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**BIOLOGIA MARINA**

# *FOSTERING RESILIENCE: PRIMING TECHNIQUES TO MITIGATE Ruditapes philippinarum VULNERABILITY TO MULTIPLE STRESSORS*

# *PROMUOVERE LA RESILIENZA: TECNICHE DI PRIMING PER MITIGARE LA VULNERABILITÀ DELLA VONGOLA Ruditapes philippinarum A STRESS MULTIPLI*

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# LIST OF ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| ABAP | 2-2′-azo-bis-(2-methylpropionamidine)-dihydrochloride |
| ACP | Acid Phosphatase |
| AChE | Acetylcholinesterase |
| AIRC | International Agency for Research on Cancer |
| AOI | Area Of Interest |
| BA | Blank area |
| BSA | Bovin Serum Albumin |
| $Ca^{2+}$ | Calcium Ion |
| CAT | Catalase |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| $Cl^-$ | Chloride Ion |
| CP | Chemical-Primed |
| CTRL | Control |
| CTRL-CH | Control under Challenge |
| DAPI | 6-diamidino-2-phenylindole |

| | |
|---|---|
| DMSO | Dimethyl sulfoxide |
| DTNB | 5,5’ -ditio-bis-2nitrobenzoic acid |
| ε | Extinction coefficient |
| EDTA | Ethylenediaminetetraacetic acid |
| $Fe^{3+}$ | Ferric Ion |
| GHG | Greenhouse Gases |
| GPx | Glutathione Peroxidases |
| GR | Glutathione Reductase |
| GSH | Reduced Glutathione |
| GSSG | Glutathione disulfide |
| GSTs | Glutathione S-Transferases |
| GWLs | Global Warming Levels |
| HCl | Chloridric acid |
| $H_2O_2$ | Hydrogen Peroxide |
| HOCl | Hypochloric Acid |
| KCl | Potassium Chloride |
| KMBA | α-keto-γ-methiolbutyric acid |
| MDA | Malondialdehyde |
| $Mg^{2+}$ | Magnesium Ion |
| MHWs | Marine Heatwaves |

| | |
|---|---|
| NaCl | Sodium Chloride |
| $NaN_3$ | Sodium azide |
| NaOH | Sodium hydroxide |
| NR | Neutral Red |
| NRRT | Neutral Red Retention Time |
| NO• | Nitric Oxide |
| •OH | Hydroxyl Radical |
| $O_2^-$ | Superoxide Anion |
| OCLTT | Oxygen and Capacity Limited Thermal Tolerance |
| $ONOO^-$ | Peroxynitrite |
| PCA | Principal Components Analysis |
| PFAS | Polyfluoroalkyl Substances |
| PFOA | Perfluorooctanoic Acid |
| PMSF | Phenylmethylsulphonyl fluoride |
| POPs | Persistent Organic Pollutants |
| ppm | parts per million |
| PSU | Practical Salinity Unit |
| RO• | Alkoxyl Radical |
| ROO• | Peroxyl Radical |

| | |
|---|---|
| ROS | Reactive Oxygen Species |
| rpm | revolutions per minute |
| SA | Sample Area |
| SOD | Superoxide Dismutase |
| SPC | Stress Priming Clams |
| SPE | Stress Priming Experiment |
| TOSC | Total Oxyradical Scavenging Capacity |
| TOSC-A | Total Oxyradical Scavenging Capacity Assay |
| V/cm | Volume/centimeters |
| w/v | weight/volume |
| λ | Wavelength |

# ABSTRACT

Global climate changes are affecting the integrity and functioning of marine ecosystems worldwide, which are threatened by the increasing the frequency and intensity of extreme events such as marine heatwaves, among others. These events may reduce the tolerance of marine species already coping with other co-occurring stressors, as chemical pollution, listed among the first five causes of biodiversity reduction. According to this multiple stressors' scenario, marine organisms will be subjected to cumulative pressures which may lead to biodiversity loss and socio-economic impacts on fishery and aquaculture: in this respect, bivalves, as filter-feeding and sedentary organisms, are highlighted as particularly vulnerable to these scenarios as also the blue-economy depends on bivalves' stocks. The present study is part of the MANILA project, which aims to improve the performance of Manila clam (*Ruditapes philippinarum*) towards environmental stressors exploring priming techniques as innovative tools to provide a possible mitigation strategy for biodiversity conservation and sustainable aquaculture management. To this end, for the first time, chemical priming techniques (using different concentrations of $H_2O_2$) have been initially tested for their capability to stimulate stress-response mechanisms as lysosomal integrity, antioxidant system, oxidative damages and cholinergic response.

Results highlighted a similar efficiency of tested $H_2O_2$ concentrations in stimulating stress-response, with more promising evidence in clams treated with 50 μM $H_2O_2$. Therefore, the highest concentration was used to prime organisms before exposing them to a multiple stressors' scenario, consisting in a combination of thermal and chemical stress.

Naïve- and primed-clams performance was compared to evaluate priming efficiency in increasing organisms' protection toward environmental disturbance: to this end, a wide panel of biological responses was evaluated, including antioxidant system, oxidative damages to DNA, cholinergic response and immunocytes functionality and organelles integrity.

Obtained results highlighted a higher tolerance to challenge in primed-clams compared to naïve ones, as supported by the lack of effects in terms of oxidative pressure and on cholinergic function; on the other hand, a generalized sensitivity of immunocytes was observed. Nonetheless, primed-clams showed a lower mortality rate compared to naïve challenged ones throughout one month of rest after the challenge, suggesting the positive effect of the tested approach and allowing to hypothesize this as a promising tool for its application in aquaculture.

*Keywords: multiple stressors, priming techniques, Ruditapes philippinarum, multi-biomarker approach*

# RIASSUNTO

I cambiamenti climatici globali stanno influenzando l'integrità e il funzionamento degli ecosistemi marini in tutto il mondo, minacciati dall'aumento della frequenza e dell'intensità di eventi estremi come le ondate di calore marine, tra gli altri. Questi eventi possono ridurre la tolleranza delle specie marine che stanno già affrontando altri fattori di stress, come l'inquinamento chimico, elencato tra le prime cinque cause di riduzione della biodiversità. Secondo questo scenario di stress multipli, gli organismi marini saranno soggetti a pressioni cumulative che possono portare alla perdita di biodiversità e a impatti socio-economici sulla pesca e sull'acquacoltura: a questo proposito, i bivalvi, in quanto organismi filtratori e sedentari, sono particolarmente vulnerabili a questi scenari, essendo anche la blue-economy dipendente dai loro stock. Il presente studio fa parte del progetto MANILA, che mira a migliorare le prestazioni della vongola di Manila (*Ruditapes philippinarum*) nei confronti dei fattori di stress ambientale, esplorando le tecniche di priming come strumenti innovativi per fornire una possibile strategia di mitigazione per la conservazione della biodiversità e la gestione sostenibile dell'acquacoltura. A tal fine, per la prima volta, sono state testate

tecniche di priming chimico (utilizzando diverse concentrazioni di $H_2O_2$) per verificare la loro capacità di stimolare meccanismi di risposta allo stress come l'integrità lisosomiale, il sistema antiossidante, i danni ossidativi e la risposta colinergica.

I risultati hanno evidenziato un'efficienza simile delle concentrazioni di H2O2 testate nello stimolare la risposta allo stress, con prove più promettenti nelle vongole trattate con 50 μM di $H_2O_2$. Pertanto, la concentrazione più alta è stata utilizzata per "primare" gli organismi prima di esporli a uno scenario di stress multipli, consistente in una combinazione di stress termico e chimico.

Le prestazioni delle vongole "naïve" e di quelle primate sono state confrontate per valutare l'efficacia del priming nell'aumentare la protezione degli organismi nei confronti delle perturbazioni ambientali: a tal fine, è stato valutato un ampio panel di risposte biologiche, tra cui il sistema antiossidante, i danni ossidativi al DNA, la risposta colinergica e la funzionalità degli immunociti e l'integrità degli organelli.

I risultati ottenuti hanno evidenziato una maggiore tolleranza alla sfida nelle vongole primate rispetto a quelle "naïve", come supportato dall'assenza di effetti in termini di pressione ossidativa e sulla funzione colinergica; d'altra parte, è stata osservata una sensibilità generalizzata degli immunociti. Ciononostante, le vongole primate hanno mostrato un tasso di mortalità

inferiore rispetto a quelle “naïve” sottoposte a challenge, per tutto il mese di riposo successivo alla challenge, suggerendo l'effetto positivo dell'approccio testato e consentendo di ipotizzarlo come uno strumento promettente per la sua applicazione nell’ambito dell'acquacoltura.

# 1- INTRODUCTION

## *1.1- MARINE HEATWAVES UNDER THE CLIMATE CHANGE SCENARIO*

Marine ecosystems are pivotal to humankind, given the essential services provided, as provisioning (fish, seafood, health products) and supporting ones (primary productivity, element and nutrient cycling, climate regulation) (Strategic Plan for Biodiversity 2011-2020, including Aichi Biodiversity Targets). Their functioning depends on the link between several diverse biotic components, whose fitness is shaped by abiotic factors, as pH/$pCO_2$, temperature, oxygen concentration and salinity, to cite a few, which can be subjected to changes brought about by both natural and anthropogenic stressors (Gattuso *et al*., 2015; Kappel, 2005; Doney *et al*., 2012).

Since the industrial revolution, human activities have produced a consistent release of greenhouse gases (GHG), mainly $CO_2$, which concentration increased from a six-thousand-year-stable level of 280 parts per million (ppm) reaching 410 ppm in 2019 (IPCC, 2023), the highest level over the past two million years; according to IPCC, 2023, this is very likely to have caused the increase of global heat content triggering the process known as global warming, the increase of mean temperatures measured over the past decades; for instance, a mean global surface warming 1.1°C was measured between 2011 and 2020 (*fig. 1.1.1*).

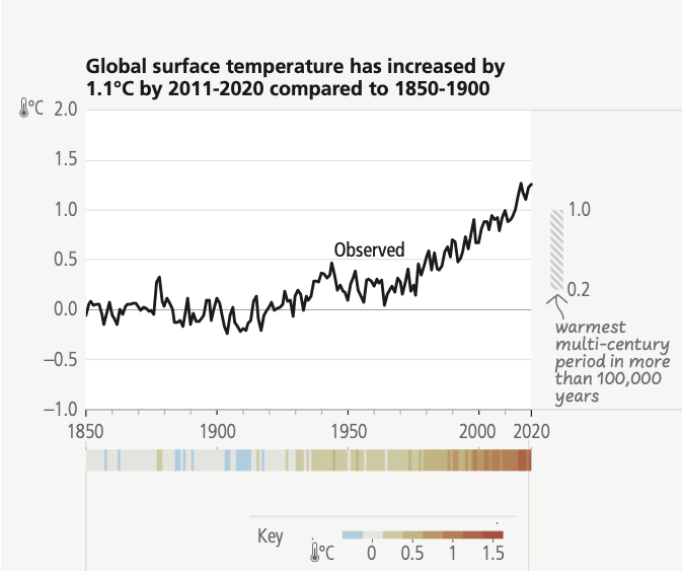



*Figure 1.1.1: Changes in global surface temperature (IPCC, 2023)*

This warming is causing severe changes to climate system, which are expected to impact human and natural systems and are projected to exacerbate and widen regional disparities in the future as shown in *figure 1.1.2* (IPCC, 2023).

Global warming is causing several physical changes such as melting glaciers, rising sea levels or changes in the hydrological cycle, such as the formation of more intense storms (Bijma *et al*., 2013); a further consequence is ocean stratification, which leads to alterations in both the availability of oxygen in the surface layers and in primary productivity (Hoegh-Guldberg, 2010; Keeling *et al*., 2010; Halpern *et al*., 2008).

Direct effects of changes in temperature may alter the physiological functions and demographic characteristics of organisms leading to alterations in size, spatial range and abundance of populations (Byrne, 2012; Doney *et al*., 2012).

At organism level, temperature plays a key role in the physiology of ectotherms, as their thermal condition changes with the environmental one (Sokolova & Lannig, 2008). Temperature directly influences oxidation rates and mitochondrial functions (e.g. production of reactive oxygen species-ROS- or phosphorylation) (Cherkasov *et al*., 2006) and higher temperature can cause changes in ventilation rates and a decrease in oxygen solubility (Kennedy and Walsh, 1997). At population level, paleontological records have shown that even the slightest change in environmental temperature of a few Celsius degrees causes a major shift in the distribution of organisms (Sokolova and Lannig, 2008).

Besides, another phenomenon occurring along with global warming is Ocean Acidification (OA), the worldwide reduction in the pH of seawater because of the absorption of large amounts of carbon dioxide by the oceans. Indeed, since the start of the industrial revolution, the world's oceans have adsorbed approximately 500 billion tons of atmospheric $CO_2$, increasing the average acidity by 30 % (Doney, Fabry *et al*., 2009).

Ocean acidification is now recognized as the main process generating changes in the biogeochemistry and functioning of marine ecosystems, affecting tens of thousands of marine species relying on carbonate to produce their shells and skeletons (Harms *et al*., 2014; Byrne, 2012). OA is therefore expected to impact

marine biodiversity and commercial fisheries worldwide, threatening a food source for hundreds of millions of people as well as a multi-billion-dollar industry (Wootton *et al*., 2008).

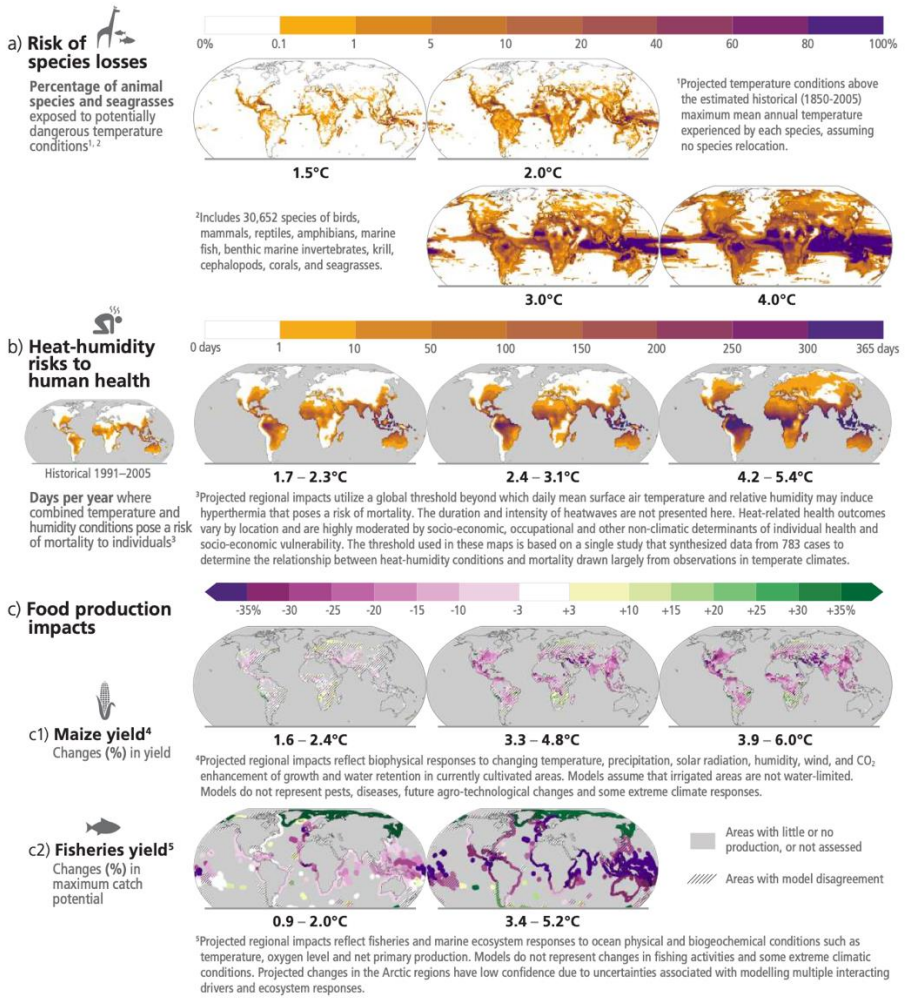



*Figure 1.1.2: Projected risks and impacts of climate change on natural and human systems at different global warming levels (GWLs) relative to 1850-1900 levels (IPCC 2023)*

Moreover, the frequency of extremely intense regional warming events, also known as marine heatwaves (MHWs), has grown in tandem with the long-term, persistent warming (Smale *et al*., 2019). Marine heatwaves are exceptional

occurrences in which the sea surface temperature rises beyond a seasonally varying threshold (often the 90$^{th}$ or 99$^{th}$ percentile of climatological series) for a minimum of five days in a row and, lasting up to months covering thousands of kilometers (Nardi *et al*., 2022; Oliver *et al*., 2019; Frölicher *et al*., 2018; Hobday *et al*., 2018; Scannell *et al*., 2016; Perkins *et al*., 2012). Since the early 20$^{th}$ century, the number of anomalous warm days has increased by 50% worldwide, and since 1982, the average intensity of MHWs has shown a linear trend of about +0.1°C every decade as shown in *figure 1.1.3* (Oliver *et al*., 2019, 2018). Additionally, by the end of the century, many oceanic regions are expected to experience a nearly permanent state of MHW (*fig. 1.1.4*) due to human impacts on climate stability (Collins *et al*., 2022; Oliver *et al*., 2019).

