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TESI DI LAUREA

How an acidified habitat influences the physiological and behavioural changes in a sea urchin species: *Arbacia lixula*

Relatore: Prof. Marco Munari
Dipartimento di Biologia

Laureando: Riccardo Bernardi Malagugini

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Abstract

Anthropic effects of industrialization and greenhouse gas emissions affect ocean ecosystems by gradually decreasing seawater pH in a phenomenon called Ocean Acidification (OA). This effect has a negative impact on most marine animals, especially calcifying organisms. Sea urchins can provide valuable insights on the impact of OA on an ecosystemic level, due to their importance as benthic grazers and the lack of many predatory species. Most of the experiments on AO have been conducted on indoor aquaria, but natural volcanic oases can simulate near-future acidified conditions. In this experiment, behavioural and physiological responses of the sea urchin *Arbacia lixula* were examined alongside natural volcanic vents in Ischia, Italy. Adult sea urchins were collected from three sites, two of which were located in Castello Aragonese (S1, ~pH 8.1 and S2, ~pH 7.7) and the third one (SP, ~pH 8.1) was alongside San Pietro Bay. Experimental design consists in exposing to two to different pH conditions (pH 8.1, pH 7.7) the individual collected from each site. Experiments were replicated at 5 selected times (October 2023, January, April, June and August 2024) over the course of two years (October 2022, August 2024). Two physiological (Excretion rate, Respiration rate) and two behavioural (Righting Time, Sheltering Time) response variables were selected; furthermore, supplementary data of benthic cover was provided to possibly link any variations to the surrounding environment or vice-versa. Hypothesis consists in different responses of urchins collected from experimental sites, possibly demonstrating physiological or behavioural adaptations to chronic exposure to reduced pH. Our results provide significant effects of pH, sites and/or their interaction for at least some of the selected times across all the response variables. These results indicate that effects of pH inevitably have effect on adults of *A. lixula*, but also that environmental complexity and the interactions with other factors could mitigate, enhance or contrast the direct effects of naturally induced acidification.

1. INTRODUCTION

1.1 The human effect

Human activities have clear, undeniable impacts on the stability of natural systems: the production of goods irrevocably relies on depletion of resources and creates pollution in the process of both manufacturing and dismantling (Panagiotopoulou et al., 2021; Yang et al., 2017). Within the last centuries, the effect of the Industrial Revolution combined with an exponential increase of the population have become more evident (Jonsson F. A., 2012). To these days, emissions have changed so drastically that on the stratigraphic composition of the ground, the human impacts are distinguishable and divided from pre-industrial era (Waters et al., 2016). To separate this chronological division, a new term has been introduced in 2000: the Anthropocene (Monastersky, 2015). Anthropic effects on global climate are measurable and recorded through international institutions like NOAA and GISS. The first systematic measurements of temperature started in the late 19th century (*Climate Monitoring*, 2024), whereas atmospheric CO_2 was first assessed in March 1958 by the Scripps institution of Oceanography, at the Mauna Loa Observatory in Hawaii led by Charles David Keeling (Mims, 2012).

Earth's Climatic history has shown that similar values of CO_2 and temperature have already been reached, like in the Pliocene epoch, over 2.6 million years ago (Tierney et al., 2020). However, the difference between naturally induced environmental change and human induced climate change is the pace at which parameters are rising, whereas one occurs over a span of a few centuries, and the other within a time frame of millions of years, leaving enough time for evolutionary adaptation (Fletcher et al., 2024; Anderson et al., 2012).

Prior to human measurements, past climatic indicators are assessed through isotopic analyses on planktonic fossil records and ice core records, which on whose estimate climatic models can predict not only previous climatic conditions, but also the possible future scenarios of anthropic activities (Kucera, 2007; Stute et al., 2001; Meure et al., 2006).

Nowadays, CO_2 levels have already skyrocketed over 420 ppm, from a pre-industrial level of 280 ppm, which have been stable for more than 6000 years prior to the Industrial Revolution (*Carbon Dioxide Now More Than 50% Higher Than Pre-Industrial Levels*, 2022). Based on the current trends, the IPCC made four possible socioeconomic pathways that predict our planet's future. These Shared Socioeconomic Pathways (SSP) are a set of scenarios used by the IPCC to explore how different socio-economic choices might influence the future global climate, ranging from sustainable development (SSP1) to continued fossil-fuel reliance (SSP5), with varying impacts on socioeconomic and environmental factors. Depicting the worst case, a possible atmospheric CO_2 would cross 700 ppm by the end of the century (*IPCC, 2021*). Provided these assets of evidence, it is pivotal to evaluate the anthropogenic impact of industrialization and greenhouse effect (Kweku et al., 2018).

1.2 Climate change

As we transition from understanding the Anthropocene, it is essential to address a significant repercussion: climate change. The greenhouse effect, intensified by human activities, results in the retention of heat within the Earth's atmosphere and drives global warming (Kweku et al., 2018). This underscores the profound impact of anthropogenic emissions of carbon dioxide. Climate change is defined by the IPCC as deviation of meteorological variables' mean and variance over a span of months or thousands of years. This definition includes rising annual temperatures and extreme weather events, but also other aspects of Earth's climate, such as humidity, Ocean Acidification (OA), atmospheric circulation, global currents, and ice cover.

Climate change is primarily driven by the greenhouse effect, which is the process that limits our planet's reflection capability of solar infrared radiation. This process effectively traps an excess of energy within the atmosphere, most of which increases the planet's temperature and is absorbed by bodies of water like the ocean, that absorbs more than 90% of this energy (Venegas et al., 2023).

Within the most important climatic effect that anthropic activities and greenhouse are altering, a critical attention have been placed on these parameters from the Shared Socioeconomic Pathways during the IPCC's Sixth assessment Report (2021):

- Global temperature is already more than one degree Celsius higher than pre-industrial levels. IPCC future scenarios are based on different levels of global temperature rise, ranging between 1.5°C (SSP 1-1.9) and 4.4°C (SSP 5-8.5)
- Ocean surface temperature is heterogeneous alongside the ocean, rising over the next century with anomalies that rise over 3 °C Celsius. Equatorial waters being more stratified and global current disrupted followed by global climate phenomenon like El Niño southern oscillation.
- Precipitations will be more frequent around 1.3 times more than today, and in the worst scenario (SSP 5-8.5), around 2.7 times more than today on average. Humidity and precipitation events will increase differently biased on geological area, with central Africa and equatorial pacific being the most likely.
- Ocean pH is predicted to decrease below 8.0 (today is around 8.05 on average) and in the SSP 5-8.5 will reach a value of 7.7.
- Ice cover is predicted to drop by 7 million square kilometers just in the arctic by the end of the century, by SSP 5-8.5 and around 2 million square kilometers in SSP 1-1.9

The interaction between atmosphere and biosphere is a fragile equilibrium that such rapid changes are already dismantling. Local extinctions, enhanced competition and arrival of alien species are ultimately leading to irreversible ecosystem shifts and loss of biodiversity (Román-Palacios & Wiens, 2020; Harley et al., 2006). This does not include just terrestrial environments, but considers coastal and oceanic ecosystem fragility, with coral reefs being the most threatened. Direct consequence of global warming is tropicalization of temperate marine areas further away from the equator, forcing migration of most heat-sensible species (Hsiang & Sobel, 2016). Coral bleaching, marine heatwaves, global currents changes and coastal erosion are already having an impact on the economy (Oliver et al., 2018; Allison et al., 2009). As an example, Hinkel et al. (2013) have estimated that migration due to coastal erosion over the 21st century could reach over one trillion US $. It has been demonstrated that environmental stressors affected by climate change have a combined effect on organisms that can be exponential, meaning that depending on individual species, ecosystem adjustments and reactions will be unpredictable in the next decades (Collins et al., 2021). It's vital to monitor, at best of our capability, environmental effects on both terrestrial and marine ecosystems, in order to determine how much climate change is affecting them.

1.3 Ocean Acidification

Between the current global threats to biodiversity, OA is one of the most concerning. The combination of more acidic water and an increased average temperature of global seawaters is linked to the constant emission of greenhouse gasses by anthropogenic activities (Reid et al., 2009). Despite the information shared by the scientific community and the already existing datasets of previous decades, the topic of global warming and OA has been taken into consideration by the public opinion only more recently (Cooney, 2010; Doney et al., 2008).

The effects of greenhouse gasses are both linked to temperature of surface waters and acidification; in particular, the emission of carbon dioxide unbalances the carbonate system: a series of chemical reactions that keeps the seawater pH globally around 8.1. The main reactions of this system are Shown in Figure 1:

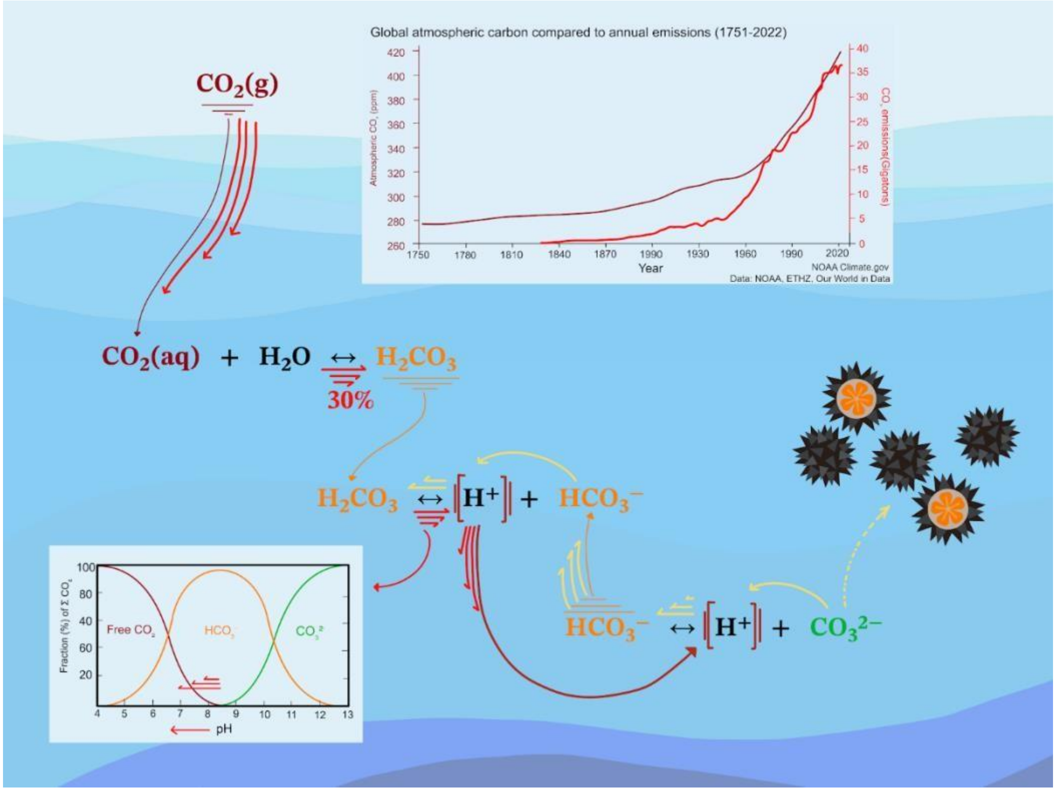


Figure 1- Visual description of the carbonate system of the ocean. The red arrows indicate the effect of anthropic CO¢ emissions and the trend in marine absorption. The yellow arrows indicate the ion counter-balancing mechanisms due to the buffer effect. pH is maintained constant thanks to the carbonate ions stock, which are used from calcifying organisms for calcium carbonate deposition. On the bottom left, there are the concentrations of carbonate system ions relative to pH acidity (Bjerrum plot). On the top right, the constant increase of atmospheric CO¢ due to anthropic industrialization (modified from Feely et al., 2004).

