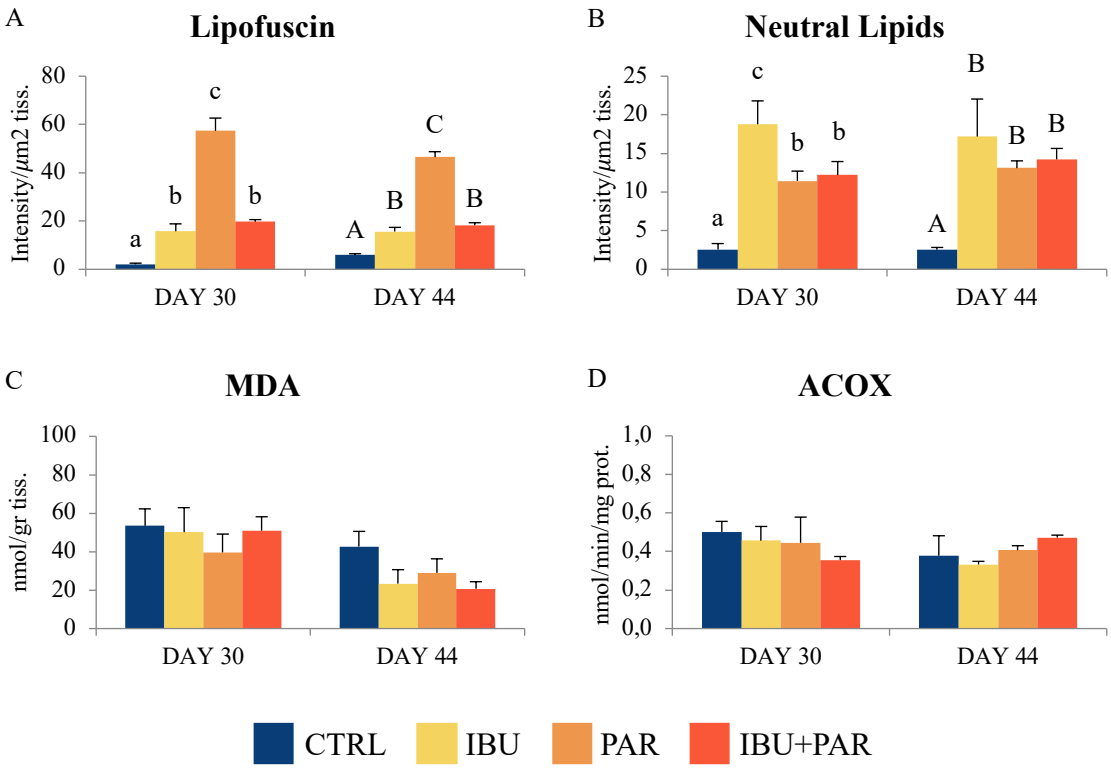


Figure 6. Lipid peroxidation products and lipid metabolism tested in digestive glands. A) Lipofuscin (intensity/µm^2), B) neutral lipids (intensity/µm^2), C) levels of malondialdehyde (MDA) (nmol/gr tiss.), D) acyl-CoA oxidase activity (ACOX) (nmol/min/mg prot.). Data are given as mean value ± standard deviation. Different letters indicate significant differences between groups of mean.

4.3 WOE Model

Obtained results were first elaborated within individual LOEs and then integrated with the WOE approach to calculate an overall level of risk of each treatment for both phases of the experiment (Figure 7).

LOE-3, which corresponds to the elaboration of the results on bioaccumulation, provided a quantitative hazard quotient (HQ) of "Major" for all the treatments during the exposure phase (Figure 7A), while the hazard came out to be "Absent" after the depuration phase (Figure 7B).

In LOE-4, regarding the variation of biomarkers, the HQ was "Moderate" for all three treatments in both the exposure (Figure 7A) and depuration phase (Figure 7B).

The final WOE integration assigned a "Moderate" risk to all the treatments during the exposure phase (Figure 7A) and a "Slight" risk to the treatments after the depuration phase (Figure 7B).

A Exposure Phase

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

B Depuration Phase

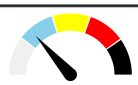
TREATMENT	LOE 3 BIOAVAILABILITY	LOE 4 BIOMARKER	WOE INTEGRATION	LEVEL OF RISK
IBU	ABSENT HQ: 0	MODERATE HQ: 203.21	SLIGHT	
PAR	ABSENT HQ: 0	MODERATE HQ: 184.62	SLIGHT	
IBU + PAR	ABSENT HQ: 0	MODERATE HQ: 192.31	SLIGHT	

Figure 7. Weighted elaboration of whole dataset for each treatment during the A) exposure phase and B) depuration phase.

5. Discussions

In recent years, ecopharmacovigilance has become a hot topic of conversation in scientific research and among legislative bodies of governments all around the world aiming to assess, understand and prevent possible adverse effects of pharmaceuticals in the environment, particularly aquatic ecosystems. Although studies to evaluate the possible environmental risks of these compounds individually have become more common to come by, mixture toxicity is still a rather unexplored issue. In field conditions, differently from patients' usage for specific diseases, non-target marine organisms are exposed to complex mixtures of APIs that can modulate the overall toxicity through different and/or overlapping pathways for which we have little to no understanding on the potential dangerous effects that they could induce (Mezzelani and Regoli, 2022).

To date, no research in the literature has yet been done concerning the bioaccumulation and ecotoxicological effects of a mixture made of the NSAID ibuprofen and the SSRI paroxetine, despite such compounds are among the most detected pharmaceuticals in wild mussels collected along the Adriatic and Tyrrhenian Sea (Mezzelani et al., 2020).

Results obtained from the present study confirmed the capability of the Mediterranean mussels *M. galloprovincialis* to accumulate IBU and PAR (Mezzelani et al., 2020), further highlighting a different bioaccumulation pattern in single compared to mixture-exposed organisms. Interestingly, when in the mixture, IBU concentrations in mussels remained below the LOD and were much lower than in organisms treated with IBU alone. Therefore, co-exposure appeared to lower IBU accumulation while PAR accumulation was left unaffected by the co-exposure. Although mechanisms regulating the mussels' uptake of pharmaceuticals mixtures is still unknown, these results point to a possible interaction between these two compounds with a competing mechanism that sees PAR prevailing on IBU, when present together. This pharmacodynamics could be explained by the fact that both these APIs compete for the same transporters to enter the cell and PAR happens to have a better affinity with them resulting in a higher uptake and impeding IBU bioaccumulation (Mezzelani and Regoli, 2022). No other study has yet investigated bioaccumulation dynamics for a mixture of these two specific pharmaceuticals, however a few studies have focused on the bioaccumulation of IBU and PAR in mixtures with other APIs.

