FARHAT-UN-NISÁ NASCIMENTO BAJWA

PHARMACEUTICAL POLLUTION IN A CHANGING CLIMATE: THE INFLUENCE OF TEMPERATURE AND SALINITY ON THE TOXICITY OF CARBAMAZEPINE IN *MYTILUS GALLOPROVINCIALIS*

UNIVERSIDADE DO ALGARVE

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PHARMACEUTICAL POLLUTION IN A CHANGING CLIMATE: THE INFLUENCE OF TEMPERATURE AND SALINITY ON THE TOXICITY OF CARBAMAZEPINE IN *MYTILUS GALLOPROVINCIALIS*

Mestrado em Sistemas Marinhos e Costeiros

Especialidade em Ecotoxicologia

Trabalho efetuado sob a orientação de:

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UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

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Pharmaceutical pollution in a changing climate: The influence of temperature and salinity on the toxicity of carbamazepine in Mytilus galloprovincialis

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Declaro ser a autora 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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Farhat-Un-Nisá Nascimento Bajwa

Faro, Portugal

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ABSTRACT

The relentless presence of contaminants of emerging concern, such as human pharmaceuticals, in marine and coastal ecosystems has made ocean pollution one of the most pressing challenges of the Anthropocene. The continuous and exacerbated use of pharmaceuticals worldwide has been linked to the ubiquitous presence of different therapeutic classes in marine matrices (*e.g.*, water, sediment and biota), with direct and indirect implications for ocean and human health. Alongside this, the era of climate change exacerbates the interplay of multiple stressors in marine systems through increased seawater temperature and salinity fluctuations, leading to unknown impacts on the marine biota. However, limited information is available on the combined effects of pharmaceuticals in a multiple-stressor marine environment. Psychotropic drugs, such as carbamazepine (CBZ), are consumed for the treatment of epilepsy and some psychiatric disorders. CBZ causes negative effects on marine biota, and its persistence in aquatic ecosystems has been widely studied. A multiple-biomarker approach was employed to assess alterations in energy balance, antioxidant and biotransformation systems, membrane damage, neurotoxicity, and genotoxicity in gills and digestive glands of marine mussels. Therefore, to better understand the combined impacts of CBZ and climate change-driven stressors (increased seawater temperature and salinity), the present study aims to assess the biochemical and genotoxic alterations through an *in vivo* experiment. For this purpose, marine mussels *M. galloprovincialis* were exposed to a combination of CBZ (nominal concentration of 5 µg/L) with different temperatures (17° and 23° C) and salinities (35 and 40) for 28 days. The outcomes provide a first insight on the mussel's responses to CBZ under a changing climate. To best our knowledge, this is the first study to explore the effects of CBZ combined with the rise of seawater temperature and salinity on marine mussels.

Keywords: Marine pollution; pharmaceuticals; carbamazepine; climate change stressors; biochemical biomarkers; genotoxicity.

RESUMO

A presença contínua de contaminantes emergentes, como fármacos, em ecossistemas marinhos e costeiros tornou a poluição marinha um dos desafios mais proeminentes do Antropoceno. Consequentemente, várias classes terapêuticas de fármacos tornaram-se onipresentes na coluna d'água, sedimentos e biota marinha, em concentrações de ng à µg/L. Apesar de ocorrer em baixas concentrações, os fármacos têm demonstrado atividade biológica em organismos marinhos, visando vias terapêuticas ou biomoléculas semelhantes em organismos não-alvo. Evidências científicas crescentes enfatizam o impacto que as diferentes classes terapêuticas de fármacos podem ter na biota marinha com relevância ecológica e socioeconômica, como os mexilhões marinhos. A necessidade de implementar medidas baseadas no potencial impacto destes em ecossistemas marinhos foi enfatizada através da implementação da "lista de vigilância" do Quadro de Água da EU, incluindo vários fármacos sob vigilância, cujas concentrações ambientais provavelmente aumentarão no futuro, como é o caso dos medicamentos psicotrópicos (*p. ex.*, antidepressivos, ansiolíticos, antiepilépticos e estabilizadores do humor).

No entanto, o efeito de um único estressor (*p. ex*., poluição por fármacos) pode afetar a biota marinha diferentemente em comparação com o efeito de uma combinação de fatores devido natureza antagônica ou sinérgica das interações (*p. ex*., fármacos em combinação com estressores relacionados à mudança climática). Desta forma, a era das alterações climáticas exacerba a interação de múltiplos fatores de stress nos ecossistemas marinhos através do aumento da temperatura e da salinidade, levando a impactos desconhecidos na biota marinha. As projeções de aumento de temperatura e mudanças na salinidade já foram experimentadas em áreas costeiras em todo o mundo, por exemplo no Mar Mediterrâneo. O aumento da temperatura é uma das maiores ameaças à região que tem tido um aumento de 0,4 °C na temperatura da água do mar. Além disso, projeta-se que a chuva diminua nesta região, contribuindo para um aumento alarmante e acelerado de mudanças dos níveis de salinidade. Estas mudanças podem, por si só, alterar os mecanismos fisiológicos de tolerância da biota marinha. No entanto, a informação existente é limitada sobre os efeitos combinados dos fármacos num ambiente marinho com múltiplos fatores de stress. A temperatura da água do mar molda a capacidade aeróbica e a homeostase fisiológica dos invertebrados marinhos, portanto, um aumento de temperatura pode levar a alterações significativas nos processos

relacionados ao balanço energético. Juntamente com isso, mudanças de salinidade têm grandes implicações na osmorregulação e na absorção de oxigênio em organismos marinhos, causando alterações significativas nas respostas bioquímicas (*p. ex*., mudanças metabólicas e alterações significantes na estabilidade da membrana lisossomal).

Fármacos psicótropos, como a carbamazepina (CBZ), são utilizados para o tratamento da epilepsia e outros distúrbios psiquiátricos, e este tornou-se um marcador de poluição antropogênica devido ao seu alto consumo e baixa eficiência de eliminação por estações de tratamento de águas residuais. Consequentemente, o CBZ foi amplamente detectada na coluna de água (até dezenas de µg/L) e na biota marinha (*p. ex.*, concentrações na faixa de ng/g peso seco em bivalves). Desta forma, os efeitos negativos da CBZ em organismos marinhos têm sido amplamente estudados nos últimos anos, no qual observou-se alterações no metabolismo energético e estresse oxidativo, redução da estabilidade da membrana, dano celular e efeitos genotóxicos em espécies de bivalves marinhos. Para melhor compreender os impactos combinados da CBZ em combinação com o aumento da temperatura da água do mar e salinidade, o presente estudo visa avaliar as alterações bioquímicas e genotóxicas através de uma exposição *in vivo*. Mexilhões marinhos *Mytilus galloprovincialis* foram expostos a uma combinação de CBZ (concentração nominal de 5 µg/L) em diferentes temperaturas (17° e 23° C) e salinidades (35 e 40) durante 28 dias. Os resultados fornecem um primeiro insight sobre as respostas desta espécie de mexilhão exposto a múltiplos fatores de stress (*p. ex.*, CBZ em combinação com alterações na temperatura e salinidade da água do mar). Este é o primeiro estudo a explorar os efeitos da CBZ combinada com o aumento da temperatura e salinidade da água do mar nos mexilhões marinhos.

Palavras-chave: Poluição marinha; fármacos; carbamazepina; mudança climática; marcadores bioquímicos; genotoxicidade.

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LIST OF ABBREVIATIONS, ACRONYMS, AND SYMBOLS

Ach	Acetylcholine
AchE	Acetylcholinesterase
AEDs	Antiepileptic drugs
ATC	Acetylthiocholine iodide
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAT	Catalase
CbEs	Carboxylesterase
CBZ	Carbamazepine
CBZE	Carbamazepine-10,11-epoxide
CDNB	1-Chloro-2,4-dinitrobenzene
CECs	Contaminants of Emerging Concern
CI	Condition Index
CNS	Central Nervous System
CO_2	Carbon dioxide
CTL	Control
CYP3A4	Cytochrome P450 3A4
CYPs	Cytochromes P450
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
EC50	Half maximal effective concentration
ETS	Electron transport system
EU	European
FMO	Flavin-containing monooxygenases
FRAP	Ferric-reducing antioxidant power
FW	Fresh weight
GABA	Gamma amino butyric acid
GLY	Glycogen
GHG	Greenhouse gases
GPx	Glutathione peroxidase

GSH	Glutathione
GSTs	Glutathione-S-Tranferases
H_2O_2	Hydrogen peroxide
K^+	Potassium
LMA	Low melting point agarose
LPO	Lipid peroxidation
MAOs	Monoamine oxidases
MDA	Malondialdehyde content
$MgSO_4$	Magnesium sulfate
MoA	Mechanisms of Action
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NATs	N-acetyltransferases
NOEC	No-observed-effect concentration
$O_2^{-\cdot}$	Superoxide anion
PCA	Principal Component Analysis
PCs	Principal components
PhACs	Pharmaceutical active compounds
PNEC	Predicted no-effect concentration
pNPB	Substrate p-nitrophenyl butyrate
PPCPs	Pharmaceuticals and personal care products
PROT	Proteins
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
STs	Sulfotransferases
TAC	Total antioxidante capacity
TBARS	Thiobarbituric acid reactive substances
TS	Increased seawater temperature and salinity
TS+CBZ	Increased seawater temperature and salinity combined with carbamazepine
U	Units
UGTs	UDP-glucuronosyltransferases
WWTPs	Wastewater treatment plants
Ɛ	Molar extinction coefficient

1. Introduction

1.1. Ocean pollution as an Anthropocene issue

The Anthropocene is marked by human influence on the environment, climate, and ecology (Bernard, 2019). Nowadays, approximately half of the world's population (*i.e.*, 3.3 billion people) live within 200 km of a coastline (IPCC, 2022,). Moreover, 15 of the 23 megacities (*i.e.,* cities with more than 8 million inhabitants) (Blackburn *et al.,* 2019) located on the coast are predicted to face a range of environmental management issues (Li, 2003; Martinez *et al.,* 2007). Global demographic trends predict an increase population living along the coastline linked to an exacerbated anthropogenic impact on aquatic ecosystems and ecosystem services (*e.g.,* ecologic and socio-economic benefits) provided by coastal and marine environments (Creel, 2003; Gaw *et al.,* 2014). Humankind has created a diverse range of synthetic chemicals that are increasing rapidly along with other global stressors, such as biodiversity loss, increased atmospheric dioxide carbon concentrations, climate change, among others (Bernhard *et al.,* 2017; de Oliveira Souza *et al.,* 2021).

Therefore, unwanted toxic and hazardous wastes are released into aquatic ecosystems by anthropogenic sources from regular human activities, such as domestic, agricultural, and industrial processes (Puri *et al.,* 2023). Thus, the presence of contaminants of emerging concern (CECs) become ubiquitous in coastal and marine ecosystems, making ocean pollution one of the main challenges of our present time (Landrigan *et al.,* 2020). CECs consist of synthetic or naturally occurring chemicals, including pharmaceuticals and personal care products (PPCPs), surfactants, plasticizers, pesticides, synthetic hormones, and flame retardants (Rosenfeld *et al.,* 2011; Quesada *et al.,* 2019). Supplements, over-the-counter drugs, and prescription pharmaceuticals comprise the diverse range of chemicals within PPCPs (Rosenfeld *et al.,* 2011). As the coastal population increases, the extensive use of pharmaceuticals rapidly grows worldwide (Busfield, 2010) linked to its environmental occurrence that has implications for human and environmental health (Klatte *et al.,* 2017; Souza *et al.,* 2020).

1.2. Global pharmaceutical market trends and expenditure

Over the past decade, worldwide medicine use has increased by 36 %, and its market exceeded 1.48 trillion U.S. dollars in 2022 (Statista, 2023). The medicine spending has been increasing at a rate of 3 – 6 % per year, which has been expected to reach 1.9 trillion U.S. dollars by 2027 (IQVIA, 2023). The global outlook predicts a volume growth of over 10 % until 2027, particularly in Latin America, Asia-Pacific, Africa, and the Middle East (IQVIA, 2023). Europe

and North America are expected to grow slower at a rate of 0.1 to 0.4 % through 2027 due to their higher per capita use of pharmaceuticals (IQVIA, 2023). Moreover, modern-day life habits have led to adverse effects on human mental health, leading to an increase in Anthropocene-related diseases with broad effects on societal health (Gluckman *et al.*, 2020), thus characterizing the last decades as the age of psychopharmacology (Braslow and Marder, 2019).

Since the early 1990s, the range of drug treatment for mental disorders and seizures expanded rapidly; one in six persons currently takes a psychotropic drug (Braslow and Marder, 2019). A growing prevalence of mental health disorders leads to an increasing demand for psychotropic drugs (antidepressants, anti-anxiety, and anti-epileptic drugs), which consumption raised 4.8 % from 2018 to 2022 (FMI, 2023). Global psychotropic drugs market is expected to reach US$ 28.8 million by 2033 compared to US$ 21.6 million in 2023 (*i.e.*, annual growth rate of 3.1 % in 10 years) (FMI, 2023). The COVID-19 pandemic has increased demand for psychotropic drugs as it resulted in higher rates of anxiety, depression, and other mental health conditions (Rachidi *et al.*, 2023).

1.3. Pharmaceutical drugs as a threat to aquatic ecosystems

Pharmaceuticals from various therapeutical classes are widely used to prevent and cure diseases globally consisting of one or several active ingredients, excipients, additives, inorganic salts, or other organic chemicals (*e.g.*, sugars, pigments, among others) (Swiacka *et al.*, 2022; Kümmerer, 2010). Pharmaceutical active compounds (PhACs) are categorized by their biological activity and purpose (*e.g.*, anti-infectious, antineoplastic, psychotropic), although classification regarding their chemical structure and behavior are often used (*e.g.*, psychotropic: antidepressants, anti-anxiety, anti-epileptic). Additionally, the compounds can also be grouped based on the drugs' mode of action (MoA) (Kümmerer, 2010).

Coastal urban areas with dense populations are 'hotspots' of pharmaceutical consumption. After consumption, PhACs are metabolized and excreted, entering the sewage system, and following to wastewater treatment plants (WWTPs), where they often undergo incomplete removal by conventional technologies due to their complex molecular structure (Zhang *et al.*, 2008). Therefore, WWTPs represent the main source of pharmaceutical input into the aquatic environment, reaching the ocean as their fate. Still, marine and coastal ecosystems have long been neglected due to the assumption that dilution could represent a safety factor as pharmaceuticals are detected in low concentrations on aquatic environments (Fabbri, 2015). In

addition, wastewater from manufacturers, production industries and landfill leachates may contribute significantly via direct pharmaceutical effluent discharge to waterbodies or accidental spillage during distribution processes (Adeola *et al.*, 2022).

Concentrations in hospital wastewater are higher than in municipal sewage. Still, the total load is much lower as the usage of pharmaceuticals in hospitals compared to public effluents is lower in high-income countries (Kümmerer, 2010). Improper disposal of human pharmaceuticals is one of the main issues for contamination of surface water or groundwater. The disposal of unused drugs via household waste or by pouring them into the toilets can lead to the presence of PhACs in effluents of the landfill (Kümmerer *et al.*, 2010). In this sense, the improper disposal and inefficient removal of pharmaceuticals and their metabolites from wastewater and via rivers receiving WWTP effluents (Benotti and Brownawell, 2007; Metcalfe *et al.*, 2011; Rodriguez-Navas *et al.*, 2013) have led to their increasing presence in the environment, posing ecological and human health concerns (González-Peña *et al.*, 2021).

Several therapeutic classes of pharmaceuticals are found in marine realms at trace levels (ng – µg/L), including antibiotics, anti-hypertensives, analgesics/anti-inflammatories, tranquillizers, β-blockers, diuretics, and psychotropic drugs (antidepressants, anti-anxiety and anti-epileptic drugs) (Álvarez-Muñoz *et al.*, 2015; Hernández-Tenorio *et al.*, 2022; McEneff *et al.*, 2013; Moreno-González *et al.*, 2016; Świacka *et al.*, 2019). The compounds with the highest occurrence are antibiotics (*e.g.*, sulfamethoxazole, trimethoprim, ciprofloxacin, erythromycin and clarithromycin), analgesics/anti-inflammatories (*e.g.*, acetaminophen, diclofenac, ibuprofen, naproxen) and psychotropic drugs (*e.g.*, carbamazepine, alprazolam, bromazepam, diazepam, lorazepam, oxazepam, citalopram), even though the occurrence and concentration of pharmaceutical classes vary in different geographical regions worldwide (Cunha *et al.*, 2017; Hernández-Tenorio *et al.*, 2022; Zheng *et al.*, 2021).