The presence of CO£²{ and HCO£{ ions allow the ocean to neutralize excess Hz ions, thereby stabilizing pH levels. However, as CO¢ levels increase and more Hz ions are produced, the buffering capacity of the carbonate system is weakened (Langdon C., 2016). One factor that further complicates the acidity levels of our oceans is that the carbonate system follows a logarithmic scale: this means that the capability of our ocean to absorb atmospheric CO¢ will decrease as rapidly as the efficiency of the buffer system (Orr et al., 2005).

It has been demonstrated that such unprecedented variation of pH can have a negative impact on pelagic and benthic ecosystems (Fabry et al., 2008). Specifically, it can alter the structure of populations, enhance habitat perturbation and composition, decrease environmental complexity and mitigate both resilience and resistance of marine communities (Harvey et al., 2016; Cornwall et al., 2024). The overall effect of ocean acidification results from the action of this phenomenon at different ecological levels:

- Ecosystem level: competition, nutrient availability, balance of the trophic web and ecosystem services. (Portner H., 2008)
- Community level: connectivity, migration, abundance and short term genetic drifts (Hale R et al., 2011; Harvey, B. P. et al., 2016)
- Individual level: Survival, growth rate, reproductive effort, (Ben P.Harvey et al., 2016)
- Cellular level: respiration, calcification rate, gene expression, reactive oxygen compound (ROS) metabolism (Hoffmann G et al., 2008).

Since some organisms are more resilient than others, it is likely that anthropogenic OA will bring some species extinct (Román-Palacios & Wiens, 2020). Current studies are trying to model current anthropogenic emission trends to predict and quantify the severity of future acidification (Denman et al., 2011; Terhaar et al., 2022). Despite the high quantity of evidence, it is still important to provide new data, especially on natural experiments that rely on environmental-induced acidification. Most of the bibliography about this topic relies on experimental designs that experimentally induce acidic conditions in artificial tanks, excluding the variability and possible resilience of natural ecosystems.

1.4 Sea urchins as a model organism

Sea urchins (genus *Echinoidea*) are considered as a valuable model organism and have been largely used to assess ecological impact of environmental stressors, for predicting habitat stability, grazing effort and other ecologically relevant aspects of marine habitats (Bögner, 2016; Dupont et al., 2010). It is demonstrated that sea urchins have a crucial activity in the trophic chain and can affect distribution and ecology of benthic communities through selective grazing and predation (Bulleri et al., 1999; Wangensteen et al., 2011). Their habitat ranges on most intertidal and subtidal environments, while abundance is usually large in number of individuals. Regardless of specimens, ecological traits like population size, distribution, ecological niche and complex life cycle of a class that is mostly sessile, have placed sea urchins in an optimal position to be considered bioindicators (Parra-Luna et al., 2020). The diversity of diet between the adult and the larval stage on both regular and irregular sea urchins increases their sensibility to environmental factors, especially during the metamorphosis and settlement, impacting their distribution across benthic environments (Brundu et al., 2016).

Moreover, sea urchins are subjected to the high variability of the surrounding environment and some species have been studied for their high tolerance to stress like heat waves and pollution (Kapsenberg & Hofmann, 2014; Stabili & Pagliara, 2015).

1.5 Calcification on sea urchins

Although calcification studies primarily utilize sea urchin pluteus larva as a model subject, the calcification process and metabolic markers are important in post metamorphic stages as well. Sea stars primarily focus skeletal structure accretion and deposition in terminal aboral centers of calcification at the ventral tip of their arms. Likewise, regular echinoids like *Arbacia lixula* and *Paracentrotus lividus* adopt modular skeletal growth (Thompson et al., 2021).

The sea urchin endoskeleton is made of porous interlocking skeletal plates that consists of an organized mesh of calcium carbonate: this pentaradial, globe shaped, hollow structure makes up the test. The arrangement of the adult plates begins during the metamorphosis of the individual, after the disassemblement of the post-oral arms and formation of the primary ambulacraria. The axial plates are organized around the most aboral extraxial elements of the tests like the periproct and genital plates (Figure 2). They divide into peri-ambulacral plates and ambulacral plates, surrounding the test. Ambulacral plates reconnect to the axial elements through ocular plates, placed between connection points of genital plates (Perricone et al., 2020). Skeletal early growth in regular sea urchin is modular, meaning that between the first two weeks after settlement, somatic growth and plate deposition is enhanced in respect to other calcified parts of the individual, which are developed more during the third and fourth week of growth instead. During somatic growth, the accretion is concentrated on the aboral side of the individual, in calcification centers right beneath the ocular plates that acts as a primer for the addition of new elements of the tests. Furthermore, skeletal deposition is likely to be associated with cell proliferation, which is mainly allocated to tissue differentiation and growth. Controversially, cell division is concentrated in the oral side of the individual, in concomitance with the formation of secondary ambulacraria and digestive system, but cell proliferation is also found beneath the accretion centers around the ocular plates (Thompson et al., 2021).

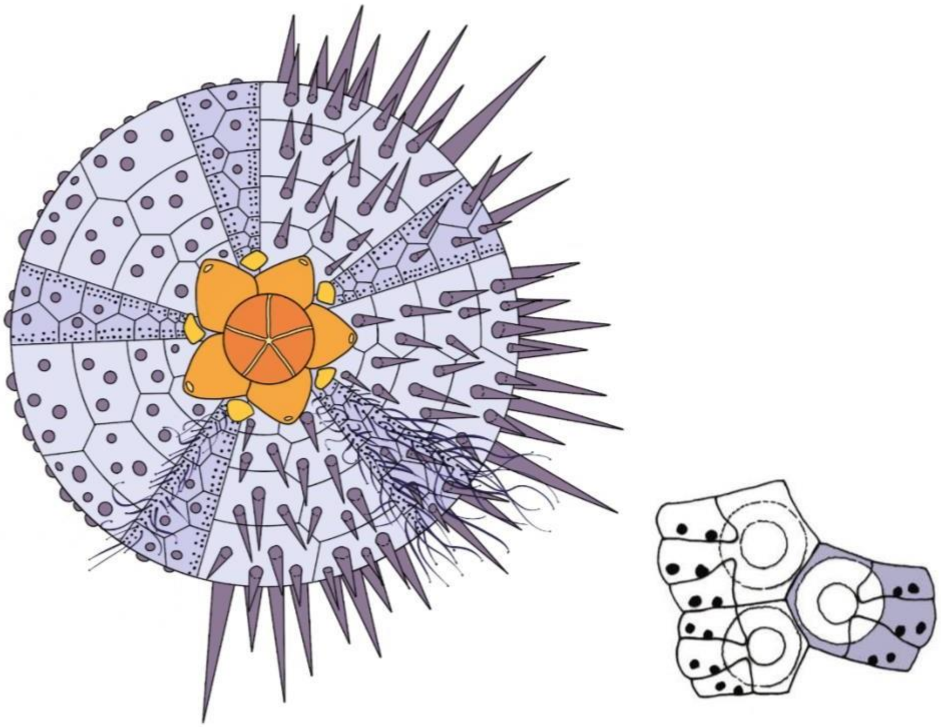


Figure 2- Skeletal plate deposition of Arbacia lixula. Ambulacral plates are coloured in dark sky blue, while the peri-ambulacral plates are lighter. In the center, ocular plates are highlighted in yellow, genital plates are highlighted in light orange, whereas central extraxial elements in dark orange (modified from Thompson et al., 2021).

From a molecular point of view, genetic regulation of calcification relies on growth promoters that modulate a chain reaction of a few hundred transcribed proteins. Echinoid endoskeleton is enclosed in an epithelial integument with an intracellular matrix acting directly on the process of calcification. Many molecular studies is concentrated on the genus *Camarodonta* (Killian & Wilt, 2008), although there are some molecular studies on *Arbacioida* (Kanold et al., 2014). Within the matrix proteins, short signal peptides have been found, alongside C-type lectin domain proteins, proline rich proteins and matrix metalloproteinase.

1.6 Effects of seawater acidification on sea urchins

It’s been studied that ocean acidification is an environmental stressor that brings a collection of physiological impairment to an individual, leaving it with the necessity of correcting several molecular patterns simultaneously (Devens et al., 2020). For sea urchins, pH-driven fragilities may result in alterations of both diet and behaviour, which could affect or be affected by the nutrient availability and the environment surrounding them (Asnaghi et al., 2013; Cohen-Rengifo et al., 2019). When looking at the assets of responses already observed (Stumpp et al., 2012; Asnicar et al., 2021), it’s necessary to understand how calcification and metabolism of the individuals are set into place.

Sea urchins, like other calcifying organisms, have similarly shown negative effects of acidification on survival, growth, settlement and other aspects of their lifecycle (Chan & Tong, 2020; Dupont et al., 2010 Byrne & Przeslawski, 2013). Although ocean acidification doesn’t leave evident marks on the adult organism, the anthropogenic driven anomalies in pH can increase the energetic cost of calcification, driving to a weakened skeletal structure, slower growth rate, behavioural anomalies and a decrease in reproductive potential (Mos et al., 2015; Rodríguez et al., 2018). As an example, a study by Migliaccio et al. (2019) shows increased production of glutathione-S-transferase and Catalase in adults exposed to acidic water (pH 7.8± 0.2).

Early life stages on the contrary, are severely impacted by pelagic pH: decrease in pluteus survival and settlement is paired by increased physiological stress and reactive oxygen species (ROS) metabolism.

It is evident that sea urchin responses to anthropogenic impacts are affecting the benthic composition and vice versa, given their important ecological role (Gianguzza et al., 2011). Unlike other calcifying organisms, altered sea urchin populations and behavioural patterns could lead to ecosystem shifts and radical rearrangement of the benthic trophic structure (Leinaas & Christie, 1996; Klumpp et al., 1993). Recent studies have shown a direct correlation between foraging preferences and ocean acidification (Burnham et al., 2021),

The majority of sea urchin analyses on the possible effects of ocean acidification occur in laboratory-closed environments, which hardly represent the complexity of natural environments; hence, open field experiments are crucial for identifying the ecological interactions between physiological, behavioural responses and the surrounding environment. Within the variety of calcifying organisms, Sea urchins are one among the most resilient to acidification (Espinel-Velasco et al., 2020), showing evidence of both acclimation and trans-generational adaptation potential in different species (Palombo et al., 2023).

