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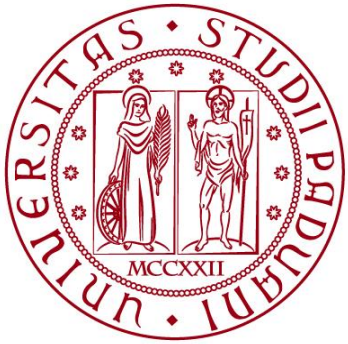

TESI DI LAUREA

Possible buffer effects of primary producers on marine invertebrates against environmental stressors

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To the child that made the choice.
To the girl that worked for it.
To the woman that made it possible.

Index

Abstract

Recent decades have highlighted how human action is bringing deterioration and change to the marine environment through the release of contaminants. By releasing oxygen, coastal photosynthetic organisms have been proven beneficial to marine animals as a mitigating factor against ocean warming and acidification. Whether or not this buffer effect is applicable against pollution has yet to be proven. Understanding this becomes pivotal for predicting future changes and enhancing current conservation and restoration strategies of primary producers-based habitats. This study investigates the potential buffer effect of primary producers against mercury pollution by increasing the oxygen concentration and examining behavioral and physiological responses. In two separate trials, the sea urchin *Paracentrotus lividus* and the clam *Ruditapes philippinarum* were exposed to the combination of 2 level of oxygen saturation (90% as control and 160% representing daily supersaturation) and 2 concentration of mercury (0 and 1 mg/L) for 7 days. At the end of exposure, regarding *P. lividus*, coelomocytes related biomarkers, individual respiration rate and behavioral traits were measured in exposed sea urchins. Results show that righting time was significantly positively influenced by the presence of a higher %DOP. Regarding *R. philippinarum*, hemocytes-related biomarkers were measured in the exposed clams. Results show a significant drop in hemocytes cell count, cell volume and cell diameter in the individuals that were exposed both to mercury and hyper-oxygenation. As the results show both a negative and a positive outcome, what we can gather is that primary producers-based habitats need to be protected and restored, and we need to find new and improved ways to deal with the release of contaminants.

Abstract esteso

L'uomo ha da sempre modificato l'ambiente per soddisfare le proprie esigenze, ma in tempi recenti, soprattutto dopo la rivoluzione industriale, queste modifiche hanno portato a fenomeni come l'inquinamento. Negli ultimi decenni studi scientifici hanno evidenziato come l'azione antropica influenzi negativamente l'ambiente marino. Uno dei principali fattori responsabili di quello che oggi chiamiamo "inquinamento" è il rilascio di contaminanti che, attraverso diversi percorsi, trovano quasi sempre una via verso il mare. Gli organismi fotosintetici che popolano le coste giocano un comprovato ruolo di mitigatori contro il riscaldamento degli oceani e l'acidificazione degli stessi: l'ossigeno rilasciato da questi organismi giova al metabolismo degli organismi esposti a questi stressor, aiutandoli a sopravvivere. Se questo effetto buffer possa essere applicato anche contro l'inquinamento deve ancora essere provato.

Comprendere la scala di questo effetto è diventato centrale per predire cambiamenti futuri, ma anche a livello legislativo per rinforzare strategie correnti per la conservazione e il restauro di ambienti ricchi di produttori primari.
Questo studio si propone di investigare il potenziale effetto buffer dei produttori primari, simulando la produzione di ossigeno, contro la contaminazione da mercurio attraverso la misura di risposte fisiologiche e comportamentali in due specie di invertebrati marini di rilievo sia economico che ecologico nel bacino Mediterraneo, il riccio di mare *Paracentrotus lividus* e la vongola *Ruditapes philippinarum*. In due esperimenti separati *P. lividus* e *R. philippinarum* sono stati esposti alla combinazione di due livelli di saturazione di ossigeno (90% come controllo e 160% come rappresentazione della super saturazione giornaliera) e due concentrazioni di mercurio (0 and 1 mg/L) per una settimana (7 giorni). Alla fine dell'esposizione, per *P. lividus* sono stati misurati biomarker associati al sistema immunitario, tratti comportamentali e fisiologici per ciascun individuo. I risultati mostrano come la presenza di una concentrazione più alta di ossigeno, sebbene non abbia influenzato significativamente i tassi di respirazione degli individui, ha invece influenzato positivamente il "righting time"sia negli individui contaminati che nei controlli. Per *R. philippinarum* sono stati misurati biomarker associati al sistema immunitario. I risultati mostrano una diminuzione significativa nel numero, diametro e volume di queste cellule negli individui esposti a contaminazione da mercurio e super saturazione. Poiché i risultati mostrano sia risposte positive che negative alla supersaturazione, le conclusioni devono essere interpretate in modo interconnesso. È fondamentale proteggere e restaurare gli habitat creati dai produttori primari, ma ciò deve essere accompagnato da sforzi paralleli per limitare il rilascio di contaminanti. Affrontare solo uno di questi aspetti potrebbe risultare dannoso e controproducente per alcuni organismi, come evidenziato in questo studio dalle risposte di *R. philippinarum*.

1.Introduction

1.1 The Anthropocene

The term Anthropocene comes from the Greek *Anthro*, meaning "human", and *cene*, meaning "new". It was first introduced at a conference in the early 2000s by Paul Crutzen (Slaughter, 2012). In its broadest form, the concept aims to define the relationship between Humanity and the Earth's ecosystem, while describing how the first affects and has been affecting the second (Lewis e Maslin, 2015; Cork et al., 2023; Head et al., 2023).

After years of deliberation, in 2016, the Working Group on the Anthropocene declared in favor of a formal conceptualization of the term. At that conference, the beginning for the Anthropocene was loosely set in the mid-50s, with the annotation to find an exact time point at a later date (Malhi, 2017).

To do so, a global marker was required to appear in the stratigraphy of the soil (Cork et al., 2023).

The mid-50s starting point coincides with the Great Acceleration, the phenomenon where industrialization began its true march towards globalization (Cork et al., 2023; Malhi, 2017). Also, the residual consequences of the nuclear bombs at the end of World War II can be found pretty much anywhere in the world (Cork et al., 2023).

However, a parallel narrative has been setting the beginning of the Anthropocene at an earlier date, spanning from just a few decades to millenias (Malhi, 2017).

Some argue the Industrial Revolution as the beginning of the Anthropocene. The counter argument is that the Industrial Revolution was not as steep as it is made to appear, as the use of coal had been present for decades before (Cork et al., 2023).

Some argue that it began when Humanity began farming, in doing so actively changing the environment they lived in (Malhi, 2017).

A third viewpoint states that the Anthropocene has not begun yet. Its beginning would coincide with a planet-wide tipping point in the ecosystem's state, with no chance of recovery (Malhi, 2017).

Truth be told, any time point presented can be argued and discarded (Cork et al., 2023).

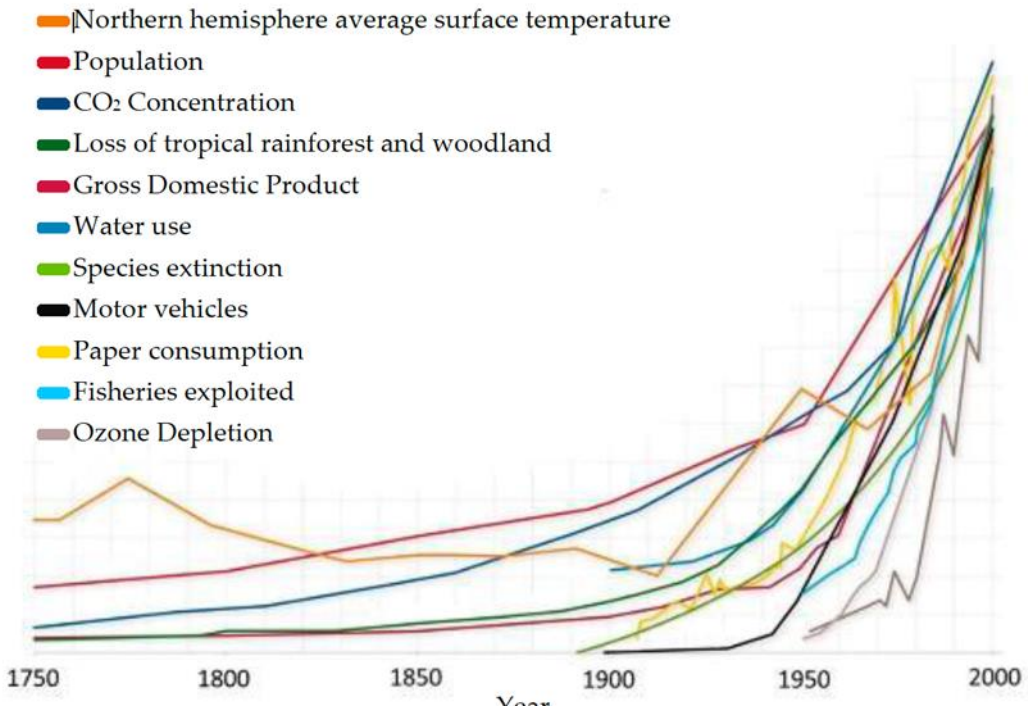

Figure 1. Mesures of Anthropocene. Global change and Earth system (Steffen 2004)