1.4. Psychopharmacology: psychotropic drugs

Psychotropic drugs are designed to affect human thinking, emotion, will, and behavior (Jin *et al.*, 2022). These drugs are designed to treat mental disorders, reduce disability, and prevent relapse through anti-psychotics, anti-depressants, mood stabilizers and anti-epileptic medications (UNODC, 2021). Psychotropic drugs are divided into different classes based on their therapeutic actions, but a particular compound is not confined to one category (Cowen, 1998). Psychotropic drugs exert their effect through actions on the neurotransmitters in the

central nervous system (CNS), including gamma amino butyric acid (GABA), acetylcholine (AChE) and the biogenic amines (monoamines) – the last has been subdivided into tryptophan-derived indoleamines (serotonin and melatonin) and tyrosine-derived catecholamines (dopamine, norepinephrine, and epinephrine) (Ebert, 2002). The neurotransmitters are released into the synaptic cleft, binding to the receptors and resulting in the transduction of the signal to the postsynaptic membrane. The signal ceases upon deactivation of the neurotransmitter activity, which occurs through enzymatic degradation, reuptake, and auto-receptor activation (Ebert, 2002).

The effects of the drugs depend on their chemical composition and the neurotransmitter's target, able to act through five main mechanisms of action: (1) increasing synthesis of neurotransmitters; (2) promoting the release of neurotransmitters into the synapse; (3) mimicking the action of the endogenous neurotransmitter at the receptor as an agonist; (4) inhibiting the action of the endogenous neurotransmitter at the receptor as an antagonist; and (5) prolonging the endogenous neurotransmitter activity by either preventing the action of degrading enzymes or preventing reuptake to the neuron (Wright and O'Neil, 2012).

1.5. Drug metabolism and excretion

Pharmaceuticals are characterized by interacting with specific physiological pathways in the target organism (Hernández-Tenorio *et al.*, 2022). Once consumed, PhACs are metabolized mainly in the liver, but it also can occur in the kidneys, lungs, skin, and gastrointestinal tract. Afterwards, the compounds are excreted from the body, in their parent form or as metabolites, following waterways to reach aquatic environments (Galwa-Widera, 2019). Hence, the processes of absorption, distribution, biotransformation/metabolism, and excretion are part of drugs' pharmacokinetics (Turfus *et al.*, 2017). Briefly, absorption transports the drug allowing it to be bioavailable (*i.e.*, the fraction of the administered drug is available to reach the systemic circulation), followed by its distribution from the systemic circulation to tissues (Currie, 2018).

Metabolic biotransformation pathways are crucial to yield biochemical conversions and drug activation in the target organism, such as to increase pharmaceutical polarity and facilitate its excretion from the organism. Biotransformation occurs in two phases, comprised of functionalization (Phase I) and conjugation (Phase II) (Turfus *et al.*, 2017). Phase I is characterized by oxidation (*e.g.*, hydroxylation, dealkylation, deamination), reduction,

hydrolysis, or hydration reactions (Turfus *et al.*, 2017). This process relies on enzymes responsible for the biotransformation of drugs within an enzymatic system of defense against most xenobiotic compounds (Stanley, 2017). It includes cytochromes P450 (CYPs), flavin-containing monooxygenases (FMO), monoamine oxidases (MAOs), cyclooxygenases, dihydrodiol dehydrogenases, nicotinamide adenine dinucleotide phosphate (NADPH), quinone oxidoreductases, alcohol dehydrogenases, and xanthine oxidase/aldehyde oxidase. Monooxygenation represents an essential step in the detoxification of many compounds. However, CYP enzymes can generate intermediates that are more toxic than the original substrate and require detoxification by Phase II enzymes (Stanley, 2017).

Phase II xenobiotic metabolism involves conjugation reactions to hydrophilic moieties, usually leading to more water-soluble and easily excretable compounds (Stanley, 2017). The reactions occur by conjugating with acetyl, sulfate, glucuronic acid, glutathione, and amino acids functional groups (Currie, 2018). These reactions are catalyzed by enzymes that are classified as products of CYP-mediated Phase I metabolism, mostly by glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), sulfotransferases (STs), and N-acetyltransferases (NATs) (Stanley, 2017). After the metabolic processes (Phase I and II), drugs are excreted, either unchanged or as a metabolite, according to their chemical properties (Currie *et al.*, 2018).

1.6. Carbamazepine

Globally, epilepsy is one of the most common neurological disorders. Over the years, several antiepileptic drugs (AEDs) have been developed, but nowadays, the treatments are carried out only by four drugs: phenobarbital, phenytoin, carbamazepine (CBZ) and valproic acid (Ambrosio *et al.*, 2002). Since the 1960s, CBZ (*5H*-dibenz[*b,f*]azepine-5-carbox-amide) has been widely prescribed for the treatment of epilepsy as the first-line drug of choice (*e.g.*, focal epileptic syndromes) and its consumption has reached up to 1,014 tons per year, becoming one of the most consumed drugs worldwide (Zhang *et al.*, 2008). CBZ is an iminodibenzyl derivative consisting of a tricyclic ring structure, similar to antidepressants (Ambrosio *et al.*, 2002). Initially, CBZ was synthesized as a potential antidepressant (Ayano, 2016), playing an essential role in the treatment of neuropathic pain and some psychiatric disorders. The therapeutic properties of the drug are recognized in most guidelines as a second-line mood stabilizer for the treatment and prevention of both phases of bipolar affective disorder (Ayano, 2016).

1.6.1. Pharmacodynamics of carbamazepine

Over the past few decades, the MoA of CBZ has been extensively explored, although it remains scarcely understood. Later advancements proposed the possibility that the different therapeutic properties of this drug result from more than one MoA. Overall, this drug blocks voltage-gated sodium (Na^+) channels in the excitatory nervous system, stabilizing the cell membranes of nerve fibers, inhibiting neuronal discharge, and reducing excitatory synaptic transmission (Grześk *et al.*, 2021). The main MoA relies on the blockage of inactivated neuronal Na^+ channels, preventing them from opening, which stops the neuronal sodium current from gaining sufficient amplitude to depolarize the nerve and inhibits the repetitive neuronal firing that occurs during a seizure. Interactions with voltage-controlled calcium (Ca^{2+}) and potassium (K^+) channels have been considered in the literature to inhibit stimulatory effects on nerves (Ambrosio *et al.*, 2002; Elliott, 1990; Okada *et al.*, 1997). Further, this drug is considered a GABA agonist, which stimulates the activation of the GABA receptor producing a sedative effect. This inhibitory neurotransmitter plays a key role in dopamine and glutamate regulation. For this reason, this medication is occasionally used in the prophylactic treatment of bipolar disorder, treating manic and depressive symptoms as it increases dopamine turnover and GABA transmission (Ambrosio *et al.*, 2002; Ayano, 2016; Okada *et al.*, 1997).

1.6.2. Pharmacokinetics of carbamazepine

Pharmacokinetic profiles can vary considerably according to the drugs' properties. For instance, some pharmaceuticals undergo incomplete metabolism, leading to the elimination of both the unmetabolized parent drug and its metabolites from the body (Zhang *et al.*, 2008). In this regard, CBZ consists of a prodrug, which can be defined as a drug substance that needs to undergo enzymatic or chemical transformation to become pharmacologically active in the organism (Vale *et al.*, 2018). As CBZ is moderately hydrophobic, most of the compound is absorbed when orally administered, and only 1 % of the dosage leaves the body unaltered (Adeola *et al.*, 2022; Zhang *et al.*, 2008). CBZ is absorbed in the gastrointestinal tract, and it is largely metabolized in the liver by CYP isoenzymes (Schwarz *et al.*, 2021), particularly by the isoform cytochrome P450 3A4 (CYP3A4), producing the active and therapeutic anticonvulsant metabolite carbamazepine-10,11-epoxide (CBZE) (Bardal *et al.*, 2011). This metabolite contributes to the therapeutic activity of the drug (Bardal *et al.*, 2011) and is almost entirely excreted in urine or feces (72 and 28 %, respectively) (Zhang *et al.*, 2008).

1.7. The presence of carbamazepine in the marine environment

1.7.1. Source and routes of carbamazepine

WWTPs play a significant role in removing pharmaceutical compounds, including sorption and biodegradation (Ulvi *et al.,* 2022). CBZ is considered persistent to conventional WWTP technologies due to its resistance to biodegradation at low concentrations and non-attachment onto sludge, resulting in its low removal efficiency (up to 20 %) (Ulvi *et al.,* 2022; Zhang *et al.,* 2008). In this sense, CBZ is one of the most frequently detected drugs in WWTP influents, effluents, groundwater, drinking water, surface water and seawater in different geographical regions around the globe, discharged via untreated, partially treated, or even treated effluents (Hernández-Tenorio *et al.,* 2022). Therefore, environmental studies have revealed (2010 – 2019) a concentration range from 18 to 7,100 ng/L in WWTP' influents, 117 to 2,499 ng/L and 20 to 7,009 ng/L in effluents (WWTPs and hospitals respectively), 0.2 to 1000 ng/L in groundwater, 1.3 to 610 ng/L in drinking water, 0.1 to 2,420 in surface waters, 0.4 to 1,400 ng/L in seawater, and 9 to 50 ng/g in sediments (Gaw *et al.,* 2014 ; Hernández-Tenorio *et al.,* 2023; Hernando *et al.,* 2006; Ulvi *et al.,* 2022; Zhou and Broodbank, 2013). Therefore, hospital and WWTP effluents represent the main sources of CBZ in the marine environment.

1.7.2. Ecotoxicological impacts of carbamazepine on the marine biota

CBZ exhibits a relatively high log octanol-water distribution coefficient (Dow) at pH 8 (3.52), in which Dow accounts for neutral and ionized compound fractions at a given pH as a useful predictor of the bioaccumulation potential of a compound (*i.e.*, high Dow value indicates an acute potential for bioaccumulation in marine organisms). PhACs have a low volatility, indicating that their distribution will occur mainly through aqueous transport but also via food chain dispersal (Fent *et al.,* 2006). CBZ has been detected at concentrations of up to 11 ng/g dry weight in wild marine bivalves (Klosterhaus *et al.,* 2013; Moreno-Gonzalez *et al.,* 2016; Rodrigues *et al.,* 2019), across coastal areas of Europe, Africa and South America (Madikizela and Ncube, 2022). For example, in Italy, this drug has been mostly detected (up to 90 % frequency) in marine mussels (Mezzelani *et al.,* 2020).

Moreover, once in the aquatic environment, pharmaceuticals may affect the same pathways in marine organisms having identical or similar target organs, tissues, cells, or

biomolecules (Fent *et al.*, 2006). CBZ has been widely investigated for both acute and chronic ecotoxicological effects, particularly in freshwater organisms. As a result, previous authors defined the concentrations that cause 50 % of effect (EC50) in non-target organisms to be in the range of mg/L, while the worst no observed effects (NOEC) and predicted no-effect concentration (PNEC) in different aquatic organisms (*e.g.*, phytoplankton, benthos, zooplankton, bivalves and fishes) were 25 µg/L and 0.42 µg/L, respectively, (Baali and Cosio, 2022; Fent *et al.*, 2006; Ferrari *et al.*, 2003; Jones *et al.*, 2002; Jos *et al.*, 2003). Thus, most acute toxicity concentrations observed are below 100 mg/L, evidencing the potential negative effects of CBZ on aquatic biota at concentrations commonly found in surface waters worldwide (Baali and Cosio, 2022; Fent *et al.*, 2006). Currently, standardized toxicity tests are in accordance with existing guidelines for traditional endpoints, such as mortality (Fent *et al.*, 2006). However, it also represents one of the main issues in the advancement of ecotoxicological studies, which demands toxicity experiments designed for specific targets of the pharmaceutical drugs considering its MoA.

A growing body of evidence reveals the ecotoxicological threat of CBZ on marine organisms (*e.g.*, bivalves, anemones, sea urchin, fishes, and amphipods), in which concentrations up to 1 µg/L can potentially alter biological responses, such as biochemical, physiological, cellular and molecular. Previous studies reported significant changes in energy balance and lysosomal membrane stability, oxidative stress, impairment of immune system, and DNA damage in marine bivalves (adults and early life stages), exposed to realistic range of a few µg/L over acute and chronic bioassays (Abdelhafidh *et al.*, 2018; Almeida *et al.*, 2014, 2015, 2017; Baali and Cosio, 2022; Brandts *et al.*, 2018; Dumas *et al.*, 2022; Freitas *et al.*, 2016; Juhel *et al.*, 2017; Oliveira *et al.*, 2017). Consequently, CBZ has been widely reported to cause toxic effects on non-target organisms, classified as "*R52/53 harmful to aquatic organisms and may cause long-term adverse effects in the aquatic environment*" (Almeida *et al.*, 2014; Tsiaka *et al.*, 2013).

1.8. Pharmaceuticals in a multiple-stressor environment

Anthropogenic activities, principally through emissions of greenhouse gases (GHG), have caused an increase of 1.1 °C of global temperatures above pre-industrial levels and are extremely likely to rise by 1.5 °C between 2030 and 2052 (IPCC, 2023). Global warming has been causing progressive changes in marine ecosystems and the socio-economic systems relying on these environments (Harley *et al.*, 2006). Thus, higher oceanic uptake of carbon

dioxide (CO_2) by the increase of GHG leads to changes in the seawater pH, salinity, and temperature (Maulvault *et al.*, 2019). In addition, climate change has increased the frequency and intensity of extreme events, including heavy precipitation and pluvial floods, river floods, droughts, storms (*e.g.*, tropical cyclones), as well as compound events (multivariate and concurrent extremes) (IPCC, 2023). The projections of temperature rise and changes in salinity have already been experienced in coastal areas across the globe, resulting in the creation of climate change "hotspots", such as in the Mediterranean Sea (IPCC, 2023; MedECC, 2020). Temperature rise is one of the biggest threats to the region that has been warming 20 % faster than the global average, inducing an increase of 0.4 °C in seawater temperature (projected to + 3.5 °C by 2100) (MSSD, 2016; IPCC, 2023). In addition, rainfall in spring and summer is expected to decrease by up to 30 % by 2080 in Southern Europe contributing to an alarming and accelerated increase in salinity trends (MSSD, 2016; Vargas-Yáñez *et al.*, 2017). Changes in these two main abiotic factors due to increased occurrence of extreme weather events (*e.g.*, long drought or heavy rainfall periods) (Harley *et al.*, 2006), can, *per se*, shift physiological mechanisms driving tolerance in marine biota (Maulvault *et al.*, 2019; IPCC, 2023), besides disrupting the behavior and fate of chemical contaminants, altering their uptake, detoxification and toxicity (Bethke *et al.*, 2023; Maulvault *et al.*, 2019; Puckowski *et al.*, 2016). Thus, climate change effects on marine and coastal organisms vary from changes in their physiology, metabolism, and ecology (Maulvault *et al.*, 2019) to changes in the chemical and physical properties of CECs, leading to disruption in partitioning, behavior and fate, reaction rates and their temporal and spatial distribution (Bethke *et al.*, 2023; Maulvault *et al.*, 2019; Puckowski *et al.*, 2016).

