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IMPACT OF CLIMATE CHANGE ON PHARMACEUTICAL MIXTURES UPTAKE AND TOXICITY ON THE MEDITERRANEAN MUSSEL *Mytilus galloprovincialis*

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Mestrado em Biologia Marinha

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Declaração de autoria de trabalho

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Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Melisa Castelli)

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Resumo

Os produtos farmacêuticos estão a emergir como contaminantes, sendo cada vez mais frequentemente detetados em ambientes aquáticos devido á sua utilização generalizada pelos seres humanos. As estações de tratamento de águas residuais (ETARs) convencionais não têm a capacidade de remover adequadamente estas substâncias, o que resulta na sua introdução e persistência nos ecossistemas marinhos, representando riscos significativos para os organismos aquáticos. Este estudo investiga a bioacumulação e os impactos biológicos de misturas farmacêuticas no mexilhão do Mediterrâneo, *Mytilus galloprovincialis*, em concentrações ambientais realistas encontradas naturalmente em águas superficiais, destacando a importância da compreensão dos efeitos dos contaminantes emergentes no contexto das alterações climáticas.

Nas últimas décadas, a presença de substâncias farmacêuticas em ambientes aquáticos tem causado uma crescente preocupação devido aos seus efeitos potencialmente nocivos nos organismos não-alvo. Esses compostos, que incluem uma ampla gama de medicamentos como analgésicos, antidepressivos, anticonvulsivos, entre outros, são frequentemente detetados em massas de água devido ao despejo contínuo de efluentes de estações de tratamento de águas residuais (ETARs). Embora as ETARs desempenhem um processo essencial para a remoção de muitos poluentes, a sua eficiência na remoção de diversos compostos farmacêuticos é limitada, permitindo que esses contaminantes entrem no ambiente aquático, e se espalhem continuamente nas bacias hidrográficas e no meio marinho.

A presença de produtos farmacêuticos em ambientes marinhos é particularmente preocupante devido à sua natureza bioativa e ao seu potencial de bioacumulação em organismos aquáticos. Estes fármacos são concebidos para serem biologicamente ativos em dosagens baixas, o que aumenta as preocupações sobre os seus possíveis impactos ambientais, mesmo que em pequenas concentrações. Além disso, muitos destes compostos não são completamente metabolizados ou degradados biologicamente e podem, mesmo após utilizados, tornar-se persistentes. .Estes compostos podem afetar negativamente os organismos aquáticos através de múltiplos mecanismos de ação, incluindo inibição de enzimas essenciais, indução de stress oxidativo e interferência nos sistemas hormonal e imunológico.

Mytilus galloprovincialis, um mexilhão mediterrâneo amplamente distribuído, é frequentemente utilizado como bioindicador para avaliar a qualidade ambiental devido à sua capacidade de filtrar grandes volumes de água e acumular contaminantes nos seus tecidos. Estudos anteriores demonstraram que vários produtos farmacêuticos podem acumular-se nos tecidos dos mexilhões, causando uma variedade de respostas

biológicas adversas que vão desde o stress oxidativo e danos genéticos, até a alterações no metabolismo lipídico e na função imunológica dos organismos.

Este estudo investiga especificamente a bioacumulação e os efeitos biológicos de uma mistura farmacêutica composta por várias classes de medicamentos encontradas em concentrações presentes no ambiente aquático em *Mytilus galloprovincialis* sob condições de alterações climáticas, considerando fatores ambientais como acidificação dos oceanos e ondas de calor marinho. Estes fatores de stress ambiental são particularmente relevantes no contexto das alterações climáticas, pois podem alterar a bioacessibilidade e a toxicidade dos contaminantes, e potenciar o seu efeito num organismos em metabolismo acrescido.

Entre os produtos farmacêuticos testados, a venlafaxina e a carbamazepina (utilizadas respetivamente como antidepressivo e anticonvulsivo) demonstram uma bioacumulação significativa em comparação com tratamentos de controlo não expostos à mistura, enquanto o ibuprofeno (anti-inflamatório), e o ramipril (vasodilatador e diurético) permanecem abaixo dos limites de deteção. Além disso, a contaminação pré-existente apresenta desafios para uma maior depuração e bioacumulação de metformina e gemfibrozil (utilizadas no tratameto da diabetes e do colesterol elevado). Estes resultados realçam a importância de considerar não só a presença de contaminantes, mas também quais os níveis de bioacumulação e o potencial de potenciais efeitos tóxicos nos organismos marinhos, mesmo em condições de hipercapnia e stress térmico.

Os resultados obtidos mostraram que a hipercapnia e as ondas de calor dinâmicas, tanto isoladamente quanto em combinação, influenciaram a bioacumulação dos antiepilépticos carbamazepina e dos antidepressivos venlafaxina. Notavelmente, níveis significativos do regulador lipídico gemfibrozil e do antidiabético metformina foram destacados em todos os tratamentos, sugerindo níveis basais potenciais desses medicamentos no ambiente marinho.

A análise das respostas celulares e bioquímicas revelou efeitos na modulação dos parâmetros imunológicos e do metabolismo lipídico, aumento da fragmentação do DNA e apoptose celular, além de um aumento do estresse oxidativo em organismos expostos aos estressores combinados das mudanças climáticas e à mistura de substâncias farmacêuticas. Interessantemente, a coexposição a ondas de calor e substâncias farmacêuticas reduziu significativamente a fragmentação do DNA, enquanto a acidificação combinada com a mistura aumentou o estresse oxidativo, sugerindo que as condições de hipercapnia podem ter um impacto mais pronunciado nos efeitos da mistura de substâncias farmacêuticas em *Mytilus galloprovincialis* em comparação com as mudanças de temperatura.

As análises químicas e de biomarcadores indicaram frequentes interações sinérgicas ou antagónicas entre os estressores das mudanças climáticas e a mistura de substâncias farmacêuticas, especialmente quando combinados. Estas descobertas sugerem ameaças potenciais para a saúde de *Mytilus galloprovincialis* em áreas costeiras com altos níveis de medicamentos e impactos climáticos concomitantes. Dada a importância ecológica desta espécie e seu valor econômico no mercado de frutos do mar, estudos de longo prazo são essenciais para elucidar as consequências ecológicas das misturas farmacêuticas sob cenários previstos de mudanças nas condições oceânicas.

Testar misturas farmacêuticas é fundamental devido à sua presença constante e aos potenciais efeitos cumulativos e interativos que possam surgir nos organismos marinhos. Considerar fatores relacionados com as mudanças climáticas, como a acidificação dos oceanos e as ondas de calor, é essencial, pois esses fatores podem modular a biodisponibilidade e a toxicidade dos contaminantes, levando a interações complexas que influenciam a saúde dos organismos e a estabilidade dos ecossistemas. Este estudo reforça a necessidade de realizar estudos de riscos ambientais abrangentes que integrem os impactos das misturas de contaminantes químicos e das alterações climáticas.

Compreender as interações entre misturas de contaminantes farmacêuticos e fatores de stress ambiental é fundamental para o desenvolvimento de estratégias de mitigação eficazes e políticas ambientais que protejam a biodiversidade marinha e garantam ecossistemas sustentáveis. Este estudo contribui para esta compreensão, fornecendo conhecimentos valiosos acerca dos mecanismos de bioacumulação e os efeitos biológicos das misturas farmacêuticas em *Mytilus galloprovincialis* no contexto das alterações climáticas.

Palavras-chave: alterações climáticas, poluição farmacêutica, múltiplos estressores, *M. galloprovincialis*

Abstract

Active pharmaceutical ingredients (APIs) are contaminants of emerging concern due to the lack of information on their presence, distribution, and ecological impacts. Marine organisms are exposed to complex mixtures of chemicals, including pharmaceuticals, which can interact and modulate overall toxicity through overlapping biological pathways. With climate change predicting ocean acidification and dynamic marine heatwaves, the impacts of pharmaceutical mixtures may differ, potentially altering their chemical composition and modulating the health of aquatic organisms.

This study aimed to assess how climate change stressors, namely decreased pH and two dynamic marine heatwaves, modulate the uptake and toxicity of an APIs mixture composed of six pharmaceuticals from different therapeutic classes at environmentally realistic concentrations, in Mediterranean mussels, *Mytilus galloprovincialis*. Measurements of drug bioaccumulation were integrated with biochemical and cellular responses. Mussels exposed to the APIs mixture accumulated significant levels of venlafaxine and carbamazepine, with hypercapnia and dynamic heatwaves, both alone and in combination, influencing their bioaccumulation patterns. Ibuprofen and ramipril were below the limit of detection, while no variations were observed for metformin and gemfibrozil: mussels from all treatments were characterized by pre-existing natural basal levels which remained unaffected throughout the experiment, thus representing a challenge for the depuration further limiting bioaccumulation of these two drugs. Along with bioaccumulation, various ecotoxicological markers were affected by the climate change stressors. Notably, there was a decline in lysosomal membrane stability and phagocytosis rate, indicating immune system impairment, primarily driven by the climate change stressors rather than by exposure to the APIs mixture. On the other hand, the effects measured in terms of increased DNA fragmentation and cell apoptosis, inhibition acyl-CoA oxidase activity and accumulation of neutral lipids, were mainly observed in response to combined stressors, namely hypercapnic, marine heatwaves and APIs mixture. Enhanced toxicity of the APIs mixture was particularly evident on oxidative stress parameters, especially under hypercapnic and combined stressor conditions.

Overall, the obtained results provide new insights into the response of aquatic organisms to pharmaceutical mixtures under scenarios of marine heatwaves and ocean acidification, highlighting their complex interactions and the need for comprehensive environmental risk assessments.

Keywords: pharmaceutical mixtures, multiple stressors, climate change, *Mytilus galloprovincialis*

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CHAPTER 2: IMPACT OF CLIMATE CHANGE ON PHARMACEUTICAL MIXTURES UPTAKE AND TOXICITY ON THE MEDITERRANEAN MUSSEL *Mytilus galloprovincialis*

List of abbreviations

CECs – Contaminants of Emerging Concerns

APIs – Active pharmaceutical ingredients

WWTPs - Wastewater treatment plants

NSAIDs - Nonsteroidal Anti-Inflammatory Drugs

VLF – Venlafaxine

CBZ – Carbamazepine

GEM – Gemfibrozil

MET – Metformin

IBU– Ibuprofen

RAM – Ramipril

HW – Marine Heatwaves

OA – Ocean Acidification

CHAPTER 1: Introduction

Challenges for the marine environment have dramatically changed in the last decade, both in terms of magnitude of disturbance and typologies of hazards. Besides traditional chemicals, contaminants of emerging concern (CECs) such as pharmaceuticals, microplastics, algal toxins, and new pathogens represent examples of occurring stressors for the health stratus of marine ecosystems. CECs are defined as 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. Additionally, global warming, primarily driven by anthropogenic activities, has resulted in significant alterations in aquatic environments, including ocean warming and ocean acidification. These environmental shifts have important implications for aquatic organisms, both directly and indirectly. One of the less studied but important indirect consequences of climate change is the alteration of chemical pollutants toxicity, including CECs.

Beyond the mere change in chemical compositions due to increased temperature or acidification processes, climate change may modulate various aspects of the aquatic species' health, including metabolic and feeding rates, consequently altering the bioaccumulation of chemicals in their tissue, potentially impacting the health of the organisms and the overall function of the ecosystem.

Despite the environmental risks posed by CECs, the limited data in the scientific literature and the inadequately documented concerns are the factors contributing to the classification of CECs as "emerging". As mentioned above, thousands of CECs have been discovered, spanning diverse chemical classifications including pharmaceuticals, personal care products (PPCPs), pesticides, hormones, flame retardants and micro and nano plastics. Among these, pharmaceuticals stand out as particularly noteworthy, due to their widespread use in healthcare and their continuous release in the aquatic system.

Therefore, it is important to address how climate change related stressors, such as the increased of heatwaves phenomena and ocean acidification, can influence the uptake of these chemicals from aquatic non-target species, altering their bioaccumulation rate and biological pathways.

1. Pharmaceuticals as CECs: sources and occurrence in aquatic ecosystems

Active pharmaceutical ingredients (APIs) are essential in modern society and have revolutionized healthcare by extending lifespans, enhancing disease prevention, and elevating overall health standards

(Mezzelani et al. 2018). However, their extensive use has led to their ubiquitous presence in aquatic ecosystems, posing potential risks and environmental concerns.

The primary source of pharmaceuticals in aquatic systems is human consumption, which results in excretion of both metabolized and unmetabolized drugs, improper disposal of unused medicines, and direct application of pharmaceutical-containing materials in agriculture and aquaculture. Additionally, their presence in the environment is also due to other pathways, including inefficient removal by wastewater treatment plants, industrial and hospital discharges, aquaculture facilities, animal farming, soil runoff, and direct discharge of untreated wastewater (Jaseem et al. 2017; Caban and Stepnowski 2021).

Wastewater treatment plants (WWTPs), not designed to fully eliminate pharmaceuticals, demonstrate low efficiencies in removing several compounds, resulting in their persistence in the environment. These treatment facilities typically handle organic substances within the milligrams per Liter range and often struggle to fully degrade pharmaceuticals due to their bio-refractory nature. Primary and secondary treatment processes offer minimal removal of these compounds, while tertiary treatments incorporating advanced filtration techniques exhibit higher removal capacities but face challenges in widespread implementation due to elevated costs (Stackelberg et al. 2004; Kümmerer 2009).

The occurrence and concentration of pharmaceuticals in the influents of WWTPs vary by region, influenced by factors such as the socioeconomic composition of the population served, consumption patterns, climatic conditions, and water usage (Stackelberg et al. 2004; Huang et al. 2018; Khasawneh and Palaniandy 2021). Conversely, the concentrations of pharmaceuticals in WWTP effluents are determined by the characteristics of the pharmaceutical compounds (e.g. volatility), the properties of the wastewater influent (e.g. alkalinity and acidity), the adsorption of pharmaceuticals to suspended solids, and the specific treatment processes employed (Huang et al. 2018).

Studies showed that the antiepileptic carbamazepine (CBZ) and the nonsteroidal anti-inflammatory (NSAID) diclofenac (DCF) were detected at higher concentration in the effluent than in the influent (Nivala et al. 2019; Martínez-Alcalá et al. 2021). For CBZ, Kruglova et al. (2014) reported that this behaviour could be explained by the presence of in the inlet conjugate compounds that during the treatment process are transformed into the parental compounds. In the case of DCF, Kimura and co-Authors (2007) demonstrated that its persistence was due to the presence of chlorine in its structure, which made it difficult to degrade during biological treatment.

However other compounds, e.g. the NSAID ibuprofen (IBU), were moderately biodegraded during the WWTP processes, showing lower concentrations in the effluent than in the influent. Nevertheless, only a few pharmaceutical compounds were monitored comparing with whole of them.

Moreover, different variables such as physical and chemical characteristic of pharmaceuticals, or abiotic parameters as temperature, pH, and photolysis by solar irradiation can lead to degradation of these molecules. However, these compounds are considered pseudo-persistent pollutants because of their continuous influx into environmental matrix (Daughton 2004; Patel et al. 2019).

Over the past three decades, traces of pharmaceuticals have been detected across various environmental compartments worldwide, encompassing surface water bodies such as lakes, rivers, streams, estuaries, and seawater, with concentrations ranging from nanograms per Liter (ng/L) to micrograms per Liter (μg/L) (Garg et al. 2023). Commonly found APIs in marine environments include NSAIDs, psychiatric drugs, antiepileptics, lipid regulators, and cardiovascular drugs, highlighting their broad environmental presence and potential ecological impacts (Godoy, Kummrow, and Pamplin 2015; Alygizakis et al. 2016; Biel-Maeso et al. 2018; Almeida et al. 2021; Ambrosio-Albuquerque et al. 2021; Mezzelani et al. 2023).

