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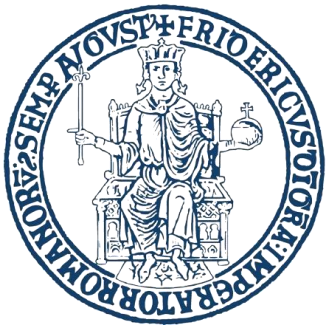

DEPARTMENT OF BIOLOGY

PHD COURSE IN BIOLOGY XXXVI CYCLE

Mytilus galloprovincialis as a model to approach ecotoxicological aspects of marine biological invasions

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Academic Year 2022/2023

Abstract

Background

In this study, crucial ecotoxicological issues have been approached by using the molluscan bivalve species *Mytilus galloprovincialis* as a model organism. A special focus has been placed on the Mediterranean Sea, which is one of the major biodiversity hotspots under threat in the world, negatively impacted by both biological invasions and anthropogenic pollution. In addition to the negative and far-reaching impacts of invasive marine species *per se*, a growing literature is raising major concerns about the effects of bioactive substances carried by allochthonous species in the colonized environments, also because their ecotoxicological potential often adds to that of anthropogenic hazardous compounds widespread in marine ecosystems. Among the bioactive metabolites from exotic species, major attention has been paid in this study to the alkaloid caulerpin from green algae of the genus *Caulerpa*, since this compound already showed a panel of biological properties suggesting a non-negligible impact on native fauna. Given the similarities in the mechanism of action of caulerpin with that of fibrate drugs, the action of caulerpin has been compared to that of fenofibrate which is widely considered dangerous for aquatic fauna. On the other hand, since caulerpin co-occurs in the marine environment with caffeine, which is an anthropogenic pollutant widely recognized as a hazard to marine animals, the possible synergistic effects of caffeine and caulerpin have been also evaluated. A further aspect of this study concerned the invasive calcareous sponge *Paraleucilla magna*, which colonizes mussels' farms in the Mediterranean. Although little is known about the sponge's chemical composition, the toxicological potential of its crude extracts has been tested.

Methodology and Principal Findings

The above issues have been approached by proposing via food pure metabolites and crude extracts to mussels, followed by biochemical analyses, NMR-based metabolomics, and histopathological examinations. Results obtained indicated that caulerpin did not affect mussel physiology, while several impacts were observed

after the exposure to fenofibrate (Chapter 2). Also, no cumulative effects were observed when mussels were exposed simultaneously to caulerpin and caffeine (Chapter 3). Finally, the study on *P. magna* (Chapter 4) clarified that the sponge's acetone extract did not affect mussels, while the diethyl ether and butanol extracts induced significant variations in osmolyte levels and biochemical markers.

Significance

Overall, the present study confirms the harmful effects of both fenofibrate and caffeine, underscoring the potential risks associated with their presence in the marine environment. The findings also support the safety profile of caulerpin, a metabolite derived from highly invasive marine species, paving the way for further investigation in support of its possible use for nutraceutical and aquaculture applications, and the sustainable exploitation of the huge biomass from invasive species. Finally, the study suggests that the invasive sponge *P. magna* may pose a real chemical threat to the Mediterranean Sea, although more comprehensive additional research to determine which specific sponge metabolites are responsible for the observed effects is needed.

Keywords

Invasive Species, Marine Pollution, *Mytilus galloprovincialis*, Biomarkers, NMR-based metabolomics, Histopathology

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CHAPTER 1

INTRODUCTION

1.1 INVASIVE SPECIES

Biological invasions pose a significant threat to the planet's biodiversity and the proper functioning of ecosystems (Simberloff et al., 2013; Tsirintanis et al., 2022; Weinstein and Turner, 2012). In addition to altering biodiversity, these invasions can cause significant economic losses, particularly in fisheries and aquaculture (Giangrande et al., 2020). This process is increasing, especially in the marine environment, where human activities such as international navigation, aquaculture, and aquariums facilitate the spread of organisms to new locations in which they would not naturally occur (Ulman et al., 2019).

The terminology regarding allochthonous species varies across taxonomic kingdoms, scientific disciplines, and linguistic and national boundaries. These species are commonly referred to as alien, allochthonous, exotic, introduced, invasive, naturalized, non-indigenous (NIS), and non-native (Galil and Goren, 2014). They are defined as "species, subspecies or lower taxa introduced outside of their natural range (past or present) and outside of their natural dispersal potential. This includes any part, gamete, or propagule of such species that might survive and subsequently reproduce. Their presence in the given region is due to intentional or unintentional introduction resulting from human activities" (Olenin et al., 2010). "Invasive alien species (IAS) are a subset of established non-indigenous species that have spread, are spreading, or have demonstrated their potential to spread elsewhere and have an adverse effect on biological diversity, ecosystem functioning, socio-economic values, and/or human health in invaded regions" (Olenin et al., 2010). Invasive species could replace the native species causing the loss of native genotypes, modifying the habitat, and influencing the food webs (Katsanevakis et al., 2014; Fanelli et al., 2015). Species of unknown origin are termed cryptogenic species (Olenin et al., 2010). On average, only 10% of alien species become invasive (Boudouresque et al., 2005).

The introduction of NIS involves four phases: arrival, settlement, expansion, and persistence (Boudouresque et al., 2005; Boudouresque and Verlaque, 2012). The first phase consists of the transfer of the organisms from the site of origin to the new site using different vectors, even if most of them face local extinction as they

are unable to adapt to the new environmental conditions. Then follows the settlement phase, in which the surviving organisms, capable of reproducing, give rise to a viable population able to survive in the new region. Once established, introduced species can grow exponentially, attempting to occupy all habitats and the entire geographical range to which they have access until a maximum limit of expansion is reached, after which the growth rate tends to slow. Finally, in the defined phase of persistence, introduced species occupy all accessible habitats and the entire accessible geographical range. During the expansion and persistence phases, the abundance of the introduced species fluctuates as most of the native species do (Boudouresque et al., 2005; Boudouresque and Verlaque, 2012). Such fluctuations are linked to predator-prey relationships, parasite-host relationships, and discontinuous propagule production strategies (Boudouresque and Verlaque, 2012). Factors influencing the success of invasive species include the magnitude of the phenomenon, such as the frequency of vectors; the abiotic and biotic characteristics of the receiving environment; and the ecological and physiological characteristics of the introduced species (such as high dispersal capacity, tolerance to extreme environmental conditions, and genetic variability) (Pulzatto et al., 2019).

1.2 INVASIVE SPECIES IN THE MEDITERRANEAN SEA

The Mediterranean Sea is particularly affected by biological invasions, resulting in the most invaded marine ecosystem in the world, with about 1000 non-indigenous species, representing more than 5% of the total species (Giangrande et al., 2020; Tsiamis et al., 2020; Zenetos et al., 2022). Climate change and the resulting increase in water temperature in the Mediterranean, which is warming faster due to its semi-enclosed conformation, make it increasingly suitable for hosting thermophilic species from tropical Indo-Pacific and Atlantic ecosystems (Azzurro et al., 2019; Coll et al., 2010). The main entry route for NIS is the Suez Canal (Lessepsian migration), which connects the Red Sea to the Mediterranean Sea, and the major introduction is represented by shipping and aquaculture (Galil et al., 2014; Tsiamis et al., 2018). NIS also arrive through the Gibraltar Strait (Raitsos et al., 2010). NIS, particularly IAS, can have both positive and negative effects on the Mediterranean

ecosystem, with the negative effects (on biodiversity, ecosystem services, and human health) outweighing the positive ones (Tsirintanis et al., 2022). Negative impacts on biodiversity include competition for resources, alteration of habitats, and predation, which can lead to the loss of native species (Katsanevakis et al., 2023). An example of local extinction is the Mediterranean mussel *Pinna nobilis*, damaged by the invasive protozoan *Haplosporidium pinnae* (Katsanevakis et al., 2022). Biofouling is the main negative impact on ecosystem services, as many IAS densely colonize aquaculture facilities, resulting in significant economic losses (Tsotsios et al., 2023). IAS have a significant impact also on tourism and cultural services, such as the occurrence of jellyfish blooms, extensive algae washing ashore, and the alteration of habitats with cultural relevance (Katsanevakis et al., 2014). Regarding human health, IAS primarily pose risks through stings, poisoning, or intoxication (Galil, 2018). On a positive note, NIS can create new habitats for various species and can improve ecosystem services in terms of water purification and climate regulation (Katsanevakis et al., 2014). Furthermore, research on IAS specimens can have a beneficial impact on cultural services, offering the potential for future exploration of molecules for pharmaceutical or industrial applications (Mollo et al., 2015; Mollo, 2022; Pereira et al., 2021; Pinteus et al., 2018).

Considering the challenges in this research field and the recent identification of around 60 new NIS in the region (Fortič et al., 2023; Langeneck et al., 2023; Tiralongo et al., 2022), a recent review (Zenetos et al., 2022) highlighted that Mollusca has the highest number of established NIS, followed by Crustacea, Pisces, Polychaeta, and Foraminifera, with others among Phytobenthos and Miscellanea. Macroalgae are one of the taxonomic groups with the highest number of allochthonous invasive species (Boudouresque et al., 2005). In particular, the alien green alga *Caulerpa cylindracea* represents one of the most threatening invasive species in the Mediterranean Sea, with impacts on native species such as the alteration of the structure of invaded assemblages (Piazzi et al., 2016).

In addition to the various impacts outlined above, the phenomenon of biological invasion should be approached from a chemoecological perspective to assess how bioactive compounds from NIS affect native communities (Mollo et al., 2008). Indeed, the introduction of bioactive natural products that native communities have

not previously encountered could trigger rapid succession events with impacts on ecosystem dynamics (Mollo et al., 2015).

1.2.1 *CAULERPA CYLINDRACEA*

Macroalgae have often caused significant invasions in marine habitats, leading to profound alterations in the structural and functional characteristics of native benthic communities (Klein and Verlaque, 2008; Navarro-Barranco et al., 2018; Piazzi et al., 2021; Williams and Smith, 2007). The green alga *Caulerpa cylindracea* (Fig. 1) is particularly noteworthy among macroalgae as one of the most invasive species recorded in the Mediterranean Sea, causing significant landscape changes to the seabed (Felline et al., 2012; Piazzi et al., 2016; Pierucci et al., 2012; Tsirintanis et al., 2022). This alga can form multilayered mats with thicknesses of up to 15 cm, trapping sediment and forming an anoxic layer underneath. Its rapidly growing stolons can incorporate other macroalgae or invertebrates (Katsanevakis et al., 2014).

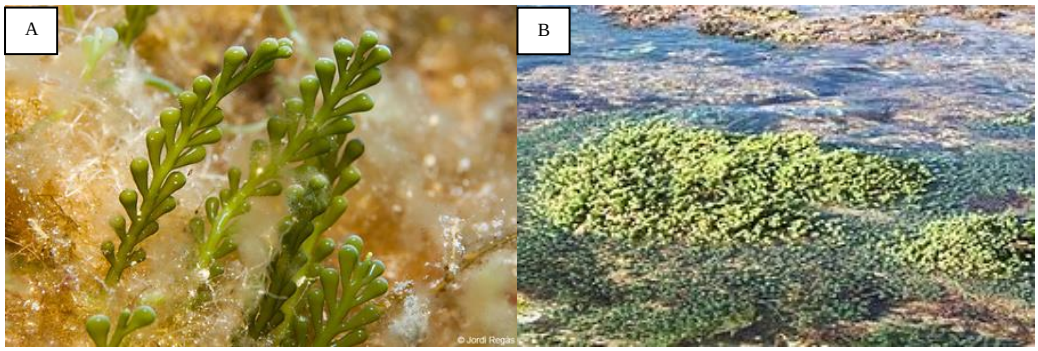

Figure 1. A: Detail of *C. cylindracea* filloids (picture from https://www.cibsub.cat/bioespecie_es-caulerpa_racemosa-27618). B: *C. cylindracea* superficial colonies.

The entry of *C. cylindracea* into the Mediterranean remains uncertain. While it may be a Lessepsian species that has colonized the eastern Mediterranean Sea since the 1920s (Piazzi et al., 2005), there is also some evidence that the species has been introduced (Klein & Verlaque, 2008).

The alga grows on a variety of substrates, including rock, dead *Posidonia oceanica* "matte", sand, mud, detritus, and coralligenous assemblages, at depths ranging from 0 to 70 m, with its highest abundance observed between 0 and 30 m. Its presence

generally resulted in a decrease in macrophyte cover (Klein & Verlaque, 2008). For instance, rhizomes of seagrass meadows *P. oceanica* showed a significant decrease in both the number of epiphytic algal taxa and their coverage due to the presence of *C. cylindracea* (Antolić et al., 2008). However, a recent long-term study revealed that healthy meadows were not affected and acted as ecological barriers against the spread of the invasive alga (Bernardeau-Esteller et al., 2020). In addition, the macrofauna on soft bottoms heavily colonized by *C. cylindracea* changed significantly, with decreases in gastropods and crustaceans and increases in polychaetas, as reported in a bay in Cyprus (Argyrou et al., 1999). Similarly, *C. cylindracea* appeared to increase meiofauna abundance while decreasing the diversity of some taxa, such as meiobenthic crustaceans in the Gulf of Taranto (Italy) (Sandulli et al., 2004).

Currently, in the Mediterranean *C. cylindracea* represents a food resource for at least 29 species, including deposit feeders, detritivores, filter feeders (among which the bivalves *Arca noae* and *Glans trapezia*), herbivores, and omnivores, thus entering the food chain (Rizzo and Vega Fernández, 2023; Terlizzi et al., 2011). In particular, the trophic relationship between *C. cylindracea* and the omnivorous white seabream *Diplodus sargus* was brought to the attention of the scientific community in 2011 (Terlizzi et al., 2011). Analyses of biomarkers in *D. sargus* tissue after ingesting *C. cylindracea* showed a reduction in the polyunsaturated fatty acids in fish tissues, activation of some antioxidant defenses, inhibition of acetylcholinesterase and acylCoAoxidase, an increase in the hepatosomatic index, and a decrease in the gonadosomatic index (Felline et al., 2012; 2014). Further studies on *D. sargus* revealed limited changes in antioxidant defenses, including glutathione reductase and glutathione levels, an elevation in the activities of cytochrome P450, glutathione S-transferase, and acylCoAoxidase, and in the gene transcription for peroxisome proliferator-activated receptor α (PPARα), cytochrome P4501A, and vitellogenin 1, indicating a cellular response to seaweed consumption (Gorbi et al., 2014). Also, activation of the antioxidant pathway was observed in individuals of *Spondyliosoma cantharus* living in sites invaded by *Caulerpa* (Box et al., 2009).

The invasion of *C. cylindracea* in the Mediterranean Sea has led to the introduction of secondary metabolites from the alga into the new ecosystem, among which the most studied are the red bisindolic alkaloid caulerpin (CAU), the sesquiterpene caulerpenyne containing a bis-enol acetate group, and the mixture of hydroxy amides caulerpicin (Defranoux and Mollo, 2020; Mollo et al., 2015). In particular, it has been observed that caulerpenyne exhibited phytotoxic effects on native phanerogams, with a probable allelopathic function (Raniello et al., 2007). CAU instead has attracted the attention of the scientific community mainly since it accumulates in the tissues of fish consuming *C. cylindracea*, thus entering the food chain, making its accumulation an indicator of exposure to the alien alga (Felline et al., 2012, 2014, 2017; Gorbi et al., 2014). Furthermore, *C. cylindracea* belongs to the group of edible algae within the genus *Caulerpa* along with *C. lentillifera* and *C. racemosa.* These algae contain relatively high levels of CAU and are known as "sea grapes", being consumed by humans as food in the Indo-Pacific and Caribbean regions (Vitale et al., 2018).

1.2.1.1 CAULERPIN

Caulerpin (CAU), a bisindolic alkaloid (Fig. 2), is the most abundant secondary metabolite of *C. cylindracea* (Terlizzi et al., 2011). This metabolite has shown several properties, including antimicrobial, antiviral, and anti-inflammatory activities (De Souza et al., 2009; Defranoux and Mollo, 2020; Lucena et al., 2018).

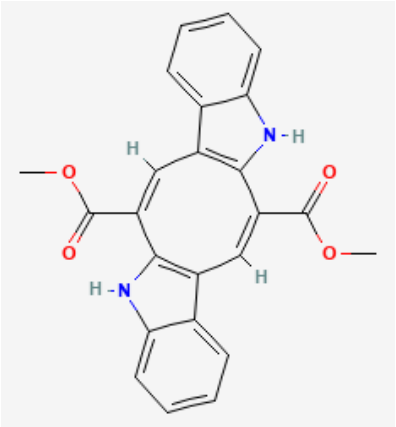

Figure 2. Structure of caulerpin (CAU).

Among the many chemical classes found in plant and algae species, alkaloids have been identified as being responsible for many of the medicinal plants' pharmacological properties (De Souza et al., 2009).

Even if some species of the genus *Caulerpa* are native to the Mediterranean Sea (e.g., *C. prolifera*), CAU has been found only in the invasive species, mainly in *C. cylindracea* (Defranoux et al., 2022), making it an "alien invasive metabolite". The interactions among different species play a key role in the structure and balance of ecosystems. Small organic molecules, serving as mediators in essential biological processes like competition, nutrition, defense, and reproduction, regulate these interactions. Therefore, the considerable natural chemical diversity is essential to ensuring the stability of ecosystems (Mollo et al., 2015). For this, it is important to underline that invasive species can impact ecosystems through bioactive secondary metabolites, which pose a challenge for native populations as they lack evolutionary adaptations to them (Mollo et al., 2008; Terlizzi et al., 2011). Thus, the alien metabolite CAU has been an object of investigation in the last decade.

Some behavioral changes in *D. sargus* were related to CAU, as its dietary administration reduced the aggressiveness of the seabream, suggesting an anxiolytic effect. This hypothesis is supported by the discovery of a significant increase in neuropeptide Y transcriptional expression in the central nervous system of seabream fed with CAU-enriched food (Magliozzi et al., 2017; 2019). Del Coco et al. (2018) observed a reduction, analyzed with ^{1}H NMR spectroscopy techniques, in the relative content of polyunsaturated fatty acids (PUFA) of interest for human nutrition after food administration of CAU, proposing CAU as responsible for the changes observed by Terlizzi et al. (2011) in fish meat. However, subsequent absolute quantifications of PUFA levels in the meat of fish fed with CAU, carried out by gas chromatography coupled to mass spectrometry (GC-MS) with the addition of an internal standard, have provided more accurate information, refuting the hypothesis that CAU induces a reduction in PUFA levels (Schiano et al., 2022). However, the effective risk posed by CAU remains uncertain.

In addition, it is worth mentioning that evidence has recently been provided for the direct binding and activation by CAU of the α and γ isoforms of peroxisome proliferator-activated receptors (PPARs), which are nuclear transcription factors

that modulate the expression of genes involved in the regulation of metabolism, behavior, reproduction, tumorigenesis, and cytochrome P450 activation (Vázquez-Carrera and Wahli, 2022; Vitale et al., 2018).

1.2.2 *PARALEUCILLA MAGNA*

Among invasive species, only a few specimens belong to the phylum Porifera (Longo et al., 2007). In particular, the only calcareous invasive sponge recorded along the Mediterranean coast is *Paraleucilla magna* (Longo et al., 2012; Occhipinti-Ambrogi et al., 2010). Aquaculture, together with shipping traffic, was identified as the most probable vector for the expansion of *P. magna*' in the Mediterranean Sea (Di Blasio et al., 2023). This species is characterized by high genetic diversity, phenotypic local adaptation, and high reproduction rates, which probably explain its strong invasive potential (Guardiola et al., 2016). *P. magna* thrives in various Mediterranean environments, including ports, marinas, and inlets, on both natural and artificial hard substrates, and in clean or polluted water. This species may also grow directly on rocks, on erect algae such as those of the *Halopteris* or *Corallina* genera, or selectively overgrow mussel shells, posing a problem for mussel farmers (Guzzetti et al., 2019). This ability to colonize native algae and filter-feeding invertebrates has drawn researchers' attention to the sponge's chemical composition (Longo et al., 2021). Furthermore, sessile marine organisms frequently use chemical defenses to prevent fouling on their surfaces. Indeed, it has been suggested that *P. magna* produces long-term antifouling compounds (Longo et al., 2021). In support of this hypothesis, the ethanolic extract from *P. magna* exhibited inhibitory effects on the settlement of *Mytilus galloprovincialis* juveniles and demonstrated toxicity towards *Artemia salina* nauplii and the microalgae *Nannochloropsis sp.* and *Tetraselmis suecica*. Furthermore, Guzzetti et al. (2019) observed that the presence of *P. magna* induced an adaptive antioxidant response in the red alga *Peyssonnelia squamaria*, with notably elevated activity levels of antioxidant enzymes, including catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase. The sponge's specific antifouling activity and its potential negative impact on native

fauna remain unclear and require further investigation. More research is needed to understand how the sponge's antifouling properties work and to assess any potential adverse effects on the surrounding marine environment.

1.3 ENVIRONMENTAL POLLUTION

Besides biological invasions, aquatic environments, especially the Mediterranean Sea, are seriously affected by increasing pollution, which represents one of the main causes of biodiversity loss and a major driver of ecosystem changes (Brumovský et al., 2017; Coll et al., 2010; Costello et al., 2023; Danovaro, 2003). This increment is strongly related to the growth of the world population and to industrial and agricultural activities (Alves et al., 2021; Zaqoot et al., 2012). Pollution is defined as the introduction of substances or energy into the environment by human activities, causing harmful effects on living resources, including humans, and disrupting environmental amenities. Aquatic environments face significant threats from a range of pollutants, including metals, industrial solvents, volatile organic compounds, agrochemicals, household products, fuel combustion by-products, nanoparticles, personal care products, antibiotics, as well as prescription and nonprescription drugs (Tonelli and Tonelli, 2020).

Environmental pollution caused by potentially toxic elements is a major source of concern, and numerous studies have addressed this issue. These studies not only highlight the observable accumulation of these elements in various aquatic compartments but also their significant impact on both freshwater and marine organisms (Beretta et al., 2014; Brumovský et al., 2017; Jones et al., 2008; Catsiki et al., 2006; Vieira et al., 2022).

Investigating the combined effects of various anthropogenic pollutants with other drivers of change, such as climate change, is gaining popularity (Coppola et al., 2017, 2022; Freitas et al., 2019; Leite et al., 2023; Paciello et al., 2023; Piscopo et al., 2021a, among others). However, there is a notable lack of literature on the combined effects of environmental pollution and bioinvasion (Rodrigues et al., 2022). Therefore, to fill this gap, more investigations into the complex interactions between environmental pollutants and compounds carried by IAS are needed.

Understanding the antagonistic or cumulative effects of pollution and bioinvasion is critical for developing effective environmental management strategies and mitigating the impact of human activities on ecosystems.

1.3.1 FENOFIBRATE

Drug pollution is an emerging environmental issue, and a wide variety of active pharmaceutical ingredients are released into the environment, posing a threat to aquatic organisms, among which is the blood lipid regulator fenofibrate (FFB) (Fig. 3) (Andreu et al., 2016; Kumar et al., 2018). Indeed, the high consumption of blood lipid regulators is leading to a more frequent presence of fibrates in natural watercourses and wastewater effluents (Rosal et al., 2010).

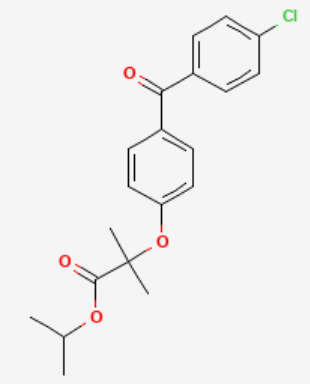

Figure 3. Structure of fenofibrate (FFB).

FFB is a prodrug hydrolyzed *in vivo* to its active form, fenofibric acid and acts as a ligand of PPARα (McKeage and Keating, 2011). Upon activation, PPARα induces the expression of genes crucial for fatty acid activation, fatty acid transport, and β-oxidation, thereby regulating lipoprotein and fatty acid metabolism (McKeage and Keating, 2011). Administering FFB to humans effectively reduces plasma triglyceride levels and increases the concentration of high-density lipoprotein cholesterol, making it valuable for conditions such as hypertriglyceridemia and atherogenic dyslipidemia (Farnier, 2008). Studies in animals fed a high-fat diet indicated that the dietary administration of FFB induced a lipid-lowering effect

increasing the activities and expression of enzymes involved in lipid metabolism (Chan et al., 2015).

Due to their presence in aquatic environments, PPARα ligands have been studied with increasing interest in fish. Studies on grass carp (*Ctenopharyngodon idella*) treated with FFB while fed a high-fat diet revealed a reduction in plasma triglycerides and cholesterol concentrations, total lipids, and the content of essential fatty acids [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] in tissues, suggesting FFB's role in regulating lipid metabolism also in grass carp (Du et al., 2008). In yellow catfish (*Pelteobagrus fulvidraco*) exposed to aqueous Zn, dietary FFB reduced hepatic lipid deposition (Zheng et al., 2015). Similarly, in rainbow trout (*Oncorhynchus mykiss*) after administration of a diet containing EPA and DHA, FFB treatment altered the content of these essential fatty acids and peroxisomal activities in various tissues (Du et al., 2004).

In addition, the potential ecotoxicity of FFB and other lipid regulators (bezafibrate and gemfibrozil) on other aquatic organisms has been carried out: exposure to lipid regulators' compounds inhibited population growth in rotifers and crustaceans, with slight effects on algae (Isidori et al., 2007); fenofibric acid exhibited toxicity to *Daphnia magna*, and adverse effects, including lethality, on zebrafish embryos (Rosal et al., 2010; Hering et al., 2021).

Overall, these findings underline the importance of assessing the environmental impact of lipid regulators on aquatic organisms.

1.3.2 CAFFEINE

Among the emerging contaminants is caffeine (CAF), which, due to its abundant and continuous use, is found ubiquitously in aquatic ecosystems, both marine and freshwater and is considered an indicator of anthropogenic contamination (Buerge et al., 2003; Vieira et al., 2022). CAF is an alkaloid present in the leaves, seeds, and fruits of approximately sixty species of plants, where it acts as a chemical defense against herbivores with higher concentrations in juvenile tissues (Ashihara et al., 2017). These concentrations significantly decline as the tissues mature, when the

physical structure becomes the primary defense against herbivores (Harborne, 2001).

CAF, IUPAC name 1,3,7-trimethylxanthine, derives from the purine base xanthine and has three methyl groups (-CH3) and three hydrogen bonds with nitrogen (N-H), which play the role of proton acceptors but lacks a group that acts as a proton donor (Carvalho et al., 2012; Karthika et al., 2013) (Fig. 4).

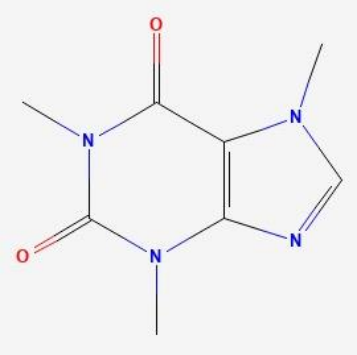

Figure 4. Structure of caffeine (CAF).