Seawater temperature shapes the aerobic capacity and physiological homeostasis of marine invertebrates (Sokolova *et al.*, 2012), thus a temperature rise can lead to significant alterations in energy-related processes, impairment of molecular and cellular pathways (*e.g.*, oxidative stress) and reproduction (Abele *et al.*, 2002; Crespo *et al.*, 2021; Monari *et al.*, 2007; Velez *et al.*, 2016). Alongside this, salinity plays a key role in controlling ecological and physiological processes in marine species. Thereby, salinity shifts in marine realms have great implications on osmoregulation and oxygen uptake causing alteration in immune and biochemical (*e.g.*, metabolic shifts and oxidative stress) responses of various species (Carregosa *et al.*, 2014; Dickinson *et al.*, 2012; Hamer *et al.*, 2008; Monari *et al.*, 2007; Pfeifer *et al.*, 2005; Reid *et al.*, 2003). Both changes in seawater temperature and salinity raise concerns about the biological consequences of their synergistic or antagonistic interaction with other co-occurring

stressors, such as pharmaceutical drugs (Almeida, *et al.*, 2021a, 2022; Arrigo *et al.*, 2024; De Marchi *et al.*, 2020; Nardi *et al.*, 2022). Previous studies on the combined effects of pharmaceuticals and warming reported an increased uptake and metabolization of drugs leading to oxidative stress and metabolism shifts (Almeida *et al.*, 2021a; Bethke *et al.*, 2023; Freitas *et al.*, 2020b; Maulvault *et al.*, 2019; Maynou *et al.*, 2021; Nardi *et al.*, 2022; Queirós *et al.*, 2021; Serra-Compte *et al.*, 2018). Moreover, salinity shifts may increase bivalves' vulnerability to other stressors (Pourmozaffar *et al.*, 2020) affecting the physical-chemical properties of pharmaceuticals inducing alterations in the uptake and retention of drugs resulting in possible higher toxicity (Almeida *et al.*, 2022; Campos *et al.*, 2016; Correia *et al.*, 2016; Freitas *et al.*, 2017, 2019a, 2020a). Yet, the physiological tolerance of marine mussels to changes in seawater temperature and salinity is still under-investigated, and most studies are performed in freshwater mussels. For this reason, insufficient scientific evidence is available on the salinity tolerance of the species *M. galloprovincialis*, which may tolerate salinity variations from 31 to 36 ppt (Cáceres-Martínez and Figueiras, 1998). Regarding thermal tolerance, Bertolini *et al.* (2023) explored tolerance landscapes in *M. galloprovincialis* reporting that this species can withstand for longer periods the increased temperature range between 25 – 28 °C, but only 1 – 2 days of and above 30 °C. Similarly, Anestis *et al.* (2007) observed a significant filtration reduction in marine mussels at temperatures above 25 °C. Likewise, Jansen *et al.* (2007) reported that respiration rates between 10 – 24 °C are within the natural range of these organisms. Thus, temperatures beyond 25 °C are a potential limit of thermal tolerance for *M. galloprovincialis*, leading to increased mortality revealing the occurrence of significant metabolic alterations in marine mussels that negatively affect their growth and reproduction (Anestis *et al.*, 2007; Jansen *et al.*, 2007).

1.8.1. The effect of combined stressors on carbamazepine: ocean warming and changes in salinity

A growing body of evidence has alerted for the potential enhancement of toxicity of pharmaceuticals, such as CBZ, in the ocean by the global warming effects (Kibria *et al.*, 2021). Yet, the combined toxicity of CBZ with increased seawater temperature (Almeida *et al.*, 2021a; Arrigo *et al.*, 2024; Nardi *et al.*, 2022; Serra-Compte *et al.*, 2018) and salinity changes (Almeida *et al.*, 2022) have been scarcely investigated in marine bivalves. Salinity fluctuations in marine realms lead to metabolic shifts and oxidative stress in bivalves at the biochemical level (Almeida *et al.*, 2022; Velez *et al.*, 2016a, 2016b; Freitas *et al.*, 2020; 2019; 2017). Yet, limited studies have been conducted on the interaction of salinity variations and the uptake of

pharmaceutical drugs by marine mussels (Almeida *et al.*, 2022). Freitas *et al.* (2017) investigated the impacts of salinity variations in *M. galloprovincialis*, combined with exposure to triclosan and diclofenac (Freitas *et al.*, 2019), and salicylic acid (Freitas *et al.*, 2020). Freitas *et al.* (2019) detected a higher uptake of diclofenac under extreme salinities (25 and 35) along with changes in the biochemical parameters (*e.g.*, increased electron transport system activity (ETS), higher cellular damage). In sum, salinity, *per se*, may drive several biochemical responses (*e.g.*, changes in metabolic activity and energy balance, induce oxidative stress, etc.) and significant alterations in the responses of marine bivalves (*e.g.*, clams and mussels) to drug exposure (Almeida *et al.*, 2022; Freitas *et al.*, 2019). However, the impacts of salinity variations on the toxicity of pharmaceuticals are still unclear.

On the other hand, ocean warming-related impacts have been widely studied in marine organisms, especially marine bivalves. Serra-Compte *et al.* (2018) revealed that CBZ (15.7 µg/L for 20 days of exposure, followed by 20 days of depuration) reaches its highest bioconcentration in water under warming conditions (22 °C), suggesting that mussels *M. galloprovincialis* are under a higher exposure to the drug at such environmental condition. Similarly, Nardi *et al.* (2022) highlight that events of temperature extremes (22.5 °C) may enhance the accumulation of CBZ (1 µg/L for 20 days of exposure) in marine mussels, which can persist even 10 days after the end of the heatwave acting as a synergistic stressor on the neuroendocrine-immune and oxidative system of the organism. However, there is a lack of knowledge regarding the impacts that the interaction of ocean warming and salinity changes on the pharmaceutical toxicity to marine mussels.

1.9. The use of biomarkers in ecotoxicological assessments

Over the past decades, the increasing anthropogenic pressure on marine and coastal ecosystems has spurred growing efforts to assess the impacts of toxic exposure on biota (De Coen *et al.*, 2000). As a result, a diverse array of molecular, biochemical, physiological and histological biomarkers has been proposed and adapted within the field of ecotoxicology. Depledge (1994) defined biomarkers as "a biochemical, cellular, physiological or behavioral variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of one or more chemical pollutants (and/or radiations)". The World Health Organization (WHO; 1993) further refined these categories, defining biomarkers of exposure as "an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in

a compartment within an organism", and biomarkers of effect as "a measurable biochemical, physiological, behavioral or other alteration within an organisms, that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease" (Martinez-Haro *et al.,* 2015).

The development and refinement of biomarkers as ecotoxicological tools were driven by the need for sensitive early-warning indicators of sub-lethal effects (Martinez-Haro *et al.,* 2015). Consequently, biomarkers offer invaluable insights into the exposure and effects of chemicals on biota, while chemical analyses are primarily limited to detecting the presence of contaminants without revealing their toxic effects within an organism (Martinez-Haro *et al.,* 2015). Moreover, chemical analyses encounter challenges, such as the lack of sufficiently sensitive techniques to detect all pollutants and their byproducts in water and sediment samples (Martinez-Haro *et al.,* 2015). Therefore, biomarkers serve as essential complements to chemical analyses in the field of ecotoxicology. Widely used and well-established biomarkers of exposure include antioxidant (*e.g.,* superoxide dismutase, catalase, etc.) and biotransformation (*e.g.,* cytochrome P450 (CYP450), glutathione-S-transferase, etc.) enzymes, and biomarker of effects include, but are not limited to, lipid peroxidation and protein carbonyl (López-Barea, 1995). Various xenobiotics detected in the marine environment demonstrated genotoxicity in organisms, leading to severe adverse effects on genetic material (López-Barea, 1995; Mouneyrac and Amiard-Triquet, 2013). Consequently, biomarkers of genotoxicity are crucial for providing in-depth information on the relationship between effects at the sub-individual levels and the population level (Mouneyrac and Amiard-Triquet, 2013). One of the most used techniques for assessing genotoxicity is the comet assay test.

The exposure to single or multiple stressors can directly or indirectly alter the energy balance in marine organisms, affecting their basal metabolism, growth, and reproduction (Holloway *et al.,* 1990). Therefore, biomarkers of energy metabolism play a pivotal role in evaluating the negative effects of stressors on important biological processes through the measurement of the mitochondrial electron transport system and energy reserve levels (*e.g.,* glycogen, lipids, and proteins) (López-Barea, 1995; Mouneyrac and Amiard-Triquet, 2013). Additionally, various biochemical biomarkers are employed to assess the induction of specific biotransformation systems or cellular alterations, such as antioxidant responses (Depledge and Fossi, 1994). Cellular oxidative damage caused by chemical pollutants is well-documented in marine organisms (López-Barea, 1995). Environmental contaminants can greatly enhance the intracellular formation of reactive oxygen species (ROS), leading to a cascade of oxidative responses (Depledge and Fossi, 1994; Ferreira *et al.,* 2021). Organisms have an integrated

antioxidant and biotransformation systems to minimize and avoid the harmful effects of ROS (Ferreira *et al.*, 2021; Regoli and Giuliani, 2014), thus antioxidant and biotransformation enzymes serve as valuable early warning biomarkers (Mouneyrac and Amiard-Triquet, 2013). However, if the stressors result in an overwhelming prooxidant condition, marine organisms may experience significant alterations at biochemical and molecular levels (Ferreira *et al.*, 2021). For this reason, it becomes imperative to evaluate alterations as well in lysosomes (*i.e.*, decreased integrity of the cellular membrane) in order to understand the organisms' protective responses to counteract the negative effects under contaminant exposure (López-Barea, 1995).

The applicability of biomarkers in ecotoxicology highlights the importance of integrative analyses encompassing total antioxidant capacity, energy balance and metabolism, alterations in lysosomes, neurotoxicity, and genotoxicity to comprehensively evaluate responses of marine organisms exposed to chemical stress (López-Barea, 1995). As a result, several biomarkers have been validated and applied in environmental biomonitoring programs (*e.g.*, Convention for the protection of the marine environment of the Northeast Atlantic (OSPAR), Programme for the Assessment and Control of Pollution in the Mediterranean region (MEDPOL)), including cytochrome P450, DNA damage, lysosomal membrane stability, and acetylcholinesterase (Martinez-Haro *et al.*, 2015; Mouneyrac and Amiard-Triquet, 2013). Therefore, the integration and implementation of biomarkers with chemical analyses in environmental programs is pivotal to identifying pollutants and sites at risk, prioritizing their management and remediation needs, particularly under a changing climate (Galloway *et al.*, 2002).

1.10. *Mytilidae* family as a bioindicator

Marine bivalves, such as mussels, clams and oysters, have high ecological and socioeconomic value. These organisms belong to the Mollusca phylum and are characterized by their wide distribution, bioaccumulation potential, well-known life cycles, sessile behavior, and filter-feeding activity (Swiacka *et al.*, 2019). Marine bivalves are continuously exposed to pharmaceuticals discharged into marine environments near coastal sites, where they can be harvested (Almeida *et al.*, 2020). For this reason, mussels play an important role in environmental monitoring and ecotoxicological programs, contributing to information on the exposure risks and effects of these compounds on the marine biota (McEneff *et al.*, 2014; Fabbri and Franzellitti, 2016).

Mytilidae is one of the most studied bivalves' families worldwide. The species belonging to this family are present on almost every continent with a stable and large population, which makes them suitable for ecotoxicological studies worldwide. Several studies have been conducted with the marine mussel *M. galloprovincialis* under pharmaceutical exposure, indicating negative effects on physiological, cellular and molecular endpoints (*e.g.*, Adeola *et al.*, 2022; Aguirre-Martinez *et al.*, 2016; Ajala *et al.*, 2022; Almeida *et al.*, 2014, 2015, 2021ab; Baali and Cosio, 2022; Bahlmann *et al.*, 2014; Boillot *et al.*, 2015; Mezzelani *et al.*, 2021; Di Poi *et al.*, 2018; Tolou-Ghamari *et al.*, 2013). The biological endpoints can be measured in tissues or body fluids (*e.g.*, gills, digestive glands, and hemocytes) of these organisms providing accurate and reliable of the exposure and effects of single or multiple stressors (Depledge, 1993; Faggio *et al.*, 2018). Each organ provides different responses because of their distinct roles and exposure levels, for instance the gills are involved in respiration and ion regulation (*i.e.*, directly in contact with external environment), while the digestive glands perform nutrient assimilation and detoxification processes (Faggio *et al.*, 2018; Franco-Martinez *et al.*, 2016).

1.11. Ecotoxicological effects of carbamazepine in the mussel *M. galloprovincialis*

Ecotoxicological assessments have confirmed that CBZ can disrupt energy metabolism, cause oxidative stress, reduction of lysosome membrane stability, cell damage and genotoxic effects in marine bivalves' species (Aguirre-Martínez *et al.*, 2016; Almeida *et al.*, 2021b, 2021c; Baali & Cosio, 2022; Brandts *et al.*, 2018; Juhel *et al.*, 2017; Lacaze *et al.*, 2015). Moreover, previous studies observed an enzymatic activation of the prodrug CBZ in marine mussels *Mytilus galloprovincialis* into its pharmacologically active metabolite (CBZ-10,11-epoxide), leading to sodium and calcium channel modulation, GABAergic effects, and serotonin release, linked to its designed MoA (Abdelhafidh *et al.*, 2018; Almeida *et al.*, 2018; Ambrósio *et al.*, 2002; Boillot *et al.*, 2015; Dumas *et al.*, 2022).

Considering the MoA of CBZ in humans, it focuses on the voltage-dependent Na+ channels and secondarily also interacts with Ca+ and K+ channels to modulate the release, uptake and receptor binding of neurotransmitters (*e.g.*, GABA), inhibit adenylase cyclase activity, decreased arachidonic acid turnover, and inhibit histone deacetylases (Almeida *et al.*, 2020; Ambrosio *et al.*, 2002). Even though the effects of CBZ in related processes and metabolic pathways have been under-investigated in marine bivalves, previous ecotoxicological studies evidenced that CBZ's MoA targets similar pathways in marine

mussels, which may be related to changes in the regulation of glycogen breakdown and concomitant control of gonad maturation in these organisms (Fabbri and Capuzzo, 2010; Martin-Diaz *et al.*, 2009).

To date, most of the findings indicate that CBZ undergoes metabolism and detoxification in marine mussels. The upregulation of CYP11 gene expression in *M. galloprovincialis* exposed to 6.3 µg/L (96 h exposure period) demonstrated the involvement of CYP450 system in the detoxification process of the mussel (Brandts *et al.*, 2018). Other studies support the detoxification process in mussels, such as Boillot *et al.* (2015) and Luis *et al.* (2016). These studies showed the occurrence of CBZ metabolites (10,11-epoxide and acridine), induction of enzymatic activity in digestive glands and mantle/gonads, and changes in the activity of GSTs (a decrease) for concentrations between 0.1 – 10 µg/L and non-environmental CBZ concentrations up to 250 mg/L (Luis *et al.*, 2016).

Moreover, oxidative stress-related studies have been widely explored in marine bivalves exposed to CBZ, evidencing the induction of oxidative stress promoted by CBZ due to the generation of ROS by biotransformation and metabolic processes (Almeida *et al.*, 2020). CYP450 (Phase I) and GST (Phase II) enzymes exert an important role in the biotransformation process of CBZ (Almeida *et al.*, 2020). Consequently, these biotransformation enzymes have been widely studied in *M. galloprovincialis* acutely or chronically exposed to CBZ. Tsiaka *et al.* (2013) reported an increase in cell death and in the levels of superoxide anions and nitric oxides in *M. galloprovincialis* hemocytes exposed to CBZ (0.01 – 100 µg/L; 1 h exposure). Moreover, a reduction in the integrity of the lysosome membrane, an increase in lipid peroxidation (LPO) levels, and an increase of antioxidant enzymes activity were observed in *M. galloprovincialis* after 7 days of exposure to 0.1 and 10 µg/L of CBZ after (Martin-Diaz *et al.*, 2009). The induction was correlated with the alteration of physiological functions in the organisms, such as feeding behavior, growth, and survival (Almeida *et al.*, 2020). Additionally, mussels exposed over 96 h to CBZ 6.5 µg/L, showed an increased DNA damage and a decrease in lysosomal membrane stability (Baali and Cosio, 2022). Yet, the genotoxicity effects of CBZ should be further explored in a long-term exposure to the drug in marine bivalves

1.12. Justification of the work

As global human population grows, there is an increasing global demand for psychotropic drugs, such as CBZ, leading to their ubiquitous presence in marine ecosystems, under a complex

interplay of environmental and anthropogenic multiple stressors. Changes in seawater temperature and salinity may change pharmaceuticals' physical and chemical properties and the species' sensitivity to the drug, which may cause changes in the uptake, retention, and detoxification of CBZ by mussels (Almeida *et al.,* 2022).

To date, the toxicity of CBZ in combination with increased seawater temperature (Almeida *et al.,* 2021a; Arrigo *et al.,* 2024; Nardi *et al.,* 2022; Serra-Compte *et al.,* 2018) and salinity (Almeida *et al.,* 2022) in marine mussels have been scarcely investigated. There is a critical knowledge gap regarding the potential synergistic interactions between these abiotic factors on marine pollution effects, which is reflected in the lack of bi-directional communication between scientists and policy-makers in implementing environmental regulations and concentration thresholds that considers the unknown impacts of the interactions among stressors under a changing climate (Puri *et al.,* 2023). The present research addresses this gap by examining the combined effects of the drug exposure under a climate change scenario on the biochemical and genotoxic toxicity in *M. galloprovincialis*

1.13. Aim of the thesis

Among the most environmentally relevant pharmaceuticals, the antiepileptic carbamazepine is a marker of anthropogenic pollution widely detected in marine matrices worldwide. However, the effect of a single stressor (*e.g.,* CBZ) on a particular organism or process is different from the effect of multiple stressors due to the antagonistic or synergistic nature of interactions (*e.g.,* combined with climate change stressors). Thus, the present study questions whether the combined effects of CBZ exposure with a rise in temperature and salinity impact the biological responses of marine mussels *M. galloprovincialis*. For this reason, this study hypothesizes that the increased seawater temperature and salinity will synergistically influence the toxicity of CBZ in both tissues analyzed (gills and digestive glands), by increasing oxidative effects and exacerbating DNA damage in *M. galloprovincialis'* hemocytes over the time of exposure (28 days) to these multiple stressors.