1.7 Arbacia lixula

The black sea urchin *Arbacia lixula* (Linnaeus, 1758) is currently one of the most conspicuous macroinvertebrates in the Mediterranean shallow rocky reefs (G. Visconti, 2012). It is a regular sea urchin of uniformly black epithelial colouration, relatively flattened test, with long, hard spines (Kanold et al., 2014) (Figure 3). Belonging to the genus *Arbacia*, it has a high thermotolerance thanks to the high plasticity and distribution of the genus, which can be found in both arctic and temperate rocky ecosystems. *A. lixula* is a thermophilous species with tropical and subtropical affinity: it moved in the Mediterranean in the late Pleistocene and can be found outside the Mediterranean as well, along the Atlantic coast of Spain, Portugal, and northwest Africa (Cruz & Xavier, 2013; O. S. Wangensteen, 2013).

Spawning period is often concentrated in summer, with gonadosomatic index peaks in May-July and lowest values in October-November. Reproductive cycle may vary among populations, and reproductive season covers a wide thermal range (G. Visconti, 2012).

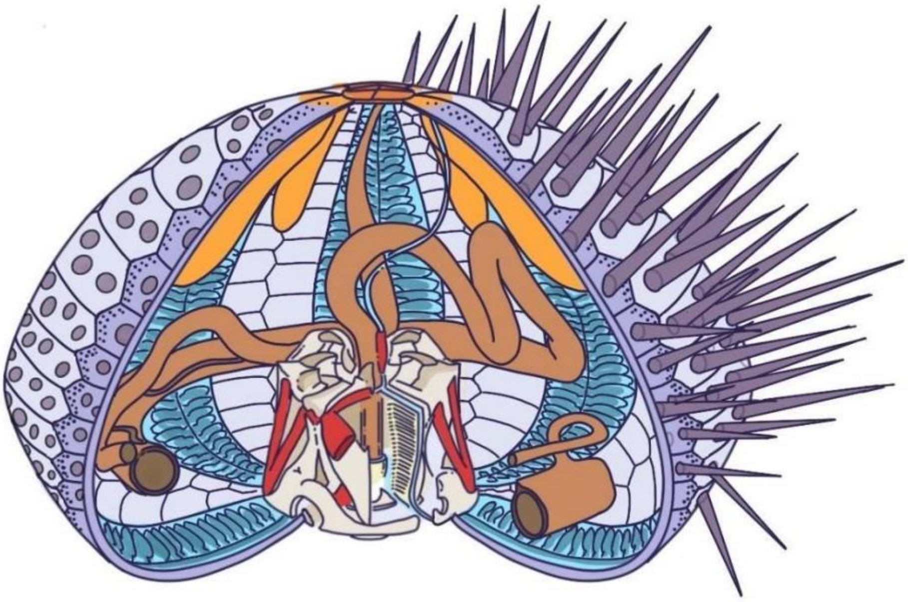


Figure 3 - Anatomy of *Arbacia lixula*: Ambulacral plates are coloured in dark sky blue, while the peri-ambulacral plates are lighter. Aristotle's lantern skeletal plates are pale white, whereas associated muscles are red. Aquiferous system and ampullae are coloured light blue. Brown indicates the digestive system while the gonads are coloured with yellow.

A. lixula doesn’t have any alimentary interest, unlike another sea urchin species found in the Mediterranean, *Paracentrotus lividus* (Bulleri et al., 1999). Both species can be found in co-occurrence: *P. lividus* ideally prefers horizontal or slightly sloping surfaces, whereas *A. lixula* can be more often found in vertical slopes, due to its higher resistance to wave action (Régis, 1979). Although these two species often coexist, the ecological niche they occupy is different. *P. lividus* chooses to feed, among the potential resources, those with a large stock (Dictyotales, in summer), abandoning them as soon as they become rarer (October, March) to move towards other resources. This is the behaviour of an apostatic generalist (Frantzis et al., 1988). *A. lixula*, instead, always relies on a sufficient abundance of encrusting Corallinaceae and appears to exhibit selective feeding (Privitera et al., 2008). Moreover, stable isotopes analyses (^{13}C and ^{15}C) show a strong tendency of *Arbacia* to carnivory (O. Wangensteen et al., 2011). *P. lividus*, having the desired abundance of *Dictyotales* only in summer (behaviour of the "selective"), becomes more "generalist" in October and to a lesser extent in March since it has access to other preferred algae (e.g. *Cystoseira sp.*). It is therefore in October that the trophic spectra of the two species may overlap the most. Nonetheless, it is suggested by Privitera et al. (2008), that in barren conditions a large trophic niche differentiation occurs, demonstrating that the two urchins play complementary and synergic roles in the maintenance of barrens in the Mediterranean.

A. lixula has a high phenotypic plasticity and therefore high adaptability and resistance to stressful environments. As a species, it's widely distributed among Mediterranean coasts including acidified and volcanic coats. Ocean acidification doesn't seem to interfere with fertilization events or early development, yet it can cause larval morphological anomalies and mortality events (Gianguzza et al., 2013). Predation of *A.lixula* is scarce, inversely proportional to its test size and splitted among few predatory species, like *Diplodus sargus*, *Diplodus vulgaris* and *Marthastreas glacialis* (Guidetti, 2004; Gianguzza et al., 2016).

Although most acidification studies on this species have focused on larval stage developments (Visconti et al., 2017), more recent studies have characterized the skeletal matrixes of the adult as well (Kanold et al., 2014). To these days, experiments in the natural environment are few and it's necessary to understand how adult individual will react in more acidified environments, to assess the adaptability of the species and/or any trans-generational effects but more importantly, to reveal any trophic change due to the plasticity of *A. lixula*'s diet. Exploring both physiological and behavioural traits of *Arbacia lixula* could clarify environmental implications and provide valuable insights to predict the future of benthic communities.

2. Aim of the study

A. Lixula doesn't have any alimentary value. Therefore, it's either used in comparison to other alimentary useful species like *P. lividus* or used as a model for in-lab acidification experimental studies (Cohen-Rengifo et al., 2019; O. S. Wangensteen et al., 2013). This approach is quite selective and usually leaves the ecological importance of this species unnoticed. Ocean Acidification and climate change are drivers of physiological and behavioural changes not only for marine invertebrates like the subject of this study, but also for entire communities (Hammill et al., 2017). This study focuses on adults of *A. lixula*, exposed to different pH conditions. The study site, Castello Aragonese (Ischia), is in fact a well-studied volcanic area with shallow underwater vents that release CO¢, originating a pH gradient varying from a pH of 7.7 to 8.1 (Gambi, 2014). We want to assess any physiological or behavioural variability between sea urchins living under different pH conditions, also exposing them reciprocally to both tested pH levels. In addition, we want to deduce if acidification can alter the benthic macroalgal cover, because significant differences in the benthic composition could therefore mean a change in diet of *A. Lixula* or vice-versa. So, the final aim of this work is to reveal potential connection between the habitat composition and the physiological and behavioural responses of relative inhabitants to acidified conditions.

Tested null Hypotheses are:

- **H0(1)**: The physiological and behavioural responses of *A. lixula* do not differ between individuals from areas with different pH levels when exposed to varying pH conditions in a controlled environment.
- **H0(2)**: The composition of algal communities does not significantly vary between areas with different pH conditions throughout the year.
- **H0(3)**: Seasonal variability does not have significant effects on the physiological and behavioural responses of *A. lixula* in relation to pH.

Our expectations are that individuals exposed to more reduced pH have slowed mobility and reduced behavioural responses, due to the physiological impairment given by acidification. At the same time, we expect sea urchins taken from acidified condition to be more acclimated than urchins taken from normal pH, therefore performing best due to chronic exposure to low pH (Palombo et al., 2023). Another expectation is that the spatial and temporal variation of the habitat would be consistent with changes in the sea urchin responses.

3. MATERIALS AND METHODS

3.1 The sampling Site

The experimental setup has been theorized based on several previous studies on a geologically interesting area, the Castello Aragonese of Ischia Island (Fig. 4) The gulf of Naples has a well-documented volcanic history, including the surrounding Phlegraean islands of Ischia, Procida, Vivara and Nisida. Ischia Island is well known for subsequent series of volcanic eruptions, which generated basaltic accumulations and detritic landslides (Sbrana et al., 2018). Evident residual activity of the geological history is the presence of several hydrothermal vents along various sites of interest on the coast of the island. In between this large number of possible candidates, the site of Castello Aragonese (40°43′57.9″ N, 13°57′51.8″ E) on the north- east side of the island was the most promising (Gambi, 2014). In fact, Castello Aragonese is the only archaeologically marine protected area of the island, therefore unaffected by any fishing activity bias. The structure of the site consists of a volcanic dome connected to the rest of the island by a road built upon an artificial bridge (Foo et al., 2018). At both sides of the connection point of this bridge to the dome, there are intense emissions of gas from the seabed, fading in intensity as the distance from the bridge increases. The gas is almost entirely composed of carbon dioxide (90–95 %) (Tedesco 1996; Hall-Spencer et al. 2008) and this characteristic makes this site a rarity among marine vents, since most of them have mixed emissions, often presenting heavy metals. The intertidal and subtidal area of Castello Aragonese has been a place of interest for many studies (M. Gambi et al., 2016; Flöter et al., 2023). The hydrothermal vents surrounding the area hosts a highly biodiverse ecosystem, with composition and abundance relative to the pH. The communities include a wide range of algae and seagrasses, an abundant benthic community of crustaceans, molluscs and polychaete (Kumar et al., 2019; M. Gambi et al., 2016; Porzio et al., 2011). Sampled individuals were near hard basaltic substrates covered ether by barrens, erect macro algae communities or turfs of filamentous algae.

