UNIVERSITÀ DEGLI STUDI DI PALERMO
Dottorato in Scienze della Terra e del Mare
Dipartimento di Scienze della Terra e del Mare
Settore Scientifico Disciplinare BIO/07

# Response of seagrasses and marine biofilms to natural acidification at $CO_2$ vents

IL DOTTORE
**Valentina Sciutteri**

IL COORDINATORE
**Prof. Alessandro Aiuppa**

IL TUTOR
**Prof. Antonio Mazzola**

CICLO XXXI
ANNO CONSEGUIMENTO TITOLO 2019

*“Nella ricerca scientifica, né il grado di intelligenza né la capacità di eseguire e portare a termine il compito intrapreso sono fattori essenziali per la riuscita e per la soddisfazione personale. Nell'uno e nell'altro contano maggiormente la totale dedizione e il chiudere gli occhi davanti alle difficoltà.”*

Rita Levi-Montalcini

# Contents

## Abstract

Over the last 200 years, about 30% of the anthropogenic $CO_2$ emissions in the atmosphere have been absorbed by the oceans, causing a decrease in the seawater pH of 0.1 unit that is impacting marine ecosystems and their functioning. Current climate predictions suggest that, unless mitigation measures will be pursued, these changes will continue and intensify. Evaluating the response of marine ecosystems to climate change is challenging, since research efforts should not only integrate the effects of global changes with regional disturbances, but also investigate the influence of biotic interactions occurring in multiple species assemblages at the same time.

The aim of this PhD thesis is to evaluate the effects of ocean acidification on marine communities in coastal ecosystems, with a special focus on *Posidonia oceanica* meadows as well as on marine biofilms community.

Based on the recent acknowledgement of shallow-water hydrothermal vents as analogues of future acidified oceans, three studies were conducted in the Panarea and Vulcano shallow vent systems (Aoelian Islands, Italy).

The first study focused on the effects of long-term acidification on *Posidonia oceanica* meadows at Panarea vents. The results obtained revealed that *P. oceanica* meadows were highly sensitive to low pH caused by the vent emissions: at these sites, meadows were less dense and characterized by shorter shoots that experienced faster leaf turnover and less grazing pressure as a consequences of the lower epiphyte biomass on the seagrass leaves. These evidences suggest that the key ecological role played by *P. oceanica* as foundation species in coastal environments could be compromised in the future acidified oceans.

The second study focused on the composition of marine biofilms in the context of ocean acidification. The composition of the biofilm community was investigated across time at different $pCO_2$/pH levels at Vulcano vents. Although the response was taxa-specific, significant shifts in the overall community were observed, with some groups such as Gammaproteobacteria resulting as potential “winners” in the future high-$CO_2$ world.

In the third study, a manipulative experiment modifying predatory pressure was conducted along a $pCO_2$/pH gradient at Vulcano vents in order to determine the possible interaction of top-down (predation) and bottom-up (ocean acidification) forces on the biochemical composition of marine biofilms. Although the patterns of different variables were not always clear due to the elevated natural variability detected in the sites, ocean acidification affected the biochemical composition of the biofilms by increasing the abundance of primary producers and enhancing the nutritional quality of the biofilms. Although top-down control was important in regulating the biofilm composition, it failed at buffering the resource effect of elevated $CO_2$ concentrations on primary producers within the biofilms. Taken together, the outcomes of these studies showed that the response of marine communities to ocean acidification is highly variable, depending not only on the environmental conditions but also on the interaction of biotic and abiotic forces.

# General Introduction

Since the beginning of the Industrial Revolution (1859), the $CO_2$ concentration in the atmosphere has increased considerably, passing from 280 ppm of the preindustrial level to 406 ppm measured in October 2018 at Mauna Loa Observatory-Hawaii (19.5 °N, 155.6 °W).

Due to the gas pressure equilibrium between the atmosphere and the oceans, over 30% of the anthropogenic $CO_2$ emissions has been absorbed by the seawater (Sabine et al. 2004).

The ultimate result of this absorption is the decrease of oceanic pH, which has already diminished by 0.1 units in the year 2000. Current climate projections coupled with simulation models estimate a pH reduction up to 0.3 units in 2100, unless carbon emissions will be substantially curbed (K. Caldeira and Wickett 2005).

Small changes in the seawater pH naturally occur in the oceans due to photosynthetic activity, upwelling, submerged volcanic activity etc., however these variations are generally localized in space and time. The anthropogenic induced ocean acidification, instead, is occurring on a global scale and at unprecedented rates, causing serious concerns regarding the response of marine biota and ecosystems, since organisms could not be able to adapt to the muted environmental conditions over such a short-time scale (Guinotte and Fabry 2008).

Initial studies on the effects of ocean acidification on marine species were based on laboratory and mesocosm experiments focusing on single species at single life stages. In this regard, the response of biological calcification to ocean acidification has been widely investigated, reporting negative impacts although with some exceptions (Ries, Cohen, and McCorkle 2009).

On the other hand, elevated $CO_2$ concentration in seawater positively affects primary producers (Connell et al. 2013). The opposite effects of $CO_2$ acting both as a stressor (lower pH on calcifying species) as well as a resource ($CO_2$ enrichment on primary producers) among different organisms have arisen new ecological questions on the response of multiple species assemblages to altered seawater carbon chemistry (Gaylord et al. 2015). Not only the effects of environmental factors, but also the influence of interactions among different organisms in the context of climate changes needs to be targeted (Russell

et al. 2012), in order to evaluate possible mechanisms of propagation or stabilization of the changes across ecosystems (Ghedini and Connell 2017).

This thesis aimed at evaluating the effects of ocean acidification on marine biota at the level of community which reflects the interaction of multiple species and allow to understand how ecological changes eventually propagate or are stabilized through ecosystems. The general hypothesis behind the research was that ocean acidification could represent a resource for primary producers such as seagrasses and microbial biofilms ($CO_2$ enrichment, bottom-up effect), and that this resource effect could be compensated by interspecific interactions such as predation (top-down control).

The experiments were conducted at $CO_2$ vent systems, which provide the opportunity to test laboratory and mesocosm derived conceptual models in natural environments (see Dahms et al. 2018; Kroeker, Gambi, and Micheli 2013; Vizzini et al. 2017 and references therein).

# Chapter 1

## Long-term effects of high $CO_2$ level exposure on *Posidonia oceanica* meadow

### 1.1 Introduction

Seagrasses are widely distributed in coastal zones of temperate and tropical areas, where they grow in meadows playing a crucial ecological role. They provide habitat and food to numerous organisms, including commercially valuable fish species, which use the intricate, dense canopy as nurseries as well as refuge areas (Duffy, 2006; Serrano et al., 2017). Along with the great variety of organisms associated with the plants themselves, seagrass beds form important ecosystems characterized by high biodiversity and elevated primary and secondary production rates, which constitute the base of complex food webs (Hemminga and Duarte 2000; S. Vizzini et al. 2002). Due to these functions, seagrass meadows provide several supporting ecosystem services (high productivity and biodiversity, nutrient cycling) essential to the delivery of regulating services such as sediment stabilization, water quality improvement, coastal erosion prevention (Ondiviela et al. 2014). Moreover, together with other vegetated coastal ecosystems, they can act as sinks for atmospheric carbon dioxide, reducing atmospheric $CO_2$ and mitigating climate change (Lau 2013).

Despite global efforts towards their conservation, seagrass meadows are facing a severe regression due to anthropogenic activities such as eutrophication, coastal urbanization and intense fishing destroying their habitat (Orth et al. 2006; Waycott et al. 2009). Changes in climate can also represent a serious long-term threat to these ecosystems (Chefaoui, Duarte, and Serrão 2018), which are already showing high vulnerability to global warming (Collier and Waycott 2014; Duarte 2002; Marbà and Duarte 2010; Unsworth, van Keulen, and Coles 2014).

There has been a general consensus that seagrass species, along with other primary producers, will be "winners" in the high-$CO_2$ world, because of their strong affinity for carbon dioxide (Connell et al. 2013; Invers et al. 2001; Jiang, Huang, and Zhang 2010; Koch et al. 2013; Zimmerman et al. 1997). This "winner" thesis is mainly based on evidence from short-term experiments, ignoring the long-term

potential mechanisms of adaptation and physiological acclimatization of natural populations (Kelly and Hofmann, 2013).

Recently, shallow-water $CO_2$ vent systems have been proposed as natural laboratories for studying the long-term effects of elevated $pCO_2$ and consequent low pH (i.e. ocean acidification) on marine biota and ecosystems (Dahms et al. 2018; Hall-Spencer et al. 2008). However, the response of seagrasses resulted to vary across species and sites (Apostolaki et al. 2014a; Guilini et al. 2017; Hall-Spencer et al. 2008; Russell, Connell, Uthicke, et al. 2013; Takahashi et al. 2015).

*Posidonia oceanica*, which represents one of the most ecologically and economically relevant coastal ecosystems in the Mediterranean Sea, i.e. *P. oceanica meadows* (Campagne et al. 2014), showed a minor increase in shoot density close to $CO_2$ vents (Guilini et al. 2017; Hall-Spencer et al. 2008). However, contrary to expectations, its metabolic activity was lower in vent sites, and primary production did not show significant variations compared to control sites (Koopmans et al. 2018). At the same time, a high-stress response of *P. oceanica* was revealed by genetic investigations (Lauritano et al. 2015), especially in case of extreme environmental conditions (Salvatrice Vizzini et al. 2010). Taken together, these results make it difficult to successfully predict the potential responses of *P. oceanica* meadows to future levels of $pCO_2$ and pH.

The aim of this study was to clarify the response of *P. oceanica* to long-term exposure to natural acidification, in order to evaluate whether elevated $CO_2$ concentration constituted a resource or a stressor for the seagrass. To do this, we compared *P. oceanica* in proximity of shallow-water $CO_2$ vents with *P. oceanica* not influenced by the vents emissions. In particular, we analysed three descriptor levels, meadow, shoot and leaf, hypothesizing that the seagrass could be affected at all levels by the long-term exposure to $CO_2$ vent emissions.

## 1.2 Materials and Methods

### 1.2.1 Study area

Panarea is the smallest of seven islands forming the archipelago of the Aeolian Islands, located in the southern Tyrrhenian Sea (Italy, Mediterranean Sea) (Fig. 1). This area has been historically known for intense submarine volcanic activity, in the form of hydrothermal fluid emissions taking place in several sites around the archipelago (e.g. Gugliandolo et al., 2006). Off the eastern coast of Panarea, numerous shallow hydrothermal vents form a geothermal field of about 2.3 $km^2$ (Esposito, Giordani, and Anzidei 2006), surrounded by the islets of Dattilo, Lisca Bianca, Bottaro and Lisca Nera constituting the remnants of a crater rim (Italiano and Nuccio 1991). The hydrothermal fluids reach the water either through fractures on the sea bottom or through the seafloor sand (diffusive permeation), from the surface up to a depth of 150 m. The fluids emissions consist of both thermal water and gases, mainly $CO_2$ (ca. 97%), with temperature of 48-54°C and pH values of 4.7-5.4. Fluids are released at a rate of $10^6$-$10^7$ l $day^{-1}$ (Italiano and Nuccio 1991). The area has also experienced parossistic events, such as that in November 2002, when an explosive outgassing phenomenon occurred at shallow depth close to Bottaro islet (eastern off Panarea). Fluids emissions were up to $10^8$–$10^9$ l $day^{-1}$ with temperature up to 50°C and pH of 5.0-5.5 (Bruno Capaccioni et al. 2005). The highly energetic activity lasted for several months up to 2003, reducing progressively in intensity (Caliro et al. 2004; Caracausi et al. 2005), but greatly affecting the surrounding environment and biota (Aliani et al. 2010; Gugliandolo, Italiano, and Maugeri 2006; Manini et al. 2008; Salvatrice Vizzini et al. 2010). This area is characterized by patchily distributed macrophytes and seagrasses meadows that grow in the vicinity of the vents area.

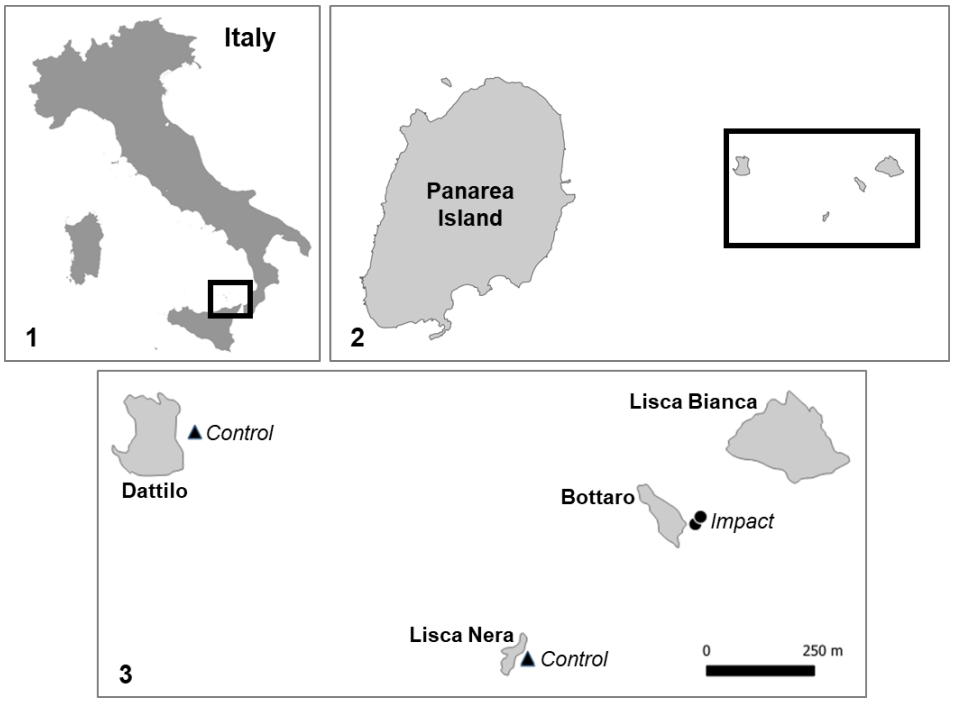


**Figure 1.** Map indicating the location of the study area near Panarea Island (Italy).

### 1.2.2 Sampling and laboratory procedures

Samplings were carried out during 2010 and 2011 summer seasons in two areas, both characterized by *P. oceanica* meadows occurring at similar depth (9-10 m), substratum type (sandy bottom) and wind exposition. The area between the islets of Bottaro and Lisca Bianca, in the proximity of the site where the 2002 parossistic event occurred, was chosen as the impact one (hereafter referred to as *impact*), because of the presence of shallow water volcanic emissions, while the area between Lisca Nera and Dattilo islets was suitable as reference (hereafter referred to as *control*), since no visible gas and fluid discharges were reported. In each area, two sites were randomly chosen.