Therefore, the main aim of the present thesis is to assess whether climate change stressors, namely increased temperature and salinity, may afford disturbances on the toxic effects of CBZ in marine mussels *Mytilus galloprovincialis.* Consequently, the specific objectives of this thesis are: (*i*) to investigate the biochemical effects of carbamazepine in marine mussels combined with increased temperature and salinity, by assessing changes in energy metabolism, antioxidant and biotransformation capacity, neurotoxicity, and oxidative

damage; and (*ii*) to assess the genotoxic potential of carbamazepine alone and combined with climate change-related stressors through the alkaline comet assay test.

The present results, discussion and conclusion of this M.Sc. thesis are part of an on-going scientific paper submission, entitled: “Climate change implications on carbamazepine toxicity in *Mytilus galloprovincialis*: Can mussels handle the combined effects?”, according to the requirements of the Science of the Total Environment Journal.

2. Material and methods

2.1. Experimental design

Mussels *Mytilus galloprovincialis* (mean length: 7.04 ± 0.33 cm; mean width: 3.31 ± 0.34 cm; mean height: 2.56 ± 0.21 cm) were collected from a coastal farm located in Sagres (West Southern Coast of Portugal) at depths between 10 and 18 m, in September 2023. Individuals (n = 300) were scrap-cleaned and transported in thermic boxes to the laboratory, where animals were allowed to acclimate over 7 days before the experiment. During acclimation, mussels were maintained in 25-L glass aquaria (2 organisms per L) containing natural seawater from Ria Formosa Coastal Lagoon (Faro, Southern Coast of Portugal) with 12:12 h photoperiod (light/dark), and under continuous aeration (salinity 36.48 ± 0.9; temperature 19.95 ± 1.0 °C; pH 7.41 ± 0.3 and oxygen saturation 95.65 ± 7.4) (Loureiro *et al.*, 2008).

Seawater was renewed every two days, followed by mussels feeding with microalgae *Tetraselmis chuii* (150.000 cells/animal/day), either during acclimation or exposure period. Mortality was daily checked both during the acclimation and exposure period. The natural environment conditions (17 °C and salinity 35) were used as a baseline compared to a climate change scenario based on the latest Intergovernmental Panel on Climate Change predicted scenario for 2100 (IPCC, 2023). After the acclimation period, the following experimental treatments were tested for 28 days: control (CTL; 0.0 µg/L CBZ) and carbamazepine (CBZ; 5.0 µg/L), both under two different environmental conditions (natural habitat 17 °C and salinity 35; predicted climate change 23 °C and salinity 40 (TS). The selection of the CBZ concentration tested in the present study was based on realistic global environmental levels of the drug reported in surface water and seawater (Gaw *et al.*, 2014; Hernández-Tenorio *et al.*, 2022; Ulvi *et al.*, 2022) as well as considering ecotoxicological bioassays conducted with bivalve mollusks (Almeida *et al.*, 2018; Mezzelani *et al.*, 2023; Moreno-González *et al.*, 2016; Nardi *et al.*, 2022; Oliveira *et al.*, 2017).

For the experimental assay, mussels were placed in glass aquaria, filled with 12.5 L of seawater (25 individuals per aquaria; 2 mussels per L), in a triplicate design (Fig. 1). Temperature and salinity of seawater from treatments simulating climate change were ensured through the preparation of a 100-L glass cistern containing seawater with the addition of commercial salt (Tropic Marin® SEA SALT) and warming with the aid of a thermostat (Eheim, 50 W). The cistern was allowed to equilibrate 24 h before every water renewal. In addition, temperature maintenance of 23 ºC was carried out by using thermostats (Eheim, 25 W) individually deployed in each test aquaria. The stock solution (CBZ) was prepared in ultrapure

Milli-Q water (10 mg/L). During the exposure period, the water was completely renewed every 48 h, and the CBZ concentration was re-established through pipetting of CBZ solution (5 µL). The CBZ analytical standard (CAS 298-46-4) was purchased from Acofarma (Spain).

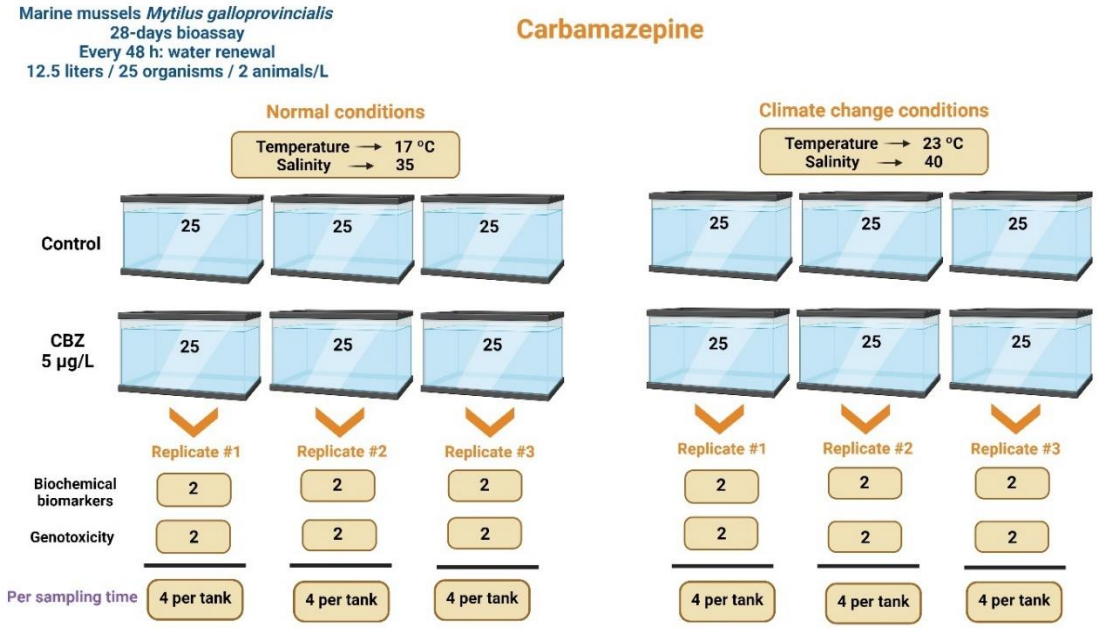

Figure 1. Experimental design of the ecotoxicological bioassay with *Mytilus galloprovincialis* unexposed (CTL) and exposed to CBZ (5 µg/L) under current (17 °C and salinity 35) and projected (23 °C and salinity 40) environmental conditions for 28 days. Created with Biorender.com.

Throughout the 28 days of the bioassay, aquaria were kept under constant aeration and controlled photoperiod. Physicochemical parameters were measured daily with the aid of a multiparametric probe (YSI, 556MPS) for current (salinity 35.84 ± 0.68, temperature 17.46 ± 0.30 °C, pH 7.95 ± 0.05 and oxygen saturation 99.84 ± 0.86) and predicted climate change (salinity 40.66 ± 0.26, temperature 23.02 ± 0.34 °C, pH 7.97 ± 0.07 and oxygen saturation 100.12 ± 0.95) conditions. Mussels were collected from the experimental setup on the 14th day (T14) and at the end of the exposure (28th day, T28) for condition index, biochemical and genotoxic analyses. Shell biometric measurements (length, width, height) were registered for the mussels applied for biochemical, genotoxic and the condition index (CI) (Gomes *et al.*, 2013) determination. Mussels from each treatment (6 mussels per tank; 18 per treatment) were individually weighed using the following ratio:

$$Condition\ index\ = \frac{\text{dry tissue weight (g)}}{\text{dry shell weight (g)}} \times 100$$

In each sampling time (T14 and T28), mussels were collected (2 mussels per aquarium; 6 mussels per treatment) followed by gills and digestive glands' dissection. Tissues were individually frozen in liquid nitrogen and stored at -80 °C for further biochemical analyses, as following: energy metabolism (electron transport system activity, ETS; total protein content, PROT; glycogen content, GLY); antioxidant capacity (the activity of the enzymes superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; and total antioxidant capacity, TAC); biotransformation activity (the activity of the enzymes glutathione-S-transferases, GSTs; carboxylesterase substrate p-nitrophenyl butyrate, CbEs-pNPB); cell membrane damage (lipid peroxidation levels, LPO); and neurotoxicity (acetylcholinesterase activity, AChE) (Fig. 2). The battery of biomarkers was assessed in aliquots of soft tissues from the same individuals. In addition, in each sampling time (T14 and T28) *M. galloprovincialis*' hemolymph (2 mussels per tank; 6 mussels per treatment) was individually extracted from the posterior adductor muscle using a sterile hypodermic syringe (2 mL), for determination of the DNA damage through the alkaline Comet assay (described in 2.2.8.).

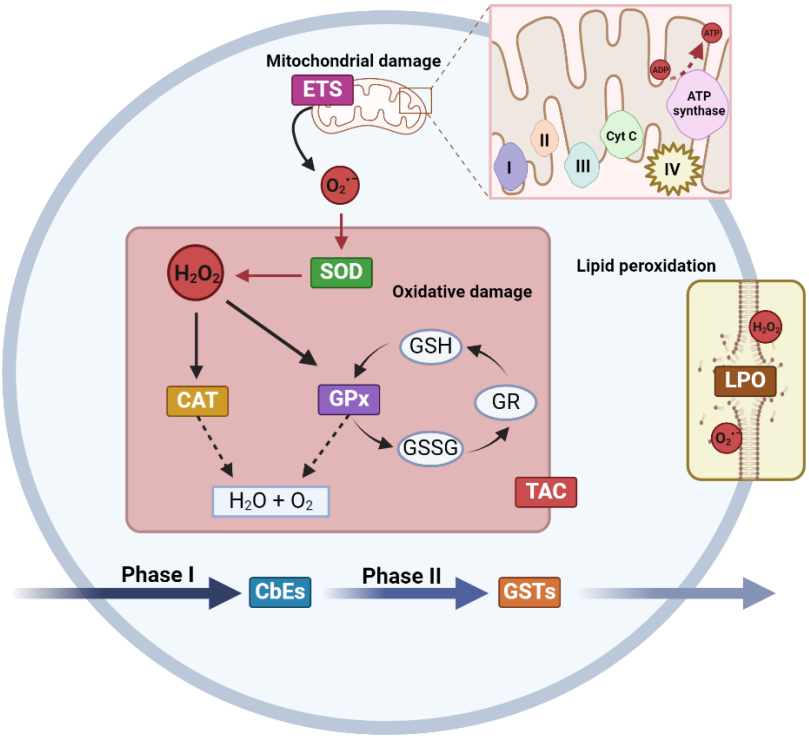

Figure 2. Multiple biomarkers analyzed in the present study: (1) energy metabolism (ETS); (2) antioxidant capacity (SOD, CAT, GPx, and TAC); (3) biotransformation activity (GSTs and CbEs); and cell membrane damage (LPO). Created with Biorender.com.

2.2. Biological responses

2.2.1. Tissue preparation

Gills and digestive glands were pooled (2 individuals per pool) and homogenized in specific buffers at a proportion of 1:3 and 1:5 (*w/v*) respectively, as described in Almeida *et al.* (2015, 2018). A potassium phosphate buffer (50 mM potassium phosphate, pH 7, EDTA 1mM, Triton X-100, 1% (v/v), DTT 1 mM) was used in the homogenization of tissues (0.66 g for gills and 0.40 g for digestive glands) aimed for the determination of PROT and GLY contents, antioxidant capacity, biotransformation and neurotoxicity activities, and LPO levels. Tissues prepared for ETS measurement (0.33 g for gills and 0.20 g, for digestive glands) were homogenized in a Tris-HCl buffer (Tris-HCl 0.1 mM with 15% (w/v) PVP, 153 mM magnesium sulfate ($MgSO_4$), 0.2% (v/v) Triton X-100 and pH 8.5). Samples were homogenized using TissueLyser II (Qiagen) for 90 s at a frequency of 20 1/s, followed by 20 min of centrifugation at 10,000 *g* (3,000 *g* for ETS activity), at 4 °C. Supernatants were individually stored at -80 °C for further biochemical analyses.

2.2.2. Metabolic capacity and energy reserves

The ETS activity was evaluated according to methods described by King Packard (1975) and De Coen & Janssen (1997) in which the absorbance was read at 490 nm, over 10 min, in a microplate reader (molar extinction coefficient (Ɛ) = 15.900 $M^{-1}cm^{-1}$), expressed as nmol/min/g fresh weight (FW) The protein content was determined following the Biuret method based on Robinson & Hogden (1940), using bovine serum albumin (BSA) as standard (0 – 40 mg/mL). The absorbance was read at 540 nm. The GLY content was quantified through the sulfuric acid method (Dubois *et al.,* 1956), using a glucose standard (0 – 5 mg/mL) and the absorbance was measured at 492 nm. Results are expressed as mg/g FW for PROT and GLY.

2.2.3. Antioxidant and biotransformation capacity

The activity of SOD (Cu – Zn) was measured based on the inhibition of pyrogallol autoxidation (Magnani *et al.,* 2000), in which the absorbance was read at 420 nm and the activity was expressed as units in units (U)/g FW, where one unit of enzyme activity indicates the inhibition of 50% of pyrogallol autoxidation. The activity of CAT was determined according to Johansson & Borg (1988), adapted by Carregosa *et al.* (2014), using standards of formaldehyde (0 – 150 µM) and the absorbance was measured at 540 nm. CAT activity was expressed as (U)/g FW, in which U is the amount of enzyme that catalyzes the formation of 1

nmol of formaldehyde per min. The activity of GPx was determined according to Paglia & Valentine (1967), at 340 nm ($\varepsilon = 6.22\ x\ 10^3\ mM^{-1}cm^{-1}$), expressed as (U)/g FW, where U represents the quantity of enzymes which catalyze the conversion of 1 nmol NADPH per min. The TAC was measured using ferric-reducing antioxidant power (FRAP) based on Benzie & Strain (1996), Capó *et al.* (2020) and Wootton-Beard *et al.* (2011). The absorbance was read at 593 nm, for 4 min, with intervals of 15 s, expressed as µmol/g FW.

The activity of GSTs was measured by the conjugation of 10 mM reduced glutathione (GSH) with 60 mM 1-Chloro-2, 4,dinitrobenzene (CDNB) ($\varepsilon = 9.6\ mM^{-1}cm^{-1}$) in a reaction mixture of 0.2 M KH_2PO_4/ K_2HPO_4 buffer (pH 7.9), at 340 nm (adapted from Habig *et al.*, 1974). The activity of GSTs was expressed in mmol/min/g. The activity of CbEs was measured at 405 nm, for 5 min, using pNPB (100 mM) as substrate, according to (Hosokawa, 2002; Solé *et al.*, 2018)The results are expressed in nmol/min/g FW for CbEs activity.

2.2.4. Lipid peroxidation

Levels of LPO were measured by the determination of thiobarbituric acid reactive substances (TBARS), according to Buege & Aust (1978). Absorbance was read at 532 nm ($\varepsilon = 1.56\ x\ 10^5\ M^{-1}cm^{-1}$). Malondialdehyde content (MDA) is presented as nmol MDA/g FW.

2.2.5. Neurotoxicity

The activity of AChE was determined according to Ellman *et al.* (1961) with modifications by Mennillo *et al.* (2017), using acetylthiocholine iodide (ATC, 5 mM) as a substrate. The absorbance of TNB (produced from the reaction between thiocholine and 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB)) was read at 412 nm for 5 min ($\varepsilon = 13.600\ M^{-1}cm^{-1}$). AChE activity is expressed as nmol/min/g FW.

2.2.6. Evaluation of the DNA damage using the Comet Assay test

The alkaline comet assay was based on the methodology described by Singh *et al.* (1988), with modifications for marine mussels *M. galloprovincialis* (Gomes *et al.*, 2013) (Fig. 3).