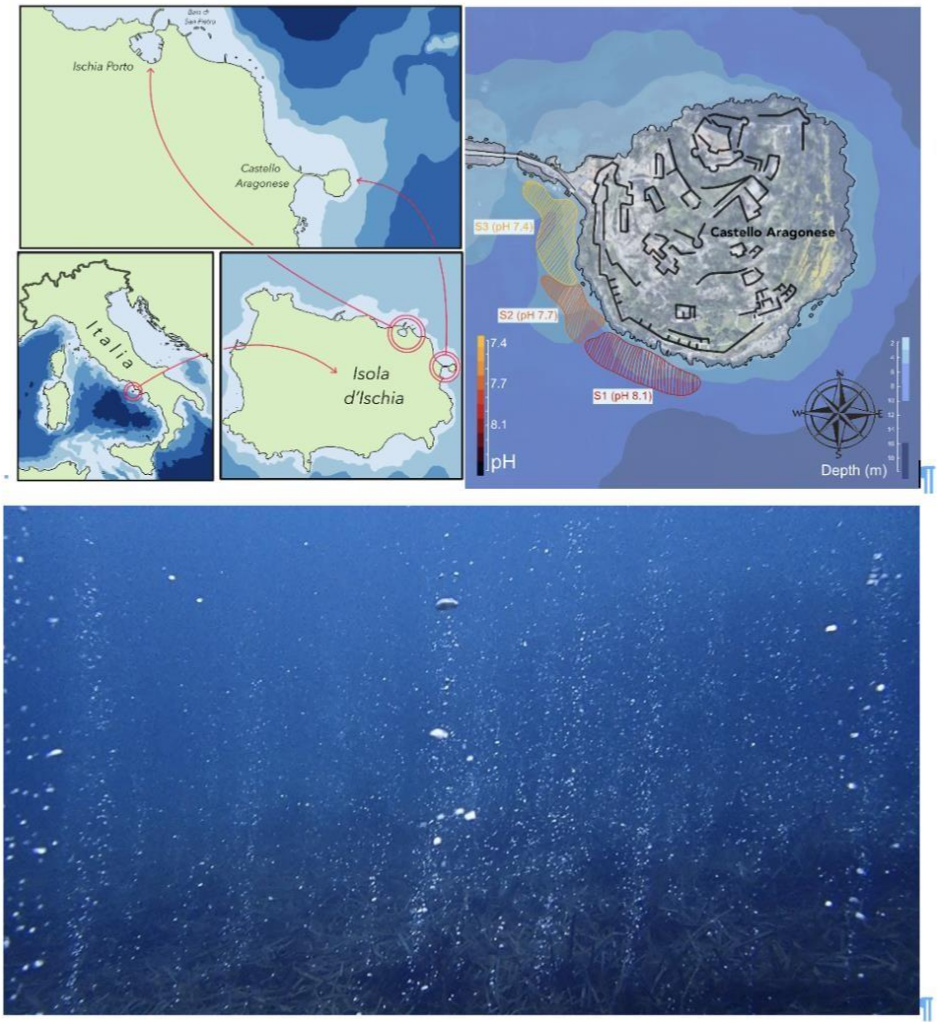


Figure 4- Geographical location of Castello Aragonese and Ischia in respect to the Italian coast (on the left), alongside the location of submerged vents areas on the south coast of Castello Aragonese (on the right). On the bottom, images of the submerged vents and constant carbon dioxide emissions.

Along the southern coast of Castello, where CO¢ emissions generate a very well-known natural pH gradient, two sampling areas were identified based on previous studies: a site with acidic pH (conventionally named S2, mean pH 7.7) and one with ambient pH (named S1, mean pH 8.1). Since these two areas are close to each other, another site was selected along the main coast of the island, 4 kilometres away from the vents' area, called San Pietro Bay (SP, pH 8.1) (Figure 4).

3.2 Experimental design

The idea of the experiment is to expose individuals of both sites to different pH levels to see if there are any differences. By exposing half of individuals from each site to ambient pH (8.1) and another half to reduced pH (7.7). We expect a physiological response of individuals in the reduced pH that enhances stress of individuals not used to acidic conditions, rather than individuals coming from S2 which are already adapted to such stressor. A total of 16 individuals were taken between 0.5 and 2 meters of depth from each site and groups of 8 individuals were used for each treatment. The experiment operated on a period of one year, with sampling approximately every 2-3 months from September 2023 to August 2024. The individual samples of *Arbacia lixula* were manipulated as little as possible to limit handling bias in the animal physiology. Once examined with non-invasive techniques, the sea urchins were brought back to the site of extraction, using a <catch-and release= modality. Since the site area has reduced dimensions, individuals from each site might have selected more than once for different sampling times and therefore the samples cannot be declared independent. Hence, tested responses were considered for each sampling time.

Hence, with this design, there is a total of 6 experimental conditions:

- Castello Aragonese, S1 individuals exposed to ambient pH (8.1).
- Castello Aragonese, S1 individuals exposed to reduced pH (7.7).
- Castello Aragonese, S2 individuals exposed to ambient pH (8.1).
- Castello Aragonese, S2 individuals exposed to reduced pH (7.7).

- San Pietro individuals exposed to ambient pH (8.1).
- San Pietro individuals exposed to reduced pH (7.7).

To assess the physiological effect of the pH-induced stress we examined 2 main response variables: respiration rate (RR) and excretion rate (ER), in terms of ammonia released by the individuals on a time span of one hour. Another aspect that pH may influence individuals is their behavioural response, which was measured through other response variables: righting time (RT) and sheltering time (ST). RT was measured at the same time of RR, inside of a respirometric chamber. For different sets of individuals, ST (negative phototaxis) was measured as well on the different pH values.

During each sampling time, photo-quadrats of the sampling areas S1, S2 and SP were taken. Like in (Hall-Spencer et al., 2008; Linares et al., 2015; Porzio et al., 2011) it has been demonstrated that acidified environments in the Mediterranean have seasonal variation; hence, we expect the photo-quadrat algal cover to differ based on sampling periods and pH. By demonstrating that the algal composition of the benthic substrate where the urchin is foraging is changing seasonally, we could further distinguish the effect of acidification from a change in diet and trophic level of the organisms.

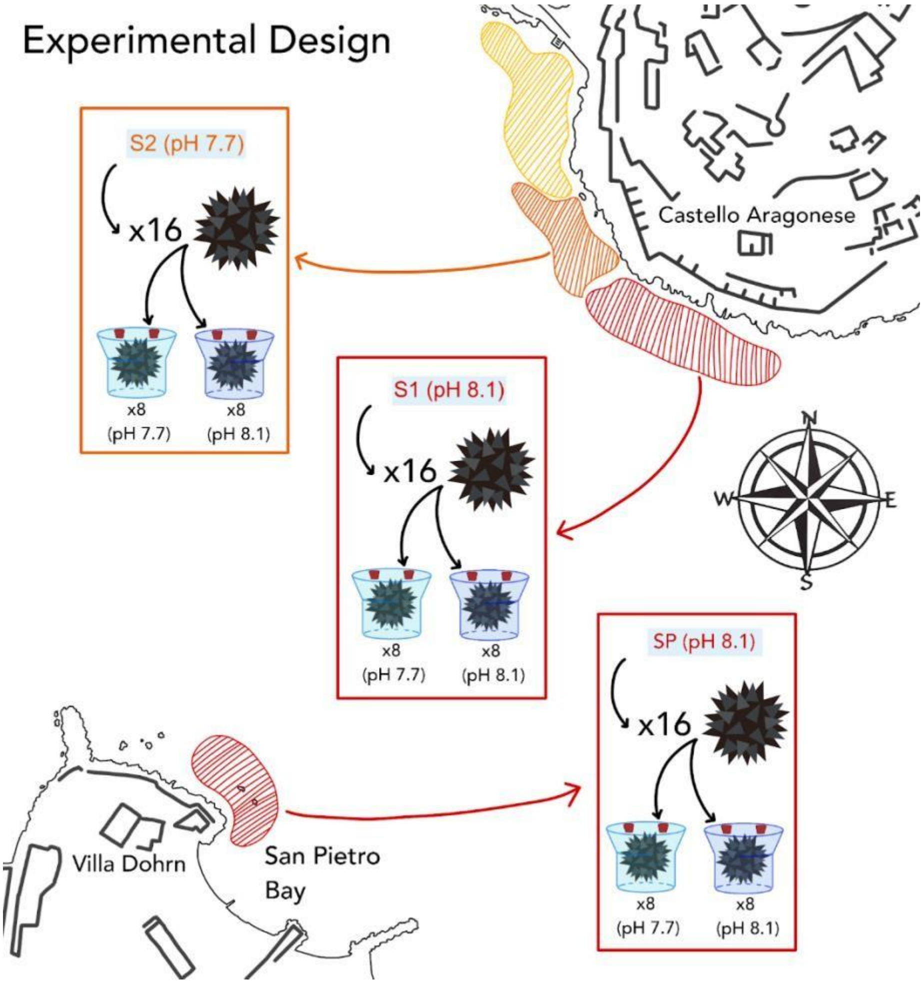


Figure 5 - Experimental design and number of replicates for each treatment from the sites S2 (orange) S1 and SP (red) respectively.

3.3 Respiration rate (RR)

Respiration is the physiological process by which an organism uptake oxygen and release carbon dioxide. Individuals of *A. lixula* were placed upside-down in respirometric chambers filled with non-filtered seawater, at different pH, collected directly from the sampling sites. To minimize photosynthetic activity of the phytoplankton, the experiments were conducted under dark conditions. Upon placement, a timer was set to record the duration required (Righting time) for the animal to revert his position in the respirometric chamber. The glass chambers were sealed hermetically to prevent the presence of air and prior to each measurement, the software was calibrated linearly using blank chambers, following the methodology of Lyndby et al. (2020).

An internal sensor in the chamber attached by transparent silica allowed real time monitoring of oxygen concentration through the Fiber-Optic Oxygen Meter Piccolo2 (Pyro Science GmbH, Aachen, Germany). Oxygen concentration (µmol L{^{1}) in each chamber was measured every twenty minutes over a one-hour trial period. The oxygen consumption over this period was used to calculate the Respiration Rate (RR). The respiration rate (RR, µmol O¢/hour) was determined using the formula by Widdows and Johnson (1988):

$$RR = 60(C_0 - C_1)(V - Va)/t_0 - t_1$$

where C_0 and C_1 represent the oxygen concentrations at the start and end of the analysis (in µmol O¢/L), V and *Va* denote the volume of the respirometric chamber (0,7 L) and the volume of the animal inside the chamber (in L), respectively, and t_0 and t_1 are the start and end times of the assay (in minutes). All data were corrected using the respective blank measurements. The oxygen consumption of sea urchins was calculated over a 60-minute period, with results expressed in µmol O¢/hour. After each Respiration Rate trial, volume of the individual *was* calculated by measuring height and width of each urchin with caliber and simplifying animal volume in a cylinder shape.

3.4 Excretion rate (ER)

Sea urchin excretion of nitrogen compounds occurs through direct release of ammonia in seawater. As known, ammonia excretion can drastically vary based on environmental conditions and is useful to calculate physiological stress and energy losses, as it reflects the rate of protein catabolism. Our expectations is that treatments will have a significant difference in excretion rate therefore establishing differently affinity to an acidic environment.

Samples for the excretion rate analyses were collected from the respirometric chambers at the end of each set in 50 ml falcons and frozen at -20 *C up to chemical analyses. Ammonia concentration was measured through spectrophotometric method and colorimetric method as described by Parsons et al., 1984.