Vertical seawater profiles (surface to the bottom) of temperature (resolution: ±0.01°C), salinity (±0.01 ppt), dissolved oxygen concentration (DO, ±0.01 mg $l^{-1}$) and pH (±0.01 units) were recorded in each site, using an HYDROLAB DS5 multiparametric probe. Measurements were performed between 10:00 and 12:00 to avoid diurnal variation. The probe was calibrated at ambient temperature with conventional buffer solutions for pH (4.00 and 7.00) and salinity (35ppt-IAPSO Seawater Standard).

To describe the characteristics of *Posidonia oceanica* meadows, we chose three descriptor levels: meadow, shoot and leaf.

The descriptors used for the meadow were density (n. shoots/$m^2$) and biomass (g DW/$m^2$). A quadrat metal frame (40 x 40 cm, n=6 replicates per site) was used to estimate *P. oceanica* density by SCUBA divers (Buia, Gambi, and Dappiano 2004). Shoots within the metal frame were carefully collected, sealed in plastic bags and transported to the laboratory, where they were dried at 60° C for 48-72 hours or until constant weight was reached, and weighted for meadow biomass estimation (g DW/$m^2$).

For the shoot and leaf levels, five shoots for each site (i.e. 10 shoots per area) were randomly selected among those collected only in 2011 sampling season. Following the methods described in Buia et al. (2004), all leaves were counted and assigned to a class category (adult, intermediate, juvenile). Length and width of leaves, length of green and brown tissues and length of sheaths were measured. Bite marks on the apex of adult and intermediate leaves were counted as well. Epiphytes on leaves were gently removed from the surface using a ceramic blade, subsequently dried at 60° C for 48-72 hours and weighted. All leaves and sheaths were dried at 60° C for 48-72 hours until constant weight was reached, and weighted.

The descriptors used for the shoot level were the following: number of leaves, leaf area ($cm^2$), leaf biomass (g DW), green tissue area ($cm^2$), brown tissue area ($cm^2$), epiphyte biomass (g DW), coefficient A (number of leaves per shoot having bite marks).

For the characterization of the leaf, the following descriptors were used according to the class category: number of leaves, leaf length (cm), leaf width (cm), green tissue length (cm), brown tissue length (cm), leaf biomass (g DW), sheath length (cm, adult leaves only), sheath biomass (g DW, adult leaves only), epiphyte biomass (g DW).

### 1.2.3 Data elaboration and statistical analysis

The statistical analysis was performed, unless otherwise stated, using the following design with "Area" (*impact* vs *control*) and "Year" (2010 vs 2011) as orthogonal and fixed factors, and "Site" as a random factor (nested in "Area" and "Year").

To evaluate the differences in the environmental seawater features (temperature, salinity, dissolved oxygen concentration and pH), ANOVA was performed on the deepest data (close to the bottom), using each vertical profile as a replicate (n=3). ANOVA was also performed on the seagrass descriptors at the meadow level (density and biomass), and post-hoc tests (Tukey HSD) were conducted when significant effects were found.

Permutational multivariate analysis of variance (PERMANOVA) was run on shoots and leaves descriptors considering the factors "Area" (fixed with two levels: *impact* vs *control*) and "Site" (random and nested in Area with two levels: 1 and 2): data were square root transformed and Monte-Carlo estimated *P*-values reported for tests with a low number of unique permutations. Since green and brown tissues covariate, only green tissue data were used for statistical purpose. Statistical analysis was performed on adult leaves exclusively. A graphical representation of the multivariate patterns was obtained by employing Principal Coordinates Analysis (PCoA) on shoots and leaves descriptors.

Univariate and multivariate analysis were performed using the statistical software StatSoft STATISTICA (Version 12) and PRIMER v6 with PERMANOVA (Plymouth Marine Laboratory, UK).

## 1.3 Results

Vertical profiles of temperature, dissolved oxygen and salinity were similar in the two areas and in the two sampling periods. On the contrary, pH values were significantly lower in the *impact* area compared to the *control* (Tab. 1).

**Table 1.** Values (means ± standard deviations) of environmental seawater features of the sampling areas. ANOVA results and post-hoc comparisons. Significant results in bold.

| Area | *Impact* | *Control* | Area | | | Year | | | Area X Year | | | Site (Area X Year) | | | PAIRWISE COMPARISONS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | df | F | p | df | F | p | df | F | p | df | F | p | |
| Temperature (°C) | 24.28 ± 0.91 | 24.41 ± 1.13 | 1 | 0.08 | 0.77 | 1 | 0.47 | 0.50 | 1 | 1.39 | 0.26 | 4 | 0.44 | 0.78 | |
| Salinity (ppt) | 38.28 ± 0.17 | 38.28 ± 0.15 | 1 | 0.01 | 0.99 | 1 | 2.00 | 0.23 | 1 | 1.00 | 0.39 | 4 | 2.00 | 0.22 | |
| Dissolved Oxygen (mg $l^{-1}$) | 4.60 ± 0.57 | 4.57 ± 0.54 | 1 | 0.01 | 0.91 | 1 | 1.69 | 0.21 | 1 | 1.24 | 0.28 | 4 | 2.30 | 0.10 | |
| pH | 7.84 ± 0.12 | 8.03 ± 0.04 | 1 | 29.70 | **0.00** | 1 | 0.10 | 0.76 | 1 | 0.00 | 0.91 | 4 | 1.90 | 0.16 | *Impact < Control* |

*P. oceanica* density resulted significantly lower in the *impact* area than in the *control* (Fig. 2a; Tab. 2). On the contrary, biomass resulted more variable overall (Fig. 2b), with significant differences between sites and for the interaction Area x Year (Tab. 2).

Regarding shoot descriptors, number of leaves resulted slightly higher in the *impact* area than in the *control* with significant differences within the areas (ANOVA, Tab. 2). All the other descriptors, as leaf area and biomass, green tissue surface, epiphyte biomass and Coefficient A, showed an opposite trend, with significantly lower values in the *impact* compared to the *control* area (ANOVA, Tab. 2). Only the length of the brown tissue did not show significant differences (ANOVA, Tab. 2). At the multivariate level, shoot descriptors were significantly different between areas (Tab. 2).

Adult leaf features revealed significant differences between areas with lower values in the *impact* than in the *control* area (PERMANOVA, Tab. 2). The only exceptions were represented by the number of leaves and the brown tissue length.

Intermediate leaves were slightly more abundant in the *impact*, however their length, width, biomass and epiphyte biomass were reduced compared to the *control*. Green tissue was longer in the *control*, while brown tissue was detected only in leaves from the impact area (Tab. 2).

Juvenile leaves were found exclusively in the *impact* area, although their number was very low. Leaves appeared very short and completely green, with no epiphytes on the surface. Width in juvenile leaves was comparable to leaf width of the others class categories (Tab. 2).

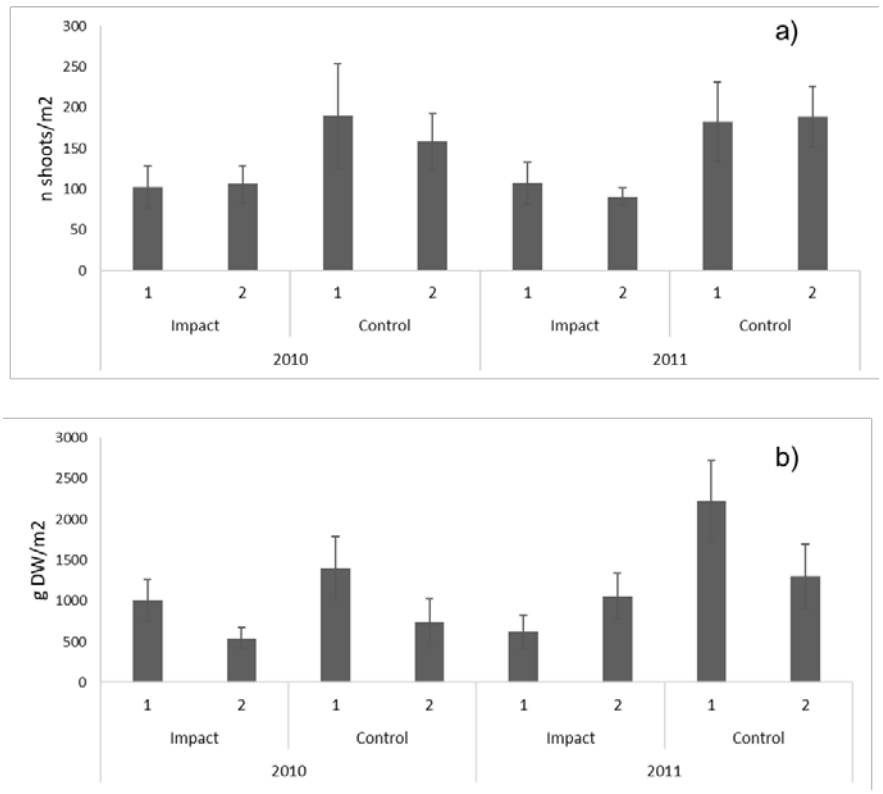


**Figure 2.** Density (a) and Biomass (b) of *Posidonia oceanica* meadow in the impact and control sites in 2010 and 2011 sampling seasons. Mean values are reported with standard deviations.

The Principal Coordinate Analysis biplots gave a clear pattern of the differences shown by PERMANOVA (Fig. 3). The two areas clustered separately both at the shoot and the leaf level; *impact* sites showed a higher dispersion than the control ones. Overall, the first two principal coordinates explained over 90% of the total variance of the shoots and leaves characterized in this study. Regarding shoot descriptors, PCO1 explained 77.3% of the total variance, while PCO2 explained 15.3%. Similar results were obtained for the leaf descriptors (PCO1=75.2% of the total variance; PCO2=16.3% of the total variance).

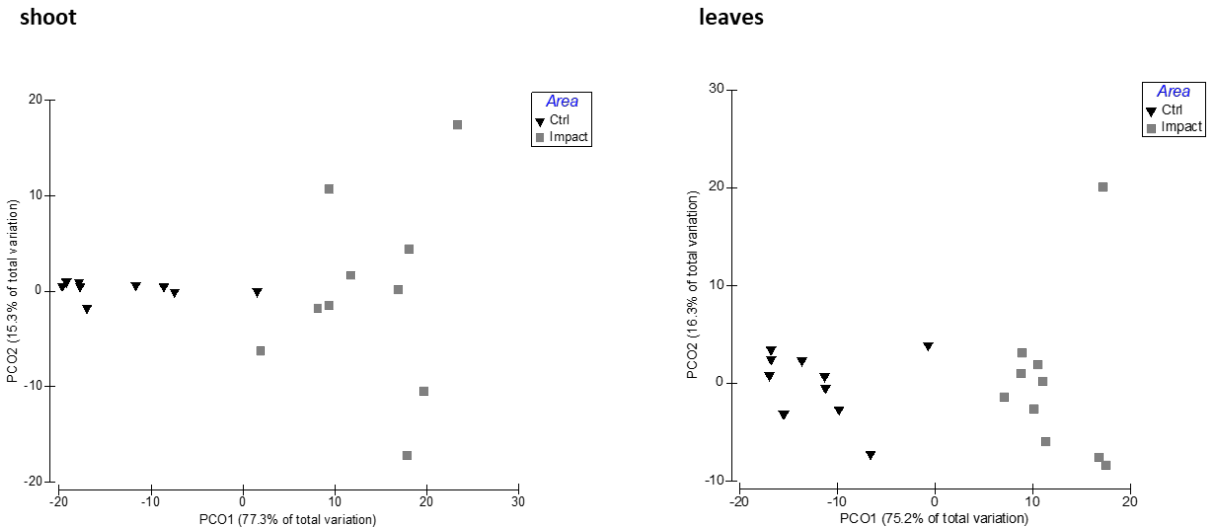


**Figure 3.** PCoA of shoot and leaves characterization in the impact and control areas.

**Table 2** Values (means ± standard deviations) of *P. oceanica* descriptors for meadow, shoot and leaf characterization (see text for details about the descriptors) in the sampling areas. ANOVA results and post-hoc comparisons for all descriptors (only on adult category for leaf descriptors). Permutational analysis of variance (PERMANOVA) results for *P. oceanica* shoot and leaf descriptors. Significant results in bold. * = no clear group order in *post-hoc* comparison.