For instance, IBU, as reviewed by aus der Beek and co-Authors (2016), was averaged at a concentration of 0.118 μg/L in surface waters globally. In other studies, high concentrations of this compounds have been found, such as in effluents of Spanish WWTPs at concentrations up to 28 μg/L (Gómez et al. 2007) and in remote areas of the Northern Antarctic Peninsula at levels ranging from not detectable up to 974 ng/L (González-Alonso et al. 2017). Moreover, in surface water of coastal areas of the Mediterranean sea, IBU levels range from 0.21 to 321 ng/L (Mezzelani et al. 2018; Togola and Budzinski 2008; Alygizakis et al. 2016). Similarly, venlafaxine (VLF), an antidepressant, is among the most ubiquitous drugs in European coastal waters, with concentrations reaching up to 291 ng/L (Alygizakis et al. 2016; Fernández-Rubio et al. 2019). The anticonvulsant carbamazepine (CBZ) is also ubiquitous in the environment due to its high resistance to biodegradation and was detected in European waters with average levels ranging from below the limit of detection (< LOD), up to hundreds of ng/L, with peaks of 1410 ng/L in the east coast of Ireland (Almeida et al. 2021). Cardiovascular drugs, though less frequently studied, are also present, with beta-blockers and angiotensin II receptor blockers detected at levels up to 10 μg/L (Godoy et al. 2015). Among antidiabetics, metformin (MET), shows concentrations in surface waters vary widely, from no detectable levels in Germany to 9200 ng/L in the USA (Ambrosio-Albuquerque et al. 2021), while gemfibrozil (GEM) has concentrations in the Mediterranean Sea ranging from 3.3 to 5.7 ng/L (Biel-Maeso et al. 2018).

1.1 Ecotoxicological effects of pharmaceuticals

Pharmaceutical compounds, designed to permeate biological membranes, have been detected in several aquatic species (Martin-Diaz et al. 2009; Almeida et al. 2014; Zenker et al. 2014; Ali et al. 2018; Elizalde-Velázquez and Gómez-Oliván 2020; Mezzelani et al. 2020; Almeida, Soares, et al. 2021; Sharma, Thakur, and Kaushik 2021; Mezzelani and Regoli 2022). These chemicals target specific metabolic and molecular pathways in humans and animals, but they often have significant side effects, affecting the same pathways in other organisms with identical or similar target organs, tissues, cells, or biomolecules (Fent et al. 2006; Mezzelani and Regoli 2022). Some species have receptors that resemble those in humans, while others differ or are absent, leading to potentially different modes of action (Fent et al. 2006). Considering their nature of biologically active molecules at low concentrations along with their ubiquitous presence in aquatic ecosystems, APIs can interact with non-target species and lead to a modulation of their biochemical and physiological processes, with uncertain long-term effects (Daughton 2004; Fent at al. 2006; Lapworth et al. 2012).

Depending on the type and concentration of pharmaceuticals, literature studies observed that the alterations involve activation of the immune system, modulation of lipid and oxidative metabolism, genotoxic effects, and impairment of the endocrine system, adversely affecting organismal homeostasis, development, behaviour, and reproduction (Moreno-González et al. 2015; Cortez et al. 2018; Mezzelani, Gorbi, and Regoli 2018; Mezzelani and Regoli 2022). The same therapeutic classes found in the environment, including NSAIDs, psychiatric drugs, antiepileptics, lipid regulators, and cardiovascular drugs, have also been detected in aquatic species (Moreno-González et al. 2015; Cortez et al. 2018; Mezzelani, Gorbi, and Regoli 2018; Mezzelani and Regoli 2022).

NSAIDs, commonly used to treat inflammation and pain and to relieve fevers, sometimes are also used for long-term treatment of rheumatic diseases. This widespread use results in substantial environmental contamination, making this class of pharmaceuticals prevalent pollutants in marine environment ecosystem. NSAIDs have been detected in a range of marine organisms, from benthic invertebrates to top predators, with evidence of a possible biomagnification (Oaks et al. 2004; Zenker et al. 2014; Mezzelani, Gorbi, and Regoli 2018).

This class of molecules exert their effects through nonselective inhibition of cyclooxygenase (COX)-1 and -2 isoforms, thereby reducing the catalytic activity involved in prostaglandin (PGs) biosynthesis from arachidonic acid (AA) phospholipids (Vane and Botting 1998; Fent et al. 2006). PGs play essential roles in various physiological processes in mammals, including hemostasis, sleep-wake regulation, smooth muscle tone, and the regulation of vascular, temperature, and immune responses. In invertebrates, PGs similarly influence oogenesis and spermatogenesis, ion transport, and defense mechanisms (Rowley et al. 2005).

The ecotoxicological effects of NSAIDs have been studied in various marine species. Although data on the presence of IBUs in marine organisms are limited, Mezzelani et al. (2018) indicated bioaccumulation of these compounds in 15.1% and 9.3% of Mediterranean mussels sampled, with concentrations ranging from a few to several hundreds of ng/g. For example, Gagné and co-Authors (2005) demonstrated the inhibition of PGs synthesis and COX activity in *Elliptio complanata* mussels following IBU injection. Gonzalez-Rey and Bebianno (2012) revealed a temporary increase in antioxidant enzyme activities along with lipid peroxide formation in *M. galloprovincialis*, suggesting IBU's ability to generate reactive oxygen species (ROS) in short-term exposure. Furthermore, alkali-labile phosphate (ALP) levels increased over time, particularly in exposed males, indicating potential reproductive impairment and indicating that IBU's role as an endocrine disruptor may have a greater impact than its short-term ROS-generating.

Among psychiatric drugs, the anticonvulsant and mood-stabilizing CBZ is one of the most frequently detected and bioaccumulated compounds in the environment. CBZ is primarily used in the treatment of epilepsy and bipolar disorders. It acts on the central nervous system by decreasing overall neuronal activity through the blockage of voltage-dependent sodium channels in excitatory neurons. The therapeutic effects of CBZ in mammals are due to its ability to block voltage-gated sodium channels, antagonize the gamma-aminobutyric acid (GABA) receptor, inhibit glutamate release, and prevent chloride entry into cells (Siebel et al. 2010).

Despite its widespread use in human therapies, CBZ is associated with several side effects, including enhancement of human erythrocyte glutathione and glutathione peroxidase, cytotoxicity at the cell membrane level, neurite swelling, hepatic toxicity (Suwalsky et al., 2006), oxidative stress, and alterations in membrane phospholipids (Santos et al. 2008).

CBZ has been measured in over 90% of mussels collected from Mediterranean coasts and various fish and shellfish species sampled in the Red Sea (Ali et al. 2018; Mezzelani et al. 2020). The high levels of CBZ, up to 3.5 ng/g d.w. in *M. galloprovincialis* collected in the Mediterranean sea, can be partly attributed to its bio-refractory properties, resulting in an average half-life of over 200 days in aquatic ecosystems (Martin-Diaz et al. 2009).

Similarities were recently discovered in modes of action of this molecule between target and non-target species: CBZ impaired the health and induced oxidative stress in marine clams *Venerupis decussata* and

V. philippinarum. Both species showed increased activities of antioxidant enzymes such as glutathione reductase (GR), superoxide dismutase (SOD), and cytochrome P450 3A4 after exposure to CBZ (Almeida et al. 2014). Additionally, CBZ lowered the health status of mussels (*M. galloprovincialis*) and challenged their antioxidant enzyme system, as evidenced by increased activities of catalase (CAT) and glutathione S-transferase (GST), along with accumulation of the oxidation product malondialdehyde (Martin-Diaz et al. 2009). At the organismal level, Oliveira and co-Authors (2017) observed alterations in mussels' physiological condition (cardiac index) and gonadosomatic index following exposure to CBZ, suggesting potential impairments in reproductive capacity and negative effects on feeding. These physiological responses may lead to a slowdown in metabolism, resulting in decreased reproductive performance and energy reserves, with long-term adverse consequences for population sustainability (Oliveira et al. 2017).

Venlafaxine (VLF) is an antidepressant acting as a serotonin-norepinephrine selective reuptake inhibitor (SNRI) that increases the availability of these neurotransmitters at the synapse by blocking their reuptake at the presynaptic terminal, resulting in enhanced stimulation of postsynaptic receptors. As already mentioned above, VLF is ubiquitous in the environment and both this chemical and its metabolite, o-desmethyl-venlafaxinehas, have been detected in various marine organisms from the Mediterranean Sea, Red Sea, and Atlantic Ocean (Álvarez-Muñoz et al. 2015; Fernández-Rubio et al. 2019; Martínez-Morcillo et al. 2020). Notably, it is one of the most frequently detected pharmaceuticals in *M. galloprovincialis* collected along the Mediterranean coast, with levels of 36.1 ng/g d.w. in the mussels collected from the Po delta (Álvarez-Muñoz et al., 2015).

The ecotoxicological effects of VLF have been documented by Lacaze et al. (2015), who observed detrimental impacts on marine mussels, including immunomodulation and DNA damage in mussel hemocytes. Additionally, their study reported a hormetic response in phagocytic activity after VLF exposure, suggesting that low-dose exposure might induce cellular stress. Hormesis, occurs when substances usually harmful at high doses show adverse effects only at lower concentrations (non-linear toxicity pattern), is often noted in immunotoxicity studies in aquatic species (Fong and Ford 2014).

Cardiovascular drugs, used to treat conditions like hypertension, myocardial infarction, heart failure, and coronary artery disease, comprehend a range of pharmaceuticals that that directly modulate the function of the heart and blood vessels. This category includes beta-blockers (more frequently found in environmental compartments), ACE inhibitors, and calcium channel blockers, often prescribed with lipid-regulating agents e.g. fibrates. Compared to other therapeutic classes, the environmental concentrations of cardiovascular drugs are relatively understudied, particularly their effects on non-target marine species (Zhang et al. 2020). ACE inhibitors, such as ramipril (RAM), inhibit both circulating and tissue angiotensin-converting enzyme (ACE), leading to decreased angiotensin II formation, reduced sympathetic activity, and lower sodium and water reabsorption in the kidneys. As a result, sympathetic activity decreases, and there is a reduction in sodium and water reabsorption from the kidneys. Furthermore, the smooth muscles in the arterioles relax, resulting in a decrease in blood pressure.

There is limited information about the bioaccumulation and potential toxicity of these drugs in marine environments. Cortez et al. (2018) found that higher concentrations of losartan (LOS), an antihypertensive drug from the angiotensin II receptor antagonist class, adversely affected the reproductive parameters of the brown mussel *Perna perna*. Their study showed that LOS exposure induced detoxification and antioxidant systems in the mussels at concentrations ranging from ng/L to μg/L, causing cyto-genotoxic effects in the gills and hemolymph. Beta-blockers have been extensively studied for their effects on aquatic organisms, particularly invertebrates and fish. In *Daphnia magna*, beta-blockers significantly impact reproduction and growth, with propranolol exposure leading to decreased fecundity and growth inhibition (Dzialowski et al. 2006). In fish early life stages, propranolol exposure resulted in growth reduction, changes in organ size, and alterations in reproductive hormone levels. Additionally, adult fish exhibited inhibited anxiety behaviour and changes in swimming behaviour when exposed to beta-blockers like diltiazem and verapamil (Zhang et al. 2020).

Lipid regulators, including statins and fibrates, are commonly used to reduce cholesterol and triglyceride levels in blood plasma. Fibrates influence lipid metabolism by altering the transcription of genes encoding proteins involved in lipoprotein metabolism. They likely activate the enzyme lipoprotein lipase, which converts very low-density lipoprotein (VLDL) to high-density lipoprotein (HDL), thus reducing plasma triglyceride levels. Fibrates bind to peroxisome proliferator-activated receptors (PPARs), nuclear receptors that regulate various cellular pathways, stimulating the expression of lipid regulatory proteins such as lipoprotein lipase. Three PPAR subtypes have been identified: PPARα, which controls hepatic lipid metabolism and is involved in peroxisome proliferation; PPARβ, which plays roles in basic lipid metabolism; and PPARγ, which is crucial for adipocyte differentiation (Staels et al. 1998; Kersten, Desvergne, and Wahli 2000).

Fibrates promote cellular fatty acid uptake, conversion to acetyl-CoA derivatives, and catabolism via beta-oxidation pathways. This, combined with reduced fatty acid and triglyceride synthesis, decreases VLDL production. Chronic fibrate exposure has been linked to hepatic damage in rats, possibly due to inhibited mitochondrial oxidative phosphorylation, and has caused massive peroxisome proliferation in rodents.

These findings increase the interest in the ecotoxicological impact of this therapeutic class of drugs (Cajaraville et al. 2003).
Among fibrates, gemfibrozil (GEM) decreases triglycerides and low-density lipoproteins by stimulating peroxisomal β-oxidation of fatty acids. Its ecotoxicity is not extensively investigated, but Schmidt et al. (2011) have discovered that GEM exposure in *Mytilus spp.* induced biomarkers of stress, including GST and levels of metallothioneins. Biomarkers of damage, such as lipid peroxidation (LPO) and DNA damage, were significantly affected, as well as the biomarker for reproduction, ALP levels, indicating the potential oxidative stress and endocrine disrupting effect of GEM (Schmidt et al. 2011). In the study by Canesi et al. (2007), GEM exposure of the bivalve *M. galloprovincialis* resulted in concentration-dependent lysosomal destabilization and extracellular lysozyme release. Additionally, *in vivo* exposure to fibrates induced significant effects on mussel digestive gland, including increase of CAT activity. These observations support the notion of GEM's impact on lysosomal integrity and the release of lysozyme, as well as its effects on tissue redox balance and peroxisomal enzymes (Canesi et al. 2007).
As already mentioned above, metformin (MET) and its major transformation product guanylurea are frequently detected in marine environments. This pharmaceutical is prescribed for type 2 diabetes and polycystic ovary syndrome. In mammals MET acts in the liver, where it enters hepatocytes via the organic cation transporter 1 (OCT1). It inhibits complex I of the mitochondrial electron transport chain, reducing glucose production and ATP levels, which are necessary for hepatic gluconeogenesis. It activates AMP-activated protein kinase (AMPK), which inhibits gluconeogenesis and lipid synthesis pathways. Moreover, MET is excreted unchanged through the bile and kidneys via the multidrug and toxin extrusion protein (MATE) (Niemuth and Klaper 2015; Ambrosio-Albuquerque et al. 2021).
Ecotoxicological studies on non-target species have shown MET's potential as an endocrine disruptor. In *Pimephales promelas*, MET exposure led to intersex conditions, where male reproductive tissues demonstrated feminization, while female tissues showed spermatogonium-stage cells, reducing fertility (Niemuth and Klaper 2015). Although MET lacks a hormone-like structure, it can impact the endocrine systems of vertebrates such as fish and mammals. Few studies on invertebrates are currently available: Koagouw and Ciocan (2018) found that *Mytilus edulis* exposed to MET exhibited increased vitellogenin mRNA expression, induce severe gonadal pathologies, and destabilization of lysosomal membranes in hemocytes, highlighting MET's potential negative effects on marine organisms.

Assessing the environmental risks associated with pharmaceuticals requires comprehensive consideration of their consumption volumes, physicochemical properties, and ecotoxicities.

Pharmaceuticals are highly soluble in water, persistent, and capable of bioaccumulating, with potential toxic effects on organisms necessitating thorough risk assessment in aquatic environments (Kümmerer 2009). These compounds exist in the environment as complex mixtures, which may exhibit different toxic effects compared to individual pharmaceuticals due to interactions among their components (Patel et al. 2019). Previous acute tests conducted on freshwater organisms often used concentrations significantly higher than those found in the environment, potentially underestimating or overestimating ecological risks (Kidd et al. 2024).

Studies on *Daphnia* and *Mytilus spp.* have demonstrated significant ecological impacts from pharmaceutical mixtures, often at lower concentrations than those of individual compounds. For instance, Cleuvers (2004) showed that mixtures of NSAIDs, including DCF, IBU, naproxen, and acetylsalicylic acid, induced toxicity in *D. magna* at environmentally relevant concentrations, whereas individual compounds did not. Similarly, Godoy et al. (2019) found that binary mixtures of MET, bisoprolol, ranitidine, and sotalol in *D. similis* and *Danio rerio* exhibited effects between those predicted by concentration addition and independent action models. This suggests that the cumulative risk from pharmaceutical mixtures may be greater than predicted based on single compound assessments.

