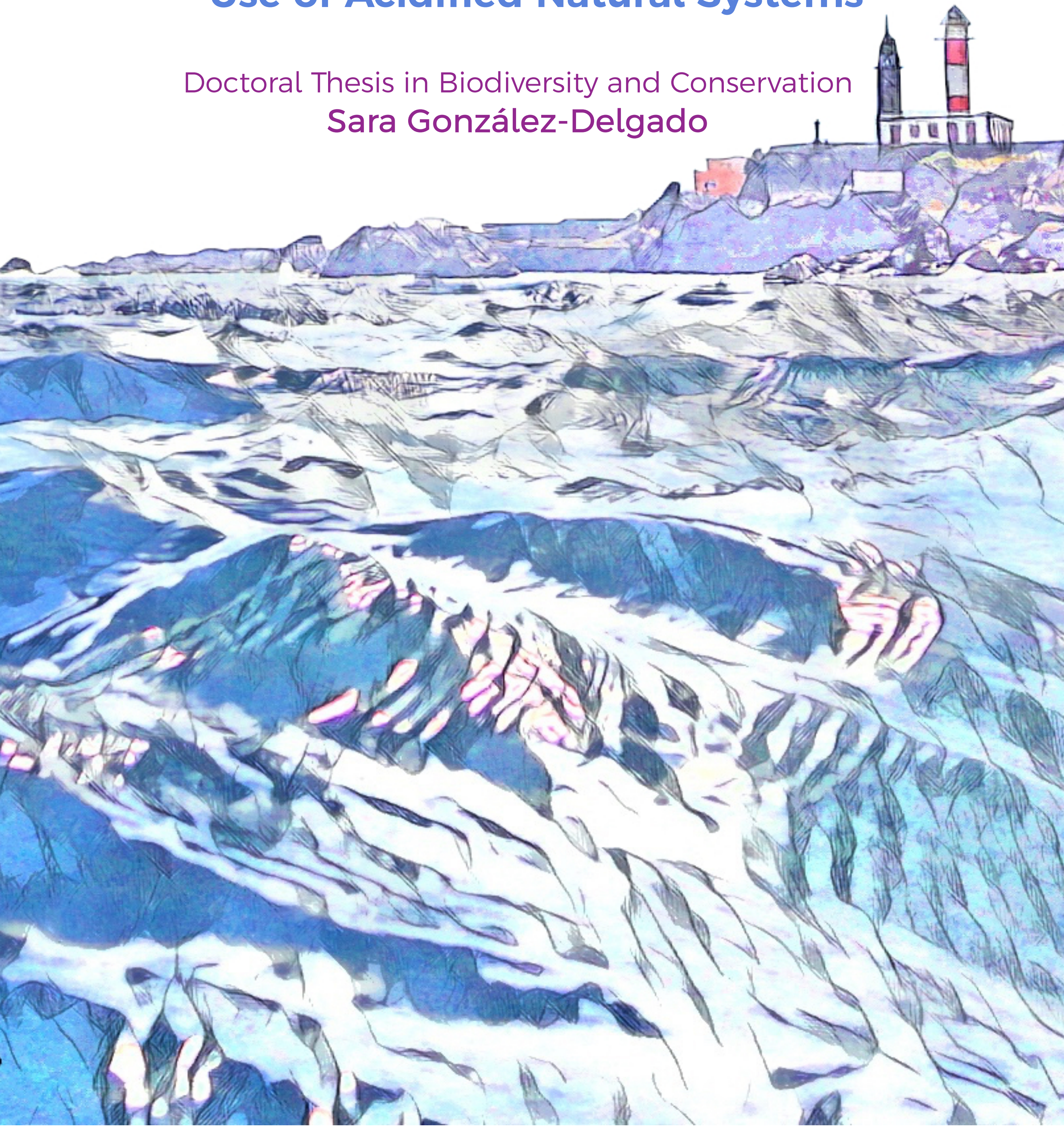
**Departamento de Biología**
**Animal, Edafología y Geología**
Universidad de La Laguna

# Marine Benthic Communities of the Future: Use of Acidified Natural Systems

Doctoral Thesis in Biodiversity and Conservation
**Sara González-Delgado**



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Que la tesis doctoral titulada 'Marine Benthic Communities of the Future: Use of Acidified Natural Systems', presentada por la graduada Dña. Sara González Delgado, ha sido realizada bajo su dirección en el Dpto. Biología Animal, Edafología y Geología de La Universidad de la Laguna y le otorgan un informe favorable para su defensa como Tesis Doctoral, en el Doctorado en Biodiversidad y Conservación de la Universidad de La Laguna.

Y para que conste a los efectos oportunos, firman la presente a día 19 de Julio de 2023.

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Fdo. Dra. Rocío Pérez Portela

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# GENERAL ABSTRACT

RESUMEN GENERAL

# GENERAL ABSTRACT

Ocean acidification (OA) is one of the most significant threats to marine organisms and is linked to climate change. It occurs when anthropogenic $CO_2$ is absorbed by the oceans, resulting in a decrease in seawater pH and the dissolution of calcium carbonate. Projections indicate that OA will exacerbate in the future, highlighting the need to understand its impact on marine ecosystems. Much of our knowledge about the effects of OA comes from laboratory experiments, as predicting responses in natural conditions is challenging. Therefore, studies focusing on species living in naturally acidified systems, such as shallow $CO_2$ seeps or vents, are becoming increasingly popular to obtain more realistic predictions.

This doctoral thesis, consisting of 5 chapters, explores the effects of ocean acidification on benthic communities in the subtropical Atlantic Ocean, using the naturally acidified $CO_2$ vent system off the southern coast of La Palma Island in the Canary Islands, Spain, as a natural laboratory.

**Chapter 1** serves as an introduction to this thesis, explaining what naturally acidified systems are and discussing the research conducted in various locations worldwide where they have been discovered. Specifically, it focuses on studies that have utilized $CO_2$ vents, which originate from volcanic activity. This chapter provides an overview of the importance, advantages, and disadvantages of using these acidified systems as natural laboratories to study OA *in situ*. It highlights that although there is no perfect analogue for future oceans, these systems help us to better understand the direct and indirect impacts of OA on different marine communities.

Among all the $CO_2$ vents in the world, one of the few naturally acidified shallow systems in the Atlantic Ocean, and the only one with subtropical communities is located off the southern coast of Fuencaliente municipality in La Palma Island, Canary Islands. **Chapter 2** of the thesis characterizes the chemical properties of this natural $CO_2$ system in La Palma. It provides

information about its volcanic and hydrological origins, as well as the different emission points along the Punta de Fuencaliente. Furthermore, it describes the carbon dynamics of the system, including variations in total inorganic carbon ($C_T$) from 2120.10 to 10784.84 µmol kg-1, alkalinity ($A_T$) from 2415.20 to 10817.12 µmol kg-1, pH from 7.12 to 8.07, aragonite saturation state (Ω) from 0.71 to 4.15, and calcite Ω from 1.09 to 6.49 units. A high $CO_2$ emission flux ranging from 2.8 to 28 kg of $CO_2$ d-1 has also been detected, making this zone an important natural carbon source. Due to its origins, this acidified system presents disadvantages as a natural laboratory for studying OA, such as natural fluctuations caused by tides or additional input of alkaline substances. Nevertheless, it creates a natural gradient of $CO_2$ or pH along the coast with chemical characteristics very similar to those predicted for future scenarios, making it an exceptional location for studying the long-term and multi-level effects of acidification on marine ecosystems.

**Chapter 3** explores rocky benthic communities along the natural pH gradient generated by the $CO_2$ vent system in front of Punta de Fuencaliente. The objective of this chapter was to understand the direct and indirect effects of OA on the diversity and species composition of these subtropical marine communities. The study utilized a high-resolution molecular technique called DNA metabarcoding, which sequences fragments of the mitochondrial gene Cytochrome C Oxidase subunit I (COI) to detect the actual species diversity in each area. In this chapter, metabarcoding analysis reveals, for the first time, high levels of taxonomic diversity in a naturally acidified area. These high levels of diversity are attributed to the detection of small and cryptic species that are undetectable by traditional techniques and are tolerant to natural acidification. The results of this chapter unveil that future subtropical communities could maintain high taxonomic diversity values under an acidification scenario, although they will tend toward miniaturization due to the dominance of small algae and invertebrate species. This will have significant consequences for benthic subtropical communities, leading to important changes in ecosystem functions.

It is not the first time that an increase in species diversity related to environmental variations has been detected. In 1978, Connell first proposed the "Intermediate Disturbance Hypothesis" (IDH), which suggests that ecosystems are more diverse when disturbances occur at intermediate scales. Chapter 4 investigates whether the IDH can be applied to a naturally acidified system at different biological organization levels (from organisms to communities) using molecular data. In La Palma's acidified system, a fluctuating pH gradient caused by tides can act as a physical disturbance to marine ecosystems. This chapter utilizes sequenced fragments of the mitochondrial COI gene from two species of sea urchins (*Arbacia lixula* and *Paracentrotus lividus*) and metabarcoding analyses of benthic communities from the previous chapter. High levels of genetic and taxonomic diversity were detected at both biological organization levels under intermediate pH fluctuation, respectively. Therefore, the results of this chapter support the validity of the IDH in marine ecosystems affected by natural pH fluctuations and at different biological organization levels.

Among the species living under natural acidification in the $CO_2$ vents of La Palma, the sea urchin *Arbacia lixula* stands out. This is because sea urchins, like other calcareous organisms, should be susceptible to acidification due to their calcareous skeletons, however, this species has been found to live apparently unaffected in both Mediterranean and Atlantic $CO_2$ vents. The final chapter 5 explores the adaptation potential of *A. lixula* populations along the natural pH gradient of La Palma Island. Using the 2bRADseq molecular technique, a total of 14,883 SNPs (Single Nucleotide Polymorphisms) were detected in 74 individuals, of which 432 loci were correlated with the pH gradient of La Palma and are considered potential SNPs under selection. Analysis of these SNPs demonstrates that despite the short distance between the studied *A. lixula* populations, significant

differences exist in the genomic structure of the populations correlated with the pH gradient. Additionally, these sequences are aligned and compared with available *A. lixula* transcriptomes, revealing 17 annotated genes involved in biological functions related to growth, development, membrane functions, and calcification. This chapter suggests that A. *lixula* can adapt to acidification and, therefore, able to withstand future changes anticipated for the oceans.

This thesis is the first to be developed at the Marine Observatory of Climate Change in Punta de Fuencaliente (OMaCC), where the naturally acidified system of La Palma is located. It emphasizes the importance of these natural laboratories in overcoming the experimental limitations of laboratory studies and contributes to understand how subtropical benthic ecosystems may change in the future. Moreover, it has uncovered evidence of local adaptation to ocean acidification in populations living in these natural laboratories. This thesis highlights the importance of these special environments and observatories for future research on the effects of OA.

## RESUMEN GENERAL

La acidificación de los océanos (AO) es una de las amenazas más importantes para los organismos marinos, y que se encuentra vinculada al cambio climático. Se produce cuando el $CO_2$ antropogénico es absorbido por los océanos, lo que provoca una disminución del pH del agua de mar y la disolución del carbonato cálcico. Las previsiones indican que el AO se agravará en el futuro, lo que subraya la necesidad de comprender su impacto en los ecosistemas marinos. Muchos de nuestros conocimientos sobre los efectos de la AO proceden principalmente de experimentos de laboratorio, ya que predecir las respuestas en condiciones naturales es todo un reto. Por ello, cada vez son más populares los estudios que estudian las especies que viven en sistemas acidificados de forma natural como los afloramientos de

$CO_2$ someros, con el fin de obtener predicciones más realistas. Esta tesis doctoral compuesta por 5 capítulos explora los efectos de la acidificación oceánica en las comunidades bentónicos subtropicales del Océano Atlántico, utilizando como laboratorio natural el sistema de afloramientos de $CO_2$ de la costa sur de la isla de La Palma en las islas Canarias, España.

El **capítulo 1** es la introducción de esta tesis, donde se explica que son los sistemas naturalmente acidificados y se dan a conocer las investigaciones llevadas a cabo en todas las zonas del mundo donde se han descubierto. Sobre todo, se discuten los estudios que han hecho uso de los afloramientos de $CO_2$, que deben su origen a la actividad volcánica de una o varias islas. Este primer capítulo ofrece una visión general de la importancia, y las ventajas y desventajas del uso de estos sistemas acidificados como laboratorio natural para el estudio *in situ* de la AO. Nos muestra que a pesar de que no existe el análogo perfecto de los océanos del futuro, estos sistemas nos ayudan a comprender mejor los impactos tanto directos como indirectos de la AO en las diferentes comunidades marinas.

De entre todos los afloramientos de $CO_2$ del mundo, se encuentra frente a la costa sur del municipio de Fuencaliente de la isla de La Palma, en las islas Canarias, uno de los pocos sistemas naturales acidificados someros del Océano Atlántico y el único con comunidades subtropicales. Este sistema natural de $CO_2$ de La Palma se caracteriza químicamente en el **capítulo 2** de la tesis. Se proporciona información sobre su origen volcánico e hídrico y los diferentes puntos de emisión existentes en toda la Punta de Fuencaliente. Además, se describe la dinámica de su sistema de carbono, existiendo variaciones de carbono inorgánico total ($C_T$) de 2120,10 a 10784,84 µmol kg-1, de alcalinidad ($A_T$) de 2415,20 a 10817,12 µmol kg-1, de pH de 7,12 a 8,07, del estado de saturación (Ω) del aragonito de 0,71 a 4,15 y Ω calcita de 1,09 a 6,49 unidades. También se detecta un alto flujo de emisiones de $CO_2$ que varía entre 2,8 y 28 kg de $CO_2$ d-1, lo que convierte esta zona además en una importante fuente de carbono natural. Debido a sus orígenes, este sistema acidificado tiene inconvenientes como laboratorio natural para el estudio de la AO, como unas fluctuaciones naturales debido a las mareas o el aporte extra de sustancia alcalinas. Aun así, se crea en la costa un gradiente natural de $CO_2$ o pH con característica químicas muy similares a los escenarios predichos para el futuro, lo que lo convierte en un lugar excepcional para estudiar los efectos a largo plazo y a distintos niveles de acidificación sobre los ecosistemas marinos.

En el capítulo 3 se exploran las comunidades bentónicas rocosas a lo largo de este gradiente natural de pH generado por el sistema de afloramientos de $CO_2$ enfrente de Punta de Fuencaliente. El objetivo de este capítulo fue conocer los efectos tanto directos como indirectos de la AO sobre las diversidad y composición de especies en estas comunidades marinas subtropicales. Para ello, se utilizó una técnica molecular de alta resolución denominada metabarcoding del ADN con un primer universal para eucariotas que secuencia fragmentos del gen mitocondrial Citocromo C Oxidasa subunidad I (COI) y que detecta la diversidad real de especies en un área determinada. En este capítulo, los análisis de metabarcoding revelan por primera vez altos niveles de diversidad taxonómica en un área naturalmente acidificada. Estos niveles de diversidad tan altos se deben a la detección de especies pequeñas y cripticas que son indetectables mediante técnicas tradicionales y que son tolerantes a la acidificación natural. Los resultados obtenidos en este capítulo desvelan que las futuras comunidades subtropicales podrían mantener altos valores de diversidad taxonómica bajo un escenario de acidificación, aunque tenderán a la miniaturización debido a la dominancia de pequeñas especies de algas e invertebrados. Esto traerá graves consecuencias a las comunidades bentónicas subtropicales puesto que conllevará a cambios importantes en las funciones de los ecosistemas.

No es la primera vez que se detecta un aumento de la diversidad de especies relacionado con variaciones ambientales. En 1978, Connell postuló por primera vez la denominada “Hipótesis de Perturbaciones Intermedias”

(HPI) que considera que los ecosistemas son inestables, siendo la diversidad de especies mayor cuando existen perturbaciones a escalas intermedias. En el **capítulo 4** se investiga si la HPI puede aplicarse a un sistema natural acidificado y a diferentes escalas de organización biológicas (desde el organismos a las comunidades), utilizando datos moleculares. En el sistema acidificado de La Palma se puede encontrar un gradiente de pH fluctuante debido a la marea, que puede actuar como perturbación física de los ecosistemas marinos. En este capítulo se usan fragmentos secuenciados del gen mitocondrial COI de dos especies de erizos de mar (*Arbacia lixula* y *Paracentrotus lividus*) y los análisis de metabarcoding de las comunidades bentónicas sacados del capítulo anterior. Para ambos niveles de organización biológica se detectaron altos niveles de diversidad genética y de diversidad taxonómica bajo una fluctuación de pH intermedia, respectivamente. Por lo tanto, los resultados de este capítulo apoyan la validez del HPI en ecosistemas marinos que se ven afectadas por fluctuaciones de pH naturales y a distinto niveles de organización biológica.

Dentro de las especies que podemos encontrar viviendo bajo acidificación natural en los afloramientos de $CO_2$ de La Palma, destaca el erizo de mar *Arbacia lixula*. Los erizos de mar, como cualquier otro organismo calcáreo deberían ser susceptible a la acidificación al poseer esqueleto calcáreo, sin embargo esta especie se ha encontrado viviendo aparentemente sin efectos negativos en los afloramientos de $CO_2$ tanto del Mediterráneo como del Atlántico. El **capítulo 5** y último, explora el potencial de adaptación de las poblaciones de *A. lixula* a lo largo del gradiente natural de pH de la isla de La Palma. Gracias a la técnica molecular de 2bRadseq se detecta en 74 individuos un total de 14,883 SNPs, de los cuales 432 loci se correlacionaron con el gradiente de pH de La Palma y por lo tanto son considerados potenciales SNPs que están bajo selección. El análisis de estos SNPs demuestra que a pesar de la corta distancia existente entre las poblaciones de *A. lixula* estudiadas, existen diferencias significativas en la estructura genómica de las poblaciones correlacionada con el gradiente de pH. Además, se alinean y se comparan estas secuencias con los transcriptomas de *A. lixula* disponibles, encontrándose 17 genes anotados implicados en funciones biológicas relacionadas con crecimiento y el desarrollo, con funciones de la membrana y relacionadas con la calcificación. Este capítulo sugiere que las poblaciones de *A. lixula* pueden adaptarse a la acidificación y, por lo tanto, resistir los futuros cambios que tendrán lugar en los océanos.

Esta tesis es la primera desarrollada en el Observatorio Marino del Cambio Climático de Punta de Fuencaliente (OMaCC), donde se encuentra el sistema natural acidificado de La Palma. Con ella, se realza la importancia de estos laboratorios naturales para hacer frente a las limitaciones experimentales de los estudios en laboratorios y contribuye a la comprensión de cómo los ecosistemas bentónicos subtropicales pueden cambiar en el futuro. Además, se descubren evidencias de una adaptación local a la acidificación oceánica en las poblaciones que viven en estos laboratorios naturales. Con esta tesis, queda evidente la importancia de estos entornos especiales y observatorios para las futuras investigaciones sobre los efectos del AO.

# CHAPTER 1

## The Importance of Natural Acidified Systems in the Study of Ocean Acidification: What Have We Learned?

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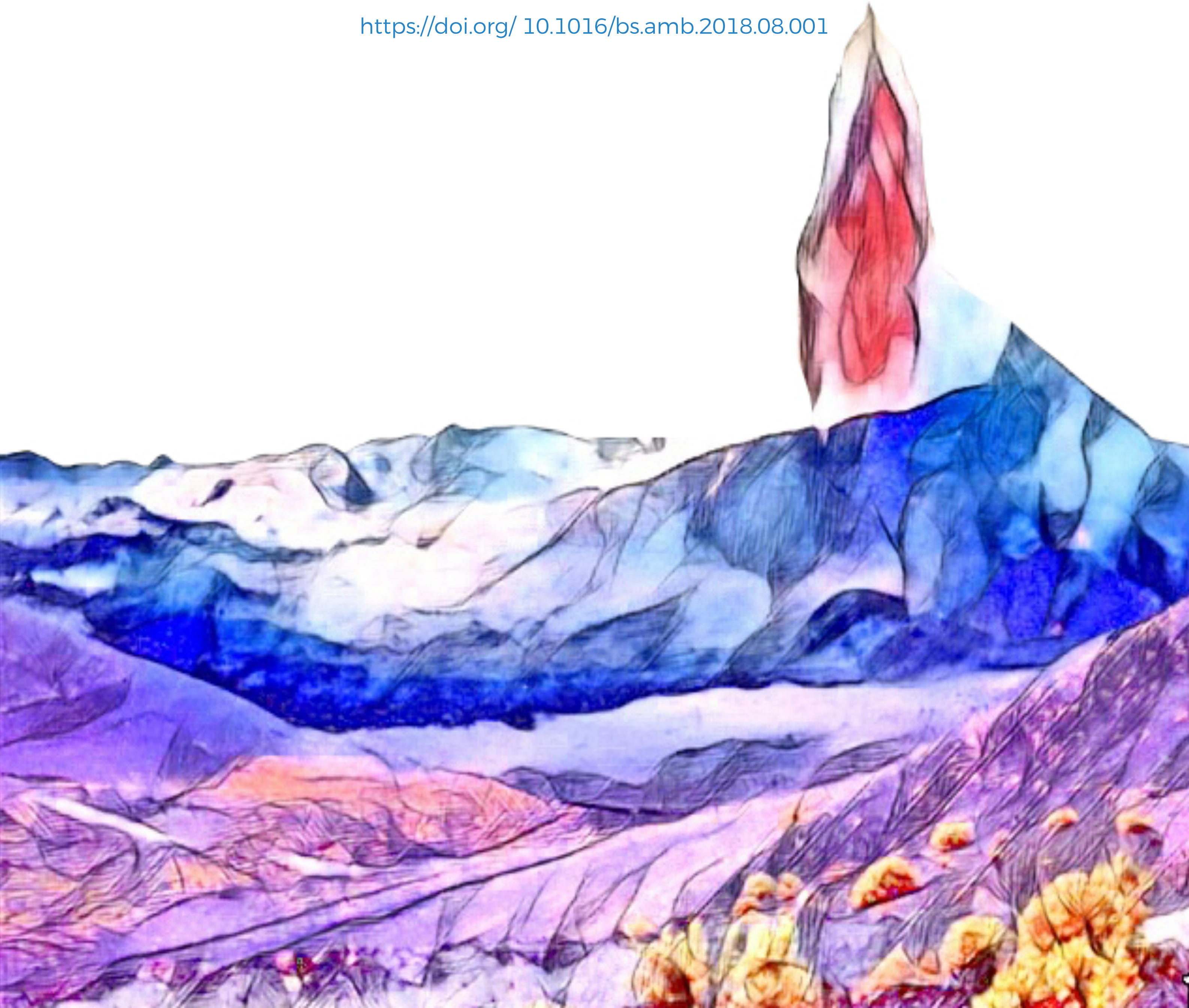


## ABSTRACT

Human activity is generating an excess of atmospheric carbon dioxide ($CO_2$), resulting in what we know as ocean acidification, which produces changes in marine ecosystems. Until recently, most of the research in this area had been done under small-scale, laboratory conditions, using few variables, few species, and few life cycle stages. These limitations raise questions about the reproducibility of the environment and about the importance of indirect effects and synergies in the results of these experiments. One way to address these experimental problems is by conducting studies *in situ*, in natural acidified areas where expected future pH conditions already occur, such as $CO_2$ vent systems. In the present work, we compile and discuss the latest research carried out in these natural laboratories, with the objective to summarize their advantages and disadvantages for research to improve these investigations so they can better help us understand how the oceans of the future will change under ocean acidification.

Keywords: Climate change, pH gradient, marine communities, ecology, $CO_2$ seeps.

## RESUMEN

La actividad humana está generando un exceso de dióxido de carbono ($CO_2$) atmosférico dando lugar a lo que conocemos como acidificación de los océanos, que está produciendo cambios en los ecosistemas marinos. Hasta hace poco, la mayor parte de la investigación en este ámbito se han llevado a cabo en condiciones de laboratorio húmedo, a pequeña escala, y estudiando pocas variables, pocas especies y pocas etapas del ciclo vital. Estas limitaciones plantean dudas sobre la reproducibilidad del medio ambiente y sobre la importancia de los efectos indirectos y las sinergias en los resultados finales de estos experimentos. Una forma de abordar estos problemas experimentales es realizar estudios *in situ* en zonas naturalmente acidificadas donde ya se dan las condiciones de pH previstas para el futuro, como los sistemas de afloramientos de $CO_2$. En el presente trabajo, recopilamos y discutimos las últimas investigaciones llevadas a cabo en estos laboratorios naturales, con el objetivo de resumir sus ventajas e inconvenientes para que mejore estas investigaciones y así nos ayude a entender mejor cómo cambiarán los océanos del futuro bajo la acidificación oceánica.

Palabras claves: Cambio climático, gradiente de pH, comunidades marinas, ecología, surgencias de $CO_2$.

## 1.1. THE QUESTION

Since the industrial revolution, human activity has produced an excess of atmospheric carbon dioxide ($CO_2$) due to the use of fossil fuels. One-third of this anthropogenic gas has been absorbed by the oceans, helping to mitigate the harmful effects of climate change in the atmosphere (Sabine et al., 2004), but the resulting changes in the carbon (C) chemistry of seawater have triggered a decrease in pH of up to 0.1 units in recent years, causing ocean acidification (OA) (Feely et al., 2004; IPCC, 2014). Acidification occurs when $CO_2$ is dissolved in seawater, forming carbonic acid ($H_2CO_3$) that rapidly dissociates into bicarbonate ($HCO_3^-$) and hydrogen atoms ($H^+$), lowering the pH of the water. The protons can combine with carbonate ion molecules ($CO_3^{2-}$), which are normally free in water, to produce more ($HCO_3^-$) and decrease the formation of calcium carbonate ($CaCO_3$). This chemical imbalance generates a series of direct and indirect negative consequences for marine life, of which organisms with external skeletons or protective shells, such as corals, crustaceans, molluscs and single- celled organisms, or those with endoskeletons, such as echinoderms, are the most affected because obtaining the $CaCO_3$ they need to form these structures becomes more difficult (Doney et al., 2012; Gattuso et al., 2013; Kroeker et al., 2010; Orr et al., 2005).

The problem is exacerbated by the continuous emission of anthropogenic gases into the atmosphere. According to recent predictions, an increase in $pCO_2$ between 750 and 900 ppm is expected by the year 2100 (Meinshausen et al., 2011) which implies a drop in seawater pH by 0.2–0.6 units in addition to a decrease in the aragonite and calcite saturation states ($\Omega$) between 1.5 and 4 units (IPCC, 2014). Preventing these changes will depend on our capacity to replace fossil fuels with renewable energy (Feely et al., 2004).

Predicting the effects of these changes in future oceans is one of the most important questions facing researchers today. Studying the effects of OA poses a great challenge because predicting future conditions is quite complex. Experimental studies carried out to date have helped us predict the possible responses of many organisms to the chemical changes produced by seawater acidification, so we have been able to estimate the likely negative effects on communities and species as well as their different life stages (Byrne et al., 2013; Dupont and Thorndyke, 2008; Hendriks et al., 2010; Kroeker et al., 2010). The most recent studies have also accounted for the interaction between OA and global warming (Byrne and Przeslawski, 2013; García et al., 2015; Harvey et al., 2013; Hernández et al., 2018b).

However, most results have so far come from experimental manipulations under laboratory conditions, i.e., in tanks or aquariums, which limits the inferences that can be drawn. The greatest challenges when predicting the effects of acidification include performing studies over large temporal or spatial scales, such as with increasing the number of studied species and the different stages of their life cycles, with studying whole populations or communities, and by including the natural variability inherent to the studied systems (Garrard et al., 2013; Hernández et al., 2016). Therefore, many questions have been raised about the reproducibility of natural conditions, about several important indirect effects such as natural variability, and about the ecological interactions between different species. Addressing these issues would ensure a more realistic prediction of the effects of acidification on marine ecosystems.

One approach to address these problems is to conduct studies in natural environments characterized by the expected future conditions, such as lower pH and lower concentrations of aragonite and calcite, which can be achieved by two methods: artificially pumping $CO_2$ into natural environments and creating mesocosms (Liu et al., 2017) or, perhaps more realistically, using areas with permanent volcanic activity that produce excess $CO_2$ known as acidified systems to study ocean acidification (AS). The first study using an AS was carried out by Hall-Spencer et al. (2008) and was widely accepted by the climate change research community. Since then, an increasing number of published studies have been performed in these areas.

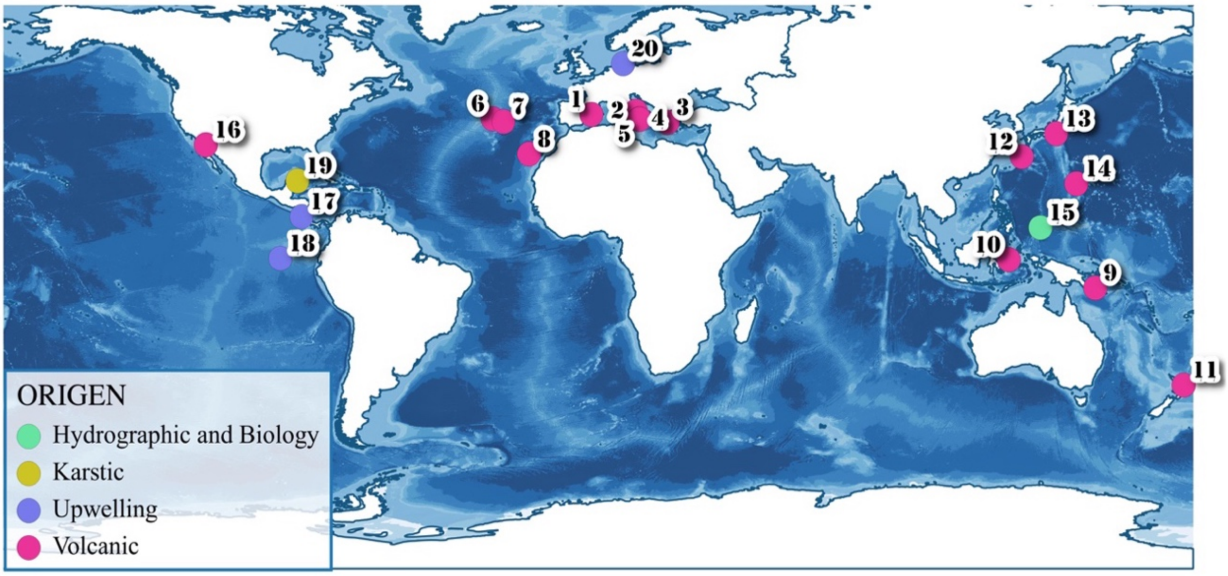
With this review, we intend to produce an exhaustive compilation of the works published to date that have been carried out in AS. We intend to compare the characteristics of the studied areas, organisms and communities and discuss the main impacts that have been found. Thus, we summarize the advantages and disadvantages of AS as well as the studied biological and ecological processes to offer a global and detailed view of the current research and provide recommendations for future studies. This review will be useful for researchers studying the impact of climate change, especially OA, on the oceans.

## 1.2. 'NATURAL LABORATORIES' IN THE STUDY OF OA

For the purposes of this review, we define an AS to be an area with a higher-than-normal $CO_2$ input that is of natural origin and sufficient to acidify the surrounding marine environment. We have selected coastal and shallow areas, not more than 200 m deep, in the euphotic zone, where the diversity of marine habitats is greatest. We have found a total of 30 AS matching this definition in different biogeographic regions (temperate, sub- tropical and tropical) whose origins are usually volcanic ($CO_2$ vents, seeps), although the origin might also be karstic (acid lagoons), biological or even generated by the upwelling of nutrient-rich water (Figure 1.1).

### 1.2.1. Carbon Dioxide Vent Systems of Volcanic Origin

Natural $CO_2$ vent or seep systems are subtidal areas affected by the emission of gases of volcanic origin, and they are rich in $CO_2$. They can be found where there is or has been recent underwater volcanic activity, such as mid-oceanic ridges or island arcs, or intra-plate magmatism (Tarasov et al., 2005). These active zones can be found in several areas of the planet; we have identified 23 such $CO_2$ vent systems where research has occurred (Figure 1.1).



| | | | | | |
|---|---|---|---|---|---|
| 1 | Columbretes Islands (Spain) | 8 | La Palma (Canary Islands) | 15 | Palau (Oceania) |
| 2 | Ischia (Italy) | 9 | Papua New Guinea | 16 | Wagner Basin (Northern Gulf of California) |
| 3 | Methana (Greece) | 10 | Indonesia | 17 | Bahia Culebra (Gulf of Papagayo) |
| 4 | Panarea (Italy) | 11 | White Island (New Zealand) | 18 | Eastern Tropical Pacific |
| 5 | Vulcano (Italy) | 12 | Iwatorishima (Japan) | 19 | Puerto Morelos (Mexico) |
| 6 | Faial (Azores) | 13 | Shikine Island (Japan) | 20 | Kiel Fjord (Baltic Sea) |
| 7 | San Miguel (Azores) | 14 | Maug (Mariana Islands) | | |

Figure 1.1. Locations in the different ocean of the 20 acidified systems to study ocean acidification (AS) that have been included in this study. Colours indicate the origin of each AS: 'Hydrographic and Biology', 'Kartic', 'Upwelling' and 'Volcanic'.

Because it is the focus of most publications, we consider the Mediterranean Sea to be the starting point for the development of this new line of research (Figure 1.1). The pioneering work on the vent system of Ischia Island (Castello d'Aragonese) in Italy was published by Hall-Spencer et al. (2008), and numerous studies have been conducted in this area since then, and even before (but see Foo et al., (2018b), Hall-Spencer and Rodolfo-Metalpa, (2012) for a more detailed information), followed by studies on the island of Vulcano (Bahia de Levante), the Panarea Islands (also Italian), Methana, in Greece and in an area near the Columbretes Islands in Spain (Figure 1.1). All these vent systems are in temperate areas and are characteristic of the Mediterranean, but they differ in their physico-chemical conditions (Table 1.1) and can be divided into two groups: one group consisting of the area near the Columbretes Islands and the Panarea Islands, which would include vents between 5 and 40 m deep with a small variation in pH of no more than 0.7 units (Linares et al., 2015; Prada et al., 2017), and a second group including all other Mediterranean vent systems, where the emissions occur less than 3 m deep but with variations in the ambient pH as well as the dissolved inorganic C (DIC) and the percent saturation (Ω) of aragonite and calcite of more than 1.5 units (Table 1.1).

The vent systems in the Atlantic Ocean include the Sao Miguel and Faial system in the Azores and the most recently discovered system on the island of La Palma in the Canary Islands, all of which are in the Macaronesian Region (Figure 1.1). The pH levels in these vent systems are not as low as those in the Mediterranean and are always above pH 7 (Table 1.1). In particular, the pH does not decrease by more than 0.2 units in the Faial vent system, and acidification only occurs below 15 m in depth (Campoy, 2015). In contrast, although more shallow (changes can already be detected on the surface), the other two vent systems show greater variations in aragonite Ω, especially the island of La Palma, and in calcite Ω in both La Palma and Sao Miguel (Campoy, 2015; Hernández et al., 2016) (Table 1.1).

In the Pacific Ocean, many natural vent systems have been studied that are characterized by high tectonic and volcanic activity (see Figure 1.1). The first three systems studied in this part of the world are in the province of Milne Bay, Papua New Guinea (PNG) (Dobu, Esá Ala and Upa-Upasina) that were also the first locations for the study of $CO_2$ emissions in tropical ecosystems supporting prolific coral reefs (Fabricus et al., 2011). We have found up to seven areas with vent systems of volcanic origin with favourable conditions for the study of OA in the Pacific Ocean (Table 1.1 and Figure 1.1), but except for PNG with more than 30 published articles and the White Islands of New Zealand with eight published articles, the other vent systems have not been used in more than one or two studies, to our knowledge. However, some of these studies were quite comprehensive and of great relevance, such as those conducted in the Marianas Islands (Enochs et al., 2015) and at Iwa Torishima in Japan (Inoue et al., 2013). The other two studies, which have gone largely unnoticed, were based in a system in Indonesia (Januar et al., 2016) and on the Mikama and Ashitsuke vent systems on Shikine Island, Japan, where only water chemistry has been studied (Agostini et al., 2015) (see Figure 1.1). We also recently became aware of a vent system in Mainit, the Philippines (Dr. Michael Roleda, personal communication) and in Roca Redonda, the Galapagos Islands, Ecuador (OA-ICC, 2018).

Lower pH, and thus differences in other chemical parameters, has been detected in the vent systems of PNG and the island of Shikine, where the ambient pH is decreased by 1.3 units (Table 1.1). In contrast, the emissions at the White Islands, New Zealand, are distinguished by the depths that they affect. The emissions do not reach the surface, but acidic conditions are present from 8 to 10 m below the surface. In addition, the location is one of the few natural $CO_2$ vent systems where an increase in ambient temperature, between 0.2 and 0.3 °C, has been detected (Brinkman and Smith, 2015) (Table 1.1). Therefore, the vent system in New Zealand is the only known AS to date that would allow the study of the combined effects of acidification and global warming. However, vents with the appropriate combined features and with no toxic gases nor higher concentrations of trace elements are very limited in extension (Tarasov, 2006).

Finally, an exceptional case of a vent system of the Gulf of California is currently the only AS at deeper levels of the euphotic layer (70 m), as it has a pH of up to 7.56 and variations in aragonite and calcite Ω values of up to 2–3 units (Prol-Ledesma et al., 2013) (Table 1.1). Another interesting deep vent system that deserves further attention is the young submarine volcano of El Hierro, where significant discharge of $CO_2$ has been recently detected (Santana-Casiano et al., 2016).

**Table 1.1.** Comparison of the physicochemical properties of all known AS, located in different parts of the world: range of depth (D(m)); temperature of the affected area ($T^a$), where 'Warmer' indicates temperatures 0.2–0.3 °C higher and 'Colder' indicates 1–5 °C lower than 'Normal'; and saturation state of aragonite (ΩAra) and calcite (ΩCal). The bottom row of the table includes predicted values according to the IPCC (2014), to compare with the predictive framework of each AS.

| | D(m) | $T^a$ | $pH_{am}$ | G pH | DIC | ΩAra | ΩCal |
|---|---|---|---|---|---|---|---|
| Mediterranean Sea | | | | | | | |
| Columbretes Islands (Spain)[1] | 5 - 40 | Normal | 8.1 | 7.95 - 7.8 | 2.3 - 2.4 | 2.26 - 1.78 | 3.51 - 2.77 |
| *Ischia (Italy)[2] | 0 - 5 | Normal | 8.2 | 8.1 - 6.6 | 1.77 – 3.16 | 3.66 - 0.19 | 5.63 – 0.3 |
| Methana (Greece)[3] | 0 - 2 | Normal | 8.1 | 8.1 - 6.53 | 2.56 - 3.21 | 4.18 - 0.09 | 6.29 - 0.13 |
| *Panarea (Italy)[4] | 8 - 23 | Normal | 8.2 | 8.17 - 7.41 | 2.11 -2.32 | 3.6 - 1.4 | 5.1 - 1.29 |
| Vulcano (Italy)[5] | 0 - 3 | Normal | 8.2 | 8.2 - 6.8 | 2.23 - 2.66 | 4.65 - 1.49 | 7 - 2.28 |
| Atlantic Ocean | | | | | | | |
| Faial (Azores)[6] | 15 - 38 | Normal | 8.1 | 8.1 - 7.88 | 2.02 - 2.17 | 4.5 - 3.54 | 5.57 - 2.78 |
| San Miguel (Azores)[7] | 0 - 10 | Normal | 8.1 | 8.1 - 7.75 | 2.06 - 2.27 | 3.13 - 1.83 | 5.3 - 2.83 |
| La Palma (Canary Islands)[8] | 0 - 5 | Normal | 8.1 | 8.0 - 7.6 | 2.81 - 3.15 | 4.35 - 1.73 | 6.65 - 2.64 |
| Pacific Ocean | | | | | | | |
| *Papua New Guinea[9] | 0 - 5 | Normal | 8.0 | 7.98 - 7.29 | 1.97 - 2.44 | 3.4 - 1.2 | 5.12 - 1.36 |
| Indonesia[10] | 0 - 4 | Normal | 8.2 | 8.01 - 7.8 | 1.90 - 1.99 | 4.85 - 2.48 | 7.29 - 3.73 |
| White Island (New Zealand)[11] | 8 - 10 | Warmer | 8.1 | 8.06 - 7.86 | 2.05 - 2.13 | 2.7 - 1.85 | 4.17 - 2.86 |
| Iwatorishima (Japan)[12] | 0 - 3 | Normal | 8.3 | 7.81 - 7.4 | 2.14 - 2.25 | 2.25 - 1.12 | 3.4 - 1.69 |
| *Shikine Island (Japan)[13] | 0 - 20 | Normal | 8.1 | 8.1 - 6.8 | 2.03 - 2.54 | 2.22 - 0.2 | 3.45 - 0.3 |
| Maug (Mariana Islands)[14] | 0 - 5 | Normal | 8.0 | 8.07 - 7.7 | 1.94 – 2.05 | 4.00 - 2.7 | 6.1 - 3.11 |
| Palau (Oceania)[15] | 0 - 10 | Normal | 8.1 | 8.05 - 7.84 | 1.77 - 1.85 | 3.73 - 2.34 | 5.51 - 3.43 |
| Wagner Basin (Northern Gulf of California)[16] | 74 - 207 | Normal | 8.2 | 7.94 - 7.56 | 2.29 - 2.33 | 3.23 - 1.19 | 4.87 - 1.83 |
| Bahía Culebra (Gulf of Papagayo)[17] | > 0.3 | Colder | 8.2 | 8.02 - 7.91 | 1.8 – 2.10 | 3.41 – 2.71 | - |
| *Eastern Tropical Pacific[18] | 0 - 5 | Colder | 8.1 | 8.07 - 7.65 | 1.61 - 2.1 | 3.09 - 2.46 | 4.26 - 3.64 |
| Caribbean Sea | | | | | | | |
| Puerto Morelos (Mexico)[19] | 4 - 7 | Colder | 8.0 | 7.29 - 6.61 | 3.18 - 3.33 | 0.87 - 0.50 | 1.32 - 0.77 |
| Baltic Sea | | | | | | | |
| Kiel Fjord (Central Europe)[20] | 1 - 20 | Colder | 8.2 | 8.1 - 7.49 | 2.04 - 2.07 | 0.79 - 0.47 | 1.36 - 0.79 |
| In the Future 2100[21] | Surface | Warmer | 7.8 – 7.4 | | - | < 3 - 2 | < 4 - 3 |

*This area includes several vent systems or upwellings spots in that location (Agostini et al., 2015; Fabricius et al., 2011; Foo et al., 2018b; Manzello, 2010; Molari et al., 2018). 1(Linares et al., 2015); 2(Hall-Spencer et al., 2008; Lombardi et al., 2011; Ricevuto et al., 2014); 3(Baggini et al., 2014); 4(Goffredo et al., 2014; Molari et al., 2018; Prada et al., 2017; Rogelja et al., 2016); 5(Boatta et al., 2013; Johnson et al., 2013); 6,7(Campoy, 2015); 8(Hernández et al., 2016); 9(Fabricius et al., 2011; Takahashi et al., 2016); 10(Januar et al., 2017); 11(Brinkman and Smith, 2015); 12(Inoue et al., 2013); 13(Agostini et al., 2015); 14(Enochs et al., 2015); 15(Shamberger et al., 2014); 16(Prol-Ledesma et al., 2013); 17(Sánchez-Noguera et al., 2018); 18(Manzello, 2010); 19(Crook et al., 2012, 2013, 2016); 20(Thomsen et al., 2010, 2013); 21(IPCC, 2014).

### 1.2.2. Natural Laboratories of Non-Volcanic Origin

The karstic or acidic lagoons of Puerto Morelos, in the Gulf of Mexico, are other areas that can be considered AS (Crook et al., 2016). They owe their origin to the chemical phenomenon of karstification, which essentially consists of the dissolution of karstic limestone by the slightly acidic rainwater that flows through caves and underground fractures into the ocean (Beddows et al., 2007). This underground acidic water is increasingly mixed with seawater in aquifers, resulting in salinity and temperature almost identical to that of the sea but with a higher DIC content and thus high alkalinity and low pH (Paytan et al., 2014). These lagoons, also called 'ojos' (eyes) by Crook et al. (Crook et al., 2012), are located in the coral reef of the Maritime National Park of the Yucatan Peninsula, so they represent a unique location for studying these habitats in the Caribbean Sea (Figure 1.1). However, due to the origin of its groundwater, the results of studies in this system must be interpreted with caution because both the present pH and $CaCO_3$ saturation values are not comparable to those expected in the future (Table 1.1), as well as the detected changes in salinity (Crook et al., 2012).

Another AS has been found in the Rocas Islands of Palau (Oceania), and it originated due to hydrographic-biological processes. Its cause is like acidic lagoons; that is, the system forms due to changes in water chemistry caused by rainwater combined with the high rate of respiration in the coral bay and the discharge of groundwater with high $CO_2$ levels (Shamberger et al., 2014). This area stands out for having a pH of approximately 7.8 without temporal fluctuations (Table 1.1).

Finally, other areas we consider to be AS are those affected by cold-water upwellings that are rich in inorganic C. To date, four such upwelling systems have been studied in very different habitats: three areas in the Eastern Tropical Pacific (ETP), i.e., the Galapagos, the Gulf of Panama and the Gulf of Chiriqui (Manzello et al., 2008), the Bay of Kiel in the Baltic Sea (Thomsen et al., 2010), as well as the Papagayo upwelling system in the north Pacific Costa

Rica (Sánchez-Noguera et al., 2018) (Figure 1.1). Due to their deep origin, the water in these systems is acidic but also colder than the ambient temperature, which can cause a decrease in environmental temperature of up to 5 °C (Table 1.1). Furthermore, there are many temporal and spatial variations that are mainly due to ocean currents, the bathymetry of the area and wind force (Manzello, 2010; Sánchez-Noguera et al., 2018; Thomsen et al., 2013).