The fact that the Anthropocene has yet to find a beginning, and in turn be defined as a true era, seems to be linked less to the scientific side of the term and more to the political one.

Picking a recent beginning for such a monumental change in the Earth's ecosystem could potentially kick off a global game of blame-placing. On the other hand, choosing to make it coincide with the dawn of Humanity would take away from the severity and the gravity of the situation Earth finds itself in (Head et al., 2023).

Lewis & Maslin, 2015 would provide terminology for Earth's current situation, giving tools to Governments to take action (Malhi, 2017).

1.2 Pollution and Contaminants

The importance of definitions and data is not limited to the concept of Anthropocene, it is pivotal to manage one of its main consequences: pollution. (Sánchez-Bayo et al., 2011).

The term pollution, according to Section 1(3) of the U.K. Environment Protection Act, 1990, means:

"The release (into any environmental medium) from any process of substances which are capable of causing harm to man or any other living organisms supported by the environment."

This could mean, namely, the eruption of a volcano, a forest fire, an earthquake, more in general natural occurrences that have large consequences. (Appannagari, 2011).

It also describes the degradation of the natural environment due to human activity (Ukaogo et al. 2011). The fallout of those activities can be divided into two categories: direct and indirect. The first one defines every change made to the environment, be it for urbanization or exploitation of resources, of which the positive and negative consequences are known. Indirect impacts are those which are not planned, and mostly arise after long periods of time. (Appannagari, 2011).
Closely linked to limiting pollution is the knowledge of the very many forms pollution can take: data shows that it is heaviest in developing countries, where the need for progress surpasses the tools to do it without disrupting the environment. (Ukaogo et al. 2011).
Pollution, while it's being addressed only in recent years, is a phenomenon that has accompanied humanity for millennia: from the toxic dyes used in the Neolithic to the very contemporary exploitation of petroleum, humans have been releasing compounds into the environment. While the studies of toxic substances in medicine began in the Renaissance, the concern for human health rose only after the Industrial Revolution, expanding the conversation to the consequences it brought to the environment. (Appannagari, 2011)
The definition of pollution given above speaks of "substances released".
To quote Paracelsus "All things are poison, and nothing is without poison". Brought to modern day, this is the very base of toxicology: it is not only the compound that matters in terms of toxicity, but also its concentration. (Sánchez-Bayo et al., 2011)
When found above its usual concentration, or in an environment different than its own, any compound can become a contaminant. The big distinction comes with what happens when the contaminant is present: many times, contaminants are present, but they don't pose a risk or a threat, whether due to their low concentration or their non-toxic nature. Other times, contaminants not only can be toxic in small quantities, but are sometimes released in the environment in concentrations that can be harmful to it and human health. (Brusseau e Artiola, 2011)
An important distinction to make is between legacy and emerging contaminants. Emerging contaminants, unlike legacy ones, are contaminants that have only in recent years begun to raise concern. This doesn't mean they haven't been present for decades, but only now the consequences are being seen and felt. Only recently are they being linked to environmental issues. There are no present regulations on their use, or how to safely dispose of them. Data is needed, research is needed, continuous reevaluation of the substances already regulated is needed. (Sauvé e Desrosiers, 2014; Brusseau e Artiola, 2011)

1.3 Mercury (Hg)

Mercury comes from both anthropogenic and natural sources: it may result from a forest fire, or a volcanic eruption, or be the result of many industrial works (Pavithra et al., 2023) (Kumar et al., 2023).

It is categorized as a toxic, non-essential metal, widespread in the aquatic environment (de Almeida Rodrigues et al, 2019). As it has no biological role to play inside an organism, it tends to accumulate in the brain, kidneys, liver and bones, presenting carcinogenic and genotoxic risks (Bjørklund et al, 2017; Kumar et al., 2023).

Mercury can enter the water column in a few different ways (Fig.2): by directly depositing on the water surface from the atmosphere, industrial runoff in rivers, and because soil and sea are physically connected, the mercury that ends up in the soil eventually finds its way in the water (Gworek et al., 2016).

Because of this, coastal environments are constantly exposed to the presence of mercury. Mostly, it enters the water column as inorganic mercury, but the presence of bacterial communities causes a process called methylation, where mercury is resuspended in its organic form, thus becoming available to organisms (de Almeida Rodrigues et al, 2019).

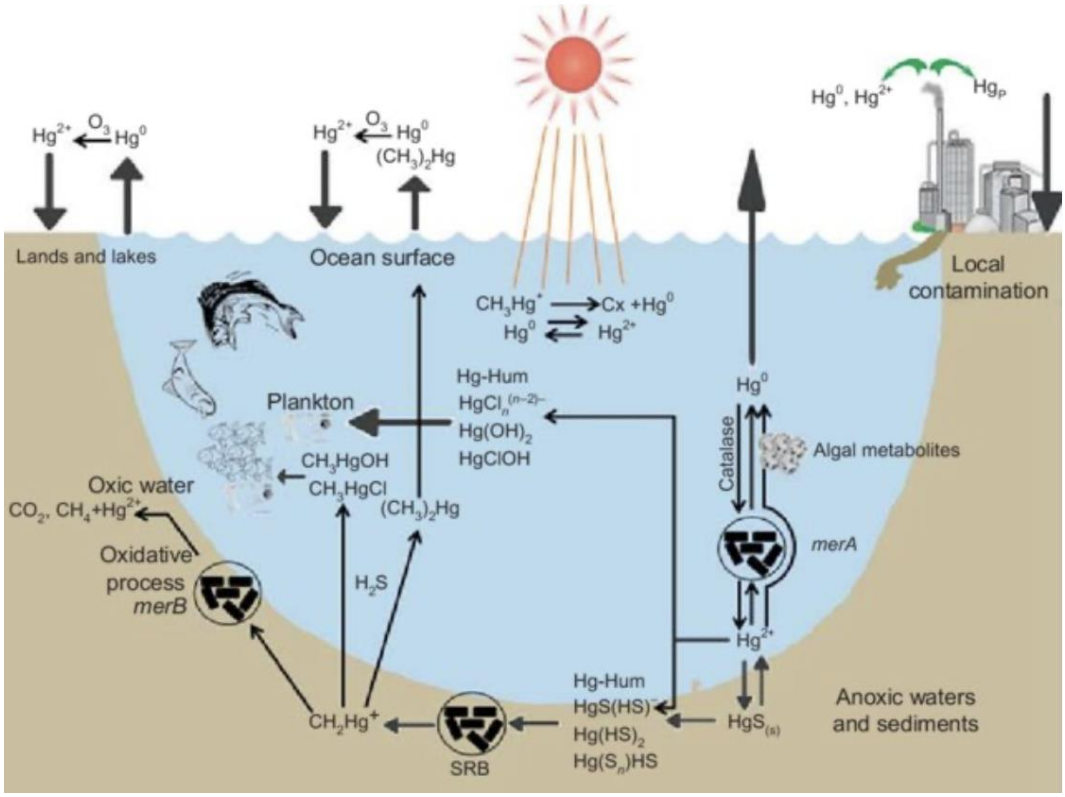

Figure 2. Fate of mercury in the environment (De et al, 2014)

Due to organisms absorbing it (bioaccumulation), it is easier to see the consequences of mercury on the organisms rather than the environment itself (Gworek et al., 2016).