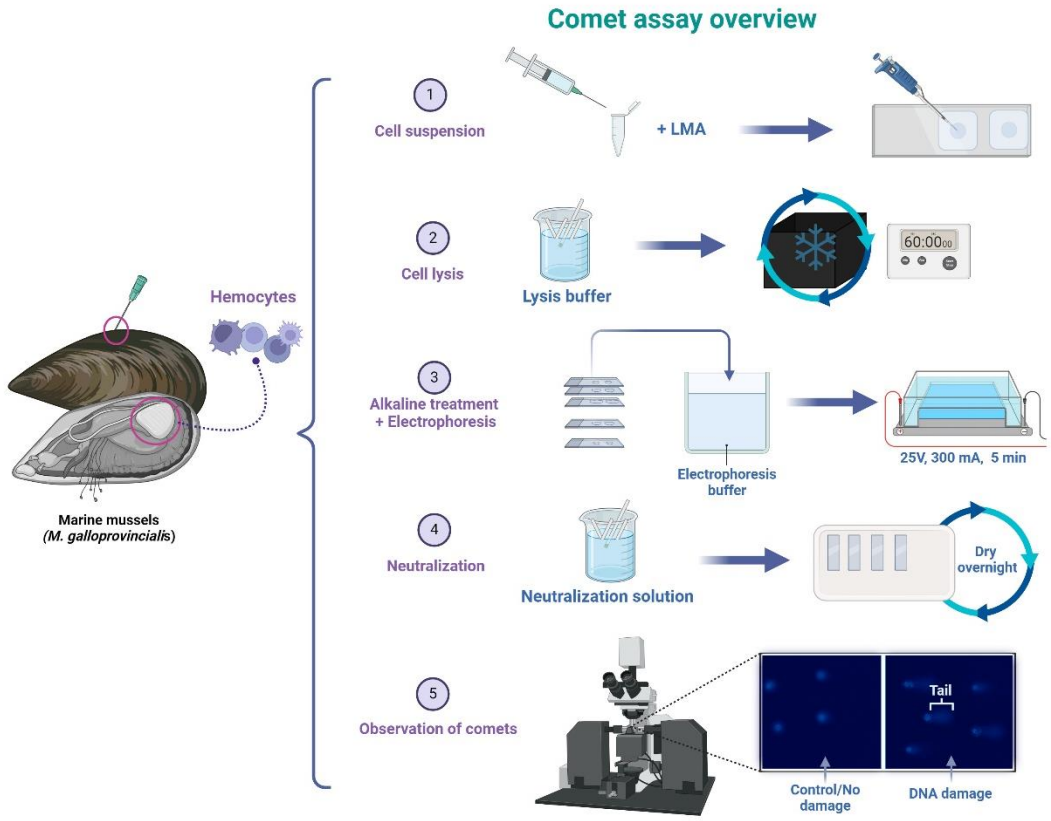

Figure 3. Comet assay test procedure adapted to *M. galloprovincialis*. Created with Biorender.com.

Hemolymph samples were centrifuged at 3,000 rpm for 3 min at 4 °C. The pellets were then suspended in 0.65% low melting point agarose (LMA) in Kenny's salt solution and cast over microscope slides submitted to a 1h lysis step. Electrophoresis was carried out for 5 min (25 V and 300 mA), followed by neutralization. For the evaluation of the DNA in the comet tail (tail DNA %), slides were stained with DAPI, and pictures were taken from 50 random cells from each slide (magnification of ×400) in an optical fluorescence microscope (Axiovert S100) coupled with a camera (Moticam 1080 HD, Motic). Scoring analysis was performed using Imaging Software Komet 7.1 (Kinetic Imaging Ltd). Results are expressed as mean tail DNA % ± STD.

2.3. Data and statistical analyses

Statistical analyses for biomarkers and genotoxicity data were performed using GraphPad Prism (version 10.2.3). Both data were tested for hypothesis considering the environmental conditions (current condition: 17 °C and salinity 35; and predicted condition: 23 °C and salinity 40), exposure to the pharmaceutical carbamazepine (CTL (0 µg/L) and CBZ 5.0

µg/L), and time (T14 and T28). Therefore, three null hypotheses were tested: *i*) for unexposed mussels, no significant differences exist between exposure time (T14 and T28) nor between environmental conditions (17 ℃ and salinity 35 vs. 23 ℃ and salinity 40); *ii*) in the presence of CBZ, no significant differences exist among exposure time (T14 and T28) neither environmental conditions (17 ℃ and salinity 35 vs. 23 ℃ and salinity 40); *iii*) for unexposed and CBZ-exposed mussels, there were no significant differences between the exposure periods (T14 and T28) and environmental conditions (17 ℃ and salinity 35 vs. 23 ℃ and salinity 40). Datasets from the biochemical (ETS, PROT, GLY, SOD, CAT, GST, GPx, CbEs-pNPB, TAC, AChE and LPO) and genotoxic responses were checked for normal distribution (Shapiro-Wilk test), followed by analysis of homogeneity of variances (Levene's test). The biochemical and genotoxic data were subjected to hypothesis testing through three-way ANOVA and the pairwise Tukey's post-hoc comparison tests was used to identify significant differences in mean among experimental treatments ($p < 0.05$). A Principal Component Analysis (PCA) was applied to explore correlations between variables (biochemical biomarkers and DNA damage) and to explain their variance (i) in both tissues (gills and digestive glands) of mussels exposed to CBZ or in combination with TS changes, over the bioassay (T14 and T28).

3. Results

3.1. Condition index

No statistically significant differences were found in the condition index over the exposure time ($p \leq 0.05$) and no mortality was detected over the 28-day exposure period.

3.2. Biological responses

3.2.1. Metabolic capacity and energy reserve content

Gills of mussels exposed to CBZ demonstrated, throughout the 28-day experiment, a metabolic capacity (ETS) similar to levels found in the CTL group ($p > 0.05$). On the other hand, at the end of the experiment, ETS activity was significantly induced in the gills of mussels exposed to TS+CBZ, with differences to the respective control at the end of the bioassay ($p < 0.05$; Fig. 4A). Moreover, mussels submitted to TS changes revealed a lower ETS compared to CTL, although not significant ($p > 0.05$). No significant differences were recorded in the ETS activity in the digestive glands of mussels among treatments and exposure periods ($p > 0.05$, Fig. 4B). Regarding the GLY content in the gills, no differences were observed within the CTL groups throughout the 28 days of exposure. However, mussels under TS changes revealed, on the 14th day, significantly lower levels of GLY compared to the respective CTL group ($p < 0.05$; Fig. 4C).

GLY content remained unchanged in the digestive glands of mussels ($p > 0.005$, Fig. 4 D). On the 28th day of exposure, gills from mussels exposed to CBZ presented a significantly lower PROT content compared to its respective control treatment ($p < 0.005$; Fig. 4E). In the digestive glands, mussels from the control groups exhibited, on the 28th day, a significant decrease in the PROT content compared to the 14th day ($p < 0.005$; Fig. 4F). After 14 days of exposure, a significant reduction of the PROT content was observed between CTL and CBZ, and CTL and TS+CBZ treatments. In addition, at the end of the exposure period, the PROT content in the CTL mussels showcased a significant difference with the TS and TS+CBZ treatments.

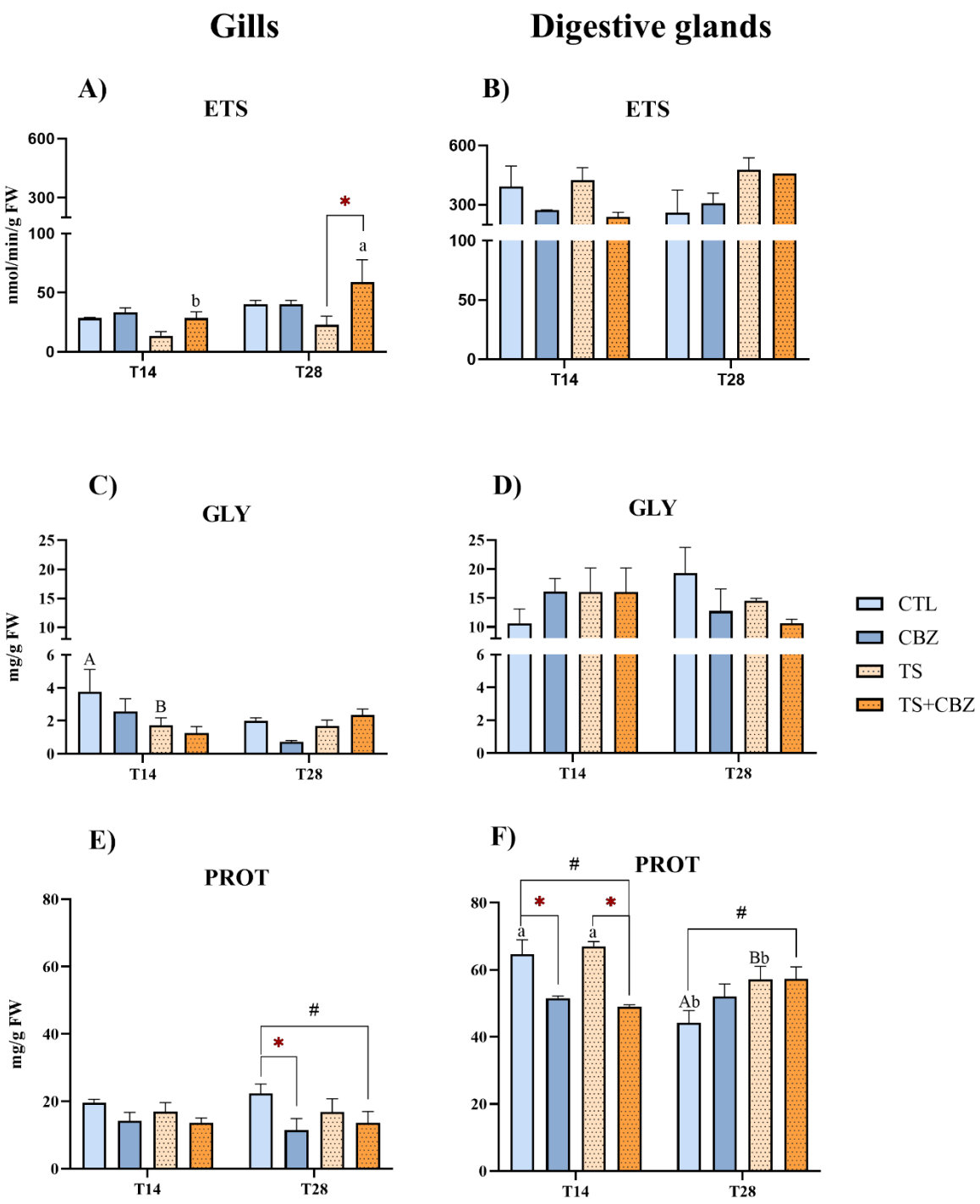

Figure 4. Biochemical biomarkers of energy metabolism in gills (A, C and E) and digestive glands (B, D and F) of *Mytilus galloprovincialis* unexposed (CTL) and exposed to CBZ (5 µg/L) under current (17 °C and salinity 35) and projected (23 °C and salinity 40; TS) environmental conditions, for 28 days. Asterisks represent statistically significant differences between CTL and CBZ (within the same time and same environmental condition). Different capital letters indicate significant differences between the same groups (CTL or CBZ), within the same exposure time (T14 or T28), under different environmental conditions (current vs.

TS). Different lowercase letters showcase the significant differences in the same groups (CTL or CBZ), between different exposure times (T14 vs. T28), under the same environmental condition (current vs. TS).

3.2.2. Antioxidant capacity

No significant differences were observed between control treatments in both tissues analyzed. SOD activity was significantly induced in mussels' gills of every CBZ and TS+CBZ treatments compared to their respective control ($p < 0.05$; Fig. 5A) at the 14th and 28th days of exposure. Regarding digestive glands, a sharp and significant increase was addressed on the 14*th* day in CTL specimens ($p < 0.05$; Fig. 5B). Throughout the 28-day experiment, CBZ-exposed mussels (CBZ and CBZ+TS) indicated a trend of lower SOD activity compared to individuals from CTL. The activity of CAT measured in gills of control mussels showed an increase over time, leading to a significant difference between T14 and T28 ($p < 0.05$; Fig. 5C). At the 14th day, gills of CBZ-exposed mussels revealed a significant increase ($p < 0.05$) in this enzyme activity compared to its respective control. On the other hand, for digestive glands, no statistically significant changes were observed in CAT activity ($p > 0.05$; Fig. 5D).
The activity of GPx determined in the gills of mussels under TS changes revealed, on the 14th day, a sharp and significant increase compared to CTL ($p < 0.05$; Fig. 5E;), which also afforded a significant difference when compared to TS+CBZ-exposed mussels ($p < 0.05$).

Over the 28-day bioassay, a trend of decrease in the GPx activity was registered for mussels under TS changes ($p < 0.05$; Fig. 5E). A statistically significant decrease was addressed in the GPx activity in the gills of CBZ-treated mussels between exposure times (T14 vs. T28). On the 28th day, the gills of CBZ-exposed mussels showed significantly lower GPx activity compared to levels reported in CTL individuals. In the digestive glands, a significant increase was observed between CBZ-treated mussels over time (T14 vs T28), disregarding the changes in TS ($p < 0.05$; Fig. 5F). Moreover, a significant rise in the GPx activity was observed between CTL and TS+CBZ-exposed mussels on the 28th day. TAC in gills of CTL showcased a slight increase over time, affording a significant difference between days 14th and 28th, as well as a difference between CTL and CBZ within the last day of the bioassay ($p < 0.05$; Fig. 5G). For digestive glands, no statistically significant differences were observed in TAC activity ($p > 0.05$; Fig. 5H).

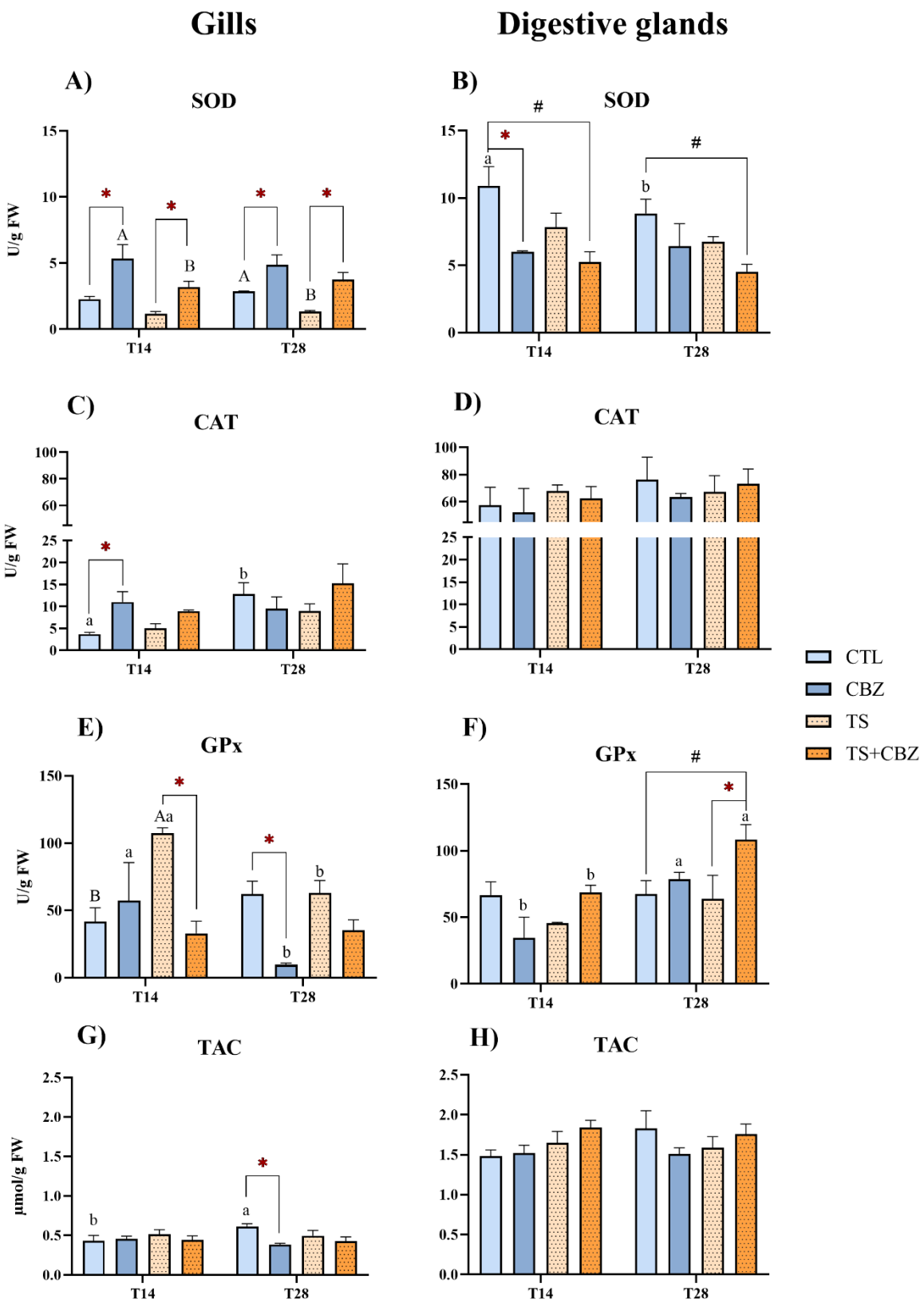

Figure 5. Biochemical biomarkers of antioxidant activities in gills and digestive glands of *Mytilus galloprovincialis* unexposed (CTL) and exposed to CBZ (5 µg/L) under current (17 °C and salinity 35) and projected (23 °C and salinity 40; TS) environmental conditions, for 28

days. Asterisks represent statistically significant differences between CTL and CBZ (within the same time and same environmental condition). Different capital letters indicate significant differences between the same groups (CTL or CBZ), within the same exposure time (T14 or T28), under different environmental conditions (current vs. TS). Different lowercase letters showcase the significant differences in the same groups (CTL or CBZ), between different exposure times (T14 vs. T28), and under the same environmental condition (current vs. TS).