Trombini et al. (2021) didn't detect any competing mechanism when they exposed the decapod *Procambarus clarkii* to a drug mixture of ibuprofen, ciprofloxacin and flumequine for 21 days at two sub-lethal doses of 10 μg/L and 100 μg/L. At both exposure concentrations IBU was accumulated in the hepatopancreas and, in the case of the highest concentration, with a similar bioconcentration factor (BCF) of the other antibiotics.

Concerning PAR, a study by Nowakowska et al. (2020) exposed *Danio rerio* larvae to a mixture of SSRIs containing paroxetine, fluoxetine, sertraline and mianserine at various concentrations (5, 10 and 25 μg/L of each compound, respectively) to test differences in bioaccumulation in larval tissues compared to single compounds. They found that BCF of PAR (as well as for the other compounds) was higher when the tested organisms were exposed to mixtures than to the single pharmaceuticals. In this case, therefore, a concentration addition model was hypothesized since SSRIs share the same mechanism of toxic action. This model was previously backed up also by Henry and Black (2007) and demonstrated by Johnson et al. (2007), even if contrasting evidence was found by Schultz et al. (2011) when exposing adult male fathead minnows (*Pimephales promelas*) to a mixture of fluoxetine and sertraline they didn't detect any additive effects on their bioaccumulation. After the depuration phase, bioaccumulation levels of both IBU and PAR went back below the limit of detection in all treatments probably related to the ability of mussels to process them through their biotransformation pathway and finally excrete them (Mezzelani and Regoli, 2022).

Among the broad selection of biological responses analyzed in this study, lysosomal membrane stability appears to be one of the most sensitive. Lysosomes play an important role in ecotoxicological terms since they are involved in the digestion and elimination processes of toxic substances and, being present in cells of the immune system, they are also responsible for cell-mediated immunity. Therefore, monitoring the integrity of their membrane represents a useful biomarker for the onset of cellular toxicity. In mussels, immune functions are linked to hemocytes, reason why NRRT is analyzed in the hemolymph, where they are circulating. A decreasing trend of the lysosomal membrane stability was observed in all treatments and was maintained even after the depuration phase reflecting stress and damage in hemocytes caused by pharmaceutical exposure. Similar effects had already been documented in mussels and clams for IBU (Mezzelani et al., 2018b, 2016a; Parolini et al., 2011; Matozzo et al., 2012; Aguirre-Martınez et al., 2013) and PAR

(Lacaze et al., 2015). No synergic effect was detected in organisms exposed to the mixture. The modulation of the immune system wasn't supported by phagocytosis activity which didn't seem to be affected by all treatments and showed lower levels compared to the control even after the depuration phase. Supporting data was found by Mezzelani et al. (2016b) while different results were previously documented by Mezzelani et al. (2018b) for IBU and Lacaze et al. (2015) for PAR, where decreasing lysosomal membrane stability was supported by an inhibition trend of phagocytosis.

The granulocyte/hyalinocyte ratio represents the difference between the two main hemocytes' subpopulations: granulocytes have higher phagocytosis capacity than hyalinocytes and normally represent the dominant cell type in hemolymph of mussels (Gorbi et al., 2012, 2013). Results didn't show any appreciable variation in the ratio between the two subpopulations at both exposure times. A similar situation was recorded by Mezzelani et al. (2016) when granulocyte-hyalinocyte ratio didn't change in IBU exposed mussels compared to the control, on the other hand data about PAR is unavailable.

Continuing with hemocyte parameters, genotoxicity was assessed through the Comet assay and micronuclei frequency. DNA damage measured with Comet assay evaluating structural integrity of DNA through its artificial denaturation. Increased levels of DNA strand breaks were recorded for organisms exposed to IBU during the exposure phase and after the depuration phase for all treatments suggesting that effects of the pharmaceuticals persist even after the compounds are excreted. Similar effects were reported in the same sentinel organism exposed to a concentrations of 25 μg/L (Mezzelani et al., 2016a) and in *M. edulis* exposed to concentrations of PAR as low as 1.5 μg/L (Lacaze et al., 2015), but also contrasting results were found by Aguirre-Martinéz et al. (2015): exposing the Asian clam (*Corbicula fluminea*) to several concentrations of IBU (0.1, 5, 10, 50 mg/L) they found no differences in DNA damage in digestive gland tissues compared to the controls. The hypothesis is that the activation of different defense mechanisms was able to prevent and protect cells form oxidative damage. Although DNA fragmentation in mussels exposed to treatments ranged between 10,7% (PAR after depuration) and 37,5% (IBU+PAR after depuration) compared to a maximum value of the CTRL of 6,4%, these values are still within the range of physiological variability of this biological response, as documented by Pisanelli et al. (2009). In an effort to characterize the natural fluctuation of basal levels of DNA fragmentation, specimens of *M. galloprovincialis* were sampled on a seasonal basis and over

a three-year period (2004-2006) from Portonovo, Ancona. Considerable variability was observed in the percentage of DNA strand breaks both seasonally and inter-annually with DNA total damage varying between 30-60% in 2004, 20-50% in 2005 and 20-35% in 2006, all percentages in line with data obtained in this study. Another work done by Bocchetti et al. (2008) found similar results for DNA fragmentation (from 26.2% to 49.8%) in the same species collected seasonally from a brackish environment in the Northern Adriatic thus corroborating the fact that analogous DNA fragmentation values are quite normal for unexposed organisms and compatible with the normal cellular status. No synergistic effect of the mixture was detected, in contrast with results obtained by Islas-Flores et al. (2017) where common carps (*Cyprinus carpio*) exposed to a mixture of ibuprofen and diclofenac (17.6 and 7.10 mg/L respectively) showed a higher rate of significant DNA damage being induced by the mixture rather than the drugs alone. Unfortunately, no comparison can be done with other mixtures made of a NSAID and a psychiatric drug because of a lack of research studies on the topic.