This raises the need for biomonitoring, where the effects of the contaminant were observed on a sample organism (Kumar et al., 2023).

The methylated mercury, Methyl mercury (MeHg) is consumed largely by plankton, in turn consumed by filter feeding organisms, benthic organisms and bigger fish. Because animals accumulate MeHg at a much faster rate than they can eliminate it, it finds its way up the trophic chain, through a phenomenon called biomagnification (Kumar et al., 2023; Pavithra et al., 2023; Gworek et al., 2016).
There are instances, however, that show how an accelerated metabolism can increase the excretion rate of the compound, lowering the concentration inside the organism (de Almeida Rodrigues et al, 2019).
It is not only MeHg that is absorbed and stored inside marine organisms, as there are many forms of Hg present in the aquatic environment. For example, the presence of ions can lead to the creation of stable forms of Hg that result in the dissolution of the solid phase of the compound. Namely, Cl^- binds to Hg, leading to the making of $HgCl_2$ (Gworek et al., 2016).
Easy to isolate, it was historically used as an antiseptic agent. It has now been decades since it was used as such, given its toxicity. Various animal models have demonstrated how it can be absorbed dermally, and its disruptive potential of enzymes, membrane transportation and structural proteins. Also, $HgCl_2$ can pass through the placenta barrier, causing severe damage to the fetus (Cappelletti et al., 2019).
Studies show that Hg is stored in many different organs, such as liver, hepatopancreas, digestive gland, brain, gills, with the highest concentration found in muscle (de Almeida Rodrigues et al, 2019).
Because of where it's stored, and the biomagnification, the largest quantities of Hg are found in large fish: their consumption is the most common way for mercury to contaminate terrestrial organisms (Pavithra et al., 2023).
Since Hg is a neurotoxic compound, the contamination of aquatic organisms is a matter of public health concern: mercury can bring degenerative changes, from the cellular to the behavioral (de Almeida Rodrigues et al, 2019).

1.4 Primary producers as buffers of anthropogenic stressors

Primary producers are organisms that, through photosynthesis, reduce inorganic carbon to organic carbon. As the name suggests, they are the primary source of organic carbon for the Ecosystem (Falkowski e Knoll, 2007). Nearly half of Earth's primary production happens in the ocean (Cloern e Jassby, 2008), carried out by phytoplankton (Paerl e Justic, 2011), and other important marine primary producers such as macroalgae and seagrasses. Oxygen is maybe the most important byproduct of a chemical reaction: since the beginning of photosynthesis, all complex life has evolved to depend on it (Falkowski e Knoll, 2007). Particularly in coastal environments, primary production rates and the accumulation of organic matter are criteria to evaluate the wellbeing of the system (Paerl, 2007), along with nutrient and oxygen input (Paerl e Justic, 2011). As it stands, primary production is controlled and made possible by different factors, such as light, nutrients, temperature and herbivory (Paerl e Justic, 2011). These factors are delicately

balanced, and one change can affect the whole system. For example, the rise in temperature can increase productivity (Häder et al., 2014).
This could have positive or negative consequences.
The most common negative example is the bloom of harmful algae: for many algal species, higher temperatures are usually the signal to start replicating. This can lead to the release of biotoxins, and/or anoxia. (Häder et al., 2014). On the flip side, seagrasses all over the coastal environment show peak productivity during the hottest hours of the day, due to direct sunlight and warmer water (Fig.3).

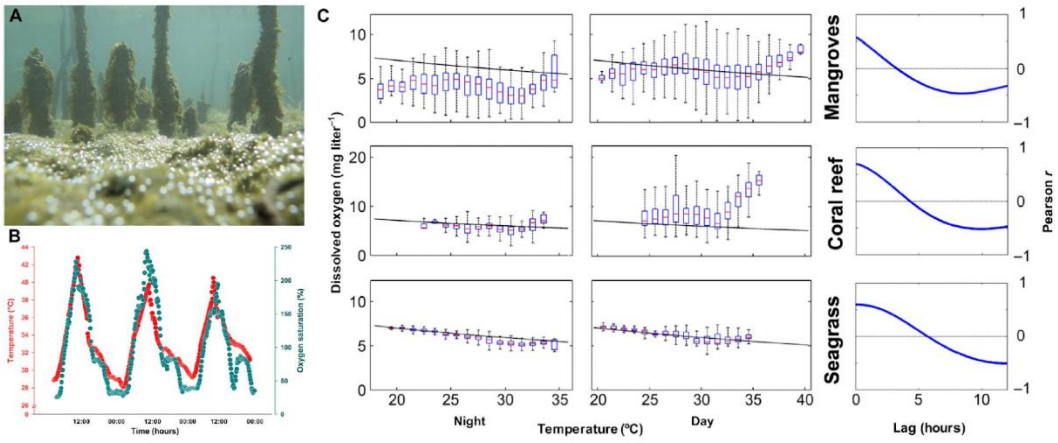

Figure 3. From Giomi et al. (2019). Dissolved oxygen concentration and water temperature in Red Sea coastal habitats were studied with the following observations: (A) Oxygen production observed in a mangrove forest floor colonized by a photosynthetic biofilm composed of cyanobacteria, algae, and microphytobenthos. (B) Diel fluctuations in seawater temperature and dissolved oxygen showed peaks of oxygen production during the hottest hours, creating local hyperoxic conditions. (C) Dissolved oxygen concentration and water temperature data recorded in the three dominant coastal habitats of the Red Sea.

The release of oxygen in those seagrass meadows seems to counterbalance the dip in oxygen levels also due to higher temperatures. (Giomi et al., 2019) The same study shows how animals that are chronically exposed to those hyper-oxygenated conditions fare significantly better against the rise in temperatures. The extra oxygen provided by the primary producers helps boost the metabolism of the animals and acts as a buffer, helping them to survive higher than normal temperatures (Giomi et al., 2019) (Fig. 4).

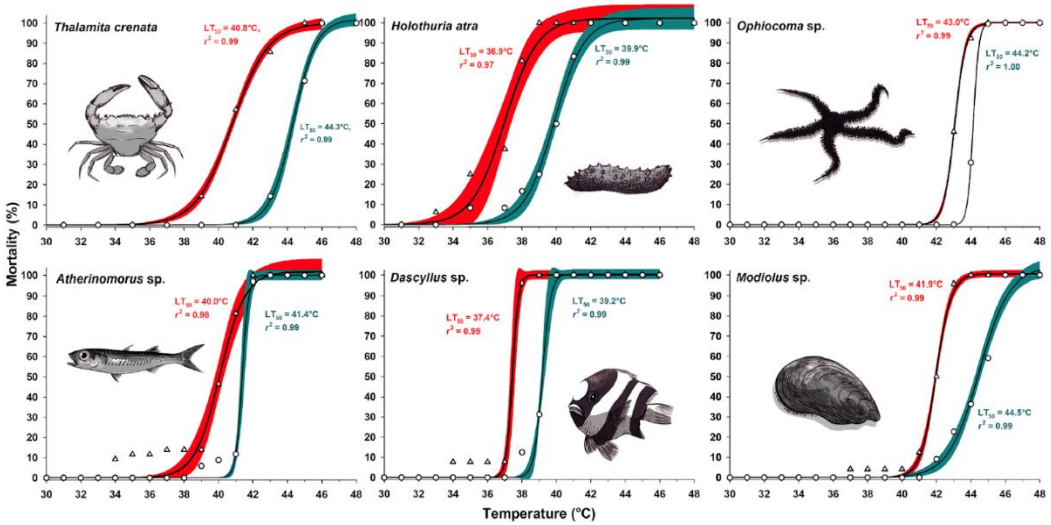

Figure 4. Figure from Giomi et al. (2019). Experimental relationship between water temperature and mortality for six marine species under simulated normoxia (triangles) and hyperoxia (circles). The filled areas represent the 95% confidence intervals (normoxia: red; hyperoxia: cyan) for each three-parameter sigmoid regression (black line). The LT50 (lethal temperature for 50% of the organisms) and R^2 values for each regression are shown in red for normoxia (97 ± 2% oxygen saturation) and cyan for hyperoxia (140 ± 3% oxygen saturation). Temperature ramping was set at 1°C every 30 minutes to mimic daily environmental warming rates. Note that the symbols may obscure some data points. Animal illustration credit: Allende Bodega Martinez.