In *Mytilus spp.*, pharmaceutical mixtures demonstrate significant effects: Ericson et al. (2010) documented reduced growth, lower byssus strength and attachment ability in mussels exposed to DCF, IBU, and propranolol, even at lower concentrations, and significant high bioaccumulation; Canesi et al. (2007) observed rapid lysosomal membrane destabilization, increased enzyme activity, and oxidative stress in *M. galloprovincialis* exposed to bezafibrate and GEM; Franzellitti et al. (2013) found that the antidepressant fluoxetine and the beta-blocker propranolol had antagonistic effects on mussels, influencing gene expression related to serotonin receptors and oxidative stress responses. Furthermore, Mezzelani et al. (2023) examined how CBZ and the cardiovascular drug valsartan interacted in *Mytilus spp.*, finding that their combination resulted in antagonistic effects, reducing CBZ's effectiveness.

However, existing tests often involve complex mixtures of drugs that occur commonly, with over half demonstrating evidence of additive effects, while fewer show synergistic or antagonistic toxicities (Martin et al. 2021). Nevertheless, these studies typically involve mixtures of fewer than five chemicals tested at unrealistically high concentrations and short periods (Cleuvers 2004; Canesi et al. 2007; Ericson, Thorsén, and Kumblad 2010; Franzellitti et al. 2013; Godoy et al. 2019; Mezzelani et al. 2023). Effective methods are needed to evaluate the ecotoxicological effects of long-term exposure to environmentally realistic concentrations of pharmaceutical mixtures (Mezzelani and Regoli 2022; Kidd et al. 2024). This becomes increasingly critical in the context of the simultaneous occurrence of chemical and physical stressors, such as those associated with climate change.

1.2 Existing regulatory guidelines

Despite their widespread use and environmental release, most pharmaceuticals are not included in environmental regulations or monitoring programs, The need to address this issue has led to international actions, including guidelines for eco-pharmacovigilance and regulatory approaches in Europe, the United States, Japan, and Australia (Mezzelani and Regoli 2022). The European Strategic Approach to Pharmaceuticals in the Environment promotes innovative, multidisciplinary approaches to identify the long-term effects of these compounds while ensuring access to safe and effective treatments (European Commission, 2019).

Eco-pharmacovigilance involves detecting, evaluating, and preventing the adverse effects of pharmaceuticals in the environment, including their toxicological effects and accumulation. It also emphasizes proper medication use, disposal, and the development of environmentally friendly medicines. Established in 2000, the European Water Framework Directive (WFD) aims to protect aquatic ecosystems and achieve good ecological and chemical status of EU waters. Despite the efforts, only 40% of EU surface waters achieved good status by 2018 (EEA Report No 7/2018). The WFD uses a holistic approach to assess environmental quality, recognizing the complexity of ecosystems and interactions between various pressures. It sets goals on a six-year cycle, with current objectives to be met by 2027; more recently, this aspect has been also considered by Marine Strategy Framework Directive (MSFD) (2008/56/EC) which specially focus on marine ecosystem.

Despite increased attention to pharmaceuticals, only 13 out of 4,000 classified substances are on the EU's dynamic watch-list of the WFD, which identifies priority substances posing significant environmental risks. The watch-list is established to identify priority substances, which are chemicals selected by the Commission due to their significant risk to the environment. The objective is to reduce these substances to their natural background levels or eliminate those that are not naturally occurring.

The first dynamic watch-list of the WFD included four pharmaceuticals: diclofenac,17-beta-estradiol (E2), 17-alpha-ethinylestradiol (EE2) and macrolide antibiotics. In October 2022, the European Commission proposed revising the list to include 24 new substances, including nine pharmaceuticals such as 17-beta estradiol, Estrone (E1), EE2, azithromycin, clarithromycin, erythromycin, carbamazepine, diclofenac, and ibuprofen, emphasizing the rising of awareness concerning the presence of pharmaceuticals in water and their effects on non-target organisms.

1.3 Ocean Warming and Marine Heatwaves: influence on pharmaceuticals toxicity

Marine heatwaves (MHWs) are defined as extended periods of abnormally elevated seawater temperatures, surpassing the 90th percentile of a 30-year historical baseline for at least five consecutive days (Hobday et al. 2016). These phenomena have become increasingly prevalent and severe due to the escalating thermal trends and climate change-related factors. In fact, the frequency of abnormally warm days annually has increased by nearly 50% since the early 20th century, with MHW intensity exhibiting a linear upward trend (Oliver et al. 2019; IPCC 2023) with projections indicating a constant aggravation of these trends, with numerous oceanic regions in near-ubiquitous MHW conditions by century's end (Oliver et al. 2019).

These perturbations will have deleterious consequences for the structural integrity, functional dynamics, and ecological services provided by marine ecosystems (Oliver et al. 2019). Significant ecological impacts have been documented, encompassing mass mortalities of emblematic taxa such as corals, gorgonians and sponges, and mussels (Oliver et al. 2019; Galil et al. 2022). Moreover, documented alterations in species distribution and abundance (Lonhart et al. 2019), coupled with shifts in community composition favouring thermophilic taxa (e.g. sea urchins, Smale et al. 2017), underscore the multifaceted repercussions of MHWs on marine ecosystems.

Furthermore, the implications of thermal stress on marine organisms extend beyond acute ecological perturbations, integrating profound impacts on organismal physiology and ecological resilience (Sokolova 2021). Elevated temperatures can change fundamental physiological processes, including metabolism, reproduction, and immune function, with far-reaching consequences for individual fitness and population dynamics (Crespo et al. 2021; Matoo et al. 2021). Organisms' capacity to acclimate or adapt to rapid changing thermal regimes plays an important role in determining the resilience of marine ecosystems to the growing impact of MHWs. Emerging evidence suggests that the long-term effects of thermal stress may manifest in delayed ecological responses, underscoring the complexity and persistence of these impacts on marine biota and ecosystem functioning. Oxidative challenge due to thermal stress in bivalves and fishes has been further evidenced by the *Nrf2*-dependent increase of antioxidants and the onset of oxidative damages as lipids peroxidation, loss of DNA integrity, nuclear abnormalities (Benedetti et al. 2022).

Recent studies have begun to explore the impact of ocean warming on the bioavailability and toxicity of pharmaceuticals in marine ecosystems. However, there is a limited literature regarding the influence of MHW scenario on pharmaceutical toxicological effects. Existing literature predominantly emphasizes the effects of ocean warming, yet it is crucial to differentiate between the two phenomena. In the case of MHW, there is a dynamic temperature fluctuation characterized by rapid increases and decreases, whereas ocean warming experiments typically maintain a steady temperature.

For instance, Freitas et al. (2020) examined the effect of a 4°C temperature rise on the toxicity of DCF and salicylic acid against *M. galloprovincialis*. Their findings indicated alterations in energy metabolism rates, antioxidant defences, and cell damage indicators in response to temperature elevation and pharmaceutical exposure individually. Although simultaneous exposure to both stressors did not enhance toxicity, elevated temperature resulted in heightened lipid peroxidation levels, suggesting hindered neutralization of oxygen free radicals produced by DCF. Previous research has demonstrated the involvement of the GST enzyme in DCF detoxification, corroborating the hypothesis that indicating that elevated temperature does not amplify its toxicity against *M. galloprovincialis* (Schmidt et al. 2011b).

Almeida et al. (2021) conducted an experiment illustrating that elevated temperature had a beneficial impact on the oxidative status of clam *V. philippinarum* when exposed to CBZ and cetirizine (CTZ), especially in combination. This highlights the activation of a defence mechanism by the bivalves under increased stress conditions. However, the authors cautioned that their study utilized relatively low concentrations of pharmaceuticals, implying that as oceanic pharmaceutical pollution increases alongside rising heatwaves, the biological response of bivalves may change.

Furthermore, Nardi et al. (2022) discovered that MHWs appeared to significantly modulate the accumulation of CBZ, accompanied by weakened immunocompetence and the onset of oxidative disturbance, ultimately leading to cellular damages and lipid metabolism disorders. These findings suggest that MHWs could impact the capability of mussels to counteract CBZ toxicity, thus affecting their vulnerability and predisposition to adverse effects from multiple stressors.

1.4 Ocean acidification: influence on pharmaceutical toxicity

Another consequence of increasing levels of carbon dioxide in the atmosphere is ocean acidification (OA). The absorption of carbon dioxide by the oceans leads to a decrease in the pH of sea water, with a consequent increase in acidity. The effects of OA on marine organisms have been studied over the last two decades (Gattuso and Hansson 2011) and has been notice how this acidification process has profound implications for marine life, particularly organisms that rely on calcareous structures, but also in several biological processes of cellular homeostasis (Tomanek 2014).

Studies have shown that OA can negatively affect metabolic processes and physiological functions in marine species, potentially leading to significant disruptions in marine ecosystems. According to Fernández-Reiriz et al. 2011, clams *Ruditapes decussatus* exposed to acidifying conditions are able to lower the standard metabolic rate, decreasing clearance, ingestion and respiration rate. Navarro et al. (2016) showed a significant reduction in clearance rate after exposing *Mytilus chilensis* to acidified seawater for 35 days; food absorption rate and efficiency were lower at high pCO_2 levels. These physiological responses resulted in a significant reduction in the energy available for mussel growth. Michaelidis et al. (2005) demonstrated that elevated CO_2 levels and a consequent decrease in seawater of pH to 7.3 may be fatal to *M. galloprovincialis*, indicating a dramatic reduction in metabolic rate and confirming that reduction in seawater pH is very harmful for shelled molluscs.

To date, only Freitas et al. (2015, 2016) has conducted an acute toxicity test of a pharmaceutical against a marine organism under acidification. The study of the biochemical changes induced by the combination of two stressors namely CBZ and reduced pH, against *Scrobicularia plana* in both acute and chronic toxicity experiments, was interestingly discovered that, during the acute test, the combined effect of CBZ and lowered pH affected the physiological and biochemical performance of *S. plana*, even if a higher mortality was observed under single stressor exposure. In long-term exposure conditions, *S. plana* exhibited higher mortality due to the cumulative effects of CBZ and acidification (Freitas et al. 2016); bivalves seem to adapt to pH drop, with onset of mortality observed only after 96 hours. This suggests that initial exposure may trigger defence mechanisms leading to the depletion of energy reserves, which became insufficient to sustain in long-term stress conditions.

Munari et al. (2018) investigated the chronic toxicity of DCF and concomitant acidification at different concentrations, against *M. galloprovincialis* and *V. philippinarum*. The clams showed less resistance to acidification as a single stressor. Combining both stressors led to genotoxic changes in mussel DNA, particularly in the gills, with more severe alterations observed in this tissue. CAT and SOD activities remained unchanged, while elevated LPO levels indicated cell damage in both mussels and clams during exposure to both stressors.

Almeida et al. (2018) conducted a 28-day toxicity test of CBZ and cetirizine (CTZ) under different pH conditions against *R. philippinarum*. A 31% decrease in CTZ accumulation occurred at lower pH, while CBZ accumulation remained unaffected due to its neutral form. However, mRNA transcription changes were observed under both stressors, indicating a biological response despite the absence of oxidative stress effects at lowered pH. Similarly, the modulation of GABA pathway in *M. galloprovincialis* (Mezzelani et al. 2021) suggested a similar CBZ' mode of action known for vertebrates (e.g. immune responses, cellular homeostasis and oxidative system represented the processes targeted by combined stressors) with an increased cellular hazard due to interactions of CBZ with acidification compared to single stressors.

Lo et al. (2021) examined the impact of acidification on fluoxetine on the sea urchin *Heliocidaris crassipina*. In addition to other parameters, the DNA damage was found to be lower when the organisms were exposed to both stressors simultaneously, possibly due to the reduced bioavailability of fluoxetine under acidified condition. The combination of acidification and fluoxetine adversely affected the embryonic development of sea urchins, particularly noticeable at lower concentrations of the tested drug.

1.5 Ocean warming and ocean acidification: influence on pharmaceutical toxicity

OA, ocean warming and MHW are concomitant changes driven by the same cause, the respective interactions and influence on biological processes are of outmost relevance to understand the implications for organisms' health. Overall, it has been extensively suggested that the reduction of seawater pH could reduce the organisms capability to cope with thermal stress, especially in marine invertebrates that lack acid-base regulation systems (Pörtner, Bock, and Mark 2017): thus, the onset of oxidative disturbance due to thermal stress could be disclosed earlier. Despite this general assumption, studies on the effects derived from the combination of temperature and pH and on the nature of these interactions, revealed that the interplay between thermal and pH stress may be subjected to different variables, but constrained by physiological aspects regarding tested life-stages, considered taxa, and more specific the level of biological organization considered and the physiological function of the analysed organ (Kroeker et al. 2013; Lefevre 2016; Giuliani et al. 2021).

As mentioned above, in this complex marine ecosystem, organisms face multiple stressors simultaneously, which can result in various additive, synergistic, or antagonistic effects (Martin et al. 2021). From a biological and environmentally perspective, even a limited disturbance caused by one stressor can indirectly impact the susceptibility of one organism toward a secondary stressor (Kroeker, Kordas, and Harley 2017). This interconnection underscores the importance of considering the combined effects of environmental stressors on marine organisms. Changes in environmental conditions, such as temperature fluctuations and ocean acidification, not only affect the physical-chemical properties of contaminants but also influence the physiological status of organisms (Nardi et al. 2018).

For instance, an increase in water temperature can enhance the metabolic activity of aquatic organisms like mussels, leading to increased feeding and adsorption of contaminants (Navarro et al. 2016). However, this relationship is not consistent for all compounds. An increase in cadmium accumulation in *M. galloprovincialis* under warming conditions was not found, suggesting that the bioconcentration capacity of each contaminant under water warming should be evaluated separately (Nardi et al. 2018; Serra-Compte et al. 2018).

Variations in the bioavailability of contaminants and their accumulation by organisms have been demonstrated to occur under different conditions, including climate change-related abiotic stressors. As mentioned earlier, ocean warming and acidification can alter the physical and chemical properties of contaminants, thereby affecting their capacity to be accumulated by organisms. However, Serra-Compte et al. (2018) observed that acidification plays a pivotal role in driving changes in bioconcentration when both OA and OW act simultaneously. This finding is consistent with other studies indicating that acidification has a more pronounced impact on calcareous organisms compared to temperature changes (Duarte et al. 2014). Additionally, research has shown that acidification exerts a greater effect than warming when both stressors are present simultaneously, as evidenced by the case of the mussel *M. chilensis*, which displayed resilience to increased temperatures but not to elevated CO_2 levels (Duarte et al. 2014).

For example, it has been found that acidification, both alone and in combination with warming, decreases the bioconcentration factor (BCF) of the antidepressant VLF, resulting in a reduced capacity of mussels to accumulate this pharmaceutical (Serra-Compte et al. 2018). However, bioaccumulation does not always correlate with pharmaceutical toxicity levels (Lopes et al. 2022). While low stress and slowed cellular metabolism can lead to contaminant accumulation, enhanced toxicity can trigger increased detoxification mechanisms, thereby reducing bioconcentration factors. However, this response varies among different pharmaceuticals. For instance, sulfonamides exhibit an opposite trend, with bioconcentration factors increasing alongside toxicity (Serra-Compte et al. 2018). Decreasing aquatic pH levels could enhance pharmaceutical bioconcentration due to a decreased metabolism. However, Serra-Compte et al. (2018) discovered that acidification also promotes mussels' excretion rates to eliminate ammonium, leading to a higher elimination percentage of these chemicals. Long-term exposure studies suggest organisms can adapt, thereby affecting bioaccumulation. The impact of temperature is complex, with elevated temperatures potentially reducing bioaccumulation despite stimulating defence mechanisms.

Effects of pharmaceuticals have been frequently modulated in marine organisms under projected ocean changes scenarios (Freitas et al. 2016, 2019; Almeida et al. 2014, 2021; Mezzelani et al. 2021; Nardi et al. 2022). However, research on the interactive effects of these multiple stressors in seawater environments are limited to a few marine species (fish, bivalves, protozoa) and drugs (mainly the widespread DCF or CBZ), highlighting the need for further investigation. In fact, there are just few studies that include realistic concentrations of mixtures of pharmaceuticals and their modulation due to climate-change related factors. Mainly all the studies focus on the combination of just few stressors and do not include realistic concentrations of mixture of APIs.