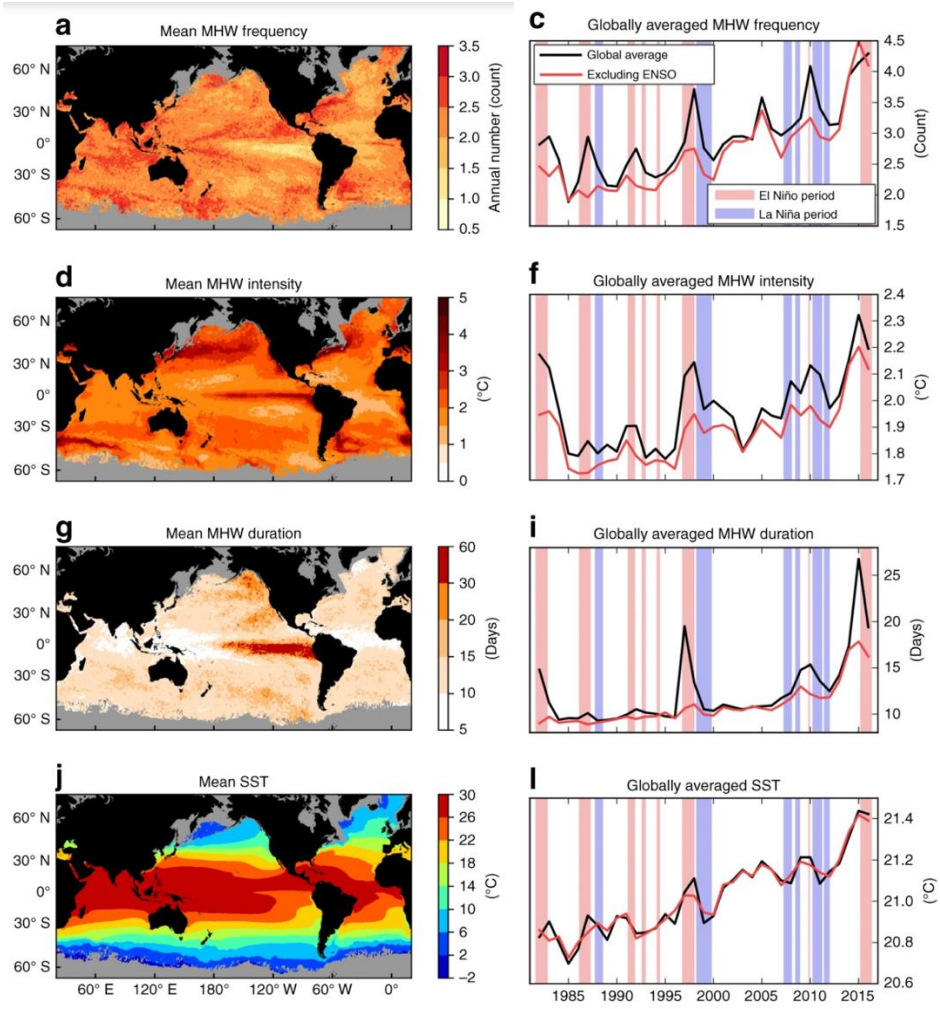



*Figure 1.1.3: Global sea surface temperature and marine heatwave properties over the period 1982-2016 (Oliver et al., 2018)*

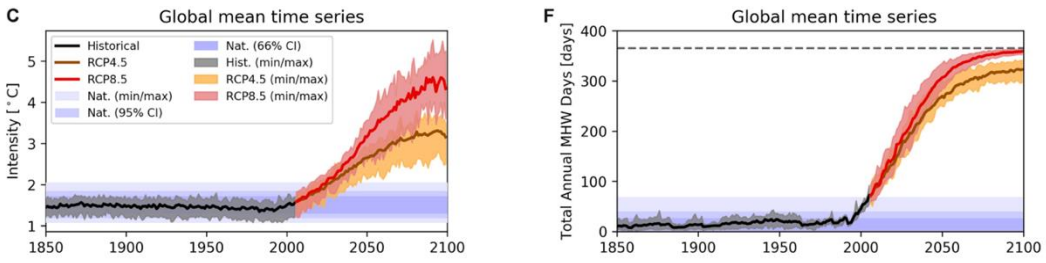



*Figure 1.1.4: Projected changes to marine heatwaves (Oliver et al., 2019)*

Current MHWs have caused entire ecosystems to suffer harsh environmental conditions, increasing the vulnerability of marine biomes and fisheries with consequently significant socioeconomic losses (Smale *et al*., 2019; Mills *et al*., 2013). As a proof, Caputi *et al*. (2016) showed 99% mortality of gastropods of the species *Haliotis roei* and severe reductions in abundance of commercial scallops and prawns in Australia after the occurrence of MWHs in 2010/2011.

The Mediterranean Sea is considered among the most vulnerable regions worldwide to climate changes, and both the frequency and intensity of MHWs have been projected to increase by 2100, with possible detrimental impacts on the services and structures of marine ecosystems as demonstrated by several mass mortality events affecting coralligenous species, *Posidonia oceanica* meadows (Rivetti *et al*., 2014; Garrabou *et al*., 2009; Cerrano *et al*., 2000) and bivalves among other taxa (Nardi *et al*., 2022; Albano *et al*., 2021; Marin *et al*., 2021; Darmaraki *et al*., 2019).

It is recognized that, in contrast to slower changes under ordinary conditions, species and biological communities experience greater effects from abrupt and brief climatic events, due to the lack of time for adaptation (Dolbeth *et al*., 2019; Oliver *et al*., 2018; Cardoso *et al*., 2008). Under this scenario, the impacts of MHWs can be more severe in animals with less

mobility and those that live near the edge of their thermal tolerance (Smale *et al*., 2019); this can be one of the reasons why introduced non-native species can outcompete their native equivalents, benefitting of a wider thermal tolerance range (Crespo *et al*., 2021; Zerebecki and Sorte, 2011). Prolonged exposure to high temperatures may represent a stressful condition for organisms which are forced to modify their energetic balance to favor stress-response mechanisms to reduce consequences of thermal stress, alterations in mitochondria functioning and increased ROS production. In this scenario, a lower amount of energy is available to maintain immune system efficiency, fertilization and reproduction rates under optimal ranges (Nardi *et al*., 2022; Crespo *et al*., 2021). According to Somero, 2012, the main factor influencing a species' tolerance to environmental variability is its physiological performance: in ectothermic organisms, metabolic rates and temperature are exponentially linked meaning that higher temperatures will lead to higher rates of cellular and physiological processes which, over time, may lead to growth limitations, reduced reproduction up to higher mortality rates. Changes in organisms' physiology may also lead to modifications in species interactions, including competition, grazing, predation, and disease dynamics, which in turn affect communities. In addition to local invasion and extinction caused by climate change, these

processes change the variety and organization of communities and can lead to shifts toward new ecosystem arrangements (Doney *et al*., 2012).

### *1.2- MULTIPLE STRESSORS AFFECTING BIODIVERSITY*

Beyond their direct effects on marine species and ecosystems, thermal stress and MHWs are of concern also for the biological implications caused by their co-occurrence along with other environmental stressors such as, hypoxia, harmful algal blooms and chemical pollutants, being these conventional (heavy metals, PAHs) or emerging contaminants like pharmaceuticals, microplastics and polyfluoroalkyl substances known as PFAS (Nardi *et al*., 2022).

The various environmental stressors linked to global climate change and human activities are having a significant negative influence on trophic and food-web dynamics, ocean systems and, ultimately, on ecological services supporting human society (Doney *et al*., 2012; Hoegh-Guldberg *et al*., 2010). Because of their higher potential for harm, synergistic interactions are especially concerning and expected to rise with stressor size which, consequently, is expected to rise with increased human activity, making ecosystem responses more unpredictable (Arrigo et al., 2020; Veiga *et al*., 2020; Hewitt *et al*., 2016; Darling *et al*., 2008; Sokolova and Lannig, 2008).

As already stated, anthropogenic climate change is predicted to cause significant increases in the intensity and duration of marine heatwaves in the 21$^{st}$ century (Oliver *et al*., 2019; Hobday *et al*., 2018; Perkins *et al*., 2012). This means that changes in environmental factors variability, in addition to average

condition shifts, will modify important biological processes and change the dynamics and structure of populations (Pansch *et al*., 2018).

In areas with less climatic variability, these variations typically have more profound biological effects because local species are exposed to temperatures that surpass their upper thermal limits and that they have never experienced previously in their life histories. The degree of event intensity, the species' sensitivity, and its ability to adjust to changing environmental conditions all affect how vulnerable benthic populations are (Smale *et al*., 2019).

In a context of multiple co-occurring stressors, temperature can alter the stability of contaminants, but also affect their accumulation and metabolism through the alteration of key biological processes, possibly increasing their toxicity. On the other hand, organisms already coping with thermal stress may experience stress-defense mechanisms unbalance and be less prone to activate detoxification mechanisms towards chemicals, leading to harsher toxic effects (Marigómez *et al*., 2017; Regoli and Giuliani, 2014; Schiedek *et al*., 2007).

For instance, it is widely acknowledged that higher temperatures accelerate ectotherms' metabolic and ventilation rates, and consequently, chemicals uptake and eventually bioaccumulation in tissues is enhanced (Cherkasov *et al*., 2007; Schiedek *et al*., 2007; Tsui and Wang, 2006).

On the other hand, Lannig *et al*. (2006), demonstrated how heavy metals contamination can negatively affect bivalves' capacity to efficiently uptake oxygen under thermal stress, with a consequent major need for active ventilation, as the case of higher cadmium bioaccumulation in *Crassostrea virginica* co-exposed to temperature increase and cadmium. Similarly, Baines *et al*., 2006, showed how increasing temperatures are positively correlated with increasing uptake of cadmium (Cd), copper (Co) and zinc (Zn) in *Mytilus edulis*. Temperature increase led to a higher cadmium uptake in *Mytilus galloprovincialis* only when warming occurred within mussels' thermal window (Nardi *et al*., 2017, 2018): indeed, a +5°C increase was positively correlated with Cd burdens in mussels' tissues only in winter (from 10°C to 15°C) and not in summer (from 20°C to 25°C).

At the same time, exposure to pollutants can impair marine ectotherms' capacity to adapt to heat stress. This can lead to deficiencies in protein synthesis (Lannig *et al*., 2006), irregular heart rates (Camus *et al*., 2004), an incapacity to acclimate to variations of temperature (Marigómez *et al*., 2017), restrictions on their ability to use energy (Cherkasov *et al*., 2006) and changes in the bioaccumulation process (Moreira *et al*., 2020).

While there is a solid body of knowledge on the effects of several classes of conventional chemicals under thermal stress, little is known for certain classes

of chemicals recognized as “emerging contaminants” (Schiedek *et al*., 2007; Yamashita *et al*., 2005). According to the Environmental Protection Agency (EPA), an emerging contaminant is a chemical or a material which is considered to provide a real or potential threat to public health or the environment; a pollutant could potentially be considered “emerging” also when a new source or pathway to humans have been found. Among them, there are per and poly-fluoroalkyl substances (PFAS), compounds characterized by fluorine-carbon bonds (Johnson *et al*., 2021; Buck *et al*., 2011), widely used in commercial products as packing material, clothes, cosmetics and as anti-fouling (Sunderland *et al*., 2019; Rodea-Palomares *et al*., 2012; Lindstrom *et al*., 2011). The most known is the perfluorooctanoic acid (PFOA), a typical persistent organic pollutant (POP), which can be accumulated in fatty tissues and is extremely water-soluble (L. Yang *et al*., 2020; Du *et al*., 2014; Suja *et al*., 2009; Lau *et al*., 2007), therefore widely distributed and with a great bio-accumulation potential (L. Yang *et al*., 2020). PFOA has been detected in several aquatic animals and marine ecosystems in recent years (M. Guo *et al*., 2019; Ahrens & Bundschuh, 2014; Rayne & Forest, 2009). It has been established that prolonged low-dose exposure to PFOA can harm organisms irreversibly causing histopathological alterations, organelles dissolution and nuclear changes (Li *et al*., 2022; Filgo *et al*., 2015; Post *et al*., 2012; Shin *et al*.,

2011). It is also included in the Stockholm convention as a compound that needs to be reduced in its production and eliminated in the proper way (UNEP); in addition, AIRC (International Agency for Research on Cancer) has listed it as a possible carcinogenic substance (AIRC, 2017).

PFOA can enter the ocean through water flow: in 2007, it was projected that rivers in Europe's coastal regions discharged roughly 30 tons of PFOA annually (Pistocchi and Loos, 2009). According to Renzi *et al*., 2013, PFOA levels in coastal marine ecosystems have been found to reach 2.15 ng/L in Orbetello lagoon while, according to Valsecchi *et al*., 2015, levels of 19.4 ng/L, and 26 ng/L in the Lagoon of Venice and Po estuary.

Prior research indicates that exposure to POPs may have detrimental consequences on a variety of aquatic and terrestrial animals, including immunotoxicity, developmental toxicity, and reproductive toxicity (Jantzen *et al*., 2017; DeWitt *et al*., 2012; C. Liu *et al*., 2007; Ankley *et al*., 2005). The immunological responses in marine organisms, such as bivalves, are perhaps the most sensitive physiological processes that POPs may disrupt (Deaton *et al*., 1999; Cajaraville *et al*., 1996): hemocytes phagocytosis reduction, alterations of multiple enzymes linked to immune functions (acid phosphatase - ACP, glutathione and lysozyme), in addition to reactive oxygen species (ROS) increase and oxidative stress damages (Tang *et al*., 2020; Liu & Gin,

2018; Sun *et al*., 2017; Croxton *et al*., 2012; Hannam, Bamber, Galloway, *et al*., 2010; Hannam, Bamber, Moody, *et al*., 2010; Bado-Nilles *et al*., 2008; Frouin *et al*., 2007)

While PFOA, and PFAS in general, effects in marine organisms are increasingly being studied, little is known on biological alterations under multiple stressors scenario.