3.2.3. Biotransformation activity

At the end of the bioassay, the gills of CBZ-treated mussels revealed a significantly lower activity of CbEs compared to the control group ($p < 0.05$; Fig. 6A). Overall, no statistically significant differences in CbEs activity were found in the mussels' digestive glands ($p > 0.05$; Fig. 6B). Similarly, GSTs activity was significantly lower among unexposed and CBZ-exposed mussels gills at the end of the bioassay (T28) ($p < 0.05$; Fig. 6C). On the 14th day, the activity of these enzymes in TS+CBZ treatment in the gills was significantly different from organisms under control environmental conditions ($p < 0.05$; Fig. 6C). Mussels submitted to CBZ and TS+CBZ experienced a significant reduction in GSTs, on the 14th day ($p < 0.05$; Fig. 6C). In contrast, digestive glands revealed that GSTs significantly increased over time in TS+CBZ treated mussels (23 °C and salinity 40) ($p > 0.05$; Fig. 6D). Additionally, at the end of the exposure period, a significant increase in the GST activity was observed among unexposed (TS changes only) and TS+CBZ-exposed mussels ($p > 0.05$; Fig. 6D).

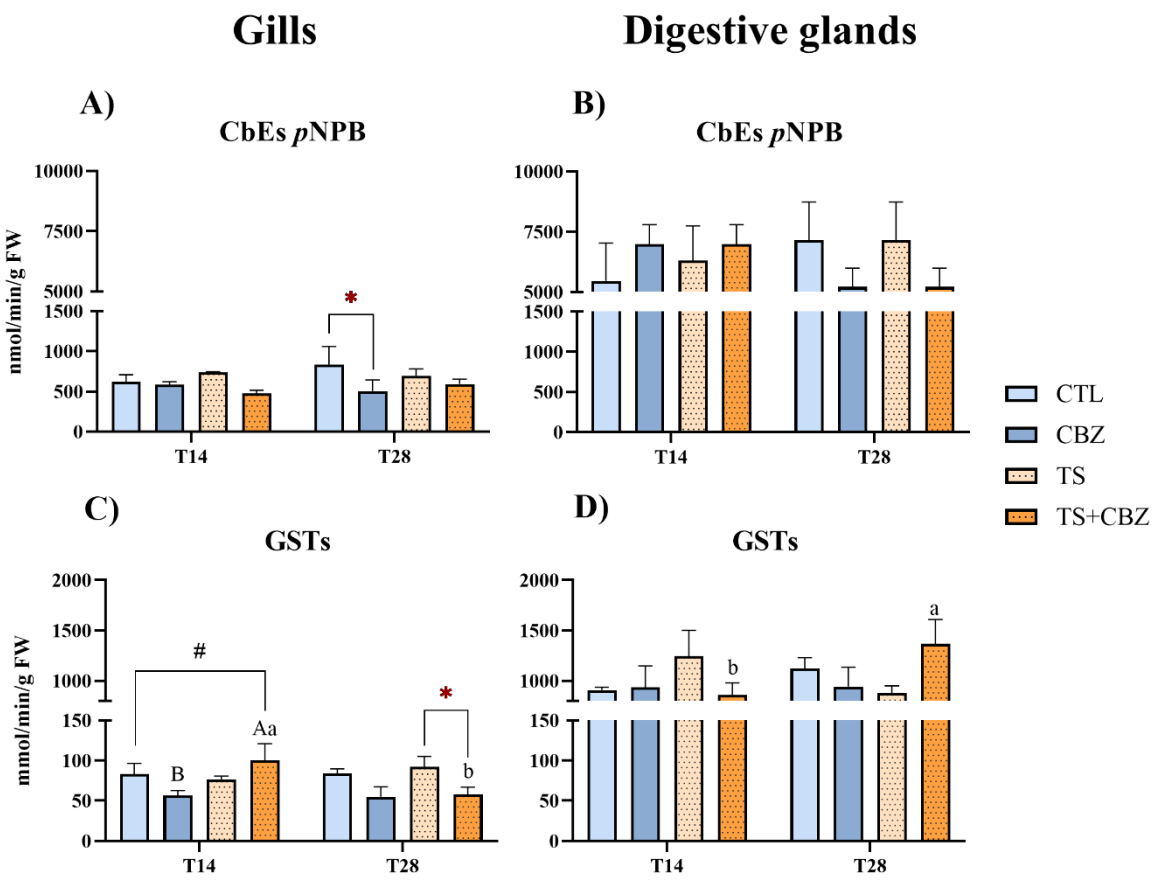

Figure 6. Biochemical biomarkers of biotransformation enzymes in gills (A, C, E and G) and digestive glands (B, D, F and H) of *Mytilus galloprovincialis* unexposed (CTL) and exposed to CBZ (5 µg/L) under current (17 °C and salinity 35) and projected (23 °C and salinity 40; TS) environmental conditions, for 28 days. Asterisks represent statistically significant differences between CTL and CBZ (within the same time and same environmental condition). Different capital letters indicate significant differences between the same groups (CTL or CBZ), within the same exposure time (T14 or T28), under different environmental conditions (current vs. TS). Different lowercase letters showcase the significant differences in the same groups (CTL or CBZ), between different exposure times (T14 vs. T28), and under the same environmental condition (current vs. TS).

3.2.4. Lipid peroxidation and genotoxicity

Levels of LPO by-products in gills did not change throughout the 28 days of a bioassay for any of the treatments ($p < 0.05$; Fig. 7A). In contrast, results obtained for digestive glands, on the 14th day, clearly depicted lower levels of LPO in CBZ-exposed groups compared to control treatment, either for those maintained at unaltered or altered-TS conditions (17 °C and

salinity 35 vs. 23 °C and salinity 40). In addition, levels of LPO by-products were significantly different between control groups under both environmental conditions (17 °C and salinity 35 vs. 23 °C and salinity 40) ($p < 0.05$; Fig. 7B).

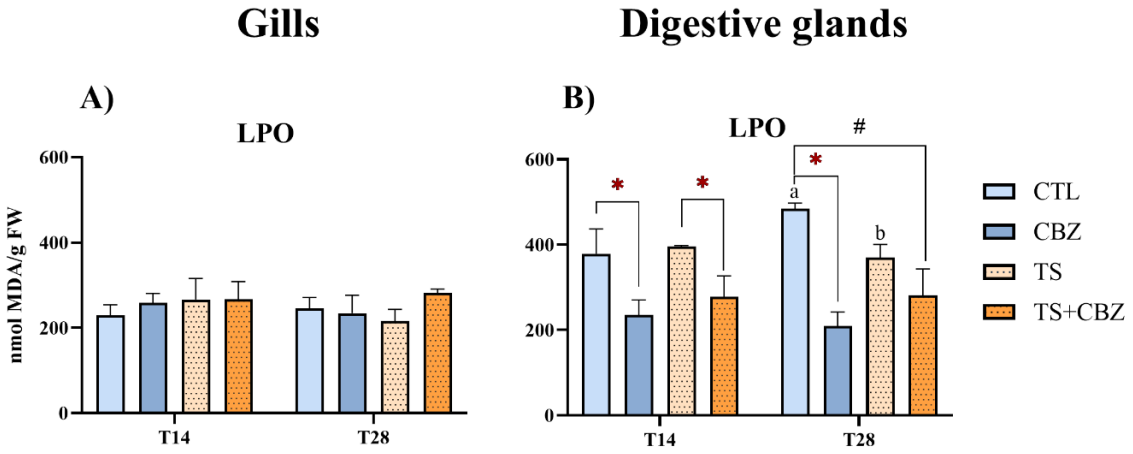

Figure 7. Biochemical biomarkers of lipid peroxidation in gills (A) and digestive glands (B) of *Mytilus galloprovincialis* unexposed (CTL) and exposed to CBZ (5 µg/L) under current (17 °C and salinity 35) and projected (23 °C and salinity 40; TS) environmental conditions, for 28 days. Asterisks represent statistically significant differences between CTL and CBZ (within the same time and same environmental condition). Different capital letters indicate significant differences between the same groups (CTL or CBZ), within the same exposure time (T14 or T28), under different environmental conditions (current vs. TS). Different lowercase letters showcase the significant differences in the same groups (CTL or CBZ), between different exposure times (T14 vs. T28), and under the same environmental condition (current vs. TS).

A higher percentage of DNA in the tail was observed in CBZ-exposed treatments throughout the bioassay, whereby, at the end of the exposure, individuals under a multiple stress condition (TS+CBZ) indicated a significant difference when compared to the respective control ($p < 0.05$; Fig. 8).

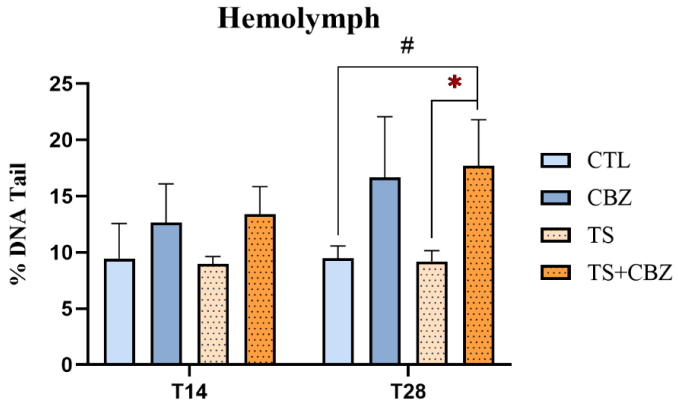

Figure 8. DNA damage measured as % DNA tail in the hemolymph of *Mytilus galloprovincialis* unexposed (CTL) and exposed to CBZ (5 µg/L) under current (17 °C and salinity 35) and projected (23 °C and salinity 40; TS) environmental conditions, for 28 days. Asterisks represent statistically significant differences between CTL and CBZ (within the same time and same environmental condition). Different capital letters indicate significant differences between the same groups (CTL or CBZ), within the same exposure time (T14 or T28), under different environmental conditions (current vs. TS). Different lowercase letters showcase the significant differences in the same groups (CTL or CBZ), between different exposure times (T14 vs. T28), and under the same environmental condition (current vs. TS).

3.2.5. Neurotoxicity

On the 14th day, mussels exposed to CBZ indicated a significant increase in AChE activity in gills compared to the control group, followed by its reduction on the last day of the experiment, which afforded significant differences with the respective control and with its time counterpart ($p < 0.05$; Figure 9A). Moreover, AChE activity in gills also demonstrated a significant difference among control groups exposed to unaltered and altered-TS conditions (17 °C and salinity 35 *vs*. 23 °C and salinity 40) at the 14th day of exposure. Similarly, CBZ-treated mussels showed statistically significant differences among environmental conditions (17 °C and salinity 35 *vs*. 23 °C and salinity 40) at the end of the exposure period (T28) ($p < 0.05$; Figure 9A). Regarding digestive glands, a high AChE activity was detected in TS+CBZ exposed mussels, on the 14th day, which afforded significant differences with its respective control group and with CBZ-exposed mussels. However, such enzyme activity significantly decreased over time ($p < 0.05$; Figure 9B) reaching levels similar to the control treatment.

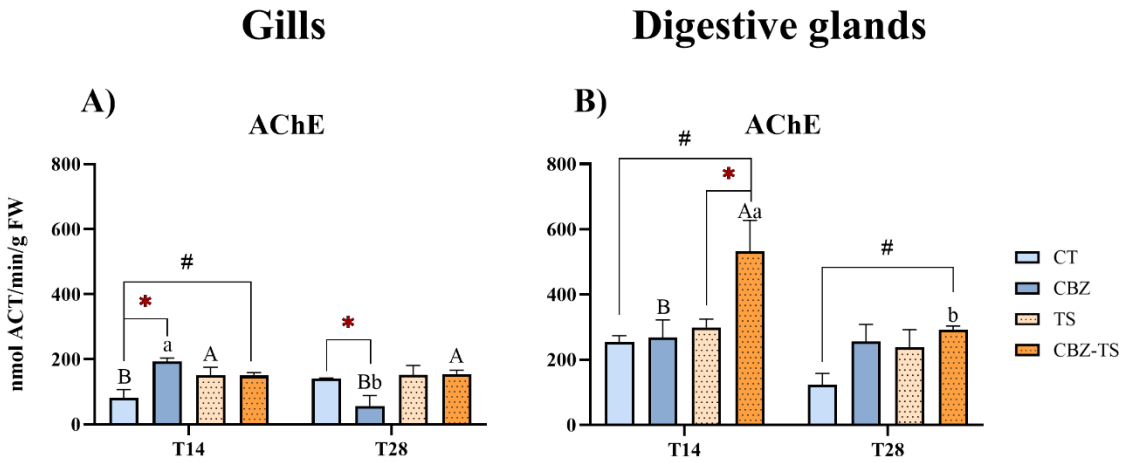

Figure 9. Biochemical biomarkers of neurotoxicity activity in gills (A) and digestive glands (B) of *Mytilus galloprovincialis* unexposed (CTL) and exposed to CBZ (5 µg/L) under current (17 °C and salinity 35) and projected (23 °C and salinity 40; TS) environmental conditions, for 28 days. Asterisks represent statistically significant differences between CTL and CBZ (within the same time and same environmental condition). Different capital letters indicate significant differences between the same groups (CTL or CBZ), within the same exposure time (T14 or T28), under different environmental conditions (current vs. TS). Different lowercase letters showcase the significant differences in the same groups (CTL or CBZ), between different exposure times (T14 vs. T28), under the same environmental condition (current vs. TS).

3.2.6. Principal Component Analysis

Variables analyzed consisted of results obtained from the biochemical biomarkers (ETS, GLY, PROT, SOD, CAT, GPx, TAC, CbEs, GSTs, AChE and LPO) in both gills (Fig. 10A) and digestive glands (Fig. 10B) and the genotoxicity data (% DNA tail) from the Comet Assay performed in the mussels' hemolymph (Fig. 10A-B). Regarding mussels' gills, the principal components (PCs) obtained corresponded to 65.50% of the total variance (PC1= 44.65% and PC2= 20.85%). Correlation coefficients were considered significant when higher than $\sqrt{(d/n)}$ (*i.e.*, *d* is the number of principal components; *n* is the number of variables), equal to 0.5 in the present data analysis.