One of the few studies on the effects of climate change-related factors was conducted by Costa et al. 2020, where the authors assessed the acute toxicity of DCF under varying conditions of acidification and warming on two species of clam, *V. philippinarum* and *V. decussatus*. The findings indicated that *V. decussatus* experienced mortality during the acclimatization period under climate change conditions, while *V. philippinarum* exhibited mortality during DCF exposure under control conditions. Both species displayed enhanced activation of defense mechanisms in response to DCF exposure, with *V. decussatus* showing increased antioxidant enzyme activity. Additionally, *V. philippinarum* demonstrated increased respiration rates and glycogen depletion under climate change conditions, highlighting species-specific metabolic responses to stress,

Furthermore, Maulvault et al. (2018) conducted a chronic toxicity test on the fish *Dicentrarchus labrax*, exposed to DCF from dietary sources (500 ± 36 ng/kg d.w.) under conditions of acidification and warming. The study revealed various biochemical stress responses due to DCF, warming, and/or acidification. These CC conditions enhanced some effects of DCF exposure, such as further reducing erythrocytes viability and increasing brain GST activity and ubiquitin synthesis in muscle. However, co-exposure to these stressors also inhibited certain molecular responses, like the inhibition of catalase (CAT) and superoxide dismutase (SOD) and reduced vitellogenin (VTG) synthesis. The Integrated Biomarker Response (IBR) index indicated a higher overall stress level when fish were exposed to both DCF and warming, while the effects of acidification were less pronounced.

These studies enlighten the need for comprehensive research on the interactive effects of multiple stressors in marine environments, underscoring the importance of considering the combined impacts of environmental changes and pharmaceutical contaminants to better understand and mitigate their possible effects on marine ecosystems. In conclusion further research is essential to fully understand their complex dynamics and interactions.

2. Ecotoxicological approaches

The use of bivalves in biomonitoring programs began in 1974 with Young et al., who used the mussel species *M. californianus* for investigating the presence of chlorinated hydrocarbons in the Southern California Bight (Young, Heesen, and McDermott 1976).

Since then, marine mussels have become one of the most widely used groups of aquatic organisms as bioindicators, owing to their wide distribution, ecological roles, commercial importance, and ability to accumulate contaminants over relatively long lifespan (Mezzelani et al. 2020).

Mussels are sessile filter-feeding mussels, highly susceptible to marine pollution, and play a crucial role in maintaining ecosystem health and therefore the overall biodiversity. They are considered valuable tools for assessing marine pollution caused by CECs due to their ability to filter large quantities of water (between 25 to 50 Liters of water per day), making them effective for monitoring and good indicators for assessing the overall ecosystem health. The Mediterranean mussel, *M. galloprovincialis,* is the most widely used species as bioindicator, able to efficiently bioaccumulates dissolved substances in its gills, digestive glands, and gonads, making it vulnerable to oxidative stress induced by contaminants accumulation (Mezzelani et al. 2020). Consequently, *M. galloprovincialis* is frequently used to assess the toxicity of CECs, such as pharmaceuticals, and the stressors associated with climate change, as demonstrated in the studies mentioned above.

To better understand the impact of chemicals on aquatic ecosystems and infer the health status of the bioindicator species, biomarker-based methodologies are employed. These methodologies assess biological responses at molecular, biochemical, cellular, physiological, or behavioral levels and are classical tools for diagnosing the stress in test organisms exposed to chemical pollution.

Environmental toxicology researchers have developed indicators to measure disturbances in specific physiological parameters. The impact of contaminants can be considered at various levels of biological organization, each with increasing ecological significance and response time: molecular, biochemical, cellular, organismal, population, and community levels. Typically, primary toxicity manifests at biochemical and molecular levels (e.g., enzyme activity modification, genotoxic damage), with subsequent cascading effects on higher levels of hierarchical organization, eventually affecting the population level (Depledge and Fossi 1994).

Overall, the use of bivalves as bioindicators and the employment of biomarkers provide critical insights into the health of marine ecosystems and the impact of pollutants, allowing for early detection and mitigation of environmental damage (Chahouri et al. 2023).

In this respect, the biomarkers used in this thesis encompass various physiological disturbances. The main investigated endpoints reflecting the onset of biochemical and cellular disturbances include immune parameters, such as lysosome membrane stability (LMS), phagocytosis capacity, and the granulocyte-to-hyalinocyte ratio, as well as genotoxicity biomarkers, such as the Comet assay, DNA diffusion assay, and micronuclei (MN) frequency, applied to mussel hemocytes.

Neurotoxicity was evaluated through acetylcholinesterase activity in mussel hemocytes and gills, indicating early damage to neuronal transmission and related structures. Indicators of lipid peroxidation, such as malondialdehyde, lipofuscin, and neutral lipid content, were measured in mussel digestive glands. Additionally, biomarkers of peroxisome proliferation were assessed by analysing the enzymatic activity of acyl-CoA oxidase, an enzyme involved in the β-oxidation of fatty acids in mussel digestive glands. This comprehensive approach provides a detailed understanding of the biological and molecular effects of contaminants and climate change related factors on marine organisms, contributing to the broader assessment of ecosystem health.

3. Objectives

The aim of this thesis was to investigate the role of climate change related stressors, namely cyclic dynamic heatwaves and pH decrease, in modulating the toxicity of APIs mixture in the Mediterranean mussels*, M. galloprovincialis*.

We examined the effects of a mixture of six pharmaceuticals commonly found in marine ecosystems at environmentally realistic concentrations: ibuprofen (IBU), carbamazepine (CBZ), venlafaxine (VLF), ramipril (RAM), gemfibrozil (GEM), and metformin (MET).

The combined effects of climate change and pharmaceutical pollution remains underexplored, with limited data on the interaction of these stressors and their cumulative effects on marine life. This research aims to fill this gap, providing valuable insights into the combined impacts of environmental changes and pharmaceutical contaminants on marine ecosystems.

4. Research questions and hypotheses

(1) How are the bioaccumulation and toxicity of pharmaceutical mixtures modulated by potential synergistic, additive, or antagonistic effects under climate change exposure scenarios for *M. galloprovincialis*?

(2) Which climate change-related stressor, namely decreased pH/increased pCO_2, cyclic dynamic heatwaves, has the greatest impact on the toxicity and bioaccumulation of the tested APIs mixture in *M. galloprovincialis*?

(3) Which molecular or biological pathway is most sensitive *in M. galloprovincialis* when co-exposed to APIs mixture and climate change stressors?

Given that *M. galloprovincialis* is a very well-known and studied species especially in the ecotoxicological analysis regarding short- and long-term exposure to pharmaceuticals and climate change related factors such as ocean warming and acidification; therefore, the following hypotheses were conceived.

(1) Co-exposure to reduced pH/increased pCO_2 and two cyclic dynamic heatwaves will synergistically increase the bioaccumulation of the APIs mixture in *M. galloprovincialis*, leading to heightened toxicity compared to exposure to a single climate change-related stressor. This synergistic effect is expected due to the combined stressors amplifying each other's impact on the organism's physiological processes.

(2) Among the climate change stressors, the exposure to two cyclic heatwaves will have the greatest impact on the toxicity and bioaccumulation of the APIs mixture in *M. galloprovincialis*. Thermal stress enhances metabolism in marine organisms, increasing ventilation and feeding rates to meet higher metabolic demands; therefore, it is expected to result in greater bioaccumulation of APIs, leading to increased toxicity, with more severe effects compared to ocean acidification alone.

(3) Oxidative stress will be increased in *M. galloprovincialis* co-exposed to the APIs mixture and climate change stressors, making its related biomarkers the more sensitive ones. Oxidative stress results from an imbalance between the production of ROS and the organism's ability to neutralize these reactive intermediates or repair the subsequent cellular damage. Therefore, we hypothesize that oxidative stress will impact lipid metabolism by modulating lipid peroxidation, compromise lysosomal stability, and possibly causing genotoxicity.

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CHAPTER 2: IMPACT OF CLIMATE CHANGE ON PHARMACEUTICAL MIXTURES UPTAKE AND TOXICITY ON THE MEDITERRANEAN MUSSEL M*ytilus galloprovincialis*

Keywords: pharmaceutical mixtures, multiple stressors, climate change, *Mytilus galloprovincialis*

Abstract

Active pharmaceutical ingredients (APIs) are contaminants of emerging concern due to the lack of information on their presence, distribution, and ecological impacts. Marine organisms are exposed to complex mixtures of chemicals, including pharmaceuticals, which can interact and modulate overall toxicity through overlapping biological pathways. With climate change predicting ocean acidification and dynamic marine heatwaves, the impacts of pharmaceutical mixtures may differ, potentially altering their chemical composition and modulating the health of aquatic organisms. This study aimed to assess how climate change stressors, namely decreased pH and two dynamic marine heatwaves, modulate the uptake and toxicity of an APIs mixture composed of six pharmaceuticals from different therapeutic classes at environmentally realistic concentrations, in Mediterranean mussels, *Mytilus galloprovincialis*. Measurements of drug bioaccumulation were integrated with biochemical and cellular responses. Mussels exposed to the APIs mixture accumulated significant levels of venlafaxine and carbamazepine, with hypercapnia and dynamic heatwaves, both alone and in combination, influencing their bioaccumulation patterns. Ibuprofen and ramipril were below the limit of detection, while no variations were observed for metformin and gemfibrozil: mussels from all treatments were characterized by pre-existing natural basal levels which remained unaffected throughout the experiment, thus representing a challenge for the depuration further limiting bioaccumulation of these two drugs. Along with bioaccumulation, various ecotoxicological markers were affected by the climate change stressors. Notably, there was a decline in lysosomal membrane stability and phagocytosis rate, indicating immune system impairment, primarily driven by the climate change stressors rather than by exposure to the APIs mixture. On the other hand, the effects measured in terms of increased DNA fragmentation and cell apoptosis, inhibition acyl-CoA oxidase activity and accumulation of neutral lipids, were mainly observed in response to combined stressors, namely hypercapnic, marine heatwaves and APIs mixture. Enhanced toxicity of the APIs mixture was particularly evident on oxidative stress parameters, especially under hypercapnic and combined stressor conditions. Overall, the obtained results provide new insights into the response of aquatic organisms to pharmaceutical mixtures under scenarios of marine heatwaves and ocean acidification, highlighting their complex interactions and the need for comprehensive environmental risk assessments.

1. Introduction

Active Pharmaceutical Ingredients (APIs) are considered contaminants of emerging concerns due to their obliquitous presence in aquatic ecosystems (Mezzelani et al. 2018). APIs enter water bodies from various sources, including human excretion, improper disposal of medications, and industrial discharges (Jaseem et al. 2017; Caban and Stepnowski 2021). Moreover, pharmaceuticals are often not fully metabolized by humans or animals, and conventional wastewater treatment plants (WWTPs) are not designed to remove them effectively, causing their persistence in the environment (Stackelberg et al. 2004; Kümmerer 2009). Indeed, incomplete removal by treatment processes of WWTPs was documented for several active principles, including the antiepileptic carbamazepine (CBZ) and the Non-steroidal Anti-inflammatory drug (NSAID) diclofenac, which showed higher concentrations in effluents compared to influents (Nivala et al. 2019; Martínez-Alcalá et al. 2021).

This continuous influx of pharmaceuticals, despite their partial degradation, has resulted in concentrations in seawater ranging from nanograms per Liter (ng/L) to micrograms per Liter (μg/L), posing concerns for aquatic organism health and leading to their classification as pseudo-persistent pollutants (Daughton 2004; Patel et al. 2019; Garg et al. 2023).

The bioaccumulation of these compounds in aquatic organisms is well-documented, with evidence showing their presence in various aquatic species ranging from invertebrates to top predators (Oaks et al. 2004; Zenker et al. 2014; Álvarez-Muñoz et al. 2015; Mezzelani et al. 2018; 2020; Ali et al. 2018; Fernández-Rubio et al. 2019; Martínez-Morcillo et al. 2020). Pharmaceuticals, being biologically active at low doses, can be toxic to non-target aquatic species due to the presence of receptors in marine organisms that are similar to those in target species. This similarity in receptors makes non-target species potentially susceptible to the same modes of action and side effects observed in humans (Daughton 2004; Fent et al. 2006; Lapworth et al. 2012).

Among the therapeutic classes most prevalent in aquatic ecosystems, nonsteroidal anti-inflammatory drugs (NSAIDs), psychiatric drugs, antiepileptics, cardiovascular drugs, and lipid regulators are particularly notable (Moreno-González et al. 2015; Mezzelani et al. 2018; Cortez et al. 2018; Mezzelani and Regoli 2022). NSAIDs like ibuprofen (IBU), widely used for anti-inflammatory purposes, have been found to bioaccumulate in marine species, leading to disruptions in physiological processes such as prostaglandin synthesis and immune responses (Gagné et al. 2005; Gonzalez-Rey and Bebianno 2012). Antiepileptics, such as CBZ, are resistant to biodegradation and persist in the environment, causing oxidative stress and reproductive impairments in mussels and clams (Almeida et al. 2014; Oliveira et al.

2017). Psychiatric drugs, including venlafaxine (VLF), have been shown to cause immunomodulation and DNA damage in marine organisms (Lacaze et al. 2015). Cardiovascular drugs, although less studied, have been linked to reproductive and developmental effects in aquatic species, with beta-blockers and ACE inhibitors (e.g., ramipril, RAM) demonstrating significant toxicological impacts (Dzialowski et al. 2006; Cortez et al. 2018). Lipid regulators like gemfibrozil (GEM) and antidiabetics such as metformin (MET) are commonly detected in aquatic environments. GEM has been shown to induce oxidative stress and endocrine disruption in marine mussels, impacting stress biomarkers and lysosomal integrity (Canesi et al. 2007; Schmidt et al. 2011). MET and its transformation product, guanylurea, also exhibit potential as endocrine disruptors, causing intersex conditions and fertility issues in fish, as well as severe gonadal pathologies and lysosomal destabilization in *Mytilus edulis* (Niemuth and Klaper 2015; Koagouw and Ciocan 2018).

The capacity of these APIs to be accumulated in marine organisms and to affect multiple molecular and cellular pathways is well-documented (Martin-Diaz et al. 2009; Almeida et al. 2014; Zenker et al. 2014; Ali et al. 2018; Elizalde-Velázquez and Gómez-Oliván 2020; Mezzelani et al. 2020; Almeida et al. 2021; Sharma, Thakur, and Kaushik 2021; Mezzelani and Regoli 2022). Organisms are typically exposed to low doses of multiple co-occurring drugs, which can interact and modulate overall toxicity through the same biological pathways (Almeida et al. 2014; Benedetti et al. 2022; Mezzelani and Regoli 2022). For example, studies on mixture have demonstrated that combinations of different classes of pharmaceuticals, e.g. NSAIDs, psychiatric and cardiovascular drugs, can lead to enhanced toxic effects, including increased mortality, behavioural changes, and impaired reproductive functions in aquatic species (Cleuvers 2004; Ericson, Thorsén, and Kumblad 2010; Canesi et al. 2007; Franzellitti et al. 2013; Mezzelani et al. 2023). However, despite the detection of pharmaceutical mixtures in marine species, the combined effects of these mixtures and their interactions with climate-change-related stressors have received limited attention (Mezzelani et al. 2023).

Climate change introduces additional challenges for the marine environment. Rising carbon dioxide (CO_2) emissions from human activities such as fossil fuel combustion and deforestation have led to significant environmental changes, including higher mean temperatures and increased frequency of marine heatwaves, short-term extreme events with seawater temperatures exceeding a seasonally varying threshold for at least five consecutive days (Oliver et al. 2019). Since the early 20th century, the frequency of these extreme warm days has increased by nearly 50%, with a linear upward trend in intensity (Hobday et al. 2016; Oliver et al. 2019). Additionally, CO_2 absorption by the ocean is causing acidification, lowering seawater pH (IPCC 2023).

These phenomena are altering the dynamics of aquatic environments, changing not only the distribution and bioavailability of pharmaceuticals but also modulating their toxicity. For example, increased temperatures can enhance the metabolic rates of aquatic organisms, leading to greater bioaccumulation of pollutants (Maulvault, Santos, et al. 2018).