### *1.3- ORGANISMS' TOLERANCE AND BIOLOGICAL RESPONSES TO ENVIRONMENTAL STRESS*

According to Skulachev *et al*., 2012, organisms are open systems that rely on outside energy to generate and maintain their bodily structures and carry out biological activities. The latter depends on energy fluxes, which directly affect an organism's fitness (Chen and Nielsen, 2019; Spotila and Standora, 1985; Pyke, 1984). Furthermore, bioenergetics is a crucial functional trait that directly connects organismal physiology with ecological and ecosystem processes. This is because energy transformations within an organism act as a focal point for the energy fluxes shaping ecosystems through interactions between organisms and the environment and among organisms, as in the trophic web (Sokolova, 2021). An animal's performance may worsen when it is under stressful conditions because of losses of energetic balance (Pörtner and Knust 2007; Hochachka and Somero 2002). Thus, organisms' tolerance limits under stress are directly linked to their metabolic performance. An environmental change is deemed stressful if it forces an organism to increase its maintenance, defense, or repair costs and lowers its net energy gain in the process. Sokolova, Sukhotin and Lannig, 2012, depicted three general aspects to keep in mind when measuring an organism's reaction to environmental stress. First, stress causes an integrated reaction in the body that concomitantly affects its molecular, cellular, and physiological levels. Second, all affected levels may undergo

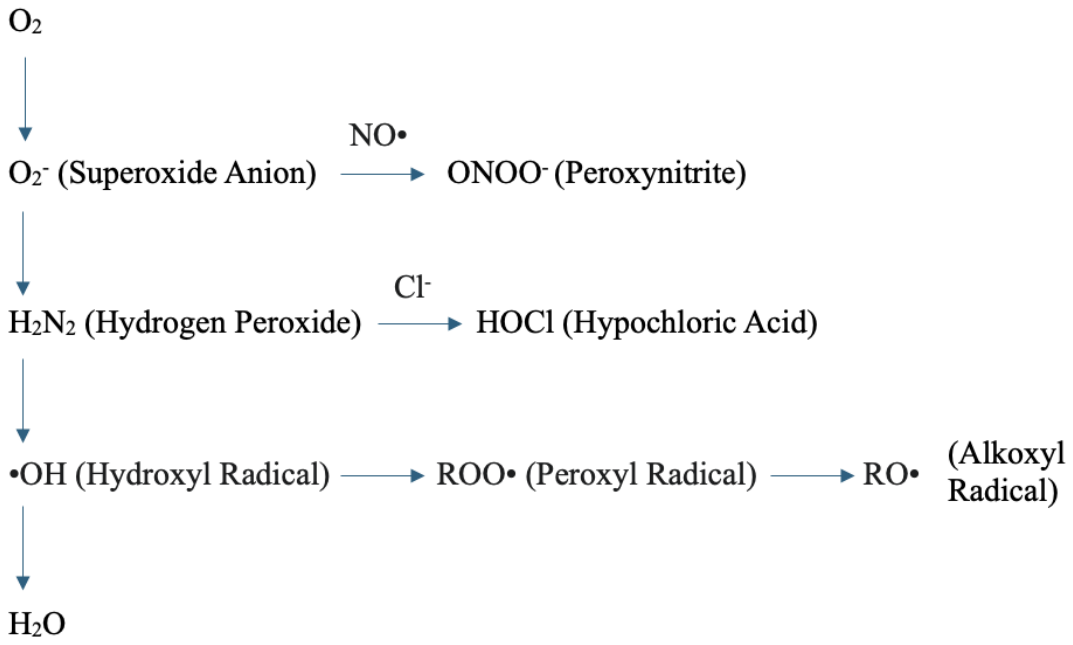
various alterations due to the dynamic nature of the stress response. Finally, the homeostatic systems may at least partially offset potential stress, even though these procedures can be metabolically expensive. The long-term impacts of stress become quantifiable as a decrease in the organism's fitness or, eventually, as mortality, when compensating for the negative stress effects is insufficient or impossible.

The kind and intensity of environmental stress determine the metabolic adaption methods applied by mollusks. Separate metabolic responses and diverse gene expression profiles are elicited by moderate and extreme stress, suggesting that the two scenarios involve separate systems of stress tolerance (Gracey *et al*., 2008; Hochachka and Somero 2002; Hochachka and Guppy 1987).

Reactive Oxygen species (ROS) in an umbrella definition for a broad range of molecular oxygen derivatives that are a direct consequence of the aerobic metabolism (Sies and Jones, 2020). ROS are produced from various cellular pathways and processes: oxidative phosphorylation, active phagocytosis, electron transport chains or as enzymes intermediates as well as in xenobiotics' metabolism (Chainy *et al*., 2016; Ray *et al*., 2012; Halliwell, 2008). ROS generation is widespread in the world's oceans, and oxidative stress plays a significant role in the stress response of marine organisms subjected to a range

of environmental stressors, including heat stress, which is increasingly more common due to climate change (Lesser, 2011).

There are several kinds of ROS, differing among each other in their cellular reactivity and toxicity to cell components as lipids, proteins and DNA (Regoli and Winston, 1999):




Their reactivity follows the diagram below:

$O_2^- < ROO\bullet < RO\bullet < ONOO^- < \bullet OH$

Since the production of ROS can normally occur under basal metabolic conditions, these do not necessarily imply toxicity since cells have developed mechanisms and antioxidants to counteract the onset of oxidative damages. Under basal conditions, there is an equilibrium between prooxidant challengers (ROS) and the efficiency of antioxidant defenses. The onset of oxidative stress

occurs when this balance is disrupted due to the increase in ROS production, the depletion of antioxidant defenses or the two happening at the same time due to various environmental stressors (heatwaves, metals and xenobiotics).

The antioxidant system comprehends (*fig. 1.3.1*):

- low molecular weight scavengers: molecules that directly bind with ROS, neutralizing them, turning themselves in the oxidized state or acting as "temporary prooxidant" radicals. Scavengers can be distinguished between water-soluble molecules in the cytoplasm (reduced glutathione GSH, ascorbic acid, uric acid) and lipid-soluble ones acting on the membranes to block lipid peroxidation (α-tocopherol, β-carotene, retinol)
- antioxidant enzymes: proteins with catalytic activity, more specific towards different substrates with which they react (Superoxide dismutase SOD, Catalase CAT, Peroxidases, Glutathione S-transferases GSTs, Glutathione reductase GR, DT- diaphorase).

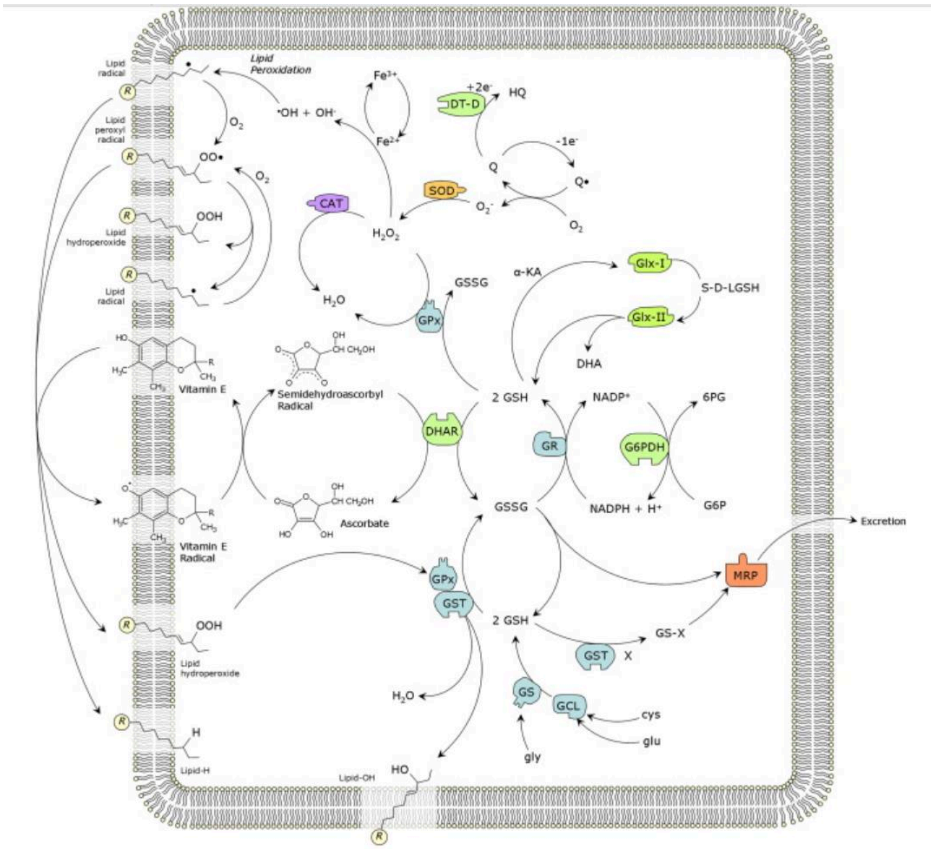



*Figure 1.3.1: Main cellular antioxidant defenses and antioxidant pathways (Regoli and Giuliani, 2014)*

Variations in antioxidant defenses in terms of catalytic activity, concentrations and gene expression have been frequently employed as biomarkers of oxidative stress conditions, since their evaluation can offer vital information about an organism's antioxidant status. Indeed, these are considered early warning signals of cellular unbalance, highly perceptive in identifying a prooxidant challenges, and helpful in comprehending the relationships and mechanisms of action of prooxidant stressors. ROS production can be enhanced when organisms are exposed to different xenobiotics (drugs, environmental

pollutants) which contribute to disrupt the complex yet sophisticated balance between prooxidant challengers and antioxidant defenses, as shown in *figure 1.3.2,* (Regoli and Giuliani, 2014).

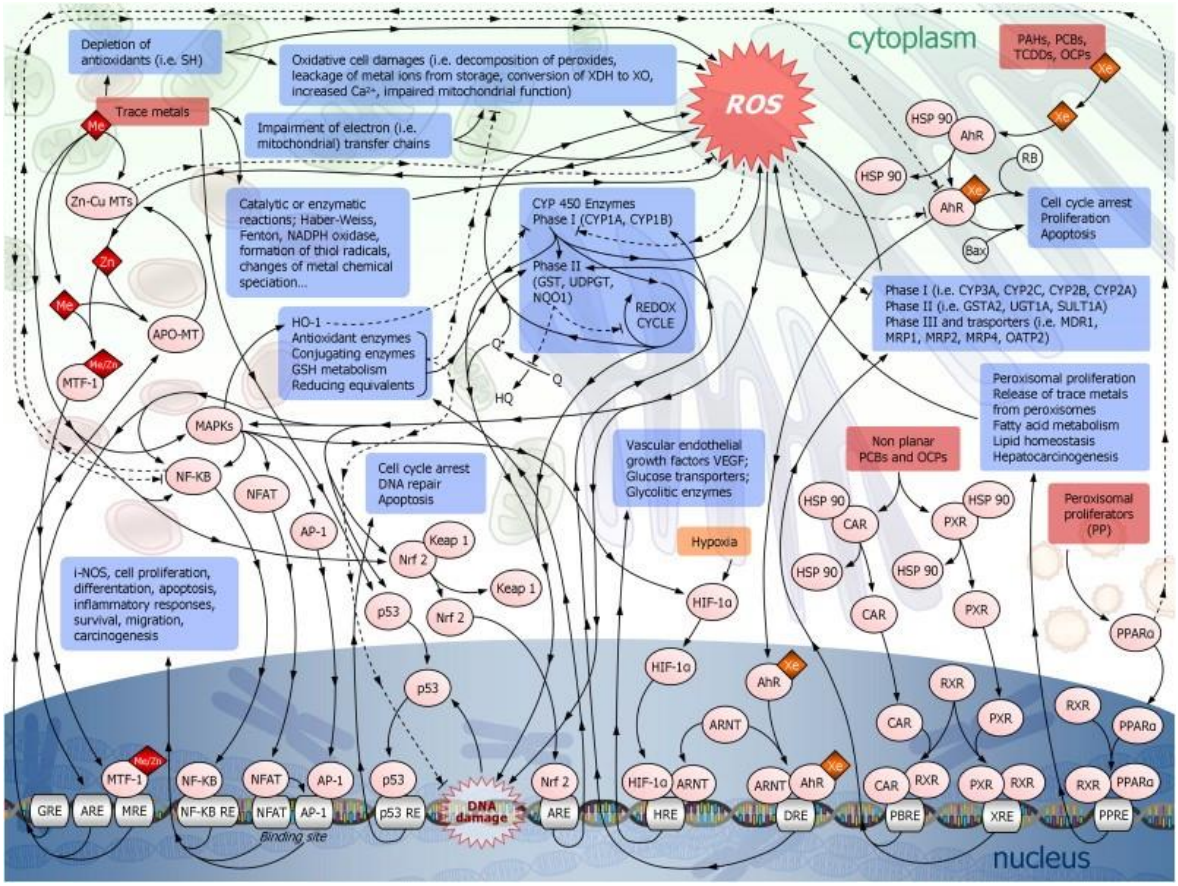



*Figure 1.3.2: Main oxidative interactions between different classes of chemicals (Regoli and Giuliani, 2014)*

Antioxidant mechanisms vary depending on the degree and length of exposure to environmental contaminants. This has significant implications for the use of different biomarkers to evaluate evidence of biological disturbance over the short, medium, and long terms (Regoli, Benedetti and Giuliani, 2011).

Regoli *et al*., 2004 and Regoli, 2000, showed how different exposure times of mussels to polluted areas determine a biphasic pattern: a first counteractive reaction of mussels to the increased prooxidant challenge, highlighting an adaptation or a compensatory tolerance toward the production of ROS in order to get over the stressful situation; nevertheless, after prolonged exposure times, antioxidants seemed to be overwhelmed, leaving the organisms more exposed to ROS damages and oxidative stress. Individual antioxidants do not change synchronously, and it is frequently the case that although some of them may change or even become depleted, others do not.

Furthermore, changes in antioxidant levels are rarely dose dependent considering the intricate network of scavengers, enzymes and regulatory feedback between these. This makes difficult to pinpoint a threshold at which the variation of an antioxidant defense can anticipate oxidative toxicity. Frequently, changes in specific antioxidants do not correspond with the onset of cellular damage, which may indicate a tolerance to contaminants exposure (Lushchak *et al*., 2009; Ventura–Lima *et al*., 2009; Box *et al*., 2007). However, since variations of antioxidant defenses are not easy to summarize in a single index due to the different oxyradicals' reactivity-toxicity and the efficiency and biological significance of an antioxidant towards them, the measurement of total antioxidant capacity is a useful tool to summarize the overall antioxidant

status. Total oxyradical scavenging capacity assay (TOSC-A) is one of the available methodologies used for a quantitative characterization of the efficiency of cellular antioxidants towards specific substrates-oxyradicals (Regoli, 2000; Regoli and Winston 1999**)**. Although TOSC-A is less sensitive than individual defense biomarkers, it has a higher prognostic value providing an integrated perspective on the overall cellular status: indeed, the onset of oxidative damages such as lysosomal alterations and genotoxic alterations have been linked to a reduced ability to neutralize ROS **(**Regoli *et al*., 2004; Gorbi and Regoli, 2003; Frenzilli *et al*., 2001; Regoli, 2000). TOSC-A has been successfully used to pollutants-mediates oxidative stress in a wide variety of marine species, including the bivalves *Mytilus galloprovincialis* and *Ruditapes philippinarum*, the fish *Anguilla Anguilla* and *Diplodus sargus*, the cnidarian *Madracis pharensis* (Nardi *et al*., 2024; Frenzilli *et al*., 2001).