Higher oxygen levels provided by macroalgal habitats also act as a buffer to the ever-increasing rate of ocean acidification (OA). (Hendriks et al., 2014).
While the evidence in favor of the buffer effect of macro primary producers against stressors such as increasing temperature and OA is present in the literature (Giomi et al., 2019; Hendriks et al., 2014), there is no record of how the buffer effect fares against contaminants.

1.5 Marine invertebrates as bioindicators

Bioindicators are organisms whose biological responses (biochemical, physiological, morphological, etc.) can provide information on the quality of the environment (Chiarelli and Roccheri , 2014). They can accumulate contaminants at concentrations higher than those found in their habitat. Impairments in physiological functions, behavioral modifications, and pollutant bioaccumulation are crucial tools in biomonitoring studies, as well as in assessing the effects of specific chemicals.
The selection of an organism as a bioindicator involves several considerations. Generally, it must possess key characteristics such as being widely distributed in the study area, readily available, easy to identify, having a relatively long life cycle, and genetically uniform populations. Additionally, there must be adequate knowledge of the species' anatomy, physiology, and ecology available from literature.
When selecting a species as a bioindicator for both field and laboratory exposures to environmental contaminants, it is essential to consider the behavior of the contaminants once released into the environment, including their mobility and

tendency to remain in the water column or accumulate in sediment. Different ecological niches of potential bioindicators entail varying exposure pathways.
Considering these features, various species of bivalve molluscs and sea urchins have proven to be excellent bioindicators in marine ecotoxicology studies. These organisms are valuable in assessing environmental quality due to their ability to reflect the presence and impact of contaminants in their habitats.

1.5.1 The sea urchin *Paracentrotus lividus* (Lamarck, 1816)

Kingdom: Animalia

Phylum: Echinodermata

Class: Echinoidea

Order: Camarodonta

Family: Parechinidae

Genus: Paracentrotus

Species: Paracentrotus lividus

Figure 5. Image of Paracentrotus Lividus

Paracentrotus lividus is a benthic marine invertebrate with a spherical body exhibiting pentaradial symmetry in adulthood and bilateral symmetry during its larval stage. Internally, it has a dermal skeleton composed of 20 porous, calcified plates fused together. The body (Fig.5) can be divided into two hemispheres: the

oral hemisphere with the mouth and the aboral hemisphere with the anus. The mouth is surrounded by the peristomial membrane, which supports the scraping apparatus known as Aristotle's lantern. This structure consists of five radially arranged calcified plates, called pyramids, which bear five pointed teeth protruding from the oral end. The alimentary canal extends from the lantern, comprising the pharynx, esophagus, stomach, and intestine. The intestine is not straight but forms loops and spirals before ending in the rectum in the aboral region.
The anus is surrounded by a membrane called the periproct, around which five genital plates are located, each with a gonopore. The largest of these plates is porous and is called the madreporite. Through the madreporite, water enters and fills the canals of the water vascular system connected to the tube feet, each ending in a suction cup. Water is forced in and out of the tube feet, allowing the animal to adhere to the substrate when the tube foot is inflated with water and to detach when it contracts. Each individual has hundreds of tube feet, which emerge through pores in the dermal skeleton. These animals cannot move efficiently on sandy substrates because the suction cups of the tube feet do not adhere well, unlike on rocky substrates. Locomotion is aided not only by the tube feet but also by the spines, which are made of calcite and organic material, and are almost symmetrically distributed in the ambulacral and interambulacral regions. All appendages, such as spines and tube feet, can regenerate if lost, and even damage to the dermal skeleton can be repaired by depositing new calcareous material (Brusca, 1996)

1.5.2 The clam *Ruditapes philippinarum* (Adams & Reeve, 1850)

Kingdom: Animalia

Phylum: Mollusca

Classe: Bivalvia

Ordine: Venerida

Famiglia: Veneroidea

Genere: Ruditapes

Specie: Ruditapes philippinarum

Figure 6. Image of Ruditapes philippinarum.

The short-necked Manila clam, *Ruditapes philippinarum*, is native to the Pacific coasts from southern Siberia to China. Today, this species is widespread along the European coasts, from Ireland to the Mediterranean, particularly in the northern Adriatic. Since its introduction to the Venice Lagoon in the early 1980s, it has largely taken over the ecological niche once dominated by the native species *Venerupis decussata*. It inhabits the mid to low intertidal zones of bays, lagoons, and estuaries, living in mud or sand, and buries itself 2–4 cm below the surface. The clam filters phytoplankton and organic detritus suspended in the water column or resuspended from the sediment. This filtration process also leads to the bioaccumulation of environmental contaminants (Ericson et al., 2010).

The shell of *R. philippinarum* consists of both organic and mineral components, comprising 5% and 95% of the dry weight, respectively. The outer proteinaceous layer (periostracum) is very thin. The mineral component is primarily calcium carbonate ($CaCO_3$), mostly in the form of aragonite, arranged in three layers: an outer prismatic layer, an intermediate lamellar layer, and a homogeneous inner layer (Mikkelsen et al., 2006; Ries et al., 2009).

R. philippinarum is a broadcast spawner. In the Venice Lagoon, spawning occurs from May to September (Meneghetti et al., 2004). Adult males and females release sperm and eggs into the water column where fertilization takes place. The fertilized eggs develop into planktonic larvae that drift with the water currents for several weeks. Within 2-4 weeks, the embryo develops into a veliger larva with a developed foot (Jones, 1993). The larvae produce byssal threads, which help the juvenile clam attach to the seafloor once a suitable substrate is found. Burrowing into the sediment allows the clam to access food and gain protection from predators. Once settled, it remains in the substrate and continues to grow into a mature clam.

1.5.3 Biomarkers in marine invertebrates

All marine organisms are potentially exposed to various stressors, both abiotic and biotic. Abiotic stressors include temperature fluctuations, oxygen levels, pH, salinity, UV radiation, and chemical contaminants. Biotic stressors encompass the presence of parasites or predators, reproduction demands, and reduced food availability. The ability of an organism to respond to these stimuli depends on its adaptive capacity, often requiring adjustments to its optimal physiological state. When an organism is under stress, changes in biological responses, from the cellular to the organism level, can be measured and used as stress indices (biomarkers).

Biomarkers have been utilized in ecotoxicology for over three decades and are defined as 'a biological response to a chemical or chemicals that gives a measure of exposure, and sometimes, also of toxic effect' (Peakall & Walker, 1994). By examining molecular, biochemical, physiological, histo-cytopathological, organismal, population, or community responses, researchers can identify exposure to specific chemicals, track spatial and temporal changes in contaminant concentrations, and assess environmental quality or the occurrence of adverse effects (Au, 2004).

Biomarkers are typically categorized into biomarkers of exposure and biomarkers of effects. Biomarkers of exposure are biological responses related to the induction or inhibition of specific enzymes involved in pollutant biotransformation and detoxification. These early signals at the molecular or cellular level indicate exposure to contaminants but do not provide information on the actual toxicological effects on the organism. They are used to monitor pollutant sources and characterize sites with unknown contamination. Biomarkers of effects, on the other hand, are associated with pathological endpoints at various levels of biological organization, from molecular to population levels (Schlenk, 1999; Lam & Gray, 2003; Broeg et al., 2005).