Concerning micronuclei frequency, significantly higher levels were shown in organisms exposed to IBU both during the exposure phase and after the depuration phase compared to the control and the other treatments supporting comparable results already present in the literature (Mezzelani et al., 2016a, 2018 a,b; Bebianno and Gonzalez-Rey, 2015; Milan et al., 2013). Since it has already been observed in previous studies that IBU isn't mutagenic (Philipose et al., 1997) the reason for an increased micronuclei frequency could be related to a modulation of the cellular cycle acting as a promoter of cellular division and cellular turnover.

In the hemolymph of treated mussels, also acetylcholinesterase activity was analyzed. This biomarker was actually tested both in hemolymph and gills in order to assess a possible different sensitivity of the tissues. Acetylcholinesterase it's an enzyme responsible for stopping the transmission of nerves' impulses and plays an important role in the neuro-muscular system in preventing continuous contractions. Its activity has been used as an effective biomarker in aquatic organisms to detect exposure to neurotoxic compounds (firstly organophosphorus compounds and carbamates, nowadays a variety of organic xenobiotics) that can inhibit its action, ultimately leading to the death of the organism (Lushchak, 2011). Results didn't show any significant difference between control and treatments throughout the whole duration of the experiment. These findings were expected for organisms exposed

to IBU since AChE's activity has not been highlighted as a primary target of NSAIDs and similar results with absent or minimal induction had already been documented in mussels (Mezzelani et al., 2016 a,b, 2018; Gonzalez-Rey and Bebianno, 2014). Although, in contrast with this, IBU was reported having neurotoxic effects in gills of *R. philippinarum* causing a marked reduction of AChE's activity (Milan et al., 2013). Less anticipated were the results for PAR for which we hypothesized a possible modulation of AChE's activity knowing its mode of action affects nervous system's pathways. Neurotoxic effects were in fact already recorded for low levels (0.03-300 μg/L) of another SSRI (fluoxetine) in *M. galloprovincialis* with significant inhibition of AChE's activity in gills (Franzellitti et al., 2014). Along the lines of previous biological responses, no significant differences emerged between mixture levels and other treatments and controls.

Imbalances between prooxidant forces and antioxidant defenses and an increase in intracellular production of reactive oxygen species (ROS) are some of the main mechanisms in modulating toxicological effects of xenobiotics in marine organisms (Regoli and Giuliani, 2014; Gorbi et al., 2013). In order to assess the oxidative status of exposed mussels, in this study we integrated data from individual antioxidant enzymes with total capability to neutralize specific oxyradicals. Results from these biomarkers evidenced an overall limited involvement of this pathway with the only statistically significant results showing induction of CAT in all treatments, during the exposure and after the depuration phase, and of GPx CHP only at day 30. Conflicting evidence from the literature found these antioxidant enzymes to be both induced (Aguirre-Martínez et al., 2015; Parolini et al., 2011) and inhibited (Gonzalez-Rey and Bebianno, 2012, 2014; Mezzelani et al., 2016a) by IBU while data about PAR is lacking. Both these enzymes have the biological role to protect the cell from oxidative damage, CAT by counteracting the production of the ROS H_2O_2 and GPx CHP, being a peroxidase, by reducing organic hydroperoxides. Their induction suggests a certain modulation of oxidative metabolism by tested pharmaceuticals, however, the lack of additional variations of the other antioxidant enzymes and the rather stable levels for total oxyradical scavenging capacity towards peroxyl and hydroxyl radicals point to the fact that the direct modulation of antioxidant defenses do not represent the main target of tested compounds.

Interestingly, at the cellular level, increased accumulation of lipid peroxidation products has been observed. Lipofuscin content, considered a biomarker indicative of

peroxidative processes, appeared remarkably increased in all treatments at both exposure times, but especially in organisms exposed to PAR. Mussels exposed to the mixture showed comparable results to those exposed to IBU, suggesting an influence only by the NSAID. Neutral lipids represent one of the main energy reserves in mussels and their increased accumulation is considered as an indicator of a disturbed lipid metabolism (Bocchetti and Regoli, 2006). All treatments showed enhanced levels of neutral lipids compared to controls, even after the depuration phase, but highest values were recorder for organisms exposed to IBU, supporting data from previous studies (Mezzelani et al., 2018b, 2022). Very little is known on NSAIDs and their possible modulation of lipid metabolism in marine invertebrates even though in mammals links between peroxisome proliferator-activated receptors, that function as regulators of lipid metabolism, and lipid-derived inflammatory mediators have already been proved (Chinetti et al., 2000).

No interactive mechanisms were detected in organisms exposed to the mixture which showed similar values of mussels exposed to PAR.

No significant changes compared to the controls were found for malondialdehyde levels. Nonetheless, Franzellitti et al. (2014) had found that concentrations of the SSRI fluoxetine caused a significant decrease in digestive gland content of MDA after *M. galloprovincialis* was treated with 0.3 and 30 ng/L. Concerning the mixture, Gonzalez-Rey et al. (2014) found discrepant results with a significant increase in MDA levels when mussels were exposed to a mixture of 250 ng/L of IBU, 250 ng/L of diclofenac and 75 ng/L of fluoxetine.

Stable acyl-CoA activity levels for IBU were supported by comparable results obtained by Mezzelani et al. (2016b, 2018b) even though measures of acyl-CoA oxidase activity inhibition in mussels treated with NSAIDs have been documented (Mezzelani et al., 2016a). Insufficient comparable data for this biological parameter is present for PAR and the mixture.

To summarize the biological responses observed in the study and depict a global picture of the environmental risk that these pharmaceuticals pose to marine invertebrates, a specific software assisted tool was developed based on the WOE approach. This model applies weighted criteria considering the toxicological relevance of measured endpoints and their magnitude of variation compared to specific thresholds. In recent years, the WOE approach has gained consensus in the scientific community for its reliability, versatility and user-friendly format that allows to process a great amount of heterogeneous data to get an

easily understandable output (Mezzelani et al., 2021; Nardi et al., 2022). The elaboration of the overall results obtained in the present study with the WOE model provided the calculation of a specific hazard index for both bioaccumulation and biomarkers before the final integration into an overall risk quotient, considering both of them. The WOE approach confirmed the lack of clear synergisms between tested compounds, in fact risk level for all treatments at day 30 was summarized as “Moderate”. The decrease of the WOE risk level to “Slight” for all treatments after the depuration phase corroborated the ability of this filter-feeding species to recover from the harmful effects caused by pharmaceuticals in a relatively short amount of time.