**MEADOW DESCRIPTORS**

| Area | *Impact* | *Control* | Area | | | Year | | | Area X Year | | | Site (Area X Year) | | | PAIRWISE COMPARISONS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | df | F | *p* | df | F | *p* | df | F | *p* | df | F | *p* | |
| **Density (shoot/m$^2$)** | 101.45 ± 21.90 | 179.66 ± 46.10 | 1 | 53.48 | **0.00** | 1 | 0.09 | 0.76 | 1 | 0.62 | 0.43 | 4 | 0.73 | 0.58 | *Impact <Control* |
| **Biomass (g/m$^2$)** | 807.54 ± 313.88 | 1418.25 ± 655.50 | 1 | 43.28 | **0.00** | 1 | 16.40 | **0.00** | 1 | 11.51 | **0.00** | 4 | 12.39 | **0.00** | *Impact <Control; 2010<2011; ** |

**SHOOT DESCRIPTORS**

| Area | *Impact* | *Control* | Area | | | Site (Area) | | | PAIRWISE COMPARISONS |
|---|---|---|---|---|---|---|---|---|---|
| | | | df | F | *p* | df | F | *p* | |
| **Leaves (n)** | 6.00 ± 2.21 | 3.90 ± 0.57 | 1 | 12.97 | **0.00** | 2 | 5.79 | **0.01** | *Control <Impact; ** |
| **Leaf Area (cm$^2$)** | 61.02 ± 26.43 | 243.02 ± 73.77 | 1 | 50.44 | **0.00** | 2 | 0.42 | 0.66 | *Impact <Control* |
| **Leaf Biomass (g DW)** | 0.29 ± 0.10 | 1.22 ± 0.44 | 1 | 38.65 | **0.00** | 2 | 0.34 | 0.71 | *Impact <Control* |
| **Green Tissue Surface (cm$^2$)** | 55.10 ± 28.27 | 236.53 ± 71.34 | 1 | 52.69 | **0.00** | 2 | 0.48 | 0.63 | *Impact <Control* |
| **Total Brown Surface (cm$^2$)** | 5.91 ± 6.49 | 6.48 ± 3.60 | 1 | 0.05 | 0.82 | 2 | 0.11 | 0.89 | |
| **Epiphyte Biomass (g DW)** | 0.03 ± 0.02 | 0.17 ± 0.10 | 1 | 19.54 | **0.00** | 2 | 1.30 | 0.29 | *Impact <Control* |
| **Coefficient A** | 0.35 ± 0.24 | 0.74 ± 0.21 | 1 | 17.22 | **0.00** | 2 | 2.39 | 0.12 | *Impact <Control* |

**PERMANOVA**

| Source | df | Pseudo-F | P(MC) |
|---|---|---|---|
| Area | 1 | 15.69 | **0.01** |
| Site (Area) | 2 | 22.68 | 0.06 |
| Residuals | 16 | | |

**Table 3** Values (means ± standard deviations) of *P. oceanica* descriptors leaf characterization (see text for details about the descriptors) in the sampling areas. ANOVA results and post-hoc comparisons for all descriptors (only on adult category for leaf descriptors). Permutational analysis of variance (PERMANOVA) results for *P. oceanica* shoot and leaf descriptors. Significant results in bold. * = no clear group order in *post-hoc* comparison.

**LEAF DESCRIPTORS**

| Area | *Impact* | *Control* | Area df | Area F | Area *p* | Site (Area) df | Site (Area) F | Site (Area) *p* | PAIRWISE COMPARISONS |
|---|---|---|---|---|---|---|---|---|---|
| **Leaves (n)** | | | | | | | | | |
| **adult** | 3.70 ± 1.64 | 2.90 ± 0.57 | 1 | 3.46 | 0.08 | 2 | 6.59 | **0.01** | * |
| **intermediate** | 1.50 ± 0.53 | 1.11 ± 0.33 | | | | | | | |
| **juveniles** | 1.14 ± 0.38 | | | | | | | | |
| **Lenght (cm)** | | | | | | | | | |
| **adult** | 23.62 ± 3.96 | 78.34 ± 19.40 | 1 | 68.88 | **0.00** | 2 | 0.12 | 0.89 | *Impact <Control* |
| **intermediate** | 14.83 ± 8.69 | 68.17 ± 26.72 | | | | | | | |
| **juveniles** | 2.24 ± 1.19 | | | | | | | | |
| **Width (cm)** | | | | | | | | | |
| **adult** | 0.57 ± 0.10 | 0.80 ± 0.05 | 1 | 44.57 | **0.00** | 2 | 0.94 | 0.41 | *Impact <Control* |
| **intermediate** | 0.57 ± 0.11 | 0.80 ± 0.06 | | | | | | | |
| **juveniles** | 0.58 ± 0.10 | | | | | | | | |
| **Sheath Lenght (cm)** | | | | | | | | | |
| **adult** | 1.90 ± 0.32 | 4.33 ± 0.45 | 1 | 172.45 | **0.00** | 2 | 0.08 | 0.92 | *Impact <Control* |
| **Sheath Biomass (g DW)** | | | | | | | | | |
| **adult** | 0.01 ± 0.01 | 0.05 ± 0.01 | 1 | 46.67 | **0.00** | 2 | 2.79 | 0.09 | *Impact <Control* |
| **Green Tissue Lenght (cm)** | | | | | | | | | |
| **adult** | 19.46 ± 7.48 | 75.69 ± 19.22 | 1 | 67.85 | **0.00** | 2 | 0.22 | 0.81 | *Impact <Control* |
| **intermediate** | 12.46 ± 9.01 | 68.17 ± 26.72 | | | | | | | |
| **juveniles** | 2.24 ± 1.19 | | | | | | | | |
| **Brown Tissue Lenght (cm)** | | | | | | | | | |
| **adult** | 4.15 ± 7.59 | 2.65 ± 1.21 | 1 | 0.37 | 0.55 | 2 | 0.82 | 0.46 | |
| **intermediate** | 2.37 ± 7.09 | 0.00 ± 0.00 | | | | | | | |
| **juveniles** | 0.00 ± 0.00 | | | | | | | | |
| **Leaf Biomass (mg DW)** | | | | | | | | | |
| **adult** | 64.73 ± 16.88 | 325.61 ± 101.17 | 1 | 58.10 | **0.00** | 2 | 0.08 | 0.92 | *Impact <Control* |
| **intermediate** | 35.39 ± 22.07 | 241.50 ± 122.89 | | | | | | | |
| **juveniles** | 5.90 ± 3.42 | | | | | | | | |
| **Epiphyte Biomass (g DW)** | | | | | | | | | |
| **adult** | 0.01 ± 0.01 | 0.05 ± 0.03 | 1 | 20.79 | **0.00** | 2 | 1.30 | 0.30 | *Impact <Control* |
| **intermediate** | 0.00 ± 0.00 | 0.02 ± 0.01 | | | | | | | |
| **juveniles** | 0.00 ± 0.00 | | | | | | | | |

**PERMANOVA**

| Source | df | Pseudo-F | P(MC) |
|---|---|---|---|
| Area | 1 | 28.50 | **0.00** |
| Site (Area) | 2 | 1.29 | 0.28 |
| Residuals | 16 | | |

## 1.4 Discussion

The analysis of seawater environmental data from the experimental areas showed values typical for the season and the region for all the parameters with the exception of pH, which showed a ΔpH of 0.2 between the *impact* and *control* areas due to the presence of the vents. Based on current predictions (Stocker et al. 2013), the pH levels recorded at Bottaro (present study) and Basiluzzo islets (Guilini et al. 2017) were comparable to those expected in 2100, confirming Panarea vent system as suitable analogue of future acidified oceans.

In this investigation, differences between *P. oceanica* meadows from the *impact* and *control* areas were clearly observed at both meadow structure as well as phenological level. A significant reduction in the density of the meadows was observed in the proximity of the volcanic vents. Biomass of the meadows was slightly lower in the *impact* compared to the *control*, however this descriptor was highly variable and reported significant results at all levels of interaction (table 2). Increase in density and biomass of *P. oceanica* were previously reported at the $CO_2$ vents off Ischia, Italy (Hall-Spencer et al. 2008), while no significant changes in density were observed near Basiluzzo Islet, Panarea vent system (Guilini et al. 2017). Opposite results have been reported for *Cymodocea nodosa* in the shallow $CO_2$ vents of Vulcano Island, Italy, Mediterranean Sea (Apostolaki et al., 2014) as well as *Cymodocea rotundata* in Milne Bay $CO_2$ vents, Pacific ocean (Takahashi et al. 2015). Literature suggests a species-specific response, but site-specific environmental features may be responsible for the differences registered for *P. oceanica* within the Panarea vent system (Bottaro Islet, this study; Basiluzzo Islet, Guilini et al. 2017). It is possible that the response of the *P. oceanica* meadow structure observed in this study may be influenced by the stressful environmental conditions caused by the 2002 explosive event occurred at Bottaro Islet (Bruno Capaccioni et al. 2005). Important variation in the seagrass growth performance (Vizzini et al. 2010) as well as in the expression of genes for antioxidant response and heat shock proteins (Lauritano et al. 2015) were reported in *P. oceanica* at Bottaro Islet after the 2002 explosive event.

The analysis on the seagrass phenology also revealed significant differences in the structure of shoot and leaf between the *impact* and *control* areas.

Leaf area and biomass close to the vents of Bottaro Islet decreased by about 4-fold compared to the *control* area. It is worth noticing that leaves from the *impact* area were not only reduced in their

dimensions and biomass; the relative proportion of green and brown tissues in leaves differed as well. In particular, brown tissue covered up to 20% (adult leaves) and 100% (intermediate leaves) of the leaf surface in the *impact* area, while it was scarcely present on leaves from the *control* sites. This pattern might be explicative for the physiological status of the seagrass. Indeed, *P. oceanica* green leaves naturally turn to a yellow-brownish color as a result of photosynthetic pigments loss. This process of natural senescence typically starts at the tip and, over the time, extends to cover almost the entire leaf, which becomes shorter and dies back. At the same time, new green leaves appear on the inner side of the shoot. The natural leaf growth pattern is the result of a balance between internal physiological mechanisms and external abiotic factors (Ott 1980). It is likely that the stresses experienced by the seagrass chronically exposed to vents emissions have accelerated the leaf growth pattern, so that the ageing process can occur more rapidly in shoots from the vent sites. This hypothesis would be further supported by the exclusive presence of juvenile leaves in the shoots close to the volcanic vents, suggesting that *P. oceanica* might try to compensate for the faster ageing by producing new leaves more rapidly. Despite the increased turnover, these leaves remained shorter and thinner than leaves at the control sites, suggesting the additional carbon from volcanic origin is not fixed into more biomass, as previously reported for the seagrass *Cymodocea nodosa* at the Vulcano vents (Apostolaki et al. 2014a). As a consequence of lower foliar surface area as well as shoot density, the canopy of *P. oceanica* close to the vents was also lower; this reduction potentially affected the ability of the seagrass to raise the local pH and buffer the effects of ocean acidification naturally (Hendriks et al. 2014). Indeed, the biomass of epiphytes on seagrass leaves was dramatically reduced in the proximity of the vents, because of the lower foliar surface area available for epiphyte colonization, as well as the rarefaction of calcified epiphytes at low pH (Cox et al. 2015; Donnarumma et al. 2014; Guilini et al. 2017; Martin et al. 2008; Nogueira et al. 2017; S. Vizzini et al. 2017). Additionally, lower epiphyte biomass might have resulted from faster leaf turnover at the vent sites, since epiphyte colonization usually increases with leaf age and reaches its maximum values on older leaves in summer (see Piazzi, Balata, and Ceccherelli 2016 and references therein).

Epiphytes colonizing the leaf surfaces can potentially reduce seagrass productivity in low light regimes, whereas they can provide a degree of photo-protection in light saturated habitats (Alcoverro, Pérez, and

Romero 2004; Cebrián et al. 1999; Costa et al. 2015). Although we did not perform any tests to demonstrate seagrass photo-inhibition, we hypothesize that in stressful conditions such as those of vent systems, the increased exposure to ambient irradiance due to the lower epiphyte coverage impaired *P. oceanica,* whose growth seems more favourable under low light conditions (Dattolo et al. 2014). As a potential consequence of the stress caused by photo-inhibition, the ageing process accelerated and brown tissue became more prevalent in leaves at the vent sites, corresponding to our observations (Munné-Bosch and Alegre 2002; Zimmermann and Zentgraf 2005). However, further studies are required to better elucidate the effects of both vents emissions and increased exposure to light irradiance on the physiological mechanisms regulating *P. oceanica* leaf growth patterns.