CAF is commonly consumed for its stimulating effects on the central nervous system, where it acts through a cAMP-dependent signal transduction mechanism, antagonizing adenosine receptors (Ialongo et al., 2023). The main sources of caffeine consumption include beverages such as tea, coffee, cola-based energy drinks, cocoa-based foods, and herbal products such as guarana (Heckman et al., 2010). It also serves as an additive in numerous food items, including baked goods, ice creams, and soft candies. CAF is often combined with synephrine in various dietary supplements designed for purposes such as weight loss and enhancing sports performance. Additionally, CAF can be found in certain medications due to its bronchodilator action, as well as in cosmetic products. On average, adults are estimated to consume up to 347 mg of CAF per day, with the primary sources being coffee (347 mg/day), followed by tea (111 mg/day), energy drinks (23.5 mg/day), cola (30.1 mg/day), and chocolate (24.9 mg/day) (EFSA, 2015). Coffee has been recognized as the most popular and widely used stimulant by humans (Lawrence et al., 2005). According to the International Coffee Organization (2023), global coffee consumption reached around 10.5 million tons in the year 2021-2022. In the Indo

Pacific region, where human consumption of the green alga *C. cylindracea* is also common, 2 kg of coffee are consumed per capita annually (Utama et al., 2021).

The main enzymatic pathway in human CAF metabolism proceeds through the formation of paraxanthine, a biologically active drug, which leads to the production of metabolites; these are excreted in the urine, thus reaching the wastewater system. Only 2% of total CAF is excreted unchanged through the urine (Grosso and Bracken, 2005). Compared to other metabolites, CAF is considered a highly degradable compound, with a removal rate of more than 95% in wastewater treatment plants (WWTPs) (Buerge et al., 2003), presenting a half-life of up to 240 days in the aquatic environment (Vieira et al., 2022). However, being continuously released from various anthropogenic sources, it is considered one of the most present and pseudo-persistent compounds in water bodies (Buerge et al., 2003). The environmental concentrations of caffeine are in the range of 2–11000 ng/L, with higher values measured in estuaries and coastal waters (Vieira et al., 2022).

Several studies have demonstrated the ecotoxicological impacts of CAF on marine organisms with increases in detoxification mechanisms and damage to the membrane of hemocytes in *M. galloprovincialis* (Capolupo et al., 2016; Munari et al., 2020); increased antioxidant capacity and metabolic activity as well as neurotoxicity and increased expenditure of energy reserves in *Ruditapes philippinarum* (Aguirre-Martinez et al., 2016; Cruz et al., 2016; Piscopo et al., 2021a); lipid peroxidation in *R. decussatus*, *R. philippinarum* and *M. galloprovincialis* (Piscopo et al., 2021b; Cruz et al., 2016; De Marchi et al., 2022); oxidative stress in the polychaetes *Diopatra neapolitana* and *Hediste diversicolor* and in the malacostracan crustacean *Carcinus maenas* (Pires et al., 2016 a, b; Aguirre-Martínez et al. 2013); decreased fertilization rate in *Paracentrotus lividus* (Aguirre-Martínez et al., 2015). Furthermore, bivalves such as *Mytilus spp.*, *Perna viridis* and *Corbicula fluminea* have been found to bioaccumulate the substance with values up to 14 ng/g (Bayen et al., 2016; Burket et al., 2019; Maruya et al., 2014). In general, this contaminant has the potential to induce alterations across various biological levels, especially following chronic exposure. In this case, it can predispose the animals to be more vulnerable to other stress factors. For this reason, given the considerable persistence in the aquatic environment, it is necessary to

extend the studies to the chronic or sub-chronic effects on aquatic organisms and outline the ecological risks connected to them also in association with other threats, such as alien invasive metabolites.

1.4 *MYTILUS GALLOPROVINCIALIS*

The mussel *Mytilus galloprovincialis* (Lamarck, 1819) is a species of bivalve mollusk belonging to the family Mytilidae. The native geographic range includes the Mediterranean Sea, the Black Sea, and the Sea of Azov, but *M. galloprovincialis* is globally distributed along coastal areas with hard substrates (Preda et al., 2012; Wenne et al., 2022). It has been introduced worldwide, probably due to shipping and aquaculture (Branch and Stefanni, 2004; Wonham, 2004).

M. galloprovincialis has huge economic relevance, being extensively cultured and commonly consumed in the region surrounding the Mediterranean Sea, the Atlantic coast, the Black Sea region, and China (Panayotova et al., 2021; Wijsman et al., 2018). For example, considering only the EU, the mussel consumption in 2020 was around 537.212 tons of live weight, with an estimated per capita consumption of 1.20 kg (EUMOFA, European Commission, 2022).

The anatomy of *M. galloprovincialis* is characterized by two mantle portions that secrete two shell valves and cover the visceral mass (Fig. 5). The mantle also contains most of the gonads. Extensions of the mantle form siphons, which facilitate the flow of water: it enters via the inhalant siphon, passes through the gills, and is then expelled through the exhalant siphon. The anterior and posterior adductor muscles control the opening and closing of the shell valves and the foot. The shell has an equivalent purplish-black morphology, with thin concentric circles representing the growth phases of the shell. The dimensions reach up to 13 cm in length. At the base of the foot is the byssus gland, responsible for the formation of the byssus, which consists of filaments that solidify in the water, allowing the fixation of the shell to the substrate. *M. galloprovincialis* is a filter-feeding organism, utilizing its lamellibranch gills for both feeding and respiratory functions.

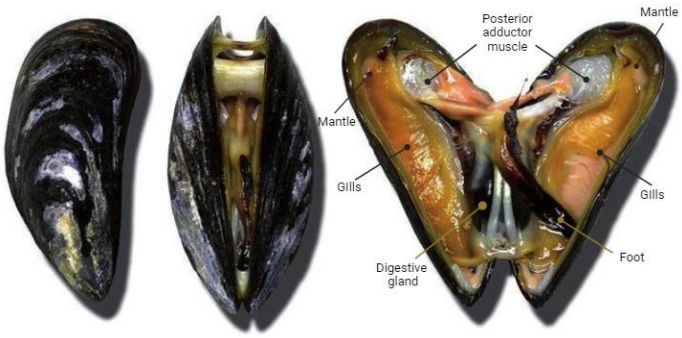

Figure.5 Anatomy of *Mytilus galloprovincialis*, modified from Irkin, 2021

The gills are formed by two curtain-like structures that conform to the shell's curvature, maximizing exposure to inhalant water flow. Each gill is composed of W-shaped (or double V) filaments, and each V is called demi-branch composed of two lamellae: an inner descending lamella and an outer ascending lamella. The space between these lamellae forms the exhalant chamber, connected to the mantle's exhalant area, while the area ventral to the filaments constitutes the inhalant chamber, linked to the mantle's inhalant region. This intricate structure allows the organism to filter plankton and suspended organic particles from the water for feeding. The nervous system of bivalves is fundamentally simple, consisting of three pairs of ganglia and several pairs of nerves (Gosling, 2003).

Frequently, this species is selected as a model organism. Indeed, mussels, particularly *M. galloprovincialis*, are well-known bioindicators due to their sedentary behavior, wide distribution, and high tolerance and serve as effective indicators of environmental pollution due to their tendency to accumulate high levels of toxic compounds. Because of their limited detoxification mechanisms, they are ideal for reflecting pollution levels in their habitats and assessing the effects of toxic compounds under laboratory conditions. Mussels react in a variety of ways to xenobiotics, and the responses can be assessed, for example, through histopathological, inflammatory lesions, oxidant enzymes, antioxidant defenses, mitochondrial function, and membrane stability analyses. Toxicity tests on mussels are inexpensive, quick, and applicable to both retrieved and laboratory-grown specimens (Curpan et al., 2022). In addition, there is a huge literature of reference

regarding their response to external stressors (Capolupo et al., 2016; Coppola et al., 2020, 2022; Freitas et al., 2019, among others).

1.5 BIOMARKERS

Biomarkers, also known as biological markers, are defined as "detectable changes at the cellular, biochemical, or molecular levels within biological media like tissues, cells, or fluids" (Hulka and Garrett, 1993). Over the past few decades, this definition has been expanded to include biological characteristic traits that can be measured objectively and used as indicators of standard biological processes or disease-related processes, as stated by Naylor in 2003. Biomarkers serve as essential instruments in research activities aimed at establishing a connection between specific biological responses and potentially hazardous agents, whether they are of a chemical, physiological, or biological nature, as outlined by the World Health Organization (WHO) in 1993. For this, applying biochemical analysis in bivalves can be useful in understanding the effects of substances of relevant interest. Exposure to stressful conditions can be associated with alterations in the metabolic rate, which can be measured by the activity of the electron transport system (ETS) (De Coen and Janssen, 1997). The activity of ETS is commonly applied as an indicator of metabolic capacity for aquatic invertebrates in response to external disturbances, and it can be used as a marker to predict changes caused by xenobiotics (Freitas et al., 2020). The balance between energy reserves [protein (PROT) and glycogen (GLY) levels] and the activity of ETS is an important tool for determining whether variation in the metabolic capacity can affect an organism's physiological and biochemical performance (Smoulders et al., 2004). For instance, metabolic changes can lead to a depletion of energy reserves with consequences on growth and reproduction processes, while an increment in mitochondrial respiration could conduce to an increase in reactive oxygen species (ROS) (Silva et al., 2021; Teixeira et al., 2017). Other sources of ROS include exposure to ionizing radiation, UV radiation, and environmental pollutants (Aranda-Rivera et al., 2022). The superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) are

the main ROS produced by metabolism (Regoli and Giuliani, 2014). Due to the presence of unpaired electrons, ROS are highly reactive, attempting to extract electrons from nearby molecules to achieve balance. This interaction generates new unstable molecules, starting a cascade that can damage crucial biological macromolecules, leading to oxidative stress (Maltings et al., 2008). To prevent the consequences of oxidative stress, organisms, including bivalves, have evolved complex enzymatic and non-enzymatic antioxidant systems to regulate their redox homeostasis (Valavanidis et al., 2006). Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are three major enzymatic defenses of the antioxidant system (Ighodaro and Akinloye, 2018). The enzyme SOD catalyzes the formation of hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) from the superoxide anion (O_2^-) (Archibald and Fridovich, 1982). CAT is the enzyme that catalyzes the reduction of H_2O_2 into water and O_2 using another H_2O_2 molecule as an electron donor or alcohols, phenols, acids, or formaldehyde (Di Giulio et al., 1989). GPx uses glutathione (GSH), which is oxidized to glutathione disulfide (GSSG), as an electron donor to catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols (Regoli and Giuliani, 2014). GPx activities in invertebrates are notably one to two orders of magnitude lower than those observed in vertebrates. In contrast, the activities of SOD and CAT in invertebrates are comparable or even higher, underscoring their significant role in providing antioxidant protection for aquatic invertebrates (Valavanidis et al., 2006). Then, GSSG is regenerated into GSH through the action of glutathione reductase (GR). Although not classified as a true antioxidant enzyme, GR is indispensable for preserving the appropriate GSH/GSSG ratio and intracellular redox status in marine organisms (Regoli and Giuliani, 2014). Indeed, the GSH/GSSH ratio can be used as an indicator of redox balance in the cells (Dafre et al., 2004). Overall, the study of the activities of SOD, CAT, GPx, and GR provides insights into an organism's response to environmental challenges since they are correlated to oxidative stress (Regoli and Giuliani, 2014).

Furthermore, biomarkers of interest also include carboxylesterases (CbEs) and glutathione S-transferases (GSTs) enzymes, which are involved in the removal of harmful substances through phase I and II biotransformation processes, respectively

(Ribalta et al., 2015). Specifically, CbEs catalyze the hydrolysis of carboxylic esters into their corresponding alcohol and carboxylic acid (Solé et al., 2018), while GSTs promote the conjugation of hydrophobic xenobiotic compounds to the hydrophilic substrate GSH, thus facilitating their elimination (Regoli and Giuliani, 2014).

When cells are unable to effectively counteract ROS, these molecules can initiate an autocatalytic process known as lipid peroxidation (LPO). In LPO, the plasmatic membrane lipids undergo oxidation, producing lipid hydroperoxides (LOOH). The high production of LOOH disrupts normal cellular metabolism, prompting adaptive responses or leading to cell death in severe cases (Regoli and Giuliani, 2014). This process is critical to understanding the impact of oxidative stress on cellular functions and the potential repercussions for organisms facing environmental challenges.

Moreover, biomarkers can assess neurotoxic effects by measuring the activity of acetylcholinesterase (AChE). The activity of AChE is the most sensitive indicator of cholinergic impairment, as it is involved in breaking down acetylcholine released into the synaptic cleft during the transmission of impulses across cholinergic synapses (Beltran and Pocsidio, 2010). For instance, in bivalves, the inhibition of AChE may be responsible for terminating the transmission of the nerve impulse gills, where the activity of AChE is primarily localized (Lionetto et al., 2013).

1.6 METABOLOMICS

Metabolomics is the study of a set of low molecular weight metabolites (metabolome) in a biological sample (Nalbantoglu and Amri, 2019). Since metabolites are normally byproducts of gene expression and cellular regulatory processes, the metabolome is extremely sensitive to environmental changes and can thus provide information about responses to environmental stress, diseases, or exposure to toxicants (Alfaro and Young, 2018). Metabolomics represents a highly applicable approach due to its ability to simultaneously characterize qualitatively and quantitatively organic molecules in a complex mixture, providing insight into the functional status of organisms (Cappello et al., 2019). One of metabolomics' notable strengths is its ability to respond quickly to stressors, which is not always

observed in the transcriptome and proteome (Viant, 2007). As a result, among -omics methodologies, metabolomics emerges as a technology capable of yielding the most functional insights (Viant, 2007). Metabolomics studies can adopt a targeted approach, requiring prior knowledge of the toxicant's metabolic effects for specific metabolite changes, or a non-targeted approach, examining the global metabolome without prior selection of specific metabolic components (Cappello et al., 2019). Nuclear Magnetic Resonance (NMR) spectroscopy is one of the main non-destructive experimental analysis techniques employed in metabolomics due to its exceptional resolution power in capturing metabolites' unique signals (Bingol and Brüschweiler, 2011; Nagana Gowda and Raftery, 2021). NMR can be combined with mass spectrometry (MS) analysis methods for the identification of unknown metabolites (Bingol et al., 2016). Over the last decades, the number of scientific publications in the metabolomics area has increased exponentially (Bingol and Brüschweiler, 2014; Cappello et al., 2019). In addition to being a valuable tool in the biomedical and diagnostic fields, it extends its applications to research in animal sciences, aquaculture, and environmental sciences. (Alfaro et al., 2018; Cappello et al., 2021; Cappello et al., 2017; Cappello et al., 2018; Cappello et al., 2013).

NMR is a spectroscopy technique that exploits the magnetic properties of certain nuclei resulting from their nuclear spin. It is based on the absorption of electromagnetic radiation in the radio frequency region by specific nuclei when placed in a magnetic field (Hammerath, 2012). An NMR spectrometer consists of three major components: a magnet, a radio frequency (RF) transmitter, and an RF receiver. A sample is placed in a tube and then inserted into the magnet. The magnet generates a stable, uniform magnetic field, causing the sample nuclei to align in a specific direction. The RF transmitter generates a radio frequency pulse that excites the nuclei and flips their alignment. Subsequently, a process called "relaxation" begins, during which the nuclei return to their alignment and emit electromagnetic radiation, which is detected by the RF receiver as a time domain emission signal known as the Free Induction Decay (FID). A Fourier transformation converts the FID into a frequency domain spectrum. The difference between a peak's corresponding RF frequency and the reference frequency is known as the "chemical

shift" (Nesbitt, 1992). Chemical shifts and their coupling constants (multiplicity, i.e., the form of a peak) are compared to existing databases for qualitative metabolite identification (Fig. 6).

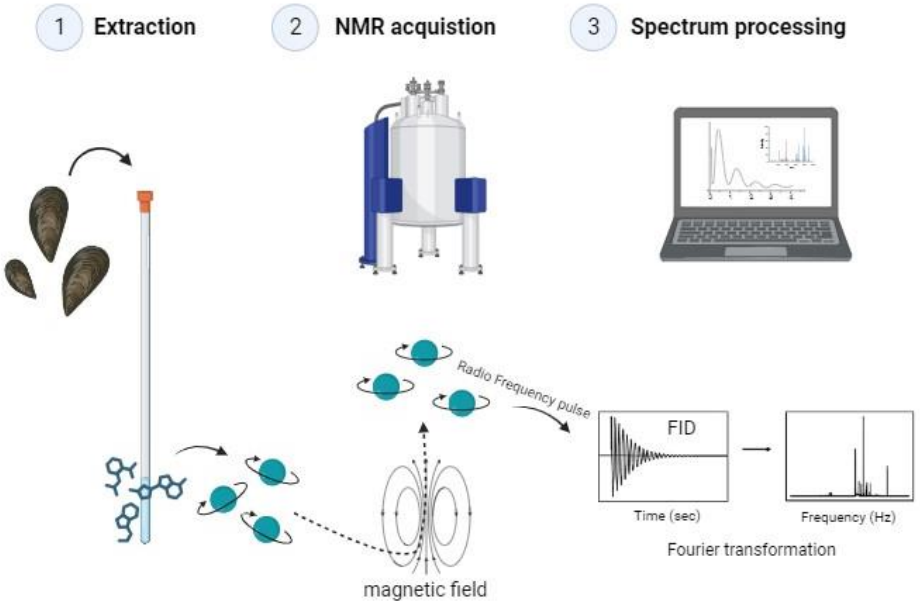

Figure 6. NMR spectra acquisition 1) biological samples are extracted providing a complex metabolite mixture. 2) during NMR acquisition a strong electromagnetic field aligns all nuclei in one direction (spin polarization). A radio frequency pulse excites the nuclei and flips their alignment. Nuclei return to their alignment emitting electromagnetic radiation, which is detected by the RF receiver as a time domain emission signal known as Free Induction Decay (FID) 3) the FID is converted via Fourier-transformation into frequencies (Hz) and yields the final metabolomics spectrum, which can be processed and analyzed for metabolite identification and abundance analyses.

NMR signals can provide both qualitative and quantitative information about each compound (Wang et al., 2021). Relative concentrations of compounds can be calculated by integrating the areas under the signals of an NMR spectrum, and the addition of a known concentration standard allows the determination of absolute concentrations (Paruzzo et al., 2020). NMR metabolomics databases and repositories have grown significantly in recent years, making it easier to identify known compounds (Bingol et al., 2016).

1.7 HISTOPATHOLOGY

Histopathology, which involves studying morphological changes related to pathological and inflammatory processes through histological techniques, is a

potent tool for evaluating the impact of contaminants on bivalves and for the biomonitoring of marine ecosystems (Bignell et al., 2008; Boscolo Papo et al., 2014; Costa et al., 2013; Yavaşoğlu et al., 2016). Histopathology is a good complement to other techniques widely used to investigate aquatic organisms' health (Bignell et al., 2011; Coppola et al., 2020a, b; Joshy et al., 2022; Leite et al., 2020a, b; Lopes et al., 2022; Pinto et al., 2019; Piscopo et al., 2021). Histological alterations can be observed in different organs, and typically, the extent of damage is correlated with the type and dosage of the substance and the duration of exposure. (Cuevas et al., 2015; Rocha et al., 2016). The digestive gland of bivalves is a frequently utilized organ for histopathological assessments, being the most sensitive to stressors (Bignell et al., 2012; Yavaşoğlu et al., 2014). This is because the digestive gland of mollusks, apart from its essential functions such as digestion, absorption, storage of nutrients, and metabolite excretion, serves as the primary organ for metabolizing, accumulating, and detoxifying pollutants (Livingstone et al., 1990). Other organs highly responsive to external hazards are the gills; given their direct exposure to the surrounding environment, they are a primary target for contaminants (Bignell et al., 2012; Rajalakshmi and Mohandas, 2005).

Some of the most common histological changes at the level of the digestive gland are infiltration of hemocytes (related to inflammation), lipofuscin aggregates (sign of oxidative processes), atrophy of the tubules' lumen, and, in the rarest cases, cellular necrosis associated with a complete loss of organization of the digestive tubule's epithelial cells (Newton and Smolowitz, 2007; Canesi et al., 2007; Coppola et al., 2022; Costa et al., 2013). In gills, some of the alterations commonly found are enlargement of the central vessel, hemocyte infiltration (both related to inflammation), lipofuscin aggregates, and cilia erosion (sign of a compromised functionality of the cells) (Costa et al., 2013; Cuevas et al., 2015; Ertürk Gürkan and Gürkan, 2021; Katalay et al., 2016; Pagano et al., 2016).

The observation of these tissues allows the calculation of a histopathological index, which combines various histopathological alterations in the target organs into a single value. This facilitates the interpretation of the health status of bivalves and standardizes the results (Costa et al., 2013; Cuevas et al., 2015).

AIM OF THE THESIS

The present thesis aims to evaluate the ecotoxicological impact of both biomolecules from marine invasive species and chemicals of anthropogenic origin. Among the invasive species, a special focus is placed on the green alga *Caulerpa cylindracea* and the calcareous sponge *Paraleucilla magna*, whose purified metabolites or crude extracts have already shown a panel of biological properties when tested in aquatic animal models. Using a multidisciplinary approach, the study aims to evaluate the effects of the chemical content of the invasive species on the bivalve mollusk *Mytilus galloprovincialis* as a key model organism. A main goal is the examination of the effects of the alkaloid caulerpin from *C. cylindracea*, a metabolite purified at the Institute of Biomolecular Chemistry (National Research Council of Italy), which has shown several biological properties in comparison with anthropogenic contaminants such as fenofibrate and caffeine. As a secondary aim, the study also addresses the toxicological potential of the crude extracts from *P. magna*, whose chemical composition is still unknown in terms of purified metabolites. Biochemical, histopathological, and NMR-based metabolomics analyses after chronic exposure to the above pure compounds and extracts were evaluated. The ultimate objective is to provide valuable insights for a better understanding and management of chemical threats to Mediterranean biodiversity and human health.

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CHAPTER 2

AN ALIEN METABOLITE VS. A SYNTHETIC CHEMICAL HAZARD: AN ECOTOXICOLOGICAL COMPARISON IN THE MEDITERRANEAN BLUE MUSSEL

This chapter is published as:

Tania Russo, Francesca Coppola, Carla Leite, Marianna Carbone, Debora Paris, Andrea Motta, Anna Di Cosmo, Amadeu M. V. M. Soares, Ernesto Mollo, Rosa Freitas, Gianluca Polese (2023). An alien metabolite vs. a synthetic chemical hazard: an ecotoxicological comparison in the Mediterranean blue mussel *Science of the Total Environment*. 892, 164476. **IF 2023 9.8 Q1 Environmental Sciences**. https://doi.org/10.1016/j.scitotenv.2023.164476

ABSTRACT

Bioactive natural products from marine invasive species may dramatically impact native communities, while many synthetic pharmaceutical drugs are released into the marine environment and have long-lasting harmful effects on aquatic life. Sometimes, metabolites from alien species and synthetic compounds share similar mechanisms of action, suggesting comparable ecotoxicological impacts. This applies to the alkaloid caulerpin (CAU) from the green algae *Caulerpa cylindracea*, highly invasive in the Mediterranean Sea, and to the synthetic lipid-lowering drug fenofibrate (FFB), both acting as agonists of peroxisome proliferator-activated receptors (PPARs). Analogies with FFB, which is widely considered hazardous to the aquatic environment, have led to concerns about the ecotoxicological potential of CAU. The problem has implications for public health as CAU is well known to enter the food web accumulating in fish of commercial importance. Here, we compared the effects of FFB and CAU through biochemical and histopathological analysis on a relevant bioindicator molluscan species, the mussel *Mytilus galloprovincialis*. Under laboratory conditions, mussels were fed with food enriched with CAU or FFB. After treatment, biochemical markers were analyzed revealing metabolic capacity impairments, cellular damage, and changes in acetylcholinesterase activity in mussels fed with FFB-enriched food. NMR-based metabolomic studies also showed significant alterations in the metabolic profiles of FFB-treated mussels. In addition, dietary administration of FFB produced morphological alterations in the mussels' gills and digestive tubules. Obtained results confirm that FFB is harmful to aquatic life and that its release into the environment should be avoided. Conversely, dietary treatment with CAU did not produce any significant alterations in the mussels. Overall, our results pave the way for the possible valorization of the huge biomass from one of the world's worst invasive species to obtain CAU, a natural product of interest in drug discovery.

KEYWORDS: invasive species, caulerpin, fenofibrate, *Mytilus galloprovincialis*, metabolomics, biochemical markers

2.1 INTRODUCTION

Biological invasions represent a major driver of ecosystem and biodiversity changes, along with habitat loss, climate change, pollution, and natural resource overexploitation (Caro et al., 2022; Nelson et al., 2006). In particular, the Mediterranean Sea is one of the marine regions most impacted by invasive species, especially due to the opening and ongoing expansion of the Suez Canal (Galil et al., 2018; Katsanevakis et al., 2013). Beyond the several impacts that invasive species can have on native communities (Shea and Chesson, 2002; Simberloff et al., 2013), growing attention in the marine literature is currently also directed to the so-called "alien metabolites": the bioactive molecules that marine invasive species carry in the new environment with potential dramatic ecological effects (Defranoux and Mollo, 2020; Mollo et al., 2015, 2008). A special focus has been placed on the bisindolic red pigment caulerpin (CAU) isolated from the highly invasive green algae *Caulerpa cylindracea*, which accumulates in the tissues of native fish feeding on the exotic alga, thus entering the food chain (Felline et al., 2017, 2014, 2012; Gorbi et al., 2014; Magliozzi et al., 2019, 2017; Raniello et al., 2007; Terlizzi et al., 2011). Evidence has been recently provided for the direct binding and the activation by CAU of the peroxisome proliferator-activated receptors (PPARs) α and γ, which are nuclear transcription factors modulating the expression of genes involved in the regulation of metabolism, behavior, reproduction, cellular differentiation, embryonic development, inflammation, and tumorigenesis (Vázquez-Carrera and Wahli, 2022; Vitale et al., 2018). This seems consistent with a direct involvement of CAU in metabolic and behavioral alterations observed in fish-eating *C. cylindracea* (Del Coco et al., 2018; Gorbi et al., 2014; Magliozzi et al., 2017; Terlizzi et al., 2011). PPAR activation, in fact, has also been associated with reproductive toxicity and endocrine disruptor activity (Nepelska et al., 2017). In the frame of a still open question, however, the harmfulness of CAU has recently been questioned, since it increases fish voracity and reproductive performance when administered via food to *Danio rerio* (zebrafish). The discovery of these properties has led proposing CAU as a possible ingredient to add to aquaculture feed (Schiano et al., 2022). This controversial issue gives urgency to proceed to an effective

comparison of any health impairments induced by CAU in aquatic animal models with those produced by a standard compound of ecotoxicological interest. Moving in this direction, here we evaluated the effects of CAU on the filter-feeder mussel *Mytilus galloprovincialis*, one of the most relevant bioindicator species in the coastal area (Coppola et al., 2020a; Kanduč et al., 2011; Pinto et al., 2019), in comparison with fenofibrate (FFB), a synthetic drug used in the treatment of hypertriglyceridemia, mixed dyslipidemia, hypercholesterolemia, type 2 diabetes and metabolic syndromes (Rosenson, 2008). There are essentially three reasons behind this decision:

- CAU and FFB share the same molecular target (PPARα) having agonist properties, while PPAR homologs were identified in a marine bivalve mollusk (Ran et al., 2021);
- FFB can be effectively chosen for comparative evaluations in ecotoxicology studies since it is released into the coastal waters from wastewater treatment plants (WWTPs) and it is widely considered harmful to aquatic life posing a major threat to aquatic ecosystems (Andreozzi et al., 2003; Du et al., 2008, 2004; Hering et al., 2021; Ido et al., 2017; Isidori et al., 2007; Jung et al., 2021; Rosal et al., 2010). The concentrations of FFB detected in the effluents from WWTPs range from 0.08 ng/L to 0.16 μg/L, reaching 70.3 ng/L in coastal areas (Afsa et al., 2020; Andreozzi et al., 2003; Ido et al., 2017; Solé and Sanchez-Hernandez, 2018; Tete et al., 2020). Moreover, FFB as well as its active form, the fenofibric acid, have been found in groundwater, surface water and drinking water in concentrations up to 1 ng/L (Ido et al., 2017; Jung et al., 2021);
- both CAU and FFB have the potential for bioaccumulation in marine organisms and persistence in the environment. Indeed, FFB has been isolated and quantified in mussels and oysters at concentrations of 0.01 and 0.03 ng/g, respectively (Maskrey et al., 2021), while CAU was found in the tissues of fish, including edible species (Felline et al., 2017; Schiano et al., 2022; Vitale et al., 2018).