Marine organisms are thus subjected to multiple stressors, including chemical pollutants and climate change-related factors. The combined effects of these stressors can lead to synergistic or antagonistic interactions, potentially intensifying the overall toxicity (Kroeker et al. 2017; Martin et al. 2021). Despite the significant implications, there are limited studies addressing the combined impacts of chemical and climate stressors on aquatic ecosystems. In addition, most studies were carried out at constant or linearly increasing temperature values (Schmidt et al. 2011a; Gazeau et al. 2014; Freitas et al. 2020; Almeida, Calisto, et al. 2021), while the effects of dynamic thermal stress, e.g. marine heatwaves, have been poorly investigated. Although several studies have already highlighted the negative impacts caused by single APIs in aquatic organisms (Canesi et al. 2007; Martin-Diaz et al. 2009; Gonzalez-Rey and Bebianno 2014; Niemuth and Klaper 2015; Lacaze et al. 2015; Mezzelani et al. 2016; Oliveira et al. 2017; Zhang et al. 2020), only limited information are available on the combined effects of climate change stressors on the responses and sensitivity of marine organisms towards environmentally realistic concentrations of APIs mixtures. To the best of our knowledge, no studies have evaluated the consequences of environmentally realistic concentrations of an APIs mixture, on bivalves acclimated to realistic climate change scenarios involving both dynamic heatwaves and acidification, acting alone and in combination. Considering that: i) most studies on drug impacts are limited to toxicity assessments under an exposure gradient, neglecting the combined effects of environmental conditions on compound behaviour and organism responses; ii) different chemicals may influence and contribute to effects in marine organisms through antagonistic, synergistic, or additive modulation of molecular and biochemical pathways; iii) climate change stressors may not only affect the sensitivity of organisms to pharmaceuticals but also change their toxicity.

The present work aims to evaluate the impacts of climate change stressors, namely ocean acidification and two marine dynamic heatwaves, on the bioaccumulation and biological responses associated with chronic pharmaceutical mixture exposure (42-day period) in the Mediterranean mussel, *M. galloprovincialis*. To this end, chemical analyses related to the bioaccumulation of the pharmaceuticals and biochemical markers related to mussels' immune capacity, genotoxicity, neurotoxicity, oxidative stress and lipid metabolism were evaluated. The present study is expected to provide a novel insight on the effects of climate change related stressors on pharmaceutical mixtures, both of emerging interest globally, thus representing a further step forward in clarifying the environmental risk correlated to combined multiple stressors.

2. Materials and methods

2.1 Experimental design

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

A total of 520 mussels were randomly distributed into 8 glass tanks, with 65 individuals per tank, in a volume of 30L of artificial seawater (Fig. 1). The experimental setup consisted of eight treatment groups: 1) representing the solvent control condition, CTRL (0.0017% of dimethyl sulfoxide, DMSO) with constant pH (8.0) and temperature (18 ± 1°C); 2) pH decrease, OA, simulating hypercapnic acidosis (pH 7.6, pCO_2 at 1700 µatm); 3) heatwaves scenario, HW, consisting of two consecutive dynamic heatwaves with peaks at 23 ± 1°C, where temperature increased daily by 1 °C until reaching the peak, maintained for 10 days, then gradually decreased until returning to the climatological mean temperature (18 ± 1°C), and held constant for an additional 6 days before the second heatwave; 4) combination of OA and HW exposure, CC; 5) APIs mixture composed by environmentally realistic concentrations of each pharmaceutical (Table 1): ibuprofen (IBU) 1.5 µg/L, carbamazepine (CBZ) 1.5 µg/L, venlafaxine (VLF) 0.3 µg/L, ramipril (RAM) 0.2 µg/L, metformin (MET) 1.5 µg/L and gemfibrozil (GEM) 0.4 µg/L; 6) combination of OA and APIs mixture; 7) combination of HW and APIs mixture; and 8) combination of CC and APIs mixture combined.

Despite the effects related to climate changes are expected to occur over the course of decades, organisms were exposed to the tested environmental conditions of APIs mixture and acidification without gradual acclimation, thus simulating sudden changes. Lowered pH was adapted from scenario RCP 8.5 and IPCC CMIP6 model projections (IPCC 2023; Kwiatkowski et al. 2020) reporting a mean pH value of 7.6 for open oceans. The experimental pH was reached by adding to each treatment ASW (pH = 8.0) small and defined amounts of CO_2-saturated ASW, obtained by bubbling pure CO_2 in ASW for at least 24h, until reaching the target pH within a couple of hours (A. Nardi et al. 2017).

Stock solutions (5000 mg/L) were prepared for each API in the mixture by dissolving drug powders in DMSO (for CBZ, VLF, IBU, RAM and GEM) or methanol (for MET). All stock solutions were then diluted with DMSO to reach a concentration of 500 mg/L. From these intermediate stocks, a final stock solution of 5mL was prepared with concentration of 90 mg/L for CBZ, IBU, and MET, 18 mg/L for VLF, 6 mg/L for RAM, and 24 mg/L for GEM. Finally, to reach the target concentrations for each pharmaceutical in the tanks (Table 1), 500 µL of the final stock were added to each aquarium.

Additionally, water changes and mixture redosing was carried out three times per week to maintain the desired concentration in each tank. Water in each treatment was changed every other day using water at the same pH and temperature to avoid fluctuations of these parameters during the exposure period. Mussels were fed 12 hours prior to water change with a commercial mixture of zooplankton for filter-feeding organisms (Easy-sps EVO).

After 42 days of exposure, for each experimental condition, 36 individuals were randomly sampled for each tank and dissected for biological and chemical analysis. For 21 organisms, hemolymph, digestive glands and gills were dissected, frozen in liquid nitrogen and stored at -80°C for histochemical and biochemical analysis. Aliquots of hemolymph for treatments were processed immediately for *in vivo* analysis of lysosomal membrane stability in hemocytes, phagocytosis activity and granulocytes-hyalinocytes ratio, while additional aliquots of hemolymph were fixed in Carnoy's solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency. 15 organisms for each treatment were also randomly selected and whole tissue was collected for chemical analysis. The remaining organisms have been processed for molecular and transcriptomic analyses, not showed in this study.

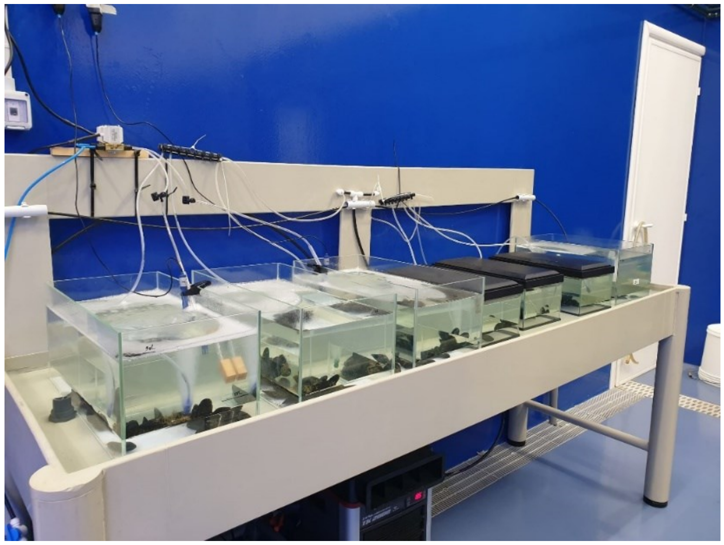
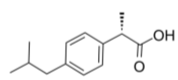
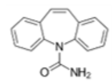
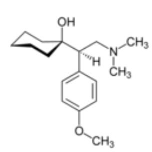
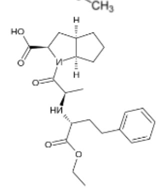
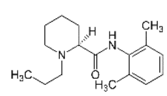
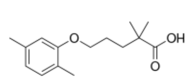


Figure 2.1 Experimental setup of 30 L tanks with mussels (n = 65) exposed to different treatments.

Table 2.1 List of therapeutic classes, chemical structures and tested dose (µg/L) of selected pharmaceuticals.

Therapeutic Group	**Compound**	**Chemical structure**	**Concentration in the APIs mixture [µg/L]**	**References**
Non-steroidal anti-inflammatory drug (NSAIDs)	**Ibuprofen (IBU)**		**1.5**	Gonzalez-Rey et al., 2015 González-Alonso et al., 2017;
Anticonvulsant	**Carbamazepine (CBZ)**		**1.5**	Moreno-González et al., 2015; Alygizakis et al., 2016; Biel-Maeso et al., 2018
Antidepressant	**Venlafaxine (VLF)**		**0.3**	Fernandez Rubio, 2019; Gomez 2021.
ACE inhibitor	**Ramipril (RAM)**		**0.2**	Zhang et al., 2020
Antidiabetic	**Metformin (MET)**		**1.5**	Ambrosio-Albuquerque et al., 2021
Lipid regulator	**Gemfibrozil (GEM)**		**0.4**	Fent et al., 2006

2.2 Chemical Analysis

Bioaccumulation of carbamazepine (CBZ), gemfibrozil (GEM), ibuprofen (IBU), metformin (MET), ramipril (RAM) and venlafaxine (VEN) was determined in the whole soft tissues of mussels on five replicates (n=5), each constituting of the tissues of 6 specimens. About 3 g of wet tissue from each replicates were homogenated in 5 mL of 100% methanol (LC-MS grade) for 10 minutes using a dispersing, stirring, homogenizing and grinding system (ULTRA TURRAX Tube Drive – IKA) at a temperature of 4°C in order to avoid oxidation or decomposition of the analytes; after centrifugation at 5000 × g for 15 min, 1 mL of samples was purified using Phree Phospholipid Cartridges (Phenomenex, US), following the procedures described by the manufacturer, in order to remove part of the protein and lipidic material, reducing interferences and increasing stability of the samples. Finally, the purified extracts were analysed using ultra-high performance liquid chromatography (UHPLC) with tandem triple quadrupole mass spectrometry (LC-MS/MS).

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

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

Six different levels of the mixture of pure standard solutions : carbamazepine 1 mgr/mL in methanol Cerilliant®(Supelco); gemfibrozil 100 mg/mL ≥ 98.5% GC (Sigma-Aldrich); ibuprofen 1 mgr/mL in methanol Cerilliant®(Supelco); metformin HCL (as free base) 1 mgr/mL in methanol Cerilliant®(Supelco); ramipril 100 mg/mL ≥ 98% HPLC (Sigma-Aldrich); venlafaxine hydrochloride 1 mg/mL in methanol Cerilliant®(Supelco) of the analytes of interest between a concentration range between 1.5 to 50 ng/mL were prepared in pure methanol (LC-MS), and each injected 3 times for each concentration level on the LC-MS system; calibration curves exhibited good linearity (R2 > 0.98) for all the compounds.

Due to the lack of appropriate Certified Standard Reference Materials (SRMs), recovery for each compound was estimated on samples of tissues of control mussels (n = 10) spiked with various concentrations of investigated molecules. The method was validated on control mussel samples fortified with pure standard solutions of target compounds at three concentration levels: 10 ng/g, 25 ng/g and 50 ng/g. To evaluate matrix effects mussel samples were prepared spiking know amount of the target analytes during the extraction procedure and the obtained signals were compared with those of the pure standard solution at the same concentrations. The mean recovery percentages obtained at various concentrations of CBZ, GEM, IBU, MET, RAM, and VEN were in the range of 90 % - 105 % (± 2%, n = 10). Method detection limits (LOD) and method quantification limits (LOQ) were determined, using spiked matrix samples, as concentrations of the calibration curves with a signal-to-noise ratio of at least 10 and 20, respectively and a percentage variation coefficient (CV %) less than respectively 10 % and 5 % on the basin of at least five replicates (n = 5). The values obtained in this way were the following: CBZ , 0.13 ng/g d.w.; GEM, 260 ng/g d.w.; IBU, 25 ng/g d.w.; MET, 0.46 ng/g d.w.; RAM 0.417 ng/g d.w. and VEN 0.623 ng/g d.w. for LOQs and CBZ, 0.013 ng/g; GEM, 36.75 ng/g; IBU, 2.54 ng/g; MET, 0.06 ng/g, RAM 0.033 ng/g and VEN, 0.07 ng/g for LODs respectively.

All determinations were carried out after verifying the absence of signals in blank solutions (reagents treated in the same conditions as the analytical standards and samples) and in the working solutions, analysed repeatedly during each analytical session. All the working solutions, which include the blank solutions, the analytical standards, the mobile phases, etc., were prepared fresh during each analytical session, previously testing all the solvents adopted, selected among those of a grade suitable for the technique analytical used (LC-MS).

Table 2.2 Additional key parameters such as collision energies (CE), entrance voltages (EV) and collision cell lens 2 (CCL2) for each compound.

Compound	Precursor ion $[M+H]^+$	Product ion	Transition m/z	CE	EV	CCL2
Ibuprofen	206.7	160.8	Quantifier	-15	9	-52
Carbamazepine	237	193.9 178.8	Quantifier Qualifier	-23 -47	29 27	-56 -104
Venlafaxine	279	261.1 58	Quantifier Qualifier	-20	1 1	-44
Ramipril	417.2	233.8 129.8	Quantifier Qualifier	-35	34 17	-124
Metformin	130.4	71.1 60	Quantifier Qualifier	-25	4 1	-28
Gemfibrozil	251	128.9 82.9	Quantifier Qualifier	-25	9 2	-36

2.3 Biomarker Analysis

Biomarkers were assessed using standardized protocols (Bocchetti and Regoli 2006; Gorbi et al. 2013; Benedetti et al. 2014).

Immunological responses were evaluated by assessing lysosomal membrane stability (LMS), the granulocyte-hyalinocyte ratio, and phagocytosis capacity in hemocytes. LMS was analyzed using neutral red retention time (NRRT). In this assay, hemocytes were incubated with a Neutral Red solution and examined microscopically at 15-minute intervals to determine the time at which 50% of cells had released the dye from lysosomes into the cytosol. For the granulocyte-hyalinocyte ratio analysis, hemolymph aliquots were spread on glass slides, fixed in Beker's fixative (+2.5% NaCl), stained with May-Grünwald Giemsa, and observed under a light microscope. Phagocytosis capacity was evaluated microscopically in hemolymph incubated for 2 hours with fluorescein labelled Zymosan A bioparticles (Invitrogen) at a 10:1 target:hemocyte ratio. Phagocytosis was expressed as the percentage of cells that internalized at least three fluorescent particles.

Genotoxicity was evaluated at chromosomal level by measuring the occurrence of micronuclei (MN) and at molecular level, as single strand breaks, by the Comet assay. Samples for micronuclei frequency evaluation were prepared from hemolymph: hemocytes were 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. In this assay, 2000 cells with preserved cytoplasm are scored for each specimen to identify micronuclei, defined as round structures smaller than 1/3 than the nucleus diameter and totally detached, on the same optical plane and clearly visible.

Comet assay was carried out on mussels' hemocytes incorporated 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. 70 randomly selected "nucleoids" per slide, with 4 replicates per treatments, were examined under fluorescence microscopy (200x magnification; Olympus BX-51). The amount of DNA damage was evaluated as the percentage of DNA migrating out of the nucleus by an image analyzer (Tritek CometScore™ software) and % Tail DNA was chosen as a reliable comet assay parameter. DNA diffusion assay is a modified version of comet assay detecting low molecular weight DNA which is lost from agarose matrix during electrophoresis (Singh 2000). Slides were prepared following the comet protocol, but electrophoresis was omitted. Apoptotic cell nuclei have a hazy or undefined outline without any clear boundary due to nucleosomal-sized DNA diffusing into agarose and a diameter 3 times over the mean nuclear diameter. The degree of DNA diffusion pattern is subdivided in 5 classes: class 1,1–5 % diffused DNA; class 2, 20 % diffused DNA around the nucleus; class 3, 50 % diffused DNA; class 4, 1–10 % diffused DNA; class 5, apoptotic cell (Cantafora et al. 2014). 100 randomly selected cells per slide were examined under fluorescence microscopy and a visual analysis was performed for evaluate the frequency of cells (%) belonging to the fifth class of damage (200x magnification; Olympus BX-51).