Excretion rate was calculated by the following:

$$ER = (C_t - C_b)\ \mathrm{x}\ V/t$$

Where <C_t= and <C_b= are, respectively, the [N- NH_4^+] in the chamber with and without sea urchin respectively, both measured at the end of the respiration trial. <V= is the chamber volume in liters (0.7 L) and <t= the incubation time in hours. Hence, ammonia excretion was expressed in terms of μg-at NH_4^+/hour.

3.5 Righting time (RT)

A clear measurable behavioural trait of sea urchins is their capability of returning to an upward position (mouth down) upon capsizing. Previous studies have shown a direct relation between physiological stressors and righting time, effectively demonstrating a negative effect (Sun et al., 2019). Rightening time is particularly important for sea urchins, because exposing the oral side can increase the risk of predation and dislocation by wave action (Percy, 1973; Zhang et al., 2017). Likewise, the individuals collected for the respiration rate were placed in the respirometric chambers upside down, and chronometrated until straightened out. In order to include individuals that did not perform experimental behaviour, arbitrary classes were attributed to the sheltering dataset accordingly to righting time range:

Table 1 - Righting classes chosen to include the individuals not exhibiting designed behaviour (represented by the highest category).

Righting time (min)	**Righting class**
1 - 3	1
4 - 6	2
7 - 9	3
10 - 15	4
16 - 20	5
21 - 30	6
> 30	7
N/A	8
> 31	9

By performing the respiration rate and the rightening time simultaneously, both physiological and behavioural traits are tested with water at the same temperature. Any individual that performed gamete emission throughout the respiration experiment was considered null and the experiment was performed again with a different individual.

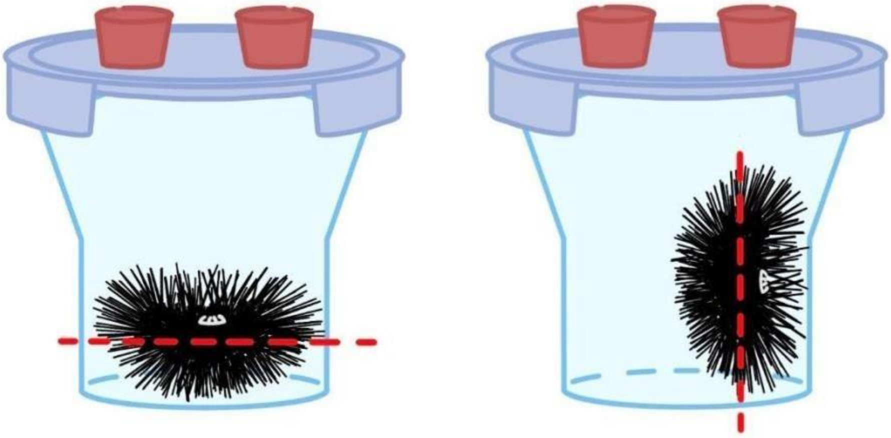


Figure 6 - Righting time experiment setup: time starts when sea urchin is placed mouth upward and the respirometric chambers are sealed (on the left), time stops when sea urchin is completely attached to the wall of the respirometric chamber or flipped completely (on the right).

3.6 Sheltering time (ST)

Sea urchins from the genus *Arbacia* have exhibited negative phototaxis, which have already been demonstrated to be another measurable variable that can be impacted by environmental stressors in sea urchins' species (Brothers & McClintock, 2015; Cohen-Rengifo et al., 2019; Asnicar et al., 2021b). Sheltering time was measured on different sets of individuals than the ones used for respiration and righting time. Experimental design was the same as previous experiments, either performed during nighttime or in a controlled environment to reduce natural sunlight. To include individuals that did not perform experimental behaviour, arbitrary classes were attributed to the sheltering dataset accordingly to sheltering time range:

Table 2 - Arbitrary sheltering classes chosen to include the individuals not exhibiting designed behaviour (represented by the highest category).

Sheltering time (min)	**Sheltering class**
0 - 0.5	1
0.51 - 1	2
1.01 - 1.5	3
1.51 - 2.0	4
2.01 - 3	5
3.01 - 4	6
4.01 - 5	7
>5	8
N/A	9

Upon placing an individual in a plastic tank, a torch (Mares EOS 10LRZ; Kriogor) was pointed from above toward the center of the individual. The beam of light was partially shaded from a non-transparent panel so that only half of the urchin was exposed to light. Sheltering time was measured until spine tips were completely shaded. Individuals that didn’t move toward dark within five minutes were considered recessive to this behaviour.

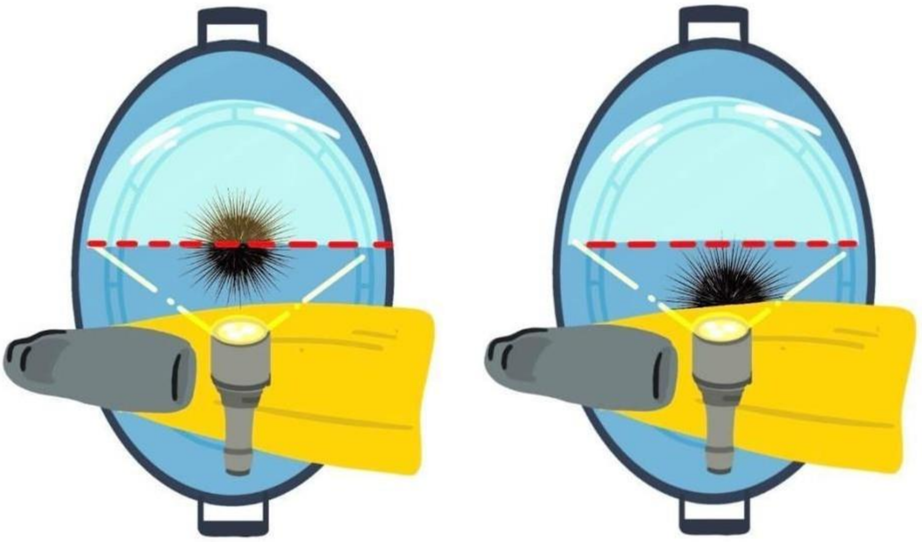


Figure 7 - Sheltering experiment setup: The time starts from the moment when the upper half of the urchin is exposed to light and finishes when the spine tips are completely shaded (respect to the red line).

4.5 Photoquadrats % cover

The Paired with the analyses of the sea urchin physiological and behavioural response, photo-quadrats of a 20 cm^2 area were randomly taken in the same sites of the sea urchin, to assess any significant effect on the benthic algal composition (Bohnsack, 1979; Foster et al., 1991). The design considers as a response variable the percentage cover of different functional groups alongside the 3 levels of factor <SITE= used for the sea urchin experiment. Five benthic cover types were previously identified and subsequently used to classify the cover in the quadrat (Farina et al., 2014; Sala et al., 2012). Five replicate photos were taken at each site and for each sampling time, approximately at the same time of the urchin collection. Photos were later examined with *ImageJ* by splitting the area into a grid of 25 squares and determining the functional group cover, by assigning values to each for a total maximum of 4 in every square of the grid.

After adjusting the values to a percentage aside from cavities and other non-visible parts of the image, functional groups were statistically examined to assess local and seasonal variation:

- Erect macro algae (*Dictyotales*, *Haptopteris*, *Padina pavonica*, *Asparagopsis*, *Flabellia petiolata*, ecc.)
- Coralline algae (*Corallina elongata*, *Jania rubens*, *Peyssonelia*, *Mesophyllum*, *Amphiroa rigida* ecc.)..
- Turfs (*Cladophoraceae*, *Rhodomelaceae*, *Bryopsis*, general filamentous algae, ecc.).
- Barrens (Bare rock, Barnacles, *Dendromorpha*, encrusting red and brown algae.
- Encrusting animals (sponges, *Cladocora caestiposa* Bryozoans, ecc.).

3.7 Statistical analyses

The test statistic for the experiments were performed for significance using PRIMER software, version 6 and R software version 4.3.1.

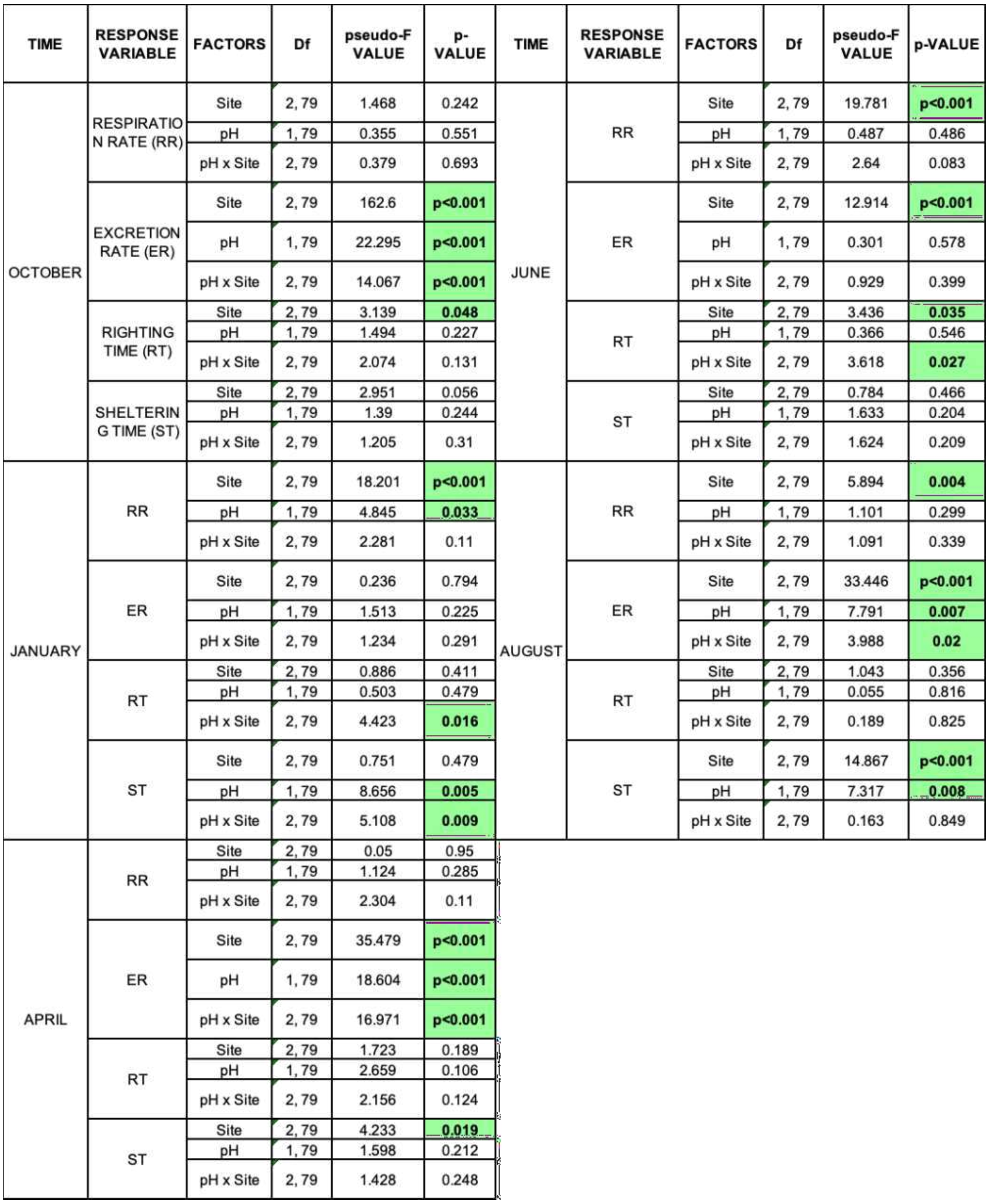
Three separated matrixes were obtained from samples, based on the number of experiments done onsite and statistical analyses were performed on each dataset separately. Assumption of data normality was rejected after the Shapiro-Wilk test for most experimental conditions; Moreover, sampling time was not considered as a factor, since sea urchin abundance in the acidified site (S2) is low and therefore samples are not independent across sampling time. Statistical analyses were accomplished for each sampling time separately, considering samples across the two years (but same sampling time) as replicates.