Overall, the present study aims at unambiguously clarifying whether CAU can be safely used as a fish feed supplement, opening new and interesting perspectives for the exploitation of the invasive algae *C. cylindracea* in aquaculture,

or whether it should be considered a risk to aquatic life, as has been widely established for FFB. For this purpose, CAU and FFB have been administered to mussels together with suspended particulate. The effects of the compound on *M. galloprovincialis* have been then compared by means of biochemical markers, metabolomics and histopathological analyses, to provide new insights into two of the major sources of biodiversity disturbance in aquatic systems: biological invasions and chemical pollution.

2.2 MATERIALS AND METHODS

2.2.1 SAMPLING OF MUSSELS AND BREEDING CONDITIONS

Mussels *(Mytilus galloprovincialis)* with a mean length of 5.6 ± 0.3 cm and a mean width of 3.4 ± 0.2 cm, were collected in October 2021 in the Ria de Aveiro lagoon, Portugal. After sampling, the bivalves were transported to the laboratory and subjected to a two-weeks period of depuration/acclimation with artificial seawater (salinity 30 ‰, 17 ± 1 °C, pH 8.0 ± 0.1) prepared with reverse osmosis water and artificial salt (Tropic Marin® SEA SALT from Tropic Marine Center), and constant aeration. Seawater was changed each 2-3 days, and mussels were fed with Algamac protein plus (150.000 cells/animal/day) starting from three days after their arrival to the laboratory.

2.2.2 ARTIFICIAL FOOD PREPARATION

Control food was prepared by soaking a combination of microalgae and probiotics (RotiBomb dry food, Algova) in acetone and then evaporating the organic solvent under reduced pressure, while treated food was made in the same manner but after dissolving CAU (1 mg/g dry food) or FFB (1 mg/g dry food) in an equal volume of acetone. Previous studies carried out on fish models (*Diplodus sargus*, *Danio rerio*) have shown significant changes in behavioral, metabolic, and molecular responses when CAU was administered at a concentration of 1mg/g dry food (Del Coco et al., 2018; Magliozzi et al., 2019; Schiano et al., 2022; Vitale et al., 2018). Therefore, the same dose of CAU has been employed in the present study to facilitate comparisons between the effects in vertebrate and invertebrate models. The use of acetone during the food preparation procedure ensured a homogeneous distribution of CAU and FFB (two compounds almost insoluble in water) within the food. In parallel, to guarantee that the preliminary treatment with acetone did not affect the organoleptic properties of the food, plain dry food was also separately administered to mussels.

2.2.3 FEEDING TREATMENTS

A total of 72 mussels were devoted to 4 different feeding treatments, including plain food, control food (CTL), food added with CAU, food added with FFB. For each treatment, three aquaria with 6 mussels each were used for a 28-days chronic dietary treatment. Mussels were fed three times per week. During the whole experiment, temperature, salinity and mortality were monitored daily, and seawater was changed each week.

2.2.4 BIOCHEMICAL MARKERS

After treatment, 3 mussels from each aquarium (9 per treatment) were immediately frozen in liquid nitrogen. Then, the soft tissues were homogenized, and aliquots of 0.5 g fresh weight (FW) were used to perform biochemical analysis. The sample extraction from each aliquot of homogenized tissue was performed by using specific buffers in a proportion of 1:2 (w/v, tissue/buffer) (Coppola et al., 2020, 2018). Samples were sonicated using, TissueLyser II (Qiagen) for 90 s and centrifuged for 20 or 10 min at 10,000 g or 3,000 g depending on the biomarker (Coppola et al., 2020a), at 4 °C. After the samples' centrifugation, about 1 mL of supernatants were collected and stored at -80 °C or immediately used.

The electron transport system (ETS) was selected to assess the metabolic capacity, following the De Coen and Janssen (1997) method. Absorbance was measured during 10 min at 490 nm with intervals of 25 s and the extinction coefficient (Ɛ) of 15,900 $(mol/L)^{-1}cm^{-1}$ was used to calculate the amount of formazan formed and results were expressed in nmol per min per g FW.

To assess energy reserves, glycogen (GLY) and total protein (PROT) contents were measured. For GLY quantification the sulfuric acid method was applied as described by DuBois et al. (1956). Glucose standards at a concentration between 0 and 5 mg/mL were used to obtain a calibration curve. Absorbance was measured at 492 nm after incubation during 30 min at room temperature. The PROT content was measured following the Biuret method (Robinson and Hogden, 1940). The calibration curve was obtained by using bovine serum albumin (BSA) as standards

from 0 to 40 mg/mL and the absorbance was read at 540 nm. Both results were expressed in mg per g FW.

Mechanisms of antioxidant defenses were assessed determining the activity of the superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) enzymes. The activity SOD was analyzed following Beauchamp and Fridovich (1971) method with adaptations accomplished by Carregosa et al. (2014). The standard curve was obtained with SOD standards between 0 and 60 U/mL. The absorbance was read at 560 nm after 20 min of incubation at room temperature. The results were expressed in U (one unit: quantity of the enzyme that catalyzes the conversion of 1 μmol of substrate per min) per g FW. The activity of CAT was quantified according to the Johansson and Borg (1988) method and adaptations accomplished by Carregosa et al. (2014). The standard curve was determined using formaldehyde standards between 0 and 150 μmol/L and the absorbance was read at 540 nm. The results were expressed in U per g FW (one unit: the quantity of enzyme that generates the formation of 1.0 nmol formaldehyde per min). The activity of GR was determined according to Carlberg and Mannervik (1985). Absorbance was measured at 340 nm, during 5 min in intervals of 15 s, using the extinction coefficient (Ɛ) 6,220 $(mol/L)^{-1}$ cm^{-1} and the activity and was expressed in U (oxidation of 1.0 μmol NADPH per min) per g FW.

Mussels' detoxification capacity was evaluated by measuring glutathione S-transferases (GSTs) and carboxylesterases (CbEs) activities. The activity of GSTs was determined based on Habig et al. (1974) method by reading the absorbance at 340 nm during 5 min in intervals of 15 s, with an extinction coefficient Ɛ = 9,600 $(mol/L)^{-1}cm^{-1}$. The activity and was expressed in U per g FW, where U represents the amount of enzyme necessary to catalyze the formation of 1 μmol of dinitrophenyl thioether per min. The activities of CbEs were determined following Hosokawa and Satoh (2001) and using the colorimetric substrates p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB). Absorbance was measured at 405 nm for 5 min in intervals of 15 s and the extinction coefficient (Ɛ) 18,000 $(mol/L)^{-1}cm^{-1}$ was used to determine the activity. The hydrolysis rate of pNPA and pNPB were expressed in nmol per min per g FW.

Redox balance was assessed by calculating the oxidized glutathione (GSSG) content, while cellular damage was investigated through lipid peroxidation levels (LPO) determination. The content of GSSG was determined as described in Rahman (2007) using GSSG as standard at a concentration from 0 to 90 µmol/L. Absorbance was read at 412 nm for 2 min in intervals of 30 s and the GSSG content was expressed in µmol per g FW. LPO levels were measured through the quantification of malondialdehyde (MDA) as reported in Ohkawa et al. (1979). The amount of MDA formed was quantified at an absorbance of 535 nm using the extinction coefficient ε = 156,000 $(mol/L)^{-1}cm^{-1}$. Results were expressed in nmol of malondialdehyde formed per g of FW.

The acetylcholinesterase (AChE) activity was evaluated to assess neurotoxicity following Ellman et al. (1961). The activity was measured using acetylthiocholine iodide (ATChI 5 mmol/L) substrates and reading the absorbance continuously for 5 min at 412 nm. The activity was expressed in nmol per min per g FW.

All biochemical parameters were run in duplicate and analyzed with the use of a microplate reader (Biotek).

2.2.5 NMR SAMPLE PREPARATION AND SPECTRA ACQUISITION

At the end of the exposure assay, three mussels per treatment (one per aquarium) were homogenized in liquid nitrogen and stored at –80 °C and used for NMR analyses. Then, tissues were lyophilized and processed to extract metabolites of interest (e.g., lipids, amino acids, carbohydrates and other small metabolites; see supplementary materials Table 1s).

Table 1s. ^{1}H and ^{13}C chemical shift assignment (δ, ppm) of metabolites found in ^{1}H-TOCSY, ^{1}H-^{13}C-HSQC and ^{1}H-^{13}C-HMBC-NMR spectra of mussel's hydrophilic extracts.

Entry	Metabolite	δ ^{1}H	δ ^{13}C	Group	Entry	Metabolite	δ^{1}H	δ^{13}C	Group
1	β-alanine	2.56 3.20		αCH βCH	16	Acetoacetate	2.25 3.43		γCH_3 αCH_2
2	Isoleucine	0.94 3.66		δCH_3	17	Glycine	3.58	42.20	αCH
3	Leucine	0.96 1.72	22.14	δCH_3 βCH_2	18	Glutamine	2.14 2.46 3.79	31.90	βCH_2 γCH_2 αCH
4	Valine	0.99 1.05	17.09 18.50	γCH_3 $\gamma' CH_3$	19	Succinate	2.41	34.50	$\alpha,\beta CH_2$
5	αGlucose	5.25	93.30	C1H	20	Aspartate	2.68-2.72 2.80-2.84	37.15	βCH β'CH
6	Threonine	1.33 3.61 4.30	20.09	γCH_3 αCH βCH	21	Betaine	3.27 3.91	53.90 67.00	NCH_3 αCH_2
7	Taurine	3.45 3.27	36.55 48.50	$S\text{-}CH_2$ $N\text{-}CH_2$	22	Tyrosine	6.90 7.90	117.00 131.80	C3,5 ring C2,6 ring
8	IMP	4.37 8.23 6.15 4.05	46.80	C8 ring C1H ribose	23	Phenylalanine	7.33 7.39 7.43	130.50 129.70 130.50	C4 ring C2,6 ring C3.5 ring
9	Lysine	1.47 1.72 1.91 3.05 3.80	29.50 39.80	γCH_2 δCH_2 βCH_2 εCH_2 αCH	24	Tryptophan	7.78 7.55 7.30 7.21	118.00 112.00 125.70	C4 ring C7 ring C6 ring C5 ring
10	Alanine	1.48 3.80	16.80 51.10	βCH_3 αCH	25	Histidine	7.79 7.02		C2H ring C4H ring
11	Arginine	1.68 1.93 3.24	25.07	γCH_2 βCH_2 γCH_3	26	Asparagine	2.86-2.90 2.95-2.97 4.02	35.40	βCH βCH αCH
12	Acetate	1.93		βCH_3	27	Choline	3.19	54.27	$N\text{-}CH_3$
13	ATP	6.14 8.55 8.27	88.00 152.40	C1H ribose NH ring C2 ring	28	NAD^{+}	9.33 9.13 8.85 8.42 8.18 6.10 6.04		N2 ring N6 ring N4 ring A8H N5 ring A1H N1H
14	UDP-glc	5.98 7.94	89.70 142.80	C1H ribose C6 ring	29	Homarine	8.72 8.54 8.04 7.99 4.36	145.00 125.00 127.00	C3H C5H C6H C4H $N(CH_3)_3$
30	Glutamate	2.10 2.36 3.79	27.51 34.70	βCH γCH_2 αCH	32	Malate	2.10 2.36	44.16	βCH β'CH
31	Fumarate	6.53		α,βC=C	33	Malonate	3.11		CH2

Combined extraction of polar and lipophilic metabolites was carried out by using methanol/water/chloroform as suggested by Beckonert et al. (2007). Polar and nonpolar fractions were transferred into different glass vials and the solvents were removed by using a rotary vacuum evaporator at room temperature. For NMR

analysis, polar fractions were resuspended in 630 µL of phosphate buffer saline (PBS, pH 7.4), adding 70 µL of $^{2}H_2O$ solution [containing 1 mM sodium 3-trimethylsilyl [2,2,3,3-2H4] propionate (TSP) as a chemical shift reference for ^{1}H spectra] to provide a field frequency lock, reaching 700 µL of total volume. Samples were loaded into the autosampler and NMR spectra were acquired on a Bruker Avance III–600 MHz spectrometer (BrukerBioSpin GmbH, Rheinstetten, Germany), equipped with a TCI CryoProbe fitted with a gradient along the Z-axis, at a probe temperature of 27 °C. In particular, standard 1D proton spectra and 2D experiments (clean total-correlation spectroscopy TOCSY and heteronuclear single quantum coherence HSQC) were acquired providing monodimensional metabolic profiles and homonuclear and heteronuclear spectra for metabolites identification. Metabolites assignments were achieved by comparing signal chemical shifts with literature and online databases. All acquired spectra were automatically reduced down to 500 integral segments of 0.02 ppm each between the 0.50–10.50 ppm spectral region, excluding the water resonance (4.50–5.15 ppm) using the AMIX 3.9.15 software package (Bruker Biospin GmbH, Rheinstetten, Germany). After reducing NMR data, bins were normalized to the total spectrum area. The obtained data format, expressed by a matrix (X matrix), was then imported into SIMCA-P+14 package (Umetrics, Umeå, Sweden) where multivariate statistical analysis was performed.

2.2.6 HISTOPATHOLOGICAL ANALYSIS

One mussel from each aquarium (three per treatment) was used for histological analysis. Mussels were fixed in Davidson's solution and gills and digestive tubules were dehydrated in ascendant ethanol, clarified in methyl benzoate and included in paraffin (Coppola et al., 2022). Sections of 5 µm were obtained with the microtome (Leica Biosystems) and stained with hematoxylin to observe the presence of morphological alterations. Histopathological indices were calculated using the following formula:

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

Where I_h is the histopathological index for the individual h; w_j the weight of the j_{th} histopathological alteration; a_{jh} is the score attributed to the h_{th} individual for the j_{th} alteration and M_j is the maximum attributable value for the j_{th} alteration (in the case in which all the alterations are present at the maximum diffusion). The I_h was determined following the concepts of the differential biological significance of each analyzed alteration (weight) and its diffusion (score). The weights range from 1 (minimum severity) to 3 (maximum severity) while the score varies from 0 (not present) to 6 (diffuse) (Costa et al., 2013). Six pictures from each tissue and sample were randomly taken with a camera (Canon, PowerShot s 50) connected to an optical microscope (Leica, DM RB) through the acquisition tool RemoteCapture, and observed to determine the diffusion score of each analyzed alteration.

2.2.7 STATISTICAL ANALYSES

Biomarkers and histopathological analysis, obtained for each treatment, were submitted to a non-parametric permutational analysis of variance (PERMANOVA + add-on in PRIMER v6) (Anderson et al., 2008). Values lower than 0.05 ($p < 0.05$) were considered significantly different. The null hypothesis tested was, for biomarkers and histopathological indices analysis, no significant differences were observed among treatments (plain food, CTL, CAU, FFB). Because no significant differences were observed between plain food and CTL treatments (see supplementary materials Table 2s), regardless of the biological response, this treatment was not represented in the graphs.

Table 2s. *p* value among plain food and CTL mussels.

	plain food vs CTL
ETS	0.1848
GLY	0.0775
PROT	0.8271
SOD	0.7393
CAT	0.3895
GR	0.0846
GSTS	0.7322
CbEs-A	0.8998
CbEs-B	0.4749
LPO	0.2506
GSSG	0.7836
AChE	0.0555
DT	0.8614
GILLS	0.5017

Multivariate statistical analysis was performed for metabolomic data as first approach, the unsupervised principal component analysis (PCA) was applied to assess class homogeneity, uncover data trends, and detect outliers (data not shown). Then, Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used to visualize class separation, clusters and the spectral variables influencing sample distribution according to the alteration of the metabolic profiles. Data visualization was achieved through scores and loadings plots, which also highlighted specific compounds as putative markers useful for classification. OPLS-DA models were validated by internal iterative cross-validation with 7 rounds of permutation test response (800 repeats), and CV-ANOVA (ANOVA testing of Cross-Validated predictive residuals). Selected isolated signals and bins with $|pcorr| \geq 0.7$ were considered for univariate statistical analysis elaborated with the OriginPro 9.1 software package (OriginLab Corporation, Northampton, USA). Statistical significance for selected metabolites was determined by parametric (ANOVA with Bonferroni correction) or non-parametric (Mann-Whitney U) tests according to the results of the normality test performed on data to evaluate each distribution (Shapiro-Wilk, Kolgomorov-Smirnov test). *P* values < 0.05 were considered as statistically significant.

2.3 RESULTS

2.3.1 BIOCHEMICAL MARKERS

2.3.1.1 METABOLIC CAPACITY AND ENERGY RESERVES

The ETS activity showed significantly higher levels in FFB mussels compared to CTL and CAU treatments (Figure 1A). Regarding GLY content, significantly higher levels were found in FFB organisms compared to the CTL (Figure 1B). Similarly, significantly higher levels of PROT were found in FFB organisms compared to CTL and CAU treatments (Figure 1C).

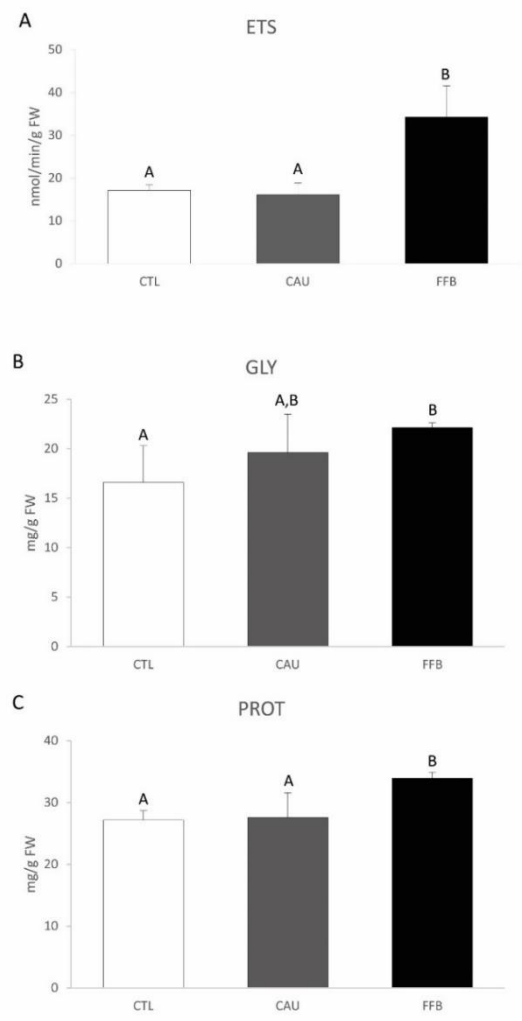

Figure 1. Metabolic capacity and energy reserve biomarkers in mussels treated with caulerpin (CAU) and fenofibrate (FFB) compared to control mussels (CTL). 1A: Electron transport system activity (ETS), 1B: Glycogen content (GLY) and 1C: total protein content (PROT). Results are mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 9$.

2.3.1.2 ANTIOXIDANT DEFENSES AND BIOTRANSFORMATION ISOENZYMES

No significant differences were found among treatments in terms of SOD and GR activities (Figures 2A and B). Regarding CAT, significantly higher activity was observed in CAU and FFB mussels compared to CTL ones (Figure 2C).

Regarding GSTs activity, CAU mussels showed significantly higher GSTs activities compared to the CTL ones (Figure 2D). The activity of CbEs – pNPA and CbEs – pNPB enzymes showed no differences among treatments (Figures 2E and F).

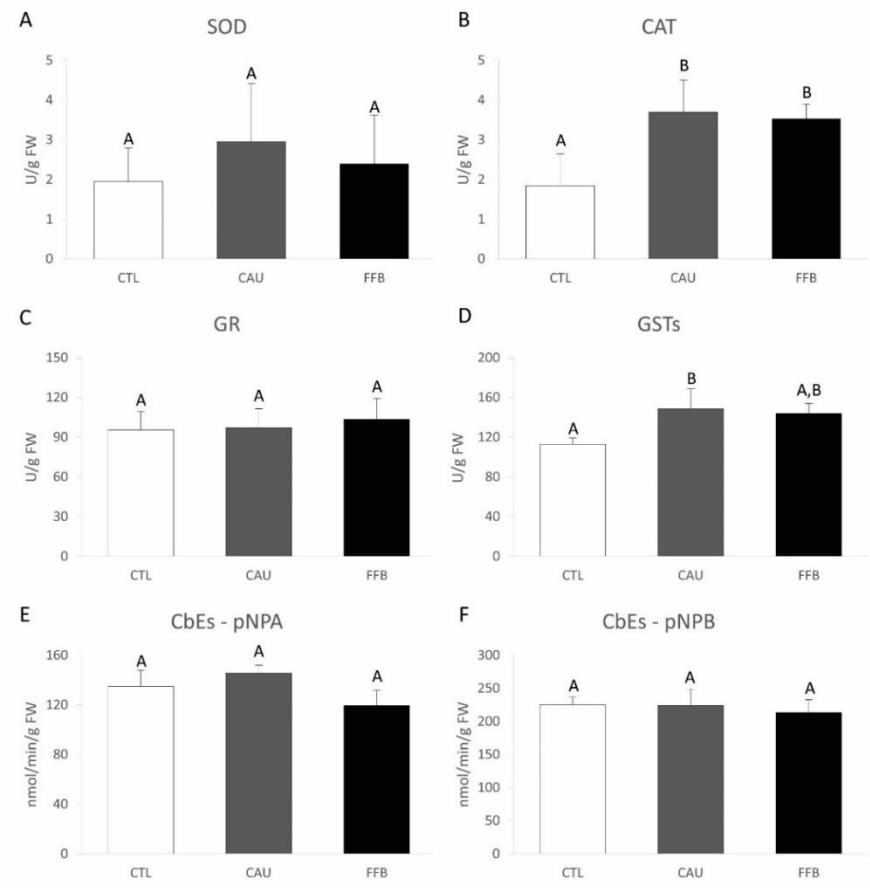

Figure 2. Antioxidant and biotransformation enzyme activities in mussels treated with caulerpin (CAU) and fenofibrate (FFB) compared to control mussels (CTL). 2A: Superoxide dismutase activity (SOD), 2B: Catalase activity (CAT), 2C: Glutathione reductase activity (GR), 2D: Glutathione S-transferases activity (GSTs), 2E: Carboxylesterases pNPA (cBES-pNPA), 2F: Carboxylesterases pNPB (cBES-pNPB). Results are mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 9$.

2.3.1.3 REDOX BALANCE AND CELLULAR DAMAGE

FFB induced a significant increase in GSSG content compared to CTL and CAU treatments (Figure 3A). Similarly, LPO levels showed a significant increment in FFB organisms compared to the CTL mussels (Figure 3B).

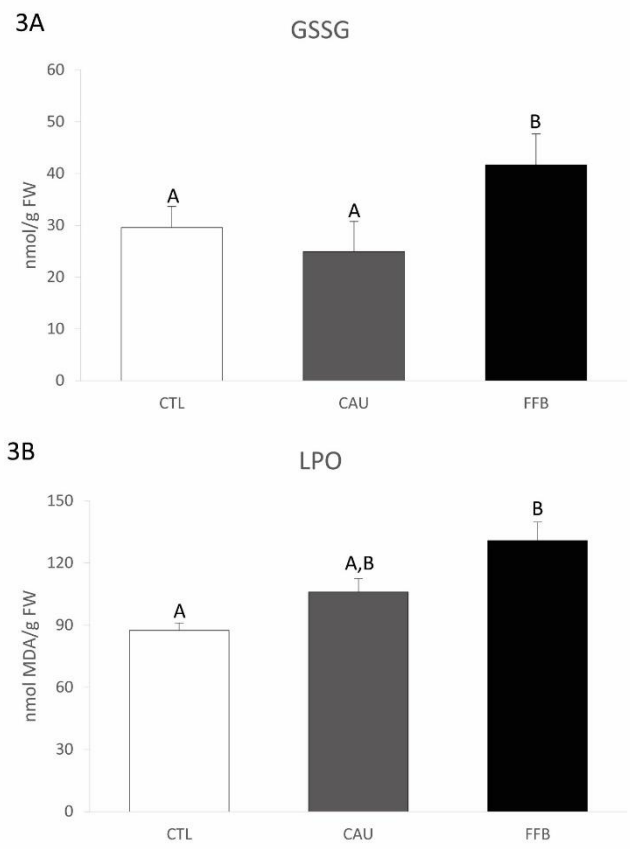

Figure 3. Redox balance and cellular damage biomarkers in mussels treated with caulerpin (CAU) and fenofibrate (FFB) compared to control mussels (CTL). 3A: Oxidized glutathione levels (GSSG), 3B: Lipid peroxidation levels (LPO). Results are mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 9$.

2.3.1.4 NEUROTOXICITY

The activity of AChE was significantly higher in FFB mussels compared to CTL and CAU ones (Figure 4).