Few predators directly consume *P. oceanica* leaf tissue, while the associated epiphytes represent the food source for a variety of organisms that are consequently attracted to the seagrass meadows (Marco-Méndez et al. 2015). Along with lower epiphyte abundance, we found a lower number of eroded leaf apexes in shoots close to the vents, suggesting a reduced exploitation of *P. oceanica* by grazers in acidified conditions, as recently observed at $CO_2$ Ischia vents (Nogueira et al. 2017). However, the role of ocean acidification on trophic cascades in *P. oceanica* meadows in vent systems require more investigation, since opposite trends have been reported at the same location (Ischia vents, Donnarumma et al. 2014). Further studies are required to analyse the nutritional quality of *P. oceanica* leaves since an increase in nutritional value (low C:N ratio) and a higher exploitation of the seagrass by herbivores are expected in vent systems (Apostolaki et al. 2014b; S. Vizzini et al. 2017)

Taken together, the seagrass parameters analysed in this study revealed a stressful response of seagrass beds close to the vents, where meadows were characterized by few shoots with shorter leaves, faster leaf turnover and lower epiphyte biomass on the foliar surface.

Our results suggest that it is possible that the key ecological role played by *P. oceanica* as a foundation species in coastal environments could be compromised in the future acidified oceans, with potential reduction of the elevated biodiversity seen in the *status quo*. The lower grazing pressure observed in this study also suggests a possible indirect propagation of the effects of ocean acidification into the food web supported by *P. oceanica* meadows, with unpredictable consequences on the stability and functioning of these ecosystems.

# Chapter 2

## Community composition of marine biofilms in the shallow-water vent system of Levante Bay (Vulcano-Aeolian Islands)

### 2.1 Introduction

Biofilms are typically defined as assemblages of microbial cells of single as well as multiple species, enclosed in a gelatinous matrix adhering to any living and inert surface (Donlan 2002). The formation of biofilms is a multi-step process where nude surfaces are initially conditioned with organic and inorganic molecules forming a primary film, which successively attracts the microbial cells. After adhesion to the surface, the organisms start producing slimy, glue-like extracellular polymers (EPS) forming the amorphous matrix surrounding their cells (Thomason and Dürr 2010). A complex, three dimensional structure including multiple layers of microcolonies (microbial cells and EPS) separated by interstitial channels characterizes mature biofilms, which constitute highly heterogenic and dynamic communities heavily attached to the surfaces (Garrett, Bhakoo, and Zhang 2008). Biofilms represent the predominant form of microbial life in the marine environments, ranging from the surface to the deep ocean, as well as in the water column where they constitute the precursor nucleus of marine snow. In the photic zone, bacteria and microalgae are the main organisms constituting marine biofilms, which include also microscopic fungi, heterotrophic flagellates and sessile ciliates (see Davey, George, and Toole 2000 and references therein).

The importance of biofilms in the ecology of benthic ecosystems is being recently recognized. Indeed, biofilms not only represent the main food source for a variety of grazers, but can actively control the development of benthic communities by influencing the settlement of algal spores and invertebrate larvae, including relevant aquaculture species such as *Mytilus galloprovincialis* (Bao et al. 2007; Hadfield 2011). Biofilms also provide valuable ecosystem services including primary production, nutrient recycling, organic matter degradation, sediment trapping (Bhaskar and Bhosle 2005; Ortega-Morales et al. 2010). Several substances with potential application in the industry of biotechnology are

isolated from microorganisms within biofilms, particularly from those living in extreme marine environments (Mancuso Nichols, Guezennec, and Bowman 2005). On the other hand, biofilms colonizing artificial surfaces such as oil and gas installations, aquaculture nets and ship hulls alter the physical and chemical properties of these structures, causing great economic losses in the maritime industry (Salta et al. 2013). Because of their ubiquity as well as ecological and economic relevance, the number of studies on marine biofilms in various research fields started growing.

In the recent years, marine biofilms have been investigated also in the context of climate change, including ocean acidification. In this regard, Lidbury and colleagues reported increased biomass as well as shifts in the assemblage of the community of biofilms along a natural $pCO_2$/pH gradient at shallow-water vents (Lidbury et al. 2012). In the same area, cholorophyll-a concentration in microphytobenthos communities were higher at low pH (7.9) compared to control pH (8.1), with changes in the composition of benthic diatom assemblages observed on both artificial (V. R. Johnson et al. 2013) as well as natural surfaces (V. Johnson et al. 2015). Although some changes in the community composition of biofilms could be the explained by taxa specific response to artificial as well as natural acidification (Taylor et al. 2014; Witt et al. 2011), microbial activities such as oxygen production would not be affected by ocean acidification (Witt et al. 2011).

More recently, Hassenruck and colleagues demonstrated that the diversity of mature biofilms in coral reef systems was scarcely influenced by pH changes related to climate changes, whereas other biotic and abiotic factors such as light exposure and grazing intensity controlled the biofilms community which, in turn, conditioned the settlement of coral larvae (Hassenrück et al. 2017).

pH variations occur naturally in the aquatic environments and are well tolerated by microorganisms, leading to the hypothesis that the degree of pH variations due to anthropogenic ocean acidification might not affect microorganisms, overall (Joint, Doney, and Karl 2011).

In order to increase the knowledge about the response of marine biofilms to ocean acidification, a colonization experiment was conducted in the shallow-water hydrothermal vent system of Levante Bay (Vulcano, Aeolian Island), a site considered as analogue of future acidified oceans (Boatta et al. 2013). The aim of this study was to evaluate the influence of hydrothermal vents emissions on the diversity and

composition of marine biofilms in subtidal systems, based on the hypothesis that the natural acidification determined by the vents emissions could affect the structure of the biofilms.

## 2.2 Materials and Methods

### 2.2.1 Study area

Vulcano Island is an active volcano belonging to the archipelago of Aeolian Islands located in the Southern Tyrrenian Sea (Mediterranean Sea, fig. 1). Since its last eruption (1888-1890), the volcano has been in state of solfataric activity, characterized by the presence of both aerial as well as submerged fumaroles mainly releasing $CO_2$ for a total of 482 t $day^{-1}$, along with $H_2S$ and other gas species to a lesser extent (Inguaggiato et al. 2012).

In Levante Bay, small fumarolic emissions occur offshore at very shallow depths (<10 m). Volcanic emissions are visible as bubble trains rising from the vents and are dominated by $CO_2$ (98-99% vol of $CO_2$), for a total estimated value of 3.6 t $day^{-1}$ of $CO_2$ (Inguaggiato et al. 2012). In the most southern point of Levante Bay, bubbling gas discharges are also characterized by a variable concentration of $H_2S$ (1.57 to 2.47% from Carapezza et al. 2011), probably derived by alkaline hydrolysis of metal sulfides promoted by weakly acidic waters (B. Capaccioni, Tassi, and Vaselli 2001). However, the concentration of $H_2S$ decreases with the distance from the vents and only a small portion of the gas enters into the aqueous phase, where it oxidizes to the non-toxic sulphate due to the high $O_2$ saturation recorded in the bay, particularly in the northern area (Boatta et al. 2013).

Due to the $CO_2$ vents in the southern part of the Levante Bay, a pH gradient (5.65 – 8.1) runs parallel to the north-eastern coast of the island, with $pCO_2$ ranging from 3361.7 ± 2971.3 µatm to 424.6 ± 61.5 µatm (Boatta 2012).

After a preliminary survey assessing the physicochemical parameters of Levante Bay, four sites were selected as suitable stations to conduct the biofilm colonization experiment (fig. 1): Vent 1 (38°24'59.05"N, 14°57'38.76"E), the main venting area characterized by intense gas flux containing both $CO_2$ and $H_2S$; Vent 2 (38°25'9.40"N, 14°57'42.14"E) about 330m north of the main venting area,

characterized by $CO_2$-dominated vents; REF 1 (38°25'14.33"N, 14°57'52.94"E) and REF 2 (38°25'17.12"N, 14°57'56.77"E.), about 560m and 760m north of the main venting area respectively, used as reference sites because of the absence of vent emissions.

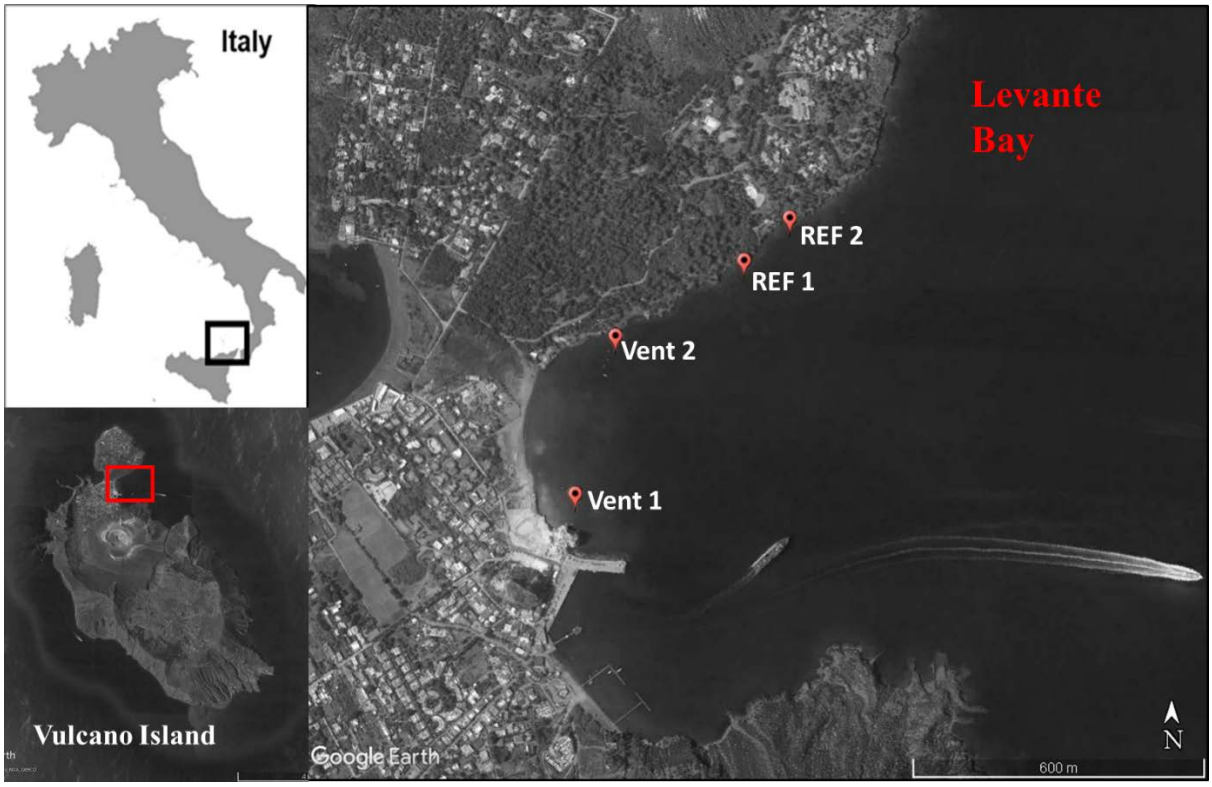


**Figure 1.** Map indicating the location of the study (Vulcano Island-Levante Bay) and the four sites chosen for the experiments.

### 2.2.2 Experimental design and sampling procedure

The taxonomic composition of marine biofilm communities along the $pCO_2$/pH gradient was assessed through a colonization experiment that took place between October and December 2016 in Levante Bay. Physicochemical parameters (temperature, pH and salinity) were recorded over the duration of the experiment.

Microscope glass slides (pre-washed with 30% hydrogen peroxide) were used as sterile substrate for the biofilm colonization. Slides were assembled into a satellite-like structure connected to a float and, on the opposite side, a dead weight to secure the full structure to the sea bottom (fig. 2). The substrates

were deployed at 3 m in the four sites across the bay (Vent 1, Vent 2, REF 1 and REF 2). Three structures (replicates n=3) were collected from each site at two times: t1 (17 days after the deployment) and t2 (57 days after the deployment, corresponding to the end of the experiment (fig. 3).

Immediately after collection, the glass slides were carefully disassembled from the structures and stored in RNA Later (Thermofisher) at -20°C until laboratory analysis.