The experimental design on sea urchins includes two factors, <pH= and <SITE=, both fixed and orthogonal. The first two matrixes were extrapolated from the respirometric chamber experiment and the sheltering experiment respectively. Within the first experiment righting time, excretion rate and respiration rate were obtained. behavioural experiment results were treated using a clustering system, to include individuals that did not perform respective behaviour. Before statistical analyses, behavioural data were divided arbitrarily in classes following biological criteria.

For each sampling time, a multivariate PERMANOVA was performed alongside Monte Carlo’s test to adjust the p-value by the number of permutations since samples taken within the first year of the experiment only included two sites. Significant results were thereafter tested with a pairwise test to assess quantitatively the interaction of each factor (Table 3). The photo-quadrat experiment was performed on the third matrix with the same statistical tests done for the sea urchins. Tests were applied on all the response variables (all functional groups) simultaneously for each time to assess general substrata differences, but also on each response variable (single functional group) to identify the influence of each component on the overall changes in algal coverage (Table 4). For the photoquadrats analysis, only a single factor was considered, i.e. <SITE=.

4. RESULTS

Table 1 – Table summarizing the results of the statistical analysis for each response variable. The table includes the following columns: <Time= (representing the time of sampling), <Response Variable= (indicating the variable of interest), <Factors= (referring to the factors included in the analysis), <pseudo-F VALUE= (level of separation between groups), <Df= (representing the degrees of freedom), and <p- VALUE= (indicating the p-value adjusted by the Monte Carlo test associated with the pseudo-F). These results provide insights into the relationship between the response variable and the factors under investigation. Significances are highlighted in green.



TIME	RESPONSE VARIABLE	FACTORS	Df	pseudo-F VALUE	p-VALUE
OCTOBER	RESPIRATION RATE (RR)	Site	2, 79	1.468	0.242
		pH	1, 79	0.355	0.551
		pH x Site	2, 79	0.379	0.693
	EXCRETION RATE (ER)	Site	2, 79	162.6	**p<0.001**
		pH	1, 79	22.295	**p<0.001**
		pH x Site	2, 79	14.067	**p<0.001**
	RIGHTING TIME (RT)	Site	2, 79	3.139	**0.048**
		pH	1, 79	1.494	0.227
		pH x Site	2, 79	2.074	0.131
	SHELTERING TIME (ST)	Site	2, 79	2.951	0.056
		pH	1, 79	1.39	0.244
		pH x Site	2, 79	1.205	0.31
JANUARY	RR	Site	2, 79	18.201	**p<0.001**
		pH	1, 79	4.845	**0.033**
		pH x Site	2, 79	2.281	0.11
	ER	Site	2, 79	0.236	0.794
		pH	1, 79	1.513	0.225
		pH x Site	2, 79	1.234	0.291
	RT	Site	2, 79	0.886	0.411
		pH	1, 79	0.503	0.479
		pH x Site	2, 79	4.423	**0.016**
	ST	Site	2, 79	0.751	0.479
		pH	1, 79	8.656	**0.005**
		pH x Site	2, 79	5.108	**0.009**
APRIL	RR	Site	2, 79	0.05	0.95
		pH	1, 79	1.124	0.285
		pH x Site	2, 79	2.304	0.11
	ER	Site	2, 79	35.479	**p<0.001**
		pH	1, 79	18.604	**p<0.001**
		pH x Site	2, 79	16.971	**p<0.001**
	RT	Site	2, 79	1.723	0.189
		pH	1, 79	2.659	0.106
		pH x Site	2, 79	2.156	0.124
	ST	Site	2, 79	4.233	**0.019**
		pH	1, 79	1.598	0.212
		pH x Site	2, 79	1.428	0.248
JUNE	RR	Site	2, 79	19.781	**p<0.001**
		pH	1, 79	0.487	0.486
		pH x Site	2, 79	2.64	0.083
	ER	Site	2, 79	12.914	**p<0.001**
		pH	1, 79	0.301	0.578
		pH x Site	2, 79	0.929	0.399
	RT	Site	2, 79	3.436	**0.035**
		pH	1, 79	0.366	0.546
		pH x Site	2, 79	3.618	**0.027**
	ST	Site	2, 79	0.784	0.466
		pH	1, 79	1.633	0.204
		pH x Site	2, 79	1.624	0.209
AUGUST	RR	Site	2, 79	5.894	**0.004**
		pH	1, 79	1.101	0.299
		pH x Site	2, 79	1.091	0.339
	ER	Site	2, 79	33.446	**p<0.001**
		pH	1, 79	7.791	**0.007**
		pH x Site	2, 79	3.988	**0.02**
	RT	Site	2, 79	1.043	0.356
		pH	1, 79	0.055	0.816
		pH x Site	2, 79	0.189	0.825
	ST	Site	2, 79	14.867	**p<0.001**
		pH	1, 79	7.317	**0.008**
		pH x Site	2, 79	0.163	0.849

4.1 Respiration Rate (RR)

In Figure 8, the results of the Respiration essay are presented:

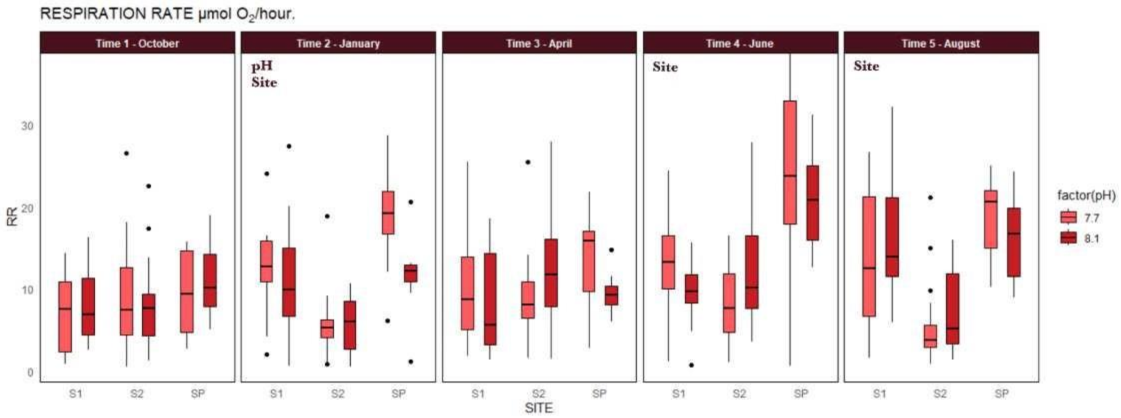


Figure 8 – Respiration rate. Boxplots highlight medians and quartiles for each experimental treatment (n=8). Lighter red indicates exposure to reduced pH (7.7), whereas darker red indicates exposure to ambient pH (8.1). S1, S2 and SP refer to the area of collection with different pH levels (S1 and SP=pH 8.1 and S2=pH 7.7). At each sampling time, labelings indicate significance of factors and/or their interaction. When factor interaction is significant, different lowercase letters indicate statistical difference between sites at reduced pH ($p<0.05$), whereas different uppercase letters indicate statistical difference between sites at ambient pH ($p<0.05$). Asterisks represent statistical differences ($p<0.05$) among pH exposure of the same site.

RR was significantly affected by both pH and Site in January, but only by Site in June and August (Fig. 8 and Table 3). In January and August, RR was generally higher in sea urchins from the ambient-pH sites (S1 and SP) when exposed to reduced pH, while lower rates were observed in sea urchins already acclimated to reduced pH (S2). When Site was a significant factor, S2 showed a consistently lower respiration rate, particularly in January and August. In contrast, in June, the control site SP displayed a higher RR compared to the Castello Aragonese sites. Additionally, in January, pH had a notable effect, with lower RR recorded in sea urchins from S2 relative to those from the other two sites.

4.2 Excretion Rate (ER)

In Figure 9, the results of the Excretion essay are presented:

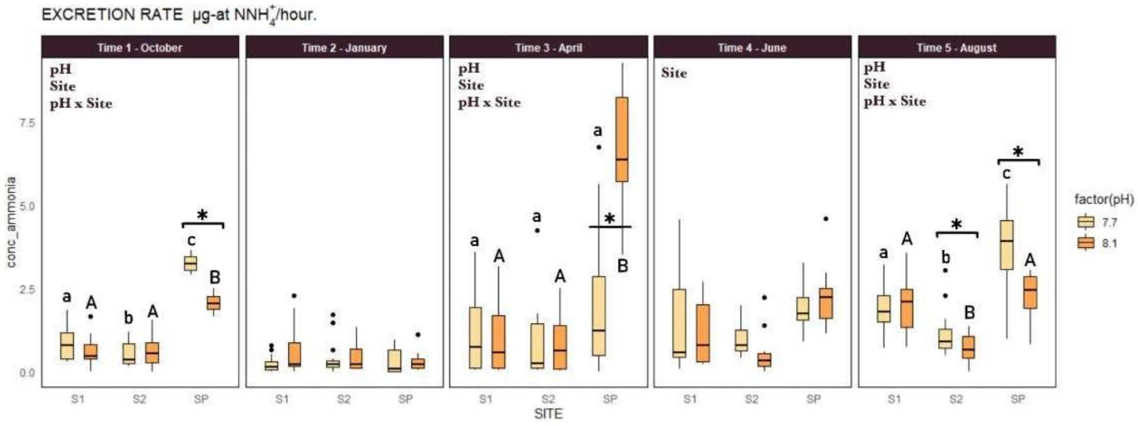


Figure 9 – Excretion rate. Boxplots highlight medians and quartiles for each experimental treatment (n=8). Lighter yellow indicates exposure to reduced pH (7.7), whereas darker yellow indicates exposure to ambient pH (8.1). S1, S2 and SP refer to the area of collection with different pH levels (S1 and SP=pH 8.1 and S2=pH 7.7). At each sampling time, labelings indicate significance of factors and/or their interaction. When factor interaction is significant, different lowercase letters indicate statistical difference between sites at reduced pH ($p<0.05$), whereas different uppercase letters indicate statistical difference between sites at ambient pH ($p<0.05$). Asterisks represent statistical differences ($p<0.05$) among pH exposure of the same site.