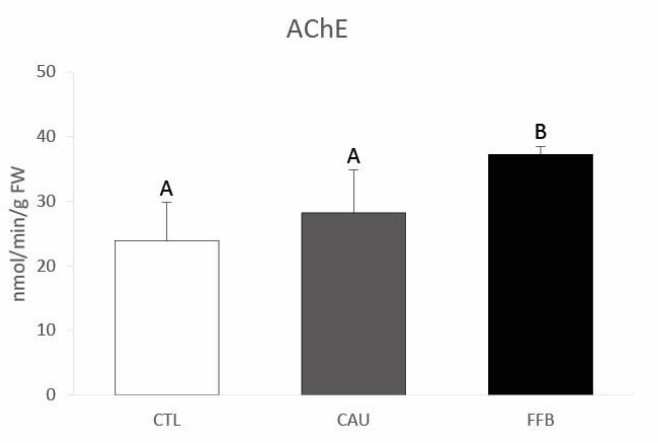

Figure 4. Acetylcholinesterase activity (AChE, neurotoxicity biomarker) in mussels treated with caulerpin (CAU) and fenofibrate (FFB) compared to control mussels (CTL). Results are mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 9$.

2.3.2 NMR-BASED METABOLOMICS

OPLS-DA performed on NMR spectra resulted in one predictive and one orthogonal component with parameters $R^2 = 0.44$ and $Q^2 = 0.001$. The scores plot in Figure 5A shows sample projection onto the principal components. The first component t[1] accounts for the main differences between FFB mussels group at t[1] negative coordinates, and the CTL class, placed at positive t[1], while the CAU category appeared in the middle. The orthogonal component to[1] expresses the intraclass inhomogeneity, mainly due to betaine variation (3.93, 3.29 ppm). The related loadings plot in Figure 5B shows the NMR variables responsible for sample projection and clustering in the model.

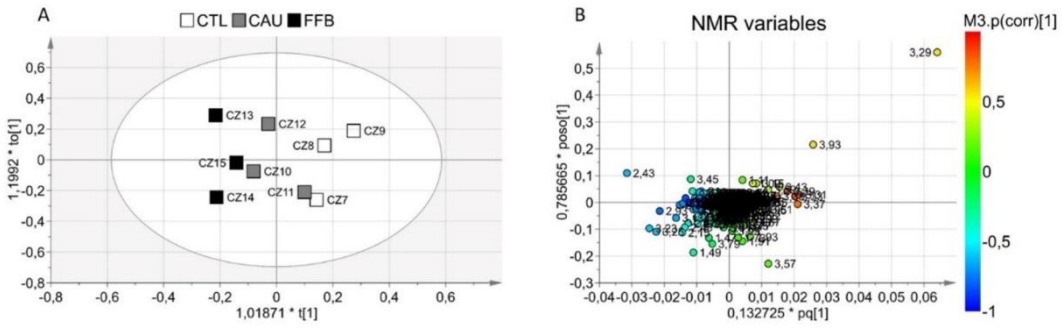

Figure 5. Metabolomics analysis of mussels treated with caulerpin (CAU, grey squares) and fenofibrate (FFB, black squares) compared to control mussels (CTL, white squares). A: Scores plot showing sample projection onto principal components, B: Loadings plot reporting the NMR variables (chemical shift) responsible for clustering in the model.

Assigning metabolites to the variables expressed in the associated loadings plot in Figure 5B, FFB group resulted in significantly higher levels of malate, asparagine, histidine, tryptophan compared to CTL as well as in significantly higher levels of homarine compared to CTL and CAU mussels (Table 1). A considerably higher content of inosine monophosphate was found in CTL compared to CAU and FFB mussels (Table 1). Total fumarate, malonate, choline and glutathione, resulted higher but not significant in FFB mussels (Table 1).

Table 1. Normalized bin mean ± standard deviation of metabolites found in *Mytilus galloprovincialis* after different feeding treatments (CTL, CAU, FFB). Significant differences ($p < 0.05$) among treatments are presented with different letters. $n = 3$.

Metabolites	Normalized bin mean ± standard deviation		
	CTL	CAU	FFB
Malate	$4.25^{-4} \pm 6.98^{-5\ A}$	$6.80^{-4} \pm 1.31^{-4\ A,B}$	$7.44^{-4} \pm 9.87^{-5\ B\uparrow}$
Asparagine	$4.32^{-4} \pm 1.69^{-4\ A}$	$8.55^{-4} \pm 2.78^{-4\ A,B}$	$10.4^{-4} \pm 2.87^{-4\ B\uparrow}$
Histidine	$4.39^{-5} \pm 3.24^{-5\ A}$	$1.29^{-4} \pm 6.26^{-5\ A,B}$	$1.59^{-4} \pm 4.14^{-5\ B\uparrow}$
Tryptophan	$9.91^{-5} \pm 3.13^{-5\ A}$	$1.99^{-4} \pm 2.85^{-5\ A,B}$	$1.93^{-4} \pm 5.53^{-5\ B\uparrow}$
Imp	$8.87^{-7} \pm 5.40^{-7\ A\uparrow}$	$1.47^{-7} \pm 2.37^{-7\ B}$	$4.33^{-8} \pm 3.78^{-8\ B}$
Homarine	$1.48^{-4} \pm 3.94^{-5\ A}$	$2.05^{-4} \pm 2.95^{-5\ A}$	$3.70^{-4} \pm 1.13^{-5\ B\uparrow}$
Fumarate	$3.02^{-6} \pm 2.96^{-6\ A}$	$1.13^{-5} \pm 6.13^{-6\ A}$	$1.24^{-5} \pm 5.87^{-6\ A}$
Choline	$22.6^{-4} \pm 1.91^{-4\ A}$	$29.5^{-4} \pm 8.19^{-4\ A}$	$31.8^{-4} \pm 7.40^{-4\ A}$
Malonate	$12.50^{-4} \pm 1.94^{-4\ A}$	$17.80^{-4} \pm 2.20^{-4\ A}$	$17.70^{-4} \pm 4.63^{-4\ A}$
Gsh	$3.85^{-4} \pm 1.26^{-4\ A}$	$5.32^{-4} \pm 1.76^{-4\ A}$	$6.52^{-4} \pm 9.77^{-5\ A}$

2.3.3 HISTOPATHOLOGICAL INDICES

Histopathological analysis showed a significantly higher histopathological index (I_h) in the gills of FFB mussels compared to CTL (Figure 6A), especially in terms of accumulation of lipofuscin and infiltration of hemocytes (Figure 7).

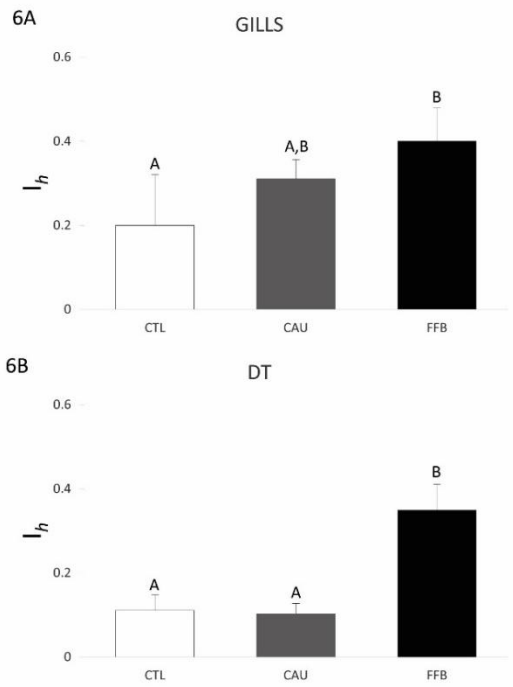

Figure 6. Histopathological indices in mussels treated with caulerpin (CAU) and fenofibrate (FFB) compared to control mussels (CTL). 6A: gills, 6B: digestive tubules (DT). Results are mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 3$.

In digestive tubules FFB induced significantly higher histological alterations compared to CTL and CAU treatments (Figure 6B), in particular, more lipofuscin aggregates and atrophy were found (Figure 7).

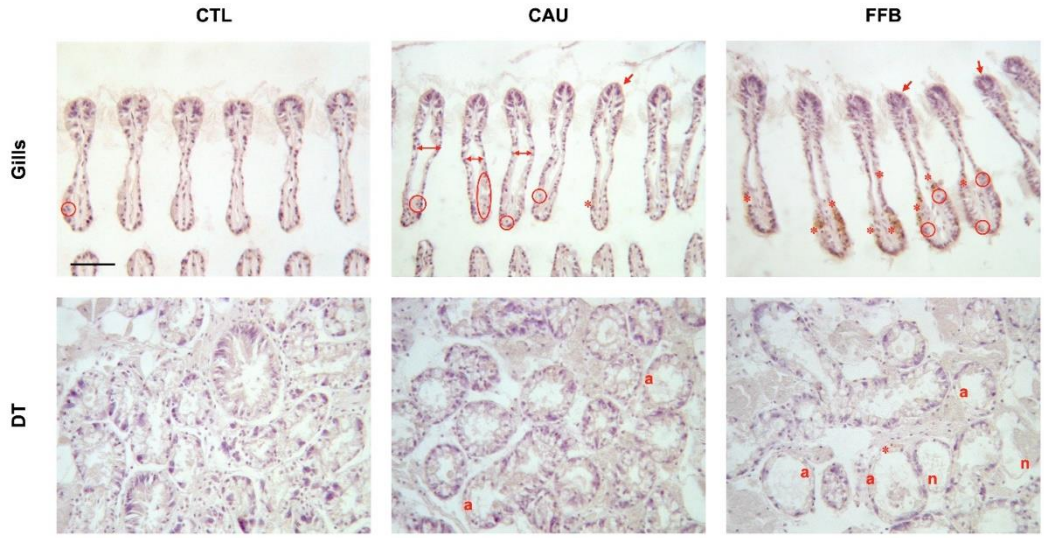

Figure 7. Micrographs of gills and digestive tubules (DT) sections of mussels after different feeding treatments: control (CTL), caulerpin (CAU), fenofibrate (FFB) stained with hematoxylin. (lipofuscin aggregates), arrows (cilia loss), double-headed arrows (enlargement of the central vessel), circles (hemocytes infiltration), a (atrophy), n (necrosis). Scale bar = 50 µm. $n = 3$.

2.4 DISCUSSION

The present study aimed to compare the effects of the natural alkaloid caulerpin (CAU) from the invasive green algae *Caulerpa cylindracea* with those of the synthetic drug fenofibrate (FFB) which is well-known for its ecotoxic potential in the aquatic environment. The two compounds share similar mechanisms of action, both acting as agonists of peroxisome proliferator-activated receptors (PPARs) (Vitale et al., 2018), suggesting comparable toxicological effects. The toxicological evaluation was carried out on the Mediterranean mussel *Mytilus galloprovincialis*, to which the compounds were administered together with food at the concentration of 1 mg/g dry food.

Obtained results showed an FFB-mediated increment in metabolic capacity, measured with high ETS activity. This finding is consistent with the high levels of malate, an intermediate of the KREBS cycle whose activity provides electrons to the ETS (Yi et al., 2015), which were revealed by the NMR-based metabolomic profile of the FFB-treated group. Accordingly, malate was found to accumulate in *M. californianus* under hypoxic stress (Bayne et al., 1976; Connor and Gracey, 2012). Instead, the treatment with CAU did not induce any significant alterations in both ETS activity and malate levels, indicating that CAU does not impact mussels' metabolic capacity. Furthermore, both FFB and CAU-treated mussels showed low levels of inosine monophosphate (IMP), a precursor of adenosine 5′-monophosphate (AMP) and guanosine 5′-monophosphate (GMP) (Lovászi et al., 2021) suggesting an increased purine expenditure during treatments with CAU and FFB. Increased metabolic capacity in mussels treated with FFB was not accompanied by a higher expenditure of GLY and PROT which, in turn, increased content under this treatment. In parallel, the high levels of free amino acids detected in FFB-treated mussels through NMR-analysis are consistent with mussels' effort to produce defensive enzymes under treatment with the drug. Accordingly, Teixeira et al. (2017) proposed that increased protein content in mussels exposed to the antihistamine cetirizine was possibly associated with the induction of defensive mechanisms. Furthermore, increased GLY content was observed in FFB-treated mussels, suggesting that GLY was not the preferential energy reserve used to fuel

up the defense mechanisms of mussels that probably stored energy to fight the stressors (Cunha et al., 2022). In addition, NMR metabolic profiles revealed high levels of homarine, a crucial osmolyte in marine bivalves (Jones et al., 2008), in FFB-treated mussels, as also occurred in *M. galloprovincialis* exposed to cadmium (Wu et al., 2017). This could be related to the fact that osmolytes, among other functions, stabilize proteins (Yancey and Siebenaller, 2015). Overall, the above findings support that bivalves use first lipids to meet their energy requirements when under stress, preserving PROT and GLY levels (Andrade et al., 2018; Velez et al., 2016). This hypothesis meets the literature, where the hypolipidemic activity of FFB was also observed in aquatic organisms (Du et al., 2008, 2004). Among fibrates, the PPAR agonist clofibrate is also known to decrease triglyceride levels in the bivalve *Dreissena polymorpha* (Lazzara et al., 2012). Instead, the lack of alteration in GLY and PROT contents in mussels treated with CAU indicates that the algal alkaloid does not affect mussels' energy reserves.

Since reactive oxygen species (ROS) are commonly associated with oxidative stress and pathologies caused by the oxidation of lipids, proteins, and DNA (Schieber and Chandel, 2014), further comparisons between CAU and FFB included the study of the antioxidant defenses. ROS generation was reported to be induced by FFB in immature rainbow trout hepatocyte cultures (Laville et al., 2004). In normal conditions, ROS levels are balanced by antioxidant defenses, including the enzymes SOD, CAT and GR (Schieber and Chandel, 2014). In the present study, although the administration of food enriched with FFB increased mussel's metabolic capacity, SOD and GR were unaltered, while CAT activity was enhanced in mussels treated with CAU or FFB. This is in line with previous findings showing that PPARα activation increases catalase expression (Shin et al., 2016). A similar result was observed in mice fed with a 0. 1% FFB diet with an increment in CAT activity (Harano et al., 2006). Conversely, Terlizzi et al. (2011) found a negative correlation between CAT and CAU as well as Gorbi et al. (2014) did not find changes in CAT activity in the Mediterranean white sea bream *Diplodus sargus* feeding on *C. cylindracea*. These contradictions can be explained by the fact that above studies were conducted on fish that had consumed *C. cylindracea*, an alga containing various bioactive secondary metabolites beyond CAU. Similarly,

regarding detoxification capacity, CAU treatment enhanced the activity of GSTs, most probably due to its known antioxidant proprieties (De Souza et al., 2009). An enhanced GSTs activity was observed in *D. sargus* fish consuming *C. cylindracea* (Felline et al., 2012), while a study on the same species showed that the consumption of the alga did not affect GSTs activity (Gorbi et al., 2014). Nevertheless, several contradictory responses of GSTs have been observed in organisms depending on the treatment time and concentration (Almeida et al., 2014; Carregosa et al., 2014; Felline et al., 2012).

Scavengers like reduced glutathione (GSH) behave as antioxidants when ROS levels rise in the cells, directly reducing reactive species and being converted to GSSG (Regoli and Giuliani, 2014). In fact, the considerable rise in GSSG levels in FFB-treated organisms suggests that a rise in ROS levels and in glutathione peroxidase activity led to the oxidation of GSH into GSSG. The scarce activation of the antioxidant system in FFB-treated mussels also induced cellular damage, highlighted by an increment in LPO. Similarly, an increase of LPO was observed both in the grass carp *Ctenopharyngodon idella* treated with FFB-enriched food (Du et al., 2008), and in zebrafish exposed to clofibric acid (Rebelo et al., 2020) demonstrating the lipids-oxidation capacity of fibrates. In the present study, treatment with CAU did not affect mussels' redox status and did not produce cellular damage. The low capacity of CAU to induce oxidative stress in mussels is consistent with previous studies showing that CAU does not stimulate ROS release in normal cells, although it was able to induce a significant increase in ROS levels in ovarian cancer cells (Ferramosca et al., 2016).

Despite the fact that AChE activity usually decreases in the presence of a neurotoxic compound (Coppola et al., 2020a; Pinto et al., 2019), FFB induced an increment in the activity of this enzyme as a possible inflammatory response, since AChE increases in inflamed tissues or cells (Rodrigues et al., 2022). Similar results were observed in mussels contaminated with Pb and the increment in AChE activity was interpreted as an attempt to hydrolyze accumulated neurotransmitters in synaptic clefts (Freitas et al., 2019). Conversely, the treatment with CAU did not produce neurotoxic effects in mussels, supporting its safety in bivalve species.

Finally, the application of classical histology techniques evidenced histopathological alterations at the level of the gills and digestive tubules of mussels treated with FFB. The increase in LPO recorded in FFB mussels was confirmed by the histological observations, with an accumulation of lipofuscin in tissues which is associated with the lipidic peroxidation process (Viarengo et al., 1990). Moreover, alterations commonly related to inflammation processes, such as the abundance of hemocytes in gills and the presence of atrophied digestive tubules (Cuevas et al., 2015), were detected in mussels treated with FFB, further confirming the harmfulness of this drug for aquatic species. Although this is the first study assessing histopathological alterations induced in bivalves by FFB and CAU, alterations similar to those induced by FFB were, however, found in bivalves (*M. galloprovincialis, Ruditapes philippinarum* and *R. decussatus*) exposed to other types of contaminants (Hg, sodium lauryl sulfate, lanthanum, caffeine) (Coppola et al., 2020a, 2020b; Pinto et al., 2019; Piscopo et al., 2021a, 2021b). Conversely, CAU did not significantly impact mussels' gills and digestive tubules morphology, compared to untreated mussels. This finding, along with previous studies showing that CAU has beneficial effects on the whole reproductive process in the zebrafish model (Schiano et al., 2022), strongly supports the harmlessness of CAU when administered via food at a concentration of 1mg/g to aquatic animals.

CONCLUSIONS

Our results revealed enhanced metabolic capacity, increased cellular damage, and changes in AChE activity, as well as morphological alterations in gills and digestive tubules after dietary administration of FFB to *M. galloprovincialis*, while no significant impairments were found in CAU-treated mussels. On the one hand, this study confirms that FFB poses serious risks to aquatic organisms. Furthermore, it supports the possible valorization and exploitation of the biomass produced by the green alga *C. cylindracea*, one of the most invasive species along the Mediterranean coasts, to obtain CAU, a non-toxic compound of interest for possible pharmaceutical and nutraceutical applications. Accordingly, CAU has already demonstrated antitumoral and anti-inflammatory properties (Cuomo et al., 2021;

De Souza et al., 2009; Yu et al., 2017). However, future challenging studies are needed to elucidate the details of the molecular pathways involved in the effects of CAU in the chosen molluscan model compared to those observed in vertebrates, in which PPAR agonists, such as FFB, regulate different crucial biological processes, including inflammation and tumorigenesis (Augimeri et al., 2020; Jin et al., 2023; Lian et al., 2018; Murphy and Holder, 2000; Vázquez-Carrera and Wahli, 2022).

ACKNOWLEDGEMENTS

We acknowledge financial support to CESAM by FCT/MCTES (UIDP/50017/2020+UIDB/50017/2020+ LA/P/0094/2020), through national funds. The chemical study performed at ICB/CNR was supported by the "National Biodiversity Future Center" (CN00000033), theme "Biodiversity", funded under the National Recovery and Resilience Plan (PNRR) (Mission 4, Component 2 Investment 1.4) supported by Next Generation EU. Francesca Coppola and Carla Leite benefit from a PhD grant (SFRH/BD/118582/2016 and 2020.05296. BD respectively).

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CHAPTER 3

EXPLORING TOXICOLOGICAL INTERACTIONS IN A CHANGING SEA: THE CASE OF THE ALKALOIDS CAFFEINE AND CAULERPIN

This chapter is published as:

Tania Russo, Francesca Coppola, Debora Paris, Lucia de Marchi, Valentina Meucci, Andrea Motta, Marianna Carbone, Anna Di Cosmo, Amadeu M. V. M. Soares, Carlo Pretti, Ernesto Mollo, Rosa Freitas, Gianluca Polese (2024). Exploring toxicological interactions in a changing sea: The case of the alkaloids caffeine and caulerpin. *Sci. Total Environ*. 912, 169190. **IF 2023 9.8 Q1 Environmental Sciences** https://doi.org/10.1016/j.scitotenv.2023.169190

ABSTRACT

The bisindolic alkaloid caulerpin (CAU) is a bioactive compound isolated from green algae of the genus *Caulerpa* that are highly invasive in the Mediterranean Sea. On the other side, the purine alkaloid caffeine (CAF) is one of the most globally consumed psychoactive substances and a widespread anthropogenic water pollutant. Both compounds display a large panel of biological properties and are well known to accumulate in the tissues of aquatic organisms and, in certain circumstances, co-occur in the human diet. On this premise, the present study aimed to investigate possible synergistic interactions between CAU and CAF by using the bivalve *Mytilus galloprovincialis* as a model organism. Mussels were exposed to CAF via medium while they were fed with food enriched with CAU. After treatments, biochemical analysis confirmed the toxic potential of CAF, with increased AChE activity and lipid peroxidation. Also, histopathological alterations were observed in the gills and digestive tubules. The NMR-based metabolomics analysis detected higher levels of free amino acids under CAF treatments. Conversely, the food administration of CAU did not affect the above toxicological biomarkers. In addition, we did not observe any cumulative effect between CAF and CAU toward increased cellular damage and neurotoxicity. On the other hand, a possible action of CAU in decreasing CAF toxicity could be hypothesized based on our results. This hypothesis is supported by the activity of CAU as an agonist of peroxisome proliferator-activated receptors (PPARs). PPARs mediate xenobiotic detoxification via cytochromes P450, which is involved in CAF metabolism. Overall, the results obtained not only rule out any cumulative adverse effects of CAF and CAU but also encourage further research to evaluate the possible use of CAU, a compound easily obtained through the valorization of biomass from invasive species, as a food additive to improve the clearance of xenobiotics.

KEYWORDS: chemical hazards, biomarkers, NMR-based metabolomics, histopathology, bivalves

3.1 INTRODUCTION

The Mediterranean Sea is constantly threatened by increasing chemical pollution related to human activities (Brumovský et al., 2017; Martínez-Megías and Rico, 2022). Among the compounds released along coastlines, the alkaloid caffeine (CAF) is ubiquitously diffused in marine environments as an emerging anthropogenic contaminant of concern (Paíga and Delerue-Matos, 2017; Quadra et al., 2020). CAF is one of the most consumed psychoactive substances in the world (Temple et al., 2017), and it is contained in several foods, medicines, and beverages (including coffee, tea, soft drinks, and energy drinks) (Afsa et al., 2020; Ferreira et al., 2005). The average daily consumption of CAF per person is estimated between 70 and 400 mg (Burge and Raches, 2003; Rigueto et al., 2020). Although CAF consumption seems to be safe up to 400 mg per day in healthy adults, concerns about the potentially harmful effects of lower doses in healthy pregnant women and children have been raised (Wikoff et al., 2017). Furthermore, there is evidence that caffeine intake at levels below what is generally considered toxic can have fatal effects on people with liver disease or cardiac conditions and poor metabolizers (Musgrave et al., 2016). In addition, evidence of the synergistic effects of CAF in association with various conventional drugs has been provided (Ialongo et al., 2023).

The most known sources of CAF in the aquatic environment are waste-water treatment plants (WWTPs), improper disposal of pharmaceutical products containing CAF, manufacturing plant wastes, and hospital trash (Vieira et al., 2022). In general, WWTPs are effective at removing CAF from wastewater, with removal rates up to 99.5% (Li et al., 2020). However, due to its widespread and increasing consumption, the amount of CAF entering aquatic environments is superior to the WWTP's degradation capacity (Quadra et al., 2020; Vieira et al., 2022). According to several studies (Afsa et al., 2020; Ali et al., 2017; Brumovský et al., 2017; French et al., 2015; Nödler et al., 2020; Paíga and Delerue-Matos, 2017; Silva et al., 2014), the concentration of CAF found in seawater samples and estuarine systems varies generally from nanograms to micrograms per liter. Additionally, bivalves and edible fish have been found to accumulate CAF in their

tissues (Bayen et al., 2016; Burket et al., 2019; Maruya et al., 2014; Lan et al., 2019; Li et al., 2020), supporting the bioaccumulation potential of CAF along the trophic chain. As a matter of concern, several studies have shown the ecotoxicological impacts of CAF on marine organisms, including mollusks, annelids, crustaceans, and echinoderms (Aguirre-Martinez et al., 2013; 2016; 2015; Capolupo et al., 2016; Cruz et al., 2016; De Marchi et al., 2022; Munari et al., 2020; Pires et al., 2016a, 2016b; Piscopo et al., 2021a, 2021b). CAF can therefore be considered an emblematic example of the anthropogenic chemical hazards to which the marine environment is exposed.

In parallel, marine ecosystems are also exposed to bioactive molecules produced by invasive species (Mollo et al., 2008, 2022). This issue is especially relevant for the Mediterranean Sea, which is one of the most invaded seas in the world, with hundreds of allochthonous species recorded along its coasts (Galil et al., 2014; Tsiamis et al., 2020, 2018; Tsirintanis et al., 2022). Among allochthonous species, the green alga *Caulerpa cylindracea* is known to contain the bisindolic alkaloid caulerpin (CAU), which has drawn the attention of the scientific community for its ecological impacts on native communities (Defranoux et al., 2022). Indeed, CAU exhibited several biological properties (De Souza et al., 2009; Defranoux and Mollo, 2020), including its ability to positively affect fish voracity and reproduction (Schiano et al., 2022). This latter finding could explain why native fish have changed their alimentary habits by including *C. cylindracea* as a major food item in their diet (Felline et al., 2012, 2017, 2014; Gorbi et al., 2014; Noè et al., 2018; Terlizzi et al., 2011).

Besides the various combined environmental impacts that we can hypothesize for CAF and CAU, it is worth mentioning that, in particular circumstances, the two compounds also co-occur in the human diet. Indeed, edible species of *Caulerpa*, collectively called “sea grapes” and containing relatively high levels of CAU, are largely consumed as food in Indo-Pacific and Caribbean regions (Vitale et al., 2018), where CAF dietary uptake is also high.

On these premises, the present study aims to explore possible toxicological interactions between CAU and CAF that could occur both in the ecological and nutraceutical fields, fully adhering to the holistic One Health Vision, in which

ecosystem health and human health are inextricably linked. The bivalve *Mytilus galloprovincialis* was selected as a model organism due to its extensive use in ecotoxicological studies and high sensitivity to chemical stressors (Capolupo et al., 2016; Coppola et al., 2020, 2022; Leite et al., 2023, 2020b). Mussels were fed with food implemented with CAU, either with or without CAF in the seawater, and biochemical, NMR-based metabolomics, and histopathological analyses were performed.

3.2 MATERIALS AND METHODS

3.2.1 SAMPLING OF MUSSELS AND MAINTENANCE CONDITIONS

Mussels *(Mytilus galloprovincialis)* with a mean length of 5.6 ± 0.3 cm and a mean width of 3.4 ± 0.2 cm, were collected in October 2021 from the Ria de Aveiro lagoon, Portugal. After sampling, the mussels were transported to the laboratory, where they were subjected to depuration/acclimation in artificial seawater (salinity 30, 17 ± 1 °C, pH 8.0 ± 0.1) prepared with reverse osmosis water and artificial salt (Tropic Marin® SEA SALT from Tropic Marine Center), and constant aeration. Seawater was changed twice per week, and mussels were fed with Algamac protein plus (150,000 cells/animal/day) 3 days after their arrival at the laboratory and then every 2-3 days.