6. Conclusions

This study tested for the first time the biological and ecotoxicological effects of the pharmaceutical mixture made of the NSAID ibuprofen and the SSRI paroxetine in the sentinel organism *M. galloprovincialis*. Overall results did not reveal clear interactive effects of the mixture.

Chemical analysis showed that, when combined, the presence of PAR reduced the mussels' uptake of IBU. This finding suggests a possible competing mechanism between the two molecules for the same cellular transporters with PAR having a higher affinity for them compared to IBU.

Although clear interactive effects were not measured, most of the biological pathways in organisms exposed to the mixture showed a modulation caused predominantly by PAR rather than IBU, corroborating the results from bioaccumulation. Those most affected by the mixture and showing significant results were the immune system, lipid and oxidative metabolism.

Such effects were still detected after the depuration phase, even if with a lower magnitude, highlighting the ability of these pharmaceuticals to perpetuate their activity despite their excretion.

Bioaccumulation data from this study suggests the need for future studies to investigate further on the uptake mechanism of these molecules from a pharmacokinetics point of view. On the other hand, the undetermined interactions between IBU and PAR should be studied and better investigated to understand their mode of action interaction.

As an added value, the elaboration of data through the WOE approach, allowed us to produce an overall risk index for the tested molecules in the marine environment.

The use of this model is crucial for the communication of complex data to the public, governments and public institutions since it can be simply understood by everyone making it easier to express the risk that these chemicals pose to the environment and hopefully speed up the process for their regulation.

References

1. 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance) (notified under document number C(2002) 3044) (OJ L 221 17.08.2002, p. 8, ELI: http://data.europa.eu/eli/dec/2002/657/oj)
2. Adeleye, A. S., Xue, J., Zhao, Y., Taylor, A. A., Zenobio, J. E., Sun, Y., Han, Z., Salawu, O. A., & Zhu, Y. (2022). Abundance, fate, and effects of pharmaceuticals and personal care products in aquatic environments. In *Journal of Hazardous Materials* (Vol. 424). https://doi.org/10.1016/j.jhazmat.2021.127284
3. Aguirre-Martínez, G. v., Buratti, S., Fabbri, E., DelValls, A. T., & Martín-Díaz, M. L. (2013). Using lysosomal membrane stability of haemocytes in Ruditapes philippinarum as a biomarker of cellular stress to assess contamination by caffeine, ibuprofen, carbamazepine and novobiocin. *Journal of Environmental Sciences (China)*, *25*(7). https://doi.org/10.1016/S1001-0742(12)60207-1
4. Aguirre-Martínez, G. v., DelValls, A. T., & Laura Martín-Díaz, M. (2015). Yes, caffeine, ibuprofen, carbamazepine, novobiocin and tamoxifen have an effect on Corbicula fluminea (Müller, 1774). *Ecotoxicology and Environmental Safety*, *120*. https://doi.org/10.1016/j.ecoenv.2015.05.036
5. Aherne, G. W., Hardcastle, A., & Nield, A. H. (1990). Cytotoxic drugs and the aquatic environment: estimation of bleomycin in river and water samples. *The Journal of pharmacy and pharmacology*, *42*(10), 741–742. https://doi.org/10.1111/j.2042-7158.1990.tb06574.x
6. Ali, A. M., Rønning, H. T., Sydnes, L. K., Alarif, W. M., Kallenborn, R., & Al-Lihaibi, S. S. (2018). Detection of PPCPs in marine organisms from contaminated coastal waters of the Saudi Red Sea. *Science of the Total Environment*, *621*. https://doi.org/10.1016/j.scitotenv.2017.11.298
7. Almeida, Â., Silva, M. G., Soares, A. M. V. M., & Freitas, R. (2020). Concentrations levels and effects of 17alpha-Ethinylestradiol in freshwater and marine waters and bivalves: A review. *Environmental Research*, *185*. https://doi.org/10.1016/j.envres.2020.109316

8. Almeida, Â., Solé, M., Soares, A. M. V. M., & Freitas, R. (2020). Anti-inflammatory drugs in the marine environment: Bioconcentration, metabolism and sub-lethal effects in marine bivalves. In *Environmental Pollution* (Vol. 263). https://doi.org/10.1016/j.envpol.2020.114442
9. Álvarez-Muñoz, D., Rodríguez-Mozaz, S., Maulvault, A. L., Tediosi, A., Fernández-Tejedor, M., van den Heuvel, F., Kotterman, M., Marques, A., & Barceló, D. (2015). Occurrence of pharmaceuticals and endocrine disrupting compounds in macroalgaes, bivalves, and fish from coastal areas in Europe. *Environmental Research*, *143*. https://doi.org/10.1016/j.envres.2015.09.018
10. Aris, A. Z., Shamsuddin, A. S., & Praveena, S. M. (2014). Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: A review. In *Environment International* (Vol. 69). https://doi.org/10.1016/j.envint.2014.04.011
11. Balabanič, D., Hermosilla, D., Merayo, N., Klemeničič, A. K., & Blanco, Á. (2012). Comparison of different wastewater treatments for removal of selected endocrine-disruptors from paper mill wastewaters. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, *47*(10). https://doi.org/10.1080/10934529.2012.672301
12. Balbi, T., Montagna, M., Fabbri, R., Carbone, C., Franzellitti, S., Fabbri, E., & Canesi, L. (2018). Diclofenac affects early embryo development in the marine bivalve Mytilus galloprovincialis. *Science of the Total Environment*, *642*. https://doi.org/10.1016/j.scitotenv.2018.06.125
13. Bebianno, M. J., & Gonzalez-Rey, M. (2015). Ecotoxicological Risk of Personal Care Products and Pharmaceuticals. In *Aquatic Ecotoxicology: Advancing Tools for Dealing with Emerging Risks*. https://doi.org/10.1016/B978-0-12-800949-9.00016-4
14. Behera, S. K., Kim, H. W., Oh, J. E., & Park, H. S. (2011). Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. *Science of the Total Environment*, *409*(20), 4351–4360. https://doi.org/10.1016/j.scitotenv.2011.07.015
15. Bidel, F., di Poi, C., Budzinski, H., Pardon, P., Callewaert, W., Arini, A., Basu, N., Dickel, L., Bellanger, C., & Jozet-Alves, C. (2016). The antidepressant venlafaxine may act as a neurodevelopmental toxicant in cuttlefish (Sepia officinalis). *NeuroToxicology*, *55*. https://doi.org/10.1016/j.neuro.2016.05.023