ER was significantly influenced by the pH*Site interaction, as well as by the individual factors Site and pH in October, April, and August, while only by Site in June (Fig. 9 and Table 3). In Fig. 9, ER values were higher during autumn, spring, and summer compared to values in sea urchins collected in winter (January). Moreover, urchins from San Pietro Bay (SP) excreted, on average, three times more ammonia than those from Castello Aragonese. In October, sea urchins from all three sites showed significant differences in ER under reduced pH conditions, whereas only SP urchins differed from those at the other two sites at ambient pH. In April, SP urchins excreted significantly more ammonia at ambient pH than those from the same site at reduced pH, as well as more than those from S1 and S2 at both pH levels. Finally, in August, S2 urchins differed from those from SP and S1 at both pH levels. When a significant pH*Site interaction was present, ER in SP urchins was affected by the pH used during trials, with a consistent trend in October and August showing higher ER at reduced pH. The opposite trend was observed in April. S2 urchins also showed significant differences in ER between pH levels, but only in August, with higher ammonia levels produced under reduced pH conditions during trials.

4.3 Righting Time (RT)

In Figure 10, the results of the Righting essay are presented:

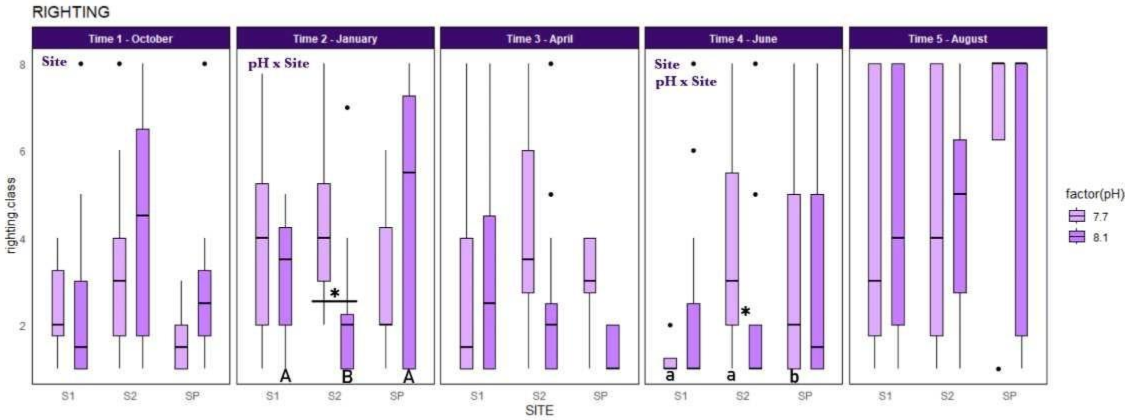


Figure 10 - Righting time. Boxplots highlight medians and quartiles for each experimental treatment (n=8). Lighter purple indicates exposure to reduced pH (7.7), whereas darker purple indicates exposure to ambient pH (8.1). S1, S2 and SP refer to the area of collection with different pH levels (S1 and SP=pH 8.1 and S2=pH 7.7). At each sampling time, labelings indicate significance of factors and/or their interaction. When factor interaction is significant, different lowercase letters indicate statistical difference between sites at reduced pH ($p<0.05$), whereas different uppercase letters indicate statistical difference between sites at ambient pH ($p<0.05$). Asterisks represent statistical differences ($p<0.05$) among pH exposure of the same site.

RT was significantly influenced by the pH*Site interaction in January and June, and by Site alone in October and June (Fig. 10 and Table 3). Specifically, urchins from S2 were significantly faster than those from ambient-pH sites when tested at ambient pH. In October, S2 urchins exposed to reduced pH were significantly slower than those at ambient pH, with no differences observed among the sites under reduced pH conditions. A similar trend was observed in June for S2 urchins, although at this sampling time, differences at reduced pH were also noted between S1 and S2 compared to SP.

4.4 Sheltering Time (ST)

In Figure 11, the results of the Sheltering essay are presented:

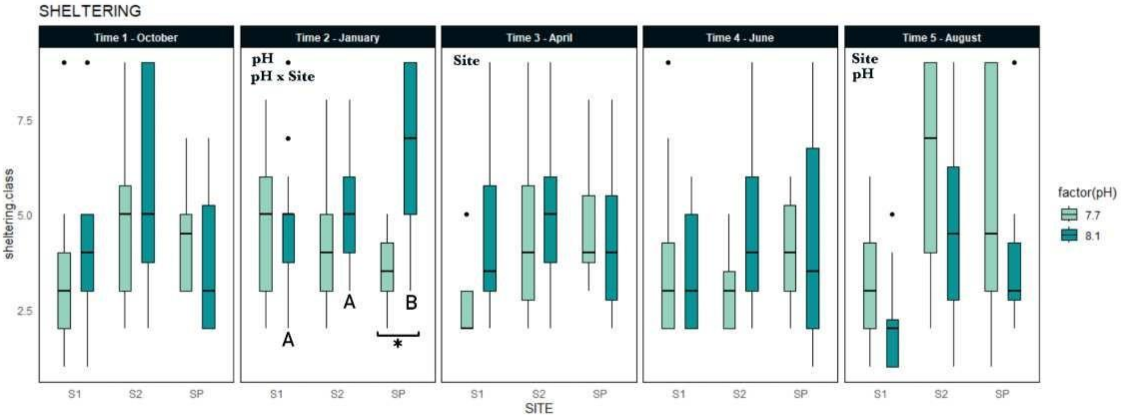


Figure 11 - Sheltering time. Boxplots highlight medians and quartiles for each experimental treatment. Lighter blue indicates exposure to reduced pH (7.7), whereas darker blue indicates exposure to ambient pH (8.1). S1, S2 and SP refer to the area of collection with different pH levels (S1 and SP=pH 8.1 and S2=pH 7.7). At each sampling time, asterisks represent significant variations of response variable among indicated factors.

ST was significantly influenced by the pH*Site interaction in January, by pH in January and August, and by Site in April and August (Fig. 11 and Table 3). In January, when the interaction effect was present, a significant difference was noted between urchins from SP and those from S1 and S2 under ambient pH, with no differences observed under reduced pH. A significant difference was also recorded for SP urchins between ambient and reduced pH. When pH alone was identified as significant by the PERMANOVA, a different response pattern to pH was seen, particularly for urchins from S2 and SP, with faster STs at reduced pH in January and faster STs at ambient pH in August.

4.5 Photoquadrats % cover

Table 4 – Table summarizing the results of the statistical analysis for the photo-quadrat differences among Sites. The table includes the following columns: <Functional Group= (representing the response variable considered), <Time= (representing the time of sampling), <Df= (representing the degrees of freedom), <pseudo-F VALUE= (level of separation between groups), and <p- VALUE= (indicating the p-value adjusted by the Monte Carlo test associated with the pseudo-F). Significances are highlighted in green.

F. Group	TIME	Df	Pseudo-F VALUE	p-VALUE	F. Group	TIME	Df	Pseudo-F VALUE	p-VALUE
ALL GROUPS	SET	1, 9	96.215	**p<0.001**	% Turfs	SET	1, 9	212.49	**p<0.001**
	NOV	2, 14	5.173	**0.007**		NOV	2, 14	6.721	**0.010**
	JAN	2, 14	4.912	**0.008**		JAN	2, 14	9.541	**0.004**
	APR	2, 14	7.731	**0.013**		APR	2, 14	8.615	**0.004**
	JUNE	2, 14	5.906	**0.008**		JUNE	2, 14	6.185	**0.015**
	AUG	2, 14	1.089	0.36		AUG	2, 14	0.391	0.682
% Erect	SET	1, 9	0.707	0.429	% Barrens	SET	1, 9	3.413	0.101
	NOV	2, 14	0.489	0.622		NOV	2, 14	6.959	**0.010**
	JAN	2, 14	4.293	**0.04**		JAN	2, 14	2.605	0.115
	APR	2, 14	6.108	**0.016**		APR	2, 14	0.709	0.515
	JUNE	2, 14	6.242	**0.016**		JUNE	2, 14	4.115	**0.045**
	AUG	2, 14	1.635	0.228		AUG	2, 14	0.943	0.421
% Others	SET	1, 9	105.3	**p<0.001**	% Animals	SET	1, 9	1	0.348
	NOV	2, 14	5.906	**0.008**		NOV	2, 14	1.024	0.388
	JAN	2, 14	105.3	**p<0.001**		JAN	2, 14	1.234	0.323
	APR	2, 14	0.634	0.55		APR	2, 14	4.525	**0.035**
	JUNE	2, 14	4.468	**0.037**		JUNE	2, 14	1.591	0.234
	AUG	2, 14	1.238	0.322		AUG	2, 14	2.248	0.153

In Figure 12, the results from the photo-quadrat experiment are presented in terms of percentage cover (%) for each functional group. Within the first time (September), photo-quadrats were taken between S1 and S2 only.