3.2.2 ARTIFICIAL FOOD PREPARATION

Control food was prepared by soaking a combination of microalgae and probiotics (RotiBomb dry food, Algova) in acetone (Sigma-Aldrich, suitable for HPLC, ≥99.9%) and then evaporating the organic solvent under reduced pressure, while treated food was made in the same manner but after dissolving caulerpin in an equal volume of acetone. The dose of CAU (1 mg/g dry food) was adopted according to Russo et al. (2023). This procedure led to a uniform mixture of CAU with the food since it is almost insoluble in water.

3.2.3 EXPERIMENTAL TREATMENTS

A total of 72 mussels were employed in this study and subjected to two different feeding conditions: control food and food enriched with CAU. The same quantity of food employed during the acclimation period has been administered daily during the treatments. In the latter case, each mussel received an estimated total amount of 2.8 µg (0.1 µg daily) of CAU.

Both feeding conditions were tested in the presence and absence of caffeine (CAF) spiked into the seawater medium at a concentration of 3 µg/L. For each of the four treatments (CTL, CAF, CAU, CAU + CAF), three tanks with six mussels each were employed for chronic treatment (28 days). Mussels were fed three times per week. At the same time, to assess the equivalence of the two types of food employed, the Algamac food used during acclimation was also administered separately to 18 mussels (*p*-values are shown in supplementary material, Table S1). During the whole chronic experiment, temperature, salinity, and mortality were monitored daily, and seawater was changed each week.

Table S1. *p* value among analysis performed on mussels fed with Algamac, the same food using during the acclimatation period, and CTL mussels after the 28 days exposure.

	Algamac *vs.* CTL
ETS	0.2309
GLY	0.2176
PROT	0.9265
SOD	0.8885
CAT	0.3521
GRed	0.1152
GSTS	0.9644
CbEs-A	0.4284
CbEs-B	0.4242
LPO	0.6812
GSSG	0.7296
AChE	0.3017
DT	0.4471
GILLS	0.3942

3.2.4 CAFFEINE QUANTIFICATION IN SEAWATER AND ORGANISMS

For the quantification of CAF in both water and tissue samples, chromatographic analyses were performed using an HPLC system with a PerkinElmer Series 200 variable flow pump coupled to a PerkinElmer UV-VIS detector (PerkinElmer), which was set at 272 nm. The system was controlled by a PerkinElmer interface module (NCI 900 Network Chromatography Interface), and chromatograms were processed by PerkinElmer TotalChrom Navigator software. Separation was carried

out on a 250 × 4.6 mm chromatographic column X-Bridge C18 5 μm (Waters) at room temperature. The mobile phase consisted of an acetonitrile-water solution (20:80, isocratic) at a flow rate of 0.8 mL/min, and an injection volume of 100 μL was used.

CAF concentration in the medium was determined by sampling 50 mL of seawater (n = 3) from each aquarium immediately after spiking and before each weekly medium renewal. The quantification and extraction analyses were already described by De Marchi et al. (2022). Briefly, the solid phase extraction (SPE) procedure was performed by using Oasis HLB Cartridges 6 cc extraction columns. Before extraction, each HLB cartridge was pre-conditioned with methanol and rinsed with deionized water. The water sample was passed through the HLB-cartridge at 1 mL/min flow rate. When the extraction was completed, the cartridge was washed with water and subsequently air-dried under a vacuum. The CAF was then eluted from the cartridge with 3 mL of methanol. The extract was completely evaporated to dryness with a stream of nitrogen at a temperature of 50 ◦C. The residue was redissolved with 500 μL of mobile phase and injected into HPLC. The detection limit was 0.02 μg/L, and the recovery was >80%. No blanks (aquaria without mussels) were used in this study since the stability of CAF in the medium was already demonstrated by the present research group (De Marchi et al., 2022; Piscopo et al., 2021a, 2021b).

At the end of the exposure time, three organisms *per* treatment were sampled and immediately frozen for CAF quantification in tissue. The method was already described in De Marchi et al. (2022). About 2 g of each sample was weighed into a centrifugal tube and added to dichloromethane-ethyl acetate (50:50%, v/v). The sample was shaken, extracted with ultrasonic, and then centrifuged at 4000 rpm for 10 min. The supernatant was collected, and the extraction procedure was repeated. The total supernatant obtained from the two extractions was dried under nitrogen flow at a temperature of 50 ◦C. The residue was redissolved with 500 μL of mobile phase and injected into HPLC. The detection limit was 0.1 ng/g for soft tissues and the recovery was >75%. CAF concentration in seawater was expressed in μg/L, while in mussels' tissues it was expressed in ng/g for fresh weight (FW).

3.2.5 BIOCHEMICAL MARKERS

At the end of the experiment, three mussels from each aquarium (nine per treatment) were immediately sacrificed in liquid nitrogen to preserve enzymatic activities. Then, the soft tissues were homogenized using a pestle and mortar in liquid nitrogen, and aliquots of 0.5 g FW were used to evaluate biochemical markers. Homogenized tissues were disrupted using a TissueLyser II (Qiagen) system for 90 s after adding specific buffers in a ratio of 1:2 (tissue/buffer) and subjected to centrifugation for 20 min at 10,000 *g* or 3,000 *g* (depending on the biomarker) at 4 °C for sample extraction (Coppola et al., 2020). Supernatants were kept at -80 °C or directly employed. Biomarkers associated with the metabolic capacity [electron transport system (ETS) activity]; energy reserves [(glycogen (GLY) and total protein (PROT) contents)]; oxidative stress [superoxide dismutase (SOD), glutathione reductase (GR) and catalase (CAT) activities; oxidized glutathione (GSSG) content]; biotransformation capacity [glutathione S-transferases (GSTs) and carboxylesterases (CbEs) activities]; cellular damage [lipid peroxidation (LPO) levels] and neurotoxicity [acetylcholinesterase (AChE) activity] were evaluated applying spectrophotometric methods.

The activity of ETS was assessed following the De Coen and Janssen (1997) method, and results were expressed in nmol per min per g FW.

Regarding energy reserves, GLY and PROT contents were measured with sulfuric acid (DuBois et al., 1956) and the Biuret methods (Robinson and Hogden, 1940), respectively. Both results were expressed in mg per g FW.

The activity of SOD was evaluated following the Beauchamp and Fridovich (1971) method, and the results were expressed in U (one unit: quantity of the enzyme that catalyzes the conversion of 1 μmol of substrate per min) per g FW. The activity of GR was defined following Carlberg and Mannervik (1985) and was expressed in U (oxidation of 1 μmol NADPH per min) per g FW. The activity of CAT was assessed according to the Johansson and Borg (1988) method with modifications from Carregosa et al. (2014), and the results were expressed in U per g FW (one unit: the quantity of enzyme that generates 1 nmol formaldehyde per min).

The activities of CbEs were determined by applying the Hosokawa and Satoh (2001) method to the substrates p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB). The hydrolysis rate of the substrates was expressed in nmol per min per g FW. The activity of GSTs was evaluated according to the Habig et al. (1974) method and was expressed in U (amount of enzyme necessary to catalyze the formation of 1 μmol of dinitrophenyl thioether per min) per g FW.

The LPO levels were tested as described by Ohkawa et al. (1979) and expressed in nmol of malondialdehyde formed per g of FW.

The content of GSSG was defined as reported in Rahman (2007) and was expressed in nmol per g FW.

The method of Ellman et al. (1961) was applied to assess AChE. The activity was expressed in nmol per min per g FW.

To ensure the reproducibility of the results, all biochemical parameters were run in duplicate in a microplate reader (Biotek).

3.2.6 NMR SAMPLE PREPARATION AND SPECTRA ACQUISITION

Three mussels per treatment were homogenized in liquid nitrogen and stored at -80 °C. Then, tissues were lyophilized and processed to extract metabolites of interest (e.g., lipids, amino acids, carbohydrates, and other small metabolites). The combined extraction of polar and lipophilic metabolites was carried out using methanol/water/chloroform, as suggested by Lindon et al. (2005). Polar and nonpolar fractions were transferred into different glass vials, and the solvents were removed using a rotary vacuum evaporator at room temperature. For NMR analysis, polar fractions were resuspended in 630 μL of phosphate buffer saline (PBS, pH 7.4), adding 70 μL of $^{2}H2O$ solution [containing 1 mM sodium 3-trimethylsilyl [2,2,3,3-2H4] propionate (TSP) as a chemical shift reference for ^{1}H spectra] to provide a field frequency lock, reaching 700 μL of total volume. Samples were loaded into the autosampler, and the NMR spectra were acquired on a Bruker Avance III–600 MHz spectrometer (BrukerBioSpin GmbH, Rheinstetten, Germany), equipped with a TCI CryoProbe fitted with a gradient along the Z-axis, at a probe temperature of 27 °C. In particular, standard 1D proton spectra and 2D

experiments [clean total-correlation spectroscopy (TOCSY) and heteronuclear single quantum coherence (HSQC)] were acquired providing 1D metabolic profiles and homonuclear and heteronuclear 2D correlation spectra for metabolites identification. The chemical shifts of identified metabolites were compared with literature and online databases. All 1D spectra were processed and data were automatically reduced in bins, arranged as a data matrix and imported for multivariate statistical analysis with projection methods.

3.2.7 HISTOPATHOLOGICAL ANALYSIS

One mussel from each aquarium (three per treatment) was fixed in Davidson's solution immediately after the experimental period for histological analysis. After 24 h of fixation, mussels were washed daily in 70% ethanol to remove the fixative. Then, gills and digestive tubules were dissected and dehydrated in ascendant ethanol, clarified in methyl benzoate, and included in paraffin (Coppola et al., 2022; Leite et al., 2023). The tissues were cut with a microtome (Leica) into 5 µm sections and stained with hematoxylin to observe the presence of histological alterations (Leite et al., 2020a). The histopathological index (I_h) was calculated following the concepts of the differential biological significance of each analyzed alteration and its degree of diffusion, according to Costa et al. (2013). Six pictures from each sample (both for gills and digestive tubules) were randomly taken with a camera (Canon, PowerShot s 50) connected to an optical microscope (Leica, DM RB) through the acquisition tool RemoteCapture (Canon, 2.7.4.23) and observed to determine the diffusion degree (from 0 to six) of each analyzed alteration.

3.2.8 STATISTICAL ANALYSES

The CAF concentration in the medium and tissues, biomarkers, and histopathological analysis, obtained for each treatment, were submitted to a non-parametric permutational analysis of variance (PERMANOVA + add-on in PRIMER v6) (Anderson et al., 2008). Values lower than 0.05 ($p < 0.05$) were

considered significantly different. The null hypothesis tested considered, for each response (CAF concentration in the medium and tissues, biomarkers, and histopathological analysis), no significant differences among treatments (CTL, CAF, CAU, CAU + CAF).

To quantify a distinct trend in the metabolic evolution under different treatments, univariate statistical analysis was applied to bins of discriminant metabolites. All acquired 1D NMR spectra were automatically reduced to 500 integral segments of 0.02 ppm each between the 0.50-10.50 ppm spectral region, excluding the water resonance (4.50-5.15 ppm) using the AMIX 3.9.15 software package (Bruker Biospin GmbH, Rheinstetten, Germany). After reducing NMR data, bins were normalized to the total spectrum area. The obtained data format, expressed by a matrix (X matrix), was then imported into the SIMCA-P+14 package (Umetrics, Umeå, Sweden) to perform multivariate statistical analysis discriminating mussels' different responses to experimental treatments according to their NMR profiles. In particular, unsupervised principal component analysis (PCA) was applied to assess class homogeneity, uncover data trends, and detect outliers. Then, Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used to visualize class separation, clusters, and the spectral variables influencing samples' distribution according to the alteration of the metabolic profile after CAF, CAU, and their combined administration. Data visualization was achieved through scores and loadings plots, which also highlight specific compounds as putative markers useful for classification. OPLS-DA models were validated by internal iterative cross-validation with 7 rounds of permutation test response (800 repeats). Selected and isolated discriminant signals with bins with $|pcorr| \geq 0.7$ were considered for univariate statistical analysis elaborated with the OriginPro 9.1 software package (OriginLab Corporation, Northampton, USA) and R software [R core team (https://www.r-project.org/)]. Statistical significance for selected metabolites was determined by a parametric ANOVA test with Fisher LSD correction for multiple comparisons, after assessing the Gaussian data distribution with a normality test (Shapiro-Wilk, Kolgomorov-Smirnov test). P values < 0.05 were considered statistically significant.

3.3 RESULTS

3.3.1 CAFFEINE QUANTIFICATION IN SEAWATER AND ORGANISMS

Caffeine (CAF) concentrations in seawater after spiking were similar to nominal concentrations, demonstrating the effective exposure of the mussels to this contaminant, as shown in supplementary materials (Table S2). Before the seawater renewal, the CAF concentrations in seawater from CAF treatments (CAF; CAU + CAF) were below the limit of quantification (LOD = 0.02 µg/L) (Table S2).
Mussels exposed to CAF (CAF; CAU + CAF) accumulated this compound in their tissues, with no differences among treatments (Table S2).

Table S2. Caffeine concentration in CAF and CAU + CAF treatments obtained for the exposure medium (collected at the beginning and end of the exposure weeks) and in *Mytilus galloprovincialis* tissues (collected at the end of the experiment). Significant differences ($p < 0.05$) among the conditions are presented with different letters. $n = 3$

Conditions	Beginning	End
Seawater (µg/L)		
CAF	2.92 ± 0.08	< LOD
CAU + CAF	2.83 ± 0.06	< LOD
***Mytilus galloprovincialis* tissue (ng/g FW)**		
CAF	-	5.00 ± 0.09[a]
CAU + CAF	-	4.93 ± 0.04[a]

3.3.2 BIOCHEMICAL MARKERS

Significantly higher ETS activity was observed in mussels exposed to CAU + CAF in comparison to CTL and CAU (Figure 1A). Organisms exposed to CAU + CAF showed significantly higher GLY levels compared to CTL (Figure 1B). No significant differences were detected in PROT content among treatments (Figure 1C).

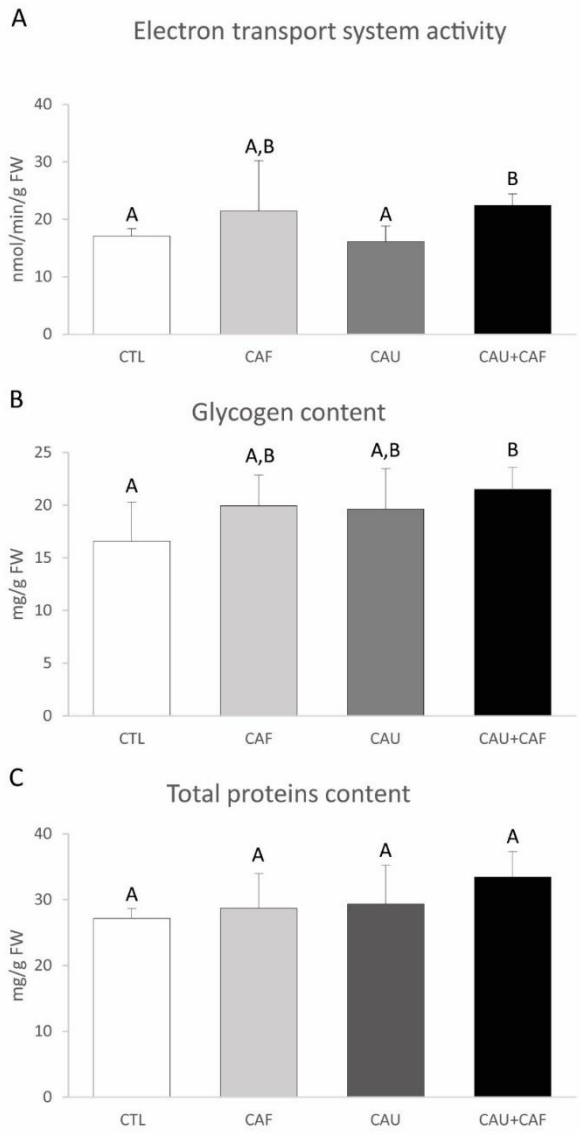

Figure 1. Metabolic capacity and energy reserve biomarkers in *Mytilus galloprovincialis* under different treatments (CTL, CAF, CAU, CAU + CAF) at the end of the experiment. A: Electron transport system activity (ETS), B: Glycogen content (GLY) and C: total protein content (PROT). Results are mean + standard deviation. Significant differences ($p < 0.05$) among the treatments are presented with different letters. $n = 9$.

The activities of SOD and GR showed no significant differences among treatments (Figures 2A and B). Significantly higher CAT activity was found in CAU compared to CTL and CAF treatments. In addition, mussels exposed to CAU + CAF revealed significantly higher CAT activity compared to those exposed to CAF (Figure 2C). The biotransformation enzymes GSTs showed significantly higher levels in exposed mussels (CAF, CAU, and CAU + CAF). Furthermore, CAU + CAF treatment induced higher GSTs activity compared to CAU (Figure 2D). Among

treatments, no significant differences were detected in CbEs – pNPA levels, while in CbEs – pNPB levels, a significantly higher value was found in CAU + CAF compared to CTL and CAU (Figures 2E and F).

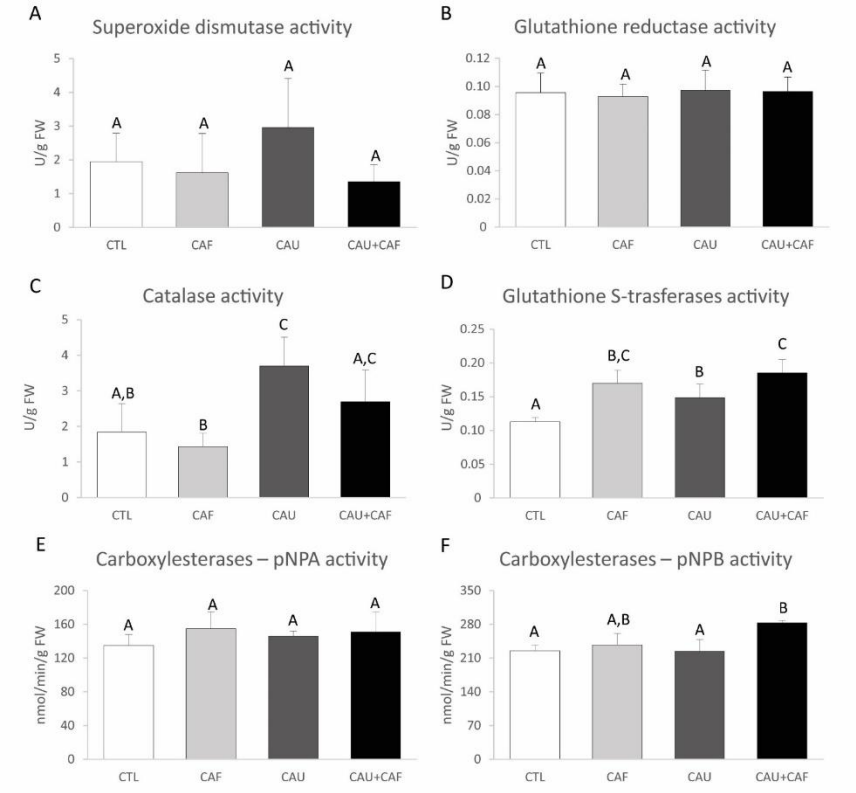

Figure 2. Antioxidant and biotransformation enzyme activities in *Mytilus galloprovincialis* under different treatments (CTL, CAF, CAU, CAU + CAF) at the end of the experiment. A: Superoxide dismutase activity (SOD), B: Glutathione reductase activity (GR), C: Catalase activity (CAT), D: Glutathione S-transferases activity (GSTs), E: Carboxylesterases pNPA activity (cBES- pNPA), F: Carboxylesterases pNPB activity (cBES- pNPB). Results are mean + standard deviation. Significant differences ($p < 0.05$) among the treatments are presented with different letters. $n = 9$.

Mussels exposed to CAF showed significantly higher LPO levels compared to CTL and CAU (Figure 3A). Furthermore, CAF and CAU + CAF-treated mussels showed significantly higher GSSG levels compared to CAU (Figure 3B). GSSG levels in CAU + CAF mussels were significantly higher compared to CTL (Figure 3B).

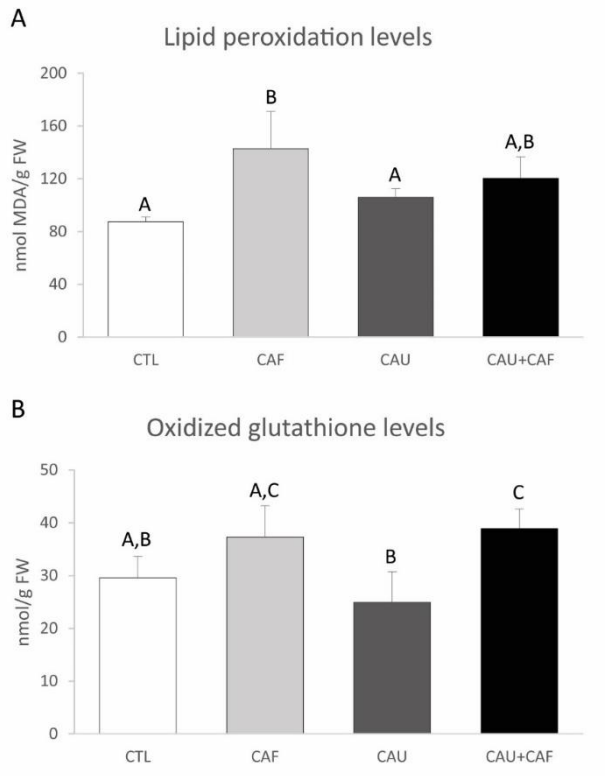

Figure 3. Cellular damage and redox balance in *Mytilus galloprovincialis* under different treatments (CTL, CAF, CAU, CAU + CAF) at the end of the experiment. A: Lipid peroxidation levels (LPO). B: Oxidized glutathione levels (GSSG). Results are mean + standard deviation. Significant differences ($p < 0.05$) among the treatments are presented with different letters. $n = 9$.

In mussels exposed to CAF, the activity of AChE was significantly higher compared to CTL (Figure 4).

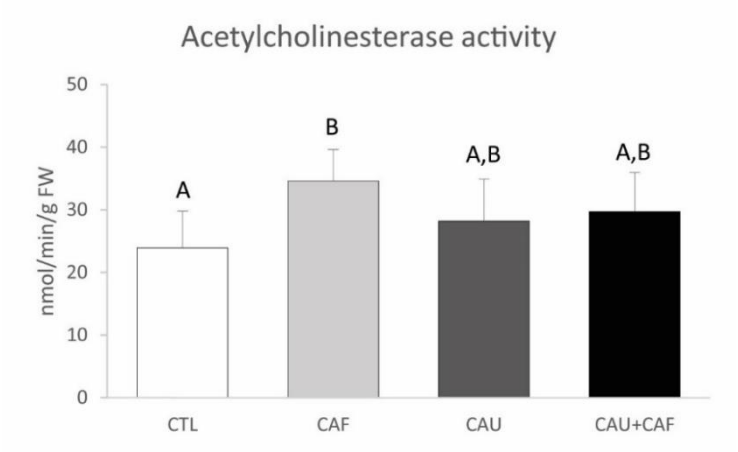

Figure 4. Acetylcholinesterase activity (AChE, neurotoxicity biomarker), in *Mytilus galloprovincialis* under different treatments (CTL, CAF, CAU, CAU + CAF) at the end of the experiment. Results are mean + standard deviation. Significant differences ($p < 0.05$) among the treatments are presented with different letters. $n = 9$.

3.3.3 NMR SPECTRAL DATA

OPLS-DA was performed on NMR data obtained from a total of *n* = 12 samples to identify and assess molecules responsible for metabolic alterations. The elaborated statistical model resulted in one predictive and one orthogonal component with parameters R^2 = 0.27 and Q^2 = 0.13. The scores plot in Figure 5A shows sample projection onto the principal components. The first component t[1] accounted for the main differences between groups. The CAF + CAU class (black squares) was located at negative t[1] values, while the other classes [except for two CAF samples (light-gray squares)] were placed at positive t[1] values. Interestingly, the CAU class (grey squares) and the CAF group (light-grey squares) were located in the central region of the scores plot, around the (0, 0) point. This would suggest that the CAU samples present some similarity with the control group (empty squares), while the CAF class (light-gray squares) is more oriented towards the CAF + CAU group. The orthogonal component t_o[1] expressed the intraclass inhomogeneity, mainly due to betaine variation (3.29 ppm) observed in the associated loadings plot of Figure 5B. It shows the NMR variables (in ppm) responsible for sample projection and clustering in the model.

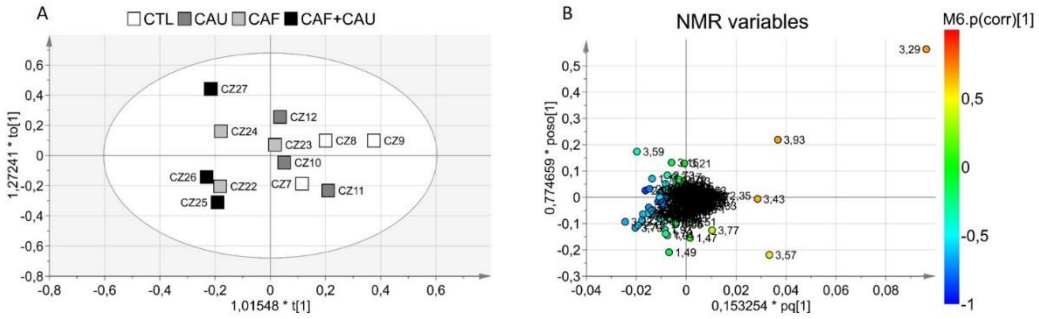

Figure 5. A: Scores plot showing sample projection onto principal components: groups represent *Mytilus galloprovincialis* after different treatments (CTL (white squares), CAF (light grey squares) CAU (grey squares), CAU + CAF (black squares)), B: Loadings plot reporting the NMR variables (chemical shift) responsible for clustering in the model.

In particular, we observed higher levels of asparagine and total glutathione (GSH) in CAU + CAF mussels compared with CTL (Table S3). Furthermore, CAU + CAF treatment induced higher levels of glutamine and pyro-glutamate compared to CAU-treated mussels and CTL (Table S3). Mussels under CAF treatments (CAF,

CAU + CAF) showed higher levels of valine and leucine compared to CTL mussels (Table S3). In addition, higher levels of an unassigned compound (unknown) were found in CTL mussels compared to the CAF and CAF + CAU groups (Table S3). No significant differences among treatments were found in homarine levels (Table S3).