Mollusks and other ectotherms are directly impacted by environmental temperature and its variations in all aspects of their metabolism (Hochachka and Somero, 2002). At optimal temperature, ROS formation is often low, and antioxidant defenses are functioning at their best; however, extremes of high and low temperature can promote ROS production, through changes of metabolic rates (Abele and Puntarulo, 2004).

In addition, seasonal variations may have an impact on animals' ROS levels and antioxidant defense mechanisms, and consequently, their physiology (Chainy *et al*., 2016). Bocchetti and Regoli, 2006, demonstrated that prooxidant conditions in *Mytilus galloprovincialis* may be triggered by abiotic factors as temperature, salinity, oxygen concentration and solar irradiance peaks which follow both the seasonal trend and the reproductive cycle. As a result, the activity of antioxidant enzymes is increased to maintain the organisms' oxidative status under balance.

In response to stressful conditions (sharp temperature, salinity changes), Carregosa *et al*., 2014, demonstrated how bivalves typically close their valves as a defense mechanism against osmotic stress. This resulted in decreased feeding activity, slower growth and respiration rates, and altered oxygen consumption. Prolonged valve closure, however, negatively impacts bivalves' performance because it stops filtration activity, which lowers energy absorption and accumulates excretion products due to the shift to the anaerobic metabolism, increasing oxidative stress (Domínguez *et al*., 2020). Rato *et al*., 2022, showed that temperature combined with low salinities on *Ruditapes decussatus* resulted to be lethal.

Evaluating the effects of environmental stressors as marine pollution and global climate change on mollusks is crucial from both an ecological and economic

perspective (Kimbro *et al*., 2009). A sizable portion of global aquaculture is centered around bivalves: 17.7 million tons produced globally worthing $29.8 billion USD (FAO, 2022). Therefore, any stressor that lowers the fitness and well-being of marine mollusks would have a significant negative impact on both natural and aquaculture populations, resulting in significant financial losses (Sokolova, Sukhotin and Lannig, 2012).

As a confirmation, one of the main factors influencing the success of non-indigenous, invasive species has long been thought to be tolerance to environmental stress (Bielen *et al*., 2016). The study of Lenz *et al*., 2011, revealed higher tolerance to stressful conditions (salinity, oxygen concentration and temperature variations) in various invasive marine species of bivalves when compared to their native equivalents.

Broad stress tolerance of an allochthonous species as well as its capacity for rapid acclimatization to fluctuating conditions represent one of the main causes of ecosystem decline and global biodiversity loss (Pyšek and Richardson, 2010, Sala *et al*., 2000).

Furthermore, abiotic stressors such as pollution (Bielen *et al*., 2016; Zenetos *et al*., 2012), heat waves combined with global warming, floods or droughts (Smale *et al*., 2019; Ferreira-Rodríguez *et al*., 2018), habitat modification (Sorte, Williams and Zerebecki, 2010) can lead invasive species to occupy

previously empty niches. Crespo *et al*., 2021, demonstrated that the invasive clam *Ruditapes philippinarum* can cope better with the global change scenario compared with its equivalent *Ruditapes decussatus*. Both are widely used in aquaculture and diffused along the European Atlantic and Mediterranean coasts (De Marchi *et al*., 2020). *R. philippinarum* can thrive in a variety of environmental conditions (14-42 PSU and 6-30°C) and habitats (Dias et al., 2019; Cordero et al., 2017; Carregosa *et al*., 2014). On the other hand, *R. decussatus* seems to have a narrower tolerance window to salinity (7-42 PSU) while it is less sensitive to high temperature. As proved by Crespo *et al*., 2021, because of reduced antioxidant and energetic investment, the native *R. decussatus* exhibited a higher amount of oxidative damage while the invasive *R. philippinarum* showed a decrease in damage levels and an enhancement of cellular energy allocation, suggesting that it benefits from higher temperatures. According to McMahon, 2002, r species-selected qualities, such as rapid population growth, early maturity and high fecundity, are primarily responsible for a species' ability to invade new areas and become more tolerable whereas k species-selected traits force native species to rely on their ability to withstand natural disturbances to be maintained.

According to the oxygen and capacity limitation of thermal tolerance (OCLTT) hypothesis, an animal's performance curve is influenced by its ability to deliver oxygen in proportion to its oxygen demand, which is dependent on temperature. It states that aerobic scope decreases above threshold temperatures due to an imbalance between oxygen supply capacity and oxygen demand (Verberk *et al*., 2016). Performance decline because of the ensuing energy shortage, which, in the long-term, limits fitness and define the animal's thermal niche. Under acute exposure to critical temperatures, aerobic metabolism cannot sustain energy demand for long time leading to organisms 'death after the first shift to anaerobic metabolism. The thermal window and niche of aerobic performance may be narrowed by a variety of stresses, including biotic variables like restricted food supply, predation pressure, or competition, and abiotic factors like $CO_2$, hypoxia, and contaminants (Pörtner, 2010, 2002, 2001).

### *1.4- BIOLOGICAL MODEL*

Bivalves are commonly used as bioindicators *in sensu lato*, as sentinel, bioaccumulator, pollution and water quality indicator species, **(**Durou, 2006**;** Lagadic *et al*., 1997), to address environmental status and effects of natural and/or anthropogenic stressors. Bivalves are excellent bioindicators because of their wide distribution, abundance, sessile habits, bioaccumulation ability and limited chemicals elimination, and responsiveness to contaminants due to their filter-feeding lifestyle (Ding *et al*., 2021; Botelho *et al*., 2019; Ward *et al*., 2019; Freitas *et al*., 2015). In June 1974, Young *et al*., employed, for the first time in biomonitoring programs, mussels of the species *Mytilus californianus* as bioindicators to monitor the presence and bioavailability of chlorinated hydrocarbons in the Southern California Bight (Young *et al*., 1976). From there on, different species of bivalves as *Mytilus galloprovincialis*, *Ruditapes philippinarum*, *Crassostrea gigas*, *Donax trunculus* among others have been used to monitor the presence of various chemicals (trace and heavy metals, PAHs, PCBs, microplastics pharmaceuticals, among others) in marine and freshwater environments, but also to explore their effects from transcriptional to physiological level (Chahouri *et al*., 2023; De Marchi *et al*., 2022; Louros *et al*., 2022; Regoli and Orlando, 1993).

According to Helmholz *et al*., 2016, bivalves are adaptable to shifting environmental conditions and enable the detection of detrimental effects

through the analysis of biological processes that may function as early warning signals, the so-called biomarkers, before these become irreversible at population and ecosystem level.-

A pivotal target organism is *Ruditapes philippinarum*, known as the Manila clam, hailing from Asia but soon spread worldwide (*figure 1.4.1*).

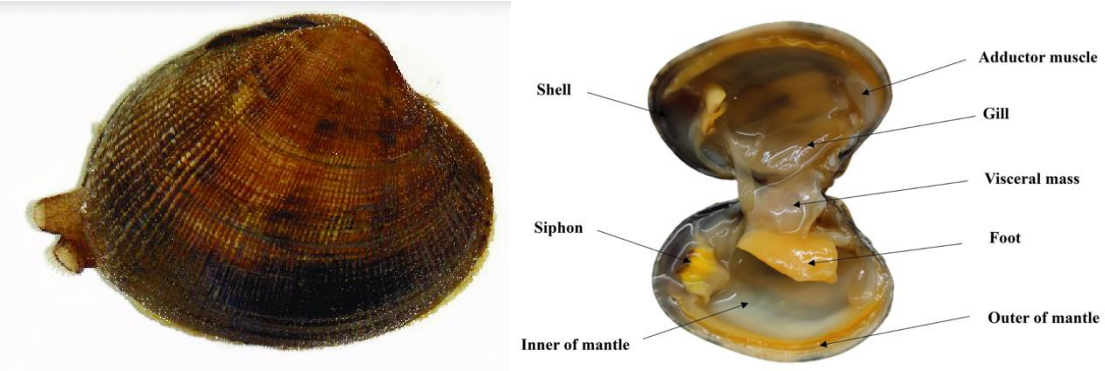



*Figure 1.4.1: The histology of the Manila clam structures (Xu et al., 2024)*

In 1972 it was imported in Europe and in 1983 introduced in the Venice Lagoon spreading along the Adriatic coasts and estuaries (Breber, 2002) due to its ability to withstand harsh environmental conditions confirming itself as an excellent bioindicator of impacted marine areas (Milan *et al*., 2015; Boscolo *et al*., 2013; Matozzo *et al*., 2010; Wang *et al*., 2019; Pranovi *et al*., 2006)

*R. philippinarum* is typical of sandy-muddy bottoms in lower intertidal and shallow subtidal waters. Adults can tolerate several salinity and temperature ranges from 12-50 PSU and 0-37°C (Kamenev and Nekrasov, 2012; Komorita *et al*., 2009; Elston *et al*., 2004; Breber, 2002).

Currently, the Manila clam is the most significant species for commercial clam landings in Europe and one of the most widely cultivated bivalve species globally (Lasner *et al*., 2021). Italy is the second largest producers of Manila clam (*Ruditapes philippinarum*) worldwide, contributing to ~80% of EU production (FAO). Most of the Italian production is based in the North Adriatic coastal lagoons, employing thousands of workers. Emilia-Romagna, Veneto and Friuli-Venezia Giulia together with Marche and Toscana cover 69% of national production (ISPRA). Despite Manila clam is highly adaptable and resistant, the ongoing environmental deterioration due to climate change and anthropic disturbance threaten wild and cultured populations (Ponti *et al*., 2017; Zentilin 2008). After the heatwave event of 2003, 66% reduction of the Manila clam stock has been observed in the Venice lagoon, highlighting the susceptibility of this species (Munari *et al*., 2011). It is very likely that such factors have contributed to the dramatic decrease in the annual Manila clam production in the Venice lagoon, from 40000 tons in 2000 down to 3000 tons in 2019 (Veneto Agricoltura 2020).

### *1.5- PRIMING TECHNIQUES IMPROVING ORGANISMS' TOLERANCE*

"Heat hardening" or "acquired stress response" is a phenomenon occurring when an organism gets acclimated to severe extreme temperatures through a brief exposure to sub-lethal temperatures which temporarily increase its thermal tolerance (Moyen *et al*., 2020). Georgoulis *et al*., 2022, postulate that frequent exposure to stress may induce "stress memory," which would enable an organism to react to comparable stress conditions more quickly and effectively.

Heat hardening activates cellular responses at transcriptional and post-transcriptional levels, such as increased antioxidant defense, more efficient electron transfer system activity, and increased heat shock response, which leads to a quicker (as opposed to days-long acclimation) return to homeostasis, enhanced cell protection, and increased survival (Georgoulis *et al*., 2021; Aleng *et al*., 2015; Malmendal *et al*., 2006; Bowler, 2005).

Different approaches are commonly employed to achieve heat tolerance including rapid heat-hardening through a single heat stress bout, which results in temporary heat tolerance lasting a few days (Sørensen *et al*., 2019; Loeschcke and Hoffmann, 2007; Rinehart *et al*., 2000), and chronic heat acclimation through serial heat-stress bouts, which results in heat tolerance lasting weeks to months (Fu *et al*., 2018; Beitinger and Bennett, 2000; Widdows J., 1976; Pickens P., 1965).

Several studies, as Moyen *et al*., 2020, Georgoulis *et al*., 2022 and Zhang *et al*., 2023, tested the effect of heat hardening on *Mytilus californianus*, *Mytilus galloprovincialis* and *Ruditapes philippinarum* respectively.

Moyen *et al*., 2020, showed that *M. californianus* survival in the intertidal zone depends on its ability to swiftly acquire heat tolerance, since 64% of consecutive heat events occur within short time frames (*i.e*., within 24–48 h). More importantly for their survival, mussels must be able to sustain this enhanced heat tolerance for at least 21 days following the initial heat-stress episode, since more than one-third (about 33%) of successive heat events occur over longer time spans (3–22 days) as pointed out in *figure 1.5.1*.

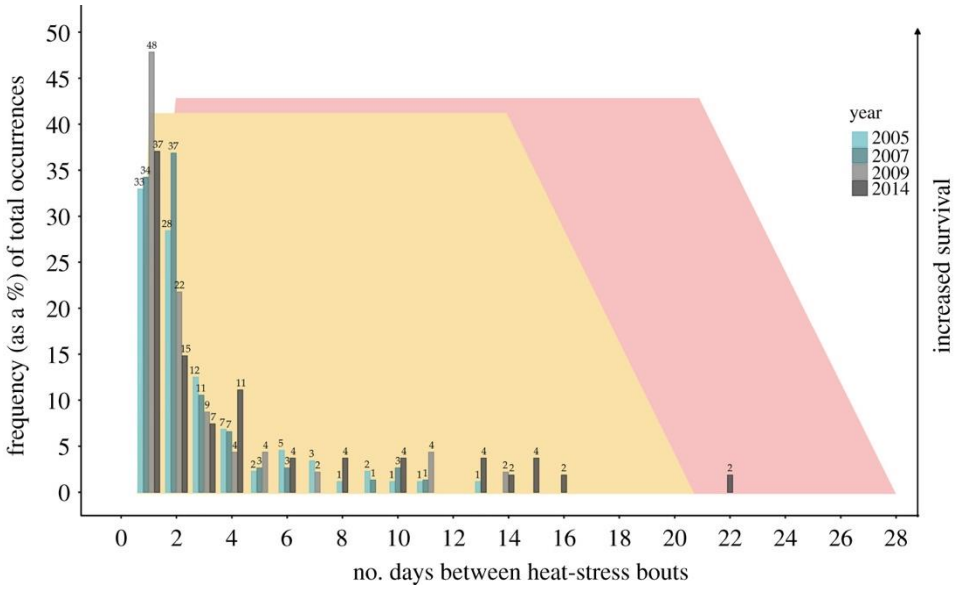



*Figure 1.5.1: Frequency of two consecutive heat-stress bouts based on field Robomussel data (Helmuth B. et al. 2016) from Moyen et al. 2020*

Mussels' heat-adapted approach, which involves increasing their heat tolerance quickly and decreasing it gradually, is in line with the frequency of successive

heat-stress episodes in the intertidal zone. Given the sessile nature (they cannot move to escape heat stress), it seems likely that mussels have acquired this heat-adapted ability. With their adaptive approach, mussels may be able to withstand most of the increasing frequency of extreme heat episodes that are anticipated to occur due to climate change (Moyen *et al*., 2020).

In addition, Georgoulis *et al*., 2022 noticed several metabolic disruptions, affecting vital pathways (succinate pathway disruption, Krebs cycle alteration and mitochondrial metabolism modification), when *M. galloprovincialis* was exposed to high temperatures. These effects were softened in mussels that had been heat-treated before being subjected to thermal stress. Overall, the metabolic responses in both heat-hardened and non-hardened mussels were different at moderate thermal stress but tended to overlap with increasing temperature, supporting 26°C as upper thermal limit of *M. galloprovincialis* (Anestis *et al*., 2007) in the context of the Oxygen and Capacity Limited Thermal Tolerance theory (OCLTT), which states that when an organism's temperature rises beyond its optimal range, there is an imbalance between oxygen supply and demand in its tissues. Thus, the capacity of heat-hardened mussels to gain and lose heat tolerance quickly and gradually may be a useful tactic for handling sporadic and frequently high temperatures, as asserted also by Moyen *et al*., 2020.

According to Zhang *et al*., 2023, temporary heat stress led to the acquisition of heat tolerance in *R. philippinarum*. Moreover, they discovered that heat tolerance was longer-lasting when comparing the survival under heat-stress scenario of Manila clams previously hardened at 30°C with the survival of non-hardened clams.