Acetylcholinesterase activity (AChE) in mussels' hemolymph and gills was used to assess neurotoxicity according to Ellman's reaction (Ellman et al. 1961). Hemolymph samples were centrifuged at 3,000 ×*g* for 5 minutes, while gills were homogenized (1:3 w/v ratio) in a 0.1 M Tris-HCl buffer (pH 7.2) with 0.25 M sucrose and centrifuged at 10,000 ×*g* for 10 minutes. Ellman's reaction relies on measuring the rate of production of thiocholine as acetylthiocholine is hydrolyzed by AChE. Thiocholine from the sample reacts with the chromogenic substrate DTNB (dinitrobenzoic acid) to produce a yellow anion (5-mercapto-2-nitrobenzoic acid and its dissociated forms), whose formation rate is measured over 1 minute at 18 ± 1 °C and λ = 412 nm using a spectrophotometer and is proportional to the acetylcholinesterase activity. AChE is expressed as nmol/min/mg of protein.

Lipofuscin and neutral lipids content was evaluated through histological analysis on cryostat sections (8 µm thick) of digestive glands (3 samples for treatment). For lipofuscin, the cryostat sections on slides were fixed using Beker's fixative and stained using Schmrol reaction before mounting with Eukitt. For neutral lipids, slides were fixed using the same Beker's fixative and then stained with the Oil Red O methods and then mounted in glycerol gelatin. For both lipofuscin and neutral lipids, four measurements were made on digestive tubules of each section. Quantification of staining intensity was performed with by an image analyzer (Image-Pro® Plus 6.2 Analysis Software) and then normalized to the area of digestive tubules.

The content of Malondialdehyde (MDA), a marker and primary product of lipid peroxidation, was determined through a conjugation reaction with 1-methyl-2-phenylindole, resulting in the formation of a compound with maximum absorbance at a wavelength of λ = 586 nm. MDA was quantified in digestive glands homogenized (1:3 w/v ratio) in 20 mM Tris–HCl (pH 7.4), centrifuged at at 3,000 ×*g* for 20 min. A conjugation reaction was performed in 1 mL reaction mixture (45 °C, 40 min), containing 10.3 mM 1-metyl-2-phenylindole (3:1, dissolved in acetonitrile/methanol), HCl 32 %, 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 ×*g* for 10 min and spectrophotometrically analyzed at λ = 586 nm. MDA concentrations were determined as a function of the 1,1,3,3-tetramethoxypropane standard curve and expressed as nmol/g tissue.

Acyl-CoA oxidase's (ACOX) activity was measured in digestive gland samples homogenized (1:5 w/v ratio) with 1 mM $NaHCO_3$ buffer with 1 mM EDTA, 0.1 % ethanol, 0.01 % Triton X-100 (pH 7.6) and centrifuged at 500 ×*g* for 15 minutes. ACOX activity was determined by monitoring the oxidation reaction of dichlorofluorescein diacetate (DCF-DA) in the presence of an external peroxidase and the addition of a specific substrate (Palmitoyl-CoA). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCFDA, 40 µM sodium azide, 0.01 % Triton X-100, 1.2 U/mL HRP in a final volume of

1.9 mL. After a pre-incubation at 25 °C for 5 minutes in the dark with 100 µL of sample, reactions were started adding 10 µL of substrate Palmitoyl-CoA 30 mM; readings were carried out at $\lambda = 502$ nm against a blank without the substrate. ACOX activity is expressed in mU mg protein^{-1}.

2.4 Statistical analysis

To evaluate the differences between the treatment groups, a two-way Analysis of Variance (ANOVA) was conducted. This analysis aimed to determine the main effects of the treatment conditions (CTRL, OA, HW, and CC) and the presence of APIs, as well as their interaction effects on the response variables. Prior to performing the ANOVA, data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test), with appropriate mathematical transformation if necessary. The ANOVA provided insight into both the individual effects of the treatments and the exposure to APIs, as well as any interaction effects between these factors. Significant results from the ANOVA were further examined using *post-hoc* pairwise comparisons with appropriate adjustments (Tukey's HSD) to control for multiple testing. Statistical significance was set at ($p < 0.05$). All analyses were performed using RStudio (4.2.2).

3. Results

3.1 Chemical analysis

Figure 2 show the levels of VLF, CBZ, GEM and MET in the whole tissue of both control and exposed mussels. Results revealed that mussels can significantly bioaccumulate VLF and CBZ (Fig. 2A, B), as indicated by the statistically significant increase in their levels in all organisms exposed to the APIs mixture, compared to those with no-APIs (< LOD). Average lower VLF levels were measured in mussels exposed simultaneously to APIs mixture and all tested climate change stressors compared to the control (Fig. 2A). CBZ bioaccumulation was not significantly affected by the co-exposure of both APIs mixture with OA, and APIs mixture with CC; while a statistically increase in CBZ levels was highlighted in mussels treated with APIs mixture with HW (Fig. 2B). Levels of GEM and MET were measured in all CTRL organisms (Fig. 2C and D), including those not treated with the APIs mixture. Results showed a statistically significant increase in GEM levels, measured in organisms exposed to APIs mixture in HW scenario compared to those treated with HW stressor alone (Fig. 2C). Higher basal levels of MET were measured in HW and OA treatments, whereas no variation was noted in CC treatments. No changes in MET bioaccumulation were measured in organisms exposed to APIs mixture alone and with one or both climate change stressors (Fig. 2D). Levels of IBU and RAM, (data not shown), were below the detection limits (< LOD 25 ng/g dry weight (d.w.) and 0.33 ng/g d.w. respectively).

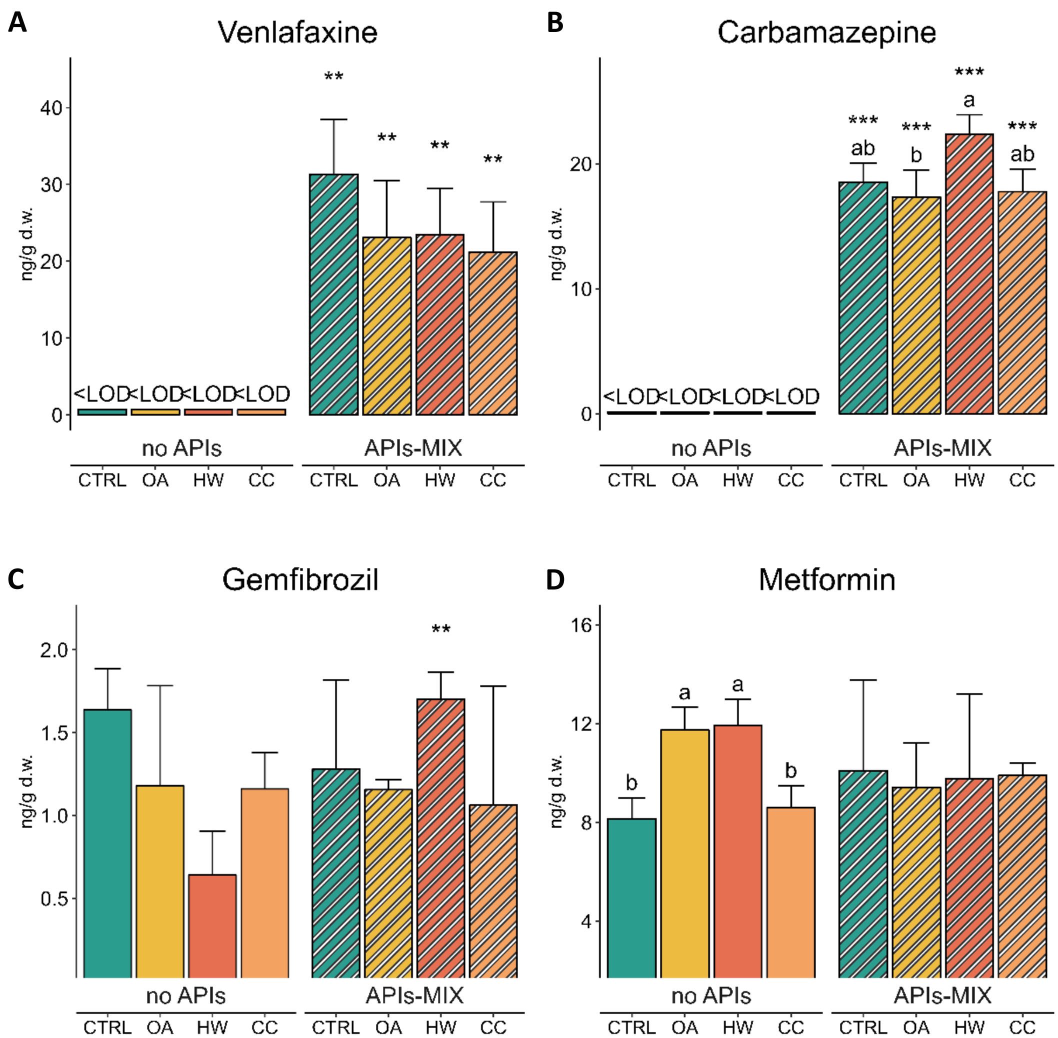


Figure 2.2 Levels of A) Venlafaxine (ng/g d.w.), B) Carbamazepine (ng/g d.w.), C) Gemfibrozil (µg/g d.w.) and D) Metformin (ng/g d.w.) in whole tissues of *M. galloprovincialis*. Data are given as mean ± standard deviation. Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (*p<0.05; **p<0.01; ***p<0.001). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

3.2 Biomarkers analysis

The results of immune parameters measured in mussel hemolymph are shown in Figure 3. A decreasing trend in lysosomal membrane stability (LMS) was measured in all organisms compared to their control (CTRL no-APIs, CTRL treated with APIs-MIX), with statistically significant reduction in mussels treated with HW and CC. A significant decline in LMS was highlighted in organisms co-exposed to APIs mixture and OA compared to those treated with OA alone (Fig. 3A).

A significant decrease in granulocytes and hyalinocytes ratio was observed in mussels treated with OA alone and in CC compared to their control (CTRL no-APIs); a significant decrease was also detected in organisms exposed to APIs mixture and both stressors (CC) compared to their control (CTRL with APIs-MIX). A significant increase in the granulocytes and hyalinocytes ratio was measured in mussels exposed to APIs mixture in both OA and HW scenarios compared to those treated with these stressors without the APIs mixture (Fig. 3B).

The phagocytosis capacity showed a significant inhibition in all mussels exposed to single (OA, HW) and combined stressors (CC) compared to their control (CTRL no-APIs); similar trend was highlighted for organisms treated to APIs MIX with OA and both stressors (APIs MIX with CC) compared to their control (CTRL treated with APIs-MIX) (Fig. 3C).

No significant difference was observed between the treatment not exposed to the APIs mixture and the treatment that was exposed to the mixture.

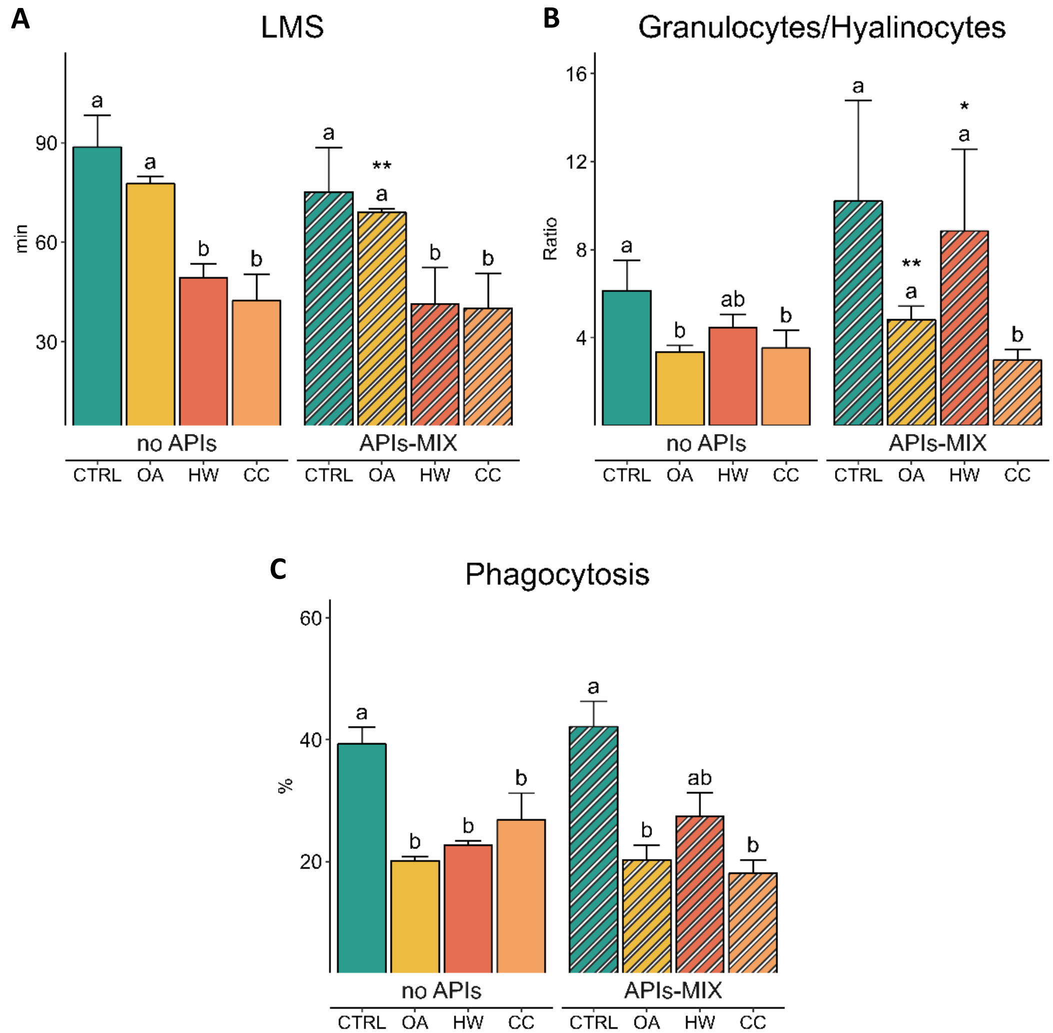


Figure 2.3 Immunological responses. A) Lysosomal membrane stability (LMS) (Minutes), B) granulocyte/hyalinocyte ratio, C) phagocytosis capacity (%) in hemocytes of *M. galloprovincialis*. Data are given as mean ± standard deviation for LMS and G/H while mean value ± SEM for phagocytosis. Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (**p*<0.05; ***p*<0.01; ****p*<0.001). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

Biomarkers of genotoxicity are shown in Figure 4. An increasing trend in micronuclei frequency was observed for all treatments compare to their controls (CTRL no-APIs, CTRL with APIs-MIX), without statistically significant differences (Fig. 4A).

A trend of increased DNA fragmentation was measured in mussels treated with one or both climate change stressors compared to their control (CTRL no-APIs); while a decrease was detected in organisms co-exposed to APIs mixture and HW compared to the others treated with APIs mixture. A significant increase in DNA fragmentation was measured in organisms exposed to APIs mixture compared to their control (CTRL no-APIs), while a significant decrease was observed for mussels treated to APIs mixture and HW scenario compared to those treated with HW alone (Fig. 4B).

Results on Diffusion Assay did not show any significant variation; except for mussels treated with APIs mixture and CC which shows a significant increase in apoptotic cells compared to their control (CC with no APIs) (Fig. 4C).