16. Bocchetti, R., & Regoli, F. (2006). Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel Mytilus galloprovincialis from Adriatic Sea. *Chemosphere*, *65*(6). https://doi.org/10.1016/j.chemosphere.2006.03.049
17. Bocchetti, R., Lamberti, C. V., Pisanelli, B., Razzetti, E. M., Maggi, C., Catalano, B., Sesta, G., Martuccio, G., Gabellini, M., & Regoli, F. (2008). Seasonal variations of exposure biomarkers, oxidative stress responses and cell damage in the clams, Tapes philippinarum, and mussels, Mytilus galloprovincialis, from Adriatic sea. *Marine Environmental Research*, *66*(1). https://doi.org/10.1016/j.marenvres.2008.02.013
18. Broeg, K., & Gorbi, S. (2011). Methods to Quantify Lysosomal Membrane Stability and the Accumulation of Lipofuscin. In *Oxidative Stress in Aquatic Ecosystems*. https://doi.org/10.1002/9781444345988.ch39
19. Carballa, M., Omil, F., Lema, J. M., Llompart, M., García-Jares, C., Rodríguez, I., Gómez, M., & Ternes, T. (2004). Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Research*, *38*(12), 2918–2926. https://doi.org/10.1016/j.watres.2004.03.029
20. Carvalho, L., Mackay, E. B., Cardoso, A. C., Baattrup-Pedersen, A., Birk, S., Blackstock, K. L., Borics, G., Borja, A., Feld, C. K., Ferreira, M. T., Globevnik, L., Grizzetti, B., Hendry, S., Hering, D., Kelly, M., Langaas, S., Meissner, K., Panagopoulos, Y., Penning, E., Rouillard, J., … Solheim, A. L. (2019). Protecting and restoring Europe's waters: An analysis of the future development needs of the Water Framework Directive. *The Science of the total environment*, *658*, 1228–1238. https://doi.org/10.1016/j.scitotenv.2018.12.255
21. Chinetti, G., Fruchart, J. C., & Staels, B. (2000). Peroxisome proliferator-activated receptors (PPARs): Nuclear receptors at the crossroads between lipid metabolism and inflammation. In *Inflammation Research* (Vol. 49, Issue 10). https://doi.org/10.1007/s000110050622
22. d'Errico, G., Nardi, A., Benedetti, M., Mezzelani, M., Fattorini, D., di Carlo, M., Pittura, L., Giuliani, M. E., Macchia, S., Vitiello, V., Sartori, D., Scuderi, A., Morroni, L., Chiaretti, G., Gorbi, S., Pellegrini, D., & Regoli, F. (2021). Application of a Multidisciplinary Weight of Evidence Approach as a Tool for Monitoring the Ecological

Risk of Dredging Activities. *Frontiers in Marine Science*, *8*. https://doi.org/10.3389/fmars.2021.765256

23. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy
24. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council
25. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy Text with EEA relevance
26. EFPIA. (2021). The Pharmaceutical Industry in Figures. *European Federation of Pharmaceutical Industries and Associations (EFPIA) Brussels Office*.
27. Estévez-Calvar, N., Canesi, L., Montagna, M., Faimali, M., Piazza, V., & Garaventa, F. (2017). Adverse effects of the SSRI antidepressant sertraline on early life stages of marine invertebrates. *Marine Environmental Research*, *128*. https://doi.org/10.1016/j.marenvres.2016.05.021
28. Fabbri, E., & Franzellitti, S. (2016). Human pharmaceuticals in the marine environment: Focus on exposure and biological effects in animal species. *Environmental Toxicology and Chemistry*, *35*(4), 799–812. https://doi.org/10.1002/etc.3131
29. Fong, P. P., & Ford, A. T. (2014). The biological effects of antidepressants on the molluscs and crustaceans: A review. In *Aquatic Toxicology* (Vol. 151). https://doi.org/10.1016/j.aquatox.2013.12.003
30. Franzellitti, S., Buratti, S., Capolupo, M., Du, B., Haddad, S. P., Chambliss, C. K., Brooks, B. W., & Fabbri, E. (2014). An exploratory investigation of various modes of action and potential adverse outcomes of fluoxetine in marine mussels. *Aquatic Toxicology*, *151*. https://doi.org/10.1016/j.aquatox.2013.11.016
31. Gomez Cortes, L., Marinov, D., Sanseverino, I., Navarro Cuenca, A., Niegowska Conforti, M., Porcel Rodriguez, E., Stefanelli, F. and Lettieri, T., Selection of substances

for the 4th Watch List under the Water Framework Directive, Publications Office of the European Union, Luxembourg, 2022, doi:10.2760/01939, JRC130252.