He *et al*., 2022 stated that Manila clams may adopt a more effective compensatory mechanism using less energy to manage heat stress when high temperatures recur. The survival analysis revealed that the temperature at which heat stress was first experienced determined the acquisition of heat tolerance, and that the survival rate was related with both temperature and time. As a confirmation, in comparison to the non-hardened clams, the heat-hardened Manila clams demonstrated superior heat endurance by altering its energy metabolism, fatty acid composition, and synthesis of antioxidant metabolites.

So far, priming techniques have been largely used in plants. Particularly, $H_2O_2$ priming is mostly performed on seeds to fight salinity stress, a growing threat for agriculture (Cai & Gao, 2020). According to Goharrizi *et al*., 2024, $H_2O_2$ priming improves antioxidant defenses, keeping the balance between ROS and scavenging species under control. Moreover, it facilitates osmoprotectants accumulation, maintaining cellular water content in salinity-induced conditions. $H_2O_2$ priming can lessen the damaging effects of salt stress on plant

growth and productivity by maintaining chloroplast integrity and photosynthetic efficiency.

## 2- AIM OF THE THESIS

Anthropogenic $CO_2$ emissions are among the main drivers of Earth's climate system change, causing the increase in frequency and intensity of severe phenomena like heatwaves—periods of elevated ocean temperature beyond a seasonal climatological threshold. Approximately 95% of the Mediterranean basin was impacted by a heatwave in the summer of 2019, especially the North Adriatic Sea, which hosts several aquaculture plants. Such an increase, along with other stressors such as chemical pollution, threatens filter-feeding and sedentary organisms' populations and stocks, possibly leading to a loss of biodiversity and socioeconomic repercussions on aquaculture. This thesis is developed in the framework of the MANILA Project ("Testing innovative strategies to mitigate shellfish vulnerability to climate change and pollutants", PRIN 2022), aimed to explore innovative approaches to enhance the performance of Manila clams, *Ruditapes philippinarum*, under stressful environmental conditions, namely priming techniques: these manipulative approaches are tailored to stimulate stress-memory mechanisms, thereby providing a temporary increase in organisms' stress tolerance. To this end, for the first time, chemical priming techniques have been initially explored and their capability to stimulate stress-response mechanisms evaluated. Then, primed organisms were subjected to a combination of thermal and chemical

stress to explore the possibility of priming to ameliorate organisms' performance and survival, with the final aim to validate and provide a tool able to mitigate the impacts of climate changes on aquaculture.

# 3- MATERIALS AND METHODS

## *3.1- COLLECTION AND ACCLIMATION*

In this study, clams of the species *Ruditapes philippinarum* were obtained from SATMAR (FR) in two distinct periods, January and March 2024, for two different experiments. Upon arrival, clams were allowed to acclimate to laboratory conditions in tanks containing artificial seawater at hatchery temperature, pH and salinity, equipped with aerators to allow adequate oxygenation. During acclimation and experimental phases, clams were fed daily with a solution of marine phytoplankton gel in a mineral suspension dissolved in Milli-Q water, and water in each tank was completely renewed every other day, after checking for mortality.

### *3.2- PILOT EXPERIMENT ON CHEMICAL PRIMING*

The fist experiment - Stress Priming Clams (SPC) – was aimed to explore the feasibility of a "chemical priming" using $H_2O_2$ to stimulate stress-response and to select the most promising technique. With this aim, 6 glass tanks (15 L) were prepared following an accurate cleansing with distilled water. Before the starting of the experiment, each tank was filled with artificial seawater at 34 ‰ salinity and held in a "water-table" to maintain a fixed temperature, as detailed below. Clams (shell length 1.92 ± 0.07 cm to 2.44 ± 0.18 cm) were allowed to acclimate to laboratory conditions: organisms were initially maintained at 12°C and temperature was gradually increased (2°C/day) to reach 18°C. After a week of acclimation at 18°C, tanks were randomly assigned to one of the following experimental treatments, each performed in duplicate (*figures 3.2.1 and 3.2.2*): CTRL, control condition; $H_2O_2$-10, exposure to 10 µM $H_2O_2$; $H_2O_2$-50, exposure to 50 µM $H_2O_2$. After 7 days of exposure, clams were sampled and tissues collected for biological analyses, as detailed in 3.4.

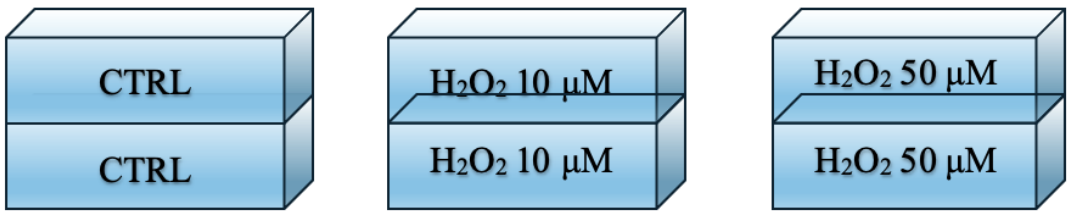



*Figure 3.2.1: Stress Priming Clams' Experimental Design*



*Figure 3.2.2: Aquariums during the acclimatation phase*

### *3.3- PRIMING EFFICIENCY EXPERIMENT*

In the Stress Priming Experiment (SPE), 6 glass tanks of 15 L capacity were prepared following an accurate cleansing with distilled water. Similarly to the pilot experiment, each tank was filled with artificial seawater (34 ‰ salinity and 20°C temperature), with each containing 60 organisms (shell length 2.3 ± 0.15 cm) for a 7 days acclimation period. After that, clams in two tanks were primed with the most suitable chemical-priming technique identified in the previous experiment, using 50 μM $H_2O_2$, for 7 days. The priming was followed by a 10-days rest, after which naïve (not-primed) and primed *R. philippinarum* were exposed to three different treatments for 7 days (*Fig. 3.3.1*), each performed in duplicates:

1. Control (CTRL): naïve clams, kept at 20°C with no chemicals
2. Control under Challenge (CTRL-CH): not-primed clams subjected to combined thermal and chemical challenge
3. Chemical-Primed (CP): 50 μM $H_2O_2$-primed clams subjected to combined thermal and chemical challenge

Combined thermal and chemical challenge consisted in a heat wave scenario (33°C) and exposure to PFOA at 2 μg/L concentration. The thermal stress scenario was selected according to Venice lagoon heatwave events occurred in summer 2021 and 2022. While, PFOA concentration in open sea water samples is usually in the order of few pg/L to ng/L (Amorim *et al*., 2024),

in estuarine areas and lagoons characterized by consistent riverine inflows (as Venice lagoon where these clams are highly cultured), PFAS concentrations in the order of hundreds of ng/L up to few tens of µg/L have been proposed (Gambaro *et al*., 2022; Bernardini *et al*., 2021). The chosen concentration, particularly high, was selected to ensure the onset of biological effects caused by PFOA, considering its scarce acute but rather chronic toxicity for aquatic organisms (Razak *et al*., 2023; Gambaro *et al*., 2022) only from a toxicological perspective and representative of highly polluted areas, far by environmentally realistic scenarios.

Throughout the experiment, mortality was daily checked. At the end of the experiment, from each experimental condition 30 clams were collected (15 from each replicate tank) to conduct biological analyses on immune responses, antioxidant system and cholinergic function. The remaining clams were used to evaluate survival rate/curves, leaving the organisms at 20°C in clean seawater for four weeks with weekly water changes and every-other-day feeding.

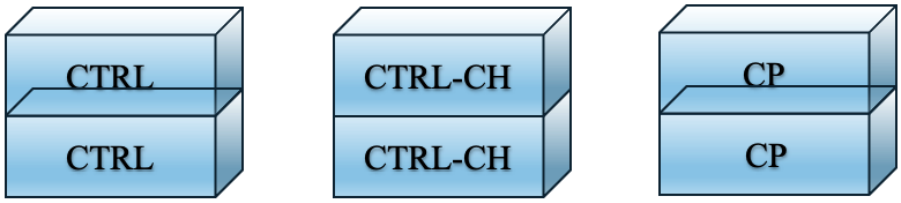



*Figure 3.3.1: Stress Priming Experiment's Experimental Design*

### *3.4- BIOLOGICAL ANALYSES*

Organisms were randomly collected from each aquarium in both experiments. A sterile syringe was used to withdraw 1 mL of hemolymph from the clam's abductor muscles as soon as these were collected, to evaluate hemocytes lysosomal membrane stability, phagocytosis rate and DNA damage. Clams' gills and digestive glands (*figure 3.4*) were collected in sterile microtubes, flash-frozen in liquid nitrogen and stored at -80°C until analyses of antioxidant system and cholinergic function, detailed below. Additionally, clams whole soft tissues were collected for lipid peroxidation analyses.



*Figure 3.4: Dissection plane and its instruments*

### *3.4.1- IN-VIVO HAEMOCYTES ANALYSES*

Immune system responses and genotoxic damage were analyzed considering lysosomal membrane stability (LMS), phagocytosis rate and comet assay. All are considered as extremely valid biomarkers which can be used as early warning signals of stress conditions and changes in the organisms' health status (Moore *et al*., 2006; Regoli, 1992).

The Neutral Red Retention Time (NRRT) assay was measured to evaluate LMS of hemocytes of *R. philippinarum* by taking an aliquot of hemolymph (1 mL) from the adductor muscle using a hypodermic syringe containing an equal volume of saline solution. The dye Neutral Red was used to evaluate the capability of the lysosomal membranes to retain it (Moore *et al*., 2004; Lowe *et al*., 1995). Hemocytes were incubated on a glass slide with a freshly prepared NR working solution (2 µL/mL filtered seawater) from a stock solution of 20 mg neutral red dye dissolved in 1 mL of dimethyl sulfoxide, and microscopically examined at 15 minutes intervals, to determine the time at which 50% of cells had lost the dye in the cytosol previously taken up by lysosomes (Gorbi *et al*., 2013).

Phagocytosis rate assay was performed following the protocol by Gorbi *et al*., 2013; 50 µL of hemolymph were dispersed on glass slides and allowed to

adhere incubating them for 15 minutes at 15 °C in the dark. Fluorescein-labelled Zymosan A bioparticles (Invitrogen) were added at 10:1 target:hemocyte ratio. After 2 h incubation at 15 °C in the dark, uninternalized particles were removed by washing with physiological solution and slides were finally fixed in Beker's fixative (+2.5% NaCl) and mounted in Eukitt. Phagocytosis was expressed as percentage of cells that internalized at least 3 fluorescent particles (positive cells), observed under a fluorescence microscope, after counting at least 300 cells for each sample.

Hemocytes, for the Comet assay, were diluted in $Ca^{2+}$/$Mg^{2+}$- free buffer at 4 °C in the dark (20 mM Hepes, 500 mM NaCl, 12.5 mM KCl, 10 mM EDTA, pH 7.3), centrifuged at 1000 rpm for 1 min at 4 °C, resuspended in 0.6% low-melting-point agarose, and added with a sandwich stratification to glass slides coated with 1% normal-melting-point agarose. After gel solidification, slides were placed into the lysing solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10% DMSO, pH 10) at 4 °C in the dark for 90 min. DNA was unwound in 75 mM NaOH, 10 mM EDTA (pH 13), and the electrophoretic migration was carried out in the same buffer at 1 V/cm for 10 min. Slides were then neutralized for 10 min in 0.4 M Tris, pH 7.5, fixed in cold methanol for 3 min at −20 °C, and dried. After staining with DAPI, 70 randomly selected

“nucleoids” per slide, and two replicates per sample, were examined under fluorescence microscopy (200 × magnification; Olympus BX-51), and the captured images (Image-Pro-Plus package) were analyzed through the Tritek CometScore™ software. The percentage of DNA in the tail was used to estimate the level of DNA fragmentation. Briefly, since for each “nucleoids” the intensity of emitted light is linearly related to the amount of DNA, the brightness of the image pixels can be directly used to extrapolate the amount of DNA damage. After the regulation of background (performed through the specific tool namely “cut off”) which allow to minimize the measurement of fuzziness, the user proceeds selecting the area of interest (AOI), to recognize the region occupied by comet. The additional step of segmentation (performed by the user) is needed to find the exactly middle of the head of the comet. After that, the software automatically applies specific algorithms, which allow to extrapolate the amount of DNA in the head and in the tail. The output is given as percentage of DNA in tail and in head.

### *3.4.2- CHOLINERGIC RESPONSE*

For AChE activity evaluation clams' hemolymph and gills were opportunely prepared as follows: the hemolymph was centrifugated at 6700 rpm for 5 minutes, then supernatants were divided in 3 aliquots and frozen at -80°C, while the gills were weighed and homogenized (1:3 w/v) in Tris-HCl 0.1 M, pH 7.2, saccharose 0.25 M, centrifugated at 12000 rpm for 10 min, then supernatants divided in 3 aliquots and stored at -80°C.

Acetylcholinesterase (AChE) activity was then spectrophotometrically assayed according to Ellman method (Ellman *et al*., 1961). Briefly, samples were incubated for enzymatic quantification with 1 mM acetylthiocholine as substrate and 0.1 mM 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB). Acetylthiocholine is hydrolyzed by AChE in acetate and thiocholine, which reacts with DTNB producing a yellow anion (5-mercapto-2-nitrobenzoato) that absorbs at 412 nm. Enzyme activity was measured at 412 nm and thus expressed as nmol/min/mg of protein.

### *3.4.3- ANTIOXIDANT SYSTEM*

For enzymatic antioxidants, digestive gland and gills samples were homogenized (1:5 and 1:3 w:v ratio, respectively) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, 1 µg/ml leupeptin, 0.5 µg/ml pepstatin, NaCl 2.5%, and centrifuged at 100 000 × g for 70 min at 4 °C. Activities were measured with a Varian (model Cary 100 Scan) spectrophotometer at a constant temperature of 18 °C ( Bocchetti *et al.*, 2008).

- Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, ε = 0.04 mM−1 cm−1) due to the consumption of hydrogen peroxide, $H_2O_2$ (12 mM $H_2O_2$ in 100 mM K-phosphate buffer pH 7.0).
- Glutathione S-transferases were determined at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH (ε = 9.6 mM−1 cm−1).
- Glutathione peroxidases (GPx) activities were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was monitored at 340 nm (ε = −6.22 mM−1 cm−1) in 100

mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM sodium azide ($NaN_3$) (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.8 mM cumene hydroperoxide as substrate, for the sum of Se-dependent and Se-independent isoforms.

- Total glutathione was analyzed in samples homogenized (1:5 w:v ratio) in 5% sulfosalicylic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37.000 × g for 15 min, before being enzymatically assayed (Akerboom & Sies, 1981).

The total oxyradical scavenging capacity (TOSC) assay was used to measure the overall capability of cellular antioxidants to absorb different forms of artificially generated oxyradicals, inhibiting the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Winston *et al.*, 1998; Regoli & Winston, 1999).

- Peroxyl radicals (ROO•) were generated by the thermal homolysis of 20 mM 2-2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4.
- Hydroxyl radicals (HO•) were produced by the Fenton reaction of iron-EDTA (1.8 μM $Fe^{3+}$, 3.6 μM EDTA) plus ascorbate (180 μM) in 100 mM K-phosphate buffer.

Ethylene formation in blank and sample reactions was analyzed at 10 minutes time intervals by gas-chromatographic analyses and the TOSC values are quantified from the equation: TOSC = 100 − (∫SA/∫BA × 100), where ∫SA and ∫BA are the integrated areas calculated under the kinetic curves for samples (SA) and blank (BA) reactions. For all the samples, a specific TOSC (normalized to total protein content) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the sample.