Some biomarkers are specific, indicating responses to a particular contaminant or class of contaminants, while others are general, reflecting overall stress conditions. Immunomarkers are general biomarkers that are highly relevant in indicating an organism's health status. They include changes in the morphological and functional characteristics of immune cells, such as blood cells or their equivalents. In bivalves for instance, haemocytes play a crucial role in various physiological processes, including immune response, nutrient transport and digestion, and shell deposition (Bigas et al., 2006; Canesi et al., 2006; Desjardins et al., 2005; Bibby et al., 2008). The immune system of invertebrates is less complex than that of vertebrates and lacks adaptive antibody-based responses and lymphoid organs (Galloway & Handy, 2003). Invertebrate immune responses include both cellular and humoral components. Cellular responses are mediated by hemocytes, which can phagocytose and/or encapsulate external pathogens. Humoral responses involve substances with bactericidal properties (Carballal et al., 1997; Lopez et al., 1997; Galloway & Handy, 2003).

Key immunomarkers in marine invertebrates, such as bivalves and sea urchins, include the total number of haemocytes/coelomocytes in the haemolymph (THC/TCC, total haemocyte/coelomocytes count), their phagocytic activity, haemocyte/coelomocyte diameter and volume, and haemocyte/coelomocyte proliferation. These immunomarkers can be influenced by changing environmental parameters and contaminants (Matozzo et al., 2003; Oweson & Hernroth, 2009; Matozzo & Marin, 2010; Matozzo et al., 2012).

In addition to immunomarkers, behavioral traits and physiological rates in marine invertebrates serve as valuable biomarkers of stress (Munari et al., 2020; Asnicar et al., 2021). Changes in behavior, such as altered feeding, burrowing, or locomotion patterns, can indicate exposure to stressors. Physiological rates, including respiration, excretion, and growth rates, provide insights into the overall health and stress levels of an organism. Monitoring these traits and rates allows researchers to assess the impact of environmental stressors on marine invertebrates and can help in identifying the presence and severity of pollution and other detrimental factors in marine ecosystems.

1.6 Aim of the work

The wellbeing of the marine environment is rapidly declining due to anthropogenic stressors. To mitigate the effects of these stressors is paramount, and the buffer effect primary producers seem to provide offers a step in the right direction. Current knowledge suggests that primary producers might play a compensatory role in mitigating the effects of climate-related stressors, contributing to ecosystem stability (Sarà et al., 2021). There is evidence indicating that the increase in oxygen levels due to primary production acts as a protective buffer, particularly during events such as heat waves. However, the available data on this topic is still limited. It is hypothesized that the accelerated metabolism of associated fauna, driven by higher oxygen concentrations, could lead to a more rapid accumulation of contaminants in their tissues (De Jonge et al., 2012; Chiarore et al., 2020). Further research and additional data collection are necessary to validate these observations and fully understand the implications.

As there is, at present, scarce data about the potential buffer effect of primary production against contaminants, my work aims to test if oxygen supersaturation could influence the consequences of mercury in two marine invertebrates species with both ecological and economical relevance, *Paracentrotus lividus* and *Ruditapes philippinarum* (Fig.6), investigating the effects at physiological and behavioral level.

The choice of these species was deliberate: *Paracentrotus lividus* is a benthic species, while *Ruditapes philippinarum* is a burrowing one. This selection allows for a comprehensive comparison between species with different ecological niches and modes of life and feeding. Additionally, both species are of significant commercial importance, making them relevant subjects for studying the impacts of

environmental stressors on economically valuable marine resources. By examining both a burrowing and a benthic species, the study aims also to preliminarily discern how different living habits may affect the organisms' responses to mercury exposure under conditions of oxygen supersaturation. This approach will help in understanding the broader implications for marine biodiversity, ecosystem health, and commercial fisheries.

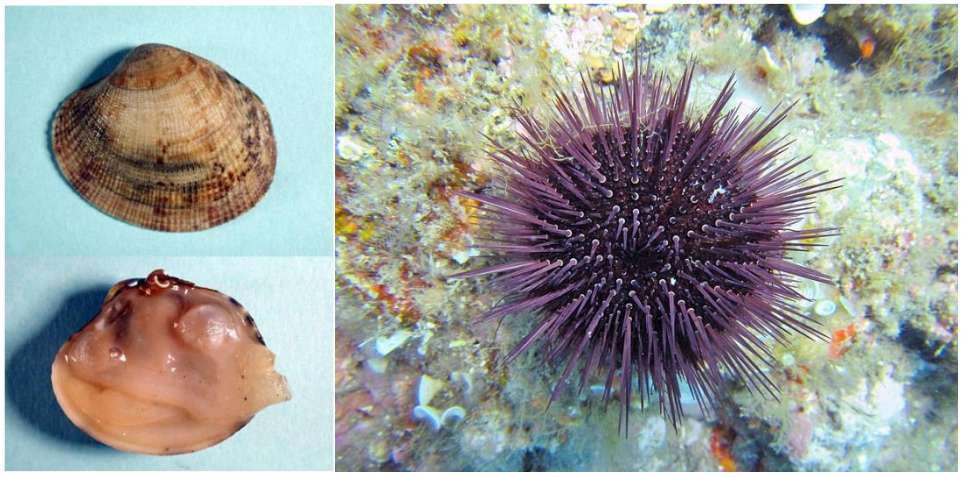

Figure 7. On the left an image of Paracentrotus lividus, by Frédéric Ducarme, 28th of July 2016. On the right, an image of Ruditapes philippinarum (https://msn.visitmuve.it/it/ricerca/schede-tematiche/specie/ruditapes-philippinarum).

2. Materials and Methods

2.1 Animal collection and feeding

Specimens of sea urchin, *P. lividus*, were collected by a scientific scuba diver in the southern part of the Venice Lagoon (45° 13’ 41” N, 12° 16' 13” E) and kept in sea water in a flowthrough tank system, being fed *Ulva sp.* every two days. Specimens of *R. philippinarum* were collected from a reference site located within a licensed clam culture area in the southern part of the Lagoon of Venice (Chioggia, Italy) and kept in sea water in a flow-through tank system. As clams are filter feeding animals, they were not fed, but survived on the organic matter present in the unfiltered seawater.

Animals of both species were maintained in these conditions for a week before starting the experiment.

2.2 Sea Urchins sex determination

Prior to the sea urchins’ trial, the sex of the animals was determined. Traditionally, the sex of sea urchin has been determined through gamete analysis. This not only is stressful for the animal, but it is often lethal. Brundu et al. (2023)

provides a non-invasive method to determine sea urchins' sex, which was implemented in this trial. Individuals were placed in holding tanks and submerged in sea water, to ensure the maximum swelling of the papillae. The five gonopores around the anus were identified, each one presenting a papillae with distinctive traits from male to female. Males have a raised protuberance, while females have a flat or sunk papillae (Fig.8).
This method has a 10% rate of failure, which was taken into account in the data analysis.

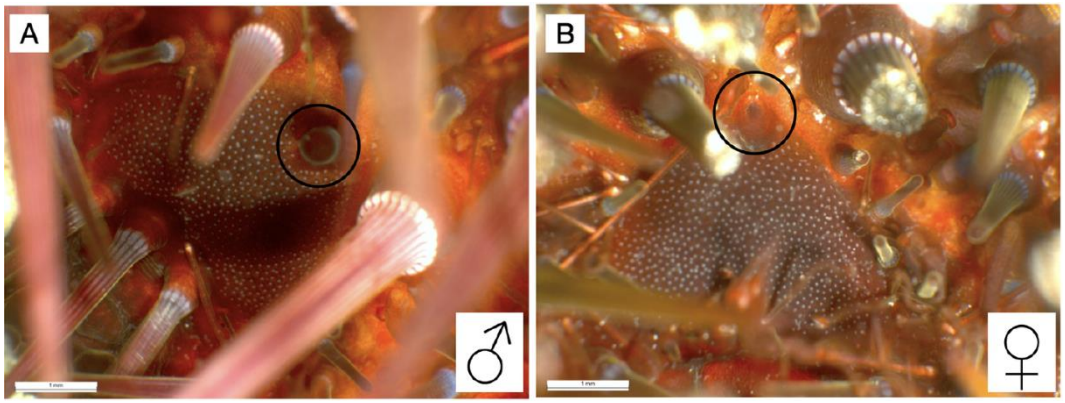

Figure 8. Image of genital papillae taken from (Brundu et al., 2023). (A) circled in black is a male papilla, recognizable because it is raised from the plaque. (B) circled in black a female papilla is shown, recognizable by the fact that it is flat to the surface of the plaque. In some cases, the female papillae is concaved inwards

After sex was determined, height and diameter of each individual was measured and 10 individuals (5 males and 5 females) were placed into each tank, for a total of 120 animals.
Since for clams we don't have a non-invasive method for sex determination, it was not taken into account in this trial. 17 individuals were randomly placed in each of the tanks, having 204 animals in total.