32. Gonzalez-Rey, M., & Bebianno, M. J. (2011). Non-steroidal anti-inflammatory drug (NSAID) ibuprofen distresses antioxidant defense system in mussel Mytilus galloprovincialis gills. *Aquatic Toxicology*, *105*(3–4). https://doi.org/10.1016/j.aquatox.2011.06.015
33. Gonzalez-Rey, M., & Bebianno, M. J. (2012). Does non-steroidal anti-inflammatory (NSAID) ibuprofen induce antioxidant stress and endocrine disruption in mussel Mytilus galloprovincialis? *Environmental Toxicology and Pharmacology*, *33*(2). https://doi.org/10.1016/j.etap.2011.12.017
34. Gonzalez-Rey, M., & Bebianno, M. J. (2013). Does selective serotonin reuptake inhibitor (SSRI) fluoxetine affects mussel Mytilus galloprovincialis? *Environmental Pollution*, *173*. https://doi.org/10.1016/j.envpol.2012.10.018
35. Gonzalez-Rey, M., & Bebianno, M. J. (2014). Effects of non-steroidal anti-inflammatory drug (NSAID) diclofenac exposure in mussel Mytilus galloprovincialis. *Aquatic Toxicology*, *148*. https://doi.org/10.1016/j.aquatox.2014.01.011
36. Gorbi, S., Avio, G. C., Benedetti, M., Totti, C., Accoroni, S., Pichierri, S., Bacchiocchi, S., Orletti, R., Graziosi, T., & Regoli, F. (2013). Effects of harmful dinoflagellate Ostreopsis cf. ovata exposure on immunological, histological and oxidative responses of mussels Mytilus galloprovincialis. *Fish and Shellfish Immunology*, *35*(3). https://doi.org/10.1016/j.fsi.2013.07.003
37. Gorbi, S., Bocchetti, R., Binelli, A., Bacchiocchi, S., Orletti, R., Nanetti, L., Raffaelli, F., Vignini, A., Accoroni, S., Totti, C., & Regoli, F. (2012). Biological effects of palytoxin-like compounds from Ostreopsis cf. ovata: A multibiomarkers approach with mussels Mytilus galloprovincialis. *Chemosphere*, *89*(5). https://doi.org/10.1016/j.chemosphere.2012.05.064
38. He, B. shu, Wang, J., Liu, J., & Hu, X. min. (2017). Eco-pharmacovigilance of non-steroidal anti-inflammatory drugs: Necessity and opportunities. In *Chemosphere* (Vol. 181). https://doi.org/10.1016/j.chemosphere.2017.04.084

39. Henry, T. B., & Black, M. C. (2007). Mixture and single-substance acute toxicity of selective serotonin reuptake inhibitors in Ceriodaphnia dubia. *Environmental Toxicology and Chemistry*, *26*(8). https://doi.org/10.1897/06-265R.1
40. Islas-Flores, H., Manuel Gómez-Oliván, L., Galar-Martínez, M., Michelle Sánchez-Ocampo, E., SanJuan-Reyes, N., Ortíz-Reynoso, M., & Dublán-García, O. (2017). Cyto-genotoxicity and oxidative stress in common carp (Cyprinus carpio) exposed to a mixture of ibuprofen and diclofenac. *Environmental Toxicology*, *32*(5). https://doi.org/10.1002/tox.22392
41. Johnson, D. J., Sanderson, H., Brain, R. A., Wilson, C. J., & Solomon, K. R. (2007). Toxicity and hazard of selective serotonin reuptake inhibitor antidepressants fluoxetine, fluvoxamine, and sertraline to algae. *Ecotoxicology and Environmental Safety*, *67*(1). https://doi.org/10.1016/j.ecoenv.2006.03.016
42. Kasprzyk-Hordern, B., Dinsdale, R. M., & Guwy, A. J. (2009). The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. *Water research*, *43*(2), 363–380.
43. Kristensen, P., Whalley, C., Zal, F. N. N., & Christiansen, T. (2018). European waters assessment of status and pressures 2018. *EEA Report*, (7/2018). https:// doi.org/10.2800/303664
44. Lacaze, E., Pédelucq, J., Fortier, M., Brousseau, P., Auffret, M., Budzinski, H., & Fournier, M. (2015). Genotoxic and immunotoxic potential effects of selected psychotropic drugs and antibiotics on blue mussel (Mytilus edulis) hemocytes. *Environmental Pollution*, *202*. https://doi.org/10.1016/j.envpol.2015.03.025
45. Lapworth D J, Baran N, Stuart, M. E., & Ward R S. (n.d.). *Emerging organic contaminants in groundwater: A review of sources, fate and occurrence*.
46. Lapworth, D. J., Baran, N., Stuart, M. E., & Ward, R. S. (2012). Emerging organic contaminants in groundwater: a review of sources, fate and occurrence. *Environmental pollution*, *163*, 287-303. http://doi.org/10.1016/j.envpol.2011.12.034
47. Linkov, I., Loney, D., Cormier, S., Satterstrom, F. K., & Bridges, T. (2009). Weight-of-evidence evaluation in environmental assessment: Review of qualitative and quantitative approaches. In *Science of the Total Environment* (Vol. 407, Issue 19). https://doi.org/10.1016/j.scitotenv.2009.05.004

48. Luo, Y., Guo, W., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J., Liang, S., & Wang, X. C. (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *The Science of the total environment*, *473-474*, 619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065
49. Lushchak, V.I. (2011). Environmentally Induced Oxidative Stress in Fish. Oxidative Stress in Aquatic Ecosystems (eds D. Abele, J.P. Vázquez-Medina and T. Zenteno-Savín). https://doi.org/10.1002/9781444345988.ch21
50. Madikizela, L. M., Ncube, S., Tutu, H., Richards, H., Newman, B., Ndungu, K., & Chimuka, L. (2020). Pharmaceuticals and their metabolites in the marine environment: Sources, analytical methods and occurrence. *Trends in Environmental Analytical Chemistry*, *28*, e00104. https://doi.org/10.1016/J.TEAC.2020.E00104
51. Martínez-Morcillo, S., Rodríguez-Gil, J. L., Fernández-Rubio, J., Rodríguez-Mozaz, S., Míguez-Santiyán, M. P., Valdes, M. E., Barceló, D., & Valcárcel, Y. (2020). Presence of pharmaceutical compounds, levels of biochemical biomarkers in seafood tissues and risk assessment for human health: Results from a case study in North-Western Spain. *International Journal of Hygiene and Environmental Health*, *223*(1). https://doi.org/10.1016/j.ijheh.2019.10.011
52. Matozzo, V., Rova, S., & Marin, M. G. (2012). The nonsteroidal anti-inflammatory drug, ibuprofen, affects the immune parameters in the clam Ruditapes philippinarum. *Marine Environmental Research*, *79*. https://doi.org/10.1016/j.marenvres.2012.06.003
53. Mazaleuskaya, L. L., Theken, K. N., Gong, L., Thorn, C. F., Fitzgerald, G. A., Altman, R. B., & Klein, T. E. (2015). PharmGKB summary: Ibuprofen pathways. *Pharmacogenetics and Genomics*, *25*(2). https://doi.org/10.1097/FPC.0000000000000113
54. Mezzelani, M., & Regoli, F. (2022). The Biological Effects of Pharmaceuticals in the Marine Environment. In *Annual Review of Marine Science* (Vol. 14). https://doi.org/10.1146/annurev-marine-040821-075606
55. Mezzelani, M., Fattorini, D., Gorbi, S., Nigro, M., & Regoli, F. (2020). Human pharmaceuticals in marine mussels: Evidence of sneaky environmental hazard along Italian coasts. *Marine environmental research*, *162*, 105137. https://doi.org/10.1016/j.marenvres.2020.105137