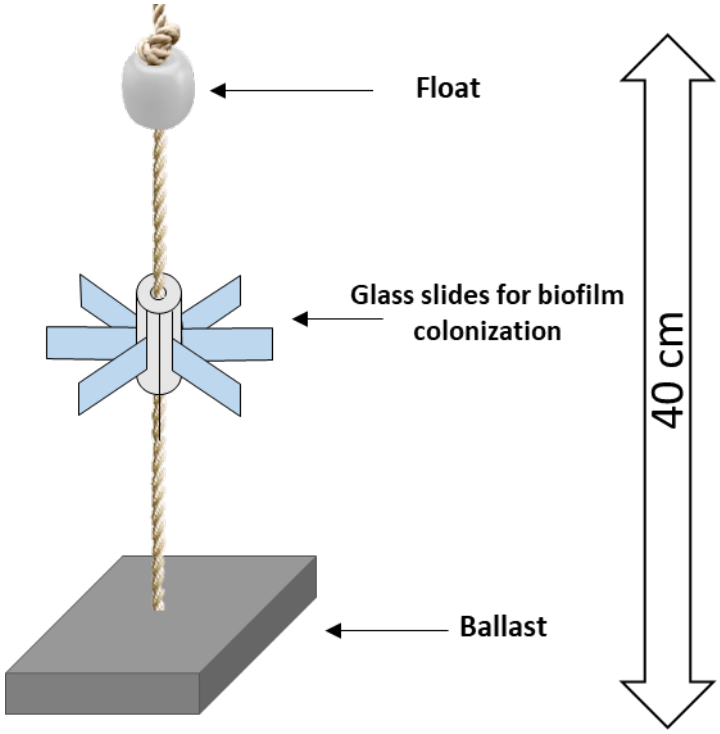


**Figure 2.** Scheme of the satellite-like structure used for the experiment.

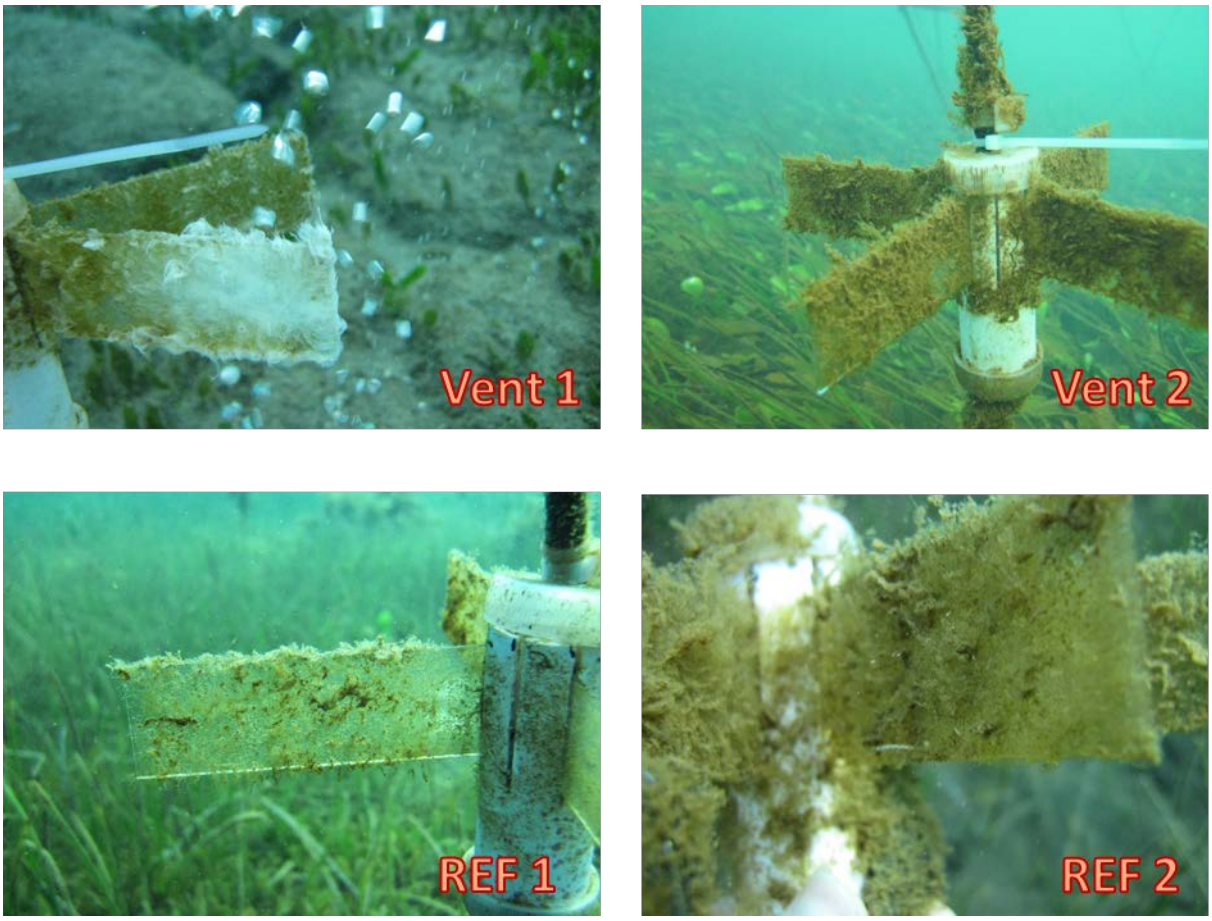


**Figure 3.** Photos of the biofilms collected from different sites in Levante Bay (Vulcano) at the end of the experiment.

### 2.2.3 Analytical methods

In order to assess the diversity and composition of prokaryotic biofilm communities from Levante Bay experiments, molecular and bioinformatic analyses were performed.

**Molecular analysis: 16S RNA extraction, amplification and sequencing.** RNA was extracted from RNA Later-stored biofilms using a phenol:chloroform extraction protocol. Briefly, 0.5g of biofilm sample were added with 850 µl of extraction buffer (50 mM Tris-HCl, 20 mM EDTA, 100 mM NaCl; pH 8.0) and 100 µl of lysozyme (100mg/ml). After incubation at 37°C for 30 minutes, samples were supplemented with 5 µl of proteinase K (20mg/ml) and incubated again as previously. This mix was then supplemented with 50 µl of SDS (20%) and incubated in a water bath at 65°C for 1 hour. RNA was extracted in a series of phenol:chloroform:isoamylalcohol (25:24:21, pH 4.3) and

chloroform:isoamylalcohol (24:21) extractions. Overnight precipitation of the extracted supernatant was performed using 3M sodium acetate and isopropanol. The precipitated sample was washed twice with 70% ice cold ethanol and resuspended in ultrapure water.

A DNAse treatment was performed in order to remove any carryover DNA from the extracted samples, using the TURBO DNAse kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The resulting RNA was used as template in a reverse transcription reaction generating cDNA by using the cDNA synthesis kit (Invitrogen, Carlsbad, CA), following the specifications of the manufacturer.

The quality of the cDNA was assessed after polymerase chain reaction amplification of 16S rRNA gene using primers Bact 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and Univ 519R (5'-ATTACCGCGGCTGCTGG- 3').

The diversity of biofilm communities from Levante Bay was evaluated by amplifying the variable 4 (V4) region of 16S rRNA transcripts using the prokaryotic universal primers (515f 5′-GTG CCA GCM GCC GCG GTA A-3′ and 806r 5′-GGA CTA CVS GGGTAT CTA AT-3′), and the HotStarTaq Plus Master Mix Kit (Qiagen, USA), under the following conditions: 94°C for 3 minutes, followed by 30 cycles at 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed.

16S rRNA amplicons were sequenced using a PMG Ion Torrent platform at the Molecular Research LP facility (Shallowater, TX, USA). Multiple PCR reactions were combined to reduce potential bias.

At the research facility, sequences were depleted of barcodes and primers, then sequences <150bp were removed along with sequences with ambiguous base calls and with homopolymer runs >6bp.

**Bioinformatics**. The 16S rRNA sequence analysis was conducted using the QIIME 1.9 software package (Caporaso et al. 2010). Chimeric sequences were removed using ChimeraSlayer (Haas et al. 2011). Operational Taxonomic Units (OTUs) were picked at 97% similarity using the "pick_open_reference_otus.py". Clustering of OTUs was performed using the 2013 Greengenes database. Taxonomic classification of clustered OTUs was done using the Ribosomal Database Project Classifier against the 2013 Greengene database (Wang et al. 2007).

### 2.2.4 Statistical analysis

The taxonomic composition of biofilms collected from Levante Bay were tested through Permutational Analysis of Variance (PERMANOVA) at different levels (Philum, Class, Genus), using a multivariate approach. Statistical differences in the physicochemical characterization of the sites were tested trough Permanova at univariate level. In all cases, a 4 x 2 orthogonal design with “Site” and “Time” set as fixed factors was used to conduct the analysis. The effect of the interaction of the two factors was tested as well. Pair-wise tests were performed on significant results. Similarities among 16S RNA transcripts was visualized using non-metric multidimensional scale (nMDS) coupled with Cluster analysis. The PRIMER+PERMANOVA v6 software package (Plymouth Marine Laboratory, UK) was used to perform the statistical elaborations.

## 2.3 Results

Environmental parameters of Levante Bay were measured frequently (at least once every two weeks) along the duration of the experiment (table 1). Temperature ranged from 18.51°C to 22.74°C, showing similar values among sites and decreasing significantly over time (table 2). pH values, ranging from 6.70 to 8.28. showed significant differences between the vent and reference sites, as well as between the vent sites 1 and 2 (table 2). Salinity was the only physicochemical parameter that remained relatively constant over time and showed similar values across all sites (table 2).

**Table 1**. Measurement of physicochemical parameters of the sites along the duration of the experiment. Values are reported as mean ± standard deviation for each site in each month.

| **October** | | | | |
|---|---|---|---|---|
| | **Vent 1** | **Vent 2** | **REF 1** | **REF 2** |
| **T [°C]** | 22.74 ± 0.35 | 22.58 ± 0.08 | 22.75 ± 0.37 | 22.65 ± 0.23 |
| **pH [unit]** | 7.39 ± 0.49 | 8.03 ± 0.13 | 8.18 ± 0.04 | 8.28 ± 0.14 |
| **Sal.[psu]** | 38.40 ± 0.00 | 38.38 ± 0.04 | 38.35 ± 0.14 | 38.41 ± 0.03 |
| **November** | | | | |
| | **Vent 1** | **Vent 2** | **REF 1** | **REF 2** |
| **T [°C]** | 21.02 ± 1.20 | 20.91 ± 1.14 | 20.88 ± 1.12 | 20.86 ± 1.09 |
| **pH [unit]** | 7.14 ± 0.46 | 7.88 ± 0.25 | 8.10 ± 0.09 | 8.14 ± 0.08 |
| **Sal.[psu]** | 38.39 ± 0.06 | 38.39 ± 0.04 | 37.64 ± 1.36 | 38.22 ± 0.32 |
| **December** | | | | |
| | **Vent 1** | **Vent 2** | **REF 1** | **REF 2** |
| **T [°C]** | 18.51 ± 0.36 | 18.35 ± 0.61 | 18.28 ± 0.63 | 18.26 ± 0.63 |
| **pH [unit]** | 6.70 ± 0.77 | 7.89 ± 0.03 | 8.07 ± 0.04 | 8.15 ± 0.04 |
| **Sal.[psu]** | 38.27 ± 0.01 | 38.27 ± 0.02 | 38.25 ± 0.06 | 38.28 ± 0.06 |

**Table 2**. Permanova analysis at univariate level on the physicochemical variable's values detected in the experimental sites (Vent 1, Vent 2, REF 1, REF 2) along the duration of the experiment (Oct = October; Nov = November; Dec = December). Significant results are in highlighted. Pair-wise tests were conducted after significant P(perm) results.

| | Temperature [°C] | | | | | pH [unit] | | | | | Salinity [psu] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** | **df** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** | **df** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** |
| **Site** | 3 | 1.33E+02 | 4.67E+02 | 0.982 | 998 | 3 | 6.41E+01 | 20,766 | **0.001** | 997 | 3 | 4.84E-01 | 0.40799 | 0.752 | 998 |
| **Time** | 2 | 12,621 | 44,293 | **0.001** | 998 | 2 | 50 | 16,247 | 0.201 | 999 | 2 | 1 | 0.47436 | 0.69 | 998 |
| **Site x Time** | 6 | 2.57E+01 | 9.02E+00 | 1 | 997 | 6 | 1.71E+01 | 0.55303 | 0.77 | 999 | 6 | 4.70E-01 | 0.39607 | 0.834 | 999 |
| **Residuals** | 20 | 0.28495 | | | | 20 | 30.887 | | | | 20 | 11858 | | | |
| **Pair-wise tests** | | **Groups** | **t** | **P(perm)** | **Unique perms** | | **Groups** | **t** | **P(perm)** | **Unique perms** | | | | | |
| | | Oct, Nov | 40,801 | **0.003** | 996 | | Vent 1, Vent 2 | 38,933 | **0.004** | 993 | | | | | |
| | | Oct, Dec | 19,311 | **0.001** | 984 | | Vent 1, REF 1 | 49,501 | **0.001** | 995 | | | | | |
| | | Nov, Dec | 57,672 | **0.001** | 999 | | Vent 1, REF 2 | 52,579 | **0.001** | 999 | | | | | |
| | | | | | | | Vent 2, REF 1 | 22,987 | **0.043** | 998 | | | | | |
| | | | | | | | Vent 2, REF 2 | 30,918 | **0.007** | 998 | | | | | |
| | | | | | | | REF 1, REF 2 | 16,853 | 0.119 | 996 | | | | | |

In the biofilms collected from Levante Bay, up to >98% of the OTUs in the community were affiliated with the domain Bacteria, while the remaining OTUs were associated to Archea and unclassified sequences. Overall, the most abundant bacterial phyla across all samples were Proteobacteria, Cyanobacteria and Bacteroidetes. Taxa such as Planctomycetes, Actinobacteria, Unclassified Bacteria, Firmicutes and Verrucomicrobia were also found, although their abundance in the community was relatively low (fig. 4) Multivariate analysis at this taxonomic level revealed, overall, significant differences between the community composition of biofilms from the vent site 1 vs biofilms from the vent site 2 as well as control site 1 (table 3).