2.3 Experimental design and setup

The two separate trials were performed back-to-back. The sea urchin trial began Nov-13th, ending Nov-20th, and the clams trial began Nov-21st , ending Nov-27th. Twelve 20L glass tanks were set up, filled with filtered sea water (FSW) and equipped with a water pump and an aerator. A second set of aerators was connected to the oxygen line, and opened when needed to pump pure oxygen into the tanks.
The temperature was kept at 16°C using the air conditioning system in the room. The temperature was kept low to mimic the winter environmental temperature and to avoid thermal shock. The animals were exposed for a week to different experimental treatments coming out from an orthogonal experimental design with two fixed factors: mercury and oxygen level. Each combination was replicated three

times. Since we tested two levels for each factor, we finally had four treatments:

- Absence of mercury (0 µg/L) + 90% O_2 saturation (24h/day)
- Presence of mercury (1 µg/L) + 90% O_2 saturation (24h/day)
- Absence of mercury (0 µg/L) + >150% O_2 saturation (5h/day)
- Presence of mercury (1 µg/L) + >150% O_2 saturation (5h/day)

Six of the tanks were hyper-oxygenated for five hours every day, keeping the %DOP above 150. By doing this, we mimic the daily oxygen fluctuations in presence of primary producers that need sunlight to perform the photosynthesis.
In six tanks, for each liter of FSW in the tank, 1µg of mercury chloride was added after the animals were placed (20 µg per tank) (Fig.9).

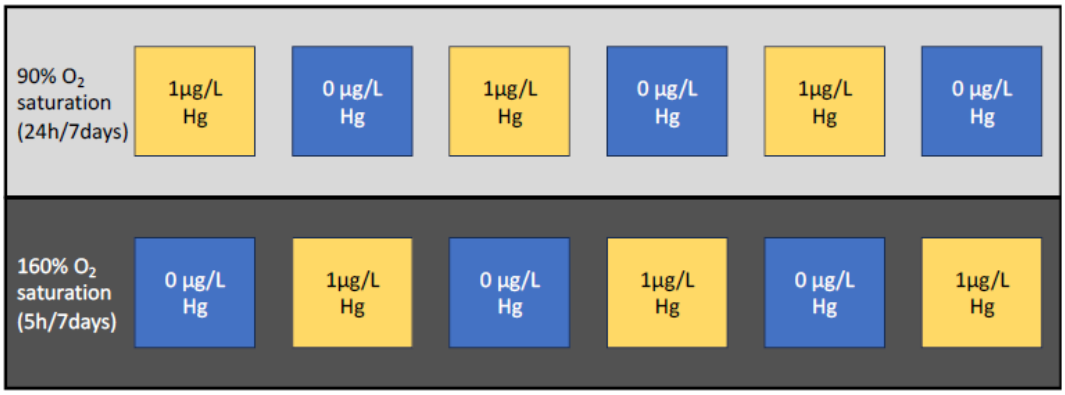

Figure 9. Schematic representation of the experimental design: the yellow squares represent the tanks where mercury was added and its concentration (1µg/L); the blue squares were the tanks where mercury was not added (0µg/L). The light gray background represents the six tanks that were kept at a normal %DOP for the entirety of the experiment; the dark gray background represents the six tanks that were hyper-oxygenated for 5 hrs each one of the 7 days.

The animals were exposed for a week to the different conditions. A multiparametric probe was used to take the measurements of %DOP, temperature and salinity. Because the tank system was not flowthrough, every two days the water in the tanks was changed: leaving the animals in the tanks, they were completely emptied and refilled with 20L of FSW. Once the tanks were filled, the mercury chloride was added. The sea urchins were fed two spoonfuls of corn and lettuce per tank every day. The clams were fed 600µL of microalgae per tank every day.

2.4 Sea urchin trial

2.4.1 Sample collection

After a week of exposure, animals were removed from the tanks and were divided into two groups:

1. individuals that would undergo the respiration, righting and sheltering trials;

2. individuals from which gonads would be removed and collected to measure bioaccumulation of the mercury chloride. From these individuals, hemolymph was also collected to analyze the number, diameter and volume of coelomocytes: the needle of a 1ml syringe was inserted on either side of the mouth of the animal, and as much hemolymph as possible was collected and analyzed with coulter counter.

2.4.2 Sea urchin respiration, righting and sheltering

a. Righting Time (RT) and Respiration Rate (RR)

Two males and two females were collected from each tank and were put into the respirometric chambers in FSW upside down. As soon as the animal was placed into the chamber, a timer was set to count how long it took to right itself and this measure was referred to as "Righting Time". Respirometric chambers were closed hermetically, avoiding the presence of air inside. The instantaneous oxygen concentration was checked through the Fiber-Optic Oxygen Meter Piccolo2 (Pyro Science GmbH, Aachen, Germany) (Fig. 10). Darkness conditions were ensured during the test to limit photosynthetic activity. At every sampling time, before the measurements, the software was linearly calibrated reading blank chambers (according to Lyndby et al., 2020).

Every twenty minutes, μmol L^{-1} of oxygen in each chamber was measured for an hour of trial. Hence, the amount of oxygen consumed in an hour was used to compute the so-called "Respiration Rate" (RR).

The respiration rate (RR, μmol O_2/hour) was calculated with the formula by Widdows and Johnson, 1988:

$$RR = [60\ (C0 - C1)\ (V - \mathrm{Va})]/(t0 - t1)$$

where "C0" and "C1" are the oxygen concentrations at the beginning and end of the analysis (in μmol O_2/L), "V" and "Va" are respectively the volume of the respirometric chamber and the volume of the animal inside the chamber (in L) and "t0" and "t1" are the start and finish times of the assay (in minutes). All data were corrected by the respective blank. Sea urchins' oxygen consumption was calculated considering time spanning from 0 to 60 minutes. Results were expressed as μmol O_2/hour.

Figure 10. Image of an individual of P. lividus inside the respirometric chamber. The chambers were hermetically closed through the use of an o-ring. In front of the chambers, the Fiber-Optic Oxygen Meter Piccolo2.

b. Sheltering Time (ST)

After the respiration, the same individuals underwent the sheltering trial: this trial measured how quickly an individual would move away from direct light to hide in the shadow. The experimental procedure took inspiration from Asnicar et al., 2021. Both righting and sheltering abilities were used as proxies of animal performance, in terms of motility.

In a tank with sea water, the sea urchins were put in the center of a tank and light was shed directly on top of them with a torch. A plank of plywood with a hole in it was placed on top of the tank, to block some of the light from the torch and create a shadowy place for the urchins to move towards (Fig. 11). The timer was stopped once the whole of the body of the urchin was in the shade and the time required to the animal to shelter was defined the “Sheltering Time”

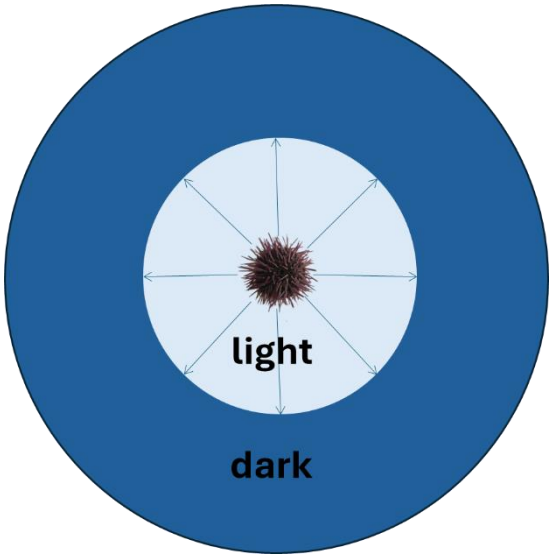

Figure 11. Schematics of the set up for the Sheltering trial: in dark blue, the part of the tank that was in shade. In light blue the part of the tank where direct artificial light was shone.