The content of malondialdehyde (MDA) was measured in whole soft tissues samples homogenized (1:5 w/v) in 20 mM Tris–HCl pH 7.4, centrifuged at 3000 × g for 20 min and then derivatized in 1 ml reaction mixture containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1), HCl 32%, 100 µl water and an equal volume of sample or standard (standard range 0–6 µM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl, pH 7.4). The tubes were vortexed and incubated at 45 °C for 40 min. Samples were cooled on ice, centrifuged at 15 000 × g for 10 min and read spectrophotometrically at 586 nm; levels of MDA were calibrated against a malondialdehyde standard curve and expressed as nmol/g tissue wet weight (Shaw *et al.*, 2004).

### *3.4.4- TOTAL PROTEIN CONTENT*

Total Protein content was analyzed according to Lowry *et al*., 1951, using Bovine Serum Albumin (BSA) as standard and reading the absorbance at 750 nm. Hemolymph, gills and digestive glands were opportunely diluted and transferred in tubes with a final volume of 500 µL (dilution 1:50 haemolymph, 1:25 gills, 1:50 digestive gland). BSA stock solution (2000 µg/mL) was used to prepare five standard solutions (20, 50, 100, 200, 400 µg/mL). Spectrophotometric determination was conducted using four different reagents. Reagent A was prepared dissolving 0.5 g $CuSO_4$ and 1 g of sodium citrate in 100 mL of Milli-Q water. Reagent B was prepared dissolving 20 g $Na_2CO_3$ and 4 g NaOH in 1000 mL of Milli-Q water. Then, 50 mL of reagent B and 1 mL of reagent A were mixed to form reagent C. 2.5 mL of the latter were added to each tube, gently vortexed and left on for 25 minutes. The last step was to prepare reagent D dissolving Folin-Ciocalteau solution in water (1:1 ratio) and add 250 µL to each tube, vortex again and wait for 50 minutes. Absorbance was read at 750 nm and, as a result, a calibration curve with its equation is obtained from the standards and Total Protein content from the samples (µg/mL).

### *3.4.5- SURVIVAL – MORTALITY (VALVE GAPING)*

Valve gaping is defined as the opening and closing of clams' valves which is fundamental in food intake and respiration controlling water flows across gills (Bamber and Wayne, 2023, Bertolini *et al*., 2022). The gaping activity is also a key pattern for the identification of dead or alive clams since living organisms are extremely sensitive to an external touch that causes them to quickly retreat inside, closing their valves. The latter does not happen in dead or almost dead organisms. Gaping activity is also used in organisms' monitoring during the exposure to various stresses in laboratory conditions. Distinguishing between dead or living organisms allows to calculate respectively the mortality or survival rate. Data were represented as:

Mortality rate (%) = (dead animals / total animals) x 100

Survival rate (%) = (live animals / total animals) x 100 (Bi *et al*., 2022).

### *3.5- STATISTICAL ANALYSIS*

All statistical analysis were performed using R software. After checking for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test), analysis of variance (ANOVA) was applied on collected results to test for differences . between experimental groups. When hypothesis of no difference between experimental treatments was rejected (significant ANOVA, $p < 0.05$), TukeyHSD *post-hoc* test, was applied to detect differences among treatments. Multivariate principal component analysis (PCA) was applied to scale the whole dataset of each experiment, visualize experimental conditions distribution and evaluate the contribution of analyzed parameters.

# 4- RESULTS

## *4.1- STRESS PRIMING CLAMS*

Below are reported the results of the experiment "SPC", in which clams were subjected to different typologies of chemical priming.

### *4.1.1- HEMOCYTES FUNCTIONALITY*

Chemical priming with $H_2O_2$ at both tested concentrations did not significantly affect hemocytes lysosomal membrane stability (*fig. 4.1.1. A*).

On the other hand, chemical priming reduced phagocytosis efficiency compared to control clams, with a statistical significance observed in clams exposed to 50 µM $H_2O_2$ (*fig. 4.1.1. B, $p < 0.05$*).

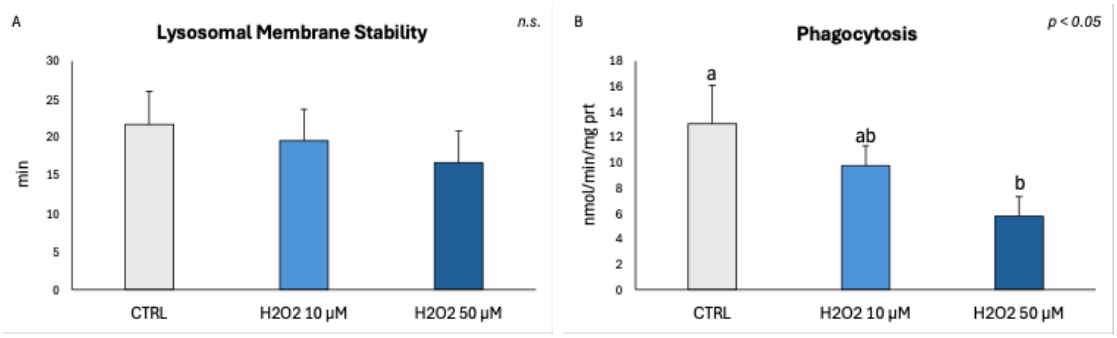



*Figure 4.1.1: Lysosomal Membrane Stability (A) and phagocytosis efficiency (B) in clams subjected to different priming conditions (CTRL: control; $H_2O_2$ 10 µM; $H_2O_2$ 50 µM). Different letters indicate significant differences between treatments. Values are expressed as mean ± SD.*

### *4.1.2- CHOLINERGIC RESPONSE*

Priming produces significant effects on AChE activity in clams' gills, where an induction of the enzyme's catalytic activity was observed in the $H_2O_2$ 50 μM treatment (*fig. 4.1.2 A, $p < 0.05$*). On the other side, AChE activity in clams' hemolymph did not reveal any statistically significant difference (*fig. 4.1.2 B*).

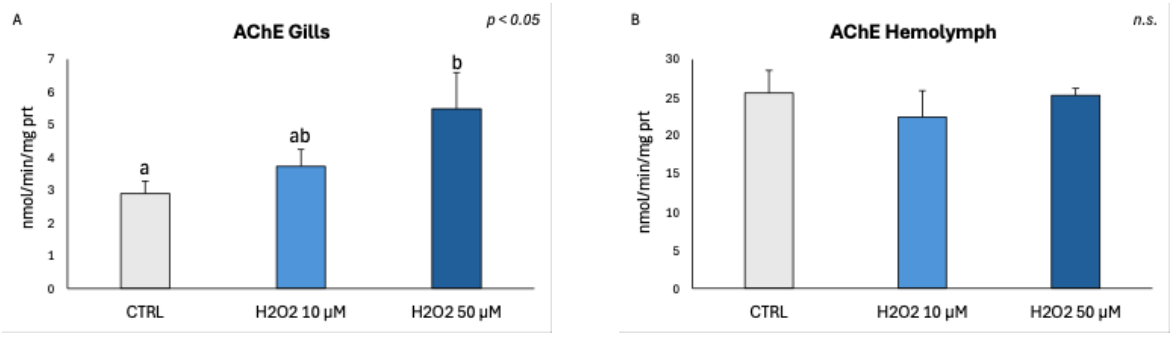



*Figure 4.1.2: AChE enzymatic activity in gills (A) and hemolymph (B) of clams subjected to different priming conditions (CTRL: control; $H_2O_2$ 10 μM; $H_2O_2$ 50 μM). Different letters indicate significant differences between treatments. Values are expressed as mean ± SD.*

### *4.1.3- ANTIOXIDANT SYSTEM*

Overall, significant differences and effects of tested priming approaches were only observed in gills of exposed clams but not in digestive glands. Chemical priming with $H_2O_2$ let to an increase of catalase activity in gills (*fig. 4.1.3 A-H, $p < 0.05$*), which was particularly evident and significant in clams primed with the highest concentration.

Similarly to catalase, glutathione S-transferases activity showed a trend of induction, with a major and significant effect in the $H_2O_2$ 50 μM treatment. On the other hand, glutathione peroxidases showed an opposite trend, with a significant inhibition in the $H_2O_2$ 50 μM treatment compared to the control. Lastly, a bell-shaped trend was observed for total glutathione, that increased compared to control in $H_2O_2$ 10 μM treatment and remained comparable to control values in $H_2O_2$ 50 μM.

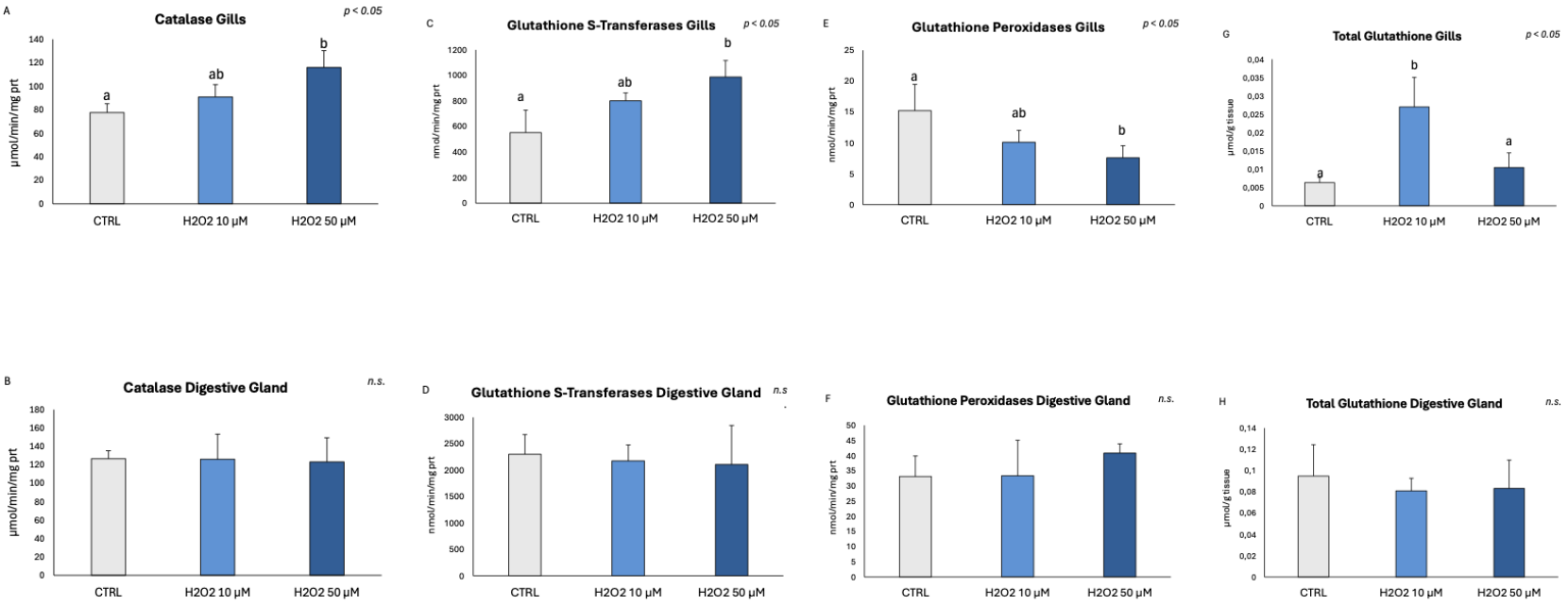
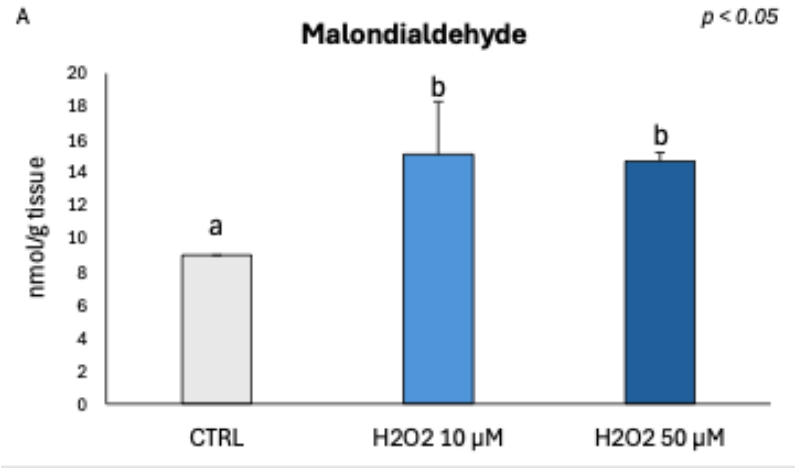



*Figure 4.1.3: Catalase enzymatic activity in gills (A) and digestive gland (B), glutathione S-transferases enzymatic activity in gills (C) and digestive gland (D), glutathione peroxidases enzymatic activity in gills (E) and digestive gland (F), total glutathione enzymatic activity in gills (G) and digestive gland (H) of clams subjected to different priming conditions (CTRL: control; $H_2O_2$ 10 µM; $H_2O_2$ 50 µM). Different letters indicate significant differences between treatments. Values are expressed as mean ± SD.*

Overall, the evaluation of malondialdehyde content in clams whole soft tissues revealed that $H_2O_2$ at both tested concentrations significantly but moderately increased lipid peroxidation (*fig. 4.1.4, $p < 0.05$*).




*Figures 4.1.4: Malondialdehyde content in whole soft tissue of clams subjected to different priming conditions (CTRL: control; $H_2O_2$ 10 µM; $H_2O_2$ 50 µM). Different letters indicate significant differences between treatments. Values are expressed as mean ± SD.*

### *4.2- PRIMING EFFICIENCY EXPERIMENT*

Below are reported the results of the experiment "SPE", in which the efficiency of priming against a multiple stressors scenario was tested.

#### *4.2.1- HEMOCYTES FUNCTIONALITY*

Combined thermal and PFOA challenge significantly reduced hemocytes lysosomal membranes stability compared to control clams (*fig. 4.2.1 A, $p < 0.05$*), with limited differences between naïve and primed clams. under challenge showed a significant reduction in hemocytes' lysosomal membranes stability. On the other hand, challenge reduced phagocytosis efficiency in naïve but not in primed clams, which showed values comparable to control clams (*fig. 4.2.1 B, $p < 0.001$*).

Additionally, challenge increased the onset of genotoxic damages, measured through Comet Assay: indeed, challenged naïve clams exhibited the highest percentage of DNA in the tail, while chemical primed showed intermediate levels, in-between control clams and challenges naïve (*fig. 4.2.1 C, $p < 0.01$*).