Table S3. Normalized bin mean ± standard deviation of metabolites found in *Mytilus galloprovincialis* under different treatments (CTL, CAF, CAU, CAU + CAF) at the end of the experiment. Significant differences ($p < 0.05$) among treatments are presented with different letters. $n = 3$.

Metabolites	Normalized bin mean ± standard deviation			
	CTL	**CAF**	**CAU**	**CAU + CAF**
asparagine	$10.30^{-4} \pm 5.63^{-4\ a}$	$18.00^{-4} \pm 2.32^{-4\ a,b}$	$11.80^{-4} \pm 5.23^{-4\ a,b}$	$20.20^{-4} \pm 3.89^{-4\ b}$
GSH	$3.85^{-4} \pm 1.26^{-4\ a}$	$5.80^{-4} \pm 6.06^{-5\ a,b}$	$5.32^{-4} \pm 1.76^{-4\ a,b}$	$7.36^{-4} \pm 1.60^{-4\ b}$
glutamine	$10.40^{-4} \pm 2.11^{-4\ a}$	$13.20^{-4} \pm 1.63^{-4\ a,b}$	$11.60^{-4} \pm 1.36^{-4\ a}$	$15.20^{-4} \pm 1.10^{-4\ b}$
pyro-glutamate	$17.10^{-4} \pm 7.82^{-5\ a}$	$18.60^{-4} \pm 2.55^{-4\ a,b}$	$17.30^{-4} \pm 1.58^{-4\ a}$	$21.60^{-4} \pm 8.11^{-5\ b}$
valine	$3.38^{-4} \pm 1.87^{-5\ a}$	$4.80^{-4} \pm 1.16^{-5\ b}$	$4.36^{-4} \pm 8.03^{-5\ a,b}$	$5.15^{-4} \pm 4.22^{-5\ b}$
leucine	$7.17^{-4} \pm 4.99^{-5\ a}$	$10.80^{-4} \pm 1.22^{-4\ b}$	$9.89^{-4} \pm 2.23^{-4\ a,b}$	$12.10^{-4} \pm 2.32^{-4\ b}$
unknown	$4.08^{-5} \pm 1.94^{-5\ a}$	$1.04^{-5} \pm 1.20^{-6\ b}$	$2.05^{-5} \pm 9.75^{-6\ a,b}$	$9.12^{-6} \pm 6.95^{-6\ b}$
homarine	$9.38^{-4} \pm 1.09^{-4\ a}$	$8.07^{-4} \pm 3.65^{-5\ a}$	$8.69^{-6} \pm 3.74^{-5\ a}$	$8.22^{-6} \pm 7.34^{-5\ a}$

3.3.4 HISTOPATHOLOGICAL OBSERVATIONS

Morphological alterations on the mussels' tissues were observed on the gills and digestive tubules of all specimens used in the experiment but with differences in terms of typology and diffusion of the alterations. Gills in CAF-exposed mussels showed more lipofuscin aggregates and enlargement of the central vessel compared to CTL, resulting in a significantly higher I_h (Figure 6A). Regarding the digestive tubules, higher alterations, mainly lipofuscin aggregates and atrophy, were observed in mussels treated with CAF (CAF, CAU + CAF) compared to both CTL and CAU-treated mussels, resulting in a significantly higher I_h (Figure 6B).

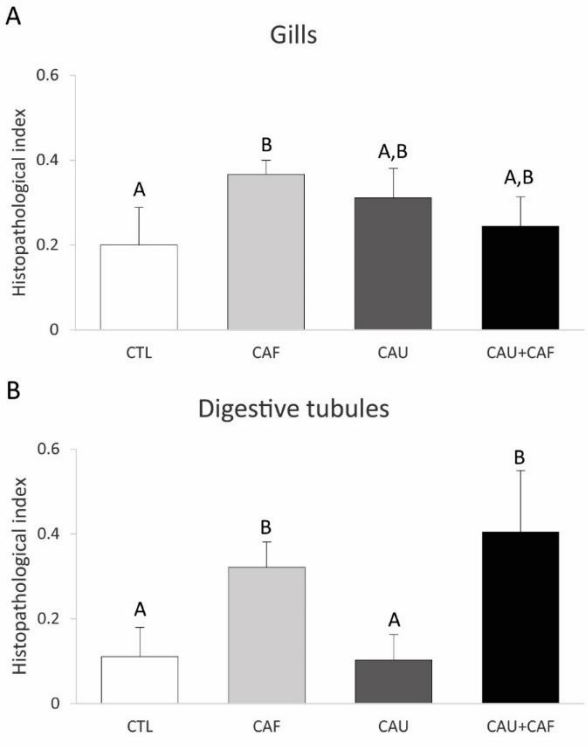

Figure 6. A: Histopathological index in gills and B: Histopathological index in digestive tubules (DT) in *Mytilus galloprovincialis* under different treatments (CTL, CAF, CAU, CAU + CAF) at the end of the experiment. Results are mean + standard deviation. Significant differences ($p < 0.05$) among the treatments are presented with different letters. $n = 3$.

3.4 DISCUSSION

Among the chemical pollutants of anthropogenic origin discharged in the marine environment from industries, agriculture, aquaculture facilities, and households, the psychoactive substance caffeine (CAF) has shown adverse impacts on aquatic species (Aguirre-Martínez et al., 2016; Capolupo et al., 2016; Cruz et al., 2016; De Marco et al., 2022; Li et al., 2020; Munari et al., 2020). The spread of CAF has global proportions, affecting the Mediterranean Sea, which is considered one of the major biodiversity hotspots in danger in the world, also due to biological invasions (Costello et al., 2023). In such circumstances, CAF coexists in the Mediterranean along with bioactive molecules produced by highly invasive marine species, including the green alga *Caulerpa cylindracea*. Despite this, little is known about the possible cumulative interactions between CAF and the so-called "alien metabolites" (Mollo et al. 2015).

The current study aimed to evaluate the possible interaction between CAF and the bisindolic alkaloid CAU, which is particularly abundant in *C. cylindracea* (Defranoux et al., 2022), by using the mussel *Mytilus galloprovincialis* as a model aquatic organism. Since CAU is well known to accumulate in the tissues of aquatic animals that feed on *C. cylindracea*, we decided to administer it via food to mussels. The hydrophobicity of the compound facilitated this mode of administration since CAU incorporated in the food particles does not dissolve in the medium. In the case of CAF, instead, we chose the administration via medium according to literature and its solubility in seawater by also assessing its accumulation in mussels' soft tissues, whose concentration under CAF treatments (CAF, CAU + CAF) resulted to be similar (4.96 ± 0.07 ng/g FW on average) after treatments. Such concentrations were not affected by the simultaneous dietary treatment with CAU. CAF concentration in the medium, measured before seawater renewal, was below the limit of detection (LOD = 0.02 µg/L), indicating that the contaminant in the seawater was filtered and accumulated by the bivalves, as also observed by Piscopo et al. (2021a, 2021b) in other bivalve species.

After treatments, mussels' metabolic capacity was evaluated by assessing the activity of the electron transport system (ETS) since previous studies showed

increased ETS in bivalves after exposure to CAF (De Marchi et al., 2022). CAU did not affect mussels' metabolism, while a significant increment of ETS was observed when CAU was administered along with CAF, suggesting a possible CAU-mediated increased metabolism in the presence of CAF.

Similarly, the levels of glycogen (GLY), which is known to increase under treatment with CAF in clams (Piscopo et al., 2021a, 2021b), only rose when mussels exposed to CAF were treated with CAU (CAU + CAF treatment). Normally, under stressful conditions, the increase in ETS activity in mussels can be associated with a decrease in GLY, since the organisms consume their reserves to satisfy the metabolic demand (Cruz et al., 2016). In this case, even if an increment in ETS activity was observed, the simultaneous increment in GLY could be explained by the peroxisome proliferator-activated receptors (PPARs) agonist function of CAU (Vitale et al., 2018). PPARs, among other functions, can regulate lipid metabolism (Vázquez-Carrera and Wahli, 2022). Although the mode of action of PPARs' ligands in mussels has not been explored yet, we can hypothesize that during the combined treatment with CAU and CAF, the mussels may have mainly used their lipid reserves to fuel up the energy requirement. On the other hand, protein levels (PROT) did not significantly change among treatments. However, NMR-based metabolomics analysis indicated that the levels of asparagine, glutamine, pyro-glutamine, valine, and leucine specifically increased in mussels treated with CAU + CAF compared to CTL. Levels of valine and leucine also increased under treatment with CAF alone, compared to CTL. Since free amino acids, among various functions, are the basic units for protein synthesis, these findings suggest that under CAF treatments (CAF, CAU + CAF), mussels were preparing for protein synthesis probably only at the end of the experiment, enabling the detection of PROT level increments through a biochemical assay. Furthermore, free amino acids and their catabolites are reported to act as osmolytes in marine invertebrates (Lange, 1963; Viant et al., 2003; Young et al., 2015), suggesting an unbalance in mussel osmolarity when in the presence of CAF. It is worth mentioning that increased free amino acid levels in mussels have been associated with environmental pollution (Cappello et al., 2013). ETS activity, energy reserves, and NMR analysis revealed

no alterations induced by the administration of CAU alone, suggesting that CAF was the compound responsible for the observed alterations.

Subsequently, superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) activities were evaluated to assess the possible activation of the antioxidant defenses enzymes under stressful conditions (Regoli and Giuliani, 2014). Although SOD and GR activities were similar regardless of the treatment, higher CAT activity was found in mussels exposed to CAU + CAF, and in particular to CAU, compared to CAF and CTL. This could be explained by considering the ability of CAU to increase CAT activity as a PPARα agonist (Shin et al., 2016).

Under all the treatments, the levels of the biotransformation enzyme glutathione-S-transferases (GSTs) also significantly increased. Furthermore, CAU + CAF treatment induced higher GSTs activity compared to CAU alone, suggesting that this enzyme was further activated by the presence of CAF, as also observed by several studies on bivalves exposed to CAF (Capolupo et al., 2016; Cruz et al., 2016; De Marchi et al., 2022). In addition, when CAF was associated with CAU, higher carboxylesterase-B (CbEs-pNPB) levels than CTL and CAU-treated mussels were observed. Again, the toxic effect was linked to the presence of CAF and was in some way increased by the treatment with CAU, which alone did not induce changes. Similarly, CbEs-pNPB levels increased in mussels exposed to other chemical hazards (Lopes et al., 2022; Queirós et al., 2021).

For an indirect sign of the presence of ROS, we examined the levels of oxidized glutathione (GSSG), which acts as an antioxidant (Regoli and Giuliani, 2014). We observed that treatments with CAF were associated with significantly higher GSSG levels compared to CAU. Moreover, GSSG levels under the combined administration of CAF and CAU were also significantly higher compared to CTL. From the above, the presence of CAF apparently enhanced the oxidation of GSH in mussels in an attempt to prevent cellular damage, confirming previous studies by Pires et al. (2016a) and Li et al. (2020). Accordingly, NMR analyses revealed significantly higher levels of total glutathione in mussels treated simultaneously with CAU and CAF. Moreover, mussels exposed to CAF showed significantly higher lipid peroxidation (LPO) levels compared to CTL and CAU. Accordingly, elevated LPO levels were observed in *R. philippinarum*, *R. decussatus*, and *M.*

galloprovincialis after CAF exposure (Aguirre-Martinez et al., 2016; Cruz et al., 2016; De Marchi et al., 2022; Piscopo et al., 2021a, 2021b). On the other hand, LPO did not increase under simultaneous treatment with CAU and CAF. This rules out cumulative or synergistic interactions between the two compounds that could increase the risk of cellular damage. It remains to be clarified, however, if the antioxidant and anti-inflammatory activities of CAU (De Souza et al., 2009; Defranoux and Mollo, 2020) have partly compensated for the oxidative stress induced by CAF.

Acetylcholinesterase (AChE) activity has been evaluated as a neurotoxicity marker. AChE activity increased in mussels treated with CAF alone compared to CTL. Likewise, De Marchi et al. (2022) observed an increase in AChE activity in both mussels and clams exposed to CAF, supporting a neurotoxic effect of this compound. Indeed, increased AChE activity was already associated with neurotoxicity in bivalves exposed to other toxic contaminants (Freitas et al., 2019) and was interpreted as an attempt to hydrolyze the neurotransmitter acetylcholine accumulated in the synaptic clefts. However, when mussels exposed to CAF were simultaneously treated with CAU, the increase in AChE activity was not observed. Again, the anti-inflammatory properties of CAU may have played a role in limiting the neurotoxic effects of CAF.

Finally, morphological observations confirmed the harmfulness of CAF. In fact, exposure to CAF led to tissue alterations in mussels' gills, especially in terms of lipofuscin aggregates and enlargement of the central vessels, which are associated with lipid peroxidation and inflammation, respectively (Fig. 7). In *R. decussatus*, CAF in combination with carbon nanotubes also induced morphological alterations in gills (Piscopo et al., 2021a). Regarding digestive tubules, the morphological alterations observed were more diffused in mussels exposed to CAF, either alone or in combination with CAU, mainly in terms of lipofuscin aggregates and atrophy, while CAU had no morphological impacts on the mussels' tissues (Fig. 7).

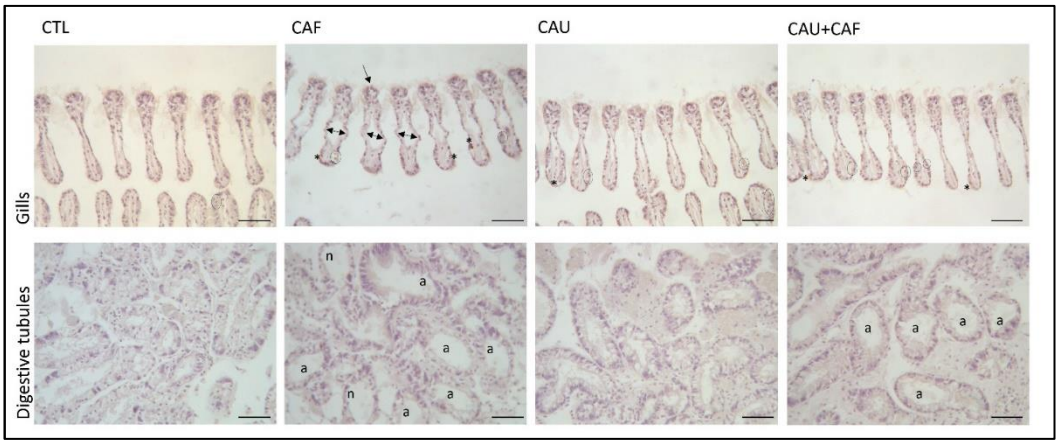

Figure 7. Representative micrographs of gills and digestive tubules (DT) in *Mytilus galloprovincialis* under different treatments (CTL, CAF, CAU, CAU + CAF) at the end of the experiment stained with hematoxylin i) Gills: lipofuscin aggregates (*); cilia loss (arrow), enlargement of the central vessel (double-headed arrows), hemocytes infiltration (circle); ii) DT: atrophy (a), necrosis (n). Scale bar = 50 µm. *n* = 3.

CONCLUSION

Pollution and biological invasions simultaneously act as major drivers of changes in the Mediterranean basin. Despite this, the combined effects of anthropogenic hazard compounds and bioactive metabolites produced by invasive species are rarely addressed in the ecotoxicological field. In this study, we focused on the possible combined impact of two alkaloids: CAF from anthropogenic sources and CAU from the highly invasive green alga *C. cylindracea*. We did not observe any cumulative effects of these compounds. On the contrary, CAU treatment of mussels simultaneously exposed to CAF tended to generate metabolic activation, as assessed by higher ETS activity, with no substantial impacts in terms of cellular damage and neurotoxicity, while CAF alone resulted in being both pro-oxidant and neurotoxic. Therefore, a possible action of CAU in modulating CAF toxicity could be hypothesized, since CAU is known to act as an agonist of PPARs, which, among other functions, mediate xenobiotic detoxification via the cytochromes P450 (Crib et al., 2005; Malliou et al., 2021; Nebert and Russell, 2002; Thomas et al., 2013; Villard et al., 2011; Werck-Reichhart and Feyereisen, 2000). Actually, P450 is involved in CAF metabolism (Temple et al., 2017). Further studies, possibly also involving model vertebrates, are needed to explore and confirm the activity of CAU

in improving the clearance of xenobiotics. This would pave the way for the possible use of CAU as a detoxifying agent in the nutraceutical field and for the sustainable exploitation of biomass from a highly invasive marine species.

ACKNOWLEDGEMENTS

We acknowledge financial support to CESAM by FCT/MCTES (UIDP/50017/2020+UIDB/50017/2020+ LA/P/0094/2020), through national funds. The chemical study performed at ICB/CNR was supported by the "National Biodiversity Future Center" (CN00000033), theme "Biodiversity", funded under the National Recovery and Resilience Plan (PNRR) (Mission 4, Component 2 Investment 1.4) supported by Next Generation EU.

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CHAPTER 4

COULD THE INVASIVE SPONGE *PARALEUCILLA MAGNA* POSE A THREAT TO MEDITERRANEAN MUSSELS?

This chapter has been prepared for submission to an international, peer-reviewed, indexed scientific Journal. Experimental activities were performed with financial support by CESAM and FCT/MCTES (UIDP/50017/2020+UIDB/50017/2020+LA/P/0094/2020), through national Portuguese funds. The chemical study performed at ICB/CNR (Pozzuoli, Naples) was supported by the "National Biodiversity Future Center" (CN00000033), theme "Biodiversity", funded under the National Recovery and Resilience Plan (PNRR) (Mission 4, Component 2 Investment 1.4) supported by Next Generation EU.

ABSTRACT

The potentially harmful impact of the invasive calcareous sponge *Paraleucilla magna* on the Mediterranean mussel *Mytilus galloprovincialis* represents the main object of this study. The sponge is currently widespread along Italian coasts, especially colonizing mussel rows as an epibiont. Since sponges are known to release bioactive/toxic molecules in the water column, the urgent problem arises of clarifying whether mussels, as active filter feeders, can undergo intoxication phenomena. We thus evaluated the biochemical, metabolomic, and histopathological responses of *M. galloprovincialis* after 28-day feeding treatments with food enriched with crude extracts (acetone, diethyl ether, and butanol) of *P. magna*. The acetone extract did not impact mussels, while treatments with sponge diethyl ether (EE) and butanol (EB) extracts revealed significant alterations in biochemical analyses such as metabolic capacity, energy reserves, antioxidant defenses, biotransformation isoenzymes, redox balance, and neurotoxicity of mussels. In particular, ETS activity and GLY levels decreased under EB treatments, while GSTs and CbEs activities were inhibited with EE treatment. In addition, EB induced higher GPx activity and LPO levels, while AChE activity was reduced under both EE and EB treatments. Metabolomic analysis using NMR spectroscopy highlighted alterations in osmolytes, nucleotides, and other metabolites, indicating stress responses, again with a different pattern among EE and EB mussels. Even so, no significant differences between treatments were observed in the histopathological indices. To what extent and at what concentration the sponge extracts and/or its purified metabolites might prove to be a risk to native species and mussel farms remains to be clarified. The present study thus paves the way for further exploration of the ecotoxicological impact of *P. magna* in the Mediterranean Sea.

KEYWORDS: invasive sponges, *Mytilus galloprovincialis*, biomarkers, NMR metabolomics, histopathology

4.1 INTRODUCTION

The Mediterranean Sea is considered one of the most vulnerable biodiversity hotspots in the world (Costello et al., 2023). Anthropogenic activities such as aquaculture and international shipping, as well as climate change with the increase in seawater temperatures, are some of the factors that promote the invasion of allochthonous species, which represent a major driver of changes seriously affecting the Mediterranean Sea (Corrales et al., 2018; Galil et al., 2014; Gorbi et al., 2014; Parravicini et al., 2015; Vitale et al., 2018). Indeed, invasive species can cause significant alterations in the structure and composition of marine ecosystems (Katsanevakis et al., 2014). For example, the green alga *Caulerpa cylindracea* is well-known for its invasiveness and effects on native species, invading meadows of native macrophytes (Pierucci et al., 2019; Bernardeau-Esteller et al., 2020) while becoming a food item for local fish and echinoderms (Felline et al., 2012, 2017, 2014; Noè et al., 2018; Terlizzi et al., 2011). In addition to highly invasive macroalgae, however, non-indigenous species in the Mediterranean Sea also encompass seagrasses, polychaetas, crustaceans, mollusks, and fishes, with approximately 10% of them becoming invasive (Giangrande et al., 2020; Zenetos et al., 2022). Among the few invasive sponges, *Paraleucilla magna* colonizes hard natural or artificial substrates in ports, marinas, or bays in both photophilous and sciaphilous habitats, with a preference for eutrophic conditions (Bachetarzi et al., 2019; Evcen and Çinar, 2020; Klautau et al., 2004; Longo et al., 2007). This species was first described in 1980 in Brazil, where it is considered a cryptogenic species due to its uncertain origin and is currently widely spread in the Mediterranean Sea (Klautau et al., 2004; Longo et al., 2012). *P. magna* fouls aquaculture facilities, especially *Mytilus galloprovincialis* (Longo et al., 2012; Tsirintanis et al., 2022), and mussel farms have been identified as possible vectors for the diffusion of this sponge along the Mediterranean coasts (Di Blasio et al., 2023). The intricate network of canals that form the body of *P. magna* provides shelter to a variety of organisms, including crustaceans, mollusks, bryozoans, and polychaetas (Padua et al., 2013). Moreover, bacteria-producing compounds with potential interest have been isolated from this species (Santos et al., 2010). Little is known, however, about

the ecological impact of *P. magna* on the Mediterranean ecological communities. In addition, since sponges are widely recognized as rich sources of bioactive metabolites and about 200 novel compounds are isolated from sponges annually (Mauduit et al., 2022), chemical warfare between *P. magna* and native species cannot be ruled out. Although the chemical composition of the sponge is almost unknown, we could hypothesize that it might release some of its metabolites in the water column, either in dissolved form or attached to particles (such as sponge cellular debris), known as exo-metabolites. These exo-metabolites could act as chemical signals, possibly influencing the surrounding marine organisms and contributing to ecosystem dynamics (Mauduit et al., 2022; Ternon et al., 2016). It is worth mentioning that the ethanol extract of *P. magna* inhibited the settlement of *M. galloprovincialis* juveniles and showed toxicity to *Artemia salina* nauplii (Longo et al., 2021). Furthermore, antioxidant defenses were induced in the red alga *Peyssonnelia squamaria* epiphytized by *P. magna* (Guzzetti et al., 2019).

Overall, the above findings support that *P. magna* makes use of chemical weapons with a potentially harmful impact on native species, including the bivalve *M. galloprovincialis*. This mollusk represents a well-established model organism because of its economic and ecological value, high sensitivity to stressors, and filter-feeding behavior (Capolupo et al., 2016; Coppola et al., 2020). In the present study, to evaluate the ecotoxicological potential of *P. magna*, biochemical, NMR-metabolomics, and histopathological analyses were performed after administering food enriched with different sponge crude extracts (acetone, diethyl ether, and butanol) to mussels. This work is also intended to serve as a preliminary screening test for future research focused on the chemical characterization of the sponge extracts and the identification of their bioactive components.

4.2 MATERIALS AND METHODS

4.2.1 SAMPLING OF MUSSELS AND MAINTENANCE CONDITIONS

Specimens of mussels *Mytilus galloprovincialis* were collected in March 2022 from the Ria of Aveiro lagoon, Portugal, with a mean length of 5.8 ± 0.3 cm and a mean width of 3.3 ± 0.2 cm. Mussels were transported to the research laboratory for depuration and acclimation procedures, and for two weeks they were kept in artificial seawater (salinity 30 ± 1) prepared with distilled water and artificial salt (Tropic Marin® SEA SALT from Tropic Marine Center) at 17 ± 1 °C, pH 8.0 ± 0.1, constant aeration, and natural photoperiod. The seawater was changed twice per week; during the first week (depuration), mussels were not fed, while in the second week (acclimation), mussels were fed with Algamac protein plus (150.000 cells/animal/day).

4.2.2 EXPERIMENTAL TREATMENTS

After depuration/acclimatation, 72 mussels were divided into 4 different feeding treatments, including control-food (CTL), *Paraleucilla magna* acetone extract-enriched food (EA), *P. magna* diethyl ether extract-enriched food (EE), and *P. magna* butanol extract-enriched food (EB). Three aquaria with six mussels each were used for each treatment. The mussels were fed three times per week, receiving approximately 5 μg of crude extracts per mussel daily. Temperature, salinity, and mortality were monitored daily throughout the experiment, and seawater was changed every seven days. In parallel, three aquaria with six mussels each were subjected to feeding treatment with RotiBomb plain food, and *p*-values (see supplementary material Table s1) showed no differences between plain food and CTL.

Table s1. *p* value among analysis performed on mussels fed with plain food and CTL food after the 28 days exposure.

	Plain food *vs.* CTL
ETS	0.8971
PROT	0.1692
GLY	0.9512
SOD	0.5508
GPx	0.0721
CbEs	0.7256
GSTs	0.0794
GSH/GSSG	0.3281
LPO	0.9510
AChE	0.0736
I_h DT	0.6277
I_h GILLS	0.6035

4.2.3 FOOD PREPARATION

The specimens of *P. magna* were sampled in January 2022 in the lake Fusaro, Italy, and submitted to subsequential extractions with different polarity solvents: acetone, diethyl ether, and butanol (Gavagnin et al., 2005) to obtain the respective extracts for the food implementation. Diethyl ether and butanol extracts are derived from the acetonic crude extract, and their composition differs based on their specific polarity and affinity with the organic solvents. Control food was prepared by soaking a combination of microalgae and probiotics (RotiBomb dry food, Algova) in acetone/methanol 2:1 (Sigma-Aldrich, suitable for HPLC, ≥ 99.9%) and then evaporating the organic solvent under reduced pressure. Treated food was prepared in the same way, but after dissolving crude extracts of *P. magna* in acetone/methanol 2:1 at a concentration of 50 mg of each extract per gram of dry food. This relatively high concentration of the extract in the food, approximately ten times higher than that occurring in the lyophilized sponge, was selected as a premise for subsequent dose-dependent evaluations.

4.2.4 BIOCHEMICAL MARKERS

On the 28th day, three mussels from each aquarium (nine per treatment) were sacrificed in liquid nitrogen to preserve enzymatic activities and to perform biochemical analysis. The soft tissues were homogenized in liquid nitrogen, and 0.5 g fresh weight (FW) aliquots were used to evaluate biochemical markers. Tissue extractions were carried out by using specific buffers, homogenizing for 90 seconds in TissueLyser II (Qiagen), and centrifuging for 20' at 10,000 g (or 3,000 g depending on the specific biomarkers examined) at 4 °C. The supernatants were kept at -80 °C until they were processed. Biomarkers associated with the metabolic capacity [electron transport system activity (ETS)]; energy reserves [total protein content (PROT) and glycogen (GLY)]; antioxidant defenses [superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities]; biotransformation capacity [carboxylesterases (CbEs) and glutathione S-transferases (GSTs) activities]; redox balance [ratio reduced glutathione/oxidized glutathione (GSH/GSSG)]; cellular damage [lipid peroxidation levels (LPO)] and neurotoxicity [acetylcholinesterase activity (AChE)] were evaluated applying spectrophotometric methods.