### 1.2.3. Caveats

Due to the nature of $CO_2$ emissions, there are factors in most AS that can affect OA research to a greater or lesser extent. These factors include pH fluctuations and thus fluctuations of the calcite and aragonite $\Omega$ values (Table 1.1), which depend on the amount of $CO_2$ and other gases emitted, the concentration of nutrients and the presence of bubbling, which can affect the AS environment both mechanically and acoustically (Table 1.2).

On the other hand, vent systems and acidic lagoons trend towards intense temporary fluctuations in pH due to coastal dynamics (Crook et al., 2012; Kerrison et al., 2011). Tides, currents, or thermoclines can temporarily dissipate the emitted $CO_2$, thus affecting the overall water chemistry. In the case of upwelling systems, punctuated, discontinuous emissions can also produce very marked fluctuations (Sánchez-Noguera et al., 2018).

One of the most important disadvantages of using AS of volcanic origin is the presence of undesirable associated chemicals. Surge emissions are composed of 90–99% $CO_2$, with the known exceptions of the Gulf of California (78%) and Maug (61.1%) (Table 1.2). Volcanic emissions are always associated with other gases or elements, such as nitrogen ($N_2$), oxygen ($O_2$), argon (Ar), dihydrogen ($H_2$), helium (He), mercury (Hg), methane ($CH_4$), hydrogen sulphide ($H_2S$) and others, which usually make up 1–20% of the remaining volume (Table 1.2). These gases, although present in small quantities, could affect research results, especially in the case of sulphides or $CH_4$. Therefore, special attention must be paid to these aspects when choosing the AS for particulate OA studies. For example, in the case of Vulcano, Italy (Boatta et al., 2013) and Greece (Baggini et al., 2014), no negative effects have been observed to be associated with these elements, but in the Shikine Islands of Japan (Agostini et al., 2015), the toxic concentrations of $H_2S$ definitely affect organisms and cannot be used for certain OA studies. Moreover, complex spatial dynamics of trace elements occur in the $CO_2$ vents that can also bias the use of $CO_2$ vents as analogues future oceans (Vizzini et al., 2013). Nevertheless, careful monitoring of such toxic emissions and elements throughout an investigation is always desirable.

Table 1.2. Comparison of the physicochemical characteristics of all known AS. Other elements that accompany the gas emissions (Other gas E); presence of bubbling in the vent zone (bubbles); high concentrations of nutrients from the vent (↑[Nu]); and minimum exposure time in years (Y. ex).

| | Origin | %$CO_2$ | Other gas E | Bubbles | ↑[Nu] | Y. ex |
|---|---|---|---|---|---|---|
| Mediterranean Sea | | | | | | |
| Columbretes Islands (Spain)[1] | Volcanic | 90 | $N_2$, $O_2$, $CH_4$ | YES | NO | - |
| *Ischia (Italy)[2] | Volcanic | 90.1 - 95.3 | $N_2$, $O_2$, Ar, $CH_4$ | YES | - | 188 |
| Methana (Greece)[3] | Volcanic | 97 - 99.1 | $N_2$, $O_2$, He, $H_2$, $CH_4$ | YES | NO | 2200 |
| *Panarea (Italy)[4] | Volcanic | 98 – 99 | $N_2$, $O_2$, Ar, $CH_4$, $H_2S$ | YES | YES | 10 |
| Vulcano (Italy)[5] | Volcanic | 98 | $N_2$, $O_2$, $H_2$, $CH_4$, $H_2S$ | YES | YES | 10000 |
| Atlantic Ocean | | | | | | |
| Faial (Azores)[6] | Volcanic | 98.6 - 99.3 | $N_2$, He, $O_2$+Ar | YES | YES | - |
| San Miguel (Azores)[7] | Volcanic | 99.9 - 99.5 | $N_2$, He, $O_2$+Ar, $CH_4$, $H_2S$ | YES | - | - |
| La Palma (Canary Islands)[8] | Volcanic | 99 | Si | NO | NO | 45 |
| Pacific Ocean | | | | | | |
| *Papua New Guinea[9] | Volcanic | 99 - 98 | $N_2$, $O_2$, $CH_4$, $H_2S$ | YES | YES | 80 |
| Indonesia (Pacific)[10] | Volcanic | - | $H_2S$ | YES | YES | 29 |
| White Island (New Zealand)[11] | Volcanic | 99 | $N_2$, Hg, $CH_4$, $SO_2$ | YES | NO | - |
| Iwatorishima (Japan)[12] | Volcanic | - | $H_2S$ | - | - | 50 |
| *Shikine Island (Japan)[13] | Volcanic | 98 | $H_2S$ | YES | - | - |
| Maug (Mariana Islands)[14] | Volcanic | 61.1 | $N_2$, Ar, $CH_4$, S | YES | - | - |
| Palau (Oceania)[15] | Hydrographic and Biology | | | NO | NO | - |
| Wagner Basin (Northern Gulf of California)[16] | Volcanic | 78 | $CH_4$ | YES | - | - |
| Bahía Culebra (Gulf of Papagayo)[17] | Upwelling | | | NO | YES | - |

| | | | | |
|---|---|---|---|---|
| *Eastern Tropical Pacific[18] | Upwelling | NO | YES | - |
| Caribbean Sea | | | | |
| Puerto Morelos (Mexico)[18] | Karstic | NO | YES | 18000 |
| Baltic Sea | | | | |
| Kiel Fjord (Central Europe)[19] | Upwelling | NO | YES | - |

*This area include several vent systems or upwellings spots in that particular location (Agostini et al., 2015; Fabricius et al., 2011; Foo et al., 2018b; Manzello, 2010; Molari et al., 2018). 1(Linares et al., 2015); 2(Foo et al., 2018b; Hall-Spencer et al., 2008; Kroeker et al., 2011; Lombardi et al., 2011); 3(Baggini et al., 2014); 4(Goffredo et al., 2014; Prada et al., 2017; Rogelja et al., 2016); 5(Boatta et al., 2013; Johnson et al., 2013); 6,7(Campoy, 2015); 8(Hernández et al., 2016); 9(Fabricius et al., 2011; Takahashi et al., 2016); 10(Januar et al., 2017); 11(Brinkman and Smith, 2015); 12(Inoue et al., 2013); 13(Agostini et al., 2015); 14(Enochs et al., 2015); 15(Shamberger et al., 2014); 16(Prol-Ledesma et al., 2013); 17(Sánchez-Noguera et al., 2018); 18(Manzello, 2010); 19(Crook et al., 2012, 2016); 20(Thomsen et al., 2010, 2013).

Another factor to emphasize that has normally been disregarded is bubbling. The gases dissolved in seawater come from continuous bubbling in areas with volcanic activity (Figure 1.2), which is a clear environmental change that is not considered in future ocean acidification scenarios and can interfere with experiments, thus affecting the results obtained in these AS. For example, continuous bubbling can both prevent the establishment of some organisms as well as generate acoustic turbulence that can alter the perception of the surrounding environment by some animals, thus affecting the development and behaviour of larvae and juveniles seeking refuge or food (Aguilar De Soto et al., 2013; Montgomery and Radford, 2017). Therefore, it is necessary to avoid this negative factor when searching for an AS. To our knowledge, the only natural vent system of volcanic origin discovered so far that does not present bubbling is south of the island of La Palma (Hernández et al., 2016) (Figure 1.2). Of course, there are also non-volcanic AS (Table 1.2).

Finally, increased nutrient concentrations have also been detected in some vent systems and in acidic lagoons, as is generally expected in areas affected by upwelling events (Table 1.2). In these cases, most authors argue that these elements do not influence investigations because they are present in very low concentrations and/or in very specific areas, such as in Ischia, Italy (Ravaglioli et al., 2017), PNG (Fabricius et al., 2015) or in Iwa Torishima (Inoue et al., 2013), among others. However, conflicting responses of different organisms, such as the excessive proliferation of Padina pavonica in the Vulcano vent system (Celis-Plá et al., 2015) or the complete disappearance of coral reefs in ETP (Manzello et al., 2014), may be favoured by the increase in nutrients, so this factor also should be taken into account.

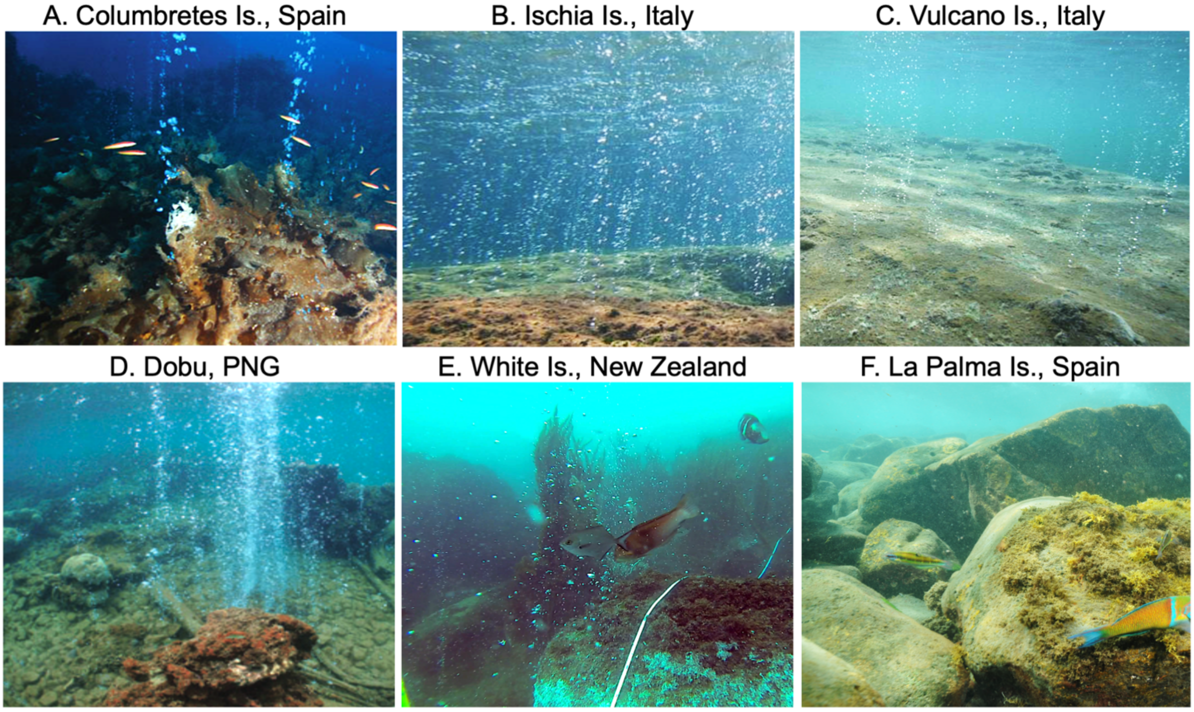


**Figure 1.2.** General view of different natural $CO_2$ vent systems: (A) Laminaria beds Columbretes Islands, Spain. (B) Macroalgae and Seagrasses at Castello, Ischia, Italy. (C) Rocky bottom in Vulcano, Italy. (D) Porite's coral reef in Dobu, Papua New Guinea, Pacific. (E) Macroalgae beds at White Island, New Zealand. (F) Macroalgae assemblages in La Palma Island, Atlantic Ocean, Spain. Panel (A): Photo: D. Kersting. Panel (B): Photo: R. Rodolfo-Metalpa. Panel (C): Photo: Maarten van Rouveroy; Panle (D): Photo: K. Fabricius. Panel (E): Photo: N. Bennett. Panel (F): Photo: J.C. Hernández.

## 1.3. RESEARCH IN ACIDIFIED SYSTEMS

Early research in as focused on the effects of acidification on species abundance and diversity and, therefore, on changes in the distribution and structure of benthic (Crook et al., 2016; Enochs et al., 2015; Fabricus et al., 2011; Hall-spencer et al., 2008; Inoue et al., 2013; Kroeker et al., 2011; Linares et al., 2015) and planktonic communities (Smith et al., 2016b; Ziveri et al., 2014) as well as microorganisms (Burrell et al., 2015; Enochs et al., 2016; Kerfahi et al., 2014; Uthicke et al., 2013). Subsequent studies focused on physiological changes in calcification (e.g., Rodolfo-Metalpa et al., 2010), photosynthesis (e.g., Russell et al., 2013), biomineralization (e.g., Collard et al., 2016; Duquette et al., 2017; Kamenos et al., 2016) and other metabolic processes (e.g., Celis-plá et al., 2015; Kumar et al., 2017b, 2017a; Uthicke and Fabricius, 2012). There have also been several studies on changes in intraspecific ecological interactions, such as herbivory (e.g., Johnson et al., 2012) and competition (e.g., Connell et al., 2013; Inoue et al., 2013; Kroeker et al., 2013b). Finally, studies have focused on early life cycle stages and reproduction, such as recruitment, colonization or juveniles (e.g., Fabricius et al., 2011; Munday et al., 2014; Porzio et al., 2013; Ricevuto et al., 2014), on ecological succession (Brown et al., 2018; Kroeker et al., 2013b, 2013a) and on changes in species reproduction (Milazzo et al., 2016) in addition to impacts on larvae (e.g., Lamare et al., 2016) (Figure 1.3; Table A.1 in Appendix A).

## 1.3.1. Main Changes in Marine Communities

The results obtained in the AS from temperate, tropical, and subtropical regions have been consistent with expectations with some interesting exceptions.

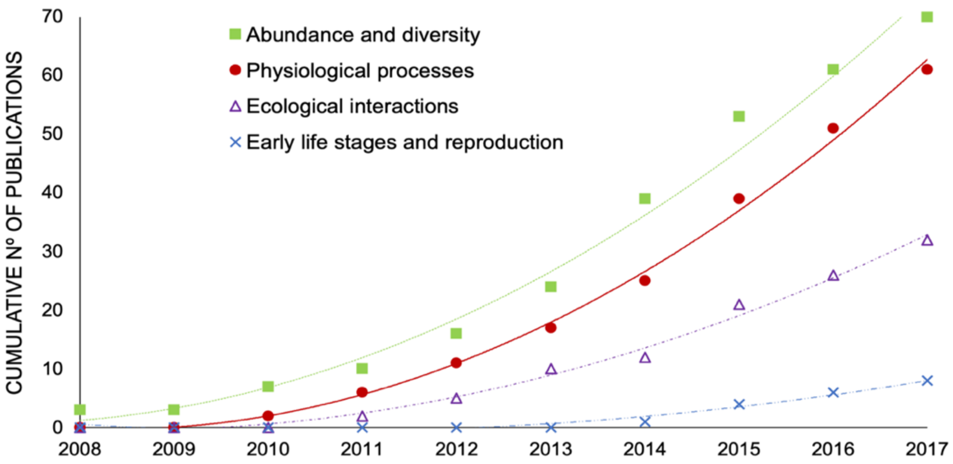


Figure 1.3. Cumulative number of published articles in the last 9 years showing research topic (Appendix A, Table A.1).

### *Algae*

Populations of calcifying red algae, especially crustose coralline algae (CCA), which form calcite crystals with Mg content, decrease and even disappear from the AS and are therefore the species most affected by acidification (e.g., Enochs et al., 2015; Fabricius et al., 2011; Johnson et al., 2013; Kamenos et al., 2016; Linares et al., 2015). In contrast, calcifying algae that form aragonite crystals, such as CCA species of the genus *Peyssonnelia* (e.g., Linares et al., 2015), brown algae (Padina spp.) (e.g., Celis-Plá et al., 2015; Goffredo et al., 2014; Johnson et al., 2012) or green algae such as *Halimeda* and *Acetabularia* (e.g., Enochs et al., 2015; Newcomb et al., 2015; Vogel et al., 2015), are more tolerant to the decrease in pH, and in many cases, with the exception of AS with large variations in aragonite Ω (such as Ischia, Methana and, to a lesser extent, Vulcano, Table 1.1), they are able to adapt and proliferate in acidified environments but with some negative cost in mineralization (Goffredo et al., 2014) (Table 1.3).

This cost is because although aragonite is more soluble than calcite, the presence of other components, such as Mg, in the mineralization favours the dissolution of the latter in acidified environments, making calcite structures with high Mg contents more vulnerable than aragonite (Koch et al., 2013). Certain brown algae, such as the genera *Lobophora, Dictyota* (e.g., Porzio et al., 2011), *Cystoseira* (e.g., Baggini et al., 2014; Celis-Plá et al., 2015), *Sargassum* (e.g., Porzio et al., 2011, 2017), *Halopteris* and *Laminaria* (e.g., Hall-Spencer et al., 2008; Linares et al., 2015) as well as some cespitose algae (e.g., Enochs et al., 2015; Porzio et al., 2011, 2013) and marine phanerogams, such as *Posidonia oceanica* (e.g., Guilini et al., 2017; Lauritano et al., 2015; Scartazza et al., 2017) or *Cymodocea* spp. (e.g., Russell et al., 2013; Takahashi et al., 2016), are also clearly favoured by the increase in $pCO_2$ and are thus more abundant in areas most affected by $CO_2$ emissions (Table 1.3).

**Table 1.3.** Summary of documented responses to the impact of acidification on macroalgae and seagrass communities according to studies in as. Symbols and abbreviations: (+) positive effect; (–) negative effect; (=) no apparent effect; CCA, crusty coralline algae; PNG, Papua New Guinea.

| | MEDITERRANEAN | | | | | ATLANTIC | | PACIFIC | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Columbretes[1] | Ischia[2] | Panarea[3] | Methana[4] | Vulcano[5] | Canaries[6] | Azores[7] | PNG[8] | White Is.[9] | Maug[10] |
| Red Algae, Calcareous | | | | | | | | | | |
| Corallinaceae | - | - = | | - | | - | - | - | | - |
| CCA | - | - | | - | - | - | | - | - | - |
| *Peyssonnelia* spp. | + | + | | | - | | | = | | |
| Non-Calcareous | | | | | | | | | | |
| Cespitose and erect fleshy algae | | + | | | + | + | | | + | + |
| Brown Algae, Calcareous | | | | | | | | | | |
| *Padina* spp. | | - | - | - | + | | | + | | - |
| Non-Calcareous | | | | | | | | | | |
| *Cystoseira* spp. | - | = | | + | + | | | | | |
| *Sargassum* spp. | | + | | | + | | | | | |
| *Dyctiota* spp. | - | + | | + | | = | | | | - |
| *Laminaria rodriguezii* | + | | | | | | | | | |
| *Halopeteris* spp. | + | + | | | - | + | | | | |
| Cespitose algae | | + | | | + | + | | | + | + |
| Green Algae, Calcareous | - | - | + | | = | | | = | | = |
| Phanerogams | | + | + | | = | | | + | | |

1(Linares et al., 2015); 2(Hall-Spencer et al., 2008; Martin et al., 2008; Porzio, et al., 2011; Johnson et al., 2012; Kroeker et al., 2013; Kroeker et al., 2013; Porzio et al., 2013; Porzio et al., 2017); 3(Goffredo et al., 2014; Lauritano et al., 2015; Guilini et al., 2017); 4(Baggini et al., 2014; Baggini et al., 2015); 5(Apostolaki et al., 2014; Celis-Plat et al., 2015; Newcomb et al., 2015; Cornwall et al., 2017); 6(Pérez, 2017); 7(Couto et al., 2010); 8(Fabricius et al., 2011; Johnson et al., 2012; Russell et al., 2013; Fabricius et al., 2015; Vogel et al., 2015; Takahashi et al., 2016); 9(Brinkman and Smith, 2015); 10(Enochs et al., 2015).

### *Benthic invertebrates*

Most changes in the abundance and diversity of benthic invertebrate communities are caused by a noticeable reduction in the number of species of sponges (Goodwin et al., 2014), decapods, calcifying polychaetes, some molluscs (e.g., Allen et al., 2016; Cigliano et al., 2010; Gambi et al., 2016; Kroeker et al., 2011) and echinoderms (e.g., Bray et al., 2014; Calosi et al., 2013a) (Table 1.4), but the reported changes are not as drastic as those in plant communities. In most groups, there are species that are able to tolerate these extreme pH conditions, such as bryozoans (Rodolfo-Metalpa et al., 2010), some species of sea urchins (e.g., Calosi et al., 2013a; Uthicke et al.,

2016) or small crustaceans (e.g., Allen et al., 2016; Cigliano et al., 2010; Kroeker et al., 2011), in addition to non-calcifying species, such as some cnidarians as *Anemonia* spp. (e.g., Borell et al., 2014; Januar et al., 2016; Suggett et al., 2012) (Table 1.4). Furthermore, among the most affected groups, such as molluscs, acidification-tolerant species are still being found, such as the bivalve *Mytilus* spp. in the Mediterranean and Baltic Seas (Ricevuto et al., 2012; Thomsen et al., 2010, 2013), the endemic Mediterranean species *Pinna nobilis* (Basso et al., 2015), the limpets *Patella* spp. (Duquette et al., 2017; Langer et al., 2014) and the gastropod *Eatoniella mortoni* (Connell et al., 2017). These organisms are interesting because they have demonstrated some resistance to decreasing pH.

Table 1.4. Summary of documented responses to the impact of acidification on invertebrate communities according to studies in as. Symbols and abbreviations: (+) positive effect; (–) negative effect; (=) no apparent effect; CCA, crusty coralline algae; PNG, Papua New Guinea.

| | MEDITERRANEAN | | | | ATLANCTIC | PACIFIC | | BALTIC |
|---|---|---|---|---|---|---|---|---|
| | Ischia[1] | Panarea[2] | Methana[3] | Vulcano[4] | Canaries[5] | PNG[6] | White Is.[7] | Kiel Fjord[8] |
| Porifera | - | | | | | - | | |
| Bryozoa | = + | | | | | | | |
| Polychaeta | | | | | | | | |
| No Calcifying | = + | = | | | | = | | |
| Calcifying | - = | - | | | | - | | |
| Echinodermata | | | | | | | | |
| *Paracentrotus lividus* | - | | - | = | = | | | |
| *Arbacia lixula* | - | | = | + | + | | | |
| *Echinometra* sp. | | | | | | + | | |
| *Evechinus chloroticus* | | | | | | | = | |
| Mollusca | - = | - | | - = | | = | = | = |
| Crustacea | | | | | | | | |
| Decapoda | - | | | | | - | | |
| Amphipoda | + | + | | | | - | | |
| Tanaidacea | + | | | | | + | | |
| Isopoda | = | | | | | | | |
| Cnidaria | | | | | | | | |
| *Anemonia* spp. | + | | | + | + | | | |
| *Balanophyllia europaea* | | = | | | | | | |

1(Hall-Spencer et al., 2008; Cigliano et al., 2010; Rodolfo-Metalpa et al., 2010; Kroeker et al., 2011; Lombardi et al., 2011; Rodolfo-Metalpa et al., 2011; Ricevuto et al., 2012; Garrard et al., 2014; Giangrande et al., 2014; Goodwin et al., 2014; Ricevuto et al., 2014; Lombardi et al., 2015;

Ricevuto et al., 2015a; Gambi et al., 2016; Lucey et al., 2016; Ricevuto et al., 2016; Turner et al., 2016); 2(Goffredo et al., 2014; Fantazzini et al., 2015; Prada et al., 2017; Molari et al., 2018); 3(Bray et al., 2014); 4(Suggett et al., 2012; Calosi et al., 2013a,b; Horwitz et al., 2014; Milazzo et al., 2014; Garilli et al., 2015; Small et al., 2015; Collard et al., 2016; Harvey et al., 2016; Ventura et al., 2016); 5(Hernández et al., 2018a; Pérez, 2017); 6(Fabricius et al., 2011; 2014; Allen et al., 2016; Uthicke et al., 2016); 7(Brinkman, 2014; Connell et al., 2017); 8(Thomsen et al., 2010; 2013).

### *Corals*

Corals are the most studied invertebrates in such studies due to their role as habitat formers that build entire ecosystems, such as the coral reefs of tropical regions. Because they are sessile and calcifying, they are highly sensitive to changes in seawater pH. According to the results obtained in different tropical AS, coral reefs tend to lose their composition and their characteristic high degree of heterogeneity (Table 1.5), especially in those environments where the pH is lower than 7.8 (Table 1.1). Hard corals, which are considered habitat-structuring or habitat-transforming organisms, tend to disappear from acidic areas, being replaced by coral species that are more resistant to acidification, such as Porites spp., or by soft corals that are capable of tolerating such conditions (e.g., Barkley et al., 2015; Crook et al., 2012, 2013, 2016; Enochs et al., 2015; Fabricius et al., 2011, 2017; Noonan et al., 2018) (Table 1.5). In broad terms, the diversity of hard corals is lost and replaced by a few species of resistant soft corals.

Table 1.5. Summary of documented responses to the impact of acidification on coral reef communities according to studies in as. Symbols and abbreviations: (+) positive effect; (–) negative effect; (=) no apparent effect; PNG, Papua New Guinea; ETP, Eastern Tropical Pacific.

| | PACIFIC | | | | | | CARIBBEAN SEA |
|---|---|---|---|---|---|---|---|
| | PNG[1] | Indonesia[2] | Iwatorishima[3] | Maug[4] | Palau[5] | ETP[6] | Puerto Morelos[7] |
| No framework-bulding | | | | | | | |
| Soft-Coral | - | = | = | | | | |
| Hard-Coral | + | | | = | + | - | = |
| Framework-building | - | = | - | - | = | - | - |

1(Fabricius et al., 2011; Wall et al., 2016; Brown et al., 2018; Fabricius et al., 2017); 2(Januar et al., 2016; 2017); 3(Inoue et al., 2013); 4(Enochs et al., 2015); 5(Barkley et al., 2015); 6(Manzello et al., 2014); 7(Crook et al., 2012; 2013; 2016).

### *Planktonic Invertebrates*

Regarding planktonic communities, changes are only known in phytoplankton (coccolithophorids) and coastal zooplankton communities (Table 1.6). More specifically, coccolithophorid communities decrease in abundance and diversity with acidification, and some species are further harmed by malformation and corrosion of their calcite skeletons (Ziveri et al., 2014). Similarly, coastal zooplankton also present lower abundance, as seen in both the Azores (Campoy, 2015) and PNG (Smith et al., 2016b, 2017), mainly due to the decreased abundance of crustaceans.

The foraminifera are another group of small organisms that have been considered important due to their calcifying ability (Table 1.6). Although this group has become practically extinct in many acidified areas (e.g., Cigliano et al., 2010; Dias et al., 2010; Uthicke et al., 2013), species have also been found that are able to adapt to extreme pH conditions (pH 7.6), such as in the deep waters of the Northern Gulf of California (Pettit et al., 2013).

### *Microorganisms*

The groups of organisms that are most favoured by OA include the biofilm-forming microorganisms (Table 1.6). These biofilms, which are mainly composed of bacteria, cyanobacteria and microalgae, increase in both species abundance and diversity (Chauhan et al., 2015; Hassenruck et al., 2017; Johnson et al., 2015; Kerfahi et al., 2014; Lidbury et al., 2012; Morrow et al., 2015), especially those species that degrade organic matter (Taylor et al., 2014). In tropical regions, this increase is worrisome because it can amplify the negative effects of acidification on other organisms, as has been observed by Enochs et al. (2016) in the vent system of the Mariana Islands, where the increase in bacterial populations that compose the coral microbiota magnifies the erosion of coral reefs.

Table 1.6. Summary of documented responses to the impact of acidification on planktonic invertebrate and microorganisms according to studies in as. Symbols and abbreviations: (+) positive effect; (–) negative effect; (=) no apparent effect; PNG, Papua New Guinea; NGC, Northern Gulf of California.

| | MEDITERRANEAN | | ATLANTIC | PACIFIC | | | |
|---|---|---|---|---|---|---|---|
| | Ischia[1] | Vulcano[2] | Azores[3] | NGC[4] | PNG[5] | White Isl.[6] | Maug[7] |
| Plankton | | | | | | | |
| Zooplankton | | | – = | | – | | |
| Phytoplankton | + | – | | | | | |
| Others | | | | | | | |
| Foraminifera | – | | | = | – | | |
| Microorganisms | = | + | | | + | + | + |

1(Cigliano et al., 2010; Dias et al., 2010; Meron et al., 2012; Di Cioccio et al., 2015); 2(Lidbury et al., 2012; Kerfahi et al., 2014; Taylor et al., 2014; Ziveri et al., 2014; Johnson et al., 2015); 3(Campoy, 2015); 4(Pettit et al., 2013); 5(Uthicke and Fabricius, 2012; Uthicke et al., 2013; Raulf et al., 2015; Smith et al., 2016b; Hassenrück et al., 2017; Marcelino et al., 2017); 6(Burrell et al., 2015); 7(Enochs et al., 2016).

### 1.3.2. Changes in the Physiological Responses in Natural Laboratories

Thanks to AS, we now know many of the physiological responses of organisms after exposure to an acidified environment for an extended period. The AS studied thus far date from at least between 10 and 18,000 years old (Table 1.2), which allows us to identify species that are already adapted to these putative future conditions and compare their ability to adapt to their ability to acclimatize through organism transplant experiments (e.g., Kumar et al., 2017b; Olivé et al., 2017).

Brown algae and phanerogams seem to thrive in acidified waters due to the extra DIC (Table 1.1) that allows them to increase their photosynthetic rate and growth (Koch et al., 2013) by overcoming their physiological C limitations; they usually present a C concentration mechanism (CCM) with low affinity for DIC (Cornwall et al., 2017). The physiology of the seaweed Sargassum vulgaris has been one of the most studied in $CO_2$ vent systems (Kumar et al., 2017a, 2017b; Porzio et al., 2017) in addition to the marine phanerogam *P. oceanica* (Hall-Spencer et al., 2008; Lauritano et al., 2015; Ravaglioli et al., 2017; Scartazza et al., 2017) and several species of *Cymodocea* (Apostolaki et al., 2014; Arnold et al., 2012; Olivé et al., 2017;

Russell et al., 2013; Takahashi et al., 2016). These species have favourably adapted to acidified environments and have increased their photosynthetic activities and energetic metabolism, which, among other changes, allows them to control ionic homeostasis and allocate resources for increased growth and cell development. The only exception is *Cymodocea nodosa* in the Mediterranean Sea, which does not present increased net productivity. This responds differently to other phanerogams and has not increased in abundance in the case of the Vulcano vent system (Olivé et al., 2017). Its response is likely influenced by other factors such as the loss of its epiphytic species, which reduces protection in eutrophic conditions (Arnold et al., 2012), or the appearance of toxic compounds of volcanic origin (Vizzini et al., 2013).

The regulation of calcification can also play a very important role in the survival of calcifying species. For example, extraordinary adaptation with no compromise to its calcified structures has been observed by the red algae *Peyssonnelia* spp. in AS (Linares et al., 2015), and in extreme cases (Ischia pH below 7.4) for other algae, aragonite concentrations have been replaced with gypsum (Kamenos et al., 2016).

Invertebrates that are resistant to changes in seawater pH have also developed different strategies to survive and adapt to these environments. They also have a great capacity to regulate extracellular ionic homeostasis or exert acid-base regulation, as demonstrated by the polychaete *Platynereis dumerilii* (Calosi et al., 2013b), the sea urchin *Arbacia lixula* (Calosi et al., 2013a; Small et al., 2016), the fan worm *Sabella spallanzanii* (Ricevuto et al., 2016) and the isopod *Dynamene torelliae* (Turner et al., 2016). In contrast, many species can redirect their metabolic energy to compensate for the problems caused by increased acidity. For example, hard corals of the genus *Porites* are able to regulate and maintain net calcification at low pH levels due to an increase in photosynthetic rates (Crook et al., 2013; Strahl et al., 2015), and the bryozoan *Schizoporella errata* increases its energy available for calcification processes by decreasing its defensive structures so as not to harm its somatic growth (Lombardi et al., 2011). There are also some species of gastropods that obtain the energy necessary to calcify in these acidic environments by decreasing the size of their shell (Garilli et al., 2015; Harvey et al., 2016). Decreases in body size and spines have also been observed in polychaetes (Gambi et al., 2016; Lucey et al., 2015) and in the sea urchin *A. lixula*, which even allows the sea urchin to grow a more resistant skeleton (Hernández et al., 2018a). The presence of external protective layers, such as in the bryozoan *Myriapora truncata* (Rodolfo-Metalpa et al., 2010) and expansion of organic layers in the mussel *Mytilus galloprovincialis* or the coral *Balanophyllia* spp. (Rodolfo-Metalpa et al., 2011) is another defence against low pH levels. Finally, some gastropods stand out for their ability to alter their mineralization processes, increasing the thickness of their shells (Langer et al., 2014; Rodolfo-Metalpa et al., 2011) or even replacing the aragonite crystals, which are more soluble under low pH, with calcite, as observed by Duquette et al. (2017) in *Patella rustica*.

### 1.3.3. Intraspecific Ecological Interactions

One of the most important advantages offered by experimentation in AS is the opportunity to observe potential changes in ecosystem function. Acidification has been demonstrated to influence herbivory dynamics (Connell et al., 2013; Nogueira et al., 2017; Ricevuto et al., 2015a, 2015b). The relationships between producers (in this case, algae) and their herbivores, such as sea urchins, molluscs, or fish, have been found to be indirectly altered in acidified ecosystems. In the Mediterranean, increased browsing by herbivores has been associated with the survival of species such as *P. pavonica* (Baggini et al., 2015; Johnson et al., 2012). Likewise, the increase in the abundance of herbivorous gastropods in vent systems has been attributed to increased quantities of cespitose algae (Connell et al., 2017) or to the seagrasses buffering effect on low pH (Garrard et al., 2014; Zupo et al., 2015).

Acidified natural environments also offer the opportunity to observe changes in the recruitment phase. There have been several studies in this regard, in both the Mediterranean (Cigliano et al., 2010; Kroeker et al., 2013b; Ricevuto et al., 2012) and tropical systems (Allen et al., 2016; Crook et al., 2016; Fabricius et al., 2015), where acidified environments have been shown to negatively alter the recruitment success of calcifying species (especially CCA and corals) and indirectly alter the recruitment success of other species that might lose their substrates in the absence of calcifying species. Changes in succession and colonization dynamics have also been studied recently (Brown et al., 2018) as have metabolic rate variations during these processes (Noonan et al., 2018). Acidification alters succession rates and influences colonization by favouring some species (e.g., biofilm-forming microorganisms) over others, which results in more homogeneous, less diverse environments in addition to a possible imbalance in the flow of energy through trophic chains (Brown et al., 2018; Vizzini et al., 2017).

Many organisms, such as fleshy algae (Kroeker et al., 2013b), phanerogams (Takahashi et al., 2016) or the corals of the genus Porites (Inoue et al., 2013), exhibit competitive advantages in acidified environments. In extreme cases, the favoured species can completely transform a habitat, as observed in the Maug vent system where macroalgae have completely dominated the ecosystem and replaced coral reefs (Enochs et al., 2015). In turn, these changes have indirect effects on other organisms, as observed in demersal zooplankton communities associated with coral reefs that lose their places of settlement and refuge in the absence of corals (Smith et al., 2016b, 2017).

Species are expected to compete with each other for resources, such as space and food, but in altered environments, such as AS, the biotic and abiotic relationships between species change markedly (Nagelkerken et al., 2016; Sunday et al., 2017). Knowing these future changes is one of the objectives of OA investigations, and although it is extremely difficult to identify the indirect, global effects of OA due to their magnitude, interesting answers are increasingly being obtained at the local level. All indications are that the changes observed in the AS will generate communities with less diversity and structural complexity, in which the primary producers will directly increase along with the herbivore populations while the predators will not be affected, thus creating decompensation in the trophic chain (Nagelkerken et al., 2016; Vizzini et al., 2017).

### 1.3.4. Reproduction and Early Life Stages

There have been several laboratory studies of the effects of acidification on the early life stages of organisms. The best studied are changes in the reproduction and development of sea urchin larvae (Dupont and Thorndyke, 2013; García et al., 2015), but even here there have been only a few publications on this line of research in AS. In a recent study, Lamare et al. (2016) observed the effects of $CO_2$ vents in echinoderm larvae in a short-term *in situ* experiment, and although they did not observe significant differences in the abundance of individuals, they did observe atrophied development and morphological anomalies in the larvae in laboratory experiments. Additionally, fish larvae exhibited changes in behaviour in acidified areas due to the loss of acoustic signals from the habitat that the species used for orientation. This effect is mainly caused by the loss of structural complexity as well as by loss of the organisms that generate environmental noise (Mirasole et al., 2017; Nagelkerken et al., 2016; Rossi et al., 2016b, 2016a).

Another discovery made possible through *in situ* studies in AS was of changes in life strategies related to parental care. The larval stages of some free-swimming invertebrates, such as some polychaetes, are more sensitive to acidification, while species with parental care and whose off- spring are more protected are favoured (Gambi et al., 2016; Lucey et al., 2015; Wäge et al., 2017). Only three studies have yielded information about impacts on reproduction. The first was carried out in Vulcano, where an increase in reproductive activity was reported for dominant fish males of acidified zones, which was contrary to expectations (Milazzo et al., 2016), and the other study, in Panarea, showed no negative effects on gametogenesis nor on embryogenesis in the scleractinic coral *Leptopsammia pruvoti* (Gizzi et al., 2017). And a third recently published paper, on *A. lixula* egg development, has shown a plastic response to low pH conditions of the jelly coat size of sea urchins from the vent site. This could be a strategy to facilitate the maintenance of gamete function, facilitating fertilization success in a low pH ocean (Foo et al., 2018a).

## 1.4. CONCLUSION AND RECOMEMENDATIONS

The above presents most of the known AS to date. A summary of the negative and positive inclination of different marine organisms due to a natural decreasing of pH is shown in Figure 1.4. The evidence strongly indicates that these acidified systems are useful for examining the direct, and more interestingly, unexpected indirect effects (e.g., community wide effects) of OA.

Despite the evident usefulness of the AS, it is necessary to carefully control certain chemical and physical variables before conducting research in these areas. Therefore, we recommend (1) thoroughly studying the area before and during the entire investigation to better understand the fluctuations in the venting intensity, the chemical composition of the water, not only in the concentrations of carbon but those of other chemical compounds, nutrients and contaminants; (2) choosing the area of interest according to the pH levels, temperature (when possible) and levels of aragonite and calcite Ω that are expected in the future to achieve the best possible predictions; and (3) complementing *in situ* studies with *in vitro* experiments that help demonstrate the causality of patterns observed in the AS.

Presently, the most abundant AS are those of volcanic origin, so we recommend continuing to search for this type of vent system, which will help expand the number of biogeographic areas and represented habitats to create a worldwide network of laboratories that can share results. Maps of volcanic arcs, faults and other areas with volcanic activity can be used to locate new potential AS. However, in this search for new areas, we must bear in mind that an appropriate AS for the study of acidification must have a constant C input that maintains the average pH levels below 8 but never lower than 7.4 to more realistically represent the putative future conditions predicted by the IPCC (2014), with aragonite Ω below 3 (tropical zones) or 2 (temperate zones) and calcite Ω below 4 (tropical zones) or 3 (temperate zones).

Another important consideration is that the system should not be subject to the influence of other chemical compounds, such as sulphur from gases of volcanic origin, nutrient-rich water, or freshwater and, if possible, mechanical-acoustic contamination due to bubbling should be avoided. In addition, the AS would ideally be in shallow coastal areas with the greatest possible representation of habitats that are easily accessible for study.

Many questions remain about the changes that are taking place in these acidified areas that can help us understand the oceans of the future. We need more information about changes at the ecosystem level as well as ecological processes and species interactions, and we must also emphasize the importance of understanding the evolutionary processes leading to adaptation in many of the organisms that live in these environments. Finally, we must strive to work in an integrative way, studying the greatest number of geographical areas and habitats; this will help us create a worldwide network of AS for studying and understanding of the oceans of the future.

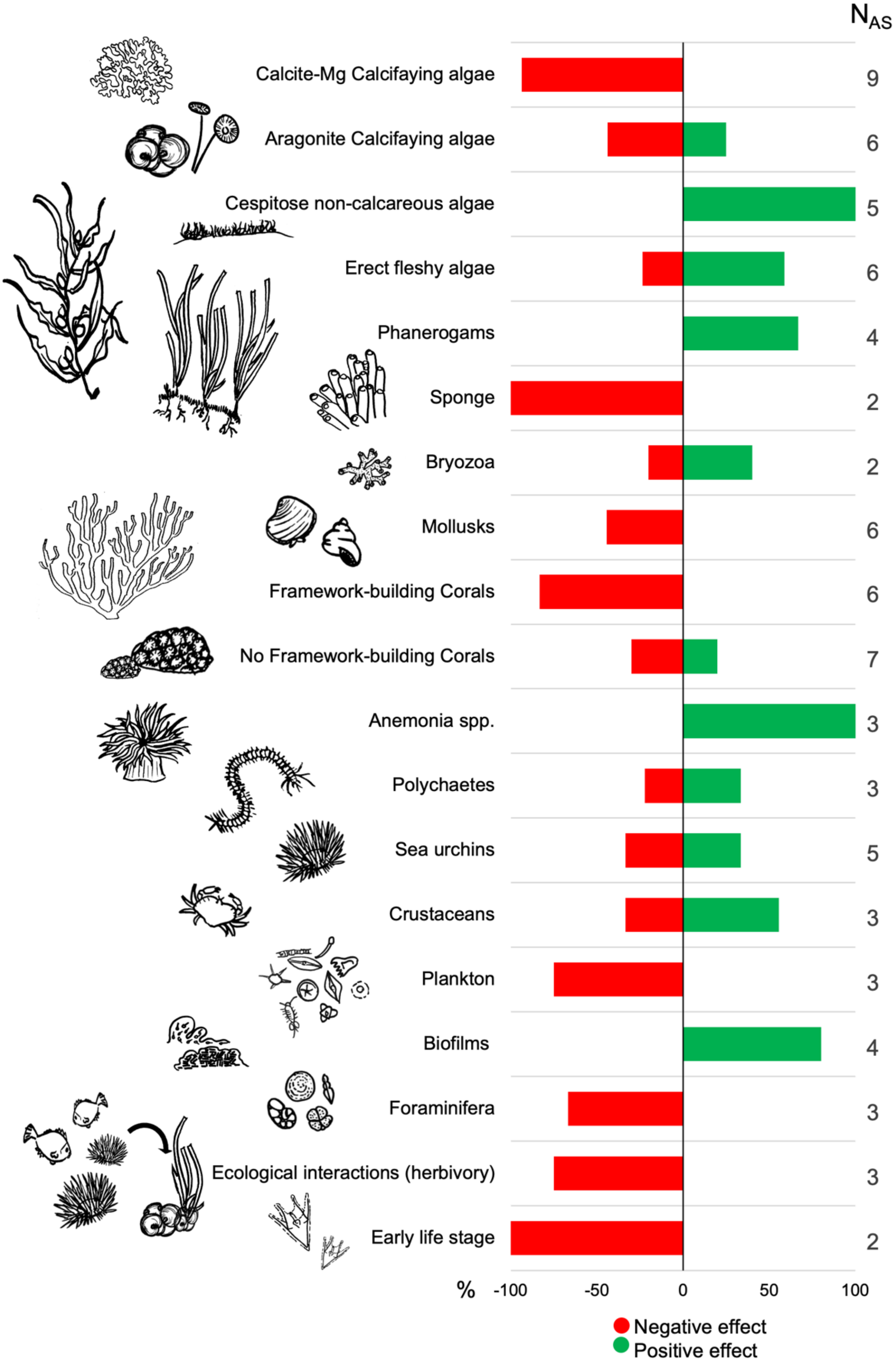


**Figure 1.4.** Summary of the positive and negative effects of OA on organisms. Percentages have been calculated based on the studies conducted at naturally acidified areas (AS). $N_{AS}$ is the number of AS where the organism has been studied.