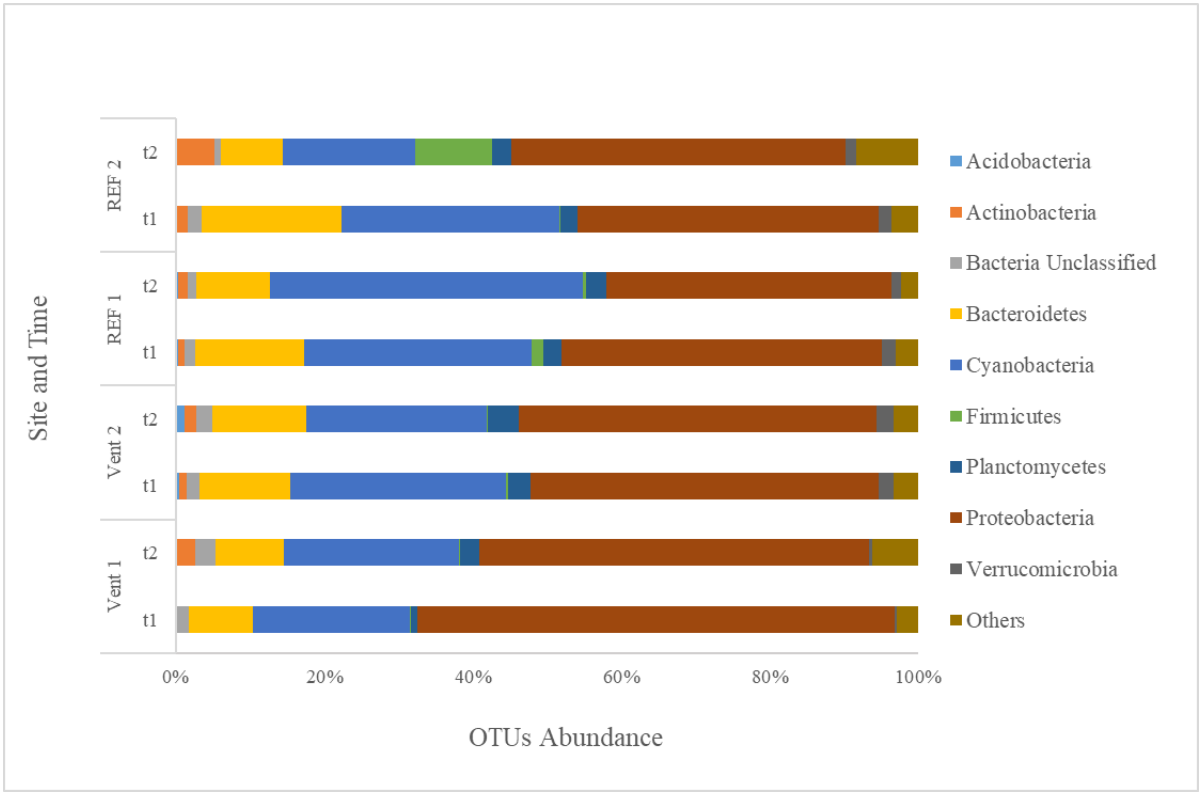


**Figure 4**. Philum level distribution of 16S rRNA transcript sequences recovered from the biofilms in Levante Bay. Phylogenetic categories representing phila that account for at least 1% (on average) of the overall abundance in all samples are shown.

At the Class level (fig. 5), Gammaproteobacteria were numerically dominant in the site Vent 1 at the beginning of the experiment (45.8% on average at t1), and were followed by Chloroplast (19.9% on average) and Alphaproteobacteria (8.1% on average). Other abundant groups in this site at experimental time t1 were Epsilonproteobacteria and Deltaproteobacteria (4.8% and 3.7%). At the end of the experiment (t2), the abundance of Gammaproteobacteria in Vent 1 decreased considerably (22% on average), while Alphaproteobacteria doubled their abundance (16.5% on average); Chloroplast, Deltaproteobacteria and Epsilonproteobacteria slightly increased towards the end of the experiment (t2, 23.1%, 5.7%, 6.2% on average respectively).

The biofilm community in Vent 2 was initially dominated by Chloroplast and Gammaproteobacteria (25.5% and 23.5% on average, respectively at t1), followed by Alpha- and Deltaproteobacteria (15.4% and 5.6% on average, respectively). After 57 days (t2), the community composition remained substantially similar overall, with slight decreases in the relative abundance of Chloroplast and Gammaproteobacteria (22.5% and 22.9% on average, respectively), and minor increases in Alpha- and Deltaproteobacteria (17% and 6.2% on average, respectively).

In REF 1, 16S rRNA transcripts from Chloroplast were always numerically dominant in the community, with their abundance increasing over the time (from 26.1% to 38.1% on average, respectively for t1 and t2). Alphaproteobacteria were the second most abundant bacterial group in this site, and remained relatively constant during the experiment (24.5% and 24.1% on average, respectively for t1 and t2). The abundance of Gammaproteobacteria in REF 1 was lower compared to the other groups in the same site, as well as compared to the abundance of Gammaproteobacteria in both Vent sites. The abundance of this taxonomic group decreased over the duration of the experiment (from 12.5% at t1, to 9.6% at t2 on average). A similar trend was observed for Deltaproteobacteria (4.3% and 3.7% on average, at t1 and t2 respectively). On the other side, Cytophagia were numerically higher in this site compared to Vent 1 and Vent 2, although their abundance slightly decreased over the time (from 5.2% at t1, to 3.9% at t2 on average).

The composition of the biofilm community in REF 2 was similar to REF 1 initially (t1), with Chloroplast constituting 25.9% (on average) of the community, followed by Alphaproteobacteria (20.3% on average), Gammaproteobacteria (14.8% on average), Cytophagia (9.2% on average) and

Deltaproteobacteria (4.1% on average). At the end of the experiment (t2), the relative abundance of Chloroplast and Gammaproteobacteria decreased (16.9% and 11% on average, respectively), as well as those of Cytophagia and Deltaproteobacteria (2.1% and 2.5% on average, respectively), whereas Alphaproteobacteria remained relatively constant (20.6% on average). On the other side, Bacilli and Betaproteobacteria, which were scarcely present at the beginning of the experiment (t1), increased numerically in mature biofilms (t2), reaching 10.3% and 10% (on average) of the overall abundance of the community, respectively.

At the Class level, the community of biofilms was significantly different in Vent 1 compared to all the other sites, whereas biofilms of Vent 2 were significantly different compared to biofilms of REF 1 (table 3).

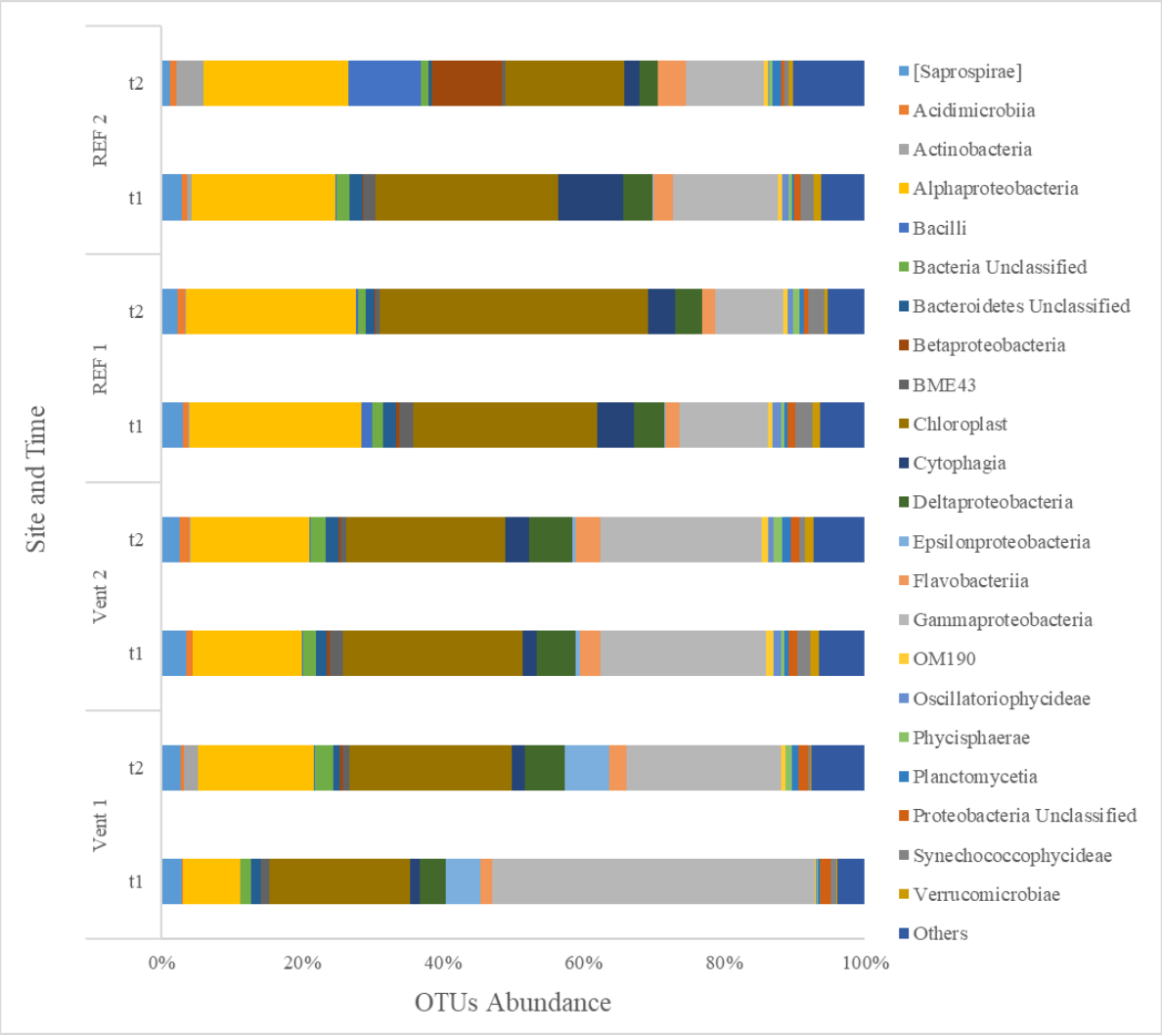


**Figure 5**. Class level distribution of 16S rRNA transcript sequences recovered from the biofilms in Levante Bay. Phylogenetic categories representing classes that account for at least 1% (on average) of the overall abundance in all samples are shown.

At the Genus level (fig. 6), sequences related to CF-26 were the most abundant (23.3% on average) in young biofilms (t1) in Vent 1; the other abundant groups were unclassified sequences of Stramenopiles and Gammaproteobacteria, representing respectively 19.3% and 14% (on average) of the overall abundance, followed by unclassified sequences affiliated with the families Rhodobacteraceae and Helicobacteraceae (4.2% and 3.7% on average, respectively), and *Marithrix* (2.4% on average). At the end of the experiment (t2), the relative abundance of these groups changed: CF-26 abundance decreased to 9.1% (on average); *Marithrix* and unclassified sequences of Gammaproteobacteria decreased to 0.7%

and 6% (on average, respectively), while the relative abundance of Unclassified Stramenopiles as well as of taxa affiliated with Rhodobacteraceae and Helicobacteraceae families increased (22.5%, 7.6% and 5% on average, respectively).

Unclassified Stramenopiles constituted the 24.7% on average of the overall abundance of young biofilms (t1) in Vent 2. Unclassified Gammaproteobacteria were the second most abundant group (6.9% on average), followed by unclassified Rhodobacteraceae (5.7% on average), *Pseudoalteromonas* (4% on average) and unclassified Alphaproteobacteria (3.2% on average). In the same site, the mature biofilm community (t2) showed a similar taxa assemblage, with the exception of *Pseudoalteromonas* whose abundance decreased considerably (<0.01% on average).

Chloroplast-related 16S rRNA transcripts from unclassified Stramenopiles constituted the major group in REF 1, with their abundance increasing from 25.1% to 37.4% over the time. Unclassified Hyphomicrobiaceae and unclassified Rhodobacteraceae were also relatively abundant, although the former decreased (from 9.5% to 5.6% on average) and the latter remained relatively constant during the experiment (from 8.1% to 8.3% on average).

The most abundant groups at the beginning of the experiment (t1) in REF 2 were unclassified Stramenopiles (24.8% on average) and unclassified Rhodobacteraceae (7.3% on average), followed by unclassified Gammaproteobacteria (5.1%), SC3-56 (5% on average) and unclassified Hyphomicrobiaceae (4.4% on average). At the end of the experiment, all these groups decreased in their abundance, while other taxa such as *Salinicoccus*, *Staphylococcus*, unclassified Alphaproteobacteria and unclassified Comamonadaceae increased greatly (6.9%, 3.1%, 5.9% and 4.8% on average, respectively).

Permanova analysis of the community composition at the highest taxonomic level (Genus) revealed highly significant differences for the factor “Site”. “Time” was also significant for the community structure, although to a lesser extent (table 3). Pair-wise tests at the level of factor “Site” revealed significant differences between Vent 1 vs all the other sites, as well between Vent 2 and REF 1 (table 3).

Similarity among communities from different sites at the genus level is shown by the nMDS plot (fig. 7), based on Bray-Curtis similarity calculated on the occurrence of sequences from OTU table. The nMDS ordination plot, coupled with Cluster analysis at 70% similarity level, showed the separation of

two main groups: samples from Vent 1 formed a unique cluster, opposite to the majority of samples from the other sites forming a unique group. Four samples (one from each site) clustered indipendently. n-MDS 2-D stress was 0.1.