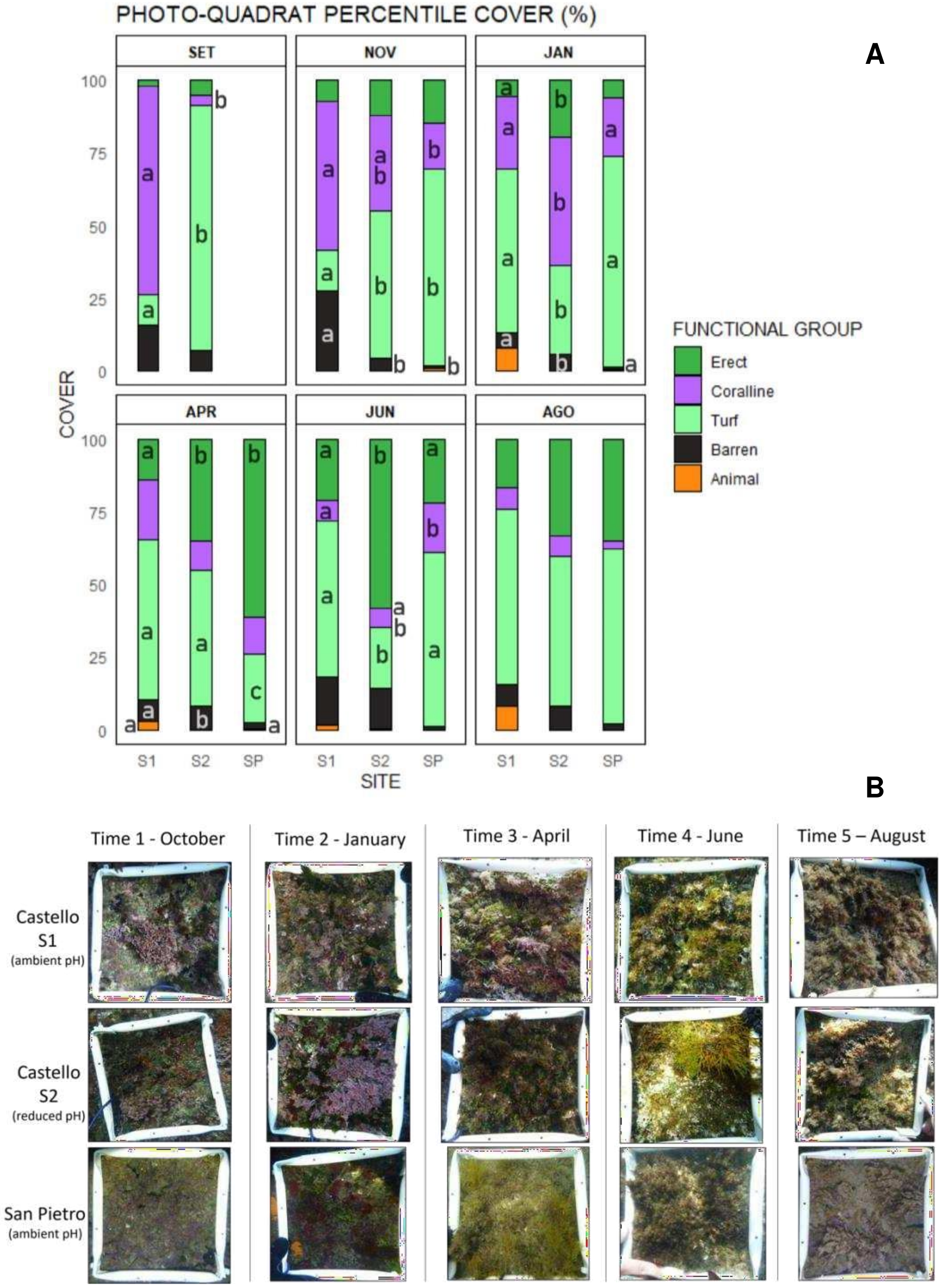


Figure 12 – A: Photo-quadrats percentage cover of functional groups. Boxplots highlight medians and quartiles for each experimental treatment. Color palette follows sampling time from September 2023 to August 2024. S1, S2 and SP refer to the area of collection with different pH levels (S1 and SP=pH 8.1 and S2=pH 7.7). At each functional group, asterisks represent significant variations of cover among sites at indicated sampling times. B: Most representative photoquadrats for each sampling time and site.

A significant difference among sites in terms of coverage was observed thanks to photo-quadrats throughout all times except in August (Fig. 12 and Table 4). S2 had higher percentage cover of erect macroalgae than S1, with both sites exhibiting higher cover toward June. Photo-quadrats taken in SP on the other hand, had both lower cover than S2 and highest cover in April. The second response variable, which included coralline algae, had oscillations depending on the site: within September, S1 cover was significantly higher than S2; November had a similar pattern, but SP photo-quadrats were presenting lower cover than S1. January on the other hand revealed the highest cover in S2. Finally, June cover presented a similar percentage cover for coralline algae within S2 and S1 but a slightly higher presence in SP.

Between the various functional groups, turf cover was the main responsible for changes across seasons. Moreover, turf cover had a drastic change between times for all sites. September showed a percentage cover 7 times higher in S2 compared toS1, which attenuated in January, where lowest turf cover was found in S2. During April, lowest percentage cover was registered in SP, whereas S1 and S2 were higher. June on the other hand, depicted S2 with the lowest cover and SP with the highest.

Barren cover presented significant differences among sites only for the months of November and June: during both times, castello aragonese sites (S1 and S2) had more barrens present than San Pietro Bay. Within Castello Aragonese, S1 presented a higher cover in the month of November, whereas a similar barren cover was registered in June. The percentage cover of Animals was only significant during the month of April, with a slightly higher cover for S1.

5 Discussion

The Understanding the impact of ocean acidification (OA) is essential, and recent studies have employed either laboratory approaches focused on individual responses without fully accounting for habitat contributions, or in situ approaches aimed at highlighting ecosystem-level effects. Natural laboratories that mimic global OA, such as the volcanic CO¢ vents in Ischia, provide valuable tools for evaluating acidification effects on marine environments, including complex ecosystem interactions. Increasingly, studies in this field use natural vents or specific emission sites as natural laboratories to predict the future impacts of acidification (Linares et al., 2015; Costa et al., 2023). For example, Teixidó et al. (2018) observed significant reductions in both predator and calcifying organism populations near low-pH sites, linking species declines to reductions in functional diversity. In this study, the volcanic vents in Ischia were utilized to better understand how the sea urchin *Arbacia lixula* might respond to future acidification scenarios in a natural context. Furthermore, connecting observations of *A. lixula*'s physiological and behavioral responses with differences in benthic composition across sites may yield valuable insights into the relationship between the diet of this omnivorous species and the structure of benthic communities (Steneck, 2020).

Sea urchin responses to acidification are complex, involving physiological impairments alongside behavioral shifts (Brothers & McClintock, 2015; Asnicar et al., 2021). These responses are also often species-specific, necessitating a detailed understanding of the species' metabolic traits. In the case of *A. lixula*, Kanold et al. (2014) identified over a hundred proteins involved in the formation of skeletal matrices and the regulation of calcification. While acidification does not cause visible somatic changes in adult sea urchins (Mos et al., 2015), it nonetheless impacts their acid-base balance.

Our study findings suggest that individuals exposed to acidified conditions exhibited a heightened metabolic rate: specifically, respiration rate (RR) was generally higher for urchins from S1 and SP exposed to reduced pH compared to ambient pH. Conversely, RR was higher for S2 urchins under ambient pH than under reduced pH. These findings align with Palombo et al. (2023), who hypothesize a transgenerational effect that may have shifted the optimal pH range for sea urchins living in S2 toward more acidic conditions. Alternatively, Kroeker et al. (2011) note daily pH variability at Ischia's acidified sites due to primary production, currents, and biological activity, which could foster greater tolerance to pH fluctuations rather than shifting the optimal pH range.

Excretion rate (ER), like RR, was generally elevated for non-acclimated urchins under reduced pH, with some exceptions, such as SP in April. Higher ammonia levels in excretions are indicative of increased protein catabolism and specialized acid homeostasis mechanisms (Catarino et al., 2012; Asnicar et al., 2021). In cases with significant results, S2 consistently had a lower ER than other sites, whereas SP had the highest rates. This may be due to its proximity to the harbor and the potential impact of pollution, which could influence *A. lixula*'s physiology directly and indirectly, possibly by reducing access to energy-rich food. In particular, the SP site has a consistently high turf cover throughout most of the year, with the exception of April.

By incorporating behavioural experiments in this study, we underpin the importance of *Arbacia lixula* to the benthic community's possible impact. More recently, behavioural responses have been used to determine consequences of a changing climate in shallow benthic communities and *Arbacia lixula*'s behaviour could ultimately increase its importance in near future acidified conditions (O. S. Wangensteen et al., 2013).

Within our results, responses showed high variability across sampling times. Nonetheless, the significance of site was observed at multiple sampling times for both RT and ST, indicating a clear correlation between site and the organism's response capability. RT showed significantly longer response times for S2 urchins in October, whereas ST showed faster response times for S1 urchins in both August and April. These findings highlight the complex interplay of area-specific factors, most likely including pH levels, and how pH shapes the habitat from which the sea urchins originate. The observed differences suggest that the organisms' responses are influenced by the origin of the sea urchins, indicating potential local adaptations or acclimation processes. The wide variability in behavioural endpoints may be explained by the difference in size between sea urchins from different sites, with S1 and SP urchins generally being larger than S2 (unpublished data).

Furthermore, in the behavioural experiments, acclimatization was not included, and individuals were tested immediately after collection. This could have further influenced the sea urchins' response to the experiment, as detachment may impair their aquiferous system. Nonetheless, the longer behavioural response times in sea urchins naturally exposed to reduced pH clearly demonstrate an altered reaction to environmental stimuli, possibly indicating a higher risk of predation. This is because the upward position of the mouth makes them more vulnerable to their main predators, *Diplodus sargus* and *Diplodus vulgaris* (Guidetti, 2004).

Photoquadrats results supported the hypothesis of a distinct benthic environment, showing variations in vegetation cover across all sampling times except August 2024 (Linares et al., 2015). Consistent with Frantzis et al. (1988), the functional group of primary interest was group 2 (other non-erect macroalgae), which included coralline algae. Coralline algae, a preferred grazing target for *Arbacia lixula*, did not exhibit a clear pattern that differentiated acidified from non-acidified sites; however, S2 consistently showed higher cover than S1 or SP at significant sampling times, except September 2023. This finding underscores the complex interplay of environmental factors, suggesting that *Arbacia*'s diet and pH are not the only stressors influencing algal cover. Additionally, sea urchin grazing intensity may vary, as Teixidó et al. (2018) demonstrated a lower sea urchin abundance at acidified sites. Conversely, significant differences in benthic cover, like those observed in our results, could more likely influence *A. lixula*'s diet based on algal availability. Such shifts could heighten *Arbacia*'s competition with other grazers, restrict growth, reproductive capacity, and potentially decrease survival.

To better assess potential dietary shifts in *A. lixula*, fecal samples were collected from all sites for future isotopic analysis. Preliminary studies of environmental cover, however, already reveal substantial differences in grazing areas. In summary, our results clearly indicate significant changes in *A. lixula*'s physiological traits and behavioral responses due to pH exposure.

Despite the anticipated negative impacts of future climate conditions, this species shows clear signs of adaptation and acclimatization to stressful environments, suggesting resilience to anthropogenic acidification. Through field observations, we aimed to capture ecological dynamics in relation to a complex, seasonally variable environment.

6 Conclusion

These experiments analyzed the consequences of ocean acidification on physiology and behaviour of *Arbacia lixula* by exploiting natural volcanic laboratories, able to replicate experimental conditions on a local environmental scale. Our results have shown that environmental stressors are influencing individual responses and altering their metabolism but have also proven signs of acclimatization to acidic environments and what are likely to be trans generational adaptations of individuals living in acidified conditions. Moreover, significance has been found on both pH, area of origin and their interaction. Further studies should focus on sea urchin’s interaction with environment and predation mechanism, correctly depicting *Arbacia's* position on the trophic chain. To test trans- generational effects, genomic data should be analysed from both areas from Castello, possibly with non-invasive methods due to the low abundance on the acidified sites. We hope that such studies will further increase our comprehension on near future acidification ecology and provide valuable insights for future studies on this topic.

7 Bibliography

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