# CHAPTER 2

## Chemical Characterization of the Punta de Fuencaliente $CO_2$-enriched System (La Palma, NE Atlantic Ocean): A New Natural Laboratory for Ocean Acidification Studies

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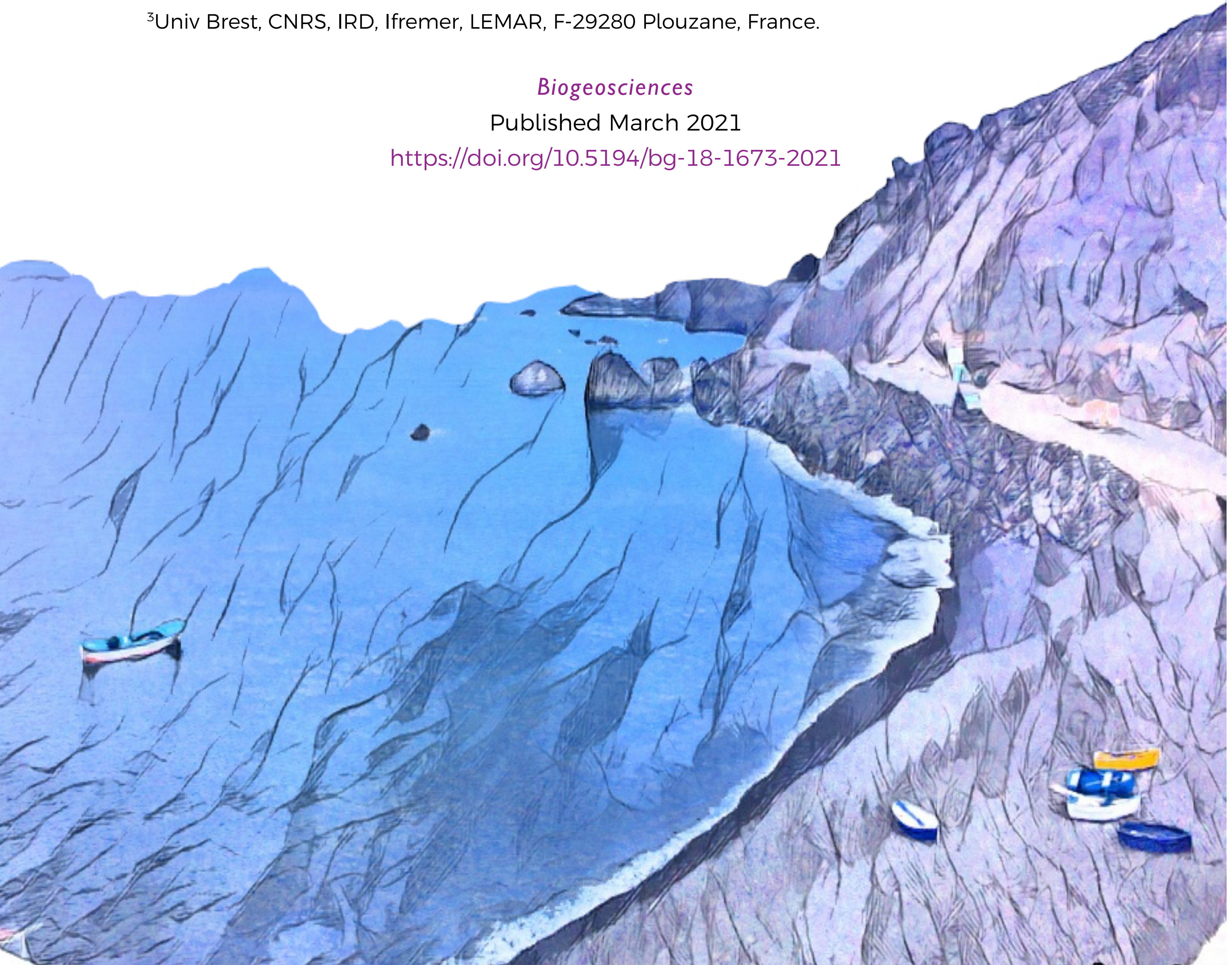


## ABSTRACT

We present a new natural $CO_2$ system located off the southern coast of La Palma Island, Canary Islands, Spain. Like $CO_2$ seeps, these $CO_2$ submarine groundwater discharges can be used as an analogue to study the effects of ocean acidification (OA) on the marine realm. With this aim, we describe the carbon system dynamics, by measuring pH, $A_T$, and $C_{T,}$ and calculating $\Omega$ aragonite and calcite. Our explorations have found several emission points with similar chemical features. Here, the $C_T$ varies from 2120.10 to 10784.84 µmol kg-1, $A_T$ from 2415.20 to 10817.12 µmol kg-1, pH from 7.12 to 8.07, $\Omega$ aragonite from 0.71 to 4.15 and calcite from 1.09 to 6.49 units. Also, the $CO_2$ emission flux varies between 2.8 to 28 kg $CO_2$ $d^{-1}$, becoming a significant source of carbon. These $CO_2$ emissions, which are of volcanic origin, acidify the brackish groundwater that is discharged into the coast and alter the local seawater chemistry. Although this kind of acidified system is not a perfect image of future oceans, this area of La Palma is an exceptional spot to perform studies aimed to understand the effect of different levels of OA on the functioning of marine ecosystems. These studies can then be used to comprehend how life has persisted through past Eras, with higher atmospheric $CO_2$, or to predict the consequences of present fossil fuel usage on the future oceans.

Keywords: Volcanic, hydrothermal, brackish water discharge, groundwater, ocean acidification, ocean chemistry.

## RESUMEN

Presentamos un nuevo sistema natural de $CO_2$ situado frente a la costa sur de la isla de La Palma, Islas Canarias. Al igual que los afloramientos de $CO_2$, estas descargas submarinas de $CO_2$ pueden ser utilizadas como un análogo para estudiar los efectos de la acidificación oceánica (OA) en el ámbito marino. Con este objetivo, describimos la dinámica del sistema de carbono, mediante la medición de pH, $A_T$ y $C_T$, y el cálculo de $\Omega$ de aragonito y calcita. Nuestras exploraciones han encontrado varios puntos de emisión con características químicas similares. Aquí, el $C_T$ varía de 2120,10 a 10784,84 µmol kg-1, $A_T$ de 2415,20 a 10817,12 µmol kg-1, el pH de 7,12 a 8,07, $\Omega$ aragonito de 0,71 a 4,15 y $\Omega$ calcita de 1,09 a 6,49 unidades. Asimismo, el flujo de emisión de $CO_2$ varía entre 2,8 y 28 kg de $CO_2$ d-1, convirtiéndose en una importante fuente de carbono. Estas emisiones de $CO_2$ acidifican las aguas subterráneas salobres que se vierten en la costa y alteran la química del agua de mar. Aunque este tipo de sistema acidificado no es una imagen perfecta de los océanos del futuro, esta zona de la palma es un lugar excepcional para realizar estudios destinados a comprender el efecto de la OA en el funcionamiento de los ecosistemas marinos. Estos estudios pueden servir para comprender cómo ha persistido la vida a lo largo de eras pasadas, con mayor $CO_2$ atmosférico, o para predecir las consecuencias del uso actual de combustibles fósiles en los océanos futuros.

Palabras claves: Volcánico, hidrotermal, descarga de agua salobre, aguas subterráneas, acidificación oceánica, química del océano.

## 2.1. INTRODUCTION

For the last decade, marine systems with natural carbon dioxide ($CO_2$) sources have been used as analogous of the acidified future oceans to understand $CO_2$ effects on organisms and marine ecosystems functioning (IPCC, 2014; Hall-Spencer et al., 2008; Foo et al., 2018b; González-Delgado and Hernández, 2018). These areas are characterized by an extra $CO_2$ input from volcanic (normally called $CO_2$ seeps), karstic or biological sources, or they originate from upwelling (González-Delgado and Hernández, 2018). Due to the origin of $CO_2$, $CO_2$ vent systems are very common and can be found all over the world from mid-oceanic ridges to oceanic islands and intra-plate magmatic areas (Dando et al., 1999; Tarasov et al., 2005). In general, the vent systems have emissions in the form of bubbles which are 90 %–99 % $CO_2$. The most notable features of these acidified systems are the fluctuation in pH, the aragonite and calcite saturation states (Ω) (declining between 1 and 3) and dissolved inorganic carbon (DIC) which increases up to 3.2 mol $Cm^{-3}$ (González-Delgado and Hernández, 2018). Moreover, there are marine shallow areas affected by $CO_2$ gas diffusive emissions through submarine groundwater discharges (SGDs) that acidify the surrounding waters (Hall-Spencer et al., 2008).

Numerous advances in ocean acidification (OA) studies have been achieved using these systems, such as in understanding the acidification effect on ecology interaction (e.g., Nagelkerken et al., 2016), physiological adaptations (e.g., Migliaccio et al., 2019) and genetic adaptations (e.g., Olivé et al., 2017). Nowadays, it is possible to better understand the direct and indirect effects of OA in marine environments due to these acidified systems; for instance, we now know that OA-related changes will be reflected in the services that ecosystems provide to us (Hall-Spencer and Harvey, 2019). Acidified systems can also be used to look back into the past of the Earth and to study how early life could have originated on the planet (Martin et al., 2008). Understanding how life has adapted in the past acidified eras can be extremely useful to understand how current life will change in the expected future (Gattuso et al., 1998).

The Canary Islands, located in the north-eastern Atlantic Ocean, are an oceanic volcanic archipelago formed by numerous hotspot island chains (Carracedo et al., 2001). The youngest islands are El Hierro at 1.1 million years and La Palma with an age of 2 million years (Carracedo et al., 2001). These islands are located to the west of the archipelago, and they are where the last historical eruptions took place. The last two were the Teneguía volcano on La Palma in 1971 and the Tagoro volcano on El Hierro in 2011 (Padrón et al., 2015; Santana-Casiano et al., 2016).

Currently, in the historic volcanic area on the south of La Palma (Cumbre Vieja volcano complex), there is a continuous degassing of $CO_2$ (Carracedo et al., 2001; Padrón et al., 2015). Correspondingly, on the nearby shore, $CO_2$ emissions have been detected recently in two different locations: the Las Cabras site (Hernandez et al., 2016) and Punta de Fuencaliente, which has already been used for OA ecological studies (Pérez, 2017; Viotti et al., 2019). However, in these works only the pH and $pCO_2$ were measured, at localized points where certain samples were taken.

The local name ‘Fuencaliente’, which translates as hot springs, refers to the thermal fresh waters that emerge at the coast. Before the conquest of the islands in 1492, its waters were used by locals for their healing properties and after that by visitors from all over the world (Soler-Liceras, 2007). However, these thermal springs were buried by the eruption of the San Antonio volcano in the 17th century. These thermal waters have been so famous and important for Fuencaliente people that there was an engineering project to dig them up (Soler-Liceras, 2007). The brackish water features measured by Soler-Liceras (2007) showed high concentrations of bicarbonate ($HCO^-_3$), sulfate ($SO^{2-}_4$) and chloride ($Cl^-$) that together with high temperatures (almost 50 °C) confirmed the influence of internal magmatic activity. Nearby, there are brackish lagoons located in the innermost part of the Echentive beach, about 200m from the coastline with diameters of 30 m and depths of up to 4m (Figure 2.2.1). Measures of oxygen isotopes 18O SMOW (Calvet et al., 2003) suggest a slight dilution of the seawater in the lagoons by inland brackish groundwater flowing into them. This indicates that in the system there are groundwater discharges, which probably come from the thermal waters studied by Soler-Liceras (2007).

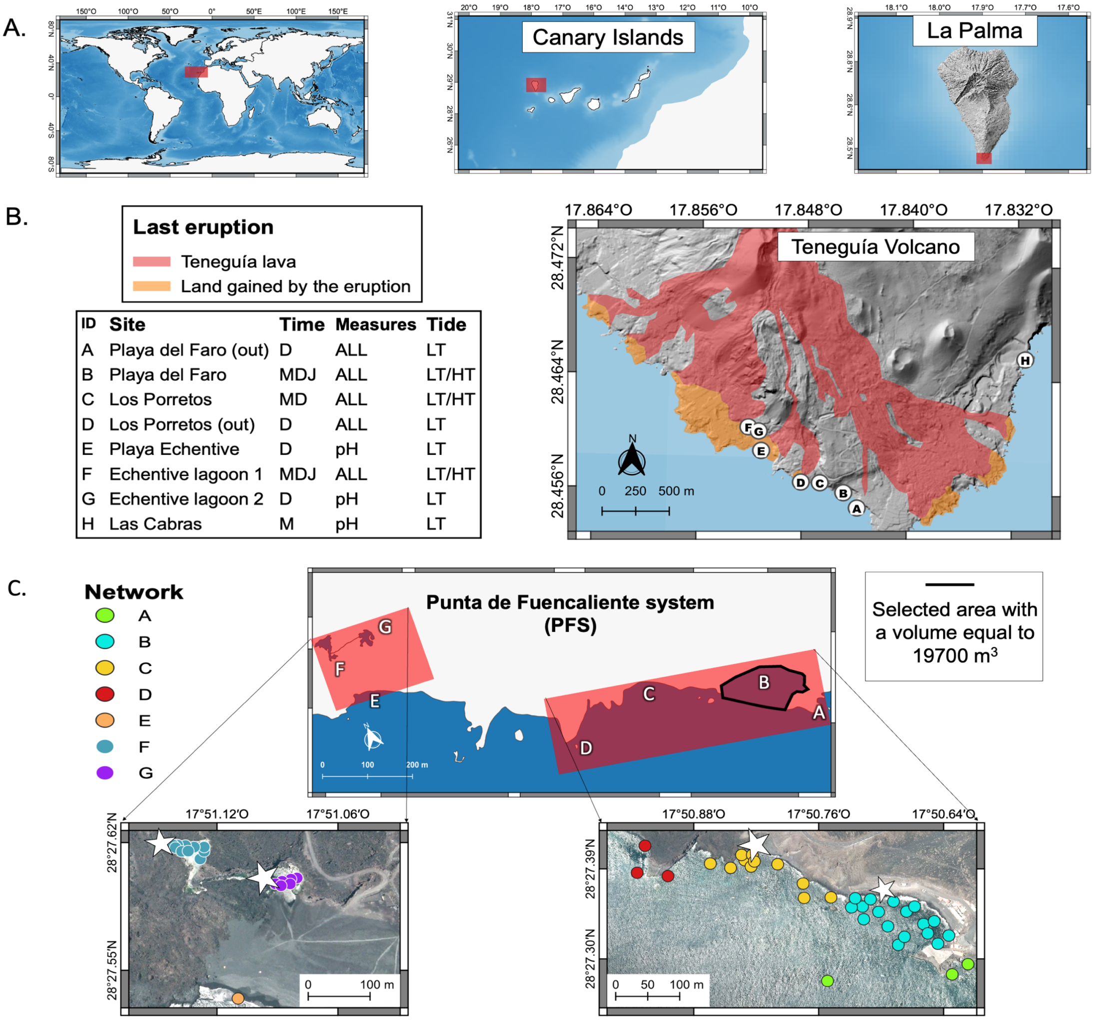
In the last 2 decades, an increasing number of studies have underlined the importance of SGD (Jeandel, 2016). SGD is an essential but poorly recognized pathway of material transport to the marine environment (Szymczycha et al., 2014). The term SGD includes the discharge of fresh groundwater to coastal seas to which recirculation of seawater often contributes (Burnett et al., 2006; Charette et al., 2016). For issues related to oceanography, the term is restricted to fluid circulation through continental shelf sediments with emphasis on the coastal zone (Burnett et al., 2006; Jeandel, 2016). One aspect that has yet not been considered is what occurs in areas where SGD is enriched by the emissions of recent volcanism or by hydrothermal activity. In these cases, these discharges can also act as sources of gases and hydrothermal emission compounds to the ocean and become points of emission of $CO_2$ that contribute to the OA. However, shallow coastal beaches and intertidal lagoons are highly dynamic systems controlled by physical processes and subjected to marine and continental influences. Processes such as the tide or the submarine groundwater discharges produce higher ranges of variation in physical and chemical parameters than processes in the open ocean water and could provide a natural environment for laboratory studies.

Hence, and with the purpose of using the Punta de Fuencaliente area as a naturally acidified laboratory, an accurate physical and chemical characterization of this area is presented in this study. The main objectives were to (1) determine the area affected by the emissions and detect new emissions points for replication studies, (2) characterize the ocean chemistry of the area, and (3) confirm the volcanic origin of the acidification.

## 2.2. MATERIAL AND METHODS

### 2.2.1. Study Area

The physical–chemical parameters were sampled across the south of La Palma, located in the west of the Canary Islands (north-eastern Atlantic Ocean) (Figure 2.1A). The sampling took places between a 0 and 2m depth, at three different times (March 2018, December 2018, and June 2019) and during low and high tide when it was necessary to assess the continuity of the natural emissions (Figure 2.1B, Table B.1. in Appendix B). Following the previous studies in the area (Hernández et al., 2016; Pérez, 2017; González-Delgado et al., 2018a, b; Hernández et al., 2018; Viotti et al., 2019), a sampling network was created for the first time. It is formed by the following sites: Playa del Faro, Los Porretos and surroundings (that together with the Las Cabras site are known as the Punta de Fuencaliente system or PFS), Playa Echentive and the two Echentive lagoons (Figure 2.1C). Scuba diving was used for sampling all bottles except in the reference station off Playa del Faro (Figure 2.1C-A), where a CTD rosette was used. For the scuba sampling, the sampling bottle was previously rinsed three times at the sampling location and then the bottle was immersed with the mouth down and turned at 1m depth for sampling. Samples were poisoned with 100 µL of saturated $HgCl_2$ solution, sealed, kept in darkness, and analysed in the laboratory. In March 2018, this was performed on the same day, while in December it was performed 2 days later. For pH, 100mL borosilicate glass bottles were filled with seawater.



| ID | Site | Time | Measures | Tide |
|---|---|---|---|---|
| A | Playa del Faro (out) | D | ALL | LT |
| B | Playa del Faro | MDJ | ALL | LT/HT |
| C | Los Porretos | MD | ALL | LT/HT |
| D | Los Porretos (out) | D | ALL | LT |
| E | Playa Echentive | D | pH | LT |
| F | Echentive lagoon 1 | MDJ | ALL | LT/HT |
| G | Echentive lagoon 2 | D | pH | LT |
| H | Las Cabras | M | pH | LT |

☆ *Mark for understanding interpolation graphs (Figs. 2.2-4)*

Figure 2.1. (A) Location of study are on North-Eastern Atlantic Ocean, in the west of the Canary Island, in the South of La Palma Island. (B) Location of the seven sampling sites (A-G) in the south of the island of La Palma. The location of Punta Las Cabras (H) considered in Hernández et al. (2016) is also included. The area covered by the last volcanic eruption, Teneguía volcano, is also indicated. (C) Location of sampling network performed in this work around the Punta de Fuencaliente system (PFS) and the selected area where the volume was calculated to $CO_2$ flux calculation. The stars are included as a help to better interpret and locate the interpolation graphics from Figure 2.2-4. The map and image base layers used are distributed under public domain (https://www.grafcan.es/).

## 2.2.2. Carbon Dioxide System Parameters

In March and December 2018, the total dissolved inorganic carbon concentration ($C_T$), total alkalinity ($A_T$), pH, salinity and temperature were measured, whilst in June 2019 only the pH and temperature were measured. Total alkalinity and $C_T$ were determined by potentiometric and coulometric methods, respectively, using a VINDTA 3C system (Mintrop et al., 2000). The calibrations were made using certified reference material batch no. 163 (González-Dávila et al., 2007). The pH was measured at a constant temperature of 25 °C within 1 h of sampling, using an Orion pH meter with a combined Orion glass electrode ($pH_{T,is}$). The calibration was performed on the total seawater scale using a Tris artificial seawater buffer (salinity 35) according to the Guide to Best Practices for Ocean $CO_2$ Measurements (Dickson et al., 2007, SOP 6a).

Salinity and temperature were measured *in situ* using a handheld conductivity meter (Hanna Instruments HI98192). Furthermore, 200mL salinity bottles were measured in the laboratory within 2 d and using a high-precision Portasal salinometer, accurate to ±0.001. The pH under *in situ* conditions, the partial pressure of carbon dioxide ($pCO_2$) and the saturation states of calcium carbonate forms ($\Omega$ aragonite and calcite) were determined from $A_T$ and $C_T$ data using the $CO_2$SYS program (Pierrot et al., 2006).

Atmospheric $CO_2$ concentrations used for flux calculations were those measured at the Izaña station on the island of Tenerife (IZO site and available in the World Data Centre for Greenhouse Gases). We used the linear interpolation method to represent the $A_T$, $C_T$, $pH_{T,is}$, $\Omega$ aragonite and calcite parameter measurement when anomalies were found.

## 2.3. RESULTS

After extensive sampling throughout the south of La Palma, we detected four areas where natural enrichment of $CO_2$ groundwater emissions occurs. These four areas, Las Cabras, Playa del Faro, Los Porretos and the two Echentive lagoons (Figure 2.1B, C), correspond to areas that were not buried by the lava during the last eruption (Teneguía volcano, 1971, Figure 2.1B). The Las Cabras site was discarded in subsequent samplings due to difficult access, the poor sea conditions and the small size of the area affected by the emissions (Hernández et al., 2016). In all cases, the anomalies were the highest during low tide (Appendix B, Figure B.1).

### 2.3.1. Temperature and Salinity

Temperature and salinity on Playa del Faro and Los Porretos do not present major changes between the different time points (Dataset online: https://doi.org/10.5194/bg-18-1673-2021). During March 2018, Playa del Faro had an average temperature of 19.00±0.20°C with colder values of 18.70°C near the shore; Los Porretos was not measured at this time. In December 2018, both Playa del Faro and Los Porretos presented an average temperature of 21.50±0.02°C. However, salinity values present a minor diminution from 37.05 to 36.51 on Playa del Faro and from 37.05 to 36.07 on Los Porretos (Dataset: https://doi.org/10.5194/bg-18-1673-2021). Both sites presented colder and slightly less saline water near the coast. Regarding the Echentive lagoons, only the biggest lagoon was measured, where the salinity varied from 31.00 to 32.00 units. The same lagoons presented warmer temperatures than the coastal waters during June 2019, 26.40±0.70 and 22.00±0.10°C, respectively.

### 2.3.2. Carbon Dioxide System Parameters

In both studied shore areas of the PFS (Playa del Faro and Los Porretos) the parameters of the carbon dioxide system, $pH_{T,is}$ (Figure 2.2A, B), $A_T$, $C_T$, and $\Omega$ aragonite and calcite (Figures 2.3, 2.4A), were strongly affected by the entrance of the SGD with less salinity.

#### *Playa del Faro*

In March 2018, the pH changed from 8.06 in offshore samples to 7.50 near the shore, reaching 7.16 and 7.13 during December 2018 and June 2019, respectively (Figure 2.2Aa). Similarly, high $A_T$ and DIC were measured throughout Playa del Faro. In March 2018, the ocean data obtained in the furthest coast station of Playa del Faro reached typical values of 2132.13 and 2418.38 μmol $kg^{-1}$ for $C_T$ and $A_T$ (Dataset online: https://doi.org/10.5194/bg-18-

1673-2021). As we approached the shore, both factors increased to values that exceeded 3100 μmol kg$^{-1}$, following an inverse distribution observed with salinity, with an increase in the $C_T$:$A_T$ ratio close to 1:1, indicating an important contribution of bicarbonate in the area (Figure 2.3Aa, b). In December 2018, the anomaly increased to over 3500 μmol kg$^{-1}$ for both parameters. As a direct consequence of the low pH values, although compensated for by the high $C_T$, $A_T$, and dissolved calcium contents (determined by ICP-MS–inductively coupled plasma mass spectrometry, data not presented), the calcite and aragonite saturation states were also affected. It was observed that the area nearest to the shore presented saturation values of calcite and aragonite that were below 1.50 (Figure 2.3Ac, d).

During high tide, the anomalies almost disappeared, which means that the tide acts as a pressure plug of the flow of this water to the coastal area. Nevertheless, we still found a mild increase in $A_T$ and $C_T$, reaching 2692.13 and 2512.35 μmol kg$^{-1}$, respectively (Figure 2.3Ba, b) and pH values of 7.75–7.85 in the sampling points closest to the coast (Figure 2.2A).

### *Los Porretos*

Los Porretos is a continuation of Playa del Faro that is also affected by the SGD with high $C_T$ and low pH. This discharge was first observed during March 2018. The measured $C_T$ exceeded 3400 μmol kg$^{-1}$, and the $pH_{T,is}$ reached 7.25 at the emission station (Figures 2.2B, 2.4Aa). In December, the sampling was repeated, observing that the most anomalous values occurred in the stations closest to the coast. The emission point presented $C_T$ concentrations of 3456.6 μmol kg$^{-1}$ (corresponding to carbon dioxide pressure values of 5200 μatm), pH values of 7.27, and 1.45 and 0.95 values of Ω calcite and aragonite, respectively (Figures 2.2B, 2.4Ac, d; Dataset online: https://doi.org/10.5194/bg-18-1673-2021).

In both beaches, the emission is acting as an important source of $CO_2$ into the atmosphere. On Playa del Faro, the partial pressures of $CO_2$ in surface waters reached up to 5000 µatm at low tide (the values in the atmosphere were between 405 and 410 µatm) (Dataset online: https://doi.org/10.5194/bg-18-1673-2021). This produced high concentration gradients that combined with high-intensity winds characteristic of the area and produced $CO_2$ fluxes that can reach up to 1 mol $m^{-2}$ $d^{-1}$ (considering its main effects during low tide and Wanninkhof, 2014, for the gas transfer velocity coefficient) that amount to 150 t $CO^2$ $yr^{-1}$.

### *Echentive lagoons*

The two lagoons at Playa Echentive (Figure 2.1C) show the maximum anomalies on the south of La Palma. They presented low salinities and low pH, below 7.5 in all stations and reaching 7.39 in the north-west during March 2018 (data only from the big lagoon) (Figure 2.2C, D). Similarly, the $C_T$ was above 9700 µmol $kg^{-1}$, with comparable values for $A_T$ (Figure 2.4Ba, b). These $C_T$ and $A_T$ concentrations together with the low pH values counteracted the saturation states of calcite and aragonite that were never below 4.35 and 2.79, respectively (Figure 2.4Bc, d). Furthermore, when both lagoons were sampled during December 2018, similar concentrations were measured at low and high tide (Figure 2.2C, D). The north-western part of the big lagoon presented the highest $C_T$ concentration (greater than 10000 µmol $kg^{-1}$), and the lowest pH reached 7.38 at low tide and 7.55 at high tide, which coincided with a decrease in salinity and a mild temperature increase (Figure 2.2C, D; Dataset online: https://doi.org/10.5194/bg-18-1673-2021). The rest of the big lagoon remained at pH 7.58, like the small lagoon with a maximum pH of 7.63. However, the small lagoon presented a lower pH range, with a minimum of 7.50 at low tide and a maximum of 7.64 at high tide in the northern part (Figure 2.2C, D).

The water levels in both lagoons were tide dependent. The entry of salty marine water during high tide reduced the anomaly caused by the intrusion of lower-salinity water rich in $C_T$ and $A_T$.

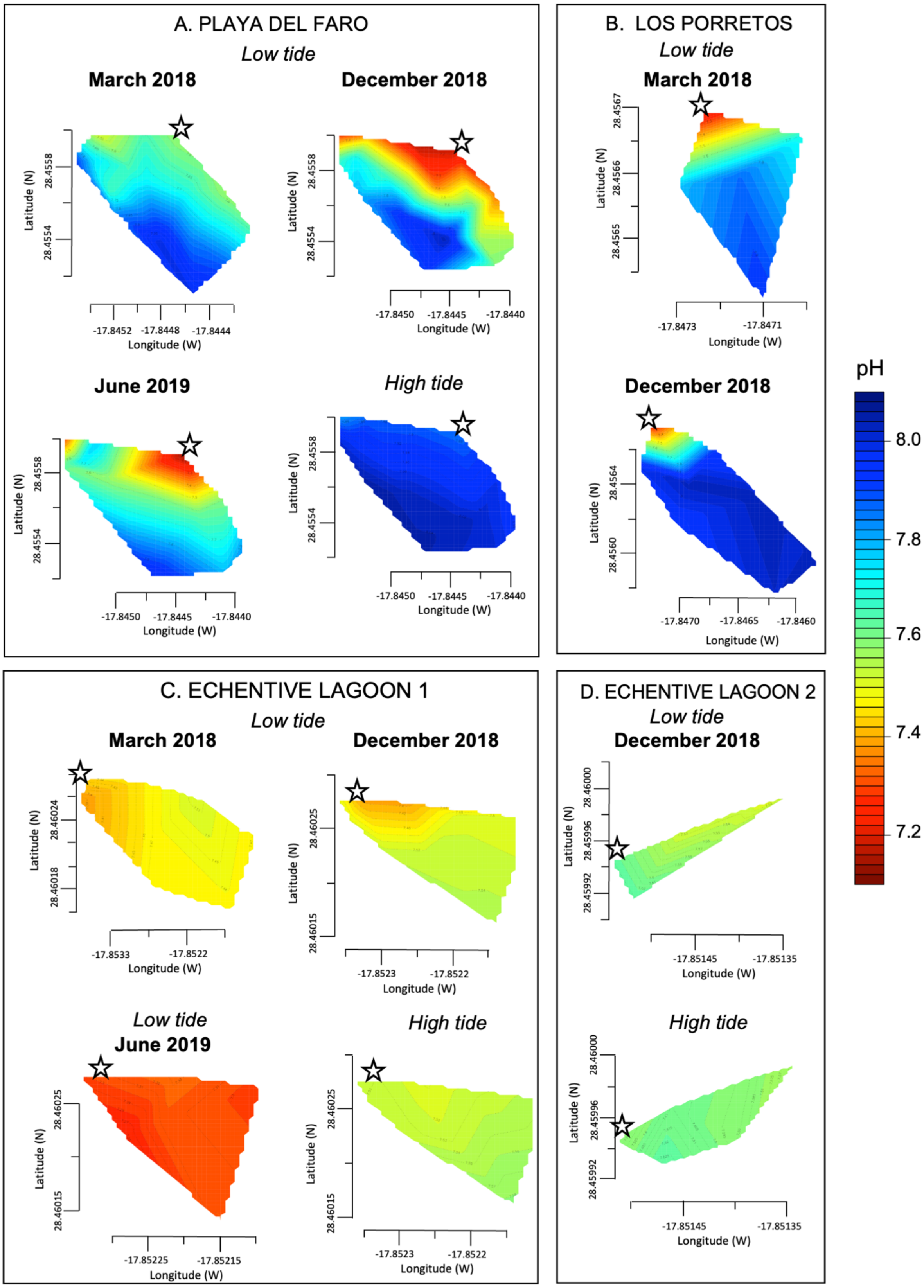


Figure 2.2. Linear interpolation graphs of pH values that were collected in March 2018, December 2018, and June 2019 during low tide (LT) and high tide (HT) in Playa del Faro (A), Los Porretos (B), Echentive lagoon 1 (C) and Echentive lagoon 2 (D). The star symbol is the reference mark on the map in Figure 2.1C.

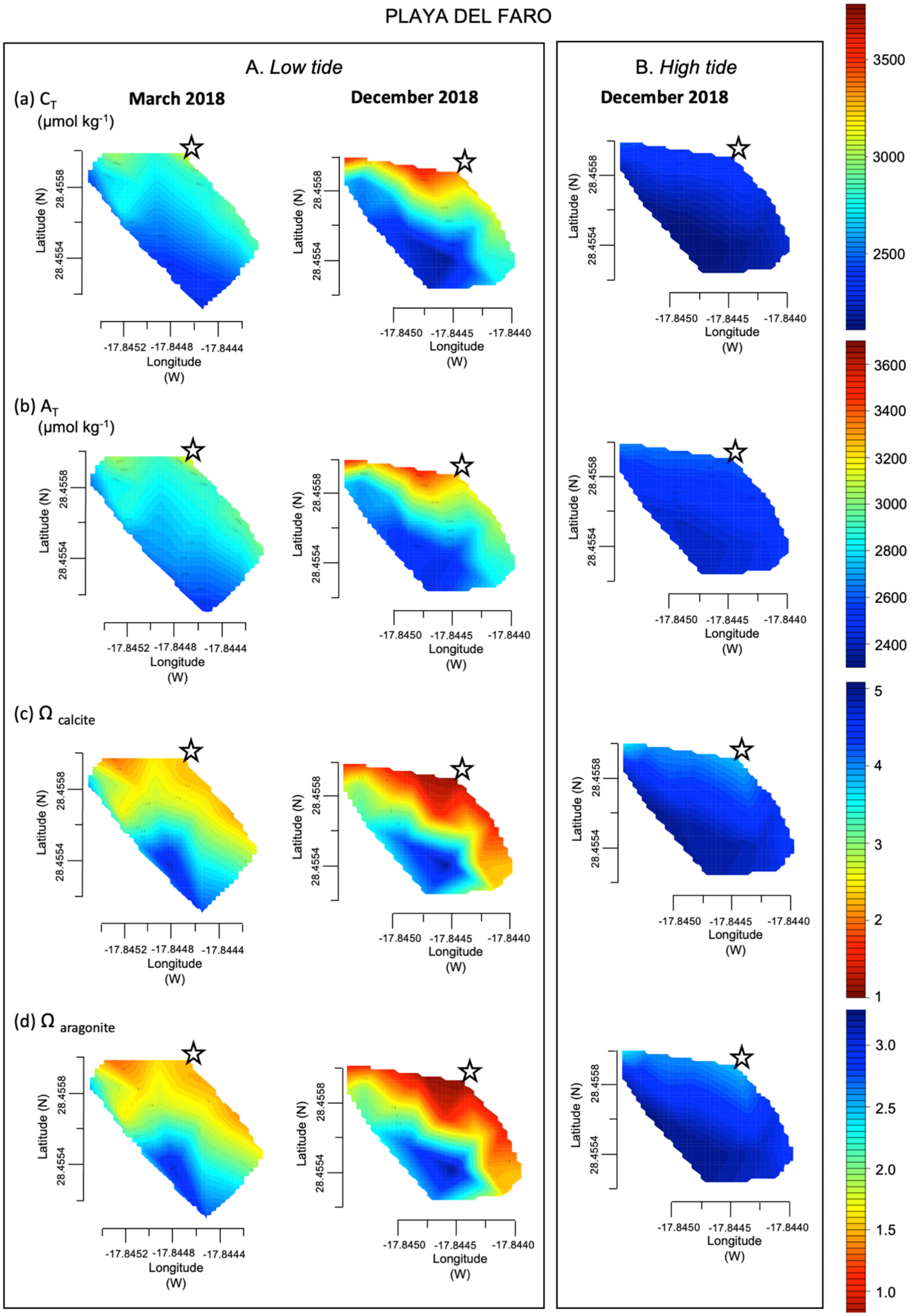


Figure 2.3. Linear interpolation graphs of $C_T$ (a), $A_T$ (b), Ωcalcite (c) and Ωaragonite (d) values during March 2018, December 2018 during low tide (A) and high tide (B) in Playa del Faro. The star symbol is the reference mark on the map in Figure 2.1C.

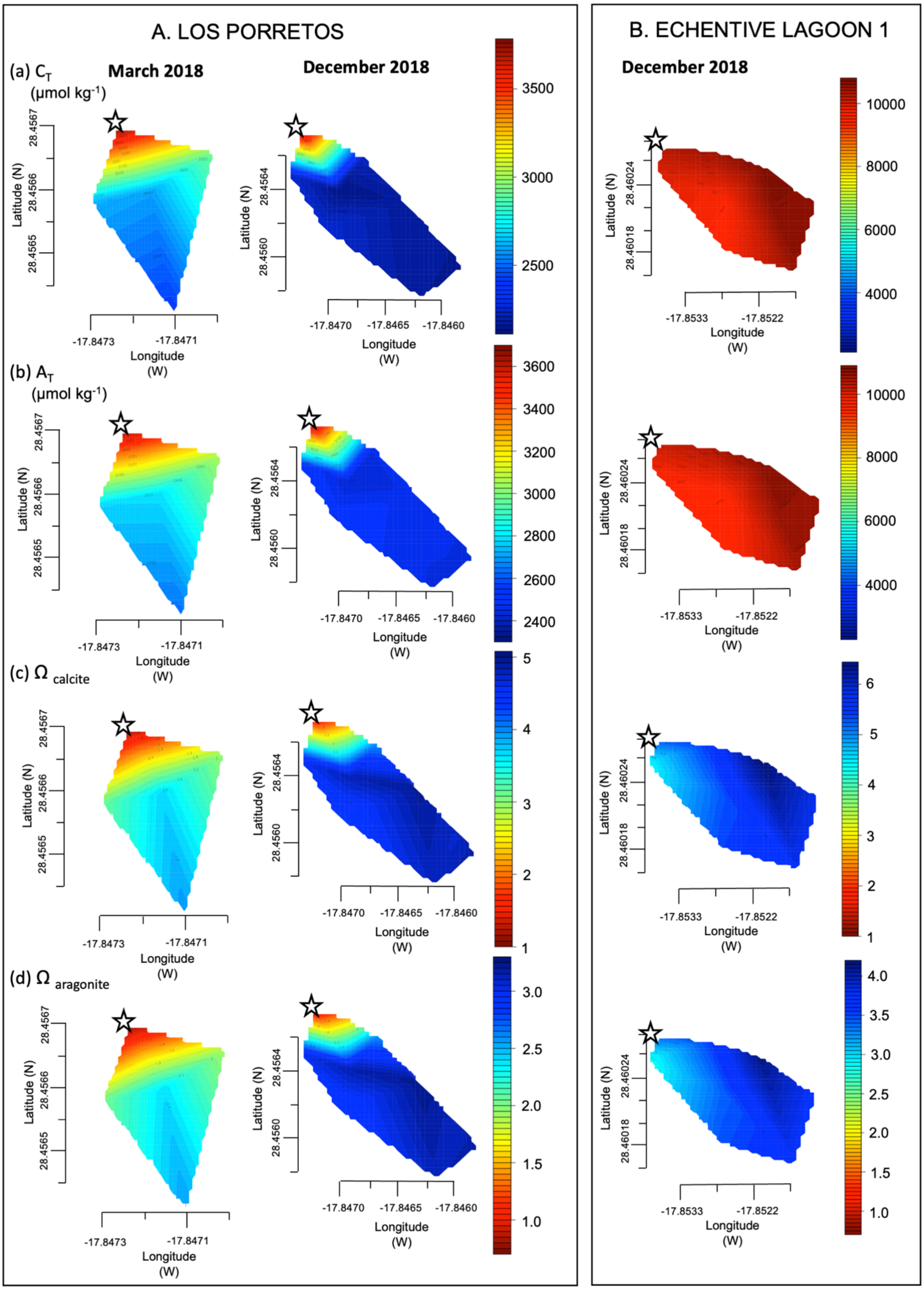


Figure 2.4. Linear interpolation graphs of $C_T$ (a), $A_T$ (b), Ωcalcite (c) and Ωaragonite (d) values during March 2018 and December 2018 during low tide (LT) in Los Porretos (A) and Echentive Lagoon 1 (B). The star symbol is the reference mark on the map in Figure 2.1C.

### 2.3.3. $CO_2$ Flux Calculation

One The $CO_2$ flux was calculated for Playa del Faro. We assumed two endmembers, the open ocean endmember, and the SGD endmember. Soler-Liceras (2007) discovered an aquifer near this area with brackish water (salinity of 30). Considering the bathymetry, the volume occupied by seawater was 19 700m$^3$. We also assumed that groundwater discharge only occurred at low tide. The average salinity changed from 36.93 (equivalent to 745.8 t of sea salt) at low tide to 37.02 at high tide (747.5 t of sea salt). The decrease in salinity at low tide could be accounted for by the emission of 57m$^3$ of brackish groundwater.

The brackish groundwater was also responsible for the $A_T$ and $C_T$ changes (Figure 2.3a, b). Alkalinity increased by 219 µmol kg$^{-1}$ from high (2465 µmol kg$^{-1}$) to low tide (2684 µmol kg$^{-1}$). Considering 57m$^3$ of brackish water, 4.40 kmol of alkalinity was required; therefore, the brackish groundwater had an $A_T$ concentration of 76 mmol kg$^{-1}$. Similarly, the $C_T$ on the beach increased by 333 µmol kg$^{-1}$, from high tide (2190 µmol kg$^{-1}$) to low tide (2523 µmol kg$^{-1}$). The brackish water caused the increase of 6.7 kmol of inorganic carbon on the beach and, hence, had an endmember concentration of 116 mmol kg$^{-1}$.

Considering the *in situ* temperature (20.67°C), the $pH_{T,is}$ decreased by 0.25 from 8.01 at high tide to 7.76 at low tide. This meant that the acidity increased by 80 %. This pH reduction meant that the water discharged on the beach had a pH of 5.57. The medium partial pressure of carbon dioxide for the area increased from 459 µatm at high tide to a value of 988 µatm at low tide. Considering an average wind speed at the beach of 7ms$^{-1}$ (https://datosclima.es/Aemethistorico/Vientostad.php, last access: 5 March 2020), Playa del Faro acts as a strong source of $CO_2$, emitting 5.70 mmol$CO_2$ m$^{-2}$ d$^{-1}$ at high tide and increasing by an order of magnitude at low tide (57 mmol$CO_2$ m$^{-2}$ d$^{-1}$; Wanninkhof, 2014). Consequently, Playa del Faro with its small area of only 0.01 km$^2$ is responsible for an atmospheric $CO_2$ emission flux varying between 2.80 and 28 kg$CO_2$ d$^{-1}$.

## 2.4. DISCUSSION

### 2.4.1. The Origin of the $CO_2$ Submarine Groundwater Discharge

Although $CO_2$ emissions on the Fuencaliente coast had already been detected (e.g., Hernández et al., 2016; Viotti et al., 2019), this is the first time that this naturally acidified system has been described chemically and physically. Previous works have focused on specific questions; Hernández et al. (2016) published for the first time the presence of $CO_2$ SGD in Fuencaliente, specifically on Las Cabras beach. Later, in the thesis by Pérez (2017), as well as in the conference papers by González-Delgado et al. (2018a, b) and in the article by Viotti et al. (2019), new points of acidification were discovered on Playa del Faro and Los Porretos. However, in none of them was a chemical characterization of the whole area made as it was here. Our results reveal the continuous influence of brackish water discharge in the acidification process of the Punta de Fuencaliente system (PFS), which had been missed before (Figure 2.5). Similarly to aerial remnant volcanic activity on La Palma that generates high $CO_2$ diffusive atmospheric concentration (Padrón et al., 2015), submarine remnant volcanic activity causes the acidification process found here, as indicated by the chemical composition of the groundwater analysed, which is less than 200m from the coast (Soler-Liceras, 2007). The activity of this SGD is comparable with other $CO_2$ vent and seep systems worldwide (references within González-Delgado and Hernández, 2018). Moreover, the presence of the acidic water flow of La Palma also has a slight resemblance to the acidification phenomenon found in Mexico, originating from a karstic groundwater discharge (Crook et al., 2012). Furthermore, the highly alkalized and bicarbonate waters found in Echentive lagoons are an artefact of water discharge from the hydrothermally affected aquifers of the area (Soler-Liceras, 2007), as found in Las Cañadas del Teide, in Tenerife (another island of the same archipelago) (Marrero et al., 2008).

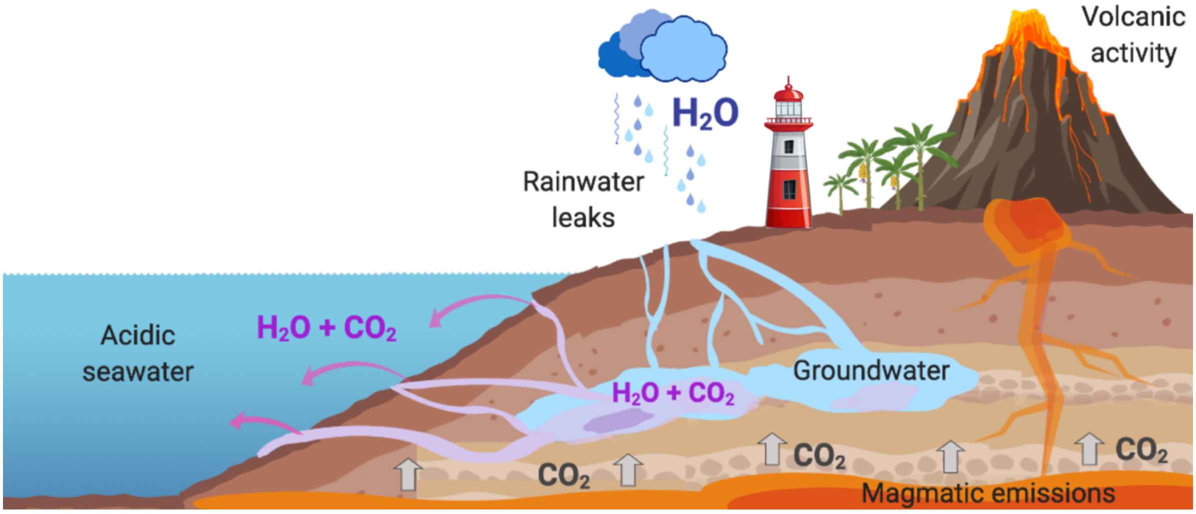


Figure 2.5. Acidification process representation of Punta de Fuencaliente system (PFS) (made with Biorender).

In the PFS there is a decrease in salinity due to brackish water discharges. Hence, there is a constant filtration of brackish acidified waters through highly permeable volcanic rocks (Carracedo et al., 2001; Marrero et al., 2008), with chemical features due to underground volcanic activity, such as a 5.57 pH and a concentration of 76 mmol $kg^{-1}$ of $A_T$ and 116 mmol $kg^{-1}$ of $C_T$. However, the effect on the surrounding seawaters depends upon tidal pressure and, more likely, upon other oceanic forces such as wind and waves (Moore, 2010; Mulligan et al., 2019).

### 2.4.2. Alteration of the Carbon Chemistry System and Implications for Organism´s Assemblages

In the case of the PFS, the water with lower salinity (36.79–36.45) and high concentrations of $C_T$ and $A_T$ affect the surroundings, decreasing the seawater pH by up to 0.8 and reducing the carbonates' saturation state by up to 1.1 for calcite and 0.7 for aragonite. This situation generates a carbon imbalance affecting carbonated organisms, especially those that precipitate aragonite on their calcareous structures (Kroeker et al., 2010). When the saturation values are below 1, the formation of carbonates is not thermodynamically possible, although certain species require much higher saturation levels

(Kroeker et al., 2010). The calcifying organisms that could live in these acidified areas may present weaker shells, skeletons and/or others solid structures, as we have recently observed in the mollusc *Phorcus sauciatus* (Viotti et al., 2019), as well as in other calcifying organisms (Pérez, 2017). This excess of $CO_2$ has also modified the community composition and trophic structure, causing a loss of ecological and functional diversity in the benthic marine ecosystem (González-Delgado et al., 2021).

In the case of Echentive lagoons, the anomaly is amplified due to a lower tidal influence and insulation. These acidified lagoons, which are at around a 200m distance from the coast (Figure 2.1), have a salinity of 32 and $C_T$ and $A_T$ concentrations 5 times higher than normal ocean values. The $C_T$ and $A_T$ concentrations are so high that they compensate for the decrease in pH with the content of carbonates in the water. These singular characteristics create a unique marine ecosystem. The environment is dominated by a biofilm of microorganisms, predominantly microalgae, cyanobacteria, and diatoms (Sangil et al., 2008) and probably other bacteria and fungi. Nonetheless, some marine invertebrates persist, such as the common errant polychaete *Eurythoe complanate* and the anemone *Actinia* sp. (Sangil et al., 2008). A more in-depth physiological study of these species could help us to better understand their adaptation process to these conditions and to give insights into what we might expect in future ocean acidification conditions, especially in the PFS area.