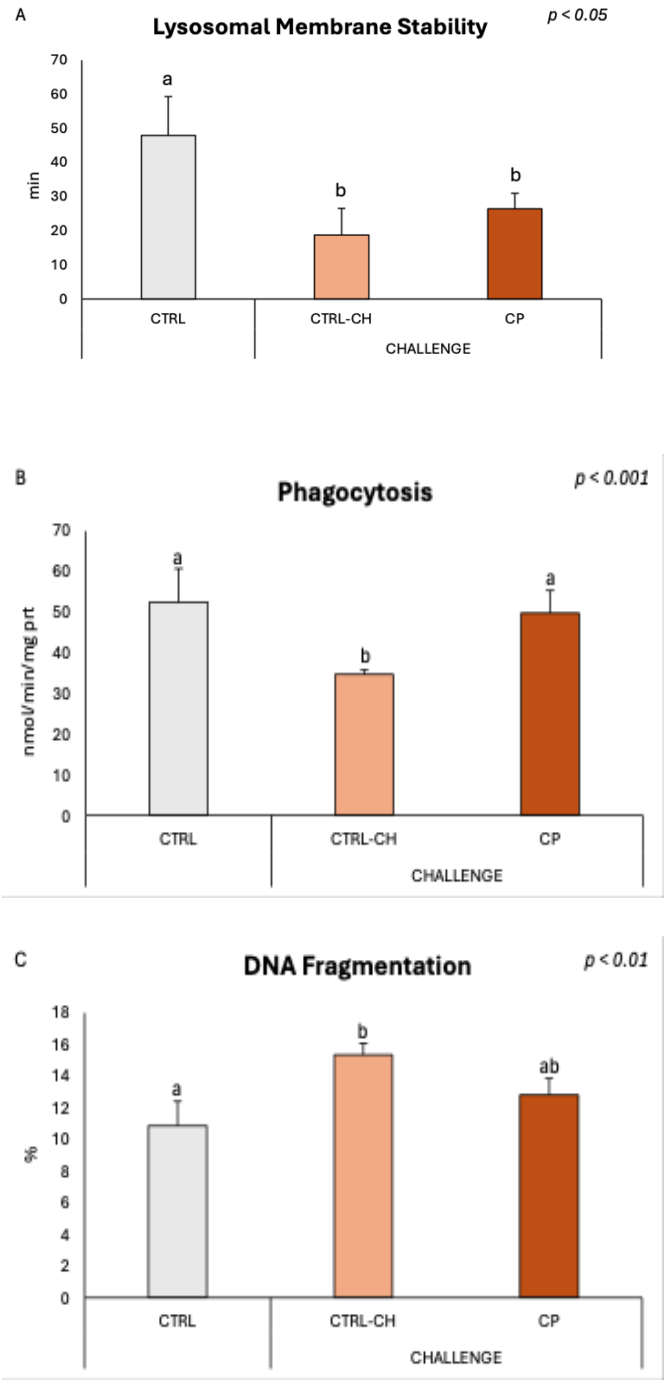



*Figure 4.2.1: Lysosomal membrane stability (A), phagocytosis efficiency (B) and DNA damage (C) in clams subjected to different treatments (CTRL: control; CTRL-CH: control under challenge; CP: chemical-primed). Different letters indicate significant differences between treatments. Values are expressed as mean ± SD.*

### *4.2.2- CHOLINERGIC RESPONSE*

AChE enzymatic activity in clams' gills showed significant differences between treatments. AChE levels are the highest in control clams with a similar trend in chemical primed ones whereas challenged control clams showed a significant decrease (*fig. 4.2.2 A, $p < 0.01$*).

AChE enzymatic activity in clams' hemolymph revealed significant differences between treatments; compared to AChE levels in gills, the CTRL-CH treatment showed the highest values, chemical primed clams had the lowest ones whereas control clams' values were in-between (*fig. 4.2.2 B, $p < 0.05$*).

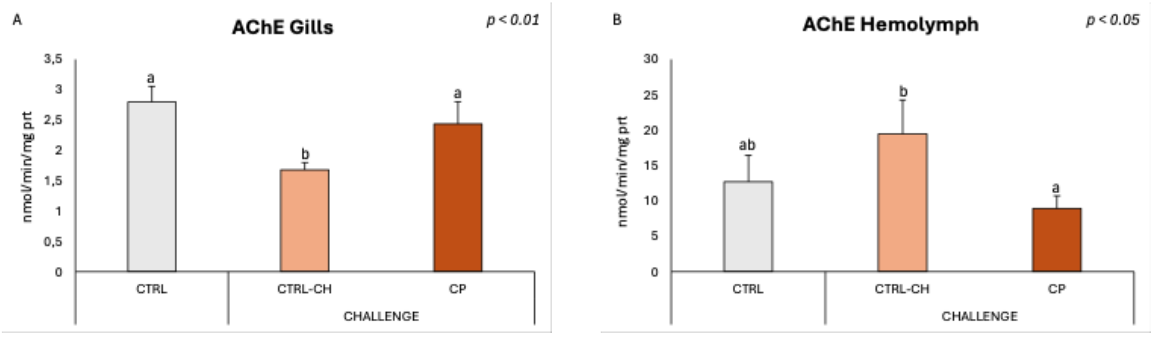



*Figure 4.2.2: AChE enzymatic activity in gills (A) and hemolymph (B) of clams subjected to different treatments (CTRL: control; CTRL-CH: control under challenge; CP: chemical-primed). Different letters indicate significant differences between treatments. Values are expressed as mean ± SD.*

### *4.2.3- ANTIOXIDANT SYSTEM*

CAT activity did not show any significant difference between treatments; however, a trend is visible with challenged control clams showing the lowest values and chemical primed ones a condition in-between (*fig. 4.2.3 A*).

Glutathione S-Transferases activity depicted a significant decrease in both challenged control clams and chemical primed ones with comparable values between them (*fig. 4.2.3 B, $p < 0.05$*).

The total antioxidant capacity against the peroxyl radical (TOSC ROO•) showed a significant increase in challenged control clams, chemical primed values are a little but still significantly higher than control ones revealing a general well-being of the organisms (*fig. 4.2.3 C, $p < 0.01$*). On the other hand, the total antioxidant capacity against the hydroxyl radical (TOSC HO•) did not show any significant difference between treatments (*fig. 4.2.3 D*).

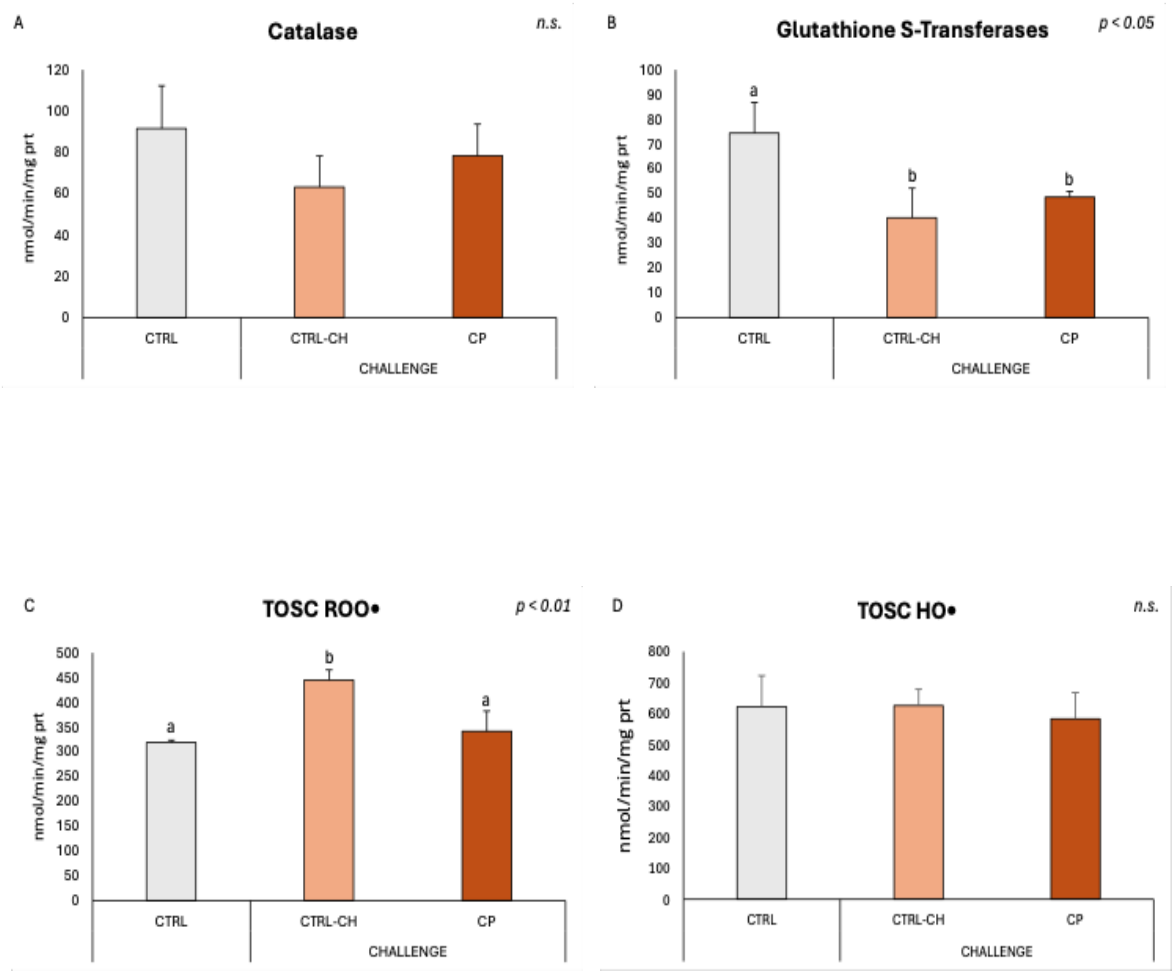



*Figure 4.2.3: Catalase enzymatic activity (A), glutathione S-transferases enzymatic activity (B), total antioxidant capacity against ROO• (C) and HO• (D) in gills of clams subjected to different treatments (CTRL: control; CTRL-CH: control under challenge; CP: chemical-primed). Different letters indicate significant differences between treatments. Values are expressed as mean ± SD.*

### *4.3- SURVIVAL RATE*

Survival was checked for one month after the end of the challenge. Naïve and chemical-primed clams showed comparable values while naïve challenged clams exhibited the highest mortality over the same period as it is shown in *figure 4.3*.

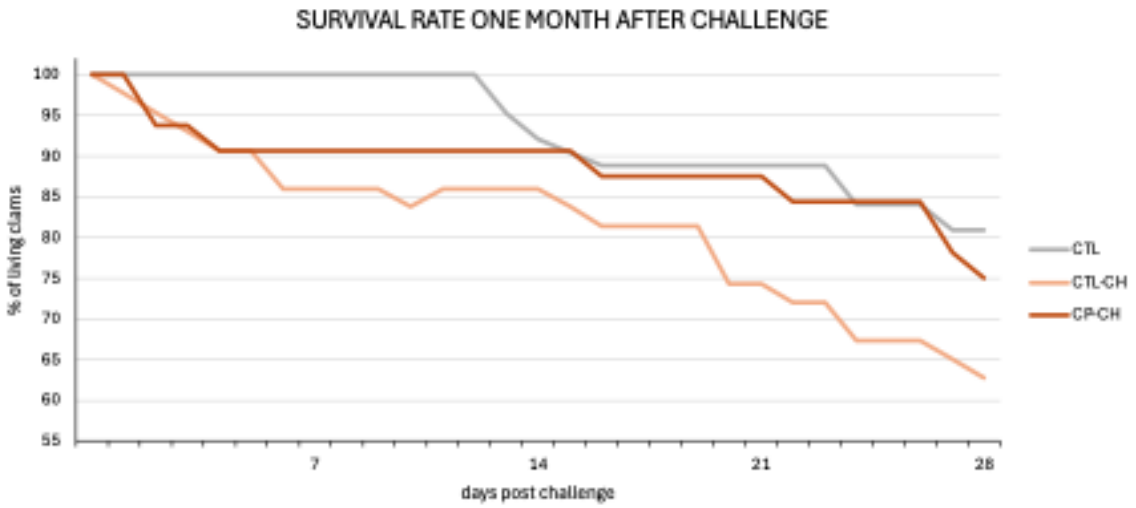



*Figure 4.3: Survival rate in clams subjected to different treatments (CTL: control; CTL-CH: control under challenge; CP-CH: chemical-primed) one month after the end of the challenge.*

### *4.4- PRINCIPAL COMPONENTS ANALYSIS*

Principal components analysis (PCA), conducted on the dataset of the different priming conditions in the SPC experiment, provided a clear pattern with two main clusters, one grouping control organisms and the other grouping primed-clams, irrespectively of tested $H_2O_2$ concentration. Overall, the major separation between the two clusters occurred along dimension 1, explaining 43% of total variance, and was mainly driven by antioxidant responses and acetylcholinesterase activity in gills, lipid peroxidation processes and hemocytes' functioning and organelles integrity (*figure 4.4*).

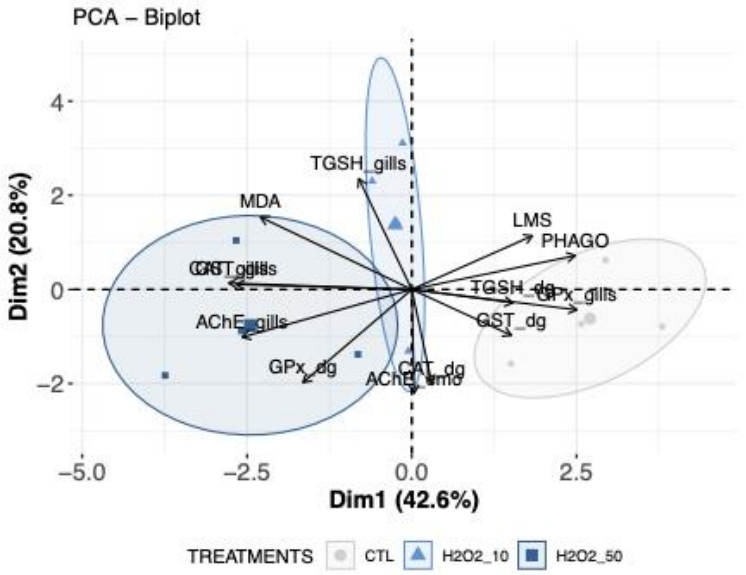



*Figure 4.4: Principal components analysis on results collected on clams exposed to different priming conditions (CTRL: control; $H_2O_2$ 10 mM; $H_2O_2$ 50 mM). Ellipses represent the 95% confidence interval of each experimental condition grouping; arrows indicate variables, and their length express their relative contribution.*

Principal components analysis was conducted also on the dataset of the responsiveness of naïve- and primed-clams toward combined thermal-chemical challenge, in the SPE phase. This provided a two-dimensional pattern (*figure 4.5*) explaining ~80% of total variance in which two main clusters were identified: one grouping naïve clams exposed to challenge, the other grouping control clams and primed-clams subjected to challenge. The clusters were mainly driven by results on hemocytes' phagocytosis activity, acetylcholinesterase in gills, antioxidants responses (TOSC ROO• and GST activity), DNA damage and lysosomal integrity in hemocytes.

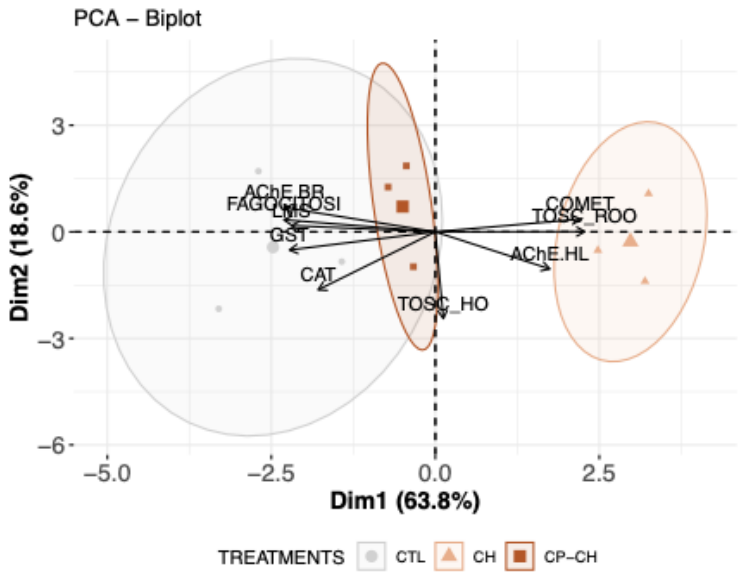



*Figure 4.5: Principal components analysis for the SPE experiment Principal components analysis on results collected on clams exposed to different challenge treatments (CTRL: control; CTRL-CH: control under challenge; CP: chemical-primed). Ellipses represent the 95% confidence interval of each experimental condition grouping; arrows indicate variables and their length express their relative contribution.*

## 5- DISCUSSION AND CONCLUSIONS

Priming techniques are manipulative approaches tailored to stimulate stress-memory mechanisms to foster organisms' stress tolerance. In this thesis, chemical priming was applied for the first time to *Ruditapes philipinnarum* to evaluate its capability to stimulate stress-response mechanisms. Initially, different priming conditions were tested to establish the most promising one.