2.5 Clams trial

2.5.1 Sample collection

After a week of exposure, the 17 animals were divided into two groups, with 1 spare for each tank:

1. Twelve animals per tank, divided into three pools of four individuals each, were used for the collection of the gills and the digestive glands for the analysis of antioxidant enzymes.
   From each of the same pools, hemolymph was also collected to analyze the number, diameter and volume of hemocytes: the needle of a 1ml syringe was inserted, and as much hemolymph as possible was collected and analyzed with Coulter Counter;

2. Four animals per tank were collected whole for the analysis of the mercury chloride bioaccumulation.

2.6 Data analysis and statistics

To assess the effects of difference in mercury and oxygen on adult sea urchin and clams performances, data were analysed using a non-parametric PERmutational ANalysis Of VAriance (PERMANOVA) test applied on the Euclidean distance matrix (Anderson, 2001). When the number of unique values from permutations was too low, the Monte-Carlo procedure was used to calculate p values. Primer software (PRIMER 6.1.10 and Permanova — University of

Plymouth, UK) was used for all statistical analyses (Clarke and Gorley, 2006), while graphs were performed with Microsoft Office Excel.

3. Results

Water chemistry characteristic during the two experiments are reported in Table 1.

Table 1. Water chemistry during the experimental period: (A) Sea Urchin trial. (B) Clam trial.

A

Hg µL / O_2 %	Temperature (°C)	%DO	PSU
0µL / 90%	14.82 ± 0.28	83.30 ± 13.93	32.06 ± 0.63
0µL / 160%	15.02 ± 0.25	173.39 ± 34.54	31.94 ± 0.69
1µL / 90%	14.80 ± 0.28	85.39 ± 10.39	32.06 ± 0.65
1µL / 160%	14.88 ± 0.22	186.34 ± 46.65	31.98 ± 0.67

B

Hg µL / O_2 %	Temperature (°C)	%DO	PSU
0µL / 90%	15.11 ± 1.88	100.66 ± 1.53	32.15 ± 0.10
0µL / 160%	15.27 ± 1.67	167.22 ± 26.26	32.15 ± 0.11
1µL / 90%	15.13 ± 1.92	100.28 ± 1.39	32.17 ± 0.07
1µL / 160%	15.32 ± 1.70	168.20 ± 27.42	32.25 ± 0.15

3.1 Sea Urchin trial

3.1.1 Sea urchin Righting Time (RT) and Respiration Rates (RR)

PERMANOVA results shown that there were no differences in RR among treatments due to the experimental factors or their interaction (Fig. 12 A), while RT was significantly influenced by different oxygen levels ($p=0.021$; $F_{(1,47)}=4.959$). From Figure 12 B it possible to note that sea urchin exposed to higher level of O_2 for 5 hours per day can rotate faster than those expose to 90% of O_2 saturation for the whole experiment.

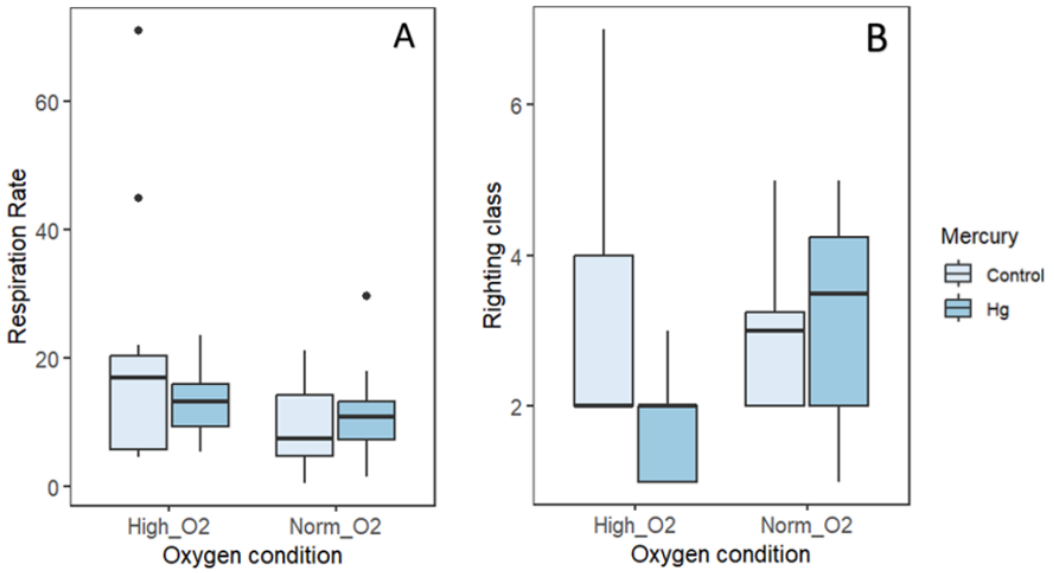

Figure 12. Respiration Rate (A) and Righting class (B) for Paracentrotus lividus exposed to the combination of two levels of O_2 saturation (90% O_2 named "Norm_O2" and 160% named "High_O") and two concentration of mercury chloride (0 µg/L named "Control" and 1 µg/L named "Hg").

3.1.2 Sea urchin Sheltering Time (ST)

The sheltering time didn't show significant differences among the four conditions. However, Figure 13 it is possible to notice a trend among the conditions, with the lowest mean values belonging to individuals exposed to mercury. Also, the hyper-oxygenated condition seemed to help animals in sheltering faster, even if there was not a significant difference with the normo-oxygenated condition.

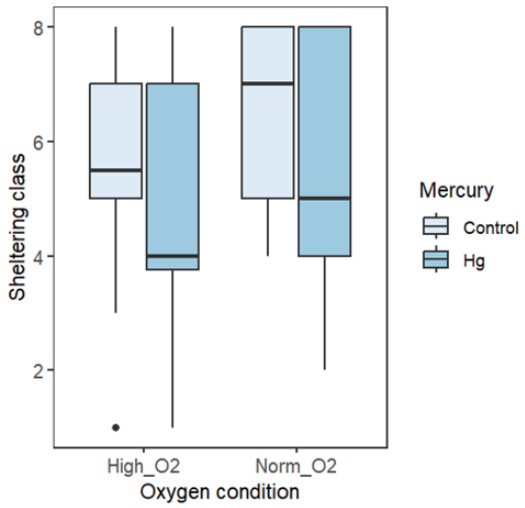

Figure 13. Sheltering Class for Paracentrotus lividus exposed to the combination of two levels of O_2 saturation (90% O_2 named "Norm_O2" and 160% named "High_O") and two concentration of mercury chloride (0 µg/L named "Control" and 1 µg/L named "Hg").

3.1.3 Sea urchin coelomocyte characteristics

PERMANOVA results didn't highlight significant differences among the coelomocytes number and characteristic, even though the mean value of the total number of coelomocytes was higher in the two conditions treated with mercury. When talking about the diameter and volume of the coelomocytes, the smallest variability showed in the normo-oxygenated individuals, whether they were treated with mercury or not. In the normo-oxygenated individuals, the mean value of the volume of the coelomocytes was very close; the largest variability came also from the volume values when looking at the hyper-oxygenated individuals treated with mercury.