### 2.4.3. La Palma as a Natural Laboratory for Marine Research

The natural $CO_2$ gradients south of La Palma have been characterized from shore to offshore, varying for $C_T$ from 2120.10 to 3794.00 µmol kg$^{-1}$, for pH from 7.12 to 8.07, for Ω aragonite from 0.71 to 3.28 and for Ω calcite from 1.09 to 5.02. This high local variability is in line with other acidified natural systems. For example, the $CO_2$ vent of Ischia (Italy) has pH levels from 6.07 to 8.17, Ω aragonite from 0.07 to 4.28 and Ω calcite from 0.11 to 6.40 (Hall-Spencer et al., 2008). The one from the island of Vulcano (Italy) has pH values between 6.80 and 8.20, Ω aragonite from 1.49 to 4.65, and Ω calcite from 2.28 to 7.00 (Boatta et al., 2013). Meanwhile the $CO_2$ seeps from Papua New Guinea have pH levels between 7.29–7.98, Ω aragonite between 1.2–3.4 and Ω calcite between 1.36–5.12 (Fabricius et al., 2011). Those from Shikine-jima (Japan) have pH values between 6.80 and 8.10, Ω aragonite from 0.20 to 2.22, and Ω calcite from 0.30 to 3.45 (Agostini et al., 2015). Although these systems are far from being perfect predictors of the ocean future due to their chemical variability and physical limitations, they have proven to be important tools for the study of ocean acidification (Foo et al., 2018b; González-Delgado and Hernández, 2018; Aiuppa et al., 2021). These naturally acidified systems, such as the Punta de Fuencaliente system (PFS), can be used as natural analogues of climate change scenarios predicted by the IPCC (2014) (Figure 2.6). Therefore, the PFS can be considered a very useful spot for large-scale and long-term adaptation experiments, as seen in other $CO_2$ systems (e.g., Ricevuto et al., 2014; Uthicke et al., 2019). Moreover, the acidified system of La Palma is highlighted by the absence of bubbling, since the volcanic degasification takes place in the aquifers and not directly on the coast as in other acidified systems of volcanic origin (e.g., Hall-Spencer et al., 2008; Fabricius et al., 2011) (Figure 2.5). This could give us new insights into the effect of acidification *in situ* avoiding the effects of bubbling (González-Delgado and Hernández, 2018). Nevertheless, several caveats for future prediction experiments should be considered, here as well as in other naturally acidified systems, especially those related to increased alkalinity values in the submarine discharge.

First, there is a clear tidal influence; this is an important force that controls the acidified brackish water discharges. Although a fluctuation in the emission is observed, normal ocean conditions can occur for a short time, about 2–4 h $d^{-1}$, during high tide and depending on the oceanic conditions (Viotti et al., 2019). The $pH_{T,is}$ is severely affected by the location, reaching down to ~ 7.2 in the emission points, so a careful selection of the study sites is recommended, depending on the study objectives (Figure 2.6). This tidal phenomenon has also been reported in other acidified natural systems such as Puerto Morelos in Mexico (Crook et al., 2012) and Ischia (Kerrison et al., 2011). However, the pH time fluctuation can be used to our advantage, as a daily and seasonal fluctuation in the pH is normal in coastal habitat environments (Hofmann et al., 2011). So, it could be considered very useful to incorporate pH variability in ocean acidification studies as environmental fluctuations that can have a large impact on marine organisms (Hofmann et al., 2011).

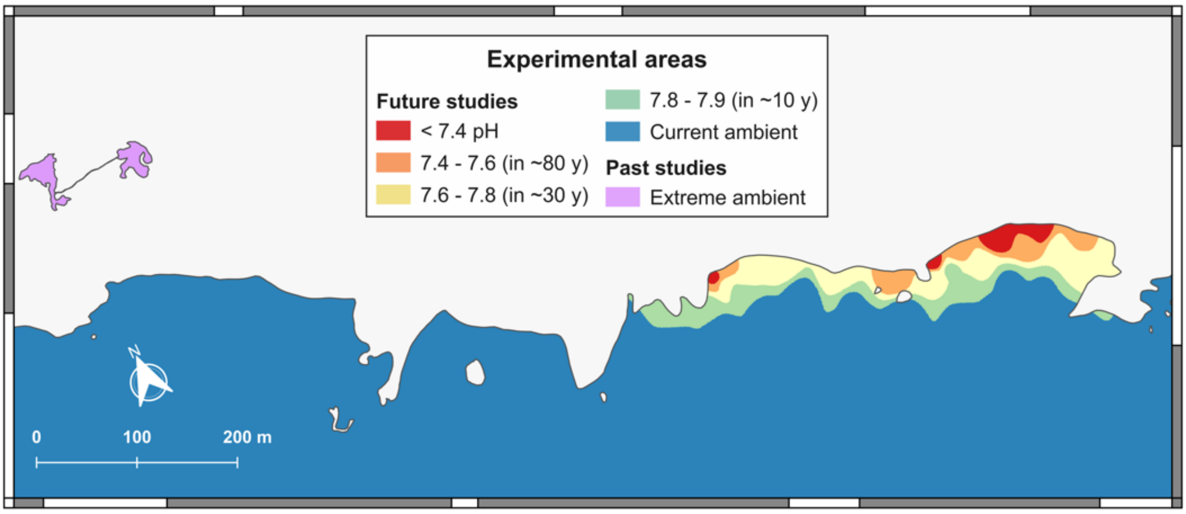


Figure 2.6. Selected areas for experimental purpose (Interpolation IDW, 4.0 of correlation with Qgis).

Second, one of the most common concerns with $CO_2$ seeps and SGD areas is the presence of other gases or elements associated with volcanic emissions, such as nitrogen ($N_2$), mercury (Hg) or methane ($CH_4$) (e.g., Fabricius et al., 2011; Boatta et al., 2013; Aiuppa et al., 2021). Although there are no traces of the presence of volcanic elements such as methane or sulfates that are harmful to marine organisms in the seawater of the PFS (Hernández et al., 2016), there is an extra supply of different elements such as Mg that comes from groundwater (Soler-Liceras, 2007). Groundwater has 10 times more magnesium than normal, but when mixed with seawater, the supply is considerably lower compared to $CO_2$. Nevertheless, Mg plays an important role in the calcification of marine organisms that have magnesite–calcite, such as echinoderms (Weber, 1969) and some Bryozoa species (Smith et al., 2006). Similarly, Hernández et al. (2016) found an increase in silicates in the nearby area of Las Cabras. In these cases, Si could participate in the calcification of diatoms (Paasche, 1973) as well as of many sponges (Smith et al., 2013). The increase in these essential elements for certain calcifying species can allow their survival and growth in the PFS while buffering the effects of acidification (Smith et al., 2016a; Ma et al., 2009). Therefore, measurements of other metals in seawater should be considered in the following studies.

The high concentration of bicarbonate in the brackish waters also implies an extra contribution of alkalinity and carbonate that can buffer the effect of acidification in the area, so it is necessary to take this into account when making predictions of the future. These values together with calcium content are especially important factors in the case of the saturation state for both calcite and aragonite, which shows high values for seawater with low pH values. Hence, even though we are dealing with a subtropical ecosystem, the values obtained in both saturation states are more similar to the predictions for a tropical ecosystem, such as the values found in Papua New Guinea seeps (Fabricius et al., 2011; IPCC, 2014). Finally, the area is not very large and only one type of rocky benthic habitat, the most typical community of the Canary Islands, is present at the PFS (Sangil et al., 2018). Therefore, all conclusions derived from this acidified system should be interpreted with caution and acknowledging local effects. Hence, it is crucial to establish a collaborative network of researchers who are working in other naturally acidified systems worldwide to have a more realistic interpretation of future ocean scenarios.

The Echentive lagoons are an oversaturated carbonate system. Like hydrothermal alkalinity vents (Martin et al., 2008), they could help us to understand early life on Earth from the Precambrian, 4000 million years ago, when the atmosphere was rich in $CO_2$ (Kasting, 1993; Nakamura and Kato, 2004) (Figure 2.6). These studies could allow us to disentangle the adaptation and evolution of marine life to the changing carbonate conditions over time (Gattuso et al., 1998).

Additionally, to our knowledge, this is the first time that a brackish water discharge altered by volcanic activity has been studied. Each studied beach with a contribution of 150 t $CO_2$ $yr^{-1}$ becomes an important source of carbon into the sea. Correspondingly, Playa del Faro is emitting 28 kg $CO_2$ $d^{-1}$ in each tidal flow to the atmosphere. This may seem very scarce compared to volcanic eruptions such as the most recent in the Canaries that occurred on the neighbouring island, El Hierro, in 2010, which was emitting $6.0 \times 10^5 \pm 1{:}1 \times 10^5$ kg $d^{-1}$, and now the emissions of the PFS are unappreciated (Santana-Casiano et al., 2016). However, the flux of $CO_2$ from La Palma seems to have started before the islands were conquered in 1493 (Soler-Liceras, 2007), being in a more advanced degassing phase than El Hierro with fewer emissions and continued over time. Therefore, if we consider its timescale, La Palma becomes a significant $CO_2$ source. For all these reasons, the PFS and the lagoons are an interesting area for future hydrological and oceanographic research, helping in new studies focusing on groundwater fluxes, the oceanic water cycle and oceanic carbon fluctuation (Moore, 2010; Santana-Casiano et al., 2016; Mulligan et al., 2019).

## 2.5. CONCLUSIONS

The studies carried out show the existence of continuous natural acidification on the southern coast of La Palma. This acidification process is caused by two natural phenomena: the discharge of submarine brackish waters from the aquifer and the magmatic emissions of $CO_2$ gas. Therefore, the monitoring of both sources is important not only from a biological point of view but also from an atmospheric, oceanographic, volcanologist and hydrological perspective. The groundwater discharges found on Playa del

Faro and Los Porretos (PFS) have similar chemical properties (even when alkalinity does not remain constant) that create a natural pH gradient analogous to future ocean conditions. Consequently, they can be used as natural laboratories to predict the effects of OA on the functioning of future oceans. In addition, the interior Echentive lagoons where the chemical alterations are intensified present the conditions capable of disentangling how life has persisted during higher-atmospheric-$CO_2$ periods on planet Earth.

# CHAPTER 3

## High Taxonomic Diversity and Miniaturization in Benthic Communities Under Persistent Natural $CO_2$ Disturbances

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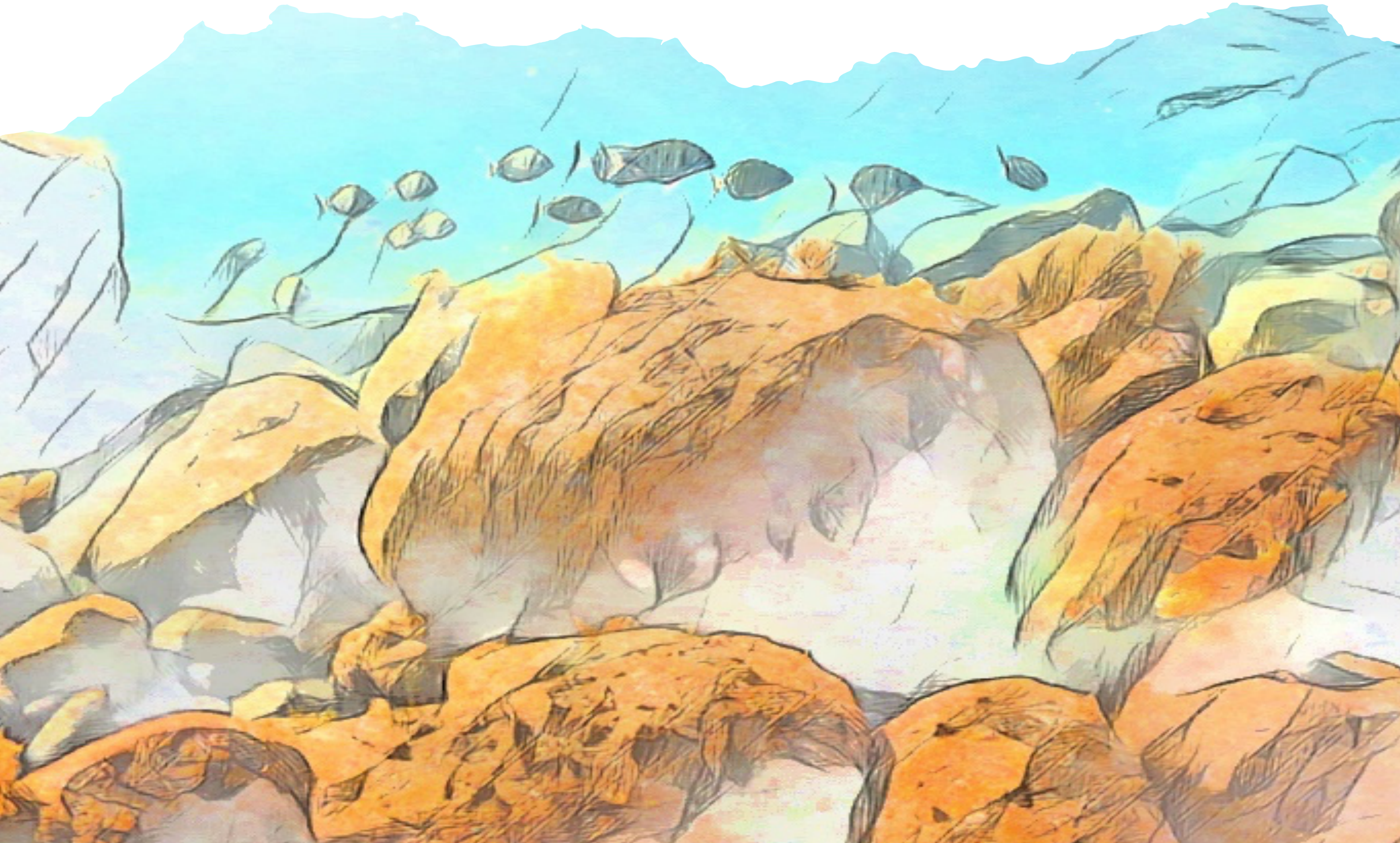

## ABSTRACT

Metabarcoding techniques have revolutionized ecological research in recent years, facilitating the differentiation of cryptic species and revealing previously hidden diversity. In the current scenario of climate change and ocean acidification, biodiversity loss is one of the main threats to marine ecosystems. Here, we explored the effects of ocean acidification on marine benthic communities using DNA metabarcoding to assess the diversity of algae and metazoans. Specifically, we examined the natural pH gradient generated by the Fuencaliente $CO_2$ vent system, located near La Palma Island (Canary Islands). High-resolution COI metabarcoding analyses revealed high levels of taxonomic diversity in an acidified natural area for the first time. This high number of species arises from the detection of small and cryptic species that were previously undetectable by other techniques. Such species are apparently tolerant to the acidification levels expected in future oceans. Hence and following our results, future subtropical communities are expected to keep high biodiversity values under an acidification scenario, although they will tend toward overall miniaturization due to the dominance of small algal and invertebrate species, leading to changes in ecosystem functions.

Keywords: $CO_2$ vent, eukaryotic metabarcoding, biodiversity, ocean acidification.

## RESUMEN

Las técnicas de metabarcoding han revolucionado las investigaciones en ecología en los últimos años al facilitar la diferenciación de especies crípticas y revelar una diversidad que hasta ahora estaba oculta. En el actual escenario de cambio climático, la pérdida de biodiversidad es una de las principales amenazas para los ecosistemas marinos. En este estudio, utilizamos la técnica de metabarcoding de ADN para evaluar la diversidad de algas y metazoos en las comunidades bentónicas marinas y explorar los efectos de la acidificación oceánica. Específicamente, examinamos el gradiente natural de pH generado por el sistema de afloramiento de $CO_2$ de la Punta de Fuencaliente, situado cerca de la isla de La Palma (Islas Canarias). Los análisis de metabarcoding de alta resolución, utilizando el marcador COI, revelaron por primera vez altos niveles de diversidad taxonómica en un área natural acidificada. Este elevado número de especies se debe a la detección de especies pequeñas y crípticas que anteriormente eran indetectables mediante otras técnicas. Estas especies aparentemente son tolerantes a los niveles de acidificación que se esperan en los océanos del futuro. Por lo tanto, nuestros resultados sugieren que las futuras comunidades subtropicales mantendrán altos valores de biodiversidad bajo un escenario de acidificación, aunque tenderán hacia una miniaturización general debido al dominio de especies pequeñas de algas e invertebrados, lo que conllevará cambios en las funciones del ecosistema.

Palabras claves: Afloramiento de $CO_2$, metabarcoding de eucariotas, biodiversidad, acidificación oceánica.

## 3.1. INTRODUCTION

Ocean acidification (OA), due to rising atmospheric $CO_2$ levels, is one of the threats faced by marine ecosystems, with potential detrimental effects on the services they offer to our society (Hall-Spencer and Harvey, 2019). The concern surrounding OA has resulted in a wave of research tackling a range of questions about the potential effects of rapid pH decrease on marine biodiversity (Linares et al., 2015; Teixidó et al., 2018), ecosystem structure (e.g., Sunday et al., 2017), trophic dynamics (e.g., Watson et al., 2017) and the resilience mechanisms of organisms (e.g., Cornwall et al., 2017).

In recent years, some researchers have focused on natural marine volcanic $CO_2$ vents as a proxy for exploring the potential effect of OA on future ecosystems (González-Delgado and Hernández, 2018; Foo and Byrne, 2021). These natural seeps are characterized by the emission of $CO_2$, which mixes with water, altering the seawater carbon system and generating pH gradients over distances of only a few hundred meters. Along these strong gradients, some sites exhibit pH values similar to those predicted by the IPCC (2021) to occur in oceans over the next few decades (González-Delgado and Hernández, 2018). Thus, these sites constitute ideal natural laboratories for applying the so-called 'substitution of space for time' approach (Hofmann et al., 2014) because their populations have spent their entire lives under conditions of decreasing pH levels.

The results collected from $CO_2$ vents predict some of the potential impacts of OA, including declines in biodiversity, simplification of the habitat structure (Linares et al., 2015; Agostini et al., 2018), and disruption of interactions within the community, ultimately to the detriment of marine ecosystem functions (Teixidó et al., 2018; Sunday et al., 2017). More specifically, the best-studied impacts are mainly related to declines in the abundance and diversity of macroorganisms with large carbon-based skeletons or biostructures (e.g., Linares et al., 2015; Kroeker et al., 2011). OA limits the amount of carbonate in seawater that is available to be deposited as $CaCO_3$ in skeletons, and studies performed under both natural and experimental conditions show that calcifying organisms, such as coralline crustose algae, mollusks, echinoderms, and reef-building cnidarians, are highly sensitive to OA (Byrne and Fitzer, 2019).

Due to the unfeasibility of obtaining a complete record of all biodiversity present in $CO_2$ vent communities, diversity estimates have been obtained based on *in situ* coverage measurements and/or species identification through morphology-based analyses, focusing on macroorganisms (e.g., Linares et al., 2015; Teixidó et al., 2018; Agostini et al., 2018). Despite the increased power and robustness of visual taxonomic identification methods, most meio-/micro-organisms generally remain unreported in such studies, which might have contributed to the underestimation of the actual levels of diversity present in benthic ecosystems (e.g., Peña et al., 2021) and, by extension, the correct prediction of OA impacts on future diversity. Although missing information has been complemented with analyses of morphological assemblies and the ecological functions of well-known groups (e.g., Teixidó et al., 2018), more accurate and universal methods for biodiversity assessment should be routinely applied in future studies to obtain a more realistic picture of the whole ecosystem. Therefore, the application of high-resolution molecular techniques capable of assessing the real extent of the biodiversity and monitoring whole community changes is an imperative need to better understand the effects of OA on marine ecosystems.

Fortunately, high-resolution techniques for marine biodiversity assessment are now available. Metabarcoding protocols developed and adapted for structurally complex, hard-bottom marine communities are among the most novel and advanced approaches for measuring marine diversity (Leray and Knowlton, 2015; Wangensteen and Turon, 2017; Wangensteen et al., 2018). These molecular techniques based on high-throughput sequencing and molecular taxonomic identification, overcome the limitations of traditional methods of morphological identification (e.g., López-Escardó et al., 2018).

These powerful tools provide accurate diversity measures of an entire community, which allows the relative abundance of the detected species to be estimated (Wangensteen and Turon, 2017), including organisms of all sizes, or fragments thereof, which tend to be overlooked when applying morphological methods of identification (e.g., Gallego et al., 2020). Additionally, metabarcoding reveals hidden biodiversity in the form of cryptic or undescribed species, which might otherwise remain undetectable by more traditional methods (Leray and Knowlton, 2016; Plaisance et al., 2021). Furthermore, the obtained DNA sequences represent objective and traceable data whose quality is independent of taxonomic expertise and the complexity of the community analyzed.

We applied high-throughput, high-resolution metabarcoding techniques to study the subtropical, hard-bottom benthic community affected by the pH gradient generated by the no bubbling $CO_2$ vent off Fuencaliente (La Palma, Canary Islands) (Figure 3.1). The features of this vent include a pH gradient that decreases from a current open-sea average value (pH ~8) at approximately 150 meters from the vent center to <7.4 at the source during low tide (González-Delgado et al., 2021). Therefore, the characteristics of this vent allowed us to test the effect of decreasing pH on marine assemblages. These seep features are like other volcanic areas such as Ischia and Papua New Guinea (e.g., Foo and Byrne, 2021; Fabricius et al., 2014) which have also been used as future ocean analogs (González-Delgado and Hernández, 2018).

In the present work, we analyze benthic community assemblies and the biodiversity levels of multicellular eukaryotes along the natural pH gradient generated by this $CO_2$ vent system, with the aim of measuring the impact of pH variation on rocky-bottom communities. Based on empirical data obtained from other studies conducted at $CO_2$ vents using morphology-based methods of macrospecies identification, we expected to observe a decline in diversity and increased simplification of benthic assemblages associated with the pH decrease (e.g., Linares et al., 2015; Kroeker et al., 2011).

We applied metabarcoding techniques to marine benthic communities to account for previously overlooked diversity, generating fine-scale taxonomic biodiversity information to make more accurate predictions of how communities will change under predicted future OA scenarios.

## 3.2. MATHERIAL AND METHODS

### 3.2.1. Description of the Experimental Area

The southern part of La Palma Island consists of a recent lineation of volcanoes referred to as the Cumbre Vieja Complex. $CO_2$ emissions have been frequented in this geologic complex at least since 1492 (González-Delgado et al., 2021). The vent analyzed in this study, located at Punta de Fuencaliente (Figure 3.1), is included within this complex, and its past volcanic eruption, before the last activity (by the end of 2021), took place in 1971. Hence, the shallow coastal benthic communities in the area are assumed to have been subject to a tide fluctuating acidification for at least 30 years (González-Delgado et al., 2021).

Samples were collected in 4 zones at different distances from the $CO_2$ vent emission point: Zone 1 (Vent: 28.45599 N, 17.84526 W) was located at the $CO_2$ vent emission point; Zone 2 (Transition25: 28.45598 N, 17.84544 W) at a transitional distance of 25 m from the emission point; Zone 3 (Transition75: 28.45595 N, 17.84570 W) at 75 m; and Zone 4 (Control: 28.45659 N, 17.84755 W) at 200 m from the vent and was considered as 'Control' condition (Figure 3.1).

The chemical characteristics of the Punta de Fuencaliente $CO_2$ vent system are briefly explained below; for more detailed information, please refer to González-Delgado et al. (González-Delgado et al., 2021). The values of pH, total alkalinity, and the saturation states of calcite and aragonite (Ω) were measured in each of the sampling zones at both high and low tides (Appendix C, Table C.1). All conditions represented by the parameters became more intense as we approached the vent emission point and during low tides: Zone 1 (Vent), pH 7.65; Zone 2 (Transition25), pH 7.88; Zone 3 (Transition75), pH ~8.09; and Zone 4 (Control), pH ~8.14 during low tide (Appendix C, Table C.1). The pH levels at the vent emission point (Zone 1) and in the transition (Zone 2 and Zone 3) were within the ranges predicted for future oceans approximately 80 years and 30 years from now, respectively (IPCC, 2021). There are no other gases or nutrients affecting this area (González-Delgado et al., 2021; Hernández et al., 2016).

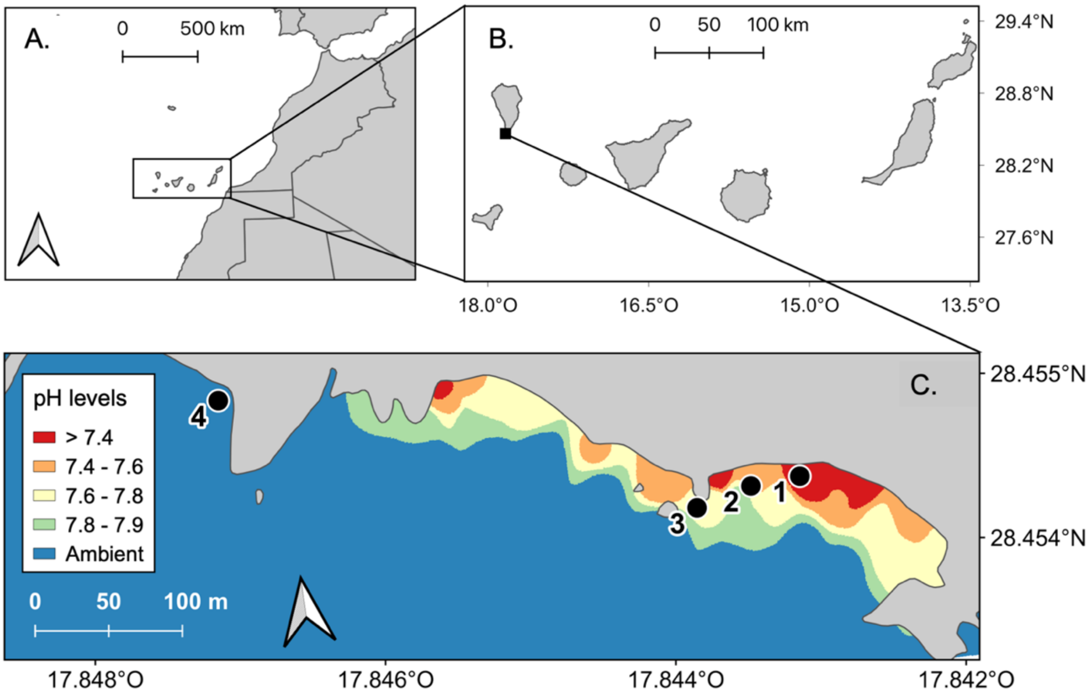

Figure 3.1. Location of the four-sampling area. (A) The Canary Islands at the northeastern Atlantic Ocean; (B) The Canary Islands, and La Palma Island; (C) Punta de Fuencaliente with the four sampling zones: Zone 1 (1-Vent: pH ~7.65) was located at the CO2 emission point; zone 2 (2-Transition25: pH ~7.88) at a transition distance of 25 m from the emission point; zone 3 (3-Transition75: pH ~8.09) at 75 m; and zone 4 (4-Control: pH ~8.14) at 200 m from the vent, The map and image base layers used are distributed under public domain (https://www.grafcan.es/). Levels of pH are represented in different colors and show the interpolation IDW, 4.0 of correlation (source González-Delgado et al., 2021).

### 3.2.2. Sampling and DNA Extraction

Following the protocol developed by Wangensteen and Turon (2017), six scrapes were collected with a chisel in 20 x 20 cm quadrats of the rocky bottom in each zone (Zone 1, Zone 2, Zone 3, and Zone 4). The samples (a total of 24) were sieved into two size fractions (> 1 mm and 64 µm – 1 mm) to separate organisms of different sizes (Wangensteen et al., 2018). Size fractionation is important when a sample has an uneven biomass, as observed among hard-bottom communities, and can allow the recovery of 30% more molecular operational taxonomic units (MOTUs) than would be obtained without fractionation (Feehan et al., 2019). For DNA extraction, 0.3 g of each homogenized size fraction (a total of 48 fractions) was extracted separately using a DNeasy PowerSoil kit (QIAGEN).

From the DNA extracts, a fragment of the cytochrome c oxidase subunit I (COI) gene was amplified with the universal Leray-XT primer set (Wangensteen et al., 2018). This primer set consists of the forward primer mlCOIintF-XT (Wangensteen et al., 2018) and the reverse primer jgHCO2198 (Geller et al., 2013) and shows high universality across eukaryotic groups (Wangensteen et al., 2018). To these primers, 8-base sample-specific tags were attached to assign sequences to the different samples. The same tag was attached at the 5’ end of both forward and reverse primers. For PCR amplification, a DNA concentration of ~10 ng/µl was used, according to measurement with a Qubit fluorometer (Thermo Fisher Scientific). PCR were made using AmpliTaq Gold DNA polymerase. Quality of amplifications was assessed by electrophoresis in agarose gels and all PCR products were purified using the MinElute PCR purification kit (QIAGEN).

A PCR-blank (elution buffer of the DNA isolation kit) and a pre-treatment negative control (a sample of sand charred in a muffle furnace at 400 °C for 24 h and sieved through the column of sieves) were amplified along with the samples. Since no bands were observed in the agarose gel, these blanks were not included in the multiplexed sequencing libraries. Additionally, to study the reproducibility of the amplification, three PCR replicates were obtained from each sample and fraction. All amplification products were pooled (Anderson et al., 2008 samples and fractions with 3 replicates each) to construct three different Illumina libraries using the NEXTFLEX PCR-Free

DNA-Seq kit (PerkinElmer). In this protocol, Illumina adapters were incorporated using a ligation procedure without any additional PCR step, thus minimizing PCR artifacts. The three libraries were pooled in equimolar amounts and sequenced in the Illumina MiSeq facility at the University of Salford (UK) using v3 chemistry, with 2x250 bp paired-end runs.

### 3.2.3. Bioinformatics Analyses

A eukaryotic metabarcoding pipeline based on OBITools (Boyer et al., 2016) was used to process the generated sequences (Wangensteen and Turon, 2017). First, the quality of the raw reads was checked using FASTQC v1.0.0, and the forward and reverse reads were trimmed to 240 and 200 bp, respectively. Thereafter, paired-end reads were assembled with the Illumina paired-end script; demultiplexing and primer removal were performed using ngsfilter; and the fragment lengths were filtered to between 303 and 323 bp with obigrep. Identical sequences were grouped using obiuniq, keeping track of their abundances, and chimeras were removed with the uchime_denovo method (Edgar et al., 2011) implemented in VSEARCH (Rognes et al., 2016). Nonchimeric sequences were then clustered using the iterative aggregation algorithm SWARM v2.0 (Mahé et al., 2015) using a distance value of d=13. The resulting output was a list of MOTUs with their representative sequences. These MOTUs were taxonomically assigned using the ecotag algorithm (Boyer et al., 2016) by comparison to a local reference database (db_COI_MBPK, Wangensteen et al., 2018) including 188,929 sequences extracted from the Barcode of Life Data Systems, BOLD and GenBank. This reference database is available online at github.com/uit-metabarcoding/DUFA. Assignments with identity percentages of less than 85% were removed since such identifications are considered questionable (Wangensteen and Turon, 2017).

We obtain a robust diversity dataset (see supplementary material online: https://doi.org/10.6084/m9.figshare.c.6461937.v1). Only MOTUs belonging to eukaryotes were selected from the final dataset for this study. MOTUs belonging to Chlorophyta were not included in the analyses because the

Leray-XT primer set is known to show significant primer bias limitations with this group, which make it unsuitable for characterizing the diversity of Chlorophyta (Wangensteen et al., 2018). Additionally, 26 MOTUs identified as planktonic groups were excluded since our focus was on the benthic community.

### 3.2.4. Statistical Analyses

Algal MOTUs were grouped into four different vegetation layers ('crustose', 'epiphytes', 'mixed turf' and 'erect macrophytes' *sensu* Piazzi et al. (2001)) to study the community composition using the sequencing depths as relative abundances (Wangensteen and Turon, 2017). Crustose algae live embedded in the rocky bottom; epiphytes grow on top of other algae; mixed turf species include small fleshy algae that form canopies; and erect macrophytes are the largest algae, which usually dominate the habitat. Metazoan MOTUs were grouped into two layers according to their preferred habitat: 'meiofauna' and 'macrofauna' *sensu* Higgins (Higgins, 1988). Additionally, algal and metazoan MOTUs were grouped into calcifying and noncalcifying systems according to their ability to deposit calcium carbonate. When a MOTU could not be clearly assigned to a specific species or genus, it was assigned to an unassigned group.

The R package vegan v2.5.5 (Oksanen et al., 2016) was used for diversity analyses. To account for samples with different sequencing depths, when comparing $\alpha$-diversity patterns, we calculated replicates of MOTU richness (number of species) applying a rarefaction method (function rarefy, 50 replicates, 8,000 reads rarefaction size, Sanders (1968)), and we compared the median values obtained. Pairwise differences in these indices among sites were evaluated using the nonparametric multiple comparison function implemented in the R package dunn.test (Dinno, 2017). To assess the change in the community composition along the $CO_2$ gradient, permutational univariate analyses of variance (PERMANOVA) were performed using the adonis function of vegan. The design consisted of a one-way model in which the '$CO_2$ gradient' factor (with four levels: '1-Vent', '2-Transition25', '3-Transition75', and '4-Control') was the tested term. Post-hoc comparisons were performed to test differences between the levels of the studied factor using the pairwise_adonis function. In all analyses, Bray-Curtis distances, and a 95% significance level (p-values < 0.05) were applied.

SIMPER analysis of species was performed to identify the species that contributed the most to the differences between levels (Venables, 2002). From the species that best contributed and represented the differences found in community composition, 8 algal and 9 metazoan species were selected as examples (considered tolerant or non-tolerant to natural acidification) and nonlinear multidimensional scaling (nMDS) was performed using the isoMDS function of MASS (Anderson et al., 2008) to visualize the differences between levels of the '$CO_2$ gradient'. Additionally, bar plots were generated in R to represent the percentages of calcifying and non-calcifying species.

## 3.3. RESULTS

### 3.3.1. Sequencing Depth

From 24 samples collected, our final curated dataset included 5,501,299 reads, with an average of 38,203 reads per sample and size fraction (where the small fraction comprised organisms between 1 mm and 64 µm in size, and the large fraction comprised organisms larger than 1 mm). Among these reads, 1,413,076 reads were classified as belonging to algae (526,837 Ochrophyta and 886,177 Rhodophyta) and 2,024,731 reads as belonging to metazoans (722,982 Annelida, 617,068 Arthropoda, 66,152 Bryozoa, 1,758 Chordata, 199,966 Cnidaria, 125,503 Echinodermata, 151,606 Mollusca and 13,908 Nematoda, 34,611 Nemertea, 858 Platyhelminthes, 43,650 Porifera, 4,654 Sipuncula and 11,646 Xenacoelomorpha) (Dataset S1 available online: https://doi.org/10.6084/m9.figshare.c.6461937.v1).

### 3.3.2. Community Diversity

A total of 186 detected molecular operational taxonomic units (MOTUs) were identified as algae, belonging to 46 families and 22 orders. Among the algal MOTUs, 25 MOTUs were classified as 'crustose' species, 73 as 'epiphytes' species, 64 as 'mixed turf' species, and 23 as 'erected macrophytes' species according to the vegetation layers (Piazzi et al., 2001) (Dataset S2 available online: https://doi.org/10.6084/m9.figshare.c.6461937.v1). A total of 631 MOTUs were identified as metazoans, belonging to 197 families, 89 orders and 15 phyla (Dataset S1 available in the online version: https://doi.org/10.6084/m9.figshare.c.6461937.v1). Among the metazoan MOTUs, 81 were grouped in the 'meiofauna' habitat layer, and 118 were grouped as 'macrofauna' (Dataset S2 available online: https://doi.org/10.6084/m9.figshare.c.6461937.v1). The metabarcoding data thus allowed us to obtain 2 to 20 times more species than was possible in previous works (Appendix C, Table C.2).

The rarefaction analysis of species richness for all MOTUs and both size fractions revealed significant differences across the levels of the '$CO_2$ gradient' factor ('1-Vent' placed at the $CO_2$ emission point, '2-Transition25' at a transition distance of 25 m to the emission point, '3-Transition75' at 75 m to the emission point and '4-Control' 200 m apart from the vent), where a significant increase in the number of species in the '2-Transtition25' zone is observed (Figure 3.2A, Table C.3A in Appendix C). For the richness of algae and their subgroups we found significant differences in the small fraction of 'crustose', the large fraction of 'epiphyte' and the small fraction of 'erected macrophytes', and slight increase was observed in the transition zone for all groups except for the 'epiphyte' algae whose diversity increased in the more acidic zone ('1-Vent') (Figure 3.2B, C and Appendix C, Table C.3B, C). For metazoans richness, the differences were significant in almost all groups (except for the small fraction of metazoans), with a high number of species in the '2-Transition25' and '1-Vent' for 'meiofauna'.

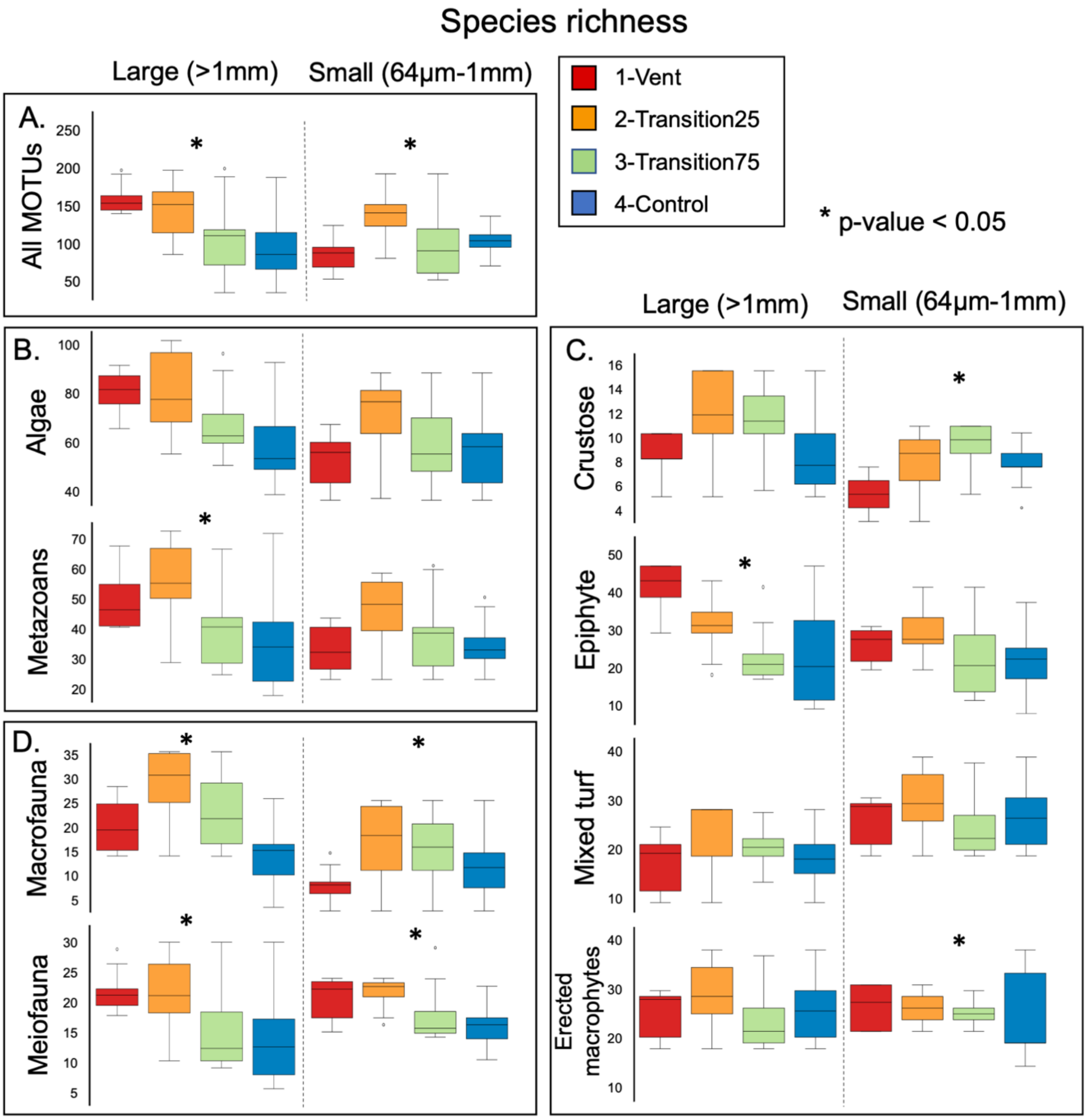

Figure 3.2. Boxplots showing the differences of the α-diversity index species richness in the natural $CO_2$ gradient (‘1-Vent’ in red, ‘2-Transition25’ in orange, ‘3-Transition75’ in green, and ‘4-Control’ in blue). (*) Significant differences among all samples measured with the dunn-test (See appendix C, Table C.3). For all MOTUs (A), for algae and metazoans (B), for algal subgroups: ‘crustose’ species, ‘epiphyte’ species, ‘mixed turf’ species and ‘erected macrophytes’ species (C) and for metazoan subgroups: ‘meiofauna’ and ‘macrofauna’ (D). In all cases the large (> 1mm) and the small fraction (64µm-1mm) are represented and separated by the dashed line.

### 3.3.3. Algal Community Assemblage

One-way PERMANOVA based on the relative read abundance of all algal datasets (Wangensteen and Turon, 2017) showed significant differences in the ‘$CO_2$ gradient’ factor in both the large and the small fraction in all vegetation layers studied (Appendix C, Table C.4A). A Post-hoc pairwise comparisons revealed significant differences in abundance between most comparisons (Appendix C, Table C.4B). In addition, multidimensional scaling (MDS) analyses show how samples and species are differently distributed along the natural $CO_2$ gradient for all vegetation layers (Figure 3.3). The greatest differences were found between the algal communities of ‘1-Vent’ and all other zones. The species that best explained the variability found in the data are summarized in the Table 3.1 (for more detail see Appendix C, Table C.5).

**Table 3.1.** Summary of SIMPER analysis results showing differences in relative average abundances and percentage contribution of the first species marking the differences in the algae subgroups (‘crustose’ species (A), ‘epiphyte’ species (B), ‘mixed turf’ species (C), and ‘erected macrophytes’ species (D)) and the metazoan subgroups (‘meiofauna‘ species (E) and ‘macrofauna’ species (F)), compared to the '$CO_2$ gradient' ('1-Vent', '2-Transition25', '3-Transition75' and '4-Control'). In this case, both fractions are combined.

| | CONTRIBUTION % | | | | | | AVERAGE ABUNDANCES | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| A. Crustose | **1 - 2** | **1 - 3** | **1 - 4** | **2 - 3** | **2 - 4** | **3 - 4** | **1** | **2** | **3** | **4** |
| *Ralfsia* sp. | 13.54 | 26.36 | 32.96 | 0 | 0 | 0 | 119.66 | 14.8 | 12.55 | 4.94 |
| *Lithophylum* sp. 2 | 7.38 | 20.32 | 33.46 | 0 | 10.16 | 9.34 | 0 | 88 | 91.71 | 70.26 |
| Peyssonnelia sp. 3 | 8.6 | 23.41 | 31.71 | 0 | 11.38 | 13.56 | 122.79 | 82.66 | 54.48 | 95.55 |
| B. Epiphyte | | | | | | | | | | |
| *Ceramium virgatum* | 12.07 | 15.11 | 12.62 | 0 | 0 | 0 | 101.62 | 9.14 | 2.33 | 0 |
| C. Mixed Turf | | | | | | | | | | |
| *Laurencia obtusa* | 5.22 | 13.05 | 12.97 | 5.73 | 0 | 0 | 161.92 | 93.83 | 61.23 | 55.73 |
| *Jania* sp. 2 | 6.55 | 16.05 | 12.86 | 0 | 0 | 0 | 14.63 | 109.66 | 129.42 | 58.96 |
| D. Erected macrophytes | | | | | | | | | | |
| *Halopteris* sp. | 32.83 | 34.44 | 43.41 | 5.83 | 0 | 7.46 | 157.1 | 54.59 | 41.59 | 4.72 |
| *Canistrocarpus cervicornis* | 29.37 | 20.82 | 29.4 | 7.82 | 0 | 11.52 | 85.31 | 177.68 | 153.11 | 188.74 |
| E. Meiofauna | | | | | | | | | | |
| *Caprella acanthifera* | 3.45 | 3.84 | 2.14 | 0 | 2.72 | 0 | 127.36 | 50.55 | 19.24 | 43.84 |
| *Timarete punctata* | 1.43 | 3.22 | 4.88 | 0 | 0 | 0 | 149.62 | 4.61 | 17.04 | 14.56 |
| *Podocerus variegatus* | 0 | 1.97 | 1.73 | 0 | 1.17 | 0 | 6.55 | 29.85 | 23.57 | 40.14 |
| *Amphipholis squamata 1* | 0 | 0 | 3.24 | 0 | 2.64 | 0 | 32.54 | 40.87 | 0 | 3.23 |
| *Amphipholis squamata 3* | 0 | 0 | 0 | 0 | 3.4 | 1.32 | 0 | 4.31 | 3.13 | 31.7 |
| *Eurythoe* sp. | 0 | 0 | 2.1 | 0 | 1.03 | 0 | 8.23 | 33.19 | 0 | 71.9 |
| F. Macrofauna | | | | | | | | | | |
| *Tubulipora* sp. 1 | 0 | 0 | 3.02 | 0 | 1.3 | 0 | 0 | 0 | 0 | 35.81 |
| *Alvania* sp. 1 | 3.74 | 0 | 1.34 | 1.18 | 1.06 | 0 | 92.43 | 34.59 | 27.22 | 37.1 |
| *Bienmidae* sp. | 0 | 0 | 1.32 | 0 | 0 | 0 | 0 | 0 | 0 | 11.11 |

In the crustose layer, coralline crustose algae (order Corallinales, e. g. *Lithophyllum* species) decreased from the '4-Control' zone to the '1-Vent' zone, and some species even disappeared in the '1-Vent' zone. Nonetheless, some other calcified crustose algae, such as *Peyssonnelia* species, were still represented in zones with decreased pH ('3-Transition75' and '2-Transition25'). Accordingly, the non-calcified *Ralfsia* sp. brown crustose algae increased in abundance in '2-Transition25' and '1-Vent' and contributed the most to these differences (Figure 3.3A, Table 3.1A).