Then, primed organisms were subjected to a combination of thermal and chemical stress to test the efficiency of priming in ameliorating clams' tolerance and survival in the context of multiple co-occurring stressors in marine ecosystems, as longer, more intense and frequent marine heatwaves and chemical pollution.

So far, chemical priming with $H_2O_2$ has been used in plants to counteract salinity stress. Several studies showed how this technique, based on the induction of the production of endogenous reactive oxygen species (Habib *et al*., 2020), can be useful in promoting antioxidant stress response as catalase, glutathione peroxidases and transferases which can eliminate ROS preventing oxidative damage (Ellouzi *et al*., 2021; Silva *et al*., 2020; Hossain *et al*., 2015). Since oxidative unbalance is widely described as one of the main mechanisms through which thermal stress and pollutants may affect marine organisms, we hypothesized that by promoting antioxidant- and stress-response mechanisms,

it could be possible to ameliorate organisms' performance under stressful conditions.

Firstly, the capability of two different $H_2O_2$ concentrations in promoting the most efficient stress-response was explored, in order to select the most promising chemical priming condition to be used to "harden" clams and prepare organisms to challenging environmental conditions. In this respect, both tested $H_2O_2$ concentrations were highlighted as effective in promoting stress-response mechanisms, with differences in certain biological responses.

Hemocytes are among the main cellular targets involved in stress-response and, in several functions, as immune defense (Gorbi *et al*., 2013). The integrity of lysosomes, organelles involved in the so-called cell autophagy that eliminates oxidative damaged organelles and proteins, is often used as a general biomarker of bivalves' health status and a variety of stressors including hyperthermia, hypoxia and chemical pollution have been highlighted as possible causes of lysosomal destabilization and damage (Moore, 2008; Moore *et al*., 2006). The results obtained in the SPC experiment did not show any significant decrease in lysosomal membrane stability in clams exposed to different $H_2O_2$ concentrations. On the other hand, phagocytosis efficiency showed a significant but very slight reduction in clams exposed to 50 μM $H_2O_2$ in the

SPC, which could suggest a lowered capability of hemocytes to counteract pathogenic insult (Allam and Raftos, 2015).

Acetylcholinesterase is a fundamental enzyme for bivalves, not only for its crucial role in cholinergic response but also for its involvement in the neuro-endocrine-immune system (Nardi *et al*., 2021). Nonetheless, AChE activity inhibition and induction have been often associated with stressful experimental conditions as chemical pollution and thermal stress (Greco *et al*., 2011). In this respect, AChE activity in clams' gills showed a remarkable induction in the $H_2O_2$ 50 μM treatment, which may be a compensatory mechanism to prevent ACh accumulation and counteract phagocytosis block (Nardi *et al*., 2021; Du *et al*., 2020; Liu *et al*., 2018). On the other hand, AChE in haemolymph was not affected.

Under basal conditions, there is an equilibrium between prooxidant challengers (reactive oxygen species, ROS) and the efficiency of antioxidant defenses. However, various environmental stressors (heatwaves, hypoxia, inorganic and organic xenobiotics) may disrupt this balance leading to the onset of oxidative stress (Regoli and Giuliani, 2014).

Among the tested priming conditions, the present study revealed a limited effect on the digestive gland coupled to a good responsiveness observed in the gills. Indeed, catalase and glutathione S-transferase activity were induced in the

$H_2O_2$ 50 μM treatment, confirming their active role as first-line antioxidants against ROS. This is in line with what has been observed in plants treated with $H_2O_2$ priming techniques (Ellouzi *et al*., 2021; Silva *et al*., 2020; Hossain *et al*., 2015). However, Alam *et al*., 2022, reported that in *R. philippinarum* exposed to different $H_2O_2$ concentrations a similar GST induction was sustained only up to 7 days of exposure, followed by a decrease of the activity, which may suggest that this initial activation can be triggered up to a certain threshold and starts to decrease when other stress mechanisms emerge.

On the other hand, glutathione peroxidases showed the opposite trend with $H_2O_2$ 50 μM treatment revealing the lowest concentrations. Since glutathione peroxidases are also involved in $H_2O_2$ transformation, the decreased trend may be explained by the fact that already other enzymes (as CAT) may have digest $H_2O_2$. This is in line with what Alam *et al*., 2022, observed in Manila clams where GPx concentrations did not show significant differences. Interestingly, total glutathione in gills showed a bell-shaped trend with the highest concentrations in the $H_2O_2$ 10 μM treatment and comparable results between naïve clams and $H_2O_2$ 50 μM treatment.

Malondialdehyde is a byproduct of different biochemical pathways, as lipid peroxidation and unsaturated fatty acids catabolism (Kaloyianni *et al*., 2009; Fogelman *et al*., 1980). It is an extremely toxic compound and is widely used

as biomarker of oxidative stress (Del Rio *et al*., 2005; Leuratti *et al*., 1998). $H_2O_2$ is to all intents and purposes a ROS due to its oxidizing capacity which can be sharpened against xenobiotics (Lushchak, 2011; O'Sullivan *et al*., 2005). Malondialdehyde content in clams' whole soft tissue showed a significant increase in both treatments at different $H_2O_2$ concentrations suggesting an increased oxidative pressure. According to He *et al*., 2022, thermal stress due to acute and repeated heatwaves caused the accumulation of MDA in Manila clams.

PCA analysis for the SPC phase offered a thorough understanding of different responses between naïve and $H_2O_2$ treated clams, regardless of the tested concentrations. In general, $H_2O_2$ priming have been demonstrated to activate antioxidant responses, AChE activity in gills, lipid peroxidation processes and hemocytes functioning and integrity.

Overall, obtained results, supported by the principal components analysis allowed to identify $H_2O_2$ 50 µM as the most promising approach to stimulate antioxidant and stress-response, with a comparable number of drawbacks as those provided by $H_2O_2$ 10 µM but with a higher capability to trigger antioxidants.

Given these results, the highest concentration was selected for testing its efficiency in empowering clams' tolerance towards multiple environmental

stressors. Thus, naïve and primed clams were exposed to a combination of thermal and chemical stress for one week to simulate a scenario of multiple environmental challenge occurring in summer in the Venice lagoon, when mass mortality of *R. philippinarum* have been reported in aquaculture facilities.

Results collected at the end of the experiment highlighted a good capability of the chemical priming in increasing clams' tolerance toward the combined challenge, with some specificities. Indeed, a comparable reduction of hemocytes lysosomal membranes stability was observed in both CTRL-CH and CP clams, confirming lysosomal sensitivity to high temperatures and pollutants. Several studies have already demonstrated that hematic parameters can be deeply altered in organisms exposed to contaminants (Su *et al*., 2017), as Li *et al*., 2022, in which a significant reduction in *R. philipinnarum* membrane stability was observed after exposure to PFOA at different concentrations (0.2 μg/L, 2 μg/L, 20 μg/L). Also, Nardi *et al*., 2017, 2022, reported lysosomal compartment sensitivity in *Mytilus galloprovincialis* co-exposed to cadmium and warming and to carbamazepine and MHW scenario.

On the other hand, phagocytosis efficiency in chemical primed clams had comparable values to naïve organisms not exposed to the challenge and higher compared to non-primed challenged organisms, suggesting a positive effect of priming in preserving granulocytes activity irrespectively of a lowered

lysosomal integrity. The effect observed in non-primed challenged clams is in line with the finding of Li *et al*., 2022, which showed a drop in phagocytosis efficiency from the third day of exposure on, in *R. philippinarum* exposed to PFOA at different concentrations (0.2 μg/L, 2 μg/L, 20 μg/L). Similarly, phagocytosis rate was observed also in *Crassostrea virginica* and *Chamelea galina* when exposed to increased temperatures (Monari *et al*., 2007; Hégaret *et al*., 2003), while Nardi *et al*., 2017 showed a slight decrease in *M. galloprovincialis* exposed to temperature increase alone but not in the co-exposure with cadmium.

Additionally, DNA integrity was assayed through Comet assay to explore the onset of genotoxic effects in hemocytes: although a generalized DNA fragmentation was observed in challenged clams, irrespectively of being primed or not, two aspects deserve attention. First, naïve showed the highest percentage of DNA in the tail (*i.e.* highest degree of fragmentation); secondly, the magnitude and extent of such increase may not imply the onset of genotoxicity but rather be the consequence of a faster cell cycle due to the thermal stress exposure, as previously observed in *M. galloprovincialis* (Nardi *et al*., 2017). Overall, CP clams had in-between values which may suggest the heatwave scenario forced an increase of clams' metabolic rate (in accordance with the OCLTT theory). On the other hand, H2O2 priming might have been

successful in stimulating the antioxidant defense system, allowing organisms to be ready to act towards prooxidant stressors. As a confirmation, Alam *et al*., 2022, did not reveal any variation in DNA integrity when *R. philippinarum* was exposed to $H_2O_2$ from day 0 to day 14 whereas they showed DNA damages only after 21 days of exposure.

Regarding the effects observed on the cholinergic system, AChE activity in both gills and hemolymph of primed clams showed values comparable with those observed in naïve clams, and in both tissues, this was statistically different from non-primed clams exposed to challenge. This allows to suggest a positive effect of priming, paralleling the trend observed also for phagocytosis. Previous studies confirm these findings, as AChE activity inhibition was observed in the hemolymph of *M. galloprovincialis* under thermal stress and cadmium exposure (Nardi *et al*., 2021). In addition, Kamel *et al*., 2012 and Greco *et al*., 2011, demonstrated that acetylcholinesterase and temperature were inversely correlated in *Mya arenaria* and *M. galloprovincialis* respectively when co-exposed to thermal stress and chemical pollution.

Redox homeostasis also suggested the efficiency of priming in increasing clams' tolerance. First, no significant differences between experimental treatments were observed for CAT activity in gills: since it is known that

thermal stress mat enhance ROS proliferation leading to the onset of oxidative stress (Xu *et al*., 2022; He *et al*., 2021; Leung *et al*., 2019; Madeira *et al*., 2016), the lack of significant effects on catalase suggest an overall good capability of clams to cope with the combined stressors scenario. On the other hand, Georgoulis *et al*., 2021, observed a sustained CAT enzymatic activity in heat-hardened mussels for *M. galloprovincialis* exposed at different temperatures (24, 26 and 28°C), demonstrating that hardened mussels exhibit a faster CAT response, acting as first-line defense against ROS production. Also, He *et al*., 2022, reported enhanced CAT enzymatic activity in Manila clams exposed to repeated heatwaves during recovery phase while in clams subjected to MHWs for the first time, CAT enzymatic activity was shown to decrease. Both studies agreed on the fact that cited organisms, subjected to both acute and repeated heatwaves scenario, were shown to enhance CAT responsiveness suggesting also the expenditure of a greater portion of their energy budget in antioxidant defenses rather than in other vital processes such as growth and reproduction.

On the other hand, glutathione S-transferases activity was significantly reduced in clams exposed to challenge, independently of being previously primed or not. GST, being a phase II detoxification enzyme, catalyzes glutathione conjugation with xenobiotics so to favor their elimination, affecting glutathione availability (Fabrello *et al*., 2021). According to Fabrello *et al*., 2021, GST in

the mediterranean mussels' digestive gland was not affected by neither PFOA at different concentrations nor the exposure time. On the other hand, Gülsever and Parlak, 2018, reported an increase in GST enzymatic activity in the digestive gland of M. galloprovincialis when it was exposed for 21 days to PFOS (2-10 mg/L). Georgulis *et al*, 2021, showed an enhanced induction of GST mRNA expression in hardened mussels throughout the experiment which may be explained by the onset of oxidative stress due to hypoxic conditions related to temperature increase, in accordance also to the OCLTT theory (Woo *et al*., 2013). Also, according to Nardi *et al*., 2022, GST enzyme activity was enhanced in mussels exposed to carbamazepine for 10 days under a heatwave scenario suggesting how biotransformation reactions may be a source of ROS which are already increased by thermal stress.

While analysis of single antioxidant defenses may provide mechanistic explanation of the effect of a certain stressor, these do not provide an integrated picture of the redox status of organisms. For this, the total oxyradical scavenging capacity against the peroxyl and hydroxyl radicals (TOSC ROO • and HO•) was analyzed, which allows to evaluate cellular capability to counteract certain ROS. Combined thermal and chemical challenge cause an increase of TOSC ROO • in naïve challenges organisms, while CP clams showed values comparable with those measured in naïve ones, suggesting a

general well-being, the lack of need to increase antioxidant defenses and thus the positive effects of priming technique. On the other hand, the total oxyradical scavenging capacity against the hydroxyl radical (TOSC HO•) did not reveal any significant difference between treatments.

The general well-being of primed organisms hypothesized given the total oxyradical scavenging capacity toward both ROO• and HO•, may suggest the baseline levels of antioxidant protection were sufficient to counteract oxidative insult, consequential to a prompt activation of antioxidant defenses following the priming. This is particularly relevant when considering the implication on the long term: indeed, the sudden increase of TOSC ROO• in non-primed clams might imply deleterious effect on the organisms' fitness, creating an imbalance between the energy rapidly allocated on the activation of defense mechanisms and those dedicated on other vital processes as growth and reproduction (He *et al*., 2022; Chainy *et al*., 2016; Pörtner and Knust, 2007; Somero, 2002).

Finally, after the challenge organisms were left in clean seawater for one month and mortality daily checked. Overall, naïve challenged clams revealed the highest percentage of dead organisms and a t a faster rate, while naïve and CP had the lowest percentages of mortality (also comparable between treatments), reinforcing the hypothesized suitability of priming techniques in fostering clams' resilience and resistance. Similarly, Georgoulis *et al*., 2021, revealed

higher survival rates in heat-hardened Mytilus galloprovincialis due to an increased thermotolerance after the exposure to sub-lethal (26°C) and lethal (28°C) temperatures. Also, Zhang *et al*., 2023 demonstrated that Manila clams, pre-hardened at 30°C, exhibited the highest survival rate over time and that the temperature of the initial heat bout can modulate organisms' heat tolerance. Several studies revealed how temperature increase may be one of the main factors affecting bivalves' mass mortality, as observed by Rahman *et al*., 2019, which showed decreased survival rates in three different molluscan species exposed to temperatures increase.

The overall observed suitability of the priming approach was summarized by PCA analysis, which offered a thorough understanding of the responsiveness of naïve and primed-clams toward combined thermal-chemical challenge. Naïve and CP clams were grouped, and these were detached from naïve clams exposed to combined challenge, revealing how priming techniques can positively modulate organisms' responses under stressful conditions.

This allows to conclude that overall chemical priming was proven to be an efficient strategy in promoting organisms' resistance and resilience to multiple stressors in laboratory conditions. Since environmental co-occuring stressors are projected to become more intense and frequent under climate changes and harsher anthropogenic pressures scenarios, this thesis allowed, for the first

time, to explore chemical priming techniques as a promising approach in *Ruditapes philippinarum*, thus representing a starting point to provide a mitigation strategy in aquaculture.

Further improvements will be directed in testing priming techniques with longer resting phases before their testing against a challenge and their application in field scenarios.

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