The activity of ETS was assessed following the De Coen and Janssen (1997) method, using p-IodoNitroTetrazolium (INT) as an artificial electron acceptor to register the electron transmission rate. The absorbance was read at 490 nm during 10 min, and the results were expressed in nmol per min per g FW.

The levels of PROT were measured using Biuret methods (Robinson and Hodgen, 1940). The absorbance was read at 490 nm, and the results were expressed in mg per g FW. The GLY contents were measured with the sulfuric acid method (Dubois et al., 1956). The absorbance was measured at 492 nm, and the results were expressed in mg per g FW.

The activity of SOD was evaluated following the Beauchamp and Fridovich (1971) method, and the absorbance was measured at 560 nm. The results were expressed in U per g FW, where U corresponds to a reduction of 50% of the substrate nitro blue tetrazolium salt (NBT). The activity of GPx was determined following the method of Paglia and Valentine (1967), using cumene hydroperoxide as a substrate. The absorbance was measured at 340 nm for 5 min, and the results were expressed in U per g FW, where U represents the amount of enzyme that catalyzes the

conversion of 1 μmol nicotinamide adenine dinucleotide phosphate (NADPH) per min.

The activity of CbEs was determined by applying Hosokawa and Satoh (2001) to the substrate p-nitrophenyl acetate. The hydrolysis rate of the substrates was measured at an absorbance of 405 nm during 5 min and was expressed in nmol per min per g FW.

The activity of GSTs was determined according to the Habig et al. (1974) method using 1-chloro-2,4-dinitrobenzene as a substrate. The absorbance was read at 340 nm, and the results were expressed in U (the amount of enzyme necessary to catalyze the formation of 1 nmol of dinitrophenyl thioether per minute) per g FW.

The content of GSH and GSSG was defined according to Rahman et al. (2007). GSH content was measured through its oxidation in 5′-thio-2-nitrobenzoic acid (TNB) by the sulfhydryl reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB). GSSG content was quantified through the measurement of NADPH consumption by glutathione reductase. The absorbance was measured at 412 nm for 2 min for both assays. The results were expressed as nmol per g of FW. The ratio GSH/GSSG was determined considering the number of thiol equivalents (GSH/2 ∗ GSSG).

The levels of LPO were tested by quantifying of the lipid peroxidation by-product malondialdehyde (MDA), according to Ohkawa et al. (1979). Absorbance was measured at 535 nm and expressed in nmol of malondialdehyde formed per g of FW.

The method of Ellman et al. (1961), modified by Mennillo et al. (2017), was applied to assess AChE using acetylthiocholine iodide (5 mM) as substrate. Enzyme activity was recorded continuously for 5 min at 412 nm and expressed in nmol per min per g FW.

To ensure the reproducibility of the results, all biochemical parameters were run in duplicate in a microplate reader (Biotek).

4.2.5 NMR SAMPLE PREPARATION AND SPECTRA ACQUISITION

Tissues from six mussels per treatment were homogenized in liquid nitrogen and stored at -80 °C for NMR analyses. Then, tissues were lyophilized and processed to extract metabolites of interest (e.g., lipids, amino acids, carbohydrates, and other

small metabolites). The combined extraction of polar and lipophilic metabolites was carried out by using methanol/water/chloroform as suggested by Lindon et al. (2005). Polar and nonpolar fractions were transferred into different glass vials, and the solvents were removed by using a rotary vacuum evaporator at room temperature. For NMR analysis, polar fractions were resuspended in 630 µL of phosphate buffer saline (PBS, pH 7.4), adding 70 µL of $^{2}H_2O$ solution [containing 1 mM sodium 3-trimethylsilyl [2,2,3,3-2H4] propionate (TSP) as a chemical shift reference for ^{1}H spectra] to provide a field frequency lock, reaching 700 µL of total volume. Samples were loaded into the autosampler, and NMR spectra were acquired on a Bruker Avance III–600 MHz spectrometer (BrukerBioSpin GmbH, Rheinstetten, Germany), equipped with a TCI CryoProbe fitted with a gradient along the Z-axis, at a probe temperature of 27°C. In particular, standard 1D proton spectra and 2D experiments [clean total-correlation spectroscopy (TOCSY) and heteronuclear single quantum coherence (HSQC)] were acquired providing monodimensional metabolic profiles and homonuclear and heteronuclear spectra for metabolites' identification. Metabolites' assignments were achieved by comparing signal chemical shifts with literature and online databases. All 1D spectra were processed, and automatically the data was reduced into bins arranged as a data matrix and imported for multivariate statistical analysis with projection methods. In addition, discriminant metabolites suggested by the statistical models were subjected to metabolic pathway analysis with MetaboAnalyst 5.0 using the *Dario rerio* library (Pang et al., 2021), since a reference database specific for *M. galloprovincialis* is still absent.

4.2.6 HISTOPATHOLOGICAL ANALYSIS

Three mussels per treatment were fixed in Davidson's solution immediately after the experimental treatments for histological observation. After 24 hours, the fixative was removed with daily washing in 70% ethanol. Gills and digestive tubules were dissected and subjected to dehydration in ascendant ethanol and clarification in methyl benzoate (Coppola et al., 2022; Russo et al., 2023). Tissues were embedded in paraffin and cut into 5 µm sections with a microtome (Leica) for

hematoxylin staining to analyze the tissue morphology and calculate the histopathological index (I_h) (Costa et al., 2013; Leite et al., 2020).

4.2.7 STATISTICAL ANALYSES

A non-parametric permutational analysis of variance was performed (PERMANOVA + add-on in PRIMER v6) on biochemical and histopathological data obtained for each tested condition (Anderson et al., 2008). Significant differences were defined as values lower than 0.05 ($p < 0.05$). The null hypothesis was that no significant differences between treatments (CTL, EA, EE, EB) were observed for each response (biomarkers and histopathological analysis).

The NMR spectral dataset was first submitted to unsupervised principal component analysis (PCA) to reduce data dimensionality into inferred variables, thus helping the identification of major trends and features, and then to supervised discriminant analysis to perform class discrimination. To that purpose, all acquired spectra were automatically reduced to 455 integral segments of 0.02 ppm each between the 0.50 –9.60 ppm spectral region, excluding the water resonance (4.50 –5.66 ppm) using the AMIX 3.9.15 software package (Bruker Biospin GmbH, Rheinstetten, Germany). After reducing NMR data, bins were normalized to the total spectrum area. The obtained data format, expressed by a matrix (X matrix), was then imported into the SIMCA-P+14 package (Umetrics, Umeå, Sweden) to perform multivariate statistical analysis discriminating mussels' different responses to experimental treatments according to their NMR profiles. In particular, PCA was applied to assess class homogeneity, uncover data trends, and detect outliers. Then, Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used to visualize class separation, clusters, and the spectral variables that influence samples' distribution according to the alteration of the metabolic profiles due to the different feeding treatments. Data visualization was achieved through scores and loadings plots, which also highlighted specific compounds as putative markers useful for classification. OPLS-DA models were validated by internal iterative cross-validation with 7 rounds of permutation test responses (800 repeats) and CV-ANOVA (ANOVA testing of Cross-Validated predictive residuals). Selected

isolated signals and bins with |pcorr| ≥ 0.5 and VIP > 1 (Variable Importance in the Projection) were considered for univariate statistical analysis elaborated with the OriginPro 9.1 software package (OriginLab Corporation, Northampton, USA) and R software [R core team (https://www.r-project.org/)]. Statistical significance for selected discriminant metabolites was determined by parametric analysis (ANOVA with Fisher correction) after a normality test was performed on the data to evaluate each distribution (Shapiro-Wilk, Kolgomorov-Smirnov test). *P* values < 0.05 were considered statistically significant.

4.3 RESULTS

4.3.1 BIOCHEMICAL MARKERS

4.3.1.1 METABOLIC CAPACITY AND ENERGY RESERVES

Significantly lower ETS activity was observed in mussels exposed to EB in comparison to CTL (Figure 1A). Mussels exposed to EB showed significantly lower GLY content compared to all conditions (Figure 2B). Lower levels were observed in the PROT content of EE-treated mussels compared to EA-treated mussels (Figure 1C).

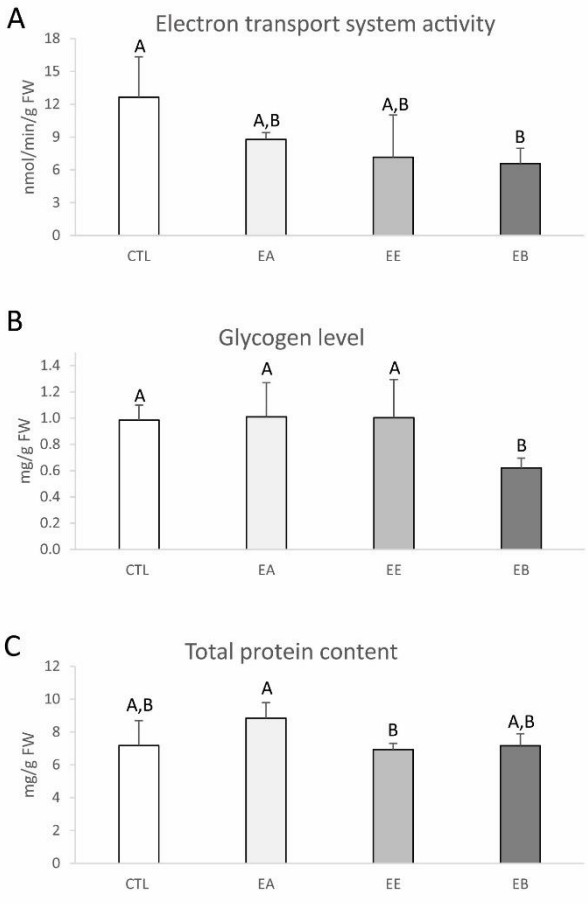


Figure 1. Metabolic capacity and energy reserve biomarkers in mussels treated with *P. magna* acetone extract (EA), diethyl ether extract (EE), and butanol extract (EB) compared to control mussels (CTL). 1A: Electron transport system activity (ETS), 1B: Glycogen content (GLY) and 1C: total protein content (PROT). The results are the mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 9$.

4.3.1.2 ANTIOXIDANT DEFENSES

SOD activity showed no significant differences among treatments (Figure 2A). On the other hand, mussels exposed to EB showed significantly higher GPx activity compared to all conditions (Figure 2B).

4.3.1.3 BIOTRANSFORMATION ISOENZYMES

Regarding GSTs, EE-treated mussels showed significantly lower levels compared to CTL and EA-treated mussels (Figure 2C). CbEs activity in EE mussels was significantly lower compared to CTL and EB (Figure 2D).

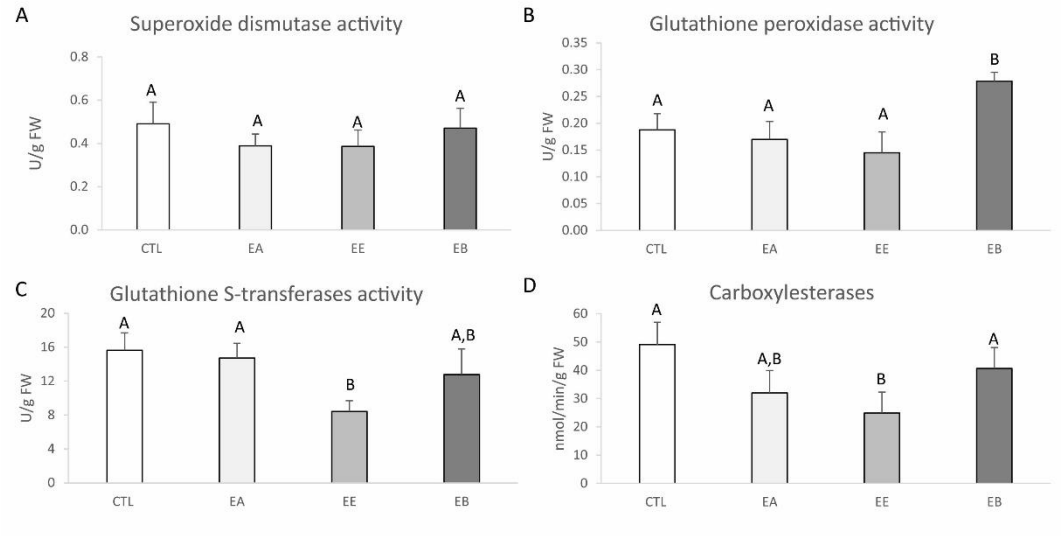


Figure 2. Antioxidant and biotransformation enzymes activities in mussels treated with *P. magna* acetone extract (EA), diethyl ether extract (EE), and butanol extract (EB) compared to control mussels (CTL). 2A: Superoxide dismutase activity (SOD), 2B: Glutathione peroxidase activity (GPx), 2C: Glutathione S-transferases activity (GSTs), 2E: Carboxylesterases activity (cBES). The results are the mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 9$.

4.3.1.4 REDOX BALANCE AND CELLULAR DAMAGE

The GSH/GSSG ratio was significantly higher in EA, EE, and EB-treated mussels compared to CTL (Figure 3A). Mussels exposed to EB showed significantly higher LPO levels compared to CTL and EE-treated mussels (Figure 3B).

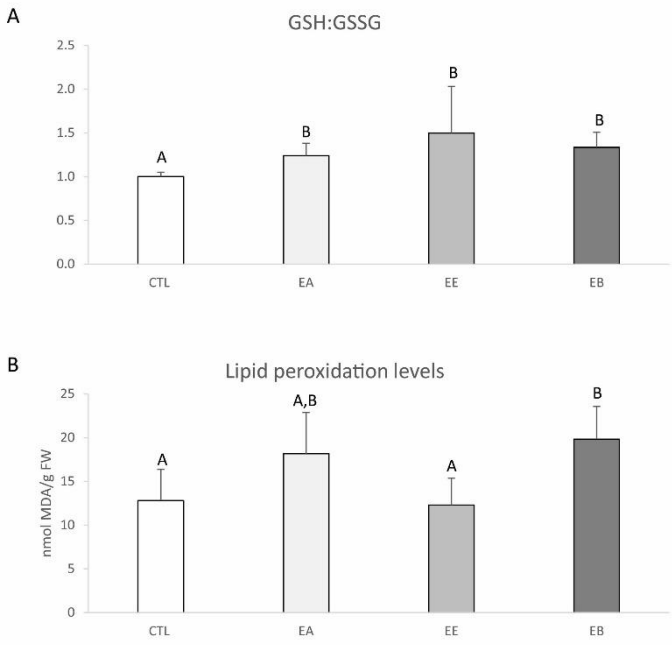


Figure 3. Redox balance and cellular damage biomarkers in mussels treated with *P. magna* acetone extract (EA), diethyl ether extract (EE), and butanol extract (EB) compared to control mussels (CTL). 3A: Ratio between reduced glutathione (GSH) and oxidized glutathione levels (GSSG), 3B: Lipid peroxidation levels (LPO). The results are the mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 9$.

4.3.1.5 NEUROTOXICITY

In mussels exposed to EE and EB, significantly lower AChE activity was detected compared to CTL and EA-treated mussels (Figure 4).

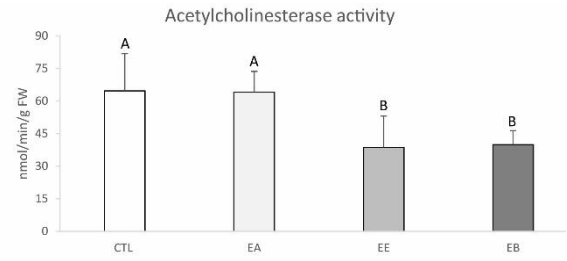


Figure 4. Acetylcholinesterase activity (AChE, neurotoxicity biomarker) in mussels treated with *P. magna* acetone extract (EA), diethyl ether extract (EE), and butanol extract (EB) compared to control mussels (CTL). The results are the mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 9$.

4.3.2 NMR METABOLOMICS ANALYSIS

OPLS-DA was performed on NMR spectra acquired from a total of 24 samples (6 per treatment). The elaborated statistical model resulted in two predictive components with parameters $R^2 = 0.39$ and $Q^2 = 0.09$. The scores plot in Figure 5A shows the sample projection onto the principal components. The first component t[1] accounts for the main differences between the EE group at t[1] negative coordinates versus the EB class, placed at positive t[1], while both the CTL and EA categories appeared in the middle of the graph. The second predictive component t[2] better differentiates both the EB and the EE classes from the CTL and EA groups. The loading plot in Figure 5B shows the NMR variables, then assigned to metabolites, responsible for sample projection in the model.

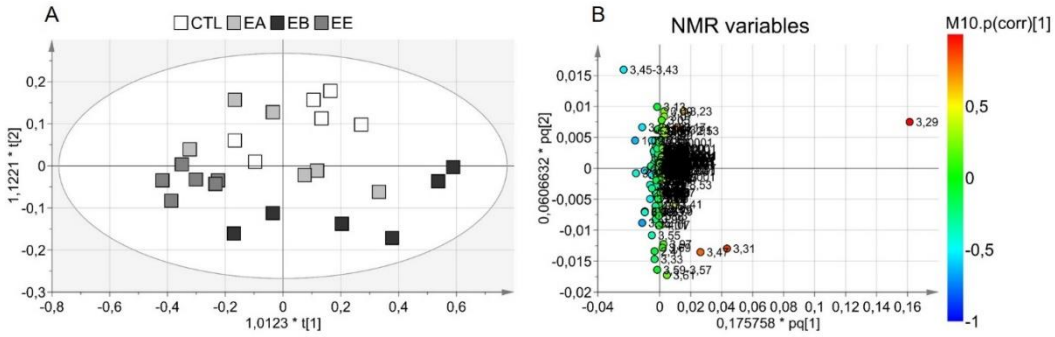


Figure 5. OPLS-DA analysis on mussels' NMR profiles. A) Scores plot showing sample projections onto principal components: groups represent mussels treated with different sponge extracts such as acetone extract (EA, light grey squares), butanol extract (EB, black squares), diethyl ether extract (EE, dark grey squares), and controls (white squares). B) Loadings plot reporting the NMR variables (chemical shift) responsible for clustering in the model.

In particular, assigning metabolites to the variables expressed in the associated loadings plot in Figure 5B, we found EE-treated mussels characterized by significantly lower levels of betaine and higher levels of glucose compared to the remaining treatments. Trigonelline levels in EE-treated mussels were significantly higher compared to CTL and EA. Glycerophosphocholine (GPC) and adenosine monophosphate (AMP) levels in EE-treated mussels were significantly higher compared to CTL. AMP in EE-treated mussels was significantly higher as well compared to EB-treated mussels. Furthermore, taurine levels in EB-treated mussels

were significantly lower than those in CTL. In EB-treated mussels, lower levels of aspartate were detected compared to all the remaining treatments. Finally, homarine levels in EB-treated mussels were significantly higher than in CTL (Table 1). In addition, further differences were observed among mussels treated with different sponge's extracts added food. Specifically, taurine resulted significantly higher in EE-treated mussels compared to EA and EB-treated mussels, while uridine resulted significantly higher only when comparing EE to EB-treated mussels; uridine diphosphate-N-Acetyl-Glucosamine (UDP-Glc-NAc) resulted significantly lower in EE-treated mussels compared to EA and EB-treated mussels.

Table 1. Normalized bin mean ± standard deviation of metabolites found in *Mytilus galloprovincialis* after different feeding treatments (CTL, EA, EE, EB). Significant differences ($p < 0.05$) among treatments are presented with different letters. $n = 6$. *GPC: glycerophosphocholine; AMP: adenosine monophosphate; UDP-Glc-NAc: uridine diphosphate-N-acetylglucosamine. $n = 6$

metabolites	**Normalized bin mean ± standard deviation**			
	CTL	**EA**	**EE**	**EB**
betaine	0.22 ± 0.04^{A}	0.20 ± 0.05^{A}	$0.13 \pm 0.02^{B\downarrow}$	0.26 ± 0.08^{A}
glucose	$0.003 \pm 3.85^{-4\,A}$	$0.004 \pm 4.28^{-4\,A}$	$0.0042 \pm 2.94^{-4\,B\uparrow}$	$0.0036 \pm 5.06^{-4\,A}$
trigonelline	$3.24^{-5} \pm 3.11^{-5\,A}$	$3.92^{-5} \pm 4.03^{-5\,A}$	$8.85^{-5} \pm 2.59^{-5\,B\uparrow}$	$6.51^{-5} \pm 4.76^{-5\,A,B}$
GPC*	$2.33^{-4} \pm 8.45^{-5\,A}$	$2.59^{-4} \pm 5.60^{-5\,A,B}$	$3.37^{-4} \pm 3.71^{-5\,B\uparrow}$	$2.78^{-4} \pm 8.58^{-5\,A,B}$
AMP*	$2.97^{-4} \pm 5.42^{-5\,A}$	$3.29^{-4} \pm 7.31^{-5\,A,B}$	$4.25^{-4} \pm 1.30^{-4\,B\uparrow}$	$2.80^{-4} \pm 3.39^{-5\,A}$
taurine	$0.062 \pm 0.004^{A,B}$	$0.057 \pm 0.003^{A,C}$	0.062 ± 0.003^{B}	$0.054 \pm 0.005^{C\downarrow}$
aspartate	$0.002 \pm 2.21^{-4\,A}$	$0.002 \pm 1.28^{-4\,A}$	$0.002 \pm 1.69^{-4\,A}$	$0.0014 \pm 1.71^{-4\,B\downarrow}$
homarine	$1.96^{-4} \pm 6.94^{-5\,A}$	$2.36^{-4} \pm 9.01^{-5\,A,B}$	$2.73^{-4} \pm 5.35^{-5\,A,B}$	$2.84^{-4} \pm 7.09^{-5\,B\uparrow}$
uridine	$4.85^{-5} \pm 2.87^{-5\,A,B}$	$4.43^{-5} \pm 2.30^{-5\,A,B}$	$5.80^{-5} \pm 2.80^{-5\,A}$	$2.19^{-5} \pm 1.24^{-5\,B}$
UDP-Glc-NAc*	$1.16^{-4} \pm 4.21^{-5\,A,B}$	$1.39^{-4} \pm 4.76^{-5A}$	$8.32^{-5} \pm 3.70^{-5\,B}$	$1.42^{-4} \pm 3.66^{-5\,A}$

In summary, EE-treated mussels showed a high content of AMP, trigonelline, GPC, and glucose, while EB-treated mussels were characterized by high levels of homarine as well as low levels of taurine and aspartate.

In addition, discriminant metabolites underwent metabolic pathway analysis to investigate their impact on the metabolic network. The analysis revealed that the pathways most involved in mussels' responses to sponge extracts included taurine and hypotaurine metabolism; alanine, aspartate, and glutamate metabolism;

glycine, serine, and threonine metabolism, together with glycerophospholipid metabolism (Figure 6).

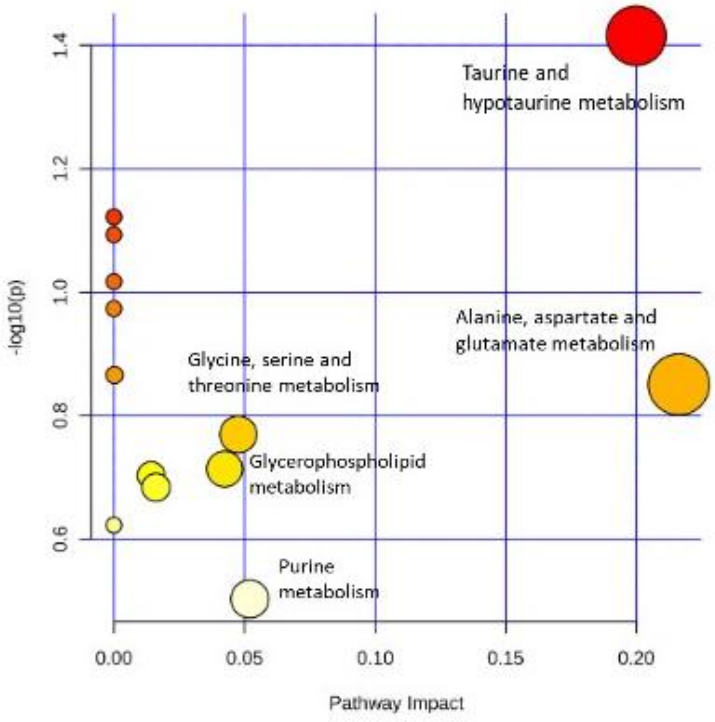


Figure. 6. The most impacted metabolic pathways are reported as circles with different sizes and colors according to their scores from the enrichment (vertical axis) and the topology analyses (pathway impact, horizontal axis). Darker circle colors indicate more significant changes in metabolites in the corresponding pathway. The size of the circle corresponds to the pathway impact score and is correlated with the centrality of the involved metabolites. The color of each metabolic pathway is related to the *P* value obtained from enrichment analysis, and its size represents the fold enrichment score i.e. −ln(*P*).

4.3.3 HISTOPATHOLOGICAL INDICES

The histological index of the gills or digestive tubules did not differ significantly between treatments. (Figure 7A and B). Nevertheless, mussels under EE treatment showed diffuse enlargement of the central vessel and hemocyte infiltration in gills, as well as extensive atrophy in digestive tubules (Figure 8).