The epiphyte layer was mostly composed of red algae from the order Ceramiales, which showed increased abundance toward the lower pH zone ('1-Vent') and showed the highest abundance in '2-Transition25'. Specifically, the species with the highest percent contribution in the '1-Vent' zone was *Ceramiun virgatum*, and that in the '2-Transition25' zone was *C. diaphanum* (Figure 3.3B, Table 3.1B).

The mixed turf layer showed changes like those observed previously; turf calcified algae from the order Corallinales decreased in abundance toward the '1-Vent' zone. However, there were some exceptions; for example, *Jania* spp. showed high abundance in both transition zones. In addition, other red algae species contributed greatly to the '1-Vent' zone, with particularly high abundance of *Laurencia* spp. being observed (Figure 3.3C, Table 3.1C).

The brown algae dominated in the erected macrophyte layer, among which *Halopteris* sp. were the greatest contributor to differentiating the '1-Vent' zone from the other zones. The opposite pattern was observed for species of Dyctiotaceae, such as *Canistrocarpus cervicornis*, which declined in abundance with decreasing pH from the '4-Control' zone to the '1-Vent' zone (Figure 3.3D, Table 3.1D).

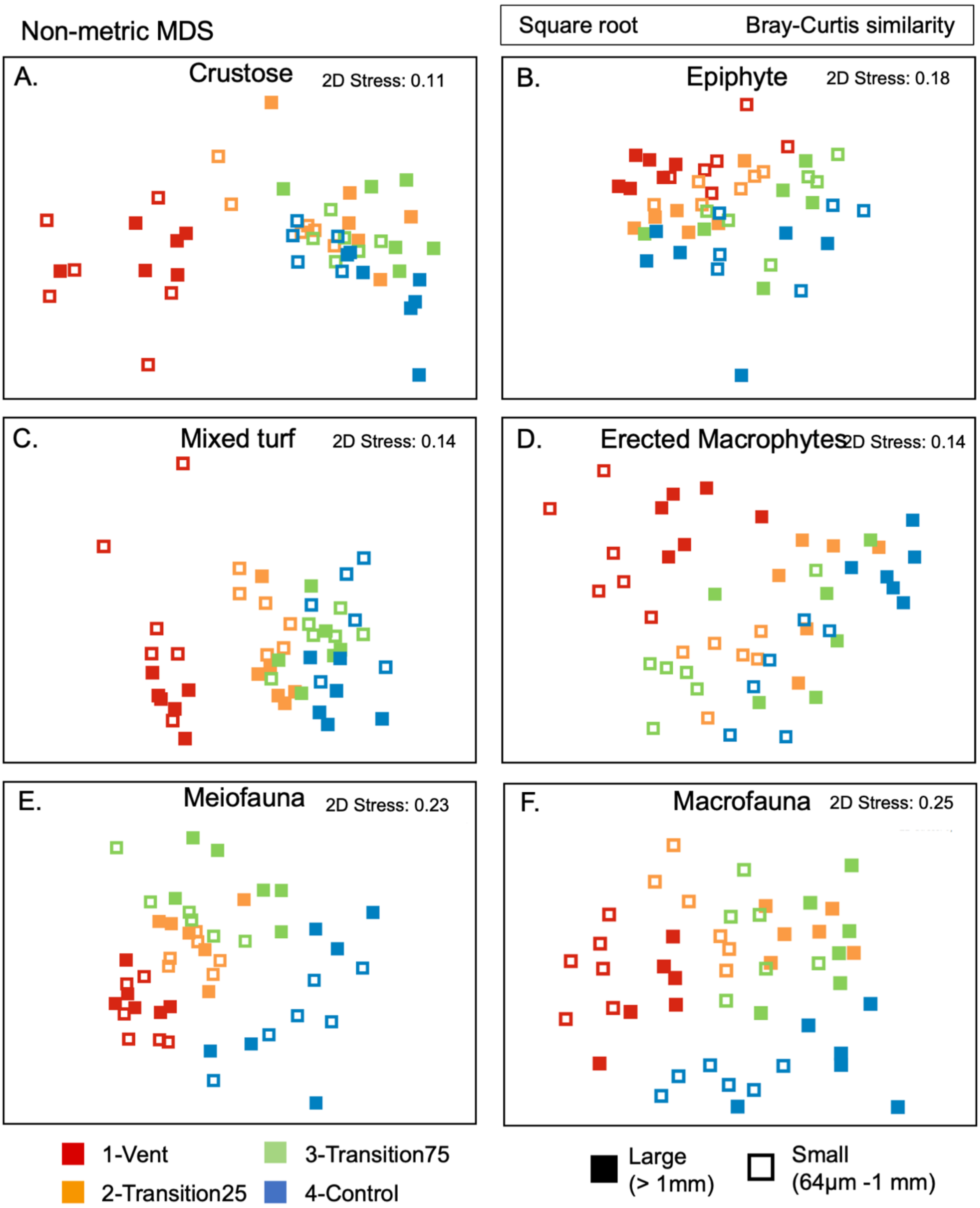

Figure 3.3. Non-metric multidimensional scaling (nMDS) plots (PERMANOVA: p<0.05, See appendix C, Table C.4) between levels of $CO_2$ gradient ('1-Vent', '2-Transition25', '3-Transition75', and '4-Control') of algae subgroups ('crustose' species (A), 'epiphyte' species (B), 'mixed turf' species (C), and 'erected macrophytes' species (D)) and metazoan subgroups ('meiofauna' species (E) and 'macrofauna' species (F)).The large fraction are organisms with size > 1 mm and the small fraction are organisms between 1 mm and 0.64 μm.

### 3.3.4. Metazoan Community Assemblage

One-way PERMANOVA based on the relative abundance data from all metazoan datasets revealed significant differences in both the large (organisms with size > 1 mm) and the small fraction (organisms between 1 mm and 0.64 μm) for the factor '$CO_2$ gradient' in the 'meiofauna' and 'macrofauna' habitat layers (Appendix C, Table C.6A). Post-hoc pairwise comparisons of both layers revealed significant differences in abundance between comparisons except for both transition in 'macrofauna' and 'Transition75' vs 'Control' in both 'meiofauna' and 'macrofauna' (Appendix C, Table C.6B).

In the meiofaunal layer, the transition zones ('2-Transition25' and '3-Transition75') retained the greatest abundance of Annelida and Arthropoda species. Species of errant Polychaeta (e.g., *Eurythoe* sp.) decreased in abundance approaching the '1-Vent' zone, while sedentary Polychaeta increased (e.g., *Timarete punctata*). The identified Arthropoda were mostly represented by amphipods (e.g., *Caprella acanthifera* and *Podocerus variegatus*) and showed increased abundance in acidified zones ('2-Transition25' and '1-Vent'). Another notable difference was found for the ophiuroids (Echinodermata) of the meiofauna layer, among which three different MOTUs of *Amphipholis* cf. *squamata* were detected. *A.* cf. *squamata* 3 contributed to differentiate the '4-Control' zone from the other zones, while there was an increase in *Amphipholis* cf. *squamata* 1 in acidified zones (Figure 3.3E, Table 3.1E).

The greatest variety and diversity of metazoans was found in the macrofaunal layer. The Arthropoda included in this layer were all decapods, whose abundance was prominent in the '2-Transition25' zone. Molluscan species also varied along the $CO_2$ gradient, among which nudibranchs and opisthobranchs were well represented in '2-Transition25', and several gastropod species (e.g., *Alvania* sp.) were more common in '1-Vent'. Some benthic substrate-attached species also varied along the $CO_2$ gradient, including Ascidiacea and Cnidaria species, which accounted for the largest differences in the percent contributions between '1-Vent' and the rest of the zones. In contrast, most of the poriferan (e.g., Biemnidae family) and bryozoan species (e.g., *Tubulipora* spp.) presented the highest abundances in the '3-Transition75' and '4-Control' zones (Figure 3.3F, Table 3.1F, for more details see Appendix C, Table C.7).

### 3.3.5. Effect on Calcifying Species

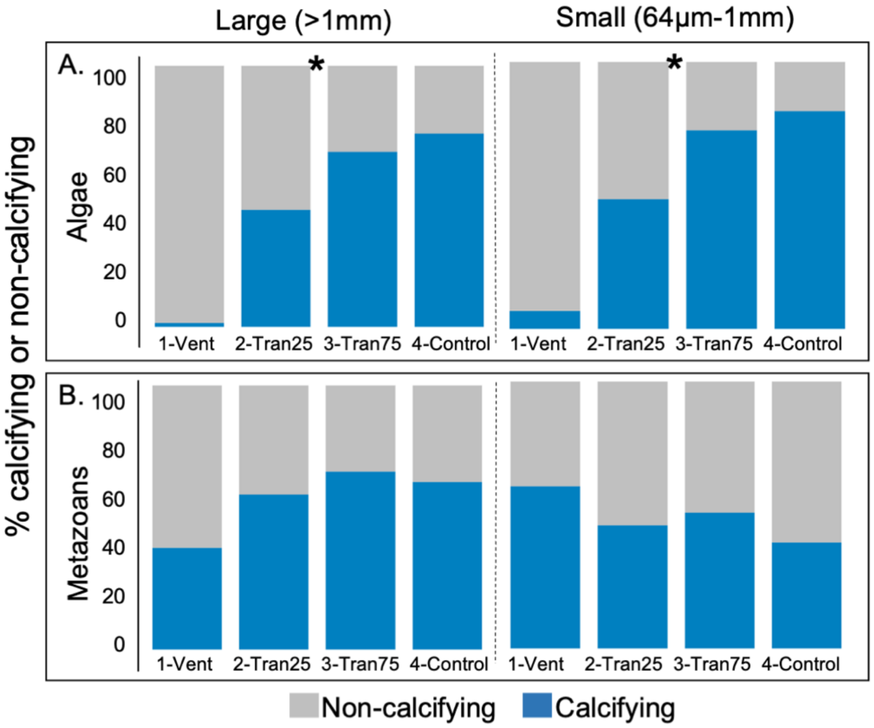

Figure 3.4. Bar-plot showing the percentage of calcifying (in blue) and non-calcifying (in grey) species along the $CO_2$ gradient ('1-Vent', '2-Transition25', '3-Transition75', and '4-Control'), in algae (A) and metazoans (B) of both large (> 1mm) and small (64µm -1mm) fraction (separated by the dashed line). Data used are the mean relative abundances ('reads') of MOTUs considered to be calcifying or non-calcifying. (*) Significant differences among all samples measured with PERMANOVA ($p < 0.05$) (See appendix C, Table C.8).

One-way permutational univariate analyses of the variance of calcifying species abundance showed a significant effect of the factor '$CO_2$ gradient' only for algae (Appendix C, Table C.8A). The abundance of calcifying algae declined following the natural gradient of $CO_2$ in both fractions (Figure 3.4). Post-hoc pairwise comparisons revealed significant differences between levels of the '$CO_2$ gradient' factor, especially between zones affected by increased $CO_2$ concentrations ('1-Vent' and '2-Transition25') and the rest of the zones, in both fractions (Appendix C, Table C.8B). There were no significant differences found among metazoan calcifying species.

## 3.4. DISCUSSION

### 3.4.1. Thorough Assessment of Community Diversity

The expectation of biodiversity loss due to increased ocean acidification was not supported by our results. Previous studies failed to detect the real species richness existing in $CO_2$ emission points due to the bias of the visual identification methods used (e.g., Linares et al., 2015; Kroeker et al., 2011). Our fine-scale taxonomic detection analysis is the only study conducted thus far to show high levels of algal and metazoan diversity based on the metabarcoding detection of small and cryptic species living under naturally acidified areas. A good example is provided by the species from the family Ceramiaceae, which were the main contributors to the high number of algae observed in the vicinity of the $CO_2$ emissions sites.

Our results confirmed high levels of species richness, especially of metazoans in the transition zone (or at intermediate pH levels) in the Fuencaliente system. From a strictly ecological standpoint, considering the $CO_2$ gradient as a disturbance, our results suggest that the 'intermediate disturbance' hypothesis (Connell, 1978) may be applicable in naturally acidified ecosystems. This hypothesis considers that species number is higher when physical or biological disturbances exist at intermediate scales. The investigated ecosystem at Fuencaliente is not an equilibrium system. Therefore, the physical-chemical processes generated by the permanent perturbation prevent a climax community from developing in the transition zones, where the pH fluctuates between 7.8 and 8.1 units, resulting in the highest levels of diversity (Sousa, 1979). Our study is the first to show this interesting pattern in a natural $CO_2$ system. At intermediate pH fluctuation (transition zone), corresponding to the scenarios predicted for 2050 (IPCC, 2021), marine communities may be unstable and show high number of species. However, by the time more extreme pH levels are reached (approximately 7.6 units as predicted for 2100 (IPCC, 2021), a new benthic community will have been established, which will be less diverse and will include species more tolerant to acidification.

### 3.4.2. Tolerant and Non-tolerant Species in the Future Subtropical Community Under OA

Beyond the observed change in beta diversity, our taxonomic analysis of benthic communities along the long-term $CO_2$ gradient at La Palma reveals tolerant and non-tolerant species under OA within the subtropical marine communities (Figure 3.5).

In the case of algae (Figure 3.5A, C), our results showed that some dictyotales species were disadvantaged in the most acidic environment, unlike other studies performed in the Mediterranean (Foo and Byrne, 2021). The typical dominance of *Canistrocarpus cervicornis* (Dictyotaceae) in our control environments (Sangil et al., 2014) was completely replaced by highly abundant *Halopteris* species (Stypocaulaceae). In particular, the rapid growth of H*alopteris* sp. (Patarra et al., 2017) and their carbon uptake mechanism could allow these species to tolerate a drop in pH. Furthermore, the unexpected disappearance of *C. cervicornis* in the most acidified zone could have been due to the indirect effect of the overgrowth of epiphytic Ceramiaceae on its thallus, as observed in a previous episode of $CO_2$ input around the Canary Islands (Tagoro Volcano, El Hierro Islands) (Sangil et al., 2016). These epiphytes take advantage of extra dissolved inorganic carbon (DIC) and grow fast over erect algae, which may be physiologically defenseless (Cornwall et al., 2017). Similarly, in agreement with other works (e.g., Linares et al., 2015; Agostini et al., 2018), our results confirmed the clear loss of calcifying algae along the subtropical $CO_2$ gradient. In the Canary Islands, the order Corallinales usually dominates rocky-bottom areas, where *Peyssonnelia* and other similar species create a calcified community (Sangil et al., 2014).

Our study indicated that under acidification, opportunistic turf algae of the Ceramiaceae family gained advantages under acidification, probably due to the benefits of their carbon uptake physiology (Connell et al., 2013), which leads to remarkable compositional changes in the community. These acidified ecosystems become structurally homogeneous as turf algae monopolize the spaces available for the growth of other species with greater ecological value, such as erect brown macroalgae, which serve as a refuge for many organisms and large net carbon sinks (Feehan et al., 2019). In addition, such alterations lead to miniaturization of the community (Pessarrodona et al., 2021). With this term we refer to a replacement of large macroalgae by small turf and epiphyte algae, which leads to changes in the community structure, in parallel with a substitution of macrofauna by meiofauna.

On the other hand, similarly to a study performed in the Columbretes Islands (Linares et al., 2015), we found that some *Peyssonnelia* species can live under high acidification, indicating the greater suitability of algae containing aragonite than those containing calcite. In contrast, unexpected perseverance of Corallinales species was observed under transition conditions, where the pH reached 7.8 units at low tide ('Transition25' zone). The perseverance of these calcifying organisms could be due to their known opportunistic nature in transient, disturbed communities (Arévalo et al., 2007). The ability of these turf coralline species to survive under acidification without showing compromised mineral skeletons has been demonstrated previously (Porzio et al., 2018). Thus, coralline algae could achieve an advantage after adapting to acidification, leading to increases in their abundance in transition zones. In subtropical ecosystems of the Atlantic Ocean, *Halopteris* sp., *Ceramiun* spp., *Laurencia* spp. and *Ralfsia* sp. will be better adapted in the predicted future affected by OA, while *C. cervicornis* and coralline algae could disappear.

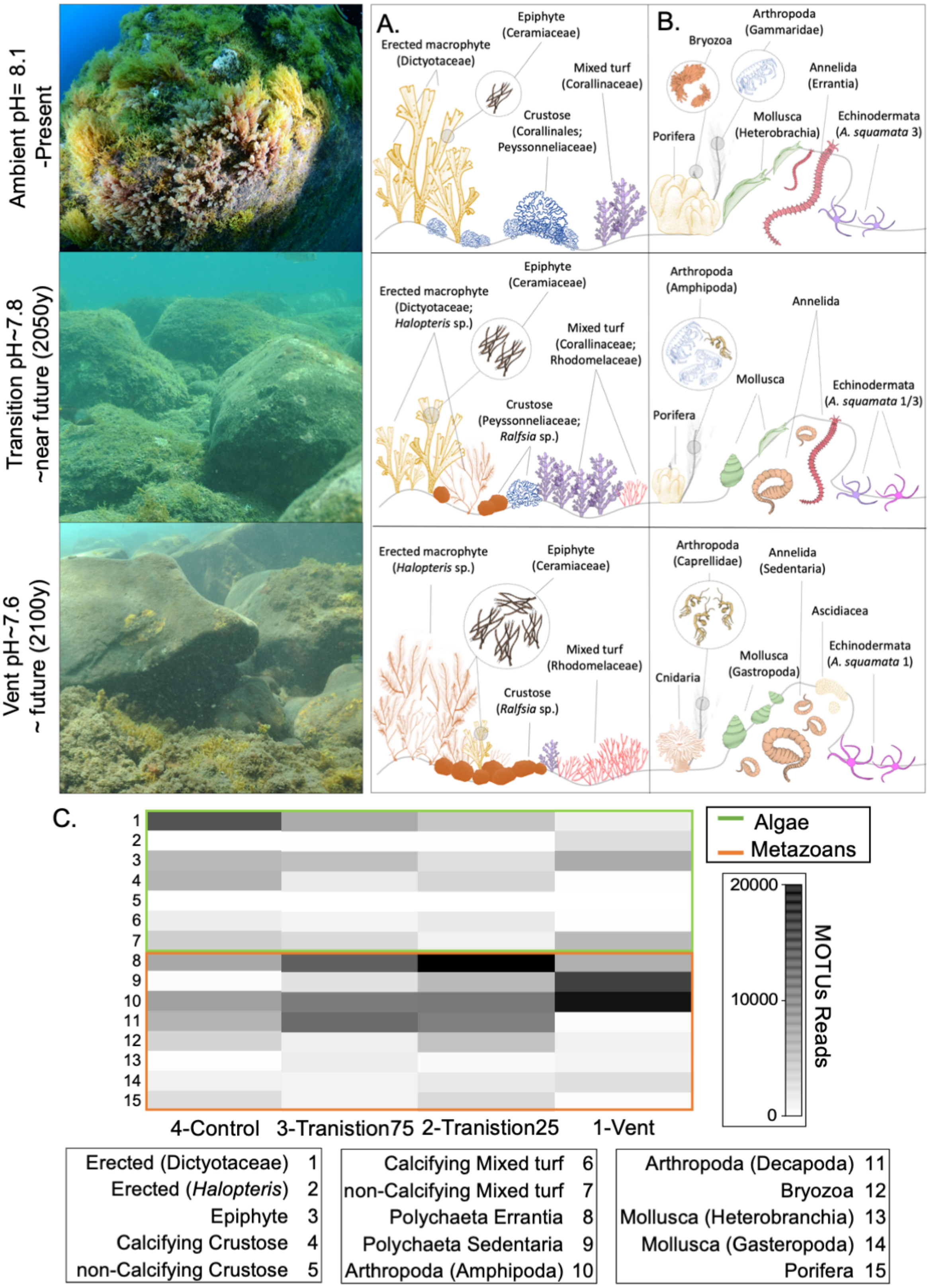

Figure 3.5. Community assemblage with different algae (A) and metazoans (B) in ambient pH (8.1 pH unit), transition zones (from 7.8 to 7.6 pH units) and vent zone (7.6 or less pH units) (photos were taken during sampling). Heatmap of the relative abundances or sum of the 'MOTU reads' of different important subgroups of algae (frame in green) and metazoans (frame in orange) along the CO2 gradient ('1-Vent', '2-Transition25', '3-Transition75', and '4-Control') (C).

Taxonomic changes in the faunal assemblage accompanied alterations of the algal communities (Figure 3.5B, C). As shown previously in other work conducted in acidified areas (e.g., Agostini et al., 2018; Fabricius et al., 2014), we found that macrofaunal species tend to decrease in abundance in high-$CO_2$ zones. Sessile organisms including ascidians, cnidarians, sponges, and calcifying bryozoans were the most affected species under acidification. As a result, the benthos lost heterogeneity and was covered by only the most competitive algae, similar to effects observed in other acidified ecosystems (e.g., Agostini et al., 2018). However, gastropods showed greater increases in abundance than other groups of mollusks, such as nudibranchs (species without calcareous shells), as we approached the $CO_2$ vent center. Despite constraints on shell calcification in acidic environments, gastropods have been shown to quantitatively increase in these areas with increases in turf and epiphyte algae (Connell et al., 2017). It has been suggested that calcifying organisms are able to acclimate better to acidifying conditions when food sources are plentiful in the environment (Clements et al., 2018). This may also explain the presence of the echinoderms *Amphipholis* species in the vent system and the increases in amphipods and sedentary polychaetes from meiofaunal habitats. In subtropical communities, herbivorous organisms and the smallest species are the most tolerant to acidification and show increased diversity in $CO_2$ vents, despite their calcified skeletons. This phenomenon, together with increases in small algae, creates a miniaturized community (Figure 3.5). Consequently, subtropical ecosystems affected by OA in the future will likely show reductions in important ecological roles despite high species richness, and these losses may lead to an imbalance in ecosystem services (Hall-Spencer and Harvey, 2019).

It is important to remark that metabarcoding is still in development and therefore has some limitations. For example, there are organisms that remain undetectable due to amplification primer bias or gaps in the reference databases (Wangensteen et al., 2018). However, this powerful technology has provided enhanced biodiversity detection for a broad range of taxonomic groups, leading to uncover hidden ecological patterns from benthic communities affected by acidification. As the use of enhanced molecular techniques for biodiversity assessment becomes widespread, we can expect that biodiversity assessment studies may be better equipped to produce stronger evidence for predicting the future trajectories of marine benthic communities and the ecological services they provide. Finally, we are also aware of the sampling limitations and lack of replication of the $CO_2$ vent of our work. However, this study might be positioned in the general OA context and compared with previous $CO_2$ vent studies. Doing so, we have found some general community changes across $CO_2$ vent studies, such as the reduction in calcified species but we have also found some important differences. For instance, a closer look to taxonomic diversity has shown high diversity of small size species under persistent low pH values (similar to values predicted for the end of the century).

## 3.5. CONCLUSION

We present a new perspective for the study of OA effects on benthic communities based on the molecular method of metabarcoding, applied in the context of a subtropical nonbubbling natural acidified system. The use of metabarcoding has revealed the important contribution of small and cryptic organisms to a high biodiversity level under acidification, despite some of these organisms hold calcified skeletons or shells, indicating that they may be well pre-adapted to the future benthic ecosystems. Our results suggest that subtropical ecosystems affected by OA in the future will tend to show miniaturization of their communities due to the dominance of small species of turf and epiphytic algae and associated small invertebrates. We recommend paying particular attention to the target species highlighted in this work, which may allow us to better understand the direct and indirect effects of OA. It would be advisable to use these species as model species since greater knowledge of their physiology, feeding, reproduction and DNA changes under acidification would help to fully understand the effects of OA.

The expanded application of constantly improving DNA-based methods will allow powerful and standardized measurements for biodiversity assessment to be performed in a range of acidified systems around the world, leading to a more realistic understanding of the future changes that will be caused by OA at a global scale.

## DATA AVAILABILITY

The raw sequencing data fastq files for this study are publicly available in the Sequence Read Archive database SRA-Genbank (Bioproject PRJNA885438). The R analysis scripts and the ngsfilter file are publicly available as a Github repository: http://github.com/Sgonzalezdelgado/MetabarcodingCO2vent. Final DNA sequence assembly online in https://doi.org/10.6084/m9.figshare.c.6461937.v1.

# CHAPTER 4

# Molecular evidence for Intermediate Disturbance Hypothesis in an Acidified Marine System

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## ABSTRACT

The Intermediate Disturbance Hypothesis (IDH), postulated by Connell (1978) for tropical forests and coral reefs, considers that ecosystems are unstable, with species diversity being higher when physical or biological disturbances occur at intermediate scales. Since then, there has been a debate about the validity of this hypothesis across marine ecosystems. In this study, we investigated whether the IDH applies at different scales of biological organization (from organisms to communities) using molecular data. As an experimental perturbation framework, we used a naturally acidified system with a sharp fluctuating pH gradient. Molecular data were obtained from a) sequences of a fragment of the mitochondrial gene Cytochrome C Oxidase subunit I (COI) in two sea urchin species (*Arbacia lixula* and *Paracentrotus lividus*) to explore genetic diversity at the organism level, and b) metabarcoding data of the entire benthic community to measure taxonomic diversity at the community level. Both sea urchin species showed the highest levels of haplotype and nucleotide diversity for COI at the intermediate pH fluctuation zone. Metabarcoding data also revealed the highest levels of taxonomic diversity (including algal and metazoan species) at the intermediate pH fluctuation zone. Our results support the validity of the IDH in benthic communities affected by strong pH fluctuations and across different levels of biological organization.

Keywords: Metabarcoding, Sanger sequencing, benthic community, sea urchins, $CO_2$ vents.

## RESUMEN

La Hipótesis de Perturbación Intermedia (HPI), postulada por Connell (1978) para bosques tropicales y arrecifes de coral, considera que los ecosistemas son inestables y que la diversidad de especies es mayor cuando existen perturbaciones físicas o biológicas a escalas intermedias. Desde entonces, existe un debate sobre la validez de esta hipótesis en los ecosistemas marinos. En este estudio, investigamos si la HPI se aplica a diferentes escalas de organización biológica (desde organismos hasta comunidades) utilizando datos moleculares. Como marco de perturbación experimental, utilizamos un sistema natural acidificado donde ocurren fluctuaciones agudas de pH. Los datos moleculares se obtuvieron de a) secuencias de un fragmento del gen mitocondrial COI en dos especies de erizos de mar (*Arbacia lixula* y *Paracentrotus lividus*) para explorar la diversidad genética a nivel de organismos, y b) datos de metabarcoding de toda la comunidad bentónica para medir la diversidad taxonómica a nivel de comunidad. Ambas especies de erizos de mar mostraron los niveles más altos de diversidad de haplotipos y de nucleótidos para el COI en la zona de fluctuación de pH intermedia. Los datos de metabarcoding también revelaron los niveles más altos de diversidad taxonómica (en especies de algas y metazoos) en la zona de fluctuación de pH intermedia. Nuestros resultados respaldan la validez de la HPI en comunidades bentónicas afectadas por fuertes fluctuaciones de pH y en diferentes niveles de organización biológica.

Palabras claves: Metabarcoding, secuenciación Sanger, comunidades bentónicas, erizos de mar, afloramiento de $CO_2$.

## 4.1. INTRODUCTION

*'I speculated whether a species very liable to repeated and great changes of conditions, might not acquire a fluctuating condition ready to be adapter to either condition'. (Charles Darwin in a letter to Carl Semper, February 6, 1881 – Darwin Correspondence Project).*

Darwin, in his insightful discussion with Carl Semper about his book (Semper, 1881), hypothesized whether a species that lives under fluctuating conditions could acquire the potential to adapt to either condition. Although his thought originated considering the evolution and adaptation of crustacean species, it also has ecological implications at different scales. Compared with stable environments, fluctuating ones might give rise to organisms with higher genetic diversity and species richness (Fox, 2013). However, to our knowledge, an official hypothesis was not postulated until 1978. John Connell found evidence for the so-called Intermediate Disturbance Hypothesis (IDH) in tropical forest and coral reef ecosystems. This hypothesis considers that ecosystems are unstable, and the abundance and distribution of species are constantly subjected to varying levels of physical and biological disturbances. Diversity and species richness are higher when disturbances occur at intermediate scales (Connell, 1978). Furthermore, it is worth noting that Paul Dayton (Dayton, 1971) previously demonstrated the role of disturbances in determining community structure and increasing species richness in intertidal habitats. However, both conceptions were only related to species richness, and no predictions were made regarding functional diversification or infraspecific genetic diversity. Only a couple of previous studies have addressed these aspects in forest environments (Molino and Sabatier, 2001; Scotti et al., 2015).

Since its formulation, the IDH has been intensely discussed, still having both defenders and detractors, based on experimental and theoretical evidence (e.g., Molino and Sabatier, 2001; Lenz et al., 2004; Gerwing et al., 2017; Fox, 2013; Bendix et al., 2017; Santos et al., 2021). Some detractors have even claimed that this hypothesis should be abandoned altogether (Fox, 2013), based on the fact that most ecological studies do not demonstrate a unimodal relationship between species diversity and disturbances. On the other hand, other ecologists consider that the model may apply to specific ecosystems and conditions (Sheil and Burslem, 2013). The most recent literature review on the topic has revealed that the IDH is generally less supported in aquatic than terrestrial ecology papers (Moi et al., 2020). However, this conclusion was merely based on counting the number of scientific papers supporting or refuting this hypothesis. Hence, the debate about the validity or invalidity of the mentioned hypothesis remains open, as it is a central theme in marine community ecology (Bertness et al., 2014).

Additionally, when considering species richness in marine ecosystems, despite considerable sampling effort, visual methods of marine taxonomic identification are not sufficient to detect most small species, leading to an underestimation of true levels of diversity (Leray and Knowlton, 2016). Fortunately, new high-resolution molecular techniques are being developed for biodiversity assessment, such as metabarcoding tools for marine communities (e.g., Wangensteen and Turon, 2017). This technique, based on high-throughput sequencing and molecular taxonomic identification, overcomes the limitations of traditional methods by objectively identifying organisms of all sizes, including cryptic or undescribed species that might otherwise remain undetectable using visual methods (e.g., Gallego et al., 2020). Therefore, these novel molecular techniques offer a whole new perspective in IDH-related research.

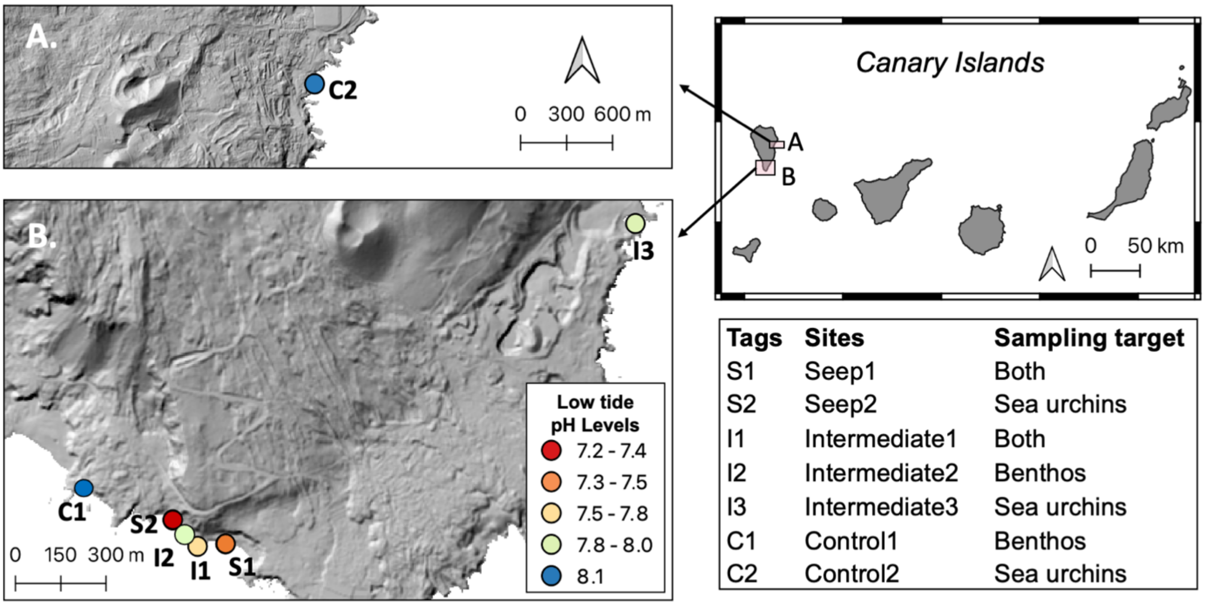
Furthermore, in the field of marine ecology, one of the recent significant shifts has been the unprecedented interest in understanding how anthropogenic climate change will affect ocean ecosystems, identifying common patterns, and determining how environmental gradients, disturbances, or stressors influence organisms, populations, and communities. Among the potential stressors on marine ecosystems, ocean acidification caused by anthropogenic $CO_2$ emissions to the atmosphere and absorbed by the oceans is currently one of the major threats (Dupont and Pörtner, 2013). Consequently, considering the above explained, marine $CO_2$ vent systems can provide an opportunity to test the IDH in relation to seawater pH fluctuations. $CO_2$ vents are shallow marine areas where constant release of $CO_2$-rich gases occurs due to volcanic activity, creating a spatial $CO_2$ gradient. In these vent systems, areas with high $CO_2$ emissions experience pH fluctuations and disturbance to the marine ecosystem, while areas with low or no emissions maintain relatively constant oceanic pH (González-Delgado and Hernández, 2018).

In this study, we aimed to determine whether the IDH applies in the $CO_2$ vent of Punta de Fuencaliente, La Palma (Canary Islands, Spain), where the different levels of pH fluctuation can be considered as a disturbance gradient (large, intermediate, and low fluctuation). We explored across different levels of biological organization, from organisms to communities, by utilizing different molecular techniques: mitochondrial gene sequencing for the organism level (infraspecific) and eukaryotic metabarcoding for the benthic community level.

## 4.2. MATERIAL AND METHODS

### 4.2.1. The $CO_2$ Vent System

The $CO_2$ vent studied is located at Punta de Fuencaliente (south of La Palma Island, Canary Islands, Spain) (see Figure 4.1 and Table 4.1), where natural acidification has occurred during the last 30 years with tidal fluctuations in three different beaches: Playa del Faro, Los Porretos, and Las Cabras (González-Delgado et al., 2021). Sites with larger pH fluctuations are called 'Vent' (pH fluctuation = 7.3-7.9), sites with intermediate fluctuations are called 'Intermediate' (pH fluctuation = 7.8-8.1), and sites with low fluctuations are referred to as 'Control' (pH around ~8.1) (see Figure 4.1).



| Tags | Sites | Sampling target |
|---|---|---|
| S1 | Seep1 | Both |
| S2 | Seep2 | Sea urchins |
| I1 | Intermediate1 | Both |
| I2 | Intermediate2 | Benthos |
| I3 | Intermediate3 | Sea urchins |
| C1 | Control1 | Benthos |
| C2 | Control2 | Sea urchins |

Figure 4.1. Sampling sites of the benthic community and sea urchins in the Punta de Fuencaliente vent system, La Palma Island (Canary Islands, northeastern Atlantic Ocean). (A) Location of the 'Control2' site. (B) Location of all the other sites. The map and image base layers used are distributed in the public domain (https://www.grafcan.es/, last accessed: 19 September 2022).

Table 4.1. Carbon system parameters obtained during low and high tides at the sampling sites in Punta de Fuencaliente (La Palma Island, Canary Islands, see Figure 1). AT = Total Alkalinity (μmol/kg), Ω = Saturation state, cal = calcite and arag = aragonite. Date from González-Delgado et al., (2021).

|  | Site | Code | pH | AT | Ωcal | Ωarag |
|---|---|---|---|---|---|---|
| Low Tide | Playa del Faro | Vent1 | 7.54±0.02 | 3252.43±42 | 2.48±0.07 | 1.62±0.04 |
|  | Los Porretos | Vent2 | 7.32±0.06 | 3256.9±217 | 1.51±0.84 | 0.99±0.05 |
|  | Playa del Faro | Intermediate1 | 7.81±0.01 | 2783.85±11 | 3.55±0.06 | 2.32±0.04 |
|  | Playa del Faro | Intermediate2 | 7.95±0.09 | 2798.68±69 | 4.73±0.69 | 3.09±0.45 |
|  | Las Cabras | Intermediate3 | 7.88 | 2613.84 | 4.32 | 2.81 |
|  | Echentive | Control1 | 8.14±0.01 | 2573.06±12 | 5.17±0.08 | 3.87±0.06 |
|  | La Bajita | Control2 | 8.14 | 2573.98 | 6.11 | 3.98 |
| High Tide | Playa del Faro | Vent1 | 7.91±0.07 | 2728.37±11 | 4.57±0.58 | 2.98±0.38 |
|  | Los Porretos | Vent2 | 7.88±0.01 | 2697.85±36 | 4.06±0.05 | 2.65±0.04 |
|  | Playa del Faro | Intermediate1 | 8.08±0.01 | 2626.33±7 | 5.57±0.11 | 3.64±0.07 |
|  | Playa del Faro | Intermediate2 | 8.15±0.01 | 2501.37±8 | 5.95±0.30 | 3.93±0.08 |
|  | Echentive | Control1 | 8.16±0.01 | 2485.96±6 | 6.09±0.12 | 3.98±0.08 |
|  | La Bajita | Control2 | 8.11 | 2559.17 | 5.76 | 3.76 |

For the study at the infraspecific level, two sea urchin species (*Paracentrotus lividus* and *Arbacia lixula*) were chosen. Despite their theoretical vulnerability to oceanic acidification due to their calcareous skeleton (Byrne and Hernández, 2020), these species inhabit the $CO_2$ vents in the Canary Islands and the Mediterranean (Foo et al., 2018b; González-Delgado et al., 2023). Individuals of both sea urchin species were collected at 5 sites: 'Vent1' located at the $CO_2$ vent of Playa del Faro (pH = 7.5-7.9); 'Vent2' located at the second $CO_2$ vent in Los Porretos (pH = 7.3-7.9); 'Intermediate1' at a transitional distance of 25 m from the Playa del Faro emission point (pH = 7.6-8.0); 'Intermediate3' at the third $CO_2$ vent in Las Cabras (pH = 7.9-8.1); and 'Control2' at a distance of 20,000 m from the vent in La Bajita (pH ~8.1) (Figure 4.1). In the case of *P. lividus*, no individuals were found in 'Intermediate3' in Las Cabras. After collection, sea urchins were dissected, and their gonads were preserved in RNAlater.

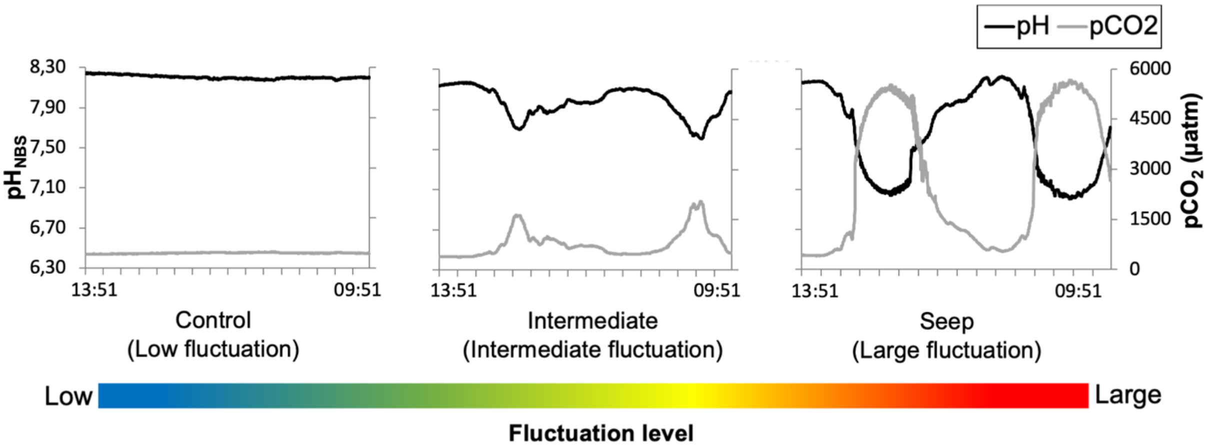


Figure 4.2. Daily pH (NBS standard pH scale – in black) and $pCO_2$ (µatm – in grey) fluctuations at the 'Control1', 'Intermediate1', and 'Vent1' sites. Modify graph from Viotti et al., (2019).

For the benthic community analyses, samples of the hard bottom community were collected at 4 sites with different levels of pH fluctuation: 'Vent1' located at the $CO_2$ vent of Playa del Faro (pH = 7.5-7.9); 'Intermediate1' at a transitional distance of 25 m from this emission point (pH = 7.8-8.1); 'Intermediate2' at 75 m (pH = 7.9-8.1); and 'Control1' at a distance of 200 m from the emission point in Echentive (pH ~8.1) (Figure 4.1). More details about the sampling points and methods applied can be found in González-Delgado et al., (2023).

The chemical characteristics of the Punta de Fuencaliente $CO_2$ vent system can be found in González-Delgado et al., (2021), and are also summarized in Table 4.1.

### 4.2.2. Sample Collection, Processing, and Sequencing

For the sea urchin samples, DNA was extracted from the gonads preserved in RNAlater using the Speedtools tissue DNA extraction kit (www.biotools.eu). A fragment of the mitochondrial gene Cytochrome C Oxidase subunit I (COI) was amplified and sequenced from 102 individuals of *A. lixula* and 75 individuals of *P. lividus*. Specific primers were designed using primer 3 software based on the complete mitochondrial genome of each species, available at GenBank (X80396.1 for *A. lixula* and NC_001572.1 for *P. lividus*) (Table 4.2). For each species separately, PCR was performed in a final volume of 20 µL, including 1 µL of REDExtract-N-ampl PCR reaction mix (www.sigma.com), 0.8 µL of each primer (10µM), 7.4 µL of ultrapure water, and 1 µL of DNA extraction. The PCR temperature cycling for fragment amplification was the same for both species: 94°C/5min-(94°C/1min-55°C/1min-72°C/1min) *35 cycles -72°C/5min. PCR products were purified and sequenced at Macrogen, Inc (Seoul, Korea). The obtained sequences were edited using Geneious software (Geneious Prime 2021.1.1) and aligned separately for *A. lixula* and *P. lividus* using the Clustal Omega alignment algorithm of Geneious. COI sequences of 905 bp and 801 bp were obtained for *A. lixula* and *P. lividus*, respectively.

Table 4.2. Sequences of the primers for each species of sea urchins: *Arbacia lixula* and *Paracentrotus lividus*.

| Species | Primers |
|---|---|
| *A. lixula* | Ar-COI-527F:TCTCTCTTTTGACCGTTTACCC/<br>Ar-COI1,521R:TTCATCAAAGGTATGGTGGG |
| *P. lividus* | Pa_103F:GTAATTATCCGAGCCGAACT/<br>Pa_992R:TTAGATCCTTGAAGGGTTGC |

The methodology for the analyses of the benthic community using metabarcoding techniques is detailed in González-Delgado et al. (2023). Briefly, DNA obtained from samples collected from rocky bottom scrapings was sequenced using a eukaryotic metabarcoding technique, also targeting a fragment of the COI gene. Throughput sequencing generated the following number of reads per eukaryotic group: 526,837 from Ochrophyta, 886,177 from Rhodophyta, 722,982 from Annelida, 617,068 from Arthropoda, 66,152 from Bryozoa, 1,758 from Chordata, 199,966 from Cnidaria, 125,503 from Echinodermata, 151,606 from Mollusca, 13,908 from Nematoda, 34,611 from Nemertea, 858 from Platyhelminthes, 43,650 from Porifera, 4,654 from Sipuncula, and 11,646 from Xenacoelomorpha. From all these reads, a total of 186 molecular operational taxonomic units (MOTUs) were identified as algae, and 631 MOTUs were identified as metazoans (see details González-Delgado et al., 2023).

### 4.2.3. Diversity Values and Statistical Analyses

For the two sea urchins, *A. lixula* and *P. lividus*, we calculated different parameters of molecular diversity from COI using DnaSP v.6 (Rozas et al., 2017). These parameters included the number of haplotypes (Nh), haplotype richness (after rarefaction to the lowest sample number (rh (15))), haplotype diversity (H), and nucleotide diversity ($\pi$), along with their standard deviation, in all sampling sites ('Vent1', 'Vent2', 'Intermediate1', 'Intermediate3' and 'Control2'). The diversity results were represented using EXCEL v16.65 and PowerPoint v16.65. For the benthic community analyses, the R package vegan v2.5.5 (Oksanen et al., 2016) was used to measure the diversity of both algae and metazoan groups. To account for samples with different sequencing depths when comparing $\alpha$-diversity patterns, we calculated MOTU density replicates by applying a rarefaction method (using the rarefy function with 50 replicates and a rarefaction size of 8,000 reads, following Sanders (1968)). Also, the mean values obtained for the density index were compared between sites ('Vent1', 'Intermediate1', 'Intermediate2', and

'Control1') using Kruskal-Wallis Chi-squared analysis (with a significant p-value < 0.05).