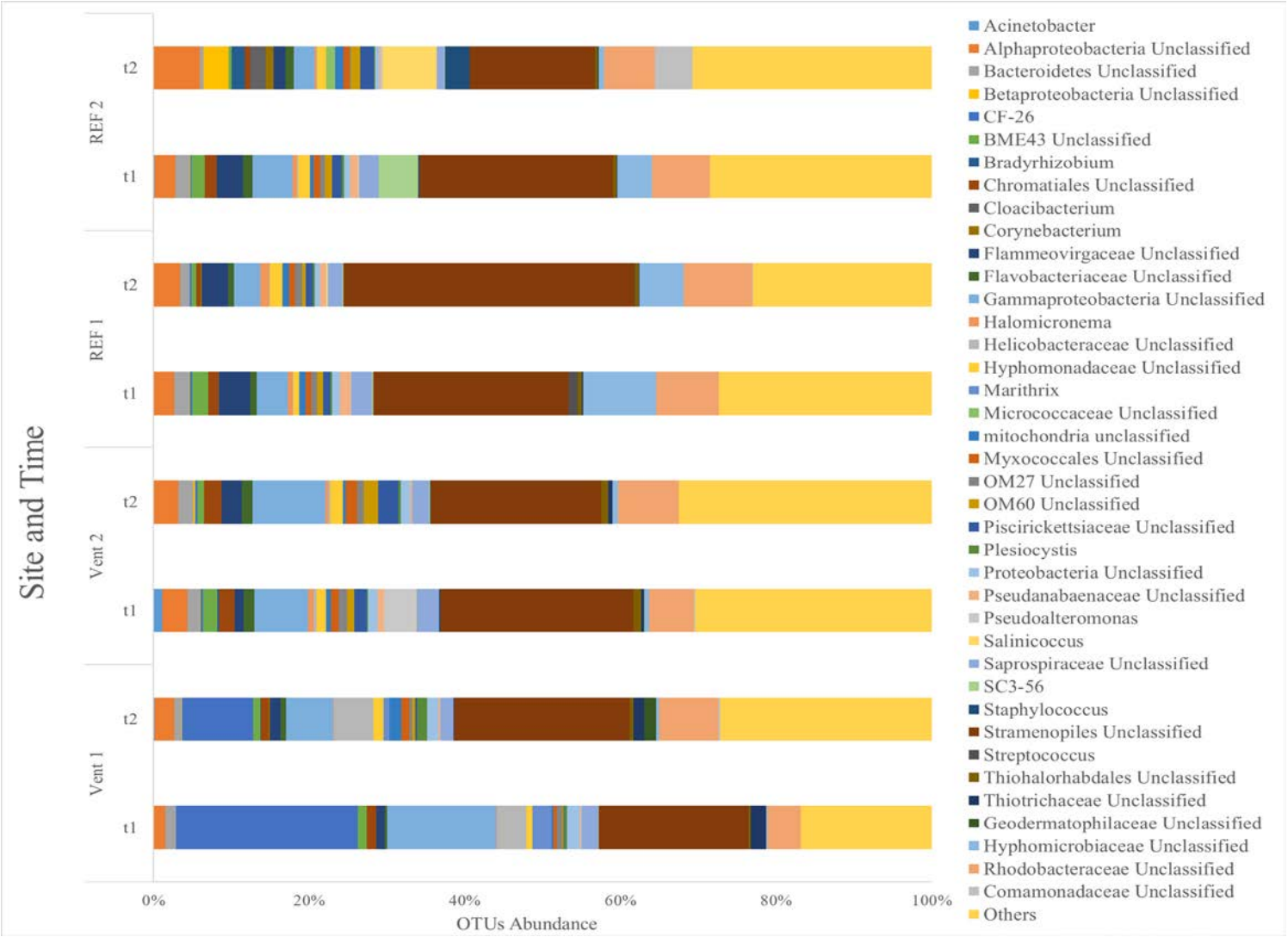


**Figure 6**. Genus level distribution of 16S rRNA transcript sequences recovered from biofilms in Levante Bay. Phylogenetic categories representing genera that account for at least 1% (on average) of the overall abundance in all samples are shown.

**Table 3**. Permanova analysis at multivariate level on the community assemblages of biofilms. Pair-wise test showed comparison between sites (Vent 1, Vent 2, REF 1, REF 2) and times (t1 and t2). Significant results are highlithed. Pair-wise tests were conducted after significant P(perm) results.

| | Philum | | | | | Class | | | | | Genus | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** | **df** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** | **df** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** |
| **Site** | 3 | 415.71 | 2.2684 | **0.007** | 997 | 3 | 1056.4 | 3.1224 | **0.001** | 999 | 3 | 1987.8 | 30.139 | **0.001** | 999 |
| **Time** | 1 | 301.28 | 1.644 | 0.17 | 999 | 1 | 806.17 | 2.3829 | 0.055 | 997 | 1 | 1423.3 | 21.579 | 0.049 | 997 |
| **Site x Time** | 3 | 230.17 | 1.2559 | 0.251 | 998 | 3 | 375.41 | 1.1097 | 0.366 | 998 | 3 | 769.23 | 11.663 | 0.29 | 998 |
| **Residuals** | 16 | | | | | 16 | | | | | 16 | | | | |
| | | | | | | | | | | | | | | | |
| **Groups** | | | **t** | **P(perm)** | **Unique perms** | | | **t** | **P(perm)** | **Unique perms** | | | **t** | **P(perm)** | **Unique perms** |
| **Vent 1, Vent 2** | | | 2.1857 | **0.003** | 984 | | | **2.4654** | 0.004 | 987 | | | 25748 | **0.002** | 985 |
| **Vent 1, REF 1** | | | 3.0676 | **0.001** | 986 | | | **3.5595** | 0.001 | 979 | | | 32537 | **0.004** | 986 |
| **Vent 1, REF 2** | | | 1.2473 | **0.231** | 988 | | | **1.7276** | 0.02 | 982 | | | 17742 | **0.004** | 986 |
| **Vent 2, REF 1** | | | 1.5517 | **0.063** | 983 | | | **1.902** | 0.003 | 989 | | | 1763 | **0.006** | 988 |
| **Vent 2, REF 2** | | | 1.1193 | 0.313 | 984 | | | 1.2403 | 0.211 | 987 | | | 10777 | 0.351 | 992 |
| **REF 1, REF 2** | | | 1.1841 | 0.277 | 989 | | | 1.2193 | 0.25 | 981 | | | 10724 | 0.354 | 983 |

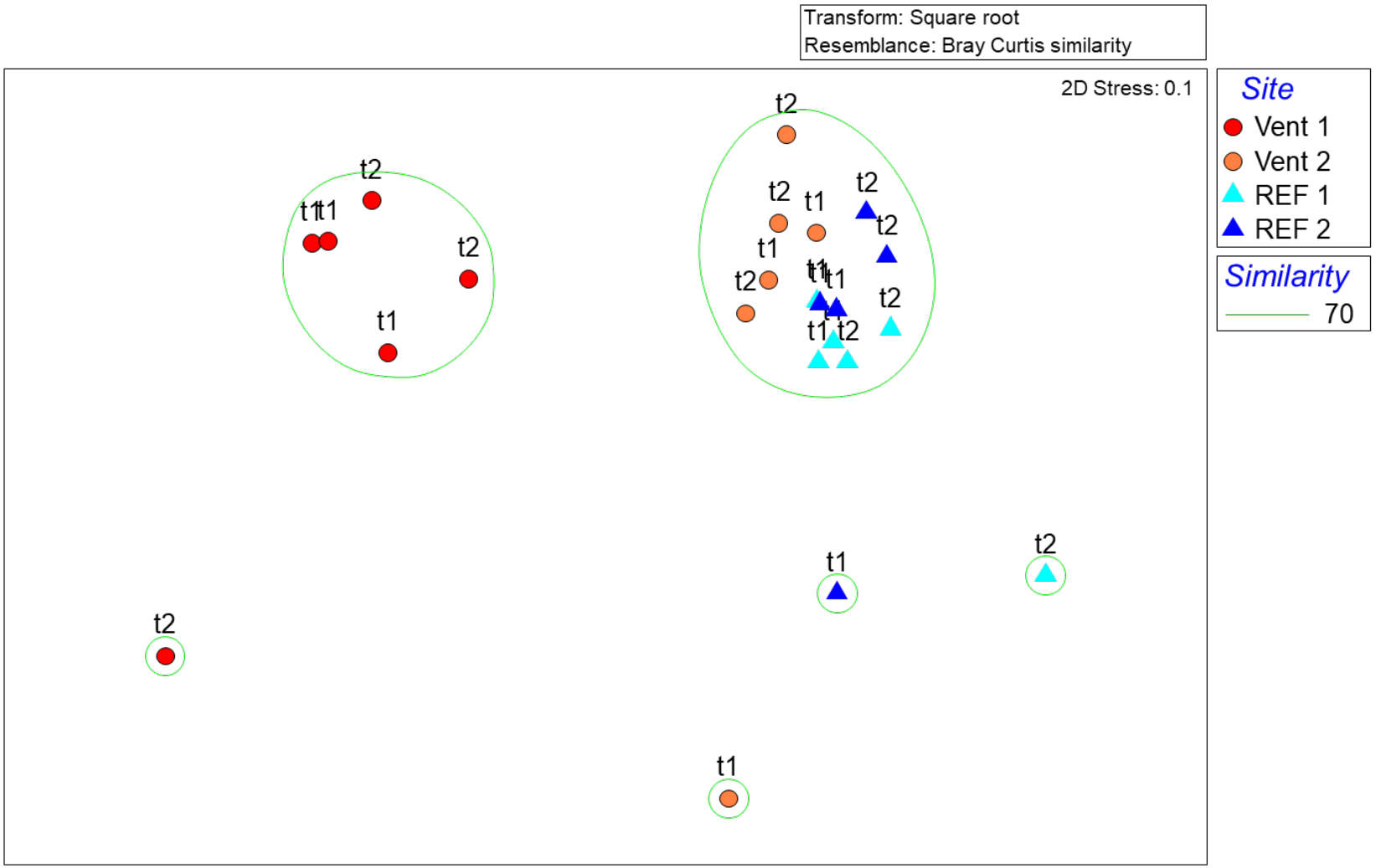


**Figure 7**. Nonmetric Multidimensional Scaling (nMDS) plot of biofilms 16S rRNA transcript sequences similarities among the four sites in Levante Bay.

## 2.4 Discussion

Marine biofilms are key components of benthic communities and provide relevant ecosystem services. However, their response to ocean acidification has not been clarified yet. This study aimed at filling this gap by investigating the community composition of biofilms exposed to natural acidification in a Mediterranean shallow-water vent system.

In this investigation on the diversity of 16S rRNA transcripts from marine biofilms, Diatoms were taxonomically identified as Chloroplast/Unclassified Stramenopiles within the phylum Cyanobacteria, as the genomes of eukaryotic plastid contain Cyanobacteria-related 16S rRNA genes due to their prokaryotic origin (Margulis 1970).

Overall, three distinct bacterial communities characterized the biofilms collected during the experiment, reflecting the environmental conditions of the Levante Bay shallow-water vents system.

Biofilms in reference sites 1 and 2 showed an assemblage typical for bacterial communities in marine environments, where Alphaproteobacteria (Rhodobacteraceae), Bacteroidetes (Cytophagia and Flavobacteria), Cyanobacteria and Diatoms are usually abundant. These communities were relatively stable in their composition over time, except in the reference site 2, where biofilms at the end of the experiment were characterized by elevated abundance of Bacilli, in particular *Salinicoccus* and *Staphylococcus* taxa, as well as members of Comamonadaceae family (Betaproteobacteria). Since these taxa typically inhabit sediments, wastewaters and activated sludge (Rosenberg 2013), their presence may indicate that a contamination from sewage or runoff from terrestrial sources might have occurred in this site before the end of the experiment, causing the shifts observed in the community.

In acidified conditions simulating the future oceanic scenario (site Vent 2), Gammaproteobacteria reached higher abundance compared to control sites, while Diatoms, Alphaproteobacteria and Bacteroidetes decreased. Single taxa response to natural acidification was highly variable and for some taxa partially in contrast with previous observations on intertidal biofilm communities from the same area (Taylor et al. 2014). These differences could be attributed to seasonal (autumn = this study; spring = Taylor et al 2014), as well as habitat (subtidal = this study; intertidal = Taylor et al 2014) effects, as marine biofilms are dynamic complex entities and rapidly respond to environmental variations. In

addition, $CO_2$ vents are highly variable environments and the effect of other variables cannot be completely excluded (Vizzini et al. 2013). Indeed, changes in the physicochemical parameters occurred over time, particularly after a strong stormy event occurred in the area at the end of November. The mixing of waters in this occasion might have temporarily affected the $pCO_2$/pH gradient in the bay and consequently the assemblage of the communities.

Among the bacterial taxa that reached higher abundance in the site Vent 2 compared to the reference sites, *Pseudoalteromonas* was one of the most evident. This taxa, belonging to Gammaproteobacteria, has been recently recognized for its ecological significance, being a highly effective biofilm former that greatly affect either positively or negatively the structure of benthic communities through the production of several low and high molecular weight compounds (Bowman 2007). It is possible that *Pseudoalteromonas* took a competitive advantage in low pH conditions by excreting more extracellular substances (Engel et al. 2014; Lidbury et al. 2012). Diatoms, along with Cyanobacteria, have been indicated as potential "winners" in the future high-$CO_2$ world, especially for the advantage they can gain by regulating their Carbon Concentrating Mechanisms (Raven et al. 2012; Sandrini et al. 2014). Contrary to expectations, the abundance of Diatoms (Stramenopiles Unclassified) did not increase in low pH conditions. It is also possible that these taxa were subjected to a selective predatory pressure, while bacteria such as *Pseudoalteromonas* could resist to grazing by secreting toxic compounds including violacein (Matz et al. 2008).