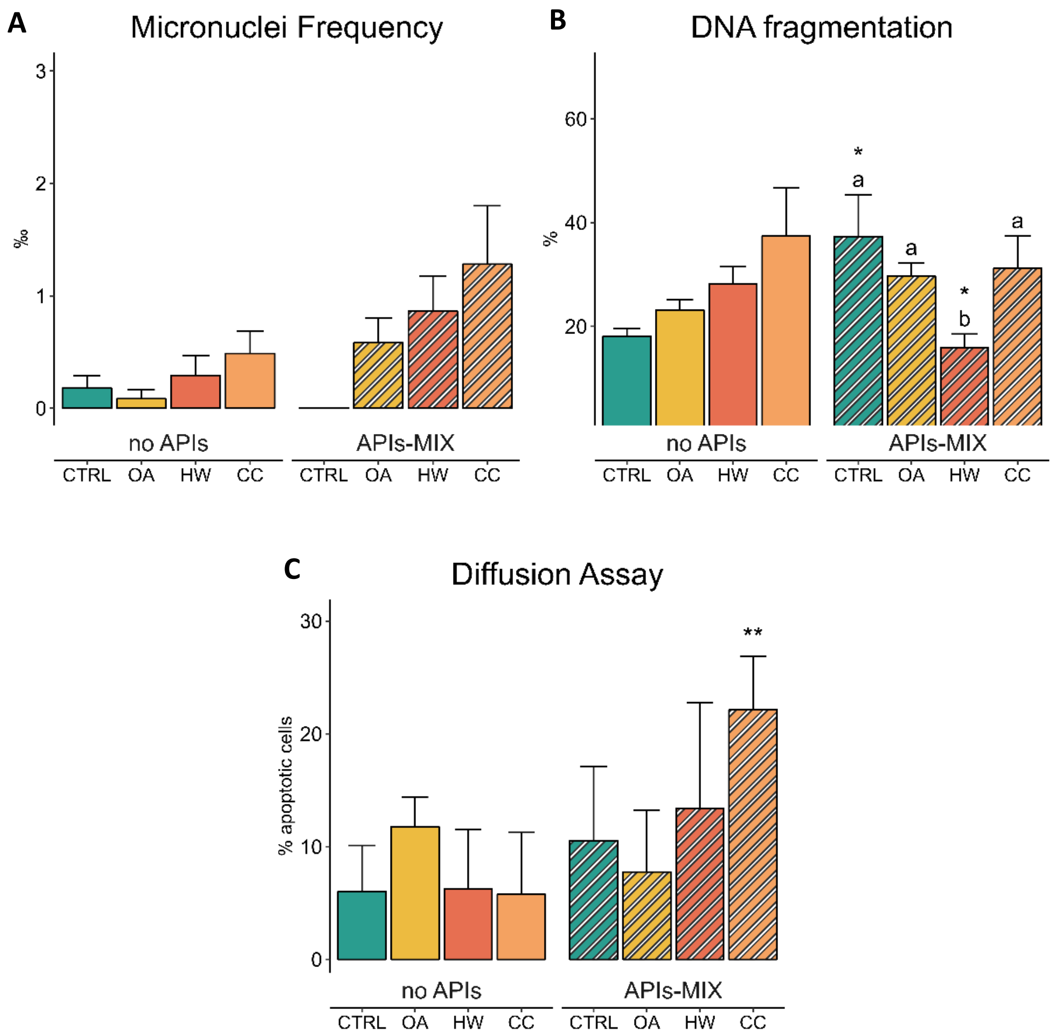


Figure 2.4 Genotoxic effects. A) Micronuclei frequency (‰), B) DNA fragmentation (Percentage of DNA in comet tail and C) Percentage of apoptotic cells (Diffusion assay) in hemocytes of *M. galloprovincialis*. Data are given as mean ± SEM. Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (*$p<0.05$; **$p<0.01$; ***$p<0.001$). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

A significant increase in acetylcholinesterase (AChE) activity in hemolymph was observed in mussels co-exposed to the APIs mixture and HW compared to those exposed to HW alone, while no significant variations were detected in other treatments (Fig. 5A).

For gills tissue, an increasing trend of AChE activity was highlighted in organisms treated with one or both climate change stressors compare to their control (CTRL no-APIs), with statistically significant variation observed in HW treatment, while no differences were measured for other treatments. Additionally, a significant increase in AChE activity was measured in mussels exposed to the APIs mixture compared to their control (CTRL no-APIs) (Fig. 5B), which however was not observed in the treatments co-exposed to OA, HW or CC.

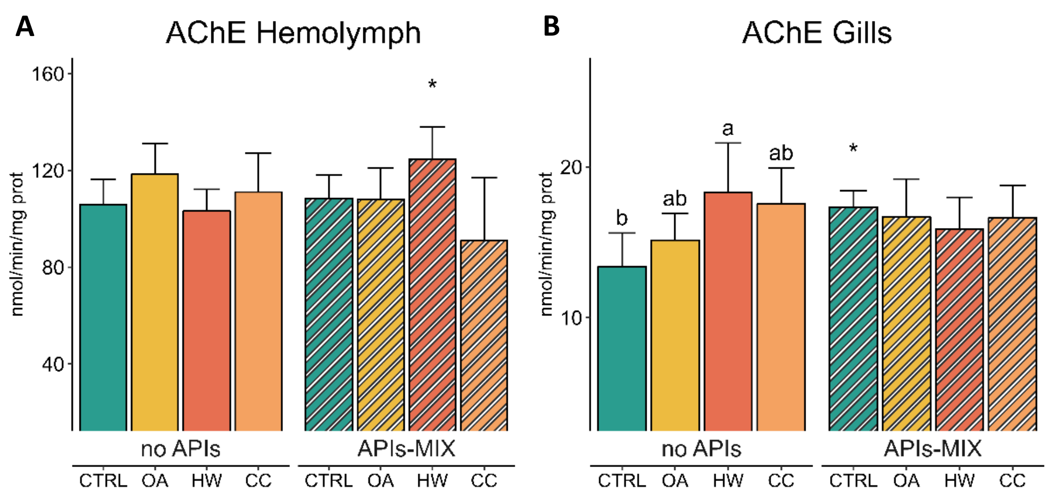


Figure 2.5 Neurotoxicity responses. A) Acetylcholinesterase activity (AChE) in hemolymph (nmol/min/mg prot.) and B) in gills (nmol/min/mg prot.). Data are given as mean ± standard deviation. Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (*$p<0.05$; **$p<0.01$; ***$p<0.001$). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

Results regarding the accumulation of peroxidation products and lipid metabolism are shown in the Figure 6. No significant variation in lipofuscin content in the mussels digestive gland was observed when they were exposed to HW and OA stressors alone and together (CC) compared to their control (CTRL no-APIs); while a significant increase in lipofuscin content was measured in organisms co-exposed to the APIs mixture in OA scenario and APIs mixture in CC scenario compared to their control exposed to the APIs mixture alone, and the control exposed to both climate stressors without APIs mixture, respectively (OA no-APIs and CC no-APIs) (Fig. 6A).

The results indicated no changes in neutral lipid content of mussels' digestive gland when exposed to single stressors (OA and HW) or both stressors (CC) compared to the control (CTRL no-APIs); however, an increase was observed in organisms simultaneously treated with APIs mixture and CC compared to controls. A statistically significant increase in lipid content was measured in both treatments exposed to the APIs mixture with HW scenario and APIs mixture with the CC scenario compared to their controls not treated with APIs mixture (HW no-APIs and CC no-APIs, respectively) (Fig. 6B).

An increasing trend for malondialdehyde (MDA) in OA and HW treatments was observed compared to their control (CTRL no-APIs); while a significant decrease was measured in mussels exposed to the APIs mixture and OA compared to those exposed to the APIs mixture alone. Additionally, a significant increase in MDA was detected in organisms treated with APIs mixture compared to the control (CTRL no-APIs) (Fig. 6C).

Acyl-CoA Oxidase (ACOX) activity was inhibited in mussels exposed to both stressors (CC) compared to their control (CTRL no-APIs); while a significant increase was observed in organisms treated with both the APIs mixture and OA, as well as the APIs mixture and HW compared to the APIs mixture alone. A significant inhibition of ACOX activity was showed in organisms exposed to the APIs mixture alone compared to their control (CTRL no-APIs), while a significant enhancement was highlighted in the APIs mixture with HW compared to its control (HW no-APIs) (Fig. 6D).

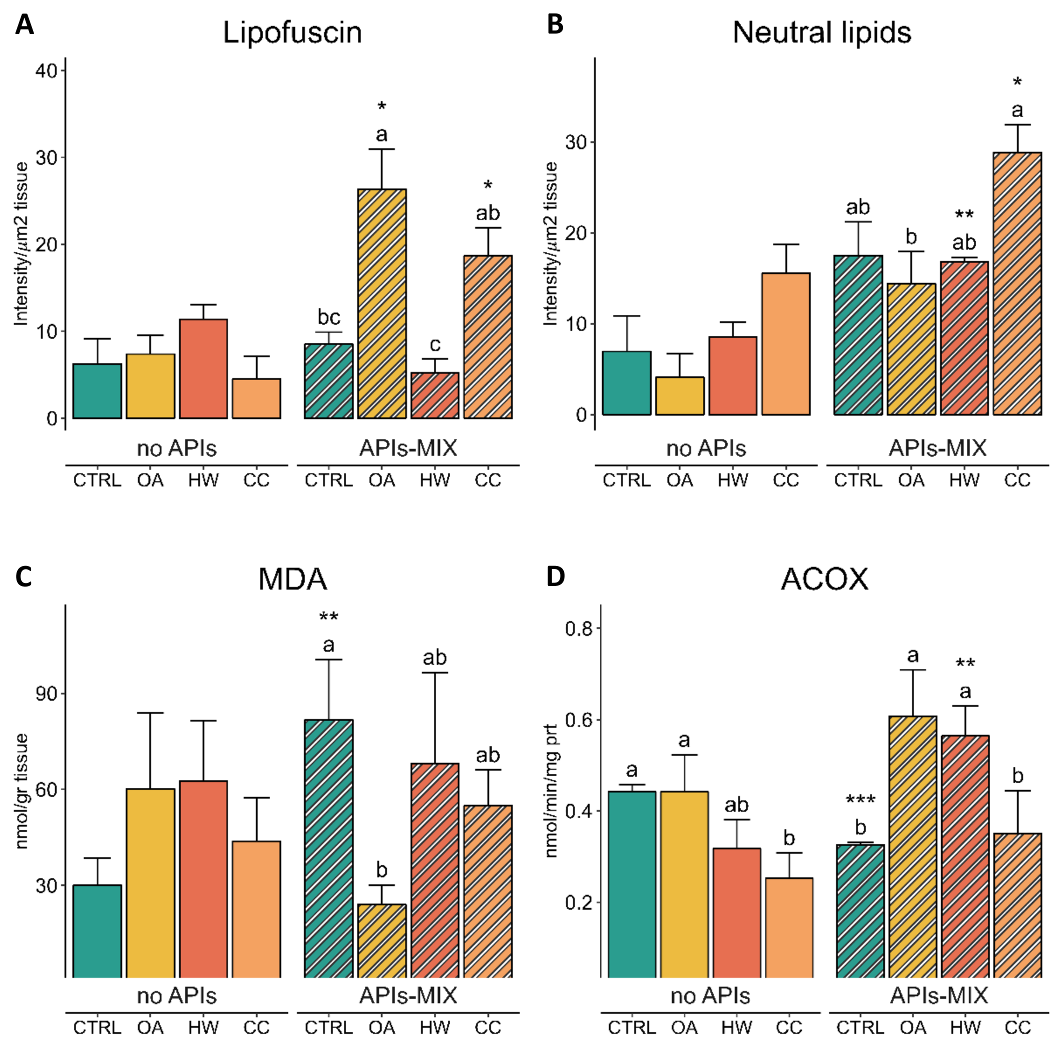


Figure 2.6 A) Lipofuscin accumulation (intensity/μm^2), B) Neutral lipids content (intensity/μm^2), C) levels of Malondialdehyde (MDA) (nmol/gr tissue) and D) Acyl-CoA oxidase activity (ACOX) (nmol/min/mg prot.) in digestive glands of *M. galloprovincialis*. Data are given as mean ± standard deviation for MDA and ACOX while mean ± SEM for lipofuscin and neutral lipids. Different letters indicate significant differences between groups of means for each condition (no APIs and APIs-MIX) based on the two-way ANOVA and Tukey HSD post-hoc tests, while asterisks indicate significant differences between treatments exposed to the same climate stressor with and without APIs-MIX (*p<0.05; **p<0.01; ***p<0.001). CTRL= Control; OA = Acidification; HW = Marine Heatwaves; CC = combination of OA and HW.

4. Discussion

Pharmaceuticals and their mixtures pose significant environmental challenges, particularly in aquatic ecosystems (Patel et al. 2019; Mezzelani et al. 2023; Kidd et al. 2024). In recent years, there has been increasing attention on the environmental monitoring of pharmaceuticals, focusing on assessing, understanding, and preventing the adverse effects of these emerging contaminants (Kümmerer 2009). However, while individual drugs are often studied, the issue of mixture toxicity remains relatively unexplored (Mezzelani and Regoli 2022; Kidd et al. 2024). Concomitantly, climate change presents a major challenge for marine ecosystems, leading to substantial alterations such as ocean warming, marine heatwaves and acidification (Oliver et al. 2019; IPCC 2023). These environmental challenges can alter the toxicity of pharmaceuticals by changing their chemical compositions due to increased temperatures or acidification processes, and by modulating various aspects of aquatic species' health, including metabolic and feeding rates, which in turn alters the bioaccumulation of chemicals in their tissues (Kroeker et al. 2017; Serra-Compte et al. 2018; Nardi et al. 2018). The present study investigated the influence of ocean acidification and marine heatwaves, both separately and together, on the bioaccumulation and toxicity of a selected APIs mixture in Mediterranean mussel *M. galloprovincialis*.

Results on chemical analysis provided novel insights into the bioaccumulation patterns of different types of drugs, belonging to diverse therapeutic classes, both in relation to their behaviour within the mixture and their response to one or more climate change-related stressors.

Findings indicated that *M. galloprovincialis* exposed to the APIs mixture significantly bioaccumulate VLF and CBZ. The levels of these drugs in mussels were modulate by climate change stressors. The general assumption was that organisms subjected to thermal stress (in our case the two dynamic HW) should exhibit enhanced metabolism, accompanied by increased ventilation and feeding rate in response to higher metabolic demands. Such changes often translate into a higher uptake of contaminants dissolved in the water column (Maulvault, Barbosa, et al. 2018). A similar pattern was observed in the results for the anticonvulsant CBZ that although not significant, showed average higher levels of this drugs in the mussels' tissues exposed to the HW scenario. This increasing trend of bioaccumulation under HW scenario was also noted by Nardi et al. (2022), with mussels exposed to 1 µg/L of the same pharmaceutical.

In contrast to this general assumption, VLF exhibited a distinct pattern with reduced levels in mussels' tissues exposed to the HW scenario. This finding is consistent with observations of Maulvault et al. (2018), in which co-exposing to increase temperatures ($\Delta T°C = +5$ °C) and to high concentration (20 µg/L) decreased VLF uptake in liver of *Argyrosomus regius* fish. The decrease observed in contaminant levels at higher temperatures may be attributed to enhanced metabolic rates, which can accelerate the elimination of contaminants through metabolism and excretion (Maulvault et al. 2018).

Moreover, ocean acidification has been demonstrated to affect aquatic organisms' physiology. Calcifying and sessile organisms like mussels are sensible to this stressor: higher energy demand for homeostatic processes provoke a depletion of growth, fitness and metabolic rates (Kroeker et al. 2013, 2017). Thus, it can be hypothesized that the reduction of mussel's biological activities may promote a decrease of contaminants uptake. Our outcomes are in line with this hypothesis, showing a slight decrease in bioaccumulation levels of VLF and CBZ when exposed in OA treatment. Similar results were highlighted in previous studies conducted by Almeida et al. (2018) on *V. philippinarum* exposed to decreased pH (7.5) and 1.0 µg/L of CBZ, and by Maulvault et al. (2018) on *A. regius* exposed to increased pCO_2 levels (~1,000 µatm) and 20 µg/L of VLF.

Levels of MET and GEM were detected in mussels' tissues not treated with the mixture, indicating pre-existing environmental contamination for both drugs. Interestingly, even after a 42-day period in pharmaceutical-free water, mussels showed the same levels of MET and GEM, suggesting their inability to eliminate these compounds. A study conducted by Meador et al. (2016) detected baseline levels of both pharmaceuticals in wild marine species collected from estuaries receiving effluents in Puget Sound, Washington, USA. The study found 1.3 ng/g of GEM in salmon and 27.8 ng/g of MET in sculpins, which are still very low values compared to the 1.6 µg/g d.w. of GEM found in the control mussels in this study, an order of magnitude greater. Additionally, baseline levels of the compounds GEM and MET were detected in *M. galloprovincialis* sampled from the Adriatic Sea, with concentrations similar to those observed in our study. Specifically, GEM was detected at approximately 2 µg/g d.w., while MET was found at around 4 ng/g d.w. (unpublished results). In our study, MET levels were three times higher than this value (11.9 ng/g d.w.), highlighting the variability in occurrence of these compounds in marine mussels.