## 4.3. RESULTS

### 4.3.1. Genetic Diversity of Sea Urchin Populations

The genetic diversity values of the *P. lividus* populations showed the highest haplotype richness, haplotype diversity, and nucleotide diversity in the 'Intermediate2' site (with a pH of 7.81 during low tide). In the case of *A. lixula*, the highest haplotype richness and haplotype diversity were obtained in the 'Intermediate3' site (with a pH of 7.88 during low tide), and the highest nucleotide diversity was found in the 'Intermediate1' site (pH 7.81 during low tide) (Table 4.3 and Figure 4.3). Therefore, the highest molecular diversity was found at the intermediate levels of fluctuation in both species (Figure 4.3).

**Table 4.3.** Genetic diversity values for *P. lividus* and *A. lixula* at each sampling site. The code of the sampling site, pH value during low tide, number of individuals per site (N), haplotype richness (rh (15)), haplotype diversity (Hd) and its standard deviation (SD (Hd)), and nucleotide diversity ($\pi$) and its standard deviation (SD ($\pi$)) are presented. The asterisk highlights the highest values per species.

| | Sites | Code | pH | N | rh(15) | Hd | SD(Hd) | $\pi$ | SD($\pi$) |
|---|---|---|---|---|---|---|---|---|---|
| *A. lixula* | La Bajita | Control2 | 8.10 | 29 | 10.48 | 0.914 | 0.00761 | 0.00410 | 0.00015 |
| | Las Cabras | Intermediate3 | 7.88 | 19 | 11.75* | 0.977* | 0.00528 | 0.00509 | 0.00022 |
| | Playa del Faro | Intermediate1 | 7.81 | 24 | 11.24 | 0.920 | 0.01000 | 0.00519* | 0.00021 |
| | Playa del Faro | Vent1 | 7.56 | 15 | 8.53 | 0.886 | 0.01601 | 0.00436 | 0.00032 |
| | Los Porretos | Vent2 | 7.32 | 15 | 7.60 | 0.866 | 0.01782 | 0.00305 | 0.00014 |
| *P. lividus* | La Bajita | Control2 | 8.10 | 29 | 11.25 | 0.894 | 0.0360 | 0.00615 | 0.00068 |
| | Las Cabras | Intermediate3 | 7.88 | - | - | - | - | - | - |
| | Playa del Faro | Intermediate1 | 7.81 | 24 | 12.48* | 0.942* | 0.0410 | 0.00729* | 0.00084 |
| | Playa del Faro | Vent1 | 7.56 | 15 | 10.65 | 0.934 | 0.0360 | 0.00702 | 0.00077 |
| | Los Porretos | Vent2 | 7.32 | 15 | 12.14 | 0.923 | 0.0600 | 0.00542 | 0.00108 |

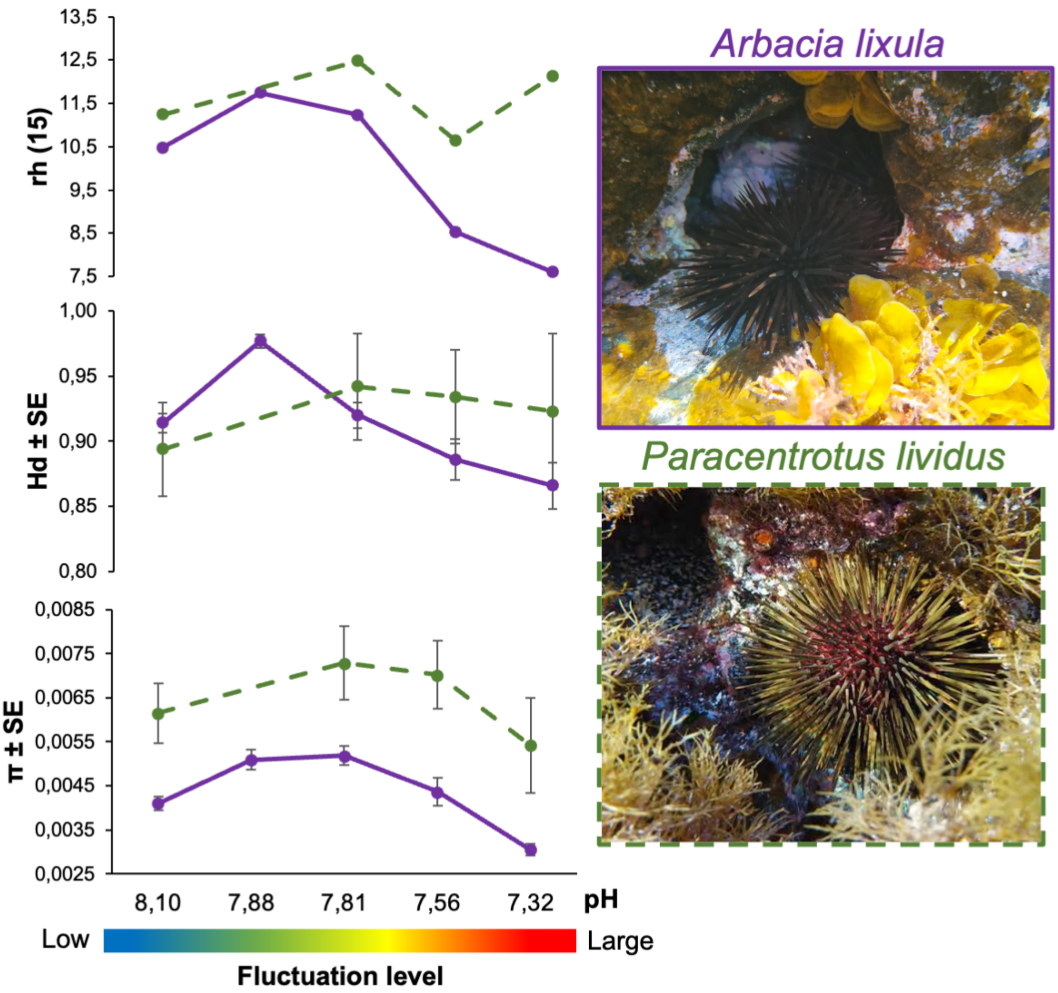


Figure 4.3. Representation of haplotype richness (rh (15)), haplotype diversity (Hd), and nucleotide diversity (π) in *P. lividus* (in green) and *A. lixula* (in purple) at the different sampling sites. The pH values shown are from low tide. Fluctuation intensity levels are show as a multicolour gradient from blue ('Low' intensity) to red ('Large' intensity).

### 4.3.2. Benthic Community Diversity

Benthic community diversity measures, based on metabarcoding, showed that the highest levels of taxonomic diversity (algae in green and metazoans in orange) were also found at the intermediate level of fluctuation (pH 7.8 during low tide) (Table 4.4; Figure 4.4). Analyses confirmed that the values between sampling points were significantly different for algae (Kruskal-Wallis Chi-squared = 4.405, df = 3, p-value = 0.01) and metazoans (Kruskal-Wallis Chi-squared = 2.381, df = 3, p-value = 0.05), especially between 'Intermediate1' (with a pH of 7.81 during low tide) and 'Intermediate2' (with a pH of 7.95 during low tide) and 'Control1' (with a pH of 8.10 during low tide) (Table 4.4).

**Table 4.4.** Pairwise Kruskal-Wallis chi-squared results (t) for the 'Algae' and 'Metazoans' groups between sites ('Control' (pH 8.1), 'Intermediate2' (pH 7.88), 'Intermediate1' (pH 7.81), and 'Vent1' (at pH 7.54)), and their associated p-value (p). The asterisk marks the significant results ($p < 0.05$). The pH values shown are from low tide.

| | | 7.54 vs 7.81 | 7.54 vs 7.88 | 7.54 vs 8.10 | 7.81 vs 7.88 | 7.81 vs 8.10 | 7.88 vs 8.10 |
|---|---|---|---|---|---|---|---|
| Algae | t | 15.406 | 7.562 | 18.056 | 2.566 | 39.102 | 10.875 |
| | p | 0.144 | 0.439 | 0.090 | 0.019* | 0.001* | 0.289 |
| Metazoans | t | 12.393 | 7.043 | 1.472 | 17.802 | 26.809 | 5.479 |
| | p | 0.227 | 0.391 | 0.150 | 0.050* | 0.015* | 0.589 |

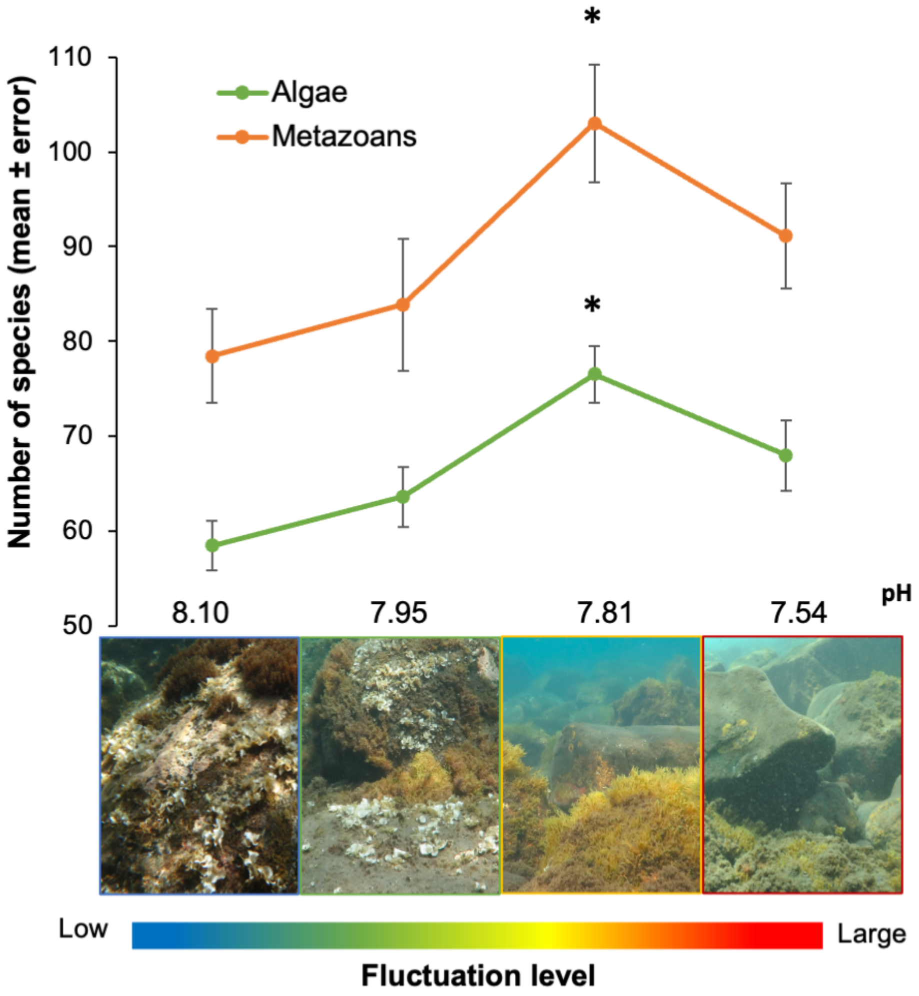


**Figure 4.4.** Number of species (mean and error) of algae (green line) and metazoans (orange line) along the pH gradient: 'Control' (pH 8.1), 'Intermediate2' (pH 7.88), 'Intermediate1' (pH 7.81), and 'Vent1' (at pH 7.54). The pH values shown are from low tide. The asterisk marks the significance of the Kruskal-Wallis chi-squared test ($p < 0.05$). A photograph of the sampling point taken during sample collection has been added below each corresponding pH value. Fluctuation intensity levels are shown as a multicolour gradient from blue ('Low' intensity) to red ('Large' intensity).

## 4.4. DISCUSSION

Considering the $CO_2$ emissions as a disturbance, our results uncover, for the first time, that the Intermediate Disturbance Hypothesis (Connell, 1978) is applicable to natural acidified ecosystems at the organismal and community levels. Furthermore, to date, most empirical tests of this hypothesis have focused on forest species (e.g., Molino and Sabatier, 2001; Scotti et al., 2015). To our knowledge, this is the second study analysing genetic diversity to test whether the IDH applies at the organismal level, and the first study examining it in a marine ecosystem.

Particularly, the two sea urchins, *A. lixula* and *P. lividus*, showed the highest levels of mitochondrial genetic diversity in the intermediate level of pH fluctuation (7.8-8.1 units) of the Punta de Fuencaliente system, despite the short geographical distances among the sampling sites and their wide dispersal capacity (e.g., Wangensteen et al., 2012; Carreras et al., 2020; Pérez-Portela et al., 2019). Previous molecular studies with the same species and markers have shown high levels of mitochondrial diversity in both species throughout their distribution ranges (Duran et al., 2004; Calderón et al., 2012; Calderón and Turón, 2010; Wangensteen et al., 2012). The comparison of the COI diversity parameters in *A. lixula* obtained here with previous analyses from 22 sites along the Atlanto-Mediterranean area, including the Canary Islands, showed values of haplotype diversity ranging between 0.825 and 0.942, and haplotype richness between 7.625 and 11.869. Hence, our results demonstrate that the intermediate sites of pH fluctuation ('Intermediate3' and 'Intermediate1' sites) in the Fuencaliente system (with pH=7.88 and pH=7.81 at low tide, respectively) retained the highest values of mitochondrial diversity. Additionally, the haplotype diversity at the 'Intermediate3' site (0.977) was the highest ever recorded in *A. lixula*, and the second-highest value of haplotype richness (11.75) in this species, being the most diverse in Tenerife (Wangensteen et al. 2012). On the other hand, at the 'Vent2' site (pH variation 7.32 - 7.88), we found the lowest values of nucleotide diversity

(0.00305) and haplotype richness (7.6), not only in the Fuencaliente vent system but also throughout the whole distribution range of this species (nucleotide diversity and haplotype richness from Wangensteen et al., (2012) = 0.00358-0.00588 and 7.625-11.869, respectively). In the case of *P. lividus*, the highest values of haplotype diversity, nucleotide diversity, and haplotype richness were also detected at the intermediate site analysed ('Intermediate1'), with the haplotype richness value at this site being the highest ever recorded for *P. lividus* (Calderón et al., 2012; Penant et al., 2013). The lowest values of these parameters were measured either the control or vent sites, but within the known range of diversity in this species (Duran et al., 2004; Calderón et al., 2012; Penant et al., 2013). Despite the differences found between both species, the highest levels of genetic diversity were recorded at the intermediate sites.

The COI is a subunit of a gene that codes for a membrane protein from the respiratory electron transport chain, involved in ATP synthesis. Although this gene has been widely used for population genetic and phylogenetic analyses in echinoderms assuming neutrality (e.g., Wangensteen et al., 2012; Pérez-Portela et al., 2017; Garcia-Cisneros et al., 2018), it is known that the mitochondrial DNA can be under natural selection (e.g., Foltz et al., 2004; Garvin et al., 2015). Hence, environmental fluctuations may determine differential selection pressures on the mitochondrial DNA, which might explain the variation found in both sea urchins at the intermediate disturbance. This genetic variation could be maintained by balancing selection that occurs when populations live across an environmental gradient or experience temporally fluctuating selection pressures (Charlesworth, 2006), as observed in the purple sea urchin, *Strongylocentrotus purpuratus*, under experimental acidification variation (Pespeni et al., 2013a).

On the other hand, a similar pattern of increased diversity at intermediate levels of disturbance was observed for both algae and metazoan communities found along the $CO_2$ gradient. Our results demonstrate that the highest levels of taxonomic benthic diversity are at the intermediate level of pH fluctuation (around 7.8-8.1 units), as postulated by the IDH, supporting the validity of this hypothesis in rocky shore ecosystems affected by pH fluctuations. Previous studies in other $CO_2$ vents may have been unable to detect increased species richness towards $CO_2$ emission points, likely due to the bias of the visual method used (e.g., Teixidó et al., 2018). Now, with the widespread use of fine-grained techniques such as DNA metabarcoding, we can expect that diversity assessment may be better equipped to both test general ecological paradigms and gather stronger evidence for predicting the future trajectories of marine benthic communities.

Our results from both the infraspecific (organism) and community levels are in line with those obtained by Scotti et al. (2015). These authors successfully tested the IDH at the canopy-gap level as a disturbance in tropical forest species and concluded that the same mechanism driving community diversity may act at the within-species level. Our study additionally supports the validity of the IDH at different biological levels in marine ecosystems and highlights the need to test whether this hypothesis applies in other marine ecosystems.

Additionally, we can conclude that the acidified system off Punta de Fuencaliente is not in equilibrium. The physicochemical processes generated by this permanent natural disturbance prevent the organisms and the community from reaching climax at sites with intermediate disturbance. This sharp natural gradient could promote increased genetic diversity in some populations, such as those of the two sea urchins studied here. The genetic variation that emerges might drive adaptive evolution (Kelly and Hofmann, 2013), with species evolving in relation to environmental variation to gain an advantage in the face of disturbance. However, when pH fluctuation conditions become extreme, strong directional selection of population genetic diversity occurs, leaving only those genetic variations favoured by extreme pH levels. Furthermore, the ability to adapt to this spatial environmental variable suggests the ability to evolve and adapt over time, thus anticipating future changes caused by OA (Hofmann et al., 2014; Calosi et al., 2017). Similarly, these intermediate fluctuations also lead to an increase in the number of species. This occurs for several reasons, mainly the reduction in competitive exclusion, as species have different tolerance regimes to acidification, and few species can compete successfully at these intermediate disturbance levels (Willig and Presley, 2018). Moreover, these intermediate fluctuations create available space within the ecosystem, allowing new species to colonize and facilitating intermittent successional processes that increase overall biodiversity. However, when fluctuating conditions become extreme, species diversity drops again because only those species that can thrive under extreme conditions become established. These species, which could be termed 'tolerant,' will be the ones that remain in extreme pH environments. Assuming that the acidified system at Punta de Fuencaliente may be an analogue to future conditions, they will be the species that could dominate the community in the future acidified ocean.

## DATA AVAILABILITY

The new haplotypes of COI are available in Genbank (OQ981110-OQ981131 for *A. lixula*) and (OQ990022-OQ990038 for *P. lividus*). The complete datasets of COI sequences per species are available in Mendeley Data (doi: 10.17632/djvn2337s6.1).

# CHAPTER 5

## Genomic Signals of Adaptation to a Natural $CO_2$ Gradient Over a Striking Microgeographic Scale

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## ABSTRACT

The black sea urchin, *Arbacia lixula,* lives in several $CO_2$ vents in the Atlantic-Mediterranean area, where pH levels decrease to values of 7.1-7.3 units, despite its supposed vulnerability to acidification. Our study aims to explore genomic signs of adaptation to low pH in *A. lixula*, to infer its potential of survival under the future ocean acidification conditions. To approach our objective, we analysed individuals of *A. lixula* living across a natural pH gradient created by a $CO_2$ vent in Fuencaliente (La Palma, Canary Islands, Spain). We applied a 2b-RADseq protocol to 74 samples collected from five sites influenced by different pH levels (7.3, 7.5, 7.8, and 7.9 pH units during low tide, some of them separated by less than 250 meter), along with a control site at pH 8.1. A total of 14,883 SNPs were isolated, and 432 loci identified as candidate SNPs under selection to pH variations with the redundancy analysis (RDA). Analysis of all SNPs revealed genomic homogeneity among sites, whereas the candidate SNPs under selection revealed genomic sub-structure among sites and along the pH gradient. Among the 432 candidate loci under selection, 17 of them had annotation from *A. lixula* transcriptomes already published. The annotated genes were involved in relevant biological functions such as membrane functioning, growth, and development. Therefore, our results suggest the presence of local adaptation in *A. lixula* populations to acidification in a $CO_2$ vent, even over distances as short as 250 meters, highlighting their potential resistance to future OA.

Keywords: ocean acidification, population genomic, adaptation, SNPs, $CO_2$ vent, 2b-RADseq, echinoderm.

## RESUMEN

El erizo de mar negro, *Arbacia lixula,* vive en varios afloramientos de $CO_2$ en el área Atlántico-Mediterránea, donde los niveles de pH disminuyen a valores de 7.1-7.3 unidades, a pesar de su supuesta vulnerabilidad a la acidificación. Nuestro estudio tiene como objetivo explorar los signos genómicos de adaptación de *A. lixula* al pH bajo, para inferir su potencial de supervivencia bajo futuras condiciones de acidificación. Para abordar esto, analizamos individuos de *A. lixula* que viven a lo largo de un gradiente de pH natural creado por el afloramiento de $CO_2$ de Fuencaliente (La Palma, Islas Canarias, España). Aplicamos un protocolo de 2b-RADseq a 74 muestras recolectadas en cinco sitios influenciados por diferentes niveles de pH (7.3, 7.5, 7.8 y 7.9 unidades de pH durante la marea baja, separados algunos de ellos por menos de 250 metros), junto con un sitio de control a pH 8.1. Se aislaron un total de 14,883 SNP y de estos se identificaron 432 SNP candidatos bajo selección debido a las variaciones de pH mediante el análisis de redundancia (RDA). El análisis de todos los SNP reveló homogeneidad genómica entre los sitios, mientras que los SNP candidatos bajo selección revelaron subestructura genómica entre los sitios y a lo largo del gradiente de pH. De los 432 loci candidatos bajo selección, obtuvimos anotación para 17 de ellos utilizando los transcriptomas de *A. lixula* ya publicados. Los genes anotados están involucrados en funciones biológicas relevantes, como el funcionamiento de las membranas, el crecimiento y desarrollo. Por lo tanto, nuestros resultados sugieren la presencia de adaptación local en las poblaciones de *A. lixula* a la acidificación en los respiraderos de $CO_2$, incluso en distancias tan cortas como 250 metros, resaltando su potencial resistencia a futuras AO.

Palabras claves: acidificación oceánica, genómica de poblaciones, afloramiento de $CO_2$, adaptación, SNPs, 2b-RADseq, equinodermos.

## 5.1. INTRODUCTION

Recently, studies dealing with the effect of ocean acidification (OA) across natural environmental gradients are becoming more popular and useful for developing realistic predictions about its potential impact (e.g., Hall-Spencer et al., 2008; Calosi et al., 2017; Rastrick et al., 2018; Teixidó et al., 2018). Analysing species' performance along natural gradients can offer the opportunity to interpret how long-term adaptations and short-term responses to environmental variables modulate individuals' sensitivity to changes and understand the biological buffering systems that species and ecosystems have to cope with environmental shifts (e.g., Calosi et al., 2017; Pereira et al., 2017; Foo and Byrne, 2021). In this sense, $CO_2$ vents have proven to be useful marine areas to develop research to predict potential impacts of decreasing pH and OA (e.g., Teixidó et al., 2020; Kumar et al., 2022; González-Delgado et al., 2023). These natural $CO_2$ constant emission points generate strong acidification gradients at microgeographical scales where other variables are stable (González-Delgado and Hernández, 2018). Worldwide, several vent sites located at tropical and temperate-subtropical latitudes are being used as natural laboratories for large-scale studies on the effects of OA, providing an opportunity to measure both rapid acclimatization processes and local adaptation patterns of the marine organisms living in these unique ecosystems (e.g., Kenkel et al., 2018; Teixidó et al., 2020; Petit-Marty et al., 2021; Kumar et al., 2022).

Several molecular studies in these natural acidified environments have led to significant discoveries, particularly in relation to gene expression. For instance, non-calcifying species such as brown algae (Kumar et al., 2020; 2022) exhibit increased expression of stress and photosynthesis related genes and proteins under acidification. Similarly, anemones show heightened expression of stress and metabolism related genes (Urbarova et al., 2019), while fishes upregulate genes associated with pH homeostasis and metabolism (Petit-Marty et. al., 2021). Regarding calcareous organisms, such as sea urchins and corals, acidification-induced alterations have been observed in genes involved in homeostasis and biomineralization (Kenkel et al., 2018; Di Giglio et al., 2020; Teixido et al., 2020). Population genetic studies using microsatellites conducted in molluscs (Harvey et al., 2016), and single nucleotide polymorphisms (SNPs) in sea urchin larvae (Uthicke et al., 2019) and adult corals (Teixidó et al., 2020) did not revealed clear adaptation patterns related to natural pH decrease. However, there is an exception in the work of Pespeni et al. (2013b) on the adult sea urchin *Strongylocentrotus purpuratus* along the cost of California. In their study, the use of SNPs enabled them to observe patterns of adaptation to local pH conditions.

In general, the adaptation and/or modulation of gene expression involved in calcification, acid-base regulation, and energy metabolism seem to play a critical role in determining calcifying invertebrate sensitivity or resistance to acidification (Kenkel et al., 2018; Padilla-Gamiño et al., 2019; Strader et al., 2020). These organisms are particularly vulnerable to the impacts of acidification due to their dependence on $CaCO_3$ for the formation of their shells or skeletons (Doney et al., 2009; Hofmann et al., 2010), although physiologic pathways plasticity and buffering mechanisms can confer some resilience to low pH (e.g., Hofmann et al., 2014; Kenkel et al., 2018; Migliaccio et al., 2019).

Echinoderms have garnered special attention in OA research due to their ecological and economic importance in many cases (e.g., Dupont et al., 2010; Strader et al., 2020). Within this invertebrate group, a wide range of both positive and negative responses to low pH conditions have been detected depending on the species (e.g., Dupont et al., 2010; Byrne and Hernández, 2020). In sea urchins, evidence indicates that OA might have sublethal but significant long-term negative effects, such as weakening of their calcareous structures, an impact that can differentially affect species ontology and life stages (Byrne and Hernández, 2020). Additionally, recent studies conducted in $CO_2$ vents have shown that some species may circumvent some negative impacts of extreme pH drops, such as *Paracentrotus lividus* which displayed variation in the immune respond (Migliaccio et al., 2019) and *Arbacia lixula* that has developed respiratory, mineralogy, and reproductive buffering systems (Calosi et al., 2013a; Bray et al., 2014; Foo et al., 2018a; Foo et al., 2020).

The black sea urchin *A. lixula*, analysed in this study, is widely distributed from the tropical and subtropical eastern Atlantic coast (Mortensen, 1935; Tommasi, 1964) to the entire Mediterranean Sea (Tortonese, 1965). This sea urchin is a keystone species in benthic ecosystems due to its browsing omnivorous feeding activity (Bulleri et al., 1999; Wangensteen et al., 2011). Therefore, due to the capacity of this species to promote changes in entire ecosystems, it has recently become the focus of several studies to predict the effects of climate change on its population dynamics (e.g., Byrne and Hernández, 2020; Wangensteen et al., 2012; Pérez-Portela et al., 2020).

During the past decade, next-generation sequencing analyses have revealed the significant plasticity of the species to enduring environmental changes across its distribution range (e.g., Pérez-Portela et al., 2020; Carreras et al., 2021). These analyses facilitated the exploration of transcriptomic responses associated with temperature changes (Pérez-Portela et al., 2020). The use of thousands of SNPs highlighted the presence of genomic substructure between the eastern and western Mediterranean populations of this species, which is correlated with environmental variables, despite high levels of gene flow (Carrera et al., 2021). Several candidate genes for local adaptation were identified, and salinity was considered the main driver of genetic structure in this species. Additionally, *A. lixula*, which inhabits continuously acidified waters in Fuencaliente, La Palma (Canary Islands), as mentioned before exhibits a high physiological capacity to tolerate acidification (Calosi et al., 2013a), despite the energetic cost it entails. These physiological costs manifest as a reduction in body size and/or the size and number of oocytes (Hernández et al., 2018a; Foo et al., 2018a; González-Delgado et al., 2018).

A genomic study of both the populations inside and outside the $CO_2$ vent, as presented here, can provide crucial insights into understanding the local adaptation patterns of *A. lixula* under different pH conditions. This information is vital for predicting the species' long-term survival in future oceans, being a valuable example to understand the ecological impacts of OA on marine invertebrate populations (Sanford and Kelly, 2011). Hence, to identify signs related to local adaptation to pH, we conducted a genomic structure analysis of the population using a 2bRad-seq method along a steep pH gradient (spanning only 150 m) in the Fuencaliente $CO_2$ vents system, located in the Canary Islands in the eastern Atlantic Ocean (González-Delgado et al., 2021). This acidified system does not only present an acidification gradient with values expected for the future (IPCC, 2021), but also exhibits daily pH fluctuations due to semidiurnal tides (González-Delgado et al., 2021). This is significant as the ocean is an open system with constant environmental fluctuations that can affect sea urchins performance, and there is a lack of related studies (Byrne and Hernández, 2020). In this natural laboratory, the 'substitution of space for time' approach (Hofmann et al., 2014) is applied, where natural spatial variation and daily pH fluctuation can be used to predict future changes in populations of marine organisms. Our initial hypothesis is that despite the large dispersal potential of *A. lixula* larvae, this species can exhibit genomic sub-structure along the pH gradient related to the differential selection pressures on its genome.

## 5.2. MATHERIAL AND METHODS

### 5.2.1. Sampling

A total of 74 individuals of *A. lixula* were collected by snorkeling from five sites along the natural pH gradient of the Punta de Fuencaliente $CO_2$ vent system, located south of La Palma Island in the Canary Islands, Spain. Additionally, a control site outside of this vent area, situated 20 km away, was also included (Figure 5.1).

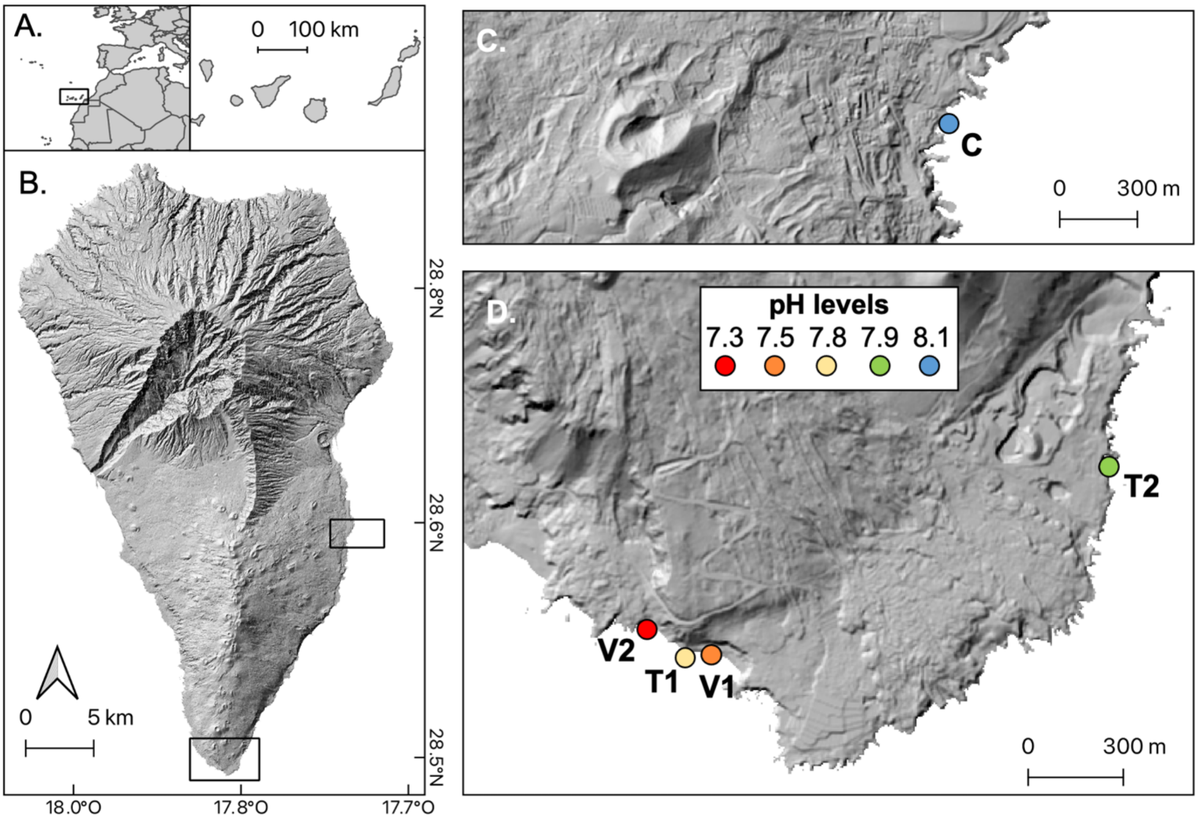

Figure 5.1. Sampling sites at La Palma. (A) The Canary Islands in the northeastern Atlantic Ocean; (B) La Palma Island; (C) La Bajita sampling site called as 'Control' (C) with pH ~8.1 units; (D) Punta de Fuencaliente with the four sampling sites: 'Vent1' (V1) with pH ~7.5 units during low tide, 'Vent2' (V2) with pH ~7.3 units during low tide; 'Transition1' (T1) with pH ~7.8 units during low tide; and 'Transition2' (T2) with pH ~7.9 units during low tide. The pH level during low tide is show by a color gradient from red (lowest pH value) to blue (current ocean pH). The map and image base layers used are distributed in the public domain (https://www.grafcan.es/, last accessed: 30 September 2022).

In this vent system, natural acidification has been occurring for the past 30 years in three different areas: Playa del Faro, Los Porretos and Las Cabras. This acidification phenomenon is a result of groundwater discharge that is being acidified by volcanic CO$_2$ emissions. This discharge into the sea mainly occurs during low tide, resulting in the lowest pH values being measured at that time (González-Delgado et al., 2021). Sea urchin Individuals were collected from two subtidal sites with the lowest pH values during low tide, henceforth called here as 'Vent' (Vent1 (V1) and Vent2 (V2) with pH 7.54 and 7.32, located on Playa del Faro and Los Porretos, respectively), two sites with intermediate pH values called as 'Transition' (Transition1 (T1) and Transition2 (T2) with pH

7.81 and 7.88, located at Playa del Faro and Las Cabras, respectively), and one site with current pH ocean conditions called as 'Control' (pH around ~8.14; C; located at La Bajita, approximately 20 km northeast of the other sites). The temperature at all sites remained constant at an average of 21°C during sampling. Sea urchins were dissected *in situ* after collection to remove their gonads, and the tissue was stored in the freezer at -20 °C with RNA later until DNA extraction.

### 5.2.2. DNA Extraction and Sequencing

Genomic DNA was extracted using a modified protocol from Panova et al. (2016). Digestion was done in sperm isolation buffer (SIB). DNA was precipitated and washed using Chloroform Isoamyl 25:1. The eluted DNA was further cleaned up using the DNA Clean and Concentrator kit -25 (Zymo). DNA quality and integrity were assessed using a Nanodrop and on a 1% agarose gel. DNA quantification was performed with a Qubit dsDNA broad range Assay Kit (Invitrogen-ThermoFisher Scientfic). DNA from 10-20 individuals from each site was extracted to construct individual 2b-RAD libraries (Wang et al., 2012) following a laboratory protocol from Galina Aglyamova and Mikhail Matz available at https://github.com/z0on/2bRAD_denovo. Equimolarly pooled libraries were then sequenced on the NovaSeq 6000 SP flowcell from Illumina platform, generating 50bp pair-end sequences, at the Science for Life Laboratory (SciLifeLab) – Genomics, SNPandSEQ Technology Platform in Uppsala University, Sweden.

The bioinformatic analyses were performed using the computer cluster 'Albiorix' at the University of Gothenburg, Sweden. The analysis followed a modified de novo pipeline available at https://github.com/crustaceana/TheFucusProject, originally developed by Mikhail Matz (https://github.com/z0on/2bRAD_denovo). Sequences were trimmed and quality filtered before clustered into RAD tags with a minimum depth of 20 reads. Genotype calling was performed with a minimum depth of 5 to call homozygotes, a maximum acceptable proportion of 0.8 for heterozygotes at a locus and minimum 20 observations for each allele across all samples. Technical replicates or repeated measurements of samples were made and used for non-parametric quantile-based recalibration of variants. Loci exceeding 75% heterozygotes were removed to avoid sequencing errors. The data were preliminarily thinned to retain one Single Nucleotide Polymorphism (SNP) per RAD fragment, where each fragment had less than 50% of missing data. Technical replicates were subsequently removed from the dataset. Further thinning was performed using Poppr (Kamvar et al., 2014) to remove loci or genotypes with > 5% missing data and to retain only informative loci with at least two differing observations. All raw 2b-RAD sequences are deposited in the Sequence Read Archive Repository at the National Centre for Biotechnology and Information (Bioproject PRJNA948020).

### 5.2.3. Detection of Potential Loci under Selection, Genetic Diversity, and Differentiation

Since, our objective was to identify signs of genomic adaptation due to selection under different pH values and distinguish them from genomic divergence caused by neutral processes (e.g., gene flow interruptions due to marine barriers), we first conducted tests to detect candidate loci (SNPs) under selection. We applied different methods to the entire database to detected potential SNPs under selection: outliers identification analyses in Arlequin v3.5 using the hierarchical island model (Excoffier and Lischer, 2010), BayeScan v2.1 using the classical island parameterisation model (Foll, 2012) and, Redundancy Analysis (RDA) (Van den Wollenberg, 1977). The RDA explores the association between genetic variation and environmental variables using the R package 'vegan' (Oksanen et al., 2016). The analysis with Arlequin was performed using 10,000 coalescent simulations and 100 demes per group without considering environmental parameters. The BayeScan analysis was performed with 20 pilot runs of 5,000 iterations each, a burn-in period of 50,000 and a total of 100,00 itineration including the information of collection site. In both cases, loci were considered outliers when the p-values < 0.05 after a Benjamini and Yekutely correction (BY corrections) (Benjamini and Yekutieli, 2001).  The RDA analysis was used to detect candidate SNPs under selection associated with natural acidification. A matrix of loci with their corresponding environmental variables was analysed using a linear regression model to identify candidate SNPs associated with pH. With all this information, we identified potential SNPs under selection and created two different datasets: a 'neutral' dataset by removing all the outliers and candidate SNPs under selection, and a 'candidate SNPs' dataset including the SNPs under selection from RDA analysis (please see the Results section).

For both datasets (neutral SNPs and candidate SNPs under selection), general descriptors of genomic diversity and equilibrium were measured. The observed (Ho) and expected (He) heterozygosity, as well as inbreeding coefficients ($F_{IS}$) and Hardy-Weinberg Equilibrium (HWE) were calculated across the five sites using GENODIVE v3.0 (Meirmans, 2020).

To explore the differences in genomic structure and significant divergence among sampling sites due to neutral processes or selection, we applied different approaches for the 'neutral and 'candidate SNPs' datasets separately. We calculated genomic distances using pairwise fixation indexes ($F_{ST}$) in Arlequin and their associated p-values with 10,000 permutations. Significant $F_{ST}$ values were identified when p-values < 0.05 after a BY correction. The $F_{ST}$ values were graphically represented on heatmaps using the R package 'ggplots2' for an easier interpretation of the results. Bayesian clustering analysis was further conducted to identify individual proportions of genomic ancestry using STRUCTURE v2.3.4 (Pritchard et al., 2000), with a burn-in length of 50,000 and 450,000 Markov Chain Monte Carlo (MCMC) chains. The software CLUMPAK (Kopelman et al., 2015) was used to average the STRUCTURE results and identify the most likely number of K genetic clusters that best described the data. Finally, the 'Adegenet' R package was used to transform the datasets into principal components and partition the variance using the Discriminant Analysis of Principal Components (DAPC) (Jombart and Ahmed, 2011). This method maximizes the discrimination between groups to identify genomic clusters, which were cross validated using the ‘Poppr’ R package (Kamvar et al., 2014).

Mantel tests were separately performed in both datasets, using the $F_{ST}$ distance matrix and a geographical distances matrix among sampling sites to determine the potential effect of the isolation by distance on population differentiation. We used the ‘vegan’ R package with 99,999 replicates to conduct these tests and calculate their associated p-values.

### 5.2.4. Candidate SNPs under pH Selection and Annotation

To identify genes under pH selection from the candidate SNPs, RAD fragments containing these candidate SNPs were aligned against reference transcriptomes available for *A. lixula* (Arbacia_lixula_Digestive_Assembly_Trinity.fasta; Arbacia_lixula_Ovary_Assembly_Trinity.fasta; Arbacia_lixula_testis_Assembly_Trinity.fasta; reference_assembly_CT+T7.fasta; reference_assembly_CT+T22.fasta) using Geneious v8.0.4. Since *A. lixula* lacks an annotated reference genome, we used transcriptomes from different tissue types as reference to obtain annotations. Annotations were extracted from their respective references available at Dryad: doi:10.5061/dryad.2rs4k (Pérez-Portela et al., 2016) and Mendeley Data: https://data.mendeley.com/datasets/5673n552yj/1 (Pérez-Portela et al., 2020).

To summarize and visualize the gene functions of the annotated genes, we explored Gene Ontology (GO) annotations (GO terms) using Revigo (http://revigo.irb.hr/). The ‘treemap’ R package (Tennekes and Ellis, 2017) was used to graphically represent the GO terms, with the size of the rectangles adjusted to reflect the frequency of each GO term, allowing us to visually identify the most common terms in our database.

## 5.3. RESULTS

### 5.3.1. General Information and Neutral and No Neutral Dataset

A mean of 75.30 reads per individual and locus was obtained. Out of the 74 individuals of *A. lixula*, a total of 14,881 loci were retained after the initial trimming and filtering. The dataset for this study is available at Mendeley Data (doi:10.17632/9phf69mt8d.1). Among all SNPs, 12 loci were identified as outliers in the Arlequin and BayeScan analyses, with only 2 of these loci being detected by BayeScan. Additionally, RDA analysis identified 432 candidate SNPs under selection, which were associated with pH (including the 2 SNPs detected by Arlequin and BayeScan). The first two axe of the RDA explained a cumulative 45.70% of the genomic variability observed in our database (Appendix D, Figure D.1) and sorted the samples along the pH gradient. After removing all loci under selection detected in the different analyses (Arlequin outliers, Bayescan and RDA), the 'neutral' dataset consisted of a total of 14,436 SNPs.

Table 5.1. Information on the sampling sites (S.): pH measurements during low tide ($pH_{lt}$), genetic features separated from the 14,436 neutral SNPs, and the 432 candidate SNPs under selection obtained from the RDA. Number of alleles ($N_{alle}$), effective number of alleles ($EN_{alle}$), observed heterozygosity (Ho), expected heterozygosity (He), and inbreeding coefficient ($F_{IS}$). Significant values ($p < 0.05$) are marked with an asterisk (*).

| | | | Neutrals (14,436 loci) | | | | | RDA 'candidate SNPs' (432 loci) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **S.** | **$pH_{lt}$** | **N** | **$N_{alle}$** | **$EN_{alle}$** | **Ho** | **He** | **$F_{IS}$** | **$N_{alle}$** | **$EN_{alle}$** | **Ho** | **He** | **$F_{IS}$** |
| **V1** | 7.54±0.02 | 19 | 1.763 | 1.225 | 0.138 | 0.157 | 0.117* | 1.775 | 1.240 | 0.149 | 0.168 | 0.112* |
| **V2** | 7.32±0.06 | 13 | 1.693 | 1.221 | 0.129 | 0.154 | 0.161* | 1.697 | 1.223 | 0.130 | 0.155 | 0.162* |
| **T1** | 7.81±0.01 | 11 | 1.645 | 1.223 | 0.131 | 0.156 | 0.161* | 1.674 | 1.222 | 0.136 | 0.158 | 0.139* |
| **T2** | 7.88±0.02 | 14 | 1.719 | 1.225 | 0.132 | 0.157 | 0.163* | 1.727 | 1.216 | 0.133 | 0.154 | 0.134* |
| **C** | 8.14±0.01 | 17 | 1.755 | 1.225 | 0.134 | 0.157 | 0.145* | 1.771 | 1.225 | 0.139 | 0.160 | 0.131* |
| *Total* | | *74* | | *1.215* | *0.133* | *0.156* | *0.149* | *2* | *1.213* | *0.137* | *0.159* | *0.135* |

For the ‘candidate SNPs’ dataset, only the 432 SNPs detected in the RDA analysis were used, as this was the only analysis that used the pH variable to detect candidate loci under selection.

In both datasets and across all sampling sites, the observed heterozygosity (Ho) (ranging from 0.129 - 0.149) was lower than the expected heterozygosity (He) (ranging from 0.154 - 0.168) displaying heterozygote deficit. The inbreeding coefficients ($F_{IS}$) were low and positive at all sampling sites, and significantly deviated from the HWE ($p < 0.05$) (Table 5.1).

### 5.3.2. Population Structure and Divergence

All analyses revealed contrasting results regarding the genomic structure between the ‘neutral’ and ‘candidate SNPs’ datasets. The 'neutral' dataset showed genomic homogeneity across the studied area, while the 'candidate SNPs' dataset exhibited significant genomic structure and divergence between sites (Figure 5.2).