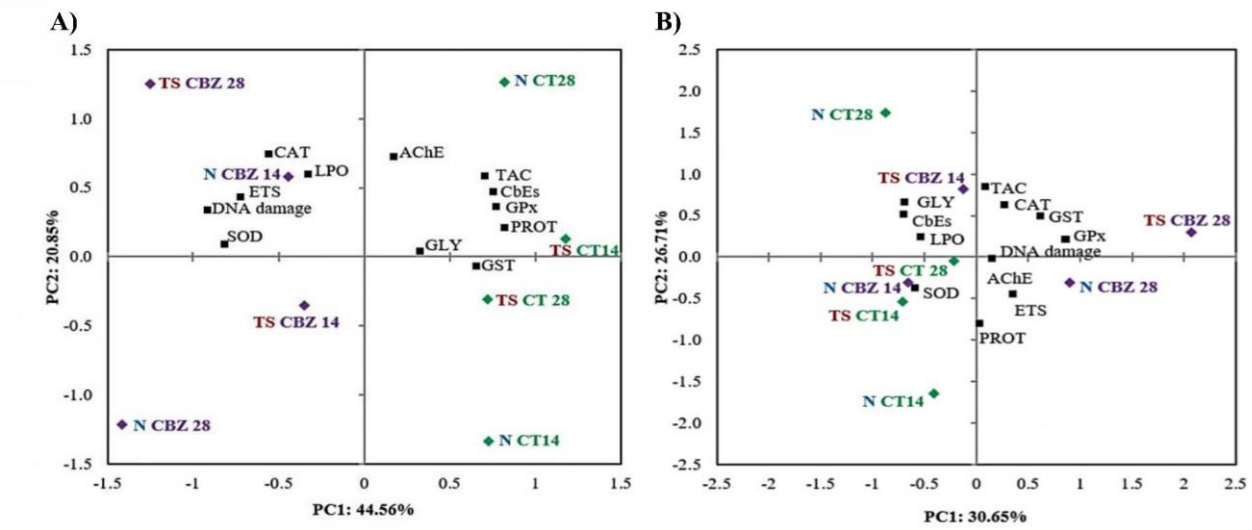

Figure 10. Principal component analysis (PCA) of a battery of biomarkers (ETS, GLY, PROT, SOD, CAT, GPx, TAC, CbEs, GST, AChE and LPO) in the gills (A) and digestive glands (B), and DNA damage (Comet Assay) in the hemolymph of *Mytilus galloprovincialis* unexposed (CTL) and exposed to CBZ (5 µg/L) under current (17 °C and salinity 35; N) and projected (23 °C and salinity 40; TS) environmental conditions, for 28 days ($p < 0.05$). PCA was performed for PC1 vs. PC2; Green and purple symbols represent the CTL and CBZ groups, respectively. The blue color corresponds to unaltered condition (N), while red color represents increased seawater temperature and salinity condition (TS).

In this sense, PC1 presented a positive significant correlation among PROT, CbEs, GSTs, TAC and GPX, whereas ETS, SOD, CAT and DNA damage were significantly negatively correlated (Fig. 10A). In PC2, LPO, TAC, CAT and AChE presented a significant positive correlation (Fig. 10A). Contrasting results were seen in the digestive glands (Fig. 10B), in which the PCs corresponded to 30.65 % and 26.71 % for PC1 and PC2, respectively. PC1 showcased fewer positive relations, including GSTs, GPx and DNA damage, while significantly negative relations were observed for GLY, CbEs, SOD and LPO (Fig. 10B). Meanwhile, PC2 had more positive relationships between GLY, CbEs, TAC and CAT, whereas only PROT was significantly negatively related (Fig. 10B).

4. Discussion

Psychotropic drugs, such as the antiepileptic CBZ, are widely detected up to tens of µg/L in the seawater column in different regions around the globe (Gaw *et al.*, 2014; Hernández-Tenorio *et al.*, 2022; Ulvi *et al.*, 2022). Environmental concerns are then rising since the global psychotropic drugs market size is expected to reach US$ 28.8 million by 2033, compared to US$ 21.2 million in 2023 (FMI, 2024) along with their low removal efficiency in WWTPs (up to 20 %) (Ulvi *et al.*, 2022; Zhang *et al.*, 2008). Previous ecotoxicological studies have confirmed significant biological alterations in marine bivalves exposed to CBZ, with changes in energy-related parameters, oxidative stress, changes in the lysosomal membrane, cell damage and DNA damage (Abdelhafidh *et al.*, 2018; Almeida *et al.*, 2021b; Baali & Cosio, 2022; Brandts *et al.*, 2018; Dumas *et al.*, 2022; Juhel *et al.*, 2017; Oliveira *et al.*, 2017). Alongside, foreseen changes in climate may exacerbate the interplay of multiple stressors in marine ecosystems through increased seawater temperature and salinity fluctuations, leading to changes in the chemical and physical properties of pharmaceuticals (Bethke *et al.*, 2023; Puckowski *et al.*, 2016), like CBZ, and the impacts on organisms' health condition (Almeida *et al.*, 2021a, 2022c; Bethke *et al.*, 2023; Correia *et al.*, 2016; Nardi *et al.*, 2022; Serra-Compte *et al.*, 2018; Velez *et al.*, 2016). Therefore, the present study provides important insights into the toxicity of CBZ in combination with seawater temperature and salinity increase in marine mussels, particularly *M. galloprovincialis*.

The results herein obtained from the multivariate analysis (PCA) showed contrasting responses among organs of mussels exposed to CBZ under different environmental conditions over time. For gills, the PC1 revealed a clear distinction among CBZ-treated and control groups, disregarding temperature and salinity to which these groups were exposed over time. Accordingly, most significant PCA responses shed light on the tissue-specific responses in mussels, in which the gills activated the metabolic capacity (ETS) and antioxidant enzymes (SOD and CAT) as a defense mechanism. While the digestive glands activated energy reserve (GLY) to prompt different defense mechanisms, such as antioxidant (SOD) and biotransformation (CbEs) enzymes, to suppress lipid peroxidation (LPO) caused solely by CBZ exposure. Moreover, a clear separation among the biological responses was detected between control and CBZ-exposed mussels after 28 days, disregarding TS conditions, in the digestive glands. Therefore, the biomarkers were shown to be modulated by the time of exposure (T14 vs. T28), suggesting a time-specific response in mussels exposed to either a single (CBZ or TS changes) or multiple stressors (TS+CBZ).

4.1. Metabolic capacity and energy reserves

Energy metabolism is considered one of the essential parameters for understanding the overall health and energy needed by organisms (De Coen & Janssen, 1997). Findings obtained herein demonstrated that the exposure to 5 µg/L CBZ alone did not interfere with the metabolic capacity (ETS) in the gills and digestive glands of marine mussels. In contrast to the present results, Oliveira *et al.* (2017) reported a reduction in the mitochondrial ETS activity in mussels *M. galloprovincialis* submitted to CBZ (0.3, 3.0, 6.0 and 9.0 µg/L) over a long-term (28 days) period, suggesting such mechanisms as a strategy to suppress energy expenditure and prevent potential cellular damages. However, it is important to underline that such results were addressed in the whole soft tissues of mussels, revealing a different pattern in the ETS activity according to the tissues herein selected for the metabolic analyses.

Climate change stressors, such as warming and salinity fluctuations, in combination with pollutants, may directly or indirectly affect the cellular respiration rate and oxygen consumption processes of marine bivalves, consequently impacting the activity of the ETS (Almeida *et al.*, 2021a, 2022; Arrigo *et al.*, 2024; Costa *et al.*, 2020a, 2020b; De Marchi *et al.*, 2020; Freitas *et al.*, 2019a, 2019b, 2020a, 2020b; Lopes *et al.*, 2022; Maynou *et al.*, 2021). When submitted to salinity 40 and temperature 23 ºC (TS treatment), gills from non-contaminated individuals indicated a slight reduction in the ETS activity, as a potential mechanism of osmotic adjustment (Almeida *et al.*, 2022). In accordance, previous studies conducted with marine clams *R. decussatus* (Velez *et al.*, 2016) and *R. phillipinarum* (Almeida *et al.*, 2022), and mussels *M. galloprovincialis* (Freitas *et al.*, 2017) demonstrated a similar pattern of decrease in the ETS activity with the increase of salinity levels (14 to 35).

Mussels exposed to TS+CBZ, at the end of the 28-day bioassay, revealed an increase of ETS activity in their gills, suggesting an attempt to boost defense mechanisms under increased toxicity of CBZ due to the interaction with variations in abiotic conditions. Similarly, Almeida *et al.* (2022) reported that clams *R. philippinarum* exposed to the combination of CBZ (1 µg/L) and cetirizine (0.6 µg/L) at higher salinity (35 in comparison with 25) had a significantly higher ETS activity than clams exposed to only CBZ over 28 days. Costa *et al.* (2020a) also observed an enhancement of ETS levels in *R. philippinarum* submitted to triclosan in combination with warming (17 °C to 21 °C) and pH changes (8.1 to 7.7) over 7 days. Therefore, under salinity fluctuations, bivalves experience valve closure and sealing as a vital response to protect the organism from osmotic stress, by separating the internal fluid from the external environment (Pourmozaffar *et al.*, 2020). However, such a strategy may also inhibit soft tissue and shell production. For example, mussels *M. galloprovincialis*, oysters *Cassostrea corteziensis* and

clams *Pinctada maxima* displayed strong reduction in oxygen consumption, energy allocation and survival rates with increasing salinity, demonstrating that osmotic costs in bivalve species may disturb critical physiological processes (Erk *et al.,* 2011). Low oxygen in cells may activate several metabolic pathways of ATP production that are less effective than oxidative phosphorylation and osmoregulation, resulting in rapidly consumed GLY reserves (Solaini *et al.,* 2010). In this sense, seawater temperature and salinity increase may enhance mussels' vulnerability to cope with stressors, exacerbating CBZ effects in these organisms, such as the accumulation of the drug in the lysosomes, as observed by Dumas *et al.* (2022), Martin-Diaz *et al.,* (2009), Nardi *et al.* (2022) and Oliveira *et al.* (2017). Therefore, climate change stressors, namely ocean warming and salinity shifts, can exert negative impacts on the metabolic capacity of organisms (*e.g.,* additional energetic costs), consequently influencing the mussels' ability to cope with other stressors like pharmaceuticals (Maulvault *et al.,* 2019).

Regarding GLY levels, no alteration was observed over the exposure time in the gills and digestive glands of mussels exposed to CBZ, regardless TS changes. Thus, the present findings indicate that CBZ solely may induce metabolic depression by resorting to glucose usage to cope with stressful conditions. Likewise, Almeida *et al.* (2014) demonstrated that different clam species *R. philippinarum* and *R. decussata* exposed to CBZ (0.03, 0.3, 3.0, 6.0 and 9.0 ug/L; 96 h) did not present alterations in GLY levels, supporting the hypothesis that concomitantly reduced oxygen consumption rate decreases energy consumption in bivalves (Solaini *et al.,* 2010). In addition, Almeida *et al.* (2015) and Oliveira *et al.* (2017) reported that clams and mussels, respectively, may change their defense strategy to resort to GLY usage when exposed to CBZ (0.03, 0.3, 3.0, 6.0, 9.0 µg/L) in acute (96 h) and chronic (28 days) bioassays.

The findings herein obtained showed that the exposure to multiple stressors did not exert additional effects on the PROT content in the gills and digestive glands of mussels, compared to the effects caused by CBZ solely. In contrast to the present findings, Almeida *et al.* (2021a) observed that the temperature rise (from 17 to 21 ºC) could hinder the effects of CBZ (1 µg/L) and CTZ (0.6 µg/L) on the PROT contents in *R. philippinarum*. Previous studies observed a reduction (Costa *et al.,* 2020a, 2020b) or increase (Freitas *et al.,* 2019a; Lopes *et al.,* 2022) in the PROT reserve of clams (*R. decussatus* and *R. philippinarum*) and mussels (*M. galloprovincialis*) exposed to the combination of pharmaceutical drugs with climate change scenario (temperature or salinity variation). Thereby, responses vary when mussels are exposed to multiple stressors (*e.g.,* abiotic changes and contaminants) to fuel up their defense mechanisms (Maulvault *et al.,* 2019).

However, in the present study, the authors hypothesize that CBZ was the only determining factor in the depletion of mussels' PROT levels at the beginning of the bioassay. By the end of the bioassay, the PROT levels were mostly affected by multiple stressors (TS and TS+CBZ), thus exposure time may influence the activation of defense mechanisms in response to stressors (*e.g.*, abiotic changes and pharmaceutical exposure). CBZ is a prodrug that requires the primary metabolic activation by the superfamily of cytochrome P450 enzymes (*e.g.*, CYP3A4 and CYP2C8) to form its bioactive metabolite CBZE, which electrophilic nature may afford interactions with proteins (Puranik *et al.*, 2013). The electrophilic binding of CBZE with proteins occurs due to the reactive properties of the metabolite that covalently attaches to the nucleophilic regions within proteins (Fuhr *et al.*, 2021), generating protein adducts (Bu *et al.*, 2005). The present results may indicate the capability of mussels to metabolize CBZ by CYP450 enzymes and the subsequent formation of CBZE, in which the presence of various metabolites (*i.e.*, CBZE, 3-hydroxycarbamazepine, and 10-hydroxy-10,11-dihydro-carbamazepine) was referred previously in marine bivalves (Abdelhafidh *et al.*, 2018; Boillot *et al.*, 2015).

4.2. Antioxidant capacity

To counteract the impact of ROS caused by the uptake and metabolization of CBZ and other stressors, organisms may undergo disturbances in their antioxidant system (Tsiaka *et al.*, 2013; Vernouillet *et al.*, 2010). In the present study, CBZ strongly induced SOD activity in the gills of mussels, regardless the changes in salinity and temperature, showcasing mussels' physiological adaptation to cope with stressful conditions through a prolonged valve closure effectively reducing the drug intake (Gosling, 2003). According to the present findings, it is hypothesized that the potential CBZ activation and generation of CBZE metabolite may lead to the induction of SOD activity in the gills, which indicates a greater demand for superoxide anion ($O_2^{-\cdot}$) reduction to hydrogen peroxide (H_2O_2) to prevent harmful effects of ROS.

In contrast to the present findings, Freitas *et al.* (2020a, 2020b) addressed an enhanced SOD activity in *M. galloprovincialis* exposed to salicylic acid (4 mg/L, 28 days) under warming (21 °C) or high salinity (35 in comparison to 25), respectively, whereas Arrigo *et al.* (2024) identified that water warming only (21 °C) affects more *M. galloprovincialis* than diclofenac (1 µg/L, 21 days) or the combined scenario. Thus, alterations in SOD levels vary according to the combination of multiple stressors (*e.g.*, warming and a specific pharmaceutical compound), according to the drug tested, its pharmacological properties and the selected tissue for analytical purposes. Marine bivalves may showcase different metabolic responses when exposed to

increased or decreased temperature and salinity levels, thus these organisms may not have a single response pattern under a multiple-stressor scenario (Almeida *et al.*, 2021a, 2022; Freitas *et al.*, 2019a, 2019b; Velez *et al.*, 2016). Thereby, various studies demonstrated that warming and salinity fluctuations *per se* or in combination with pharmaceuticals strongly modulate SOD levels in marine bivalves (Carregosa *et al.*, 2014; Correia *et al.*, 2016; Freitas *et al.*, 2019a). Alongside this, the current analyses were performed on specific tissues in mussels, whereas previous research examined whole bivalve tissue, which may also explain variations in results regarding SOD levels (Almeida *et al.*, 2021a, 2022; Freitas *et al.*, 2020b).

In the present findings, CAT activity was higher in the gills of CBZ-treated mussels at the 14th day. Although an absence of response was observed in the digestive glands over exposure time. Similarly, Almeida *et al.* (2015) did not observe changes in CAT activity of clams *R. philippinarum* exposed to CBZ (0.0 – 9.0 µg/L, whole tissue) over 28 days of exposure time (except lower CAT at CBZ 0.03 µg/L). While Martin-Diaz *et al.* (2009) detected a significant increase in the digestive glands, mantle and gonads of mussels *M. galloprovincialis* exposed to CBZ (10 µg/L, 7 days). Marine mussels exposed to other drugs demonstrate an inhibition (Franzellitti *et al.*, 2011) or induction (Canesi *et al.*, 2007) of their CAT activity due to the drug specificity, concentration and tissue tested. CAT decomposes the H_2O_2 produced by SOD into water and oxygen (Regoli & Giuliani, 2014). Thereby, it may be hypothesized the activation of GPx activity as the route for hydrogen peroxide elimination. In corroboration with the present findings, Freitas *et al.* (2020a) reported higher SOD activity and lower CAT levels in *M. galloprovincialis* exposed to salicylic acid (4 mg/L, 28 days). Therefore, lower CAT activity may be attributed to an inhibitory effect induced by the excess of H_2O_2 produced by the higher SOD activity as a compensation mechanism of response between SOD and CAT activities (Gonzalez-Rey & Bebianno, 2013, 2014). Additionally, mussels exposed to TS+CBZ had an increase in their CAT activity over the exposure time potentially suggesting a delayed demand of H_2O_2 detoxification (Regoli & Giuliani, 2014), which reflects CBZ metabolism that promotes an intracellular formation of ROS (Mezzelani *et al.*, 2021). Similar results were observed by Nardi *et al.* (2022) on *M. galloprovincialis* exposed to CBZ (1 µg/L) in combination with marine heat waves (22.5 °C, 20 days).