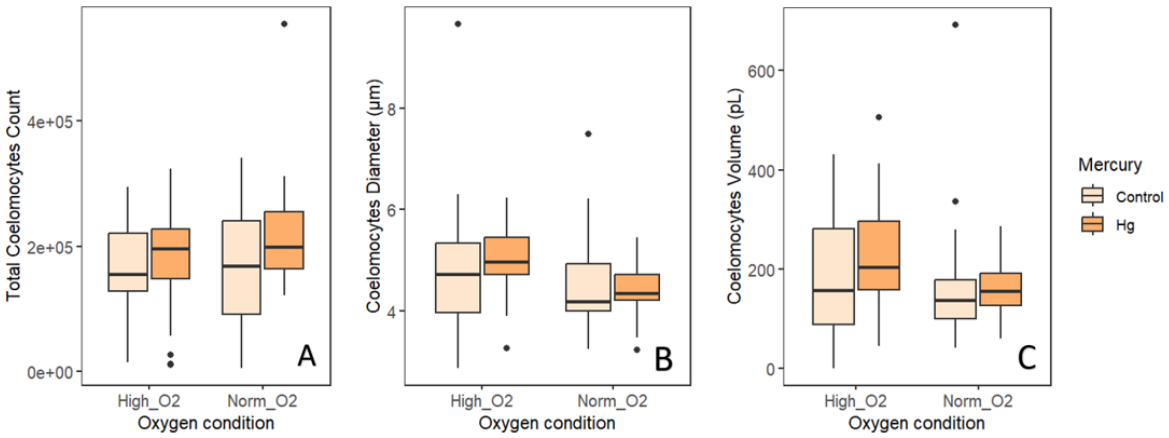

Figure 14. Total Coelomocytes Count (A), Coelomocytes Diameter (B) and Coelomocytes Volume (C) for Paracentrotus lividus exposed to the combination of two levels of O_2 saturation (90% O_2 named "Norm_O2" and 160% named "High_O") and two concentration of mercury chloride (0 µg/L named "Control" and 1 µg/L named "Hg").

3.2 Clams trial

3.2.1 Clams hemocytes characteristics

For what concern clam haemocytes, PERMANOVA didn't highlight any significant alterations in their number due to the experimental condition (Fig. 15 A). On the contrary both their diameter (Fig. 15 B) and volume (Fig. 15 C) were significantly influenced (respectively: p= 0.0248, $F_{(1,35)}$= 5.372; p=0.006, $F_{(1,35)}$=8.761) by the 7 days exposure to mercury independently by the level of oxygen clams experienced during the experiments with haemocytes then those from clam not exposed to $HgCl_2$.

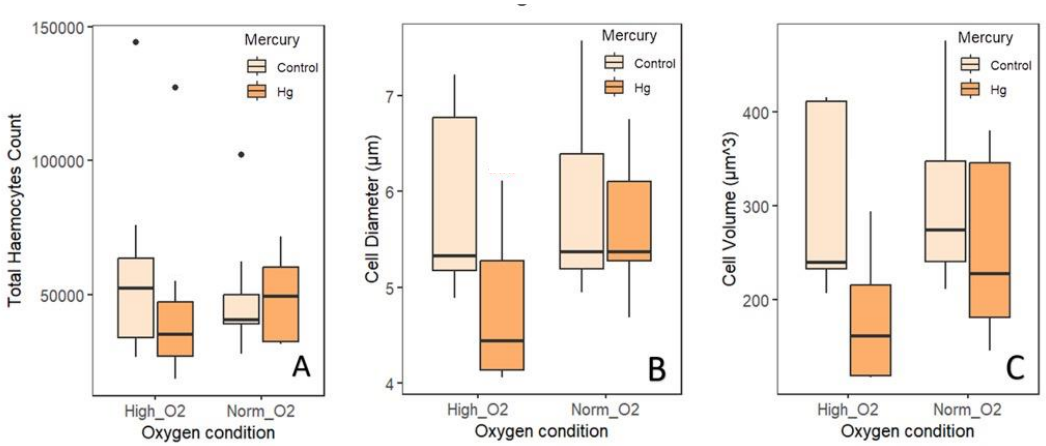

Figure 15. Total Haemocytes Count (A), Haemocytes Diameter (B) and Haemocytes Volume (C) for Paracentrotus lividus exposed to the combination of two levels of O_2 saturation (90% O_2 named "Norm_O2" and 160% named "High_O") and two concentration of mercury chloride (0 µg/L named "Control" and 1 µg/L named "Hg").

4. Discussion

Righting and sheltering were investigated because they are behaviors that insure the survival of *P. lividus* in their natural environment.

P. lividus survives by hiding. The presence of light makes it easier for predators to spot and attack it, which is why *P. lividus* prefers the shadows (Boudouresque e Verlaque, 2020). The speed with which this species can flee direct sources of light is linked to the animal's survival in the wild, and the slower the animal moves, the more vulnerable it is to predators. There are few things that can eat a sea urchin when it is anchored to the substrate, as the only soft bit of flesh is the one around the mouth. Because *P. lividus* feeds off the algae on the substrate, the mouth and the soft tissue are at all times protected, unless the sea urchin finds itself wrong-side up. The amount of time it then takes to right itself can make the difference between survival and becoming someone's dinner.

From the obtained results, we can deduce that in hyper-oxygenated conditions there is a faster response to environmental stressors such as direct light and flipping over. Even though there is no statistical difference, the mean values of the time it took the individuals that were exposed to a higher %DOP to both right themselves and find shelter were lower than the normo-oxygenated individuals. This suggests that higher concentrations of oxygen might help boost the behavioral responses versus lower concentrations, highlighting the importance of the presence of primary producers as oxygen releasers.

Coelomocytes are part of the sea urchin immune response. Their cellular origin is not clear, but they have been used as stress indicators when testing different stress markers (Matranga et al., 2000). Considering the analysis of the coelomocytes,

there was no detectable difference among the conditions. This suggests no measurable effect on these cells due to higher %DOP or mercury contamination. This is also shown in Matranga et al. (2000), where they compared coelomocytes of *P. lividus* from polluted and unpolluted areas.
It is important to consider that the lack of observable effects of mercury in our results may be attributable to the concentration of mercury used in this study (1μg/L). To gain a more comprehensive understanding of mercury's potential impact, future research should explore the effects of higher concentrations as well. Investigating a broader range of mercury concentrations could provide valuable insights into its biological and environmental effects and help establish safety and toxicity thresholds, particularly in the context of changing environmental variables predicted by climate change scenarios. Additionally, understanding the role of primary producers in mitigating mercury's harmful effects, beyond their oxygen production, is crucial. Long-term studies are also needed to assess the impact of chronic mercury exposure.
The hemolymph analysis on the *R. philippinarum* pools shows a significant effect caused by the interaction between mercury and oxygen. It is known that a higher %DOP has a boosting effect on animals' metabolisms, which can be helpful when it comes to adapting to climate change. This, however, could not be beneficial when in the presence of contaminants. In our case, in fact, mercury exposed, hyper-oxygenated pools show a significant drop in the volume, diameter and cell count of the hemocytes. Interestingly, mercury has no effect in the condition of normal oxygen saturation. This indicates that low concentration of mercury (as the one that we chose for the experiment) becomes detrimental under oxygen supersaturation in water, probably because of the increased metabolism of animals which in turn enhances mercury uptake, as guessed in previous studies (Chiarore et al, 2020).
However, the differences in responses between the two species underscore that the effects of stressors and their interactions with other environmental factors are species-specific. This emphasizes the diversification of organisms' adaptations, complicating the prediction of large-scale effects of environmental changes. The species-specific nature of these responses highlights the need for tailored conservation strategies and suggests that a one-size-fits-all approach may not be effective in managing the impacts of environmental stressors on biodiversity.

5. Conclusion

This study demonstrates that higher percentages of dissolved oxygen (DOP) enhance the motility of *P. lividus* individuals when exposed to such conditions. Previous research, such as Giomi et al. (2019), has highlighted the benefits of hyper-oxygenation in mitigating thermal stress on a physiological level, emphasizing its significance in the context of climate change.

Additionally, this study reveals a previously underexplored dichotomy: while more oxygen can be beneficial, it can also be harmful under certain conditions. Increased

oxygen availability can boost metabolism, leading to faster uptake of pollutants (Chiarore et al., 2020). Our findings show that hyper-oxygenated *R. philippinarum* individuals exhibit significant differences in hemocyte levels between those exposed to mercuric chloride and those not exposed, with the most severe effects occurring under oxygen supersaturation conditions.

The findings of this study lead us to conclude that:

1) Primary producers alone might not be able to counteract the negative effects of pollution. It is crucial not only to protect habitat-forming primary producers, which offer various ecosystem services, but also to reduce the levels of pollutants in the environment.
2) Preventing the introduction of pollutants by banning certain harmful molecules and improving wastewater treatment plans is essential for mitigating pollution and preserving marine biodiversity.

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