The $F_{ST}$ values of the ‘neutral’ SNPs were very low (ranging from 0 to 0.004) and most of them were non-significant, except for the comparison between the ‘Vent1’ and ‘Control’ sites ($F_{ST}$ = 0.002 and p-value = 0.003), ‘Vent1’ and ‘Transition1’ sites ($F_{ST}$ = 0.002 and p-value = 0.035), and ‘Vent1’ and ‘Transition2’ ($F_{ST}$ = 0.001 and p-value = 0.044) (Figure 5.2A, and Appendix D, Table D.1). However, in the ‘candidate SNPs’ dataset, the $F_{ST}$ values were higher (ranging from 0 to 0.048) and most of them were significant. The sites with the lowest pH values, ‘Vent1’ and ‘Vent2’ (~7.5 and ~7.3 pH unit during low tide, respectively), showed significantly higher $F_{ST}$ values compared to sites with higher pH values. These significant comparisons included ‘Vent1’ and ‘Transition1’ sites ($F_{ST}$ = 0.013 and p-value = 0.020), ‘Vent1’ and ‘Transition2’ sites ($F_{ST}$ = 0.048 and p-value = 0.001), ‘Vent1’ and ‘Control’ sites ($F_{ST}$ = 0.045 and p-value = 0.001), ‘Vent2’ and ‘Transition2’ sites ($F_{ST}$ = 0.040 and p-value = 0.001), and ‘Vent2’ and ‘Control’ sites ($F_{ST}$ = 0.039 and p-value = 0.001). Also, the ‘Transition1’ site (with pH ~7.8 unit during low tide) was significant different from the ‘Control’ sites ($F_{ST}$ = 0.011 and p-value = 0.017) (Figure 5.2B, Appendix D, Table D.1). On the other hand, sites with similar pH levels exhibit the lowest $F_{ST}$ values, such as the ‘Vent1’ and ‘Vent2’ sites ($F_{ST}$ = 0.008 and p-value = 0.052), ‘Transition1’ and ‘Transition2’ sites ($F_{ST}$ = 0.003 and p-value = 0.249) and ‘Transition2’ and ‘Control’ sites ($F_{ST}$ = 0.000 and p-value = 0.710).

Similarly, the Bayesian clustering approach from STRUCTURE revealed genomic homogeneity from the ‘neutral’ SNPs across the five sampling points, with most sampling sites dominated by only one cluster, except for two individuals from V1 (Figure 5.2C). However, the ‘candidate SNPs’ dataset showed different contribution of three genetic clusters, displaying a clear pattern of differentiation along the pH gradient (Figure 5.2C). The most common cluster at ‘Vent1’ and ‘Vent2’ was the least frequent at the ‘Control’ and ‘Transition2’ sites, while the ‘Transition1’ exhibited a mixed structure with all three clusters.

The DAPC analysis showed a coherent pattern to with the $F_{ST}$ and STRUCTURE results. No distinct clustering among sites was observed in the ‘neutral’ SNPs dataset (Figure 5.2D). However, from the 'candidate SNPs' dataset, three clusters were identified in accordance with the pH gradient: a cluster encompassing the 'Control' and 'Transition2' sites (with the highest pH levels), the 'Transition1' site (with intermediate pH levels of ~7.8), and a cluster comprising individuals from the two most acidified points, 'Vent1' and 'Vent2' (Figure 5.2E).

The non-significant results of the Mantel test (r = -0.468, p = 0.843 for the 'neutral' dataset, and r = 0.160, p = 0.326 for the 'candidate SNPs' dataset) dismissed isolation by distance as a reason for genomic divergence among sampling sites.

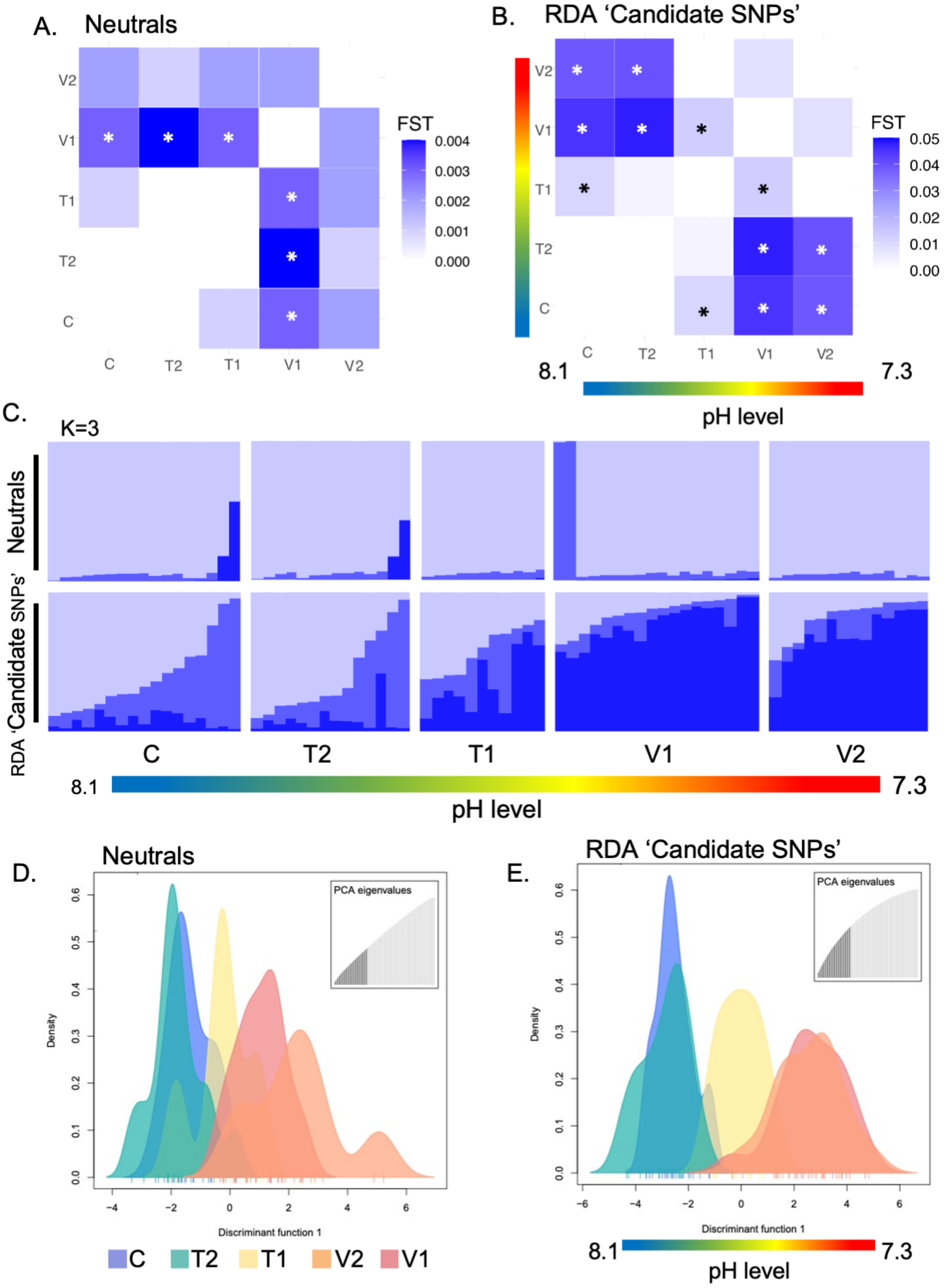

Figure 5.2. Genomic differences among sites. (A) Heatmap based on the pairwise $F_{ST}$ distance from the 'neutral' dataset, and (B) the 'candidate SNPs' dataset. Significant $F_{ST}$ values ($p < 0.05$) are with an asterisk (*). (C) STRUCTURE results (K= 3) from the 'neutral' and 'candidate SNPs'. (D) DAPC graph results using the 'neutral' and (E) the 'candidate SNPs' dataset. 'Vent1' (V1) with pH ~7.5 units during low tide, 'Vent2' (V2) with pH ~7.3 units during low tide, 'Transition1' (T1) with pH ~7.8 units during low tide, 'Transition2' (T2) with pH ~7.9 units during low tide, and 'Control' (C) with pH ~8.1 units.

### 5.3.3. Annotation of Candidate Loci under Selection

A total of 185 2bRAD fragments showed blast hit with contigs of the *A. lixula* reference transcriptomes. Among them, 14 contigs (genes) were annotated, and were associated with 31 GO terms, including 15 biological processes, 3 cellular components, and 13 molecular functions (Figure 5.3, and more details in Appendix D, Table D.2).

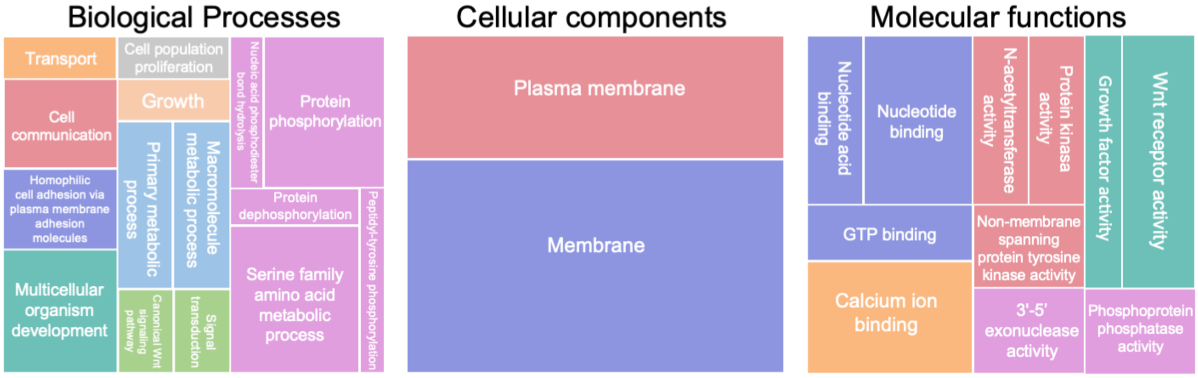

**Figure 5.3.** Gene ontology (GO) treemaps for genes annotated in *A. lixula* represented separately for 'Biological processes', 'Cellular components', and 'Molecular functions'. The size of the rectangles reflects the frequency of occurrence for each specific GO term.

Most of these annotated genes were involved in growth and development, such as ‘multicellular organism development’ (associated with the FRIZZLED7 gene), ‘growth’ and ‘growth factor activity’ (associated with the VEGF gene), and ‘cell population proliferation’ (associated with the protein DVR-1 gene). Additionally, membrane-related functions were identified, such as ‘cell communication,’ ‘homophilic cell adhesion via plasma membrane,’ ‘calcium ion binding,’ ‘plasma membrane,’ and ‘membrane’ (associated with the FREM2 gene), as well as ‘transport’ and ‘membrane’ (associated with the UGT1 gene). Furthermore, functions related to protein phosphorylation, serine family amino acid metabolic process, protein serine/threonine kinase activity, ATP binding (associated with the CSNK1G3 gene), protein dephosphorylation, and phosphoprotein phosphatase activity (associated with the DUSP1L gene), and protein kinase activity (associated with the C5orf38 gene) were identified. Other relevant gene pathways included those related to 3'-5' exonuclease activity and nucleic acid phosphodiester bond hydrolysis (from an unknown gene). Finally, the Wnt activity pathway was represented by the biological process 'canonical Wnt signaling pathway' and the molecular function 'Wnt receptor activity,' which will be discussed further in this paper (see GO term in Figure 5.3 and more details in Appendix D, Table D.2).

## 5.4. DISCUSSION

Our data suggest genomic adaptation to pH at microgeographical scales in *A. lixula*, despite the existence of high levels of gene flow due to the high dispersal capacity of its larvae (Pedrotti, 1993; Wangensteen et al., 2013; Pérez-Portela et al., 2019).

Adaptation to divergent environmental conditions has been observed in several marine invertebrate species (e.g., Pespeni et al., 2013; Bitter et al., 2019; Teixidó et. al. 2020, among others), including sea urchins along the European coast (Carreras et al., 2020; 2021). In the sea urchin *Paracentrotus lividus*, local adaptation to maximum salinity and maximum temperature was identified as a driver of divergence at the Atlantic-Mediterranean transition, while Mediterranean populations displayed specific adaptation to low temperatures (Carreras et al., 2020). Similarly, in *A. lixula*, salinity has also exerted significant selective pressure on certain genes, leading to population sub-structuring along the Atlantic-Mediterranean area (Carreras et al., 2021), despite high levels of gene flow indicated by neutral markers (Wangensteen et al., 2012; Pérez-Portela et al., 2019; Carreras et al., 2021). This pattern of local adaptation in *A. lixula* may contribute to the species' adaptive potential over its large geographical range, complemented by transcriptomic plasticity in response to abrupt temperature changes (Pérez-Portela et al., 2020). Hence, previous research in *A. lixula* has highlighted its genomic and phenotypic plasticity in response to environmental shifts.

According to the general plasticity previously observed in *A. lixula*, our current study reveals that this sea urchin can rapidly adapt to pH changes along the natural pH gradient found in Fuencaliente (La Palma). We have identified significant effects of pH changes on specific genes in *A. lixula*, resulting in differential allele frequencies in certain genomic markers and genomic sub-structuring of its populations, even when these populations are located just 100-200 meters apart in the $CO_2$ vent area. Additionally, we have detected reduced values of genomic diversity in all sites analyzed, including the control site. The observed and expected heterozygosity values (Ho and He, respectively) from SNPs were considerably lower than those reported in previous genetic studies of *A. lixula*. For instance, Pérez-Portela et al. (2019) reported means of 0.627 for Ho and 0.861 for He, using microsatellites, while Carreras et al. (2021) found means of 0.379 for Ho and 0.441 for He for SNPs. In our study, the means were 0.137 for Ho and 0.159 for He for the entire dataset. Consistent with our findings, lower-than-expected levels of heterozygosity and significant positive values of $F_{IS}$ were measured in all. Our data provide further evidence that populations of *A. lixula* do not conform to Hardy-Weinberg equilibrium, as previously reported in this species (Pérez-Portela et al., 2019; 2020; Carreras et al., 2021), which is a common pattern in marine invertebrates (Olsen et al., 2020). However, the vent area exhibited lower levels of genomic diversity compared to other areas within the species' distribution range, something that contrast with the high levels of diversity found for the mitochondrial gene cytochrome oxidase one I (COI) analyzed in the Chapter 4 of this thesis.

Regarding the genomic structure of the populations, the diversity of bioinformatic analyses applied, based on genomic distances, Bayesian clustering, or DAPC, showed contrasting results between the neutral SNPs and the candidate SNPs databases. For the neutral SNPs, we have observed a general homogeneity among sampling sites exposed to different pH values. However, for the candidate SNPs, significant genomic structure and greater divergence were detected among sites, indicating substructure. The frequencies of genomic clusters showed a gradual shift along the pH gradient. The substructure observed in the candidate SNPs dataset suggests that pH selection is the main driver of this divergence, rather than geographical clustering or isolation by distance. This is supported by the fact that two of the sampling points, the 'Control' site (pH=8.2) and 'Transition 2' (pH=7.9), were located further from the main $CO_2$ emission than the other sites. The substructure obtained from the candidate SNPs exists despite the evidence of high levels of gene flow indicated by neutral markers, coherent with the high dispersal potential of the *A. lixula* planktotrophic larvae, which can disperse thousands of kilometers (Wangensteen et al., 2013; Pérez-Portela et al., 2019; Carreras et al., 2021).

Although the reduced number of genes under selection for pH in *A. lixula* that could be annotated in this study, we have identified relevant GO terms associated with these genes, particularly related with growth, development, and calcification, among other functions. Our results are also in line with previous studies that have identified genes associated with skeletal growth and biomineralization in sea urchins under acidic conditions (e.g., Pespeni et al., 2013a, b; Padilla-Gamiño et al., 2013; Devens et al., 2020; Di Giglio et al., 2020). Decreasing pH generates difficulties in acid-base regulation and maintenance of ionic homeostasis in sea urchins (Calosi et al., 2013a; Byrne and Hernández, 2020). This may explain why most of the functions identified in our study are related to cell communication and cellular membranes, two crucial components for maintaining homeostasis, together with genes involved in different metabolic pathways that may be linked to the energetic demands associated with the stress responses. Similar findings have been reported in other calcifying invertebrates such a bivalves or corals (Bitter et al., 2019; Teixidó et al., 2020) and vertebrates (Petit-Marty et al., 2021) under acidification conditions. The allele frequency changes due to selection that we found in *A. lixula* may have phenotypic effects, as individuals living in the

Fuencaliente vent under the lowest pH conditions are significantly smaller in size and have a more robust skeleton than those living at control pH conditions (Hernández et al., 2018; Sosa, 2021 and authors unpublished data).

Therefore, our findings are consistent with other observations regarding the strong selective effect of pH on calcifying invertebrates, both in experimental conditions (e.g., Pespeni et al., 2013a; Padilla-Gamiño et al., 2019; Devens et al., 2020; Strader et al., 2020) and natural pH gradients (e.g., Pespeni et al., 2013b; Bitter et al., 2019; Uthicke et al., 2019; Teixidó et al., 2020; Petit-Marty et al., 2021). Pespeni et al. (2013a) observed strong selective effect of pH in the sea urchin *Strongylocentrotus purpuratus*, with changes in allelic frequencies in hundreds of genes related to various functional classes of proteins, after only a few generations in laboratory experiments. Furthermore, patterns of adaptation to local pH conditions were observed in natural populations of the same species along the California coast (Pespeni et al., 2013b). In contrast, other invertebrate species showed lower adaptation potential along natural $CO_2$ vents, such as those in Papua New Guinea (Uthicke et al., 2019) and Ischia (Teixidó et al., 2020). For instance, the effects of pH decrease on the population structure of the coral *Astroides calycularis* in the Ischia $CO_2$ vent (Italy) was not clear, as significant high $F_{ST}$ values were recorded between sites under different pH values but also among control populations (Teixidó et al., 2020). This study is an example of the challenges in identifying local adaptation patterns in species with very limited dispersal potential, as the genomic structure resulting from isolation by distance intertwines with adaptation, making it difficult to distinguish the genomic patterns shaped by both forces separately. In our study we have eliminated the confounding factor of geographic isolation by examining a species with a broad dispersal potential. This emphasize that the adaptive capacity may rely on the species itself rather than in its dispersal capability. It is also interesting to demonstrate that corals and sea urchins, both calcareous and *a priory* harmed by OA, show opposite genetic adaptation strategies. Our study has shown the large adaptability and plasticity of *A. lixula* to OA but compared to the coral *A. calycularis* (Teixidó et al., 2020). This great adaptability, along with the foreseeable population increase expected due to ocean warming (e.g., Pérez-Portela et al., 2020; Wangensteen et al., 2013; Visconti et al., 2017), highlights the resilience of *A. lixula*.

## DATA AVAILABILITY

The raw sequencing data fastq files for this study are publicly available from the Sequence Read Archive database SRA-GenBank (Bioproject PRJNA948020). The file containing all samples with their corresponding environmental variables and the genpop file are available on Mendeley Data: doi:10.17632/9phf69mt8d.1. The R analysis scripts are available in Github repository: https://github.com/Sgonzalezdelgado/Arbacia2bRadCO2Vent.

# CONCLUSIONS

## CONCLUSIONES

# CONCLUSIONS

## *Conclusion 1*

There are around 30 locations in the world that are considered 'natural laboratories' for studying the effects of ocean acidification (OA). Most of these locations correspond to $CO_2$ vents or seeps systems of volcanic origin, located in the Mediterranean Sea and the Pacific Ocean. Most negative and positive responses observed in marine organisms in these natural systems are consistent with the results obtained from experiments, although there are some exceptions, such as the adaptation of algae of the genus *Peyssonelia* without compromising their calcareous structure, or the tolerance of some species of sea urchins, bivalves, and gastropods who can regulate their metabolism and calcification. Although they are not perfect analogues of future conditions, the acidified natural systems have allowed researchers to go further in the study of OA, observing possible implications for marine ecosystem functions, long-term changes, and adaptations of organisms, as well as indirect effects such as species interactions due to resource reallocation in the benthic communities.

## *Conclusion 2*

The first comprehensive physicochemical description of the naturally acidified system on the southern coast of La Palma, known as Punta de Fuencaliente system (PFS), is provided. The acidification process in the PFS is influenced by the discharge of brackish water affected by magmatic $CO_2$ gas emissions associated with volcanic activity. These discharges of acidic and brackish waters in the PFS, especially in Playa del Faro and Los Porretos, create a natural pH gradient that resembles future ocean conditions, making them valuable natural laboratories for predicting the effects of ocean acidification on future marine ecosystems. In addition, the Echentive lagoons, where these chemical alterations intensify, allow us to understand how life has persisted during periods of higher atmospheric $CO_2$ levels on Earth, offering a window into understanding early life forms and their adaptation to changing carbonate conditions over time.

*Conclusion 3*

The technique of metabarcoding introduces a new perspective on the effects of ocean acidification on subtropical marine benthic communities. Taxonomic detection at a fine scale using metabarcoding has revealed high levels of diversity of algae and metazoans in areas with intermediate acidification (pH around 7.8 units) in the PFS, which is due to the detection of small organisms and cryptic species living under this natural acidification.

*Conclusion 4*

In the acidic zones of the PFS, the composition of algal communities changes, with fast-growing algae without calcareous structures and small-sized algae such as turf and epiphytic algae becoming predominant. These changes in algal communities indirectly cause changes in faunal composition, either due to increased food availability or suitable habitat substrates, being the meiofauna species the dominant over macrofauna. Some of these species tolerate acidification despite having calcified skeletons or shells, such as small gastropods and echinoderms. These findings suggest that future subtropical ecosystems affected by ocean acidification may show a tendency towards miniaturization, dominated by small species of carpet and epiphytic algae and their associated small acid-tolerant invertebrates.

*Conclusion 5*

This thesis reveals for the first time that the Intermediate Disturbance Hypothesis (IDH) applies to acidified ecosystems at the organism and community levels, considering natural $CO_2$ emissions as the disturbance. There is an increase in species diversity of algae and invertebrates at sites in the PFS that experience moderate fluctuations of pH. An increase in the genetic diversity of populations of the sea urchins *Arbacia lixula* and *Paracentrotrus lividus* has also been detected at these sites with intermediate disturbances. It is concluded that the natural disturbance resulting from physicochemical processes occurring on the coasts of La Palma prevents organisms and the community from reaching a climax state. Therefore, at moderate levels of disturbance, diversity at both the organism and community levels increases before declining under intense acidification.

### *Conclusion 6*

The study of population genomics of *A. lixula* in the PFS has revealed that this species of sea urchin has the genomic capacity to adapt to changes in pH, despite high gene flow facilitated by the dispersal ability of its larvae. The genomic diversity of its populations is reduced in all analyzed sites, including the control site when compared to previous studies. While neutral SNPs generally show homogeneity among sampling sites exposed to different pH values, candidate SNPs under selection exhibit significant genomic structure and higher divergence, indicating pH-driven substructuring rather than geographical clustering or isolation by distance. This genomic substructure of populations, which exists even at short distances of 100-200 meters in areas with $CO_2$ emissions, results from significant changes in allele frequencies on specific genes of *A. lixula*. Some of these genes are associated with growth, development, and calcification functions, among others, indicating their involvement in the adaptation process. The adaptability and plasticity of *A. lixula* to ocean acidification, along with the projected population increase due to ocean warming, indicate its potential to succeed in future oceanic conditions.

## CONCLUSIONES

### *Conclusión 1*

Existen alrededor de 30 lugares en el mundo que se consideran ‘laboratorios naturales’ para el estudio de los efectos de la acidificación de los océanos (OA). La mayoría se corresponden con sistemas de afloramientos de $CO_2$ de origen volcánico, localizados en el Mar Mediterráneo y el Océano Pacífico. La mayoría de las respuestas negativas y positivas de los organismos marinos observadas en estos sistemas naturales van a acorde con los resultados conseguidos con los experimentos, aunque hay algunas excepciones como la adaptación de las algas del género *Peyssonelia* sin comprometer su estructura calcárea, o la tolerancia de algunas especies de erizos de mar, bivalvos y gasterópodos que tienen la capacidad de regular su metabolismo y la calcificación. A pesar de que no son análogos perfectos a las condiciones futuras, gracias a los sistemas naturales acidificados, se ha conseguido dar un paso más allá en las investigaciones de la AO, observando las posibles implicaciones en las funciones de los ecosistema marinos, lo cambios y las adaptaciones de los organismos a largo plazo, así como los efectos indirectos como podría ser las interacciones entre especies debido a la reasignación de recursos en las comunidades bentónicas.

### *Conclusión 2*

Se proporciona la primera descripción fisicoquímica exhaustiva del sistema natural acidificado de la costa sur de La Palma, conocido como sistema de Punta de Fuencaliente (PFS). El proceso de acidificación en el PFS está influido por la descarga de agua salobre afectadas por las emisiones magmáticas de gas $CO_2$ asociadas a la actividad volcánica. Estas descargas de aguas ácidas y salobres en el PFS, especialmente en la Playa del Faro y Los Porretos, crean un gradiente natural de pH que se asemeja a las condiciones futuras del océano, lo que los convierte en valiosos laboratorios naturales para predecir los efectos de la acidificación oceánica de los futuros ecosistemas marinos. Además, las lagunas Echentive, donde se intensifican estas alteraciones químicas, permiten comprender cómo ha persistido la vida durante los periodos de mayores niveles de $CO_2$ atmosférico en la Tierra, ofreciendo una ventana para entender las primeras formas de vida y su adaptación a las cambiantes condiciones carbonatadas a lo largo del tiempo.

### *Conclusión 3*

Gracias a la técnica de metabarcoding, se introduce una nueva perspectiva sobre los efectos de la acidificación oceánica en las comunidades bentónicas marinas subtropicales. La detección taxonómica a escala fina mediante metabarcoding, revela altos niveles de diversidad de algas y metazoos en las zonas con una acidificación intermedia (pH alrededor de 7.8 unidades) en el PFS, que se deben a la detección de pequeños organismos y especies crípticas que viven bajo esta acidificación natural.

*Conclusión 4*

En las zonas ácidas del PFS, la composición de las comunidades de algas cambia predominando aquellas de rápido crecimiento, sin estructuras calcáreas y de pequeño tamaño como las algas cespitosas y epífitas. Estos cambios de las comunidades de algas son los que ocasionan los cambios en la composición faunística de forma indirecta ya sea por un aporte mayor de alimento o sustrato habitable, siendo las especies de la meiofauna dominantes por encima de la macrofauna. Algunas de estas especies toleran la acidificación a pesar de poseer esqueletos calcificados o conchas como los pequeños gasterópodos y equinodermos. Estos hallazgos sugieren que los futuros ecosistemas subtropicales afectados por la acidificación de los océanos pueden mostrar una tendencia a la miniaturización, dominada por pequeñas especies de algas tapizantes y epífitas y sus pequeños invertebrados asociados tolerantes a la acidificación.

*Conclusión 5*

En esta tesis se revela por primera vez que la Hipótesis de la Perturbación Intermedia (HPI) es aplicable a ecosistemas acidificados a nivel de organismo y de comunidad, considerando las emisiones naturales de $CO_2$ como la perturbación. Existe un incremento de la diversidad de especies de algas e invertebrados en los sitios del PFS que tienen unos niveles moderados de fluctuaciones de pH. También se detecta un aumento de la diversidad genética de las poblaciones de los erizos de mar *Arbacia lixula* y *Paracentrotrus lividus*, en estos sitios con perturbaciones intermedias. Se concluye que la perturbación natural resultante de los procesos fisicoquímicos que ocurren en las costas palmeras impide que los organismos y la comunidad alcancen un estado de clímax y por ello, a niveles medios de perturbación, la diversidad tanto a nivel de organismos como de toda la comunidad se incrementa antes de empezar a declinar bajo una intensa acidificación.

*Conclusión 6*

El estudio de la genómica poblacional de *A. lixula* realizado en PFS revela que esta especie de erizo de mar tiene la capacidad genómica de adaptarse a los cambios de pH, a pesar del alto flujo génico facilitado por la capacidad de dispersión de sus larvas. La diversidad genómica de sus poblaciones está reducida en todos los sitios analizados, incluido el sitio de control al comparar con trabajos anteriores. Mientras que los SNP neutrales muestran en general una homogeneidad entre los sitios de muestreo expuestos a diferentes valores de pH, los SNP candidatos bajo selección encontrados muestran una estructura genómica significativa y una mayor divergencia, lo que indica una subestructuración impulsada por la selección de pH, en lugar de agrupamiento geográfico o aislamiento por distancia. Esta subestructura genómica de las poblaciones que existe incluso en distancias cortas de 100-200 metros en las zonas donde existe emisiones de $CO_2$, son el resultado de cambios en la frecuencia alélica en genes específicos de *A. lixula*. Algunos de estos genes están asociados a funciones de crecimiento, desarrollo y calcificación, entre otras, indicando su participación en el proceso de adaptación. La adaptabilidad y plasticidad de *A. lixula* a la acidificación oceánica, junto con el aumento de la población previsto debido al calentamiento del océano, indican su potencial para prosperar en las futuras condiciones oceánicas.

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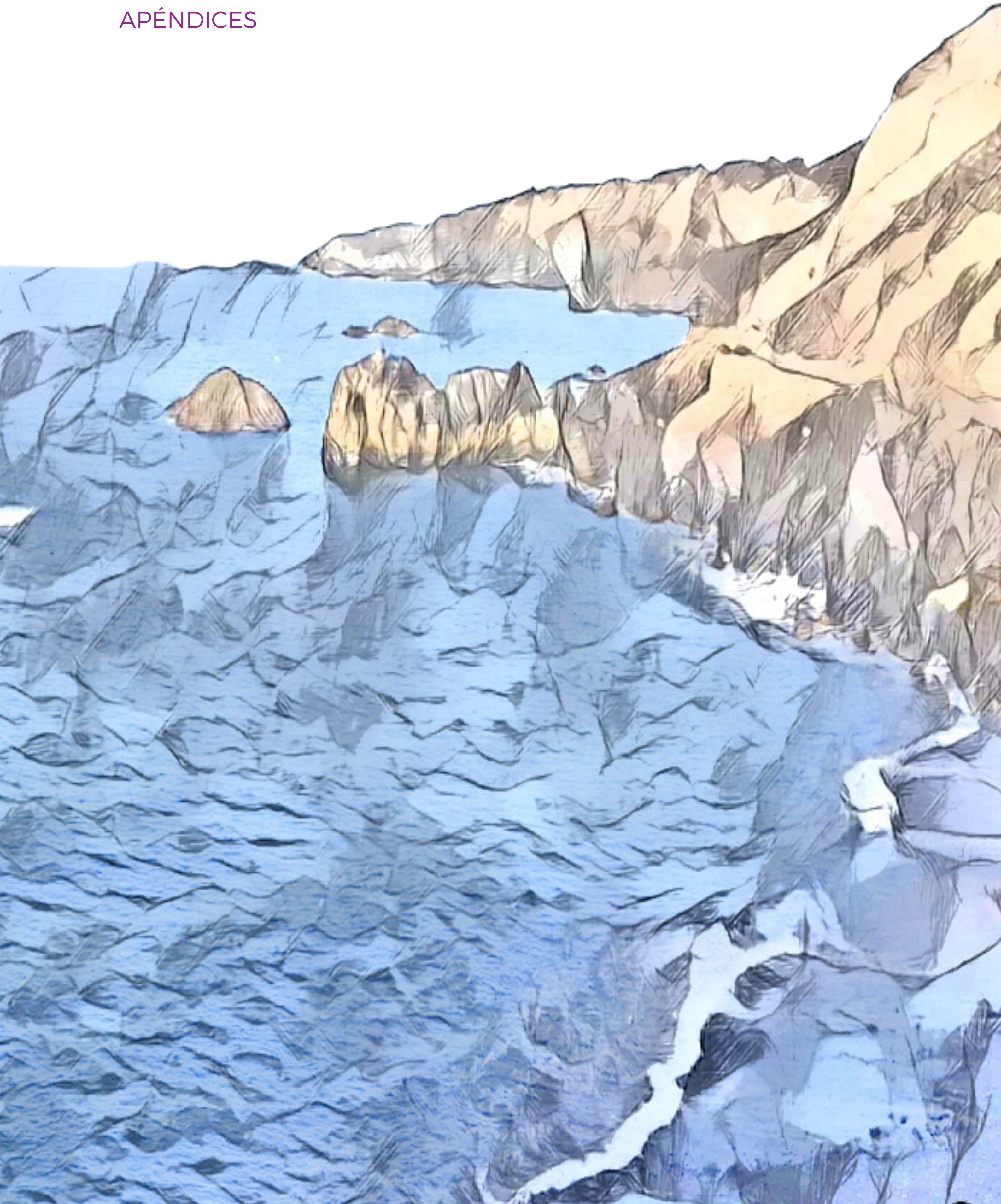
# APPENDICES

## APÉNDICES



# APPENDIX A. Chapter 1

**Table A.1.** List of the publications used for Figure 1.3.

| | |
|---|---|
| *Abundance and diversity* | |
| 2008 | Hall-Spencer et al., 2008, Manzello et al., 2008 and Martin et al., 2008, |
| 2010 | Cigliano et al., 2010, Dias et al., 2010, Gambi et al., 2010 and Thomsen et al., 2010. |
| 2011 | Fabricius et al., 2011, Kroeker et al., 2011 and Porzio et al., 2011. |
| 2012 | Crook et al., 2012, Johnson et al., 2012, Lidbury et al., 2012, Meron et al., 2012, Ricevuto et al., 2012 and Suggett et al., 2012. |
| 2013 | Calosi et al., 2013b, Inoue et al., 2013, Johnson et al., 2013, Pettit et al., 2013, Porzio et al., 2013, Russell et al., 2013, Thomsen et al., 2013 and Uthicke et al., 2013. |
| 2014 | Apostolaki et al., 2014, Baggini et al., 2014, Bray et al., 2014, Fabricius et al., 2014, Garrard et al., 2014, Giangrande et al., 2014, Goffredo et al., 2014, Goodwin et al., 2014, Kerfahi et al., 2014, Manzello et al., 2014, Morrow et al., 2015, Ricevuto et al., 2014, Shamberger et al., 2014, Taylor et al., 2014 and Ziveri et al., 2014. |
| 2015 | Baggini et al., 2015, Barkley et al., 2015, Burrell et al., 2015, Campoy, 2015, Chauhan et al., 2015, Di Cioccio et al., 2015, Fabricius et al., 2015, Hall-Spencer & Allen, 2015, Johnson et al., 2015, Linares et al., 2015, Lucey et al., 2015, Nagelkerken et al., 2016, Newcomb et al., 2015 and Raulf et al., 2015. |
| 2016 | Enochs et al., 2016, Gambi et al., 2016, Golbuu et al., 2016, Januar et al., 2016, Lucey et al., 2016, Rogelja et al., 2016, Smith et al., 2016b and Takahashi et al., 2016. |
| 2017 | Connell et al., 2017, Cornwall et al., 2017, Guilini et al., 2017, Hassenrück et al., 2017, Januar et al., 2017, Marcelino et al., 2017, Nogueira et al., 2017, Prada et al., 2017, and Smith et al., 2017. |
| *Physiological processes* | |
| 2010 | Couto et al., 2010 and Rodolfo-Metalpa et al., 2010. |
| 2011 | Lombardi et al., 2011a,b,c and Rodolfo-Metalpa et al., 2011. |
| 2012 | Arnold et al., 2012, Hahn et al., 2012, Meron et al., 2012, Suggett et al., 2012 and Uthicke & Fabricius, 2012. |
| 2013 | Calosi et al., 2013a, Crook et al., 2013, Inoue et al., 2013, Johnson et al., 2013, Meron et al., 2013 and Vizzini et al., 2013. |
| 2014 | Borell et al., 2014, Bray et al., 2014, Brinkman, 2014, Fabricius et al., 2014, Horwitz et al., 2014, Goffredo et al., 2014, Langer et al., 2014 and Milazzo et al., 2014. |
| 2015 | Basso et al., 2015, Brinkman & Smith, 2015, Celis-plá et al., 2015, Fantazzini et al., 2015, Garilli et al., 2015, Johnson et al., 2015, Lauritano et al., 2015, Lombardi et al., 2015, Newcomb et al., 2015, Ricevuto et al., 2015a, Small et al., 2015, Strahl et al., 2015, Turner et al., 2016, Vogel et al., 2015. |
| 2016 | Collard et al., 2016, Enochs et al., 2016, Harvey et al., 2016, Januar et al., 2016, Kamenos et al., 2016, Noona & Fabricius, 2016, Ricevuto et al., 2016, Takahashi et al., 2016, Turner et al., 2016, Uthicke et al., 2016, Ventura et al., 2016 and Wall et al., 2016. |
| 2017 | Cornwall et al., 2017, Duquette et al., 2017, Guilini et al., 2017, Kumar et al., 2017a,b, Mirasole et al., 2017, Olivé et al., 2017, Porzio et al., 2017, Prada et al., 2017 and Ravaglioli et al., 2017. |
| *Ecological interactions* | |
| 2011 | Fabricius et al., 2011 and Porzio et al., 2011. |
| 2012 | Arnold et al., 2012, Kroeker et al., 2012 and Suggett et al., 2012. |
| 2013 | Calosi et al., 2013b, Inoue et al., 2013, Kroeker et al., 2013a,b and Porzio et al., 2013. |
| 2014 | Donnaruma et al., 2014, Munday et al., 2014. |
| 2015 | Baggini et al., 2015, Celis-plá et al., 2015, Enochs et al., 2015, Fabricius et al., 2015, Linares et al., 2015, Nagelkerken et al., 2016, Raulf et al., 2015, Ricevuto et al., 2015b and Zupo et al., 2015. |
| 2016 | Allen et al., 2016, Crook et al., 2016, Rossi et al., 2016a,b and Smith et al., 2016a. |
| 2017 | Brown et al., 2017, Fabricius et al., 2017, Nagelkerken et al., 2017, Nogueira et al., 2017, Scartazza et al., 2017, and Vizzini et al., 2017. |
| *Early life stages and reproduction* | |
| 2014 | Munday et al., 2014 |
| 2015 | Campoy, 2015, Lucey et al., 2015 and Valvassori et al., 2015. |
| 2016 | Lamare et al., 2016 and Milazzo et al., 2016. |
| 2017 | Smith et al., 2017 and Wäge et al., 2017. |

## APPENDIX B. Chapter 2

**Table B.1.** Summary of the sampling methodology, with the locations sampled ('Sites'), the date of each sampling ('Date'), whether the sampling was done during the low (LT) or high (HT) tide and whether the parameters measured ('Measures') were all (ALL) or only the pH (pH).

| Sites | Date | Tide | Nº | Measures |
|---|---|---|---|---|
| Playa del Faro | mar-18 | LT | 23 | All |
| Playa del Faro | dec-18 | HT | 17 | All |
| Playa del Faro | dec-18 | LT | 19 | All |
| Playa del Faro | jun-19 | HT | 11 | pH |
| Playa del Faro | jun-19 | LT | 11 | pH |
| Los Porretos | mar-18 | LT | 5 | All |
| Los Porretos | dec-18 | LT | 14 | All |
| Los Porretos | dec-18 | HT | 10 | All |
| Echentive lagoon 1 | mar-18 | LT | 8 | All |
| Echentive lagoon 1 | dec-18 | LT | 10 | All |
| Echentive lagoon 1 | dec-18 | HT | 10 | All |
| Echentive lagoon 1 | jun-19 | LT | 6 | pH |
| Echentive lagoon 2 | dec-18 | LT | 6 | pH |
| Echentive lagoon 2 | dec-18 | HT | 6 | pH |

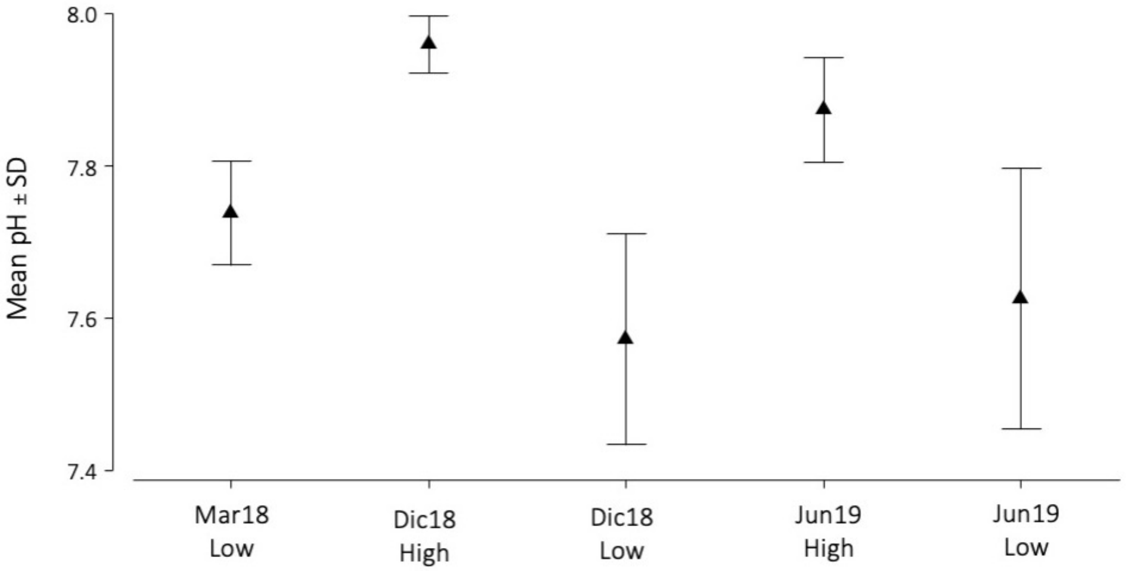


**Figure B.1.** Graph representing the tidal fluctuation (Low and High tide) of the mean pH with standard deviation (SD) at Playa del Faro, during March 2018 (Mar18), December 2018 (Dec18) and June 2019 (Jun19).

## APPENDIX C. Chapter 3

**Table C.1.** Carbon system parameters obtained during low and high tides in the four zones '1-Vent', '2-Transition25', '3-Transition75', and '4-Control' in Punta de Fuencaliente. TA = Total Alkalinity (μmol/kg), Ω = Saturation state, cal = Calcite, arag = Aragonite.

| | Low Tide | | | | High tide | | | |
|---|---|---|---|---|---|---|---|---|
| | **pH** | **TA** | **Ω cal** | **Ω arag** | **pH** | **TA** | **Ω cal** | **Ω arag** |
| 1-Vent | 7.65±0.02 | 3057.69±24.5 | 2.84±0.11 | 1.85±0.07 | 8.05±0.04 | 2615.64±10.5 | 5.24±0.35 | 3.42±0.23 |
| 2-Transition25 | 7.81±0.05 | 2771.46±40 | 4.12±0.38 | 2.685±0.24 | 8.11±0.01 | 2546.63±7.5 | 5.78±0.11 | 3.77±0.07 |
| 3-Transition75 | 8.09±0.02 | 2578.52±12 | 5.64±0.08 | 3.675±0.06 | 8.14±0.01 | 2522.57±6 | 5.93±0.12 | 3.87±0.08 |
| 4-Control | 8.14±0.02 | 2498.28±15 | 5.92±0.15 | 3.87±0.09 | - | - | - | - |

**Table C.2.** Number of Rhodophyta (R), Ochrophyta (O), Chorophyta (C), total algae (TA) and Metazoan (M) MOTUs found at different $CO_2$ vent systems which were sampled with traditional methods; and the number of species found in the present work using metabarcoding.

| $CO_2$ Vents | Methods | R | O | C | TA | M | Sources |
|---|---|---|---|---|---|---|---|
| La Palma (Spain) | COI Metabarcoding | 143 | 43 | - | 186 | 636 | Present study |
| Columbretes (Spain) | Traditional | 47 | 22 | 10 | 79 | 33 | (1)* |
| Ischia (Italy) | Traditional | 71 | 16 | 15 | 101 | 82 | (2)* |
| Methana (Greek) | Traditional | 7 | 9 | 2 | 18 | - | (3)* |

*(1) Linares et al., 2015; (2) Kroeker, et al., 2011; (3) Baggini et al., 2014.