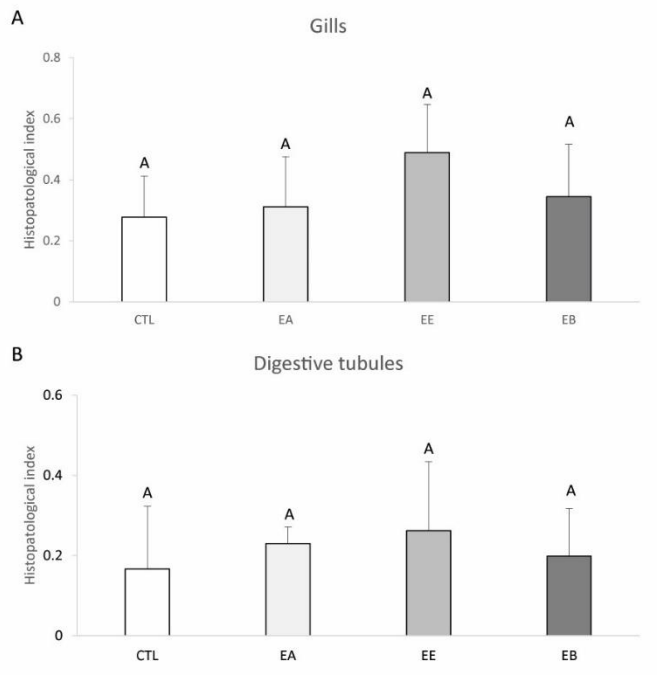
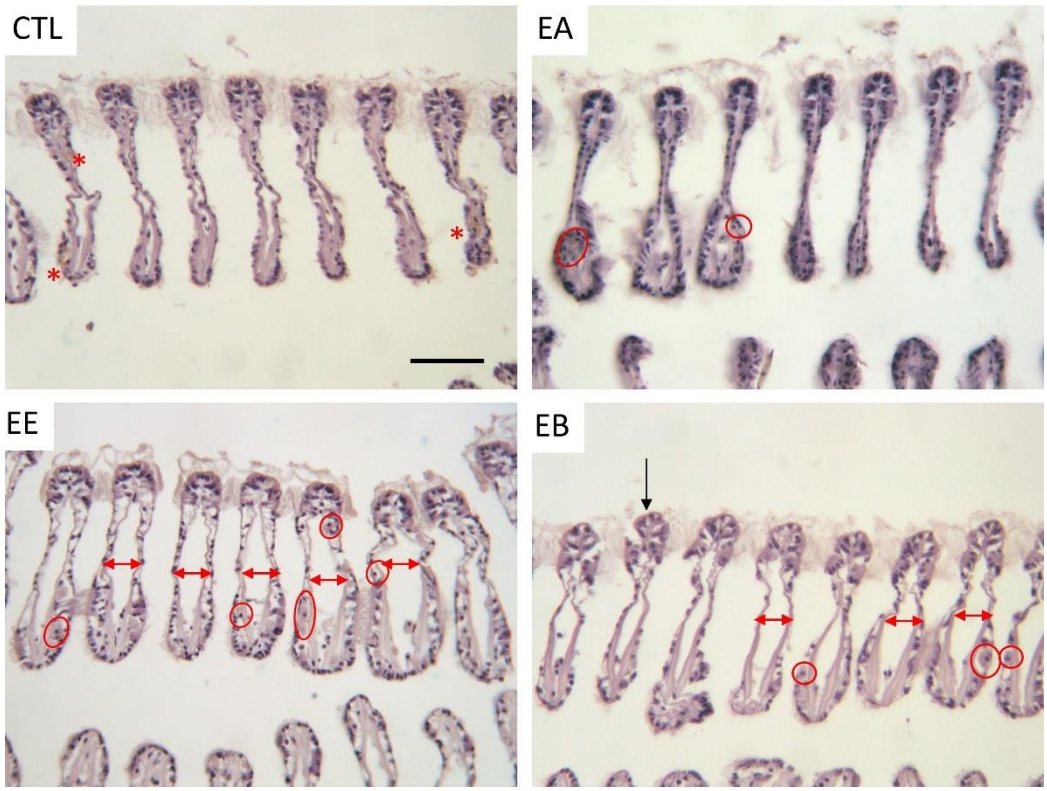


Figure 7. Histopathological indices in mussels treated with *P. magna* acetone extract (EA), diethyl ether extract (EE), and butanol extract (EB) compared to control mussels (CTL). 6A: gills; 6B: digestive tubules. The results are the mean + standard deviation. Significant differences ($p < 0.05$) among treatments are presented with different uppercase letters. $n = 3$.



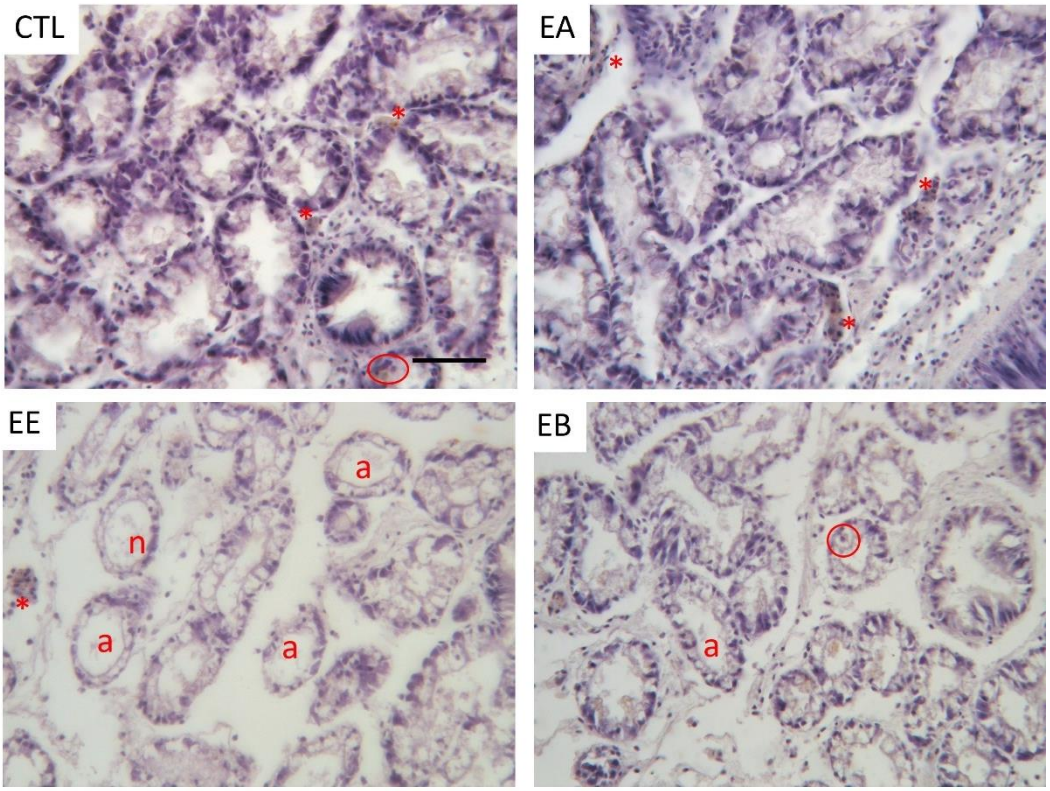


Figure 8. Micrographs of gills and digestive tubules sections of mussels after different feeding treatments: control (CTL), acetone extract (EA), diethyl ether extract (EE), and butanol extract (EB) stained with hematoxylin. *(lipofuscin aggregates), black arrow (cilia lost), double-headed arrows (enlargement of the central vessel), circles (hemocytes infiltration), a (atrophy), n (necrosis). Scale bar = 50 µm, *n* = 3.

4.4 DISCUSSION

The Mediterranean Sea, renowned for its rich biodiversity, faces a critical threat from the invasion of allochthonous species (Costello et al., 2023; Galil et al., 2014). Among the invasive species, the calcareous sponge *Paraleucilla magna* is currently widespread in the Mediterranean Sea (Tsirintanis et al., 2022).

Recently, it has been proposed that *P. magna* may produce antifouling molecules that inhibit the growth of fouling organisms (Longo et al., 2021). Given that the antifouling activity of natural substances is frequently linked to their toxicity, it is reasonable to assume that they may exert other harmful effects on local communities. In particular, a matter of concern is that the sponge frequently colonizes mussels' aquaculture facilities (Longo et al., 2012). The latter evidence further supports the selection of the bivalve *Mytilus galloprovincialis* in the present study to elucidate the potentially harmful impact of *P. magna* on the species it infests. Herein, the biochemical, NMR-based metabolomics, and histopathological responses of *M. galloprovincialis* to 28-day food treatment with different invasive sponge's crude extracts (acetone, diethyl ether, or butanol at a concentration of 50 mg/g dry food) are reported.

Biochemical analysis

The feeding treatment with acetone crude extract (EA) did not significantly impact the mussels' physiology. However, exposure to a food added with diethyl ether (EE) and butanol (EB) extracts elicited significant and distinct responses. This could be explained by considering that the use of acetone as a solvent allows the extraction of a wider panel of compounds with extremely different polarities. This implies relatively lower levels of single bioactive metabolites in EA compared to EE and EB.

In particular, electron transport system (ETS) activity decreased in EB-treated mussels, indicating a reduction in metabolism and energy production (De Coen and Janssen, 1997). This decrease was accompanied by an elevation in glycogen (GLY) expenditure, suggesting that mussels were likely keeping their valves closed while utilizing energy reserves as a stress response. This behavior aligns with the known

physiological protective mechanisms of bivalves, such as shell closure, under stressful conditions (Gosling, 2003). In addition, Ortmann and Grieshaber (2003) demonstrated that valve closure was associated with reduced metabolism. On the other hand, EE-treated mussels maintained a normal metabolic status but showed a decline in protein (PROT) content, specifically when compared to EA-treated mussels. The decrease in PROT levels could imply a reduction in protein synthesis, potentially affecting cellular and structural functions. Differences in PROT content in EA *vs.* EE treatments suggest a potential synergistic/antagonistic effect of the complex mixture of bioactive compounds present in the different crude extracts. In addition, in EE *vs.* CTL, there was a decrease in the activity of glutathione-S-transferases (GST) and carboxylesterases (CbEs), enzymes involved in the detoxification and biotransformation of hydrophobic compounds. The decrement in GST and CbEs undermines the hypothesis of overexpression of detoxifying enzymes in response to potentially toxic compounds. On the contrary, EB-treated mussels showed no impacts on GST and CbEs activities, suggesting that less hydrophobic compounds do not affect such enzymatic activities. Further investigations are needed to illuminate the observed responses to the different components of the extracts.

Even if the superoxide dismutase (SOD) activity was not affected by the treatments, the differential activity of the two crude extracts determined a significant increase in glutathione peroxidase (GPx) activity only in mussels exposed to EB, which were enhancing antioxidant defenses to counteract oxidative stress by oxidizing glutathione (GSH) into glutathione disulfide (GSSG). The increased GSH/GSSG ratio in EA, EE, and EB indicates a more reducing cellular environment (Regoli et al., 2011), suggesting an adaptive response to stressors. Thus, even if GPx activity increased in EB-treated mussels, these were able to maintain high levels of GSH. Again, similar results were observed by Guzzetti et al. (2019) in algae epiphytized by *P. magna*, in which both GPx activity and GSH levels increased. Despite the decrease in detoxification and biotransformation enzymes observed in EE-treated mussels, the lipid peroxidation (LPO) levels did not increase, probably thanks to the reducing environment characterized by a high GSH/GSSG ratio. Conversely, despite the observed activation of the antioxidant system, lipid peroxidation

occurred in EB-treated mussels, indicating the potential presence of prooxidant compounds in this extract.

A neurotoxic effect, highlighted by an inhibition in acetylcholinesterase (AChE) activity, was observed in both EE and EB-treated mussels. In EE-treated mussels, this inhibition could be correlated with the inhibition of biotransformation enzymes, which were therefore unable to promote the elimination of potential neurotoxic compounds. In EB-treated mussels, instead, this result was consistent with the decrement in ETS; in fact, the valve movement is normally inhibited as a defense mechanism and is associated with a reduction in metabolism, and AChE is supposed to regulate, among other functions, the valve closing by controlling adductor muscle functions (Beltran and Pocsidio, 2010; Corsi et al., 2007).

Metabolomic analysis

The NMR-based metabolomic study revealed a pattern similar to the biochemical investigation, with no significant changes between CTL and EA-treated mussels and a distinct response in the remaining treatments. Glycerophospocholine (GPC), a degradation product of phosphatidylcholine, one of the main membrane phospholipids (Brandão et al., 2015; Facchini et al., 2018), increased in EE-treated mussels, suggesting damages at the cellular membrane level. Nevertheless, LPO did not increase under the above treatments; thus, lipid peroxidation was probably prevented thanks to the high GSH/GSSG balance, with GSH being able to neutralize ROS. Additionally, GPC also serves as osmolytes (Brandão et al., 2015), along with trigonelline (Cappello et al., 2018), a ubiquitous metabolite in marine invertebrates belonging to alkaloids, whose levels also increased under EE-added food treatment, indicating an osmotic imbalance. These increases were followed by a decrease in betaine levels, which Liu et al. (2015) previously linked to osmotic stress in mussels. On the other hand, EB-treated mussels were characterized by high levels of homarine, which, together with trigonelline, betaine, taurine, and aspartate, represent some of the main marine species' osmolytes, small molecules involved in the maintenance of homeostasis in cells (Burg and Ferraris, 2008; Cappello et al., 2018; Liu et al., 2014; Preston, 1993). Homarine levels also increased in the Manila clam *Ruditapes philippinarum* exposed to copper (Zhang et

al., 2011) and in mussels exposed to polystyrene microplastics (Cappello et al., 2021), proving that their fluctuations can be related to a stressful condition. Instead, taurine and aspartate decreased in EB-treated mussels. A similar pattern was observed by Liu et al. (2014) in mussels exposed to pathogens, where the levels of homarine increased while those of aspartate, taurine, and betaine decreased, suggesting that in response to external stressors, mussels appear to regulate osmolytes' levels following a non-linear pattern. Moreover, in clams exposed to heavy metals, a depletion in taurine levels was also observed (Liu et al., 2011). Taurine is involved in the detoxification of xenobiotics and the neutralization of toxic aldehydes (Schaffer et al., 2014), thus, its depletion could have been caused by its consumption during the detoxification process by binding to toxic compounds found in the EB extract, allowing their elimination. Free amino acids, besides acting as osmolytes, are intermediaries in nitrogen excretion, energy sources, biosynthetic precursors (e.g., in gluconeogenesis), and building blocks for proteins, playing important roles in the physiology of marine invertebrates (Noor et al., 2020). Thus, the observed decrement in aspartate in EB-treated mussels could also be associated with the decrease in ETS and energy reserves observed with biochemical analysis. The decrement in aspartate levels was also observed by Cappello et al. (2017) in mussels exposed to drospirenone. Marine mollusks, like other marine invertebrates, use large amounts of osmolytes to protect themselves from fluctuations in extracellular osmolarity (Yancey et al., 1982). The observed variations in the present study are probably attributable to osmotic stress caused by the presence of compounds in the extracts that could alter the ionic equilibrium or cause damage to cell membranes, which can lead to the release of high quantities of ions into the intercellular spaces (Ammendolia et al., 2021). In summary, both EE and EB-treated mussels showed metabolomic signs of osmotic unbalance, even if the osmolytes involved were different. Overall, results regarding osmolytes levels were confirmed by the analysis of the metabolic pathways, showing that taurine, hypotaurine, and free amino acids were the most impacted pathways in mussels exposed to the sponge’s crude extracts.

Furthermore, EE-treated mussels showed high levels of the nucleotide adenosine monophosphate (AMP), whose increase has extensively been associated with

different stressful conditions in mussels exposed to various insults (e.g., environmental pollution, heat, pathogens) (Watanabe et al., 2015; Azizan et al., 2023; Liu et al., 2015). Adenosine plays key roles in both energy transfer and signal transduction (in the cyclic AMP form); thus, its alterations could implicate changes in both metabolic and signaling pathways. In addition, the elevated glucose levels observed in EE-treated mussels may be attributed to an augmentation in energy reserve compounds, providing a stored energy source available for the activation of defense mechanisms. Similar results were observed by Digilio et al. (2016) in mussels exposed both to copper and temperature challenges, as well as by Cappello et al., (2013) in mussels sampled at a polluted site. Another differential response in EE and EB-treated mussels was found in uridine and uridine diphosphate-N-Acetyl-Glucosamine (UDP-Glc-NAc) levels. In fact, uridine levels were higher in EE-treated mussels compared to those in EB-treated mussels, while UDP-Glc-NAc resulted in lower levels in EE compared to both EA and EB-treated mussels. High uridine levels were also observed in mussels exposed to environmental pollution (Watanabe et al., 2015), while the levels of UDP-Glc-NAc have not yet investigated. Uridine and glucose are involved in the synthesis of UDP-Glc-NAc, which in turn is the precursor of chitin (Nord, 1968), an important polymer for the formation of shells in bivalves (Weiner et al., 1984), thus their high level associated to the low level of UDP-Glc-NAc could imply that under treatment with EE the synthesis of chitin was lower when compared to EB-treatment.

Histopathological indices

Histopathological indices in gills and digestive tubules did not show a significant difference between treatments, although EE-treated mussels displayed more signs of inflammation, such as extensive enlargement of the central vessel of gills and hemocytes infiltration (Pagano et al., 2016). However, the lack of statistically significant differences in histopathological parameters between treatments suggests that, despite biochemical and metabolomic changes, the effects may not have been severe enough to cause a visible impact at the morphological level. The same result was observed by Leite et al. (2023) in mussels exposed to dysprosium, where

despite inducing several alterations in biochemical markers, the above element did not induce significant alterations in the gills of the mussels.

CONCLUSION

In conclusion, exposure to EE and EB-added food induced significant variations in the biochemical and metabolomic parameters of mussels, indicating complex and differential responses to the type of exposure. However, it is noteworthy that, despite these variations, the impacts were not so extensive as to induce histopathological alterations in the examined tissues. It is also possible that the observed biochemical changes may have effectively revealed sub-individual chemical responses earlier than potential morphological impacts, which might have become significant with extended exposure, suggesting the possibility of more pronounced long-term effects. The distinct response of mussels to the two types of implemented foods may be attributed to the nature of the metabolites extracted with the two different solvents. The present results suggest that both diethyl ether and butanol extracts may contain metabolites with bioactive activity worth exploring. Taken together, these findings underscore the complexity of interactions between mussels and exposure agents, including marine invasive species, with possible implications for cellular health and adaptation. Finally, the present study paves the way for further research aimed at understanding in detail the ecological impacts of the invasive sponge *P. magna*, involving the study of the sponge's metabolites released in the water column and possibly filtered by mussels (exometabolome). If confirmed, the uptake of toxic compounds released by the sponge could represent a serious threat to mussels' farms and Mediterranean Sea fauna, with both economic and ecological outcomes.

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CHAPTER 5

CONCLUSION

Various biological and chemical stressors are currently increasing the vulnerability of ecological systems, with critical implications for human society as well. Despite this, the assessment of the cumulative harmful effects caused by simultaneous or sequential exposure to different stressors is not always given due consideration in the literature (Rodrigues et al., 2022). This is also true for marine ecosystems that face continuous and simultaneous exposure to several drivers of change, which include both biological and chemical pollution. Indeed, biopollution by alien species is one of the main threats to the Mediterranean Sea (Occhipinti-Ambrogi, 2021). Furthermore, an increasing literature is also starting to consider the chemical threat posed by marine invasive species to native communities (Mollo et al. 2015). Frequently, to this type of environmental contamination is added chemical pollution of anthropogenic origin, with a variety of chemical hazards continuously released into the sea (Brumovský et al., 2017; Costello et al., 2023). In this study, we attempted to clarify the extent to which differential exposure to chemical contaminants of different natures contributes to increasing the vulnerability of living organisms subjected to such exposures. We first compared the effects of the alkaloid caulerpin from invasive algae of the genus *Caulerpa* to the anthropogenic hazard compound fenofibrate by using the mollusk *Mytilus galloprovincialis* as a model organism. The comparison between these two compounds was motivated by their shared mechanisms of action as ligands for peroxisome proliferator-activated receptor alpha (PPAR-α) (Vitale et al., 2018). Fenofibrate administration resulted in enhanced metabolic capacity, increased cellular damage, and morphological alterations, affirming the serious risks it poses to aquatic organisms. Conversely, caulerpin, derived from the invasive green alga *Caulerpa cylindracea*, did not produce significant impairments in the assessed parameters. This ranks caulerpin as a non-toxic compound of considerable interest for potential applications in pharmaceutical and nutraceutical sectors, given its well-established anti-tumoral and anti-inflammatory properties (De Souza et al., 2009). Further research focused on the co-exposure of caulerpin with the alkaloid caffeine, which represents a further aquatic pollutant widespread in coastal water with a toxic potential for invertebrates (Li et al., 2020). The simultaneous exposure to caulerpin and caffeine, however, did not produce any cumulative effects. Notably, caulerpin treatment

showed potential in modulating the toxicity of caffeine, since in the combined treatment, cellular damage (LPO) and neurotoxicity (AChE activity), induced by mere exposure to caffeine, were not observed. A detoxifying effect may be hypothesized for caulerpin, possibly due to its agonistic action on PPARs, which are nuclear transcription factors known for mediating xenobiotic detoxification. This possible action of caulerpin as a detoxifying agent, if confirmed, could pave the way for the sustainable exploitation of biomass from invasive *Caulerpa* species. Finally, we explored the impacts of crude extracts from the non-indigenous invasive *Paraleucilla magna* on mussels. Although this sponge is known to foul Mediterranean bivalve aquaculture facilities (Longo et al., 2012), nothing is known about the types of molecules that are released in the water column by the sponge (exometabolome) and could pose a real threat to native fauna and mussels' farms. Our preliminary evaluations of the effects produced by the sponge crude extracts on the mussels revealed significant biochemical and metabolic variations. However, histopathological alterations were not induced in the examined tissues. These preliminary results deserve further exploration to understand the actual ecological impacts of *P. magna* on the Mediterranean native communities.

Overall, as already extensively documented in the literature across a wide range of insults (Capolupo et al., 2016; Cappello et al., 2021; 2017; Coppola et al., 2020, 2022; Curpan et al., 2022; De Marco et al., 2022; Freitas et al., 2019; among many others), *M. galloprovincialis* has been confirmed in this study to be an excellent model organism for assessing the effects of both anthropogenic hazards and pollutants from invasive species, providing valuable insights into the complex interplay between organisms and the multiple drivers of change they face. This assessment has been carried out using a multidisciplinary approach that included a combination of biochemical analyses, NMR-based metabolomics, and histopathological tools to gain a broader understanding of the topic. The innovation of this thesis work also lies in the fact that exposure to insoluble molecules or extracts was made possible by incorporating them into the food administered to the mussels. In this perspective, in addition to the novel data about the possible effects of pollutants of different origins on the exposed populations, the study also provides information of interest to the nutraceutical sector.

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ACRONYMS

AChE	acetylcholinesterase
AMP	adenosine 5′-monophosphate
CAF	caffeine
CAT	catalase
CAU	caulerpin
CbEs	carboxylesterases
CTL	control
DHA	docosahexaenoic acid
DT	digestive tubules
EA	acetone extract
EB	butanol extract
EE	ether extract
EPA	eicosapentaenoic acid
ETS	electron transport system
FFB	fenofibrate
FID	free induction decay
FW	fresh weight
GC	gas chromatography
GLY	glycogen
GMP	guanosine 5′-monophosphate
GPC	glycerophosphocholine
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
GSTs	glutathione S-transferases
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
IAS	invasive alien species
I_h	histopathological index

IMP	inosine monophosphate
LOD	limit of detection
LOOH	lipid hydroperoxides
LPO	lipid peroxidation
MDA	malondialdehyde
MS	mass spectrometry
NIS	non-indigenous species
NMR	nuclear magnetic resonance
OPLS-DA	orthogonal partial least squares discriminant analysis
PBS	phosphate buffer saline
PCA	principal component analysis
pNPA	p-nitrophenyl acetate
pNPB	p-nitrophenyl butyrate
PPAR	proliferator-activated receptor
PROT	total protein
PUFA	polyunsaturated fatty acids
RF	radio frequency
ROS	reactive oxygen species
SOD	superoxide dismutase
TOCSY	clean total-correlation spectroscopy
UDP-Glc-NAc	uridine diphosphate-N-acetylglucosamine
WWTPs	wastewater treatment plants

RINGRAZIAMENTI

Esprimo la mia profonda gratitudine al mio supervisore il Professore Gianluca Polese per avermi dato l'opportunità di perseguire questo dottorato sotto la sua guida e per aver reso questo percorso non solo formativo, ma anche entusiasmante. È stato fondamentale nell'insegnarmi ad individuare soluzioni e opportunità anche in situazioni difficili.

Un sentito ringraziamento va al Dott. Ernesto Mollo per avermi ospitato nel suo gruppo di ricerca presso l'ICB - CNR. Questa esperienza è stata parte integrante del mio progetto di dottorato, e gli sono estremamente riconoscente per il suo continuo aiuto e la sua disponibilità. Allo stesso modo, la mia gratitudine si estende alla Dott.ssa Marianna Carbone e alla Dott.ssa Letizia Ciavatta per il loro contributo prezioso ed alla Dott.ssa Debora Paris e al Dott. Andrea Motta per la loro collaborazione in questa ricerca e il supporto nelle analisi metabolomiche.

Un apprezzamento speciale è destinato alla Professoressa Rosa Freitas, che mi ha permesso di svolgere una parte fondamentale della mia ricerca di dottorato nel suo laboratorio presso l'Università di Aveiro, offrendomi supporto costante. La collaborazione che abbiamo mantenuto nel corso degli anni è per me motivo di profonda stima e ammirazione.

Estendo il mio ringraziamento alla Dott.ssa Francesca Coppola; la sua prontezza nel fornire aiuto, il suo sostegno incrollabile e l'amicizia sono stati per me indispensabili.

Ringrazio sinceramente anche la Professoressa Anna Di Cosmo per il suo aiuto e i suoi insegnamenti, e per le inestimabili esperienze condivise durante questi anni.

Un profondo riconoscimento va al nostro coordinatore di dottorato, il Professore Sergio Esposito, che ha supervisionato questa esperienza, fornendo informazioni e assistenza quando richieste.

Desidero inoltre esprimere la mia gratitudine a tutti i miei colleghi, molti dei quali definirei più amici che collaboratori, ai Ricercatori e ai Professori che ho incontrato durante questo percorso, i quali hanno tutti contribuito in qualche modo al suo successo.

Ringrazio la mia famiglia numerosa e il mio compagno di vita, Mirko, il cui sostegno fornito in tutti questi anni è stato indescrivibile e inestimabile.

Infine, un ringraziamento speciale va ai miei amici e a tutti coloro che mi hanno supportata in vari modi durante questi anni.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my supervisor, Professor Gianluca Polese, for providing me with the opportunity to pursue this PhD under his guidance and mentorship and for making this journey not only educational but also stimulating. He has been instrumental in teaching me to identify solutions and opportunities, even in challenging situations.

A heartfelt thank you goes to Dr. Ernesto Mollo for hosting me in his research group at ICB-CNR. This experience has been integral to my project, and I am extremely grateful for his continuous help and availability. Similarly, my gratitude extends to Dr. Marianna Carbone and Dr. Letizia Ciavatta for their valuable contributions and to Dr. Debora Paris and Dr. Andrea Motta for their fundamental contributions to this research and their assistance in metabolomic analyses.

Special appreciation is reserved for Professor Rosa Freitas, who allowed me to conduct a crucial part of my PhD research in her laboratory at the University of Aveiro, providing me with continuous support. The collaboration we have maintained over the years is for me a source of deep respect and admiration.

I extend my gratitude to Dr. Francesca Coppola; her prompt help, unwavering support, and friendship have been indispensable to me.

Sincere acknowledgement also goes to Professor Anna Di Cosmo for her help and teachings and the invaluable experiences shared during these years.

A profound appreciation is due to our PhD coordinator, Professor Sergio Esposito, who supervised this experience, providing information when needed.

I also want to express my gratitude to all my colleagues, many of whom I would describe more as friends than collaborators, and to the researchers and professors I have encountered during this journey, all of whom have contributed to its success.

I thank my large family and my life partner, Mirko, whose support over all these years has been indescribable and invaluable.

Finally, special thanks to my friends and everyone who has sustained me in various ways.

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