56. Mezzelani, M., Gorbi, S., & Regoli, F. (2018a). Pharmaceuticals in the aquatic environments: Evidence of emerged threat and future challenges for marine organisms. *Marine Environmental Research*, *140*, 41–60. https://doi.org/10.1016/J.MARENVRES.2018.05.001
57. Mezzelani, M., Gorbi, S., da Ros, Z., Fattorini, D., d'Errico, G., Milan, M., Bargelloni, L., & Regoli, F. (2016a). Ecotoxicological potential of non-steroidal anti-inflammatory drugs (NSAIDs) in marine organisms: Bioavailability, biomarkers and natural occurrence in Mytilus galloprovincialis. *Marine Environmental Research*, *121*. https://doi.org/10.1016/j.marenvres.2016.03.005
58. Mezzelani, M., Gorbi, S., Fattorini, D., d'Errico, G., Benedetti, M., Milan, M., Bargelloni, L., & Regoli, F. (2016b). Transcriptional and cellular effects of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in experimentally exposed mussels, Mytilus galloprovincialis. *Aquatic Toxicology*, *180*. https://doi.org/10.1016/j.aquatox.2016.10.006
59. Mezzelani, M., Gorbi, S., Fattorini, D., d'Errico, G., Consolandi, G., Milan, M., Bargelloni, L., & Regoli, F. (2018b). Long-term exposure of Mytilus galloprovincialis to diclofenac, Ibuprofen and Ketoprofen: Insights into bioavailability, biomarkers and transcriptomic changes. *Chemosphere*, *198*. https://doi.org/10.1016/j.chemosphere.2018.01.148
60. Mezzelani, M., Nardi, A., Bernardini, I., Milan, M., Peruzza, L., d'Errico, G., Fattorini, D., Gorbi, S., Patarnello, T., & Regoli, F. (2021). Environmental pharmaceuticals and climate change: The case study of carbamazepine in M. galloprovincialis under ocean acidification scenario. *Environment International*, *146*. https://doi.org/10.1016/j.envint.2020.106269
61. Milan, M., Pauletto, M., Patarnello, T., Bargelloni, L., Marin, M. G., & Matozzo, V. (2013). Gene transcription and biomarker responses in the clam Ruditapes philippinarum after exposure to ibuprofen. *Aquatic Toxicology*, *126*. https://doi.org/10.1016/j.aquatox.2012.10.007
62. Monserrat, J. M., Letts, R. E., Ferreira, J. L. R., Ventura-Lima, J., Amado, L. L., Rocha, A. M., Gorbi, S., Bocchetti, R., Benedetti, M., & Regoli, F. (2011). Biomarkers of Oxidative Stress: Benefits and Drawbacks for their Application in Biomonitoring of

Aquatic Environments. In *Oxidative Stress in Aquatic Ecosystems*. https://doi.org/10.1002/9781444345988.ch23

63. Monserrat, J. M., Martínez, P. E., Geracitano, L. A., Lund Amado, L., Martinez Gaspar Martins, C., Lopes Leães Pinho, G., Soares Chaves, I., Ferreira-Cravo, M., Ventura-Lima, J., & Bianchini, A. (2007). Pollution biomarkers in estuarine animals: Critical review and new perspectives. In *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology* (Vol. 146, Issues 1-2 SPEC. ISS.). https://doi.org/10.1016/j.cbpc.2006.08.012
64. Munari, M., Marin, M. G., & Matozzo, V. (2014). Effects of the antidepressant fluoxetine on the immune parameters and acetylcholinesterase activity of the clam Venerupis philippinarum. *Marine Environmental Research*, *94*. https://doi.org/10.1016/j.marenvres.2013.11.007
65. Nardi, A., Mezzelani, M., Costa, S., d'Errico, G., Benedetti, M., Gorbi, S., Freitas, R., & Regoli, F. (2022). Marine heatwaves hamper neuro-immune and oxidative tolerance toward carbamazepine in Mytilus galloprovincialis. *Environmental Pollution*, *300*. https://doi.org/10.1016/j.envpol.2022.118970
66. Notch, E. G., Miniutti, D. M., & Mayer, G. D. (2007). 17α-Ethinylestradiol decreases expression of multiple hepatic nucleotide excision repair genes in zebrafish (Danio rerio). *Aquatic Toxicology*, *84*(3). https://doi.org/10.1016/j.aquatox.2007.06.006
67. Nowakowska, K., Giebułtowicz, J., Kamaszewski, M., Adamski, A., Szudrowicz, H., Ostaszewska, T., Solarska-Dzięciołowska, U., Nałęcz-Jawecki, G., Wroczyński, P., & Drobniewska, A. (2020). Acute exposure of zebrafish (Danio rerio) larvae to environmental concentrations of selected antidepressants: Bioaccumulation, physiological and histological changes. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, *229*. https://doi.org/10.1016/j.cbpc.2019.108670
68. OECD (2021), *Health at a Glance 2021: OECD Indicators*, OECD Publishing, Paris, https://doi.org/10.1787/ae3016b9-en
69. Ojemaye, C. Y., & Petrik, L. (2019). Pharmaceuticals in the marine environment: A review. *Environmental Reviews*, *27*(2), 151-165. https://doi.org/10.1139/er-2018-0054
70. Ojemaye, C. Y., & Petrik, L. (n.d.). *Pharmaceuticals in the marine environment: A review 2*. https://mc06.manuscriptcentral.com/er-pubs