**Table C.3.** (A) Kruskal-Wallis test for all MOTUs, algae and its subgroups and metazoans and its subgroup species richness (number of species per sample) in the natural $CO_2$ gradient as factor '$CO_2$ Gradient' with 4 levels ('1-Vent', '2-Transition25', '3-Transition75' and '4-Control') in both fraction size (small fraction: organisms with size > 1 mm; and large fraction: organisms between 1 mm and 0.64 μm). (B) Results of medians comparisons between levels of the factor '$CO_2$ Gradient' of significant Kruskal-Wallis tests.

| A. Kruskal-Wallis | Large Fraction | | | Small Fraction | |
|---|---|---|---|---|---|
| | **df** | **Chi-squared** | **p** | **Chi-squared** | **p** |
| All MOTUs | 3 | 9.190 | 0.03* | 6.828 | 0.05* |
| Algae | 3 | 6.797 | 0.08 | 5.78 | 0.12 |
| Metazoans | 3 | 7.565 | 0.06 | 10.853 | 0.01* |
| Crustose | 3 | 5.847 | 0.12 | 11.484 | 0.01* |
| Epiphyte | 3 | 10.576 | 0.01* | 5.270 | 0.15 |
| Mixed turf | 3 | 4.811 | 0.19 | 5.797 | 0.12 |
| Erected Macrophyte | 3 | 2.837 | 0.42 | 8.133 | 0.04* |
| Macrofauna | 3 | 7.661 | 0.05* | 9.237 | 0.03* |
| Meiofauna | 3 | 10.846 | 0.01* | 9.179 | 0.03* |

| **B. Medians comparisons** | | | **1 - 2** | **1 - 3** | **1 - 4** | **2 - 3** | **2 - 4** | **3 - 4** |
|---|---|---|---|---|---|---|---|---|
| All MOTUs | A | t | 0.285 | 1.899 | 2.553 | 1.613 | 2.267 | 0.653 |
| | | p | 0.387 | 0.057 | 0.032* | 0.080 | 0.035* | 0.308 |
| | B | t | -2.511 | -0.735 | -0.755 | 1.776 | 1.755 | -0.020 |
| | | p | 0.036* | 0.277 | 0.337 | 0.113 | 0.079 | 0.491 |
| Metazoans | B | t | -3.184 | -0.898 | -1.143 | 2.286 | 2.041 | -0.245 |
| | | p | 0.004* | 0.221 | 0.190 | 0.033* | 0.041* | 0.403 |
| Crustose | A | t | -2.547 | -3.065 | -1.181 | -0.517 | 1.366 | 1.884 |
| | | p | 0.016* | 0.006* | 0.142 | 0.302 | 0.128 | 0.059 |
| Epiphyte | A | t | 1.593 | 2.738 | 2.860 | 1.144 | 1.266 | 0.122 |
| | | p | 0.111 | 0.009* | 0.012* | 0.151 | 0.153 | 0.451 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Erected Macrophytes | B | t | -2.688 | -0.554 | -0.862 | 2.134 | 1.826 | -0.307 |
| | | p | 0.021* | 0.347 | 0.291 | 0.049* | 0.067 | 0.379 |
| Macrofauna | A | t | -1.474 | -0.143 | 1.289 | 1.330 | 2.764 | 1.433 |
| | | p | 0.211 | 0.443 | 0.118 | 0.137 | 0.017* | 0.151 |
| | B | t | -2.705 | -2.475 | -1.188 | 0.286 | 1.516 | 1.229 |
| | | p | 0.021* | 0.023* | 0.141 | 0.387 | 0.129 | 0.164 |
| Meiofauna | A | t | 0.266 | 2.092 | 2.728 | 1.825 | 2.461 | 0.635 |
| | | p | 0.394 | 0.036* | 0.019* | 0.051 | 0.021* | 0.314 |
| | B | t | -0.926 | 1.502 | 1.646 | 2.428 | 2.572 | 0.144 |
| | | p | 0.212 | 0.099 | 0.099 | 0.022* | 0.030* | 0.442 |

**Table C.4.** (A) One-way permutational multivariate analyses of variance of all algae species data set in the natural $CO_2$ gradient as factor '$CO_2$ Gradient' with 4 levels ('1-Vent', '2-Transition25', '3-Transition75' and '4-Control'), and separated in 4 groups of vegetation ('crustose' (crus), 'epiphyte' (Epi), 'mixed turf' (Mtu) and 'erected macrophytes' (Erec) and two size fractions (small fraction: organisms with size > 1 mm; and large fraction: organisms between 1 mm and 0.64 µm). (B) Results of pairwise comparisons for levels of the factor '$CO_2$ Gradient'.

| A. Source of variation | | | Large Fraction | | | | Small Fraction | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | df | SS | MS | Pseudo-F | p | SS | MS | Pseudo-F | p |
| Crus | Gradient | 3 | 2.495 | 0.832 | 8.2913 | 0.001* | 1.7931 | 0.598 | 4.156 | 0.001* |
| | Residual | 20 | 2.006 | 0.100 | | | 2.8766 | 0.144 | | |
| Epi | Gradient | 3 | 1.492 | 0.497 | 4.887 | 0.001* | 1.381 | 0.460 | 4.095 | 0.001* |
| | Residual | 20 | 2.036 | 0.102 | | | 2.248 | 0.112 | | |
| Mtu | Gradient | 3 | 1.906 | 0.635 | 10.661 | 0.001* | 1.679 | 0.560 | 6.154 | 0.001* |
| | Residual | 20 | 1.192 | 0.060 | | | 1.819 | 0.091 | | |
| Erec | Gradient | 3 | 0.856 | 0.285 | 6.172 | 0.001* | 0.876 | 0.292 | 5.163 | 0.001* |
| | Residual | 20 | 0.924 | 0.046 | | | 1.131 | 0.057 | | |

| B. Groups | | | 1 - 2 | 1 - 3 | 1 - 4 | 2 - 3 | 2 - 4 | 3 - 4 |
|---|---|---|---|---|---|---|---|---|
| Crus | Large fraction | t | 5.097 | 4.508 | 7.535 | 0.641 | 4.499 | 2.955 |
| | | p | 0.005* | 0.005* | 0.005* | 0.703 | 0.005* | 0.011* |
| | Small Fraction | t | 2.512 | 3.477 | 3.843 | 0.452 | 1.286 | 1.394 |
| | | p | 0.061 | 0.010* | 0.028* | 0.908 | 0.350 | 0.331 |
| Epi | Large fraction | t | 6.133 | 4.940 | 4.310 | 3.495 | 1.837 | 2.139 |
| | | p | 0.004* | 0.004* | 0.004* | 0.004* | 0.058 | 0.051 |
| | Small Fraction | t | 2.738 | 3.395 | 2.758 | 2.336 | 2.160 | 1.790 |
| | | p | 0.005* | 0.007* | 0.005* | 0.009* | 0.005* | 0.030* |
| Mtu | Large fraction | t | 9.442 | 14.065 | 10.154 | 4.198 | 4.292 | 2.252 |
| | | p | 0.005* | 0.005* | 0.005* | 0.008* | 0.005* | 0.020* |
| | Small Fraction | t | 5.391 | 8.363 | 5.587 | 3.065 | 3.182 | 1.473 |
| | | p | 0.004* | 0.004* | 0.006* | 0.012* | 0.004* | 0.092 |
| Erec | Large fraction | t | 24.133 | 9.987 | 26.938 | 1.419 | 1.124 | 1.292 |
| | | p | 0.007* | 0.007* | 0.007* | 0.294 | 0.294 | 0.294 |
| | Small Fraction | t | 8.787 | 7.430 | 13.634 | 5.117 | 2.096 | 11.558 |
| | | p | 0.005* | 0.005* | 0.005* | 0.018* | 0.094 | 0.006* |

**Table C.5.** Result of SIMPER analyses showing the differences in average transformed abundances and contribution percentage ('Contribution %') of algae species of (A) Crustose, (B) Epiphyte, (C) Mixed Turf and (D) Erected macrophytes, compared the '$CO_2$ Gradient' ('1-Vent', '2-Transition25', '3-Transition75' and '4-Control'). Large fraction are organisms with size > 1mm and small fraction are organisms between 1mm and 0.64 µm.

| AVERAGE ABUNDANCES | | | | | CONTRIBUTION % | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **A. Crustose** | 1 | 2 | 3 | 4 | 1 & 2 | 1 & 3 | 1 & 4 | 2 & 3 | 2 & 4 | 3 & 4 |
| Large fraction | | | | | | | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Ralfsia sp.* | 68.12 | 0.00 | 0.00 | 0.00 | 13.54 | 13.68 | 15.74 | - | - | - |
| *Phymatolithon sp. 2* | 9.90 | 68.84 | 50.69 | 0.00 | 12.17 | 10.28 | - | - | 18.09 | 14.29 |
| *Lithophylum sp. 1* | 0.00 | 49.54 | 55.97 | 0.00 | 10.44 | 10.97 | - | - | 13.58 | 14.18 |
| *Peyssonnelia sp. 3* | 53.74 | 39.82 | 16.19 | 54.17 | 8.60 | 10.32 | 9.80 | - | 11.38 | 13.56 |
| *Peyssonnelia sp. 7* | 20.17 | 45.56 | 20.38 | 28.57 | 7.87 | 5.25 | 6.96 | - | 8.36 | 6.06 |
| *Lithophylum sp. 2* | 0.00 | 39.18 | 42.05 | 70.26 | 7.38 | 8.05 | 16.42 | - | 10.16 | 9.34 |
| *Pneophyllum sp.* | 0.00 | 35.63 | 38.90 | 49.01 | 7.00 | 7.51 | 11.57 | - | 6.36 | 5.97 |
| *Peyssonnelia sp. 1* | 31.96 | 9.68 | 5.72 | 0.00 | 6.48 | 6.57 | 7.58 | - | - | - |
| *Peyssonnelia sp. 4* | 28.41 | 9.52 | 30.25 | 0.00 | 5.69 | 5.81 | 6.49 | - | - | 7.37 |
| *Lithothamnion sp.* | 9.97 | 29.92 | 29.04 | 2.26 | 5.56 | 5.64 | - | - | 6.80 | 6.74 |
| *Padina gymnospora* | 13.39 | - | - | 19.11 | - | - | 6.07 | | | - |
| *Hildenbrandia sp. 2* | 21.80 | - | - | 0.00 | - | - | 5.00 | | | - |
| Small fraction | | | | | | | | | | |
| *Phymatolithon sp. 2* | 0.00 | 50.55 | 42.42 | 8.88 | - | 11.28 | - | - | - | - |
| *Lithophylum sp. 2* | 0.00 | 48.82 | 49.66 | 0.00 | - | 13.29 | - | - | - | - |
| *Lithothamnion sp.* | 0.00 | 51.55 | 16.74 | 6.82 | - | 3.96 | - | - | - | - |
| *Ralfsia sp.* | 51.54 | 14.80 | 12.55 | 4.94 | - | 12.68 | 17.22 | - | - | - |
| *Peyssonnelia sp. 3* | 69.05 | 42.84 | 38.29 | 41.38 | - | 12.27 | 17.04 | - | - | - |
| *Lithophylum sp. 1* | 23.16 | 34.17 | 63.00 | 79.31 | - | 13.09 | 21.91 | - | - | - |
| *Pneophyllum sp.* | 0.00 | 43.27 | 56.59 | 58.12 | - | 15.06 | 18.32 | - | - | - |
| *Peyssonnelia sp. 7* | 12.18 | - | 20.08 | 0.00 | - | 6.49 | - | - | - | - |
| *Padina gymnospora* | 6.14 | - | 0.00 | 17.05 | - | - | 5.43 | - | - | - |
| **B. Epiphyte** | **1** | **2** | **3** | **4** | **1 & 2** | **1 & 3** | **1 & 4** | **2 & 3** | **2 & 4** | **3 & 4** |
| Large fraction | | | | | | | | | | |
| *Ceramium virgatum* | 56.92 | 9.14 | 0.00 | 0.00 | 6.30 | 7.21 | 6.86 | - | - | - |
| *Platysiphonia sp.* | 18.69 | 50.75 | 19.75 | - | 5.31 | - | - | 7.39 | - | - |
| *Kallymeniaceae sp. 2* | 8.37 | - | 63.45 | 51.62 | - | 7.14 | 5.34 | - | - | - |
| *Heterosiphonia sp. 1* | 22.63 | 32.30 | 53.15 | | - | 5.05 | - | 6.38 | - | - |
| *Centroceras sp.1* | - | 63.98 | 33.52 | - | - | - | - | 5.68 | - | - |
| *Dasya sp. 2* | - | 31.44 | 11.02 | - | - | - | - | 5.19 | - | - |
| Small fraction | | | | | | | | | | |
| *Ceramium diaphanum* | 10.50 | 59.44 | 10.13 | 22.68 | 6.45 | - | - | 7.87 | 5.38 | - |
| *Ceramium virgatum* | 44.70 | 0.00 | 2.33 | 0.00 | 5.77 | 7.90 | 5.76 | - | - | - |
| *Platysiphonia sp.* | 8.17 | 43.90 | 12.37 | 9.20 | 5.05 | - | - | 6.92 | 5.32 | - |
| *Kallymeniaceae sp. 2* | 11.32 | 47.24 | 67.05 | - | 4.64 | 7.81 | - | - | - | - |
| *Heterosiphonia sp. 1* | - | 22.66 | 38.80 | 2.36 | - | - | - | 5.54 | - | 6.82 |
| *Dasya sp. 6* | - | - | 3.56 | 32.79 | - | - | - | - | - | 5.98 |
| *Centroceras sp.1* | - | - | 30.09 | 48.66 | - | - | - | - | - | 5.28 |
| **C. Mixed Turf** | **1** | **2** | **3** | **4** | **1 & 2** | **1 & 3** | **1 & 4** | **2 & 3** | **2 & 4** | **3 & 4** |
| Large fraction | | | | | | | | | | |
| *Palisada flagellifera* | 9.29 | 59.54 | 14.12 | 0.00 | 6.85 | - | - | 8.70 | 8.21 | - |
| *Laurencia obtusa* | 85.81 | 45.29 | 34.94 | 32.39 | 5.22 | 6.29 | 6.09 | - | - | - |
| *Jania sp. 2* | 1.01 | 42.80 | 61.54 | 55.07 | 5.21 | 6.97 | 6.14 | - | - | - |
| *Jania sp. 4* | 2.96 | - | 61.54 | 48.84 | - | 7.34 | 5.25 | - | - | - |
| *Corallina sp.* | 0.00 | - | 57.80 | 49.86 | - | 7.15 | 5.63 | - | - | - |
| *Gelidiella sp. 2* | 0.00 | 2.93 | 3.75 | 46.68 | - | - | 5.36 | - | 6.01 | 6.97 |
| *Palisada sp. 2* | - | 44.44 | 15.05 | 5.88 | - | - | - | 5.93 | 5.65 | - |
| *Hypnea sp.* | - | 37.66 | 0.00 | 0.00 | - | - | - | 5.89 | 5.00 | - |
| *Jania sp. 3* | - | 53.54 | 58.30 | 0.00 | - | - | - | - | 7.24 | 9.25 |
| *Ceratodictyon sp.* | - | - | 14.74 | 55.22 | - | - | - | - | - | 6.56 |
| Small fraction | | | | | | | | | | |
| *Jania sp. 2* | 13.62 | 66.86 | 67.88 | 3.89 | 7.39 | 7.34 | - | - | 9.07 | - |
| *Jania sp. 4* | 2.16 | 50.30 | 68.14 | 59.51 | 6.55 | 8.71 | 7.61 | - | - | - |
| *Jania sp. 5* | 7.58 | 47.57 | 65.26 | 48.11 | 5.47 | 7.59 | 5.28 | - | - | - |
| *Laurencia obtusa* | 76.11 | 48.54 | 26.29 | 23.34 | - | 6.76 | 6.88 | 5.73 | - | - |
| *Palisada sp. 2* | - | 38.56 | 8.86 | - | - | - | - | 6.48 | - | - |
| *Chondrophycus sp.* | - | 61.86 | 36.79 | 10.59 | - | - | - | 6.31 | 7.25 | - |
| *Amphiroa sp. 2* | - | 35.59 | 11.13 | 23.09 | - | - | - | 5.00 | 5.01 | - |
| *Ceratodictyon sp.* | - | 9.53 | - | 51.40 | - | - | - | - | 6.04 | - |
| **D. Erected macrophytes** | **1** | **2** | **3** | **4** | **1 & 2** | **1 & 3** | **1 & 4** | **2 & 3** | **2 & 4** | **3 & 4** |
| Large fraction | | | | | | | | | | |
| *Halopteris sp.* | 85.02 | 28.77 | 21.69 | 0.00 | 19.45 | 18.43 | 24.68 | - | - | - |
| *Canistrocarpus cervicornis* | 48.14 | 95.69 | 87.07 | 96.40 | 16.35 | 11.63 | 14.12 | - | - | - |
| *Dictyota fasciola* | 52.66 | 24.43 | 27.21 | 25.46 | 9.89 | 7.46 | 8.66 | - | - | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Dictyota ciliolata* | 0.00 | 22.47 | 26.66 | 22.52 | 7.41 | 7.64 | 6.29 | - | - | - |
| *Dictyota sp .4* | 37.71 | 37.31 | 25.54 | 9.52 | 6.62 | 6.51 | 8.20 | - | - | - |
| *Taonia sp.* | 19.97 | 0.00 | 0.00 | 0.00 | 6.50 | 5.52 | 5.52 | - | - | - |
| *Asparagopsis taxiformis* | 55.67 | 40.35 | - | 40.09 | 5.86 | - | 6.56 | - | - | - |
| *Dictyota ciliolata 2* | 31.24 | 23.01 | 36.05 | 41.90 | 5.35 | 6.99 | 5.88 | - | - | - |
| *Sargassum sp.* | 5.19 | - | 28.07 | - | - | 6.54 | - | - | - | - |
| *Lobophora canariensis* | 2.02 | - | 21.42 | - | - | 5.63 | - | - | - | - |
| *Taonia atomaria* | 25.03 | - | 8.70 | - | - | 5.37 | - | - | - | - |
| Small fraction | | | | | | | | | | |
| *Halopteris sp.* | 72.08 | 25.82 | 19.90 | 4.72 | 13.38 | 16.01 | 18.73 | 5.83 | - | 7.46 |
| *Canistrocarpus cervicornis* | 37.17 | 81.99 | 66.04 | 92.34 | 13.02 | 9.19 | 15.28 | 7.82 | - | 11.52 |
| *Dictyota sp. 4* | 25.93 | 44.37 | 18.81 | 12.89 | 10.62 | 8.87 | 7.22 | 12.94 | - | 8.65 |
| *Dictyota fasciola* | 58.62 | 25.32 | 25.05 | 27.91 | 9.89 | 11.63 | 9.39 | 7.51 | - | 8.76 |
| *Dictyota ciliolata* | 2.22 | 24.24 | 17.93 | 17.57 | 6.54 | 5.08 | - | 7.89 | - | 7.42 |
| *Asparagopsis taxiformis* | 66.96 | 71.23 | 89.51 | 57.16 | 6.34 | 7.42 | 7.52 | 7.66 | | 13.78 |
| *Taonia sp.* | 21.88 | 2.71 | 0.00 | 2.51 | 5.94 | 6.56 | 5.70 | - | - | - |
| *Taonia atomaria* | 27.14 | 22.72 | 17.67 | 7.26 | 5.76 | 6.50 | 6.71 | 5.88 | - | 6.60 |
| *Dictyota ciliolata 2* | 21.38 | 39.59 | 26.27 | 38.60 | 5.67 | - | 5.20 | 6.51 | - | 6.32 |
| *Sargassum sp.* | 0.00 | - | 18.52 | - | - | 5.70 | - | - | - | - |
| *Lobophora littlerorum* | - | 24.20 | 5.23 | - | - | - | - | 7.85 | - | - |
| *Dictyotalean sp.* | - | 17.42 | 9.63 | - | - | - | - | 8.78 | - | - |
| *Lobophora canariensis* | - | - | 24.85 | 20.62 | - | - | - | - | - | 5.98 |

**Table C.6.** (A) One-way permutational multivariate analyses of variance of all metazoan species data set in the natural $CO_2$ gradient as factor '$CO_2$ Gradient' with 4 levels ('1-Vent', '2-Transition25', '3-Transition75' and '4-Control') and separated in 3 groups ('Meiofauna' (Meio) and 'Macrofauna' (Macr), and two size fractions (small fraction: organisms with size > 1 mm; and large fraction: organisms between 1 mm and 0.64 µm). (B) Results of pairwise comparisons for levels of the factor '$CO_2$ Gradient'.

| A. Source of variation | | | Large Fraction | | | | Small Fraction | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | df | SS | MS | Pseudo-F | p | SS | MS | Pseudo-F | p |
| Meio | Gradient | 3 | 1.741 | 0.580 | 3.708 | 0.001* | 1.903 | 0.634 | 4.683 | 0.001* |
| | Residual | 20 | 3.131 | 0.157 | | | 2.710 | 0.135 | | |
| Macr | Gradient | 3 | 1.956 | 0.652 | 2.896 | 0.001* | 2.152 | 0.717 | 2.931 | 0.001* |
| | Residual | 20 | 4.503 | 0.225 | | | 4.895 | 0.245 | | |

| B. Groups | | | 1 - 2 | 1 - 3 | 1 - 4 | 2 - 3 | 2 - 4 | 3 - 4 |
|---|---|---|---|---|---|---|---|---|
| Meio | Large fraction | t | 6.501 | 5.101 | 4.921 | 0.838 | 2.641 | 1.359 |
| | | p | 0.002* | 0.002* | 0.003* | 0.621 | 0.007* | 0.232 |
| | Small Fraction | t | 6.210 | 4.850 | 4.727 | 1.014 | 5.099 | 3.516 |
| | | p | 0.002* | 0.002* | 0.002* | 0.423 | 0.002* | 0.002* |
| Macr | Large fraction | t | 4.494 | 2.271 | 2.677 | 1.739 | 2.095 | 1.542 |
| | | p | 0.003* | 0.004* | 0.003* | 0.046* | 0.011* | 0.052 |
| | Small Fraction | t | 3.628 | 2.772 | 2.944 | 1.348 | 1.690 | 1.129 |
| | | p | 0.003* | 0.011* | 0.001* | 0.027* | 0.002* | 0.235 |

**Table C.7.** Result of SIMPER analyses showing the differences in average transformed abundances and contribution percentage ('Contribution %') of metazoan species of (A) Meiofauna and (B) Macrofauna, compared the '$CO_2$ Gradient' ('1-Vent', '2-Transition25', '3-Transition75' and '4-Control'). Large fraction are organisms with size > 1mm and small fraction are organisms between 1mm and 0.64 µm.

| AVERAGE ABUNDANCES | CONTRIBUTION % |
|---|---|

| **A. Meiofauna** | **1** | **2** | **3** | **4** | **1 & 2** | **1 & 3** | **1 & 4** | **2 & 3** | **2 & 4** | **3 & 4** |
|---|---|---|---|---|---|---|---|---|---|---|
| Large fraction | | | | | | | | | | |
| *Lumbrineris perkinsi* | 0.00 | 13.97 | - | - | 5.67 | - | - | - | - | - |
| *Chondrochelia dubia* | 31.24 | 12.81 | - | - | 2.15 | - | - | - | - | - |
| *Caprella acanthifera* | 58.39 | 23.93 | 16.03 | 43.84 | 2.11 | 2.77 | 2.14 | - | 2.72 | - |
| *Dexamine spinosa 4* | 25.17 | 43.76 | - | 27.50 | 1.90 | - | - | - | 2.40 | - |
| *Ampithoe ramondi* | 37.81 | 0.00 | 0.00 | - | 1.66 | 2.58 | | - | - | - |
| *Timarete cf. punctata* | 80.43 | 4.61 | 12.74 | 7.81 | 1.43 | 1.42 | 1.73 | - | - | - |
| *Dorvillea sp.* | 0.00 | 23.37 | - | 11.44 | 1.35 | - | - | - | 5.83 | - |
| *Polynoe sp.* | 43.77 | 70.87 | - | 8.13 | 1.17 | - | - | - | 2.10 | - |
| *Corophium volutator* | 11.50 | - | 0.00 | - | - | 5.57 | - | - | - | - |
| *Podocerus variegatus* | 6.55 | 29.85 | 23.57 | 40.14 | - | 1.97 | 1.73 | - | 1.17 | - |
| *Capitella cf. capitata* | 18.65 | - | 0.00 | 0.00 | - | 1.51 | 1.61 | - | - | - |
| *Naineris cf. laevigata* | 46.16 | 43.10 | 31.26 | 7.03 | - | 1.04 | | - | 2.61 | - |
| *Ototyphlonemertes (Macintoshi) macintoshi* | 4.93 | - | - | 10.42 | - | | 6.45 | - | - | - |
| *Dexamine spinosa 3* | 12.54 | - | - | 3.85 | - | - | 3.46 | - | - | - |
| *Eunice gagzoi* | 24.39 | 22.28 | - | 38.67 | - | - | 3.43 | - | 3.75 | - |
| *Amphipholis cf. squamata 1* | 32.54 | 40.87 | - | 3.23 | - | - | 3.24 | - | 2.64 | - |
| *Cephalothrix simula* | 36.61 | - | - | 0.00 | - | - | 1.12 | - | - | - |
| *Amphipholis cf. squamata 3* | - | 4.31 | - | 15.34 | - | - | - | - | 3.40 | - |
| *Eurythoe cf. complanata* | - | 33.19 | - | 28.82 | - | - | - | - | 1.03 | - |
| Small fraction | | | | | | | | | | |
| *Tetrastemma cf. coronatum* | 0.00 | 18.62 | - | 17.53 | 2.44 | - | 1.85 | - | - | - |
| *Leodice harassii* | 1.41 | 11.68 | - | - | 1.69 | - | - | - | - | - |
| *Desmodora sp.* | 12.66 | 14.57 | - | - | 1.36 | - | - | - | - | - |
| *Caprella acanthifera* | 68.97 | 26.62 | 3.21 | - | 1.34 | 1.07 | - | - | - | - |
| *Cephalothrix rufifrons* | 14.98 | 52.41 | 28.26 | 19.35 | - | 2.88 | - | - | 2.01 | 1.90 |
| *Eunice gagzoi* | 8.52 | - | 11.51 | 25.81 | - | 1.96 | 1.52 | - | - | - |
| *Lumbrineris perkinsi* | 0.00 | - | 10.24 | - | - | 1.85 | - | - | - | - |
| *Timarete cf. punctata* | 69.19 | - | 4.30 | 6.75 | - | 1.79 | 3.15 | - | - | - |
| *Ampithoe ramondi* | 23.95 | - | - | 0.00 | - | - | 2.70 | - | - | - |
| *Eurythoe cf. complanata* | 8.23 | - | - | 43.08 | - | - | 2.10 | - | - | - |
| *Tetrastemma sp.* | 0.00 | 4.11 | 5.05 | 50.00 | - | - | 1.49 | - | 1.69 | 1.91 |
| *Dexamine spinosa 2* | 21.72 | 20.60 | - | 0.00 | - | - | 1.17 | - | 1.12 | - |
| *Ampithoe cf. helleri* | - | 24.60 | - | 0.00 | - | - | - | - | 4.98 | - |
| *Polynoe sp.* | - | 79.35 | 72.85 | 15.97 | - | - | - | - | 2.75 | 3.03 |
| *Baseodiscus cf. delineatus* | - | 12.20 | - | 0.00 | - | - | - | - | 2.43 | - |
| *Ototyphlonemertes (Macintoshi) macintoshi* | - | 13.02 | - | 28.82 | - | - | - | - | 1.54 | - |
| *Dorvillea sp.* | - | - | 18.76 | 20.12 | - | - | - | - | - | 5.25 |
| *Polycirrus denticulatus* | - | - | 11.60 | 0.00 | - | - | - | - | - | 2.86 |
| *Amphipholis cf. squamata 3* | - | - | 3.13 | 16.36 | - | - | - | - | - | 1.32 |
| **B. Macrofauna** | **1** | **2** | **3** | **4** | **1 & 2** | **1 & 3** | **1 & 4** | **2 & 3** | **2 & 4** | **3 & 4** |
| Large fraction | | | | | | | | | | |
| *Calcinus tubularis* | 14.52 | 54.69 | - | - | 2.89 | - | - | - | - | - |
| *Hippolyte cf. leptocerus* | 31.64 | 3.40 | 0.00 | 4.59 | 2.55 | 1.16 | 2.05 | - | - | - |
| *Hoploplana sp.* | 18.76 | 6.60 | - | 8.51 | 2.44 | - | 1.01 | - | - | - |
| *Mannesia sabadiega* | 4.31 | 31.22 | - | 0.00 | 1.80 | - | - | - | 2.66 | - |
| *Acanthonyx lunulatus 1* | 22.61 | 50.28 | 31.38 | 15.86 | 1.63 | 2.00 | - | 1.65 | 2.59 | - |
| *Tedania (Tedania) cf. ignis* | 22.05 | 9.79 | - | 0.00 | 1.46 | - | 1.45 | - | - | - |
| *Xantho hydrophilus* | 9.75 | 47.96 | 35.31 | - | 1.06 | - | - | 1.99 | | - |
| *Phorbas dives* | 0.00 | 19.78 | 23.20 | 5.54 | - | 2.18 | - | - | 1.41 | - |
| *Acanthochitona crinita* | 9.73 | - | 29.00 | - | - | 1.81 | - | - | - | - |
| *Tubulipora sp. 3* | 10.51 | - | 29.49 | - | - | 1.72 | - | - | - | - |
| *Coscinasterias tenuispina* | 4.94 | - | 34.50 | - | - | 1.71 | - | - | - | - |
| *Tricolia pullus* | 20.52 | - | 0.00 | - | - | 1.37 | - | - | - | - |
| *Paracentrotus lividus* | 9.76 | 15.05 | 31.29 | 20.33 | - | 1.11 | 1.78 | 2.51 | 1.18 | - |
| *Synalpheus gambarelloides* | 9.39 | - | - | 23.81 | - | - | 2.17 | - | - | - |
| *Tubulipora sp. 1* | 0.00 | - | - | 35.81 | - | - | 1.78 | - | - | - |
| *Diplosoma listerianum 2* | 23.45 | - | - | 0.00 | - | - | 1.48 | - | - | - |
| *Alvania sp. 1* | 34.08 | - | - | 0.00 | - | - | 1.34 | - | - | - |
| *Aplysia punctata* | - | 15.33 | 0.00 | - | - | - | - | 1.63 | | - |
| *Tritia cuvierii* | - | 18.95 | - | 0.00 | - | - | - | - | 3.14 | - |
| *Halichondria (Halichondria) melanadocia* | - | 20.99 | - | 26.67 | - | - | - | - | 1.95 | - |
| *Stelletta hispida* | - | 14.20 | - | 7.68 | - | - | - | - | 1.78 | - |
| *Pagurus cuanensis* | - | 2.08 | - | 19.38 | - | - | - | - | 1.43 | - |
| *Bienmidae sp.* | 0.00 | 0.00 | 0.00 | 11.11 | - | - | 1.32 | - | - | - |

| Small fraction | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Alvania sp. 1* | 58.35 | 34.59 | 27.22 | 16.10 | 3.74 | - | - | 1.18 | 1.06 | - |
| *Microcosmus squamiger* | 11.21 | 6.88 | 9.68 | 0.00 | 2.56 | 1.78 | 3.38 | - | - | - |
| *Mannesia sabadiega* | 6.15 | 24.41 | 9.30 | - | 2.32 | | - | 1.03 | - | - |
| *Alvania sp. 2* | 8.75 | 49.05 | - | - | 1.46 | - | - | - | - | - |
| *Favorinus sp.* | 0.00 | 11.53 | - | - | 1.37 | - | - | - | - | - |
| *Cylista cf. laceratus* | 15.48 | 4.76 | - | - | 1.20 | - | - | - | - | - |
| *Xantho hydrophilus* | 0.00 | 26.15 | 23.07 | 0.00 | 1.00 | 1.93 | - | - | - | - |
| *Pisa cf. tetraodon* | 0.00 | 3.39 | 14.72 | - | - | 2.96 | - | 2.32 | - | - |
| *Dynamene magnitorata* | 0.00 | - | 20.06 | 2.70 | - | 2.85 | - | - | - | - |
| *Acanthonyx lunulatus 1* | 3.83 | - | 37.84 | 4.75 | - | 2.84 | - | - | - | - |
| *Ophlitaspongia papilla* | 12.31 | - | 0.00 | - | - | 2.65 | - | - | - | - |
| *Synalpheus gambarelloides* | 0.00 | 45.39 | 31.91 | 18.36 | - | 1.16 | - | 1.63 | 2.82 | - |
| *Tubulipora sp. 3* | 0.00 | - | 34.19 | 40.74 | - | 1.12 | 1.50 | - | - | - |
| *Paracentrotus lividus* | 0.00 | 17.60 | 32.71 | 20.79 | - | 1.09 | 1.87 | 1.92 | 1.01 | - |
| *Coscinasterias tenuispina* | 3.88 | - | 33.09 | - | - | 1.07 | - | - | - | - |
| *Stelletta hispida* | 0.00 | 13.80 | - | 10.60 | - | - | 3.59 | - | 1.06 | - |
| *Haminoea hydatis* | 2.94 | 17.64 | 0.00 | 12.28 | - | - | 1.62 | 1.11 | 1.76 | - |
| *Costasiella cf. Virescens* | 0.00 | - | - | 19.68 | - | - | 1.40 | - | | - |
| *Jujubinus cf. vexationis* | 12.08 | - | - | 0.00 | - | - | 1.37 | - | | - |
| *Tubulipora sp. 1* | 0.00 | 0.00 | - | 37.10 | - | - | 1.32 | - | 1.30 | - |
| *Inachus cf. dorsettensis* | - | 14.28 | 6.26 | - | - | - | - | 2.25 | - | - |
| *Halichondria (Halichondria) melanadocia* | - | 13.91 | 7.67 | 11.52 | - | - | - | 1.94 | 2.74 | - |
| *Tubulipora sp. 2* | - | 34.56 | 28.13 | - | - | - | - | 1.93 | - | - |
| *Anthelia sp.* | - | 0.00 | - | 14.84 | - | - | - | - | 2.99 | - |
| *Scopalina sp. 2* | - | 15.30 | - | 0.00 | - | - | - | - | 2.67 | - |

**Table C.8.** (A) One-way permutational multivariate analyses of variance of the calcifying classification in algae and metazoan, in the natural ‘$CO_2$ Gradient’ as factor with four levels (‘1-Vent’, ‘2-Transition25’, ‘3-Transition75’, and ‘4-Control’) in both fraction size (small fraction: organisms with size > 1 mm; and large fraction: organisms between 1 mm and 0.64 μm). (B) Results of pairwise comparisons for levels of the factor ‘$CO_2$ Gradient’ in algae.

| A. Source of variation | | LARGE FRACTION | | | | SMALL FRACTION | | | |
|---|---|---|---|---|---|---|---|---|---|
| Algae | **df** | **SS** | **MS** | **Pseudo-F** | **p** | **SS** | **MS** | **Pseudo-F** | **p** |
| Gradient | 3 | 0.229 | 0.076 | 0.703 | 0.001* | 0.010 | 0.033 | 0.644 | 0.001* |
| Residual | 20 | 0.097 | 0.004 | | | 0.006 | 0.003 | | |
| | 20 | | | | | | | | |
| Metazoan | **df** | **SS** | **MS** | **Pseudo-F** | **p** | **SS** | **MS** | **Pseudo-F** | **p** |
| Gradient | 3 | 0.039 | 0.013 | 0.196 | 0.233 | 0.015 | 0.014 | 0.109 | 0.489 |
| Residual | 20 | 0.159 | 0.008 | | | 0.116 | 0.006 | | |
| | 20 | | | | | | | | |

| **B. Groups Algae** | | **1 - 2** | **1 - 3** | **1 - 4** | **2 - 3** | **2 - 4** | **3 - 4** |
|---|---|---|---|---|---|---|---|
| Large fraction | t | 26.350 | 42.647 | 2.834 | 14.625 | 0.626 | 0.493 |
| | p | 0.002* | 0.002* | 0.002* | 0.002* | 0.730 | 0.522 |
| Small Fraction | t | 59.363 | 33.788 | 6.580 | 12.828 | 0.582 | 3.410 |
| | p | 0.002* | 0.002* | 0.005* | 0.005* | 0.495 | 0.086 |

## APPENDIX D. Chapter 5

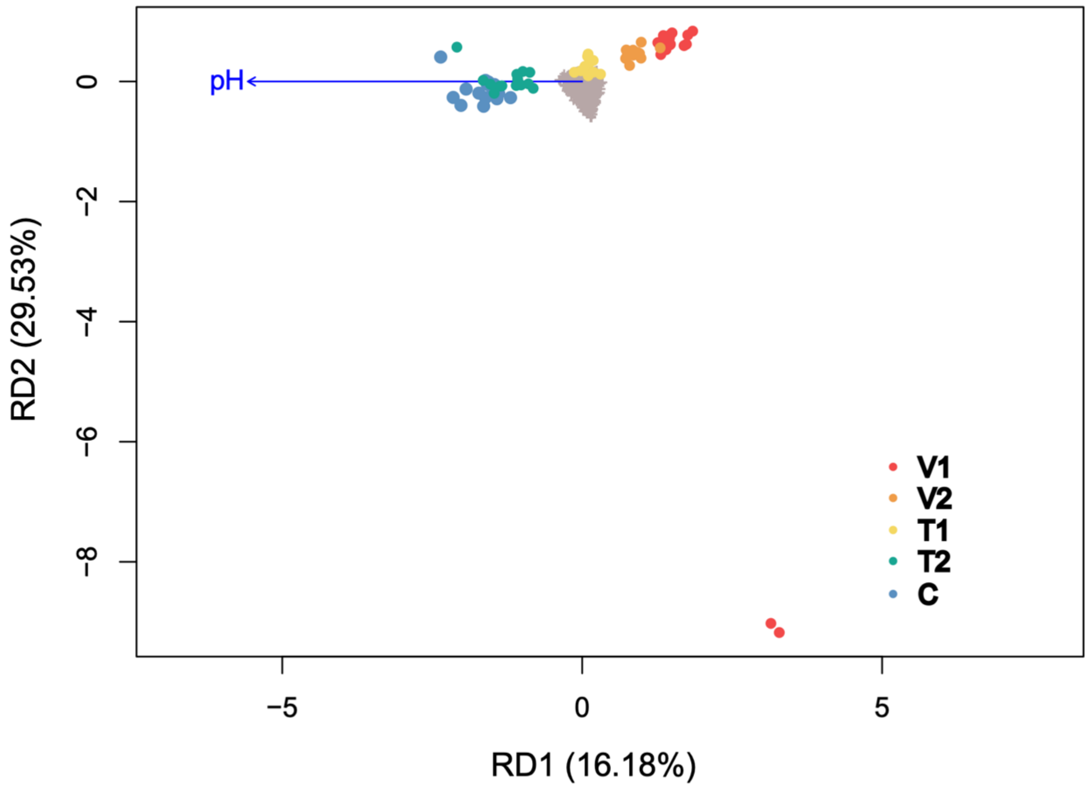


Figure D.1. Redundancy Analysis (RDA) performed on pH variation data and SNP loci. Coloured are SNPs considered 'candidate SNPs' associated with pH variation. Each sea urchin population has a different colour following the colour coding for each pH level in figure 5.1).

Table D.1. Values of $F_{ST}$ among populations from 'all', 'neutral', 'candidate SNPs' datasets, with pairwise comparison among population. (*) the significant p-value after FDR correction. C: Control, T1: Transition1, T2: Transition2, V1: Vent1, V2: Vent2.

| Site | $F_{ST}$ all | p-value | $F_{ST}$ neutral | p-value | $F_{ST}$ candidate | p-value |
|---|---|---|---|---|---|---|
| V1 - V2 | 0.002 | 0.250 | 0.002 | 0.327 | 0.008 | 0.052 |
| V2 - T1 | 0.004 | 0.143 | 0.003 | 0.161 | 0.013 | 0.020* |
| V2 - T2 | 0.006 | 0.001* | 0.004 | 0.056 | 0.048 | 0.001* |
| V2 - C | 0.005 | 0.002* | 0.003 | 0.053 | 0.045 | 0.001* |
| V1 -T1 | 0.002 | 0.037* | 0.002 | 0.035* | 0.000 | 0.626 |
| V1 - T2 | 0.003 | 0.002* | 0.001 | 0.044* | 0.040 | 0.001* |
| V1 - C | 0.004 | 0.001* | 0.002 | 0.003* | 0.039 | 0.001* |
| T1 - T2 | 0.000 | 0.347 | 0.000 | 0.572 | 0.003 | 0.249 |
| T1 - C | 0.002 | 0.059 | 0.001 | 0.125 | 0.011 | 0.017* |
| T2 - C | 0.000 | 0.925 | 0.000 | 0.940 | 0.000 | 0.710 |

**Table D.2.** List of the 31 GO functions associated to 14 genes found in *Arbacia lixula* populations living along $CO_2$ gradient ordered by ontology.

| Go terms | Gene | Name |
|---|---|---|
| Biological Process | | |
| GO:0006468 | casein kinase i isoform gamma-3 isoform 1 | protein phosphorylation |
| GO:0009069 | casein kinase i isoform gamma-3 isoform 1 | serine family amino acid metabolic process |
| GO:0006470 | dual specificity protein phosphatase 1-like | protein dephosphorylation |
| GO:0007154 | frem2_lytva ame: full=extracellular matrix protein 3 ame: full=frem2 homolog flags: precursor | cell communication |
| GO:0007156 | frem2_lytva ame: full=extracellular matrix protein 3 ame: full=frem2 homolog flags: precursor | homophilic cell adhesion via plasma membrane adhesion molecules |
| GO:0007275 | Frizzled7 [Paracentrotus lividus] | multicellular organism development |
| GO:0060070 | Frizzled7 [Paracentrotus lividus] | canonical Wnt signaling pathway |
| GO:0043170 | gag-pol polyprotein | macromolecule metabolic process |
| GO:0044238 | gag-pol polyprotein | primary metabolic process |
| GO:0008283 | protein dvr-1 homolog precursor | cell population proliferation |
| GO:0044765 | udp-galactose translocator 1-like | transport |
| GO:0090305 | uncharacterized protein loc100893986 | nucleic acid phosphodiester bond hydrolysis |
| GO:0018108 | uncharacterized protein loc577155 | peptidyl-tyrosine phosphorylation |
| GO:0007165 | vegf precursor | signal transduction |
| GO:0040007 | vegf precursor | growth |
| Cellular Component | | |
| GO:0005886 | frem2_lytva ame: full=extracellular matrix protein 3 ame: full=frem2 homolog flags: precursor | plasma membrane |
| GO:0016021 | frem2_lytva ame: full=extracellular matrix protein 3 ame: full=frem2 homolog flags: precursor | membrane |
| GO:0016020 | udp-galactose translocator 1-like | membrane |
| Molecular function | | |
| GO:0004674 | casein kinase i isoform gamma-3 isoform 1 | protein serine/threonine kinase activity |
| GO:0005524 | casein kinase i isoform gamma-3 isoform 1 | ATP binding |
| GO:0003677 | compass-like protein | DNA binding |
| GO:0008080 | diamine acetyltransferase 2-like | N-acetyltransferase activity |
| GO:0004721 | dual specificity protein phosphatase 1-like | phosphoprotein phosphatase activity |
| GO:0005509 | frem2_lytva ame: full=extracellular matrix protein 3 ame: full=frem2 homolog flags: precursor | calcium ion binding |
| GO:0042813 | Frizzled7 [Paracentrotus lividus] | Wnt receptor activity |
| GO:0000166 | nek protein | nucleotide binding |
| GO:0003676 | uncharacterized protein loc100893986 | nucleic acid binding |
| GO:0008408 | uncharacterized protein loc100893986 | 3'-5' exonuclease activity |
| GO:0004715 | uncharacterized protein loc577155 | non-membrane spanning protein tyrosine kinase activity |
| GO:0004672 | uncharacterized protein loc593493 | protein kinase activity |
| GO:0008083 | vegf precursor | growth factor activity |

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Universidad de La Laguna

# Marine Benthic Communities of the Future: Use of Acidified Natural Systems

Doctoral Thesis in Biodiversity and Conservation

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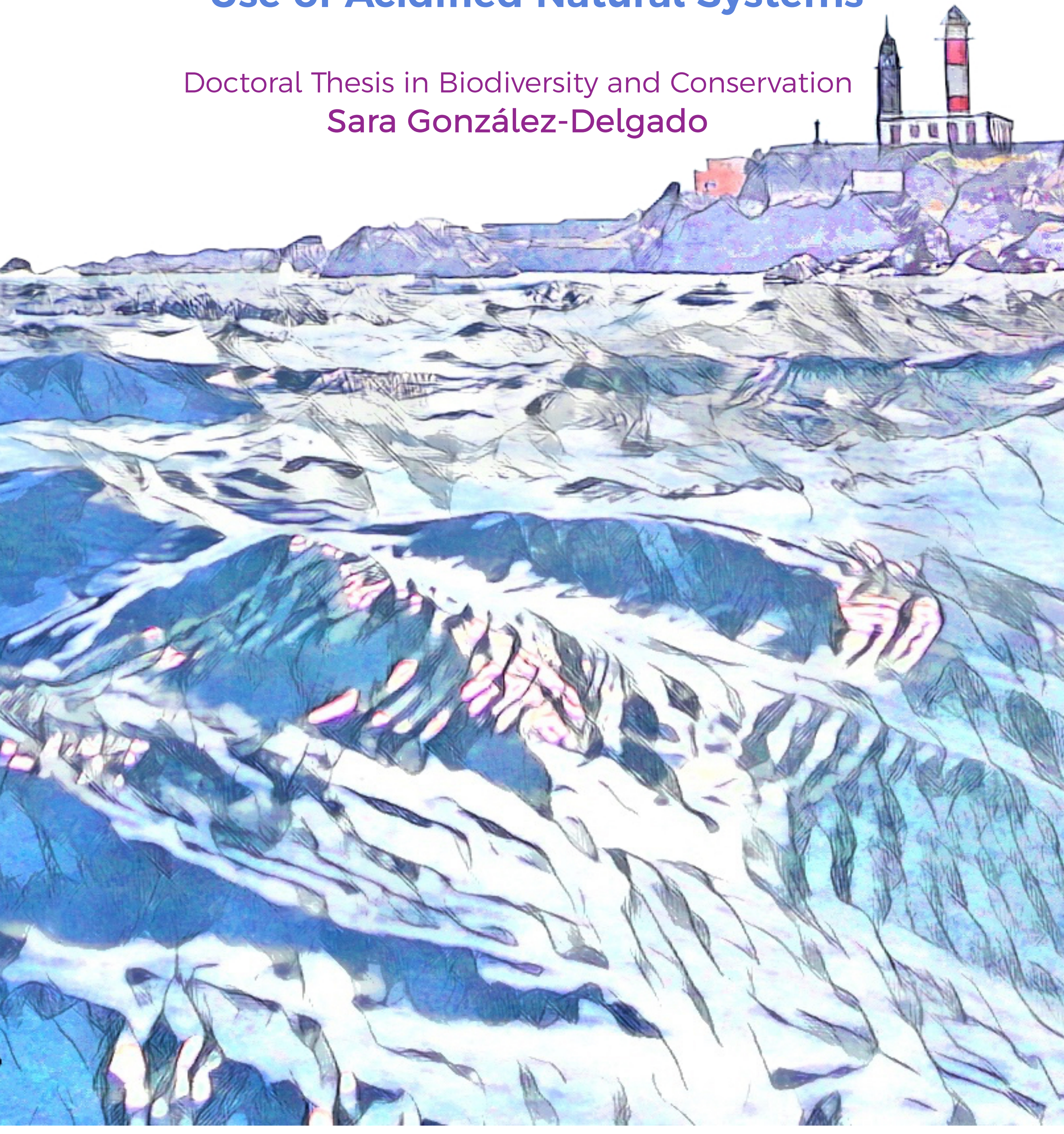

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