The enzyme GPx also plays a key role in reducing H_2O_2 and other peroxides. In the present study, an induction of GPx activity by TS changes was observed in the gills, followed by a decrease over exposure time. The present findings indicate that TS changes *per se* activate GPx to avoid further damage and eliminate excessive ROS. Therefore, these abiotic stressors can exacerbate cellular oxidative stress on marine mussels (Abele, 2002). Likewise, Freitas *et*

al. (2020a) and Nardi *et al.* (2022) reported, respectively, significant induction of GPx activity when non-contaminated mussels *M. galloprovincialis* were exposed to warming (21 °C; 28 days), and to a marine heat wave (22.5 ºC; 20 days). Additionally, previous studies demonstrated that exposure to multiple stressors, such as ocean warming (Almeida *et al.*, 2021a; Arrigo *et al.*, 2024; Costa *et al.*, 2020a, 2020b; Lopes *et al.*, 2022; Nardi *et al.*, 2022) and salinity fluctuations (Freitas *et al.*, 2019, 2020b), strongly affects the modulation of GPx activity in marine bivalves. Alongside this, a significant induction of GPx activity by TS+CBZ in the digestive glands over the exposure period was observed. Digestive glands, in *M. galloprovincialis*, are considered the main detoxification organ, which may be a plausible explanation for the more pronounced induction of GPx activity in this tissue than in the gills of TS+CBZ treated mussels (Gomes *et al.*, 2014). On the other hand, Freitas *et al.* (2020b) observed that non-contaminated *M. galloprovincialis* submitted to higher salinity (35 compared to 25) presented an enhanced response in comparison to the lack of responses when mussels were exposed to the combination of salicylic acid (4.0 mg/L) and increased salinity. A similar inhibitory response was demonstrated by Costa *et al.* (2020a) in clams *R. decussatus* exposed to triclosan (1 µg/L) and increased temperature (21 °C) for 7 days. Thereby, the combination of stressors generates the highest stress levels in marine bivalves triggering diverse responses to cope with harmful conditions, such as an induction (Queirós *et al.*, 2021) or inhibition (Freitas *et al.*, 2019a; Pirone *et al.*, 2019) of GPx levels in these organisms.

TAC was assessed to understand the overall mussels' ability to scavenge or neutralize free radicals or ROS. In the present study, no significant changes in TAC levels were addressed in both the gills and digestive glands over exposure time. Similarly, Brandts *et al.* (2018) observed no differences in TAC levels of the gills or digestive glands of *M. galloprovincialis* acutely exposed (96h) to CBZ (6.3 µg/L) and nanoplastics (0.005, 0.05, 5 and 50 mg/L). Although TAC was not activated, the previously reported results, in corroboration with the PCA, show that mussels activated their antioxidant system when exposed to CBZ or TS+CBZ.

4.3. Biotransformation capacity

The enzymes GSTs are multifunctional enzymes involved in the detoxification processes occurring in phase II, catalyzing the conjugation of toxic compounds to reduce GSH (Regoli & Giuliani, 2014). In the present study, an absence of changes in GSTs levels were observed in CBZ-treated mussels in both gills and digestive glands over the exposure time. GSTs are part of a large intracellular detoxification system that includes enzymes like CYP3A4, and consequently are directly involved in thiol metabolites formed from CBZ oxidation (*i.e.*,

CBZE) (Patsalos *et al.,* 2018). The reduction or lack of changes in GSTs activity may be justified through the GSH sequestration by the metabolite CBZE within CBZ metabolism, in which the catalyzation of CBZ by CYP3A4 produces the electrophilic metabolite CBZE (Almeida *et al.,* 2014; Boillot *et al.,* 2015), resulting in the formation of GSH (Bu *et al.,* 2005). Thereby, GSH acts as a nucleophile to sequester harmful reactive metabolites (Bu *et al.,* 2005). In corroboration with the present findings, previous authors highlighted no GSTs activation over acute and chronic exposure to CBZ (0.0 – 9.0 µg/L) in mussels *M. galloprovincialis* (Oliveira *et al.,* 2017), clams *V. decussatus* and *V. philippinarum* (Almeida *et al.,* 2014), and *R. philippinarum* (Aguirre-Martínez *et al.,* 2016). Nevertheless, previous studies observed an inhibition (Martin-Diaz *et al.,* 2009) or induction (Abdelhafidh *et al.,* 2018; Juhel *et al.,* 2017) of GSTs activity in marine bivalves exposed to CBZ. The difference in response may be explained by the different concentrations of CBZ, exposure time (*i.e.,* acute *vs.* chronic) and tissues analyzed, such as whole tissue (Almeida *et al.,* 2014; Oliveira *et al.,* 2017) *vs.* gills and digestive glands (Abdelhafidh *et al.,* 2018; Aguirre-Martínez *et al.,* 2016; Juhel *et al.,* 2007; Martin-Diaz *et al.,* 2009).

Additionally, GSTs were induced in the gills at the 14th day and in the digestive glands at the 28th day when exposed to TS+CBZ. In marine bivalves, gills are considered the first organ of contact with the contaminant, which may justify a higher response at the beginning of the exposure period in this tissue (Franco-Martinez *et al.,* 2016). While the digestive glands are a major detoxification tissue due to their mixed-function oxidase system (Faggio *et al.,* 2018; Solé & Livingstone, 2005). Thus, both tissues play a key role in unravelling the toxic effects and MoA of drugs in marine bivalves, as reported by Gonzalez-Rey *et al.* (2014) in *M. galloprovincialis* submitted to the pharmaceutical mixture (ibuprofen and diclofenac, 250 ng/L each) over 15 days. Mussels' ability to metabolize and biotransform drugs, such as CBZ, through phase I and/or phase II reactions has been previously demonstrated by Martin-Diaz *et al.* (2009) in *M. galloprovincialis* exposed to CBZ (0.1 and 10.0 µg/L, 7 days), and Boillot *et al.* (2015) in the same species exposed to CBZ and 10,hydroxy-10,11-dihydro-carbamazepine (10 µg/L, 7 days).

Thereby, the results may evidence that abiotic changes play a key role in triggering mussels' responses exacerbating CBZ impacts in GSTs activity. Previous studies reported a similar activation of GSTs activity in marine bivalves exposed to pollutants in combination with warming (Almeida *et al.,* 2021a; Costa *et al.,* 2020a, 2020b; Cunha *et al.,* 2024; Nardi *et al.,* 2022; Queirós *et al.,* 2021) and salinity (Carregosa *et al.,* 2014; De Marchi *et al.,* 2020; Freitas *et al.,* 2019), corroborating with our results.

The CbEs enzymes also play a role in detoxification processes by catalyzing the biotransformation of chemicals (*e.g.*, carboxyl acid ester, amide or thioester groups), thus preventing further damage to the organisms' cells (Solé *et al.*, 2018; Yan, 2014). The results obtained herein demonstrated a reduction in CbEs levels in the gills of mussels submitted to CBZ, and in the digestive glands of mussels exposed to both CBZ and TS+CBZ. The findings may be explained by the MoA of the drug, which involves sodium channel blockade impacting the detoxification process (Ambrósio *et al.*, 2002; Tolou-Ghamari *et al.*, 2013), consequently affecting CbEs levels. Alternatively, it may be hypothesized that CbEs reduced activity under multiple stressors may be an enzyme compensatory mechanism strategy (Solé *et al.*, 2018). By contrast, Queirós *et al.* (2021) reported a tendency to CbEs activation in *M. galloprovincialis* exposed to cisplatin (10, 100, 500 and 1000 ng/L), regardless of the temperature scenario. Yet, the response of this enzyme in marine mussels exposed to pharmaceuticals under a changing climate should still be further investigated.

4.4. Oxidative damage

In case of failure of the defense mechanisms, namely antioxidant and biotransformation enzymes, the excess of ROS produced can lead to cell damage through the oxidation of lipids and proteins (Regoli & Giuliani, 2014). In this study, LPO levels were reduced in the digestive glands of mussels exposed to CBZ and TS+CBZ over the exposure time. The results herein presented may be justified by a quick-triggered defense mechanism of mussels to these stressors. Similar responses were detected in *M. galloprovincialis* (whole tissue) acutely and chronically exposed to CBZ (0.0 – 9.0 µg/L) (Oliveira *et al.*, 2017). Similarly, a significant decrease in *R. decussatus* (whole tissue) to CBZ (0.0 – 9.0 µg/L, 96 h) (Almeida *et al.*, 2014) and *R. philippinarum* (whole tissue, 28 days) to the highest concentration of CBZ (9.0 µg/L) (Almeida *et al.*, 2015) was reported by previous authors. Likewise, Aguirre-Martínez *et al.* (2016) reported a reduction in *V. philippinarum* (whole tissue) to CBZ (0 – 50 µg/L, 35 days). By contrast, other studies observed greater levels of LPO in clams R. *philippinarum* (Almeida *et al.*, 2014) and mussels *M. galloprovincialis* (Martin-Diaz *et al.*, 2009; Tsiaka *et al.*, 2013) exposed to different concentrations of CBZ.

Thus, the difference in response may be justified by the concentration of CBZ and tissues (whole *vs.* gills/digestive glands) to effectively overcome oxidative stress. In concordance with our hypothesis, Martin-Diaz *et al.* (2009) demonstrated an increase in the gills and inhibition in the mantle/gonads of mussels exposed to CBZ (0.1 and 10 µg/L, 7 days), which highlights the effects of lipid peroxidation in different tissues. The results herein

presented greater LPO levels in the CTL groups may be explained by higher constitutive lipid peroxidation in mussels, as previous studies obtained similar results in *M. galloprovincialis* (Oliveira *et al.*, 2017) and *R. philippinarum* (Almeida *et al.*, 2015). Therefore, the present findings demonstrated that antioxidant defenses actively prevented LPO in the gills and digestive glands of *M. galloprovincialis*, supported by the PCA responses. Similar results were observed by Brandts *et al.* (2018) in the same species exposed to CBZ (6.3 µg/L, 96 h).

4.5. DNA damage

Regarding genotoxicity, the results herein presented the induction of DNA damage in *M. galloprovincialis* chronically exposed to both CBZ and TS+CBZ. Although lipid peroxidation did not occur in those treatments, the antioxidant defenses were not enough to prevent DNA damage in marine mussels (Dalhus *et al.*, 2009). Thereby, the observed damage might be linked to excessive ROS production and failed defense mechanisms (antioxidant and biotransformation systems) to adequately protect the cell against harmful conditions (Halliwell & Gutteridge, 2015). It may be hypothesized that CBZ may affect mussels' defense system as DNA damage increases over time. In corroboration with the present results, Brandts *et al.* (2018) observed enhanced DNA damage in *M. galloprovincialis* exposed to CBZ (6.3 µg/L, 96 h). On the other hand, previous genotoxicity assessments did not address DNA damage in CBZ-treated bivalves (Aguirre-Martínez *et al.*, 2016; Juhel *et al.*, 2017; Lacaze *et al.*, 2015; Martin-Diaz *et al.*, 2009).

The different ranges of concentrations of CBZ applied in the ecotoxicological assessments may justify the contrasting results. Still, the present study revealed a maximized DNA damage in mussels' hemocytes when exposed to multiple stressors (TS+CBZ). Accordingly, PCA corroborates with the presented hypothesis by evidencing that ETS, SOD, CAT and DNA damage indicate to be positively related, indicating the possible reduction of energy metabolism and activation of the defense system to protect the cells from further DNA damage. However, the activation of antioxidant enzymes (*e.g.*, SOD and CAT) was not enough to suppress DNA damage. The antioxidant and biotransformation enzymes presented a positive correlation in the digestive glands, associated to the successful, but delayed, activation of the defense system to protect the cells from further oxidative and genotoxic damage.

4.6. Neurotoxicity

CBZ affects membrane potentials and neuronal excitability through its mode of action. Thus, the possibility of neurotransmission effects was assessed through the determination of

AChE activity. This enzyme is actively involved in the hydrolysis of acetylcholine (Ach) and is widely used to assess the effects of various pollutants in marine invertebrates (Canty *et al.*, 2007). In the present study, AChE was induced by CBZ and TS+CBZ, followed by an inhibition over time in both gills and digestive glands. Similar results were observed in clams *(Ruditapes philippinarum)* exposed to carbamazepine 15 µg/L over 14 days (Trombini *et al.*, 2019). Contrasting, green mussels (*Perna viridis)* exposed CBZ (1, 10, 96 ng/L) and other contaminants exhibited a lack of AChE activity in comparison to CTL group over 7 days of exposure (Juhel *et al.*, 2017). Likewise, *M. galloprovincialis* exposed *in vitro* to CBZ observed a lack (62.5, 125 and 250 mg/L) or inhibition (500 and 1000 mg/L) of AChE activity (Luis *et al.*, 2016). However, the effects observed on the neurotransmission activity of marine bivalves may vary according to the concentration, mixture and exposure time. Thus, the present findings showcase that CBZ appears to influence AChE activity in *M. galloprovincialis,* similarly to its MoA in vertebrates, by decreasing the nerve impulses and consequently producing a neurotoxic effect on marine invertebrates (Aguirre-Martínez *et al.*, 2016).

In the present study, the variation in levels of AChE activity, characterized by an initial rise followed by its inhibition at the end of the bioassay, may potentially be associated with the binding of CBZ to adenosine receptors. Previous studies have indicated that CBZ interacts with adenosine receptors, specifically adenosine A1 receptors, resulting in alterations in receptor function and expression (Daval *et al.*, 1989). CBZ has been proven to enhance the number of adenosine A1 receptors in humans, a phenomenon similar to the impact of caffeine, which acts as an adenosine antagonist (Marangos *et al.*, 1985). This increase in adenosine A1 receptors could potentially impact the regulation of acetylcholine release. Thereby, CBZ has been shown to replicate its MoA in marine mussels *M. galloprovincialis*, by hindering adenosine binding at a nominal concentration (5 µg/L). In corroboration with the present findings, Cunha *et al.* (2024) observed similar results in *M. galloprovincialis* exposed to caffeine (0.5; 1.0; 5.0; 10.0 µg/L) under current (17 °C) and projected (21 °C) temperature conditions over 28 days. Authors hypothesize that caffeine binds to adenosine receptors without activating them, thus hindering adenosine binding and consequently increasing AChE activity. Hence, the rise of AChE levels observed in mussels following exposure to CBZ could potentially be attributed to the distinctive pharmacological MoA exhibited by this drug towards adrenergic and adenosine receptors, its impact on AChE levels, as well as the biochemical reactions triggered by the exposure to CBZ alone or in combination with TS changes (TS+CBZ) in mussels.

5. Conclusion

The present findings shed light on the toxicological responses of marine pollution by CBZ in combination with climate change stressors, namely increased seawater temperature and salinity, in the Mediterranean mussel *M. galloprovincialis*. Overall, mussels demonstrated tissue-specific responses, revealing the disruption of the energetic, antioxidant and biotransformation systems in gills exposed to the nominal concentration of 5 µg/L CBZ, regardless of temperature and salinity. In contrast, responses in digestive glands reflected the synergistic effects of the interplay of stressors (TS+CBZ) that modulated protein content, antioxidant defenses and neurotoxicity, culminating in heightened DNA damage in mussels' hemocytes.

As the toxicological effects of combined exposure to pharmaceuticals and climate change stressors on marine invertebrates remain unclear, it is relevant to emphasize the need for harmonization in the forthcoming ecotoxicological assessments regarding a multiple-stressor approach by using similar and calibrated analytical research methodologies (*i.e.*, tissues selected for analyses; time of exposure; acclimation to the climate change stressors during the experiment) for a comprehensive interpretation of the gathered data for this biological model.

Long-term variations of salinity and temperature per se were shown to disrupt the physiological health of marine mussels, consequently leading to ecotoxicological disruption when simultaneously exposed to an emerging contaminant. Given this species' importance in ecosystem functioning, its economic and cultural relevance, and the growing overlap of anthropogenic stressors in marine realms, it becomes urgent to conduct intercalibrated research to build a consistent dataset on the toxicity of pharmaceuticals under predicted climate change scenarios.

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