71. Parolini, M. (2020). Toxicity of the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) acetylsalicylic acid, paracetamol, diclofenac, ibuprofen and naproxen towards freshwater invertebrates: A review. In *Science of the Total Environment* (Vol. 740). https://doi.org/10.1016/j.scitotenv.2020.140043
72. Parolini, M., Binelli, A., & Provini, A. (2011). Chronic effects induced by ibuprofen on the freshwater bivalve Dreissena polymorpha. *Ecotoxicology and Environmental Safety*, *74*(6). https://doi.org/10.1016/j.ecoenv.2011.04.025
73. Parra-Saldivar, R., Castillo-Zacarías, C., Bilal, M., Iqbal, H. M. N., & Barceló, D. (2021). Sources of pharmaceuticals in water. In *Handbook of Environmental Chemistry* (Vol. 103, pp. 33–47). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/698_2020_623
74. Petrovic, M., Eljarrat, E., Lopez De Alda, M. J., & Barceló, D. (2004). Endocrine disrupting compounds and other emerging contaminants in the environment: A survey on new monitoring strategies and occurrence data. In *Analytical and Bioanalytical Chemistry* (Vol. 378, Issue 3). https://doi.org/10.1007/s00216-003-2184-7
75. Philipose, B., Singh, R., Khan, K. A., & Giri, A. K. (1997). Comparative mutagenic and genotoxic effects of three propionic acid derivatives ibuprofen, ketoprofen and naproxen. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, *393*(1–2). https://doi.org/10.1016/S1383-5718(97)00095-8
76. Pisanelli, B., Benedetti, M., Fattorini, D., & Regoli, F. (2009). Seasonal and inter-annual variability of DNA integrity in mussels Mytilus galloprovincialis: A possible role for natural fluctuations of trace metal concentrations and oxidative biomarkers. *Chemosphere*, *77*(11). https://doi.org/10.1016/j.chemosphere.2009.09.048
77. Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., & Regoli, F. (2011). Assessing sediment hazard through a weight of evidence approach with bioindicator organisms: A practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. *Chemosphere*, *83*(4). https://doi.org/10.1016/j.chemosphere.2010.12.064
78. Regoli, F., & Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. *Marine Environmental Research*, *93*. https://doi.org/10.1016/j.marenvres.2013.07.006

79. Regoli, F., & Winston, G. W. (1999). Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. *Toxicology and Applied Pharmacology*, *156*(2). https://doi.org/10.1006/taap.1999.8637
80. Regoli, F., d'Errico, G., Nardi, A., Mezzelani, M., Fattorini, D., Benedetti, M., di Carlo, M., Pellegrini, D., & Gorbi, S. (2019). Application of a weight of evidence approach for monitoring complex environmental scenarios: The case-study of off-shore platforms. *Frontiers in Marine Science*, *6*(JUL). https://doi.org/10.3389/fmars.2019.00377
81. Roark, A. M. C. (2020). Endocrine disruptors and marine systems. In *Encyclopedia of the World's Biomes* (Vols. 5–5). https://doi.org/10.1016/B978-0-12-409548-9.12426-1
82. Saaristo, M., Craft, J. A., Lehtonen, K. K., & Lindström, K. (2010). An endocrine disrupting chemical changes courtship and parental care in the sand goby. *Aquatic Toxicology*, *97*(4). https://doi.org/10.1016/j.aquatox.2009.12.015
83. Santos, L. H. M. L. M., Araújo, A. N., Fachini, A., Pena, A., Delerue-Matos, C., & Montenegro, M. C. B. S. M. (2010). Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. In *Journal of Hazardous Materials* (Vol. 175, Issues 1–3). https://doi.org/10.1016/j.jhazmat.2009.10.100
84. Sauvé, S., & Desrosiers, M. (2014). A review of what is an emerging contaminant. *Chemistry Central journal*, *8*(1), 15. https://doi.org/10.1186/1752-153X-8-15
85. Schultz, M. M., Painter, M. M., Bartell, S. E., Logue, A., Furlong, E. T., Werner, S. L., & Schoenfuss, H. L. (2011). Selective uptake and biological consequences of environmentally relevant antidepressant pharmaceutical exposures on male fathead minnows. *Aquatic Toxicology*, *104*(1–2). https://doi.org/10.1016/j.aquatox.2011.03.011
86. Schulze, T., Weiss, S., Schymanski, E., von der Ohe, P. C., Schmitt-Jansen, M., Altenburger, R., Streck, G., & Brack, W. (2010). Identification of a phytotoxic photo-transformation product of diclofenac using effect-directed analysis. *Environmental Pollution*, *158*(5). https://doi.org/10.1016/j.envpol.2009.12.032
87. Ștefan, M. G., Kiss, B., Gutleb, A. C., & Loghin, F. (2020). Redox metabolism modulation as a mechanism in SSRI toxicity and pharmacological effects. In *Archives of Toxicology* (Vol. 94, Issue 5). https://doi.org/10.1007/s00204-020-02721-6

88. Sumpter, J. P., Donnachie, R. L., & Johnson, A. C. (2014). The apparently very variable potency of the anti-depressant fluoxetine. *Aquatic Toxicology*, *151*. https://doi.org/10.1016/j.aquatox.2013.12.010
89. Świacka, K., Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., & Świeżak, J. (2022). Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms – Current state of knowledge. In *Journal of Hazardous Materials* (Vol. 424). https://doi.org/10.1016/j.jhazmat.2021.127350
90. The Medicines Utilisation Monitoring Centre. National Report on Medicines use in Italy. Year 2021. Rome: Italian Medicines Agency, 2022
91. Tran, B. X., Ha, G. H., Vu, G. T., Nguyen, L. H., Latkin, C. A., Nathan, K., McIntyre, R. S., Ho, C. S., Tam, W. W., & Ho, R. C. (2019). Indices of change, expectations, and popularity of biological treatments for major depressive disorder between 1988 and 2017: A scientometric analysis. *International Journal of Environmental Research and Public Health*, *16*(13). https://doi.org/10.3390/ijerph16132255
92. Trombini, C., Kazakova, J., Montilla-López, A., Fernández-Cisnal, R., Hampel, M., Fernández-Torres, R., Bello-López, M. Á., Abril, N., & Blasco, J. (2021). Assessment of pharmaceutical mixture (ibuprofen, ciprofloxacin and flumequine) effects to the crayfish Procambarus clarkii: A multilevel analysis (biochemical, transcriptional and proteomic approaches). *Environmental Research*, *200*. https://doi.org/10.1016/j.envres.2021.111396
93. United Nations Department of Economic and Social Affairs, Population Division (2022). *World Population Prospects 2022: Summary of Results*. UN DESA/POP/2022/TR/NO. 3.