This pre-existing contamination could be correlated with the poor removal efficiency of these molecules in WWTPs. For instance, Desbiolles et al. (2018) observed that the concentration levels of MET remained unchanged between the inlets and outlets of the treatment facility. Furthermore, the fact that organisms exposed to the APIs mixture showed the same levels of MET and GEM as those not treated with the pharmaceutical mixture suggests that mussels may reach a saturation point for these molecules, beyond which additional exposure does not result in higher bioaccumulation levels. However, future research is needed to verify this theory and to elucidate the mechanisms underlying the persistence of these drugs in non-target marine organisms.

IBU and RAM were the only pharmaceuticals in the mixture detected below the limit of detection (< LOD) after exposure, suggesting limited accumulation by mussels. A survey of wild mussels from the Adriatic Sea also found no bioaccumulation of these compounds (< LOD) (unpublished results). Similar findings were reported by Mezzelani et al. (2016), who observed IBU levels below the LOD in *M. galloprovincialis* exposed to 0.5 μg/L of IBU for 14 days.

Despite below the level of detection, for these drugs ecological concerns cannot be excluded. Additional studies also are needed to identify drug metabolites and evaluate their potential impacts.

In addition to elucidate the bioaccumulation behaviour of the mixture exposed to climate change stressors, various biochemical and cellular biomarkers were evaluated, thereby offering a more holistic assessment of the potential toxicity of the APIs mixture modulate by climate change stressors.

Among the broad selection of biological responses analysed in this study, the modulation of the immune system was evaluated through the assessments of lysosomal membrane stability (LMS), granulocytes-hyalinocytes ratio, and the phagocytosis capacity in hemocytes.

The neutral red retention assay is widely used to assess lysosomal membrane stability in hemocytes of invertebrate, particularly in bivalves. Lysosome are cellular organelles containing enzymes that degrade cellular components, and LMS is a sensitive marker for cell damage as lipid peroxidation caused by ROS can destabilize these membranes (Viarengo et al. 2007; Gorbi et al. 2013).

Obtained results showed that LMS was compromised in all treatments involving thermal stress without a clear influence of the APIs mixture, confirming the findings reported in the hemolymph of other invertebrate species (Beesley et al. 2008; Matozzo et al. 2012). Previous studies have also observed lysosomal membrane destabilization due to thermal stress and concurrent pH decrease. For instance, Matozzo et al. (2012) reported decreased NR uptake in *M. galloprovincialis* exposed to low pH (7.4) and high temperatures (28°C) for 7 days. Nardi et al. (2017) similarly found that exposure to acidification conditions (7.4) and thermal stress (15°C during the winter period) over a 28-day period in *M. galloprovincialis* resulted in significant lysosomal membrane instability, with the most pronounced effects observed in organisms subjected to both stressors.

Our results on LMS showed no change in OA treatments compared to control. This trend is in line with the results of Mezzelani et al. (2021), where mussels exposed to OA treatments (pH 7.6) showed unaltered LMS values compared to controls. Interestingly, LMS was significantly altered in organisms co-exposed to the mixture and OA compared to OA alone, thus revealing the deleterious effects caused by hypercapnic conditions in the presence of the API mixture. Similarly, Mezzelani et al. (2021) also reported lysosomal membrane destabilization in treatments co-exposed to OA and CBZ (1 μg/L) for 28 days, supporting our findings, where the combination of OA with the API mixture significantly reduced the LMS.

Our study further elucidates immune system modulation, evidenced by a significant decrease in the granulocyte-to-hyalinocyte ratio and concurrent inhibition of phagocytosis capacity. In mussels, hemocytes are the primary immune cells, with granulocytes predominating in the hemolymph of *M. galloprovincialis*. Granulocytes play a crucial role in cell-mediated immunity through efficient phagocytosis and cytotoxic reactions, while hyalinocytes specialize in coagulation and encapsulation processes. Therefore, the observed decrease in phagocytosis may be linked to the reduced number of granulocytes, as indicated by the decreased G/H ratio (Gorbi et al., 2013).

Mezzelani et al. (2021) and Nardi et al. (2022) also observed decreases in the granulocyte-to-hyalinocyte ratio and phagocytic capacity in mussel hemocytes exposed to OA and HW scenarios alone, consistent with our findings under the same environmental stressors, specifically under condition of OA, HW and CC scenario.

Climate change stressors appear to be the primary factor influencing immune system modulation, decreasing LMS (especially under HW scenarios), the granulocyte-to-hyalinocyte ratio and inhibiting phagocytic capacity (under both climate change stressors alone and combined), with or without APIs mixture. Despite unclear mechanisms underlying these pathways, this emphasizes the sensibility of these immune biomarker in respect to climate change stressors. Notably, no significant effects were observed on these parameters in treatments exposed solely to environmentally realistic concentrations of the pharmaceutical mixture, suggesting that these mixtures alone, without climate change-related stressors, do not significantly impact the immune parameters. It is noteworthy that the APIs mixture showed no significant effect on LMS, despite individual drugs like the antidepressant VLF, anticonvulsant CBZ, and the Non-Steroidal Anti-Inflammatory drug IBU when dosed alone, have shown to decrease LMS (Matozzo et al. 2012; Mezzelani et al. 2016, 2021; Nardi et al. 2022; Rafiq et al. 2023). This observation suggests a potential antagonistic mechanism within the mixture, which warrants further investigation.

Considering the hemocyte parameters, genotoxicity was assessed through the Comet assay, diffusion assay and micronuclei frequency. Micronuclei (MN) are extranuclear bodies formed when chromosome fragments or whole chromosomes fail to incorporate into daughter nuclei during cell division, indicating

DNA damage and chromosomal instability, often linked to reactive ROS effects (Depledge and Fossi 1994; Viarengo et al. 2007).

The results revealed a trend of increased MN frequency in treatments co-exposed to the APIs mixture and both climate change stressors, though this increase was not statistically significant. This contrasts with previous research showing a significant rise in MN frequency in mussels exposed to CBZ and OA (Mezzelani et al. 2021). Additionally, studies have demonstrated that individual pharmaceuticals can increase MN frequency in non-target species: for instance, Mezzelani et al. (2016) investigated NSAIDs and found that IBU, present in our selected APIs mixture, significantly raised MN frequency in mussel hemolymph; similarly, Mezzelani et al. (2023) reported a similar effect with the cardiovascular valsartan. The observed increase in MN frequency in these cases could potentially be linked to changes in cellular turnover, supported by molecular evidence of enhanced protein turnover through upregulation of ubiquitin-protein transferase regulatory activity. Given that CBZ is a known cell cycle inhibitor (Pérez Martín et al. 2008), it is plausible to hypothesize that the presence of different pharmaceuticals with demonstrated varied modes of action could influence MN frequency. Thus, while individual pharmaceuticals have shown the capability to increase micronuclei frequency, potentially affecting cell cycle dynamics, our study suggests the possibility of antagonistic interactions targeting specific cellular mechanisms to modulate these responses.

Conversely, the observed increase in DNA damage in hemocytes, as indicated by the Comet assay following exposure to the APIs mixture, might be attributed to a cumulative effect of each molecule within the mixture. Specifically, VLF, GEM, and IBU exposure have been reported to elevate DNA strand breaks in marine organisms exposed to the single compounds (Lacaze et al. 2015; Mezzelani et al. 2016; Nardi et al. 2022). Interestingly, DNA damage significantly decreased under co-exposure to HW scenario and APIs mixture compared to both the APIs mixture alone and the HW scenario alone. This suggests a potential antagonistic interaction between these stressors. However, the measured DNA fragmentation levels in mussels exposed to various treatments (ranging from 15.9% to 37.3%) fall within the range of physiological variability observed in natural uncontaminated organisms, as reported by Bocchetti et al. (2008) and Pisanelli et al. (2009). These seasonal studies showed DNA fragmentation levels in *M. galloprovinciali*s ranging from 20 to 50%, indicating that the observed values are not alarming and are typical for this parameter.

The DNA diffusion assay, another genotoxic endpoint, was performed on mussel hemocytes to quantify the percentage of apoptotic cells. Apoptosis, a critical process of programmed cell death, plays a vital role in maintaining cellular homeostasis and responding to stress. By quantifying apoptotic cells, it's possible to estimate the extent of cellular damage and stress responses induced by environmental conditions and contaminants (Singh 2005; Cantafora et al. 2014; Mottola et al. 2022). Results indicate a notable increase in the percentage of apoptotic cells in mussels co-exposed to climate change stressors with the APIs mixture. In contrast, treatments involving either CC stressors alone or the APIs mixture alone exhibited lower percentages of apoptotic cells. The increased apoptosis observed with combined stressors suggests that simultaneous exposure to multiple stressors amplifies the stress effect on mussel hemocytes, potentially exceeding the capacity of cellular defence mechanisms.

Acetylcholinesterase (AChE) is a critical enzyme in neurotransmission, essential for breaking down acetylcholine to regulate nerve impulses. It plays a key role in terminating synaptic transmission and maintaining precise muscle function at cholinergic synapses and neuromuscular junctions and its inhibition can lead to prolonged nerve stimulation, muscle hyperactivity, and potential paralysis. Therefore, AChE activity is widely used as a sensitive biomarker for neurotoxicity assessment of xenobiotics, measured in the hemolymph and gills of bivalves (Viarengo et al. 2007; Gorbi et al. 2013).

As demonstrated in previous studies, exposure to compounds such as the antidepressant fluoxetine (Franzellitti et al. 2014), and NSAIDs IBU and paracetamol inhibits the activity of AChE in mussels' gills (Mezzelani et al. 2016). Conversely, other pharmaceuticals, including the antiepileptic CBZ (Siebel et al. 2010; Mezzelani et al. 2021), the NSAID diclofenac (Gonzalez-Rey and Bebianno 2014), and the cardiovascular valsartan (Zhang et al. 2020; Mezzelani et al. 2023), have shown the ability to enhance AChE activity, likely interacting with ion channels, neurotransmitter systems, and receptors critical for AChE function. Our results demonstrated that APIs mixture caused a significant induction of AChE activity. This increase was observed in the hemolymph of mussels exposed to the APIs mixture under HW conditions compared to HW conditions alone, and in the gills of mussels exposed to the APIs mixture alone compared to the control condition without the mixture. These findings suggest that the combined effects of pharmaceuticals in the mixture may influence AChE activity in marine environments, though further studies are needed to elucidate these mechanisms of action.

Modulation of oxidative and lipid metabolisms was assessed in mussel's digestive glands, through histological analysis of lipofuscin and neutral lipids, alongside quantification of malondialdehyde content and determination of ACOX activity.

Histological analyses revealed a variable trend in lipofuscin accumulation, which is considered a marker of autophagic processes in Mediterranean mussels and of peroxidative damage (Bocchetti et al. 2008).

Our results showed that co-exposure to the APIs mixture and OA significantly enhanced lipofuscin accumulation, potentially indicating a synergistic effect between the drugs under hypercapnic conditions. Similarly, mussels co-exposed to APIs mixture and CC scenario exhibited increased lipofuscin accumulation, suggesting that the observed effects were primarily driven by OA rather than HW scenario. Both groups had significantly higher levels of lipofuscin compared to mussels exposed to APIs alone and APIs in the HW scenario, suggesting that the combination of OA and APIs may have a synergistic effect that enhances lipofuscin formation in lysosomes. This observation is consistent with findings by Mezzelani et al. (2021), who noted that co-exposure to hypercapnic conditions and CBZ enhanced lipofuscin levels, suggesting that the interplay of these stressors could intensify oxidative stress in cells.

Correlated to lipofuscin levels, changes in levels of malondialdehyde (MDA) accumulation were observed in mussels exposed to the APIs mixture. MDA, a final product of polyunsaturated fatty acids peroxidation, is a well-known biomarker of oxidative stress in *Mytilus spp*. (Viarengo et al. 2007; Bocchetti et al. 2008). An opposite trend was observed between MDA and lipofuscin levels with MDA levels higher in mussels exposed to APIs alone but significantly lower in those exposed to both APIs and OA, in contrast lipofuscin content significantly increased in mussels co-exposed to the APIs mixture and OA compared to those exposed to APIs alone. This is consistent with the roles of these molecules in lipid peroxidation processes, where MDA acts as an intermediate product and, as a reactive toxic metabolite, is usually rapidly degraded and converted to lipofuscin (Viarengo et al., 2007). This negative relationship has also been confirmed by previous studies (Franzellitti et al. 2014; Rafiq et al. 2023). Moreover, our results showed a significant increase in MDA content in treatments exposed to the APIs mixture alone, in contrast with previous studies where single exposure to VLF (0.5, 5, and 10 ng/L for 7 days) and CBZ (1 μg/L for 28 days) did not result in any significant increase in MDA content (Franzellitti et al. 2014; Mezzelani et al. 2021; Rafiq et al. 2023).This suggests the potential for a synergistic or additive effect within the studied pharmaceutical mixture.

The accumulation of neutral lipids in *Mytilus'* digestive gland, which is one of the primary energy reserve in bivalves, is considered an indicator of disturbed metabolism (Bocchetti and Regoli 2006). Changes in neutral lipid levels can be linked to the inhibition or increase in the activity of peroxisomal enzymes, showing opposite trends for these parameters. Peroxisomal enzymes such as acyl-CoA oxidase (ACOX) play a critical role in lipid metabolism by modulating key pathways like the β-oxidation of very long-chain fatty acids, the metabolism of important lipid derivatives such as prostaglandins and leukotrienes, and the synthesis of plasmalogens and cholesterol, which are precursors to steroid hormones (Ortiz-Zarragoitia and Cajaraville 2006).

Our results indicate a modulation of lipid metabolism in mussels exposed to the APIs mixture, evidenced by a significant decrease in ACOX activity and an increase in neutral lipid levels. This suggests that the mixture alone, and in combination with the climate change stressors, could inhibit β-oxidations, leading to increased accumulation of energy reserves in mussels. This is consistent with the findings of Mezzelani et al. (2018) and Nardi et al. (2022), where exposure to carbamazepine (CBZ) alone and several NSAIDs, namely ibuprofen (IBU), diclofenac, and ketoprofen, resulted in increased neutral lipid levels and a concomitant decrease in ACOX activity. Conversely, mussels co-exposed to the APIs mixture and individual stressors exhibited an induction of ACOX activity, indicating a potential enhancement in the β-oxidation of fatty acids, resulting in lower neutral lipid levels.

Overall, these findings confirm that the co-occurrence of multiple stressors can significantly modulate a wide range of biomarkers, particularly those related to oxidative stress and lipid metabolism. Supporting the hypothesis that the combined effects of pharmaceuticals and climate change-related stressors enhance oxidative stress in mussels, demonstrating the complex interactions and combined risks posed by multiple environmental stressors on marine organisms.

5. Conclusion

In conclusion, this study provided clear evidence of the interactive effects between climate change stressors and an APIs mixture in the sentinel organism *M. galloprovincialis*.

Obtained results showed that hypercapnia and dynamic heatwaves, both individually and in combination, influenced the bioaccumulation of the antiepileptic CBZ and the antidepressant VLF. Notably, significant levels of the lipid regulator GEM and the antidiabetic MET were highlighted in all treatments, suggesting potential basal levels of these drugs in marine environment.

Analysis of cellular and biochemical responses revealed effects in modulation of immune parameters and lipid metabolism, increase in DNA fragmentation and cell apoptosis, and an increase of oxidative stress in organisms exposed to combined climate change stressors and to APIs mixture. Interestingly, co-exposure to heatwaves and APIs decreased DNA fragmentation significantly, while acidification combined with the mixture enhanced oxidative stress, suggesting that hypercapnic conditions may have a stronger impact on the effects of the APIs mixture on *M. galloprovincialis* compared to temperature changes.

Both chemical and biomarker findings indicated frequent synergistic and antagonistic interactions between climate change stressors and the APIs mixture, particularly when combined. These findings suggest potential threats to the health of *M. galloprovincialis* in coastal areas with high drugs levels and concurrent climate change impacts. Given the ecological significance of this species and its economic importance in the seafood market, further long-term studies are essential to elucidate the ecological consequences of pharmaceutical mixtures under predicted scenarios of oceanic changes.

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