Biofilms from Vent 1 showed a unique assemblage characterized by elevated abundance of Gammaproteobacteria and, to a lesser extent, Epsilonproteobacteria. Both these taxa are usually dominant in microbial communities of shallow and deep-sea hydrothermal vents. Gammaproteobacteria are usually more prevalent in lower sulfide habitats, while Epsilonproteobacteria dominate in higher sulfide habitats (Giovannelli et al. 2013; O'Brien et al. 2015). The relatively higher abundance of *Thiotrix*-related CF-26 and *Marithrix* (Gammaproteobacteria) compared to unclassified Helicobacteraceae (Epsilonproteobacteria) is likely due to the lower concentration of sulfide at Vent 1 compared to other geothermal habitats colonized by these organisms, such as deep-sea hydrothermal vents. Slides collected from Vent 1 appeared covered with white filaments, possibly indicating the presence of *Thiothrix*-like bacteria, as well as brown mats, which were mainly constituted of

Cyanobacteria (unclassified Stramenopiles). The simultaneous $H_2S$ and $CO_2$ inputs from hydrothermal vents allowed the assemblage of a mixed community where the distribution of organisms with different metabolic strategies followed a geochemical gradient. Indeed, the micro-spatial variation of these two kind of biofilms on the same slide was exceptionally evident: the portion of substrate that was directly exposed to the hydrothermal emissions was covered with white chemotrophic biofilms; the rest of the surface, which was less exposed to the vent emissions, was covered with brown mixed photo- and heterotrophic biofilms (fig. 3). Similar community assemblages were previously found in sediments and fluids from nearby shallow-water vent systems (Lentini et al. 2014).

The results of this study showed important shifts in the assemblage of biofilm community along a natural $pCO_2$/pH gradient. Although single taxa response was highly specific and in some cases in contrast with previous findings, the overall community assemblage was significantly affected by the exposure to the vent emissions, providing evidences that anthropogenic ocean acidification can affect these microbial communities.

Based on the analysis of the community composition of biofilms from this study, Gammaproteobacteria can be identified as potential "winners" in subtidal biofilms of the future high-$CO_2$ world, while other taxa such as Alphaproteobacteria and Bacteroidetes result as potential "looser". Although shallow-water vents are recognized as natural laboratories for testing the effects of anthropogenic ocean acidification on marine biota, the influence of other environmental variables such as inputs of metals and nutrients through the vent emissions cannot be excluded (S. Vizzini et al. 2013). Moreover, natural variability also in relation to weather events can affect the output of investigations conducted in natural environments. Studies of the response of marine communities, including biofilms, to climate changes cannot ignore the effects of biotic factors such as interspecific interactions (Russell et al. 2012). Future investigations of the response of biofilm communities to ocean acidification should focus on the effects of multiple abiotic as well as biotic drivers, in order to provide more realistic predictions on the diversity and functioning of these communities in the future climate context.

# Chapter 3

## Effects of bottom-up and top-down forces in the biochemical and community composition of biofilms along a *p*$CO_2$/pH gradient

### 3.1 Introduction

Elevated $CO_2$ concentrations resulting from anthropogenic activities are causing changes not only in the atmosphere but also in the oceans, where the pH has already decreased by 0.1 units (Ken Caldeira and Wickett 2003). Unless mitigation measures will be pursued, these changes are expected to dramatically increase in the coming centuries (Bopp et al. 2013), as well as to combine with concurrent regional changes, especially in coastal areas (Boyd and Hutchins 2012; Russell and Connell 2012).

The response of marine organisms to environmental changes is influenced not only by the climate variation itself, but also by the interactions taking place among organisms (Kroeker, Kordas, & Harley, 2017). Although studies on single-species response in the context of global changes are valuable, they are not sufficient for making predictions at a bigger ecosystem scale. Investigations at the level of community including the effects of both biotic factors (competition, predation, etc.) as well as environmental changes (acidification, pollution, etc.), are strongly recommended in this regard (Russell et al. 2012).

In marine coastal ecosystems, microbial biofilms play a key role by initiating biological colonization on new surfaces, contributing to primary production and representing a major food source for a variety of grazers (Jenkins et al., 2001; Qian et al.,2007). Despite their ecological importance, the mechanisms regulating the composition and potential functioning of marine biofilms in the context of climate change are still poorly known. Shifts in the community composition of biofilms were attributed to lowered pH in natural as well as artificial environments (Lidbury et al. 2012; Taylor et al. 2014; Witt et al. 2011). Even though microorganisms could potentially adapt to abrupt environmental changes, microbe-driven processes such as primary productivity, nutrient cycling, bacterial respiration, etc. could be affected (Das and Mangwani 2015; Joint, Doney, and Karl 2011). Also, the majority of studies so far have investigated the effects of climate changes exclusively, without considering the influence of concurrent factors

deriving from interactions among the organisms. Top-down control on biofilms, for instance, is fundamental in structuring biofilm community and, consequently, benthic ecosystems: predators that selectively remove filamentous spores provide space for the settlement of habitat forming species which promote the diversity of these ecosystems (coral reefs, temperate reefs, see Russell, Connell, Findlay, et al. 2013 and references therein).

Elevated $pCO_2$ can act both as a resource ($CO_2$ enrichment increasing primary production) as well as a stressor (low pH impacting biological calcification and causing physiological stress), making it difficult to predict the structure of future ecosystems. Laboratory experiments proved that the combination of elevated $pCO_2$ and temperatures increased biofilms primary productivity while, at the same time, decreased grazers consumption to a certain extent depending on the acclimation experienced by the grazers (Russell, Connell, Findlay, et al. 2013). Even in naturally acidified conditions (i.e., shallow $CO_2$ vents) where organisms had sufficient time to adapt to environmental conditions, the outbreak of primary producers and herbivores due to elevated $pCO_2$ could not be balanced by carnivores which instead collapsed in low pH conditions; the overall community resulted simplified in its trophic structure and function (Vizzini et al. 2017)

Given the relevance of marine biofilms in structuring coastal ecosystems and the scarce information about their response to ocean acidification as well as its combination with other factors, we aimed at investigating the different contribution of bottom-up (acidification) and top-down forces on the biochemical composition of biofilms. The hypothesis behind the investigation was that bottom-up forces could enhance biofilm's biomass and nutritional quality, and that top-down control (grazing) could be able to compensate this enhancement. Considering the recent acknowledgement of shallow-water $CO_2$ vents as analogues of future acidified oceans (Dahms et al. 2018; Hall-Spencer et al. 2008), a manipulative experiment with biofilms differentially exposed to grazing pressure along a $pCO_2$/pH gradient was conducted in the volcanic vents system of Levante Bay (Vulcano Island).

## 3.2 Materials and Methods

### 3.2.1 Study area

Vulcano Island is an active volcano belonging to the archipelago of the Aeolian Islands located in the Southern Tyrrhenian Sea (Mediterranean Sea). In the Levante Bay, small fumarolic emissions occur offshore at shallow depths. Volcanic emissions are visible as bubble trains rising from the vents and are dominated by $CO_2$ (98-99% vol of $CO_2$), for a total estimated value of 3.6 t $day^{-1}$ of $CO_2$ (Inguaggiato 2012).

Due to the $CO_2$ vents in the southern part of the Levante Bay, a pH gradient (5.6 – 8.1) runs parallel to the north-eastern coast of the island, with $pCO_2$ ranging from 3361.7 ± 2971.3 µatm to 424.6 ± 61.5 µatm (Boatta 2012).

### 3.2.2 Experimental design and sampling procedure

The relative effects of bottom-up (ocean acidification) and top-down (grazing) forces on the biochemical composition of biofilms were investigated through a field experiment carried out from October to December of 2016 in Levante Bay, Vulcano Island (fig. 1). Throughout this period, grazer abundance was manipulated along the $pCO_2$/low pH north-eastern gradient of the bay and exactly in a $CO_2$-dominated vent area (hereafter Vent, 38°25'9.40"N, 14°57'42.14"E) characterized by low pH (7.8-7.9) , and two reference sites (hereafter REF 1, 38°25'14.33"N, 14°57'52.94"E; REF 2, 38°25'17.12"N, 14°57'56.77"E), characterized by pH values typical of the Mediterranean Sea waters (8.1 on average).

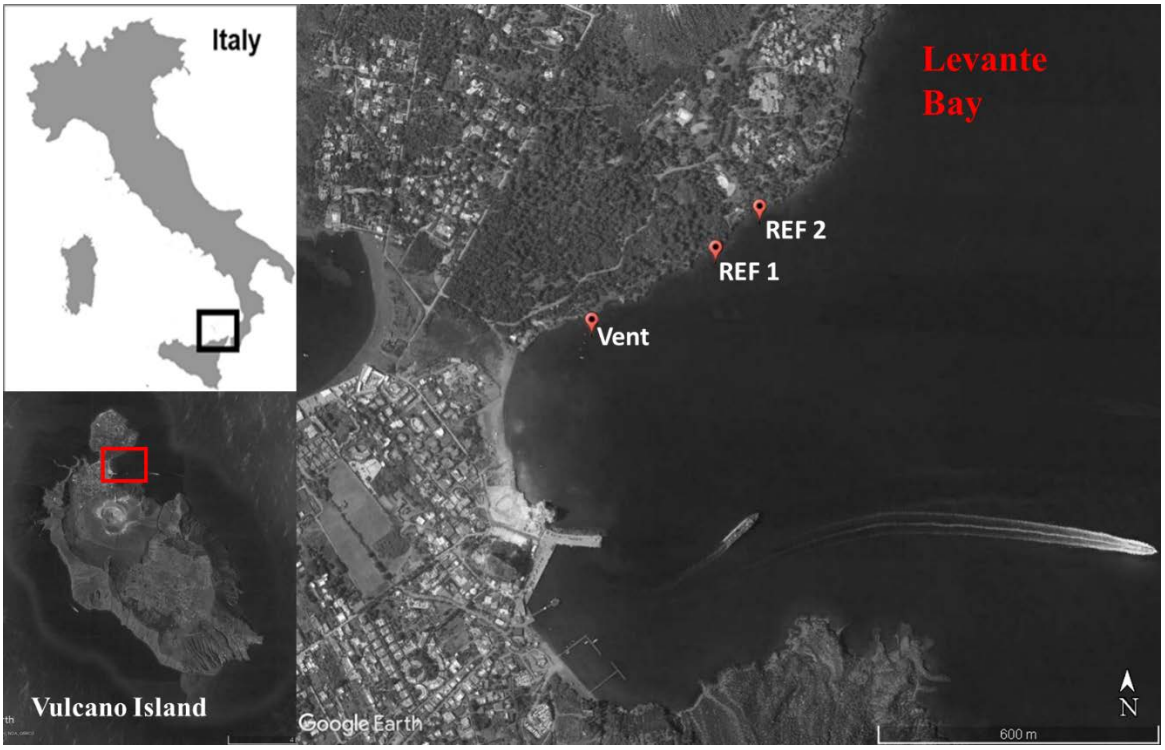


**Figure 1.** Map indicating the location of the study (Vulcano Island-Levante Bay) and the three sites where the experiment was conducted.

Glass panels (26x8 cm) were used as substrata for the biofilm colonization and were inserted into a plastic frame, connected to a ballast to secure the structure to the sea-bottom, as well as two floats on the opposite side keeping the whole structure in a vertical position within the water column (fig. 2). Before being deployed, all panels were washed with 30% peroxide hydrogen in order to provide a sterile surface for the biofilm colonization.

Grazers were manipulated with exclusion cages made of clear polyethylene mesh attached to the plastic frame (4x4 mm mesh, treatment “Exclusion”, fig.2 bottom left). Uncaged panels (treatment “Control”, fig. 2 up) were used as control plots. To evaluate the potential bias of the cage itself, a procedural control with an open cage was used as well (treatment “Procedural Control”, fig. 2 bottom right).

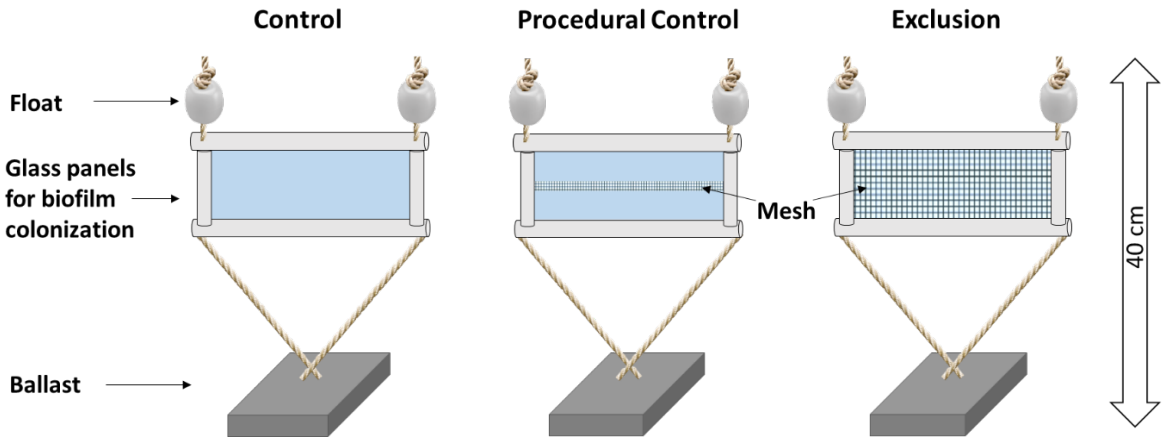


**Figure 2.** Scheme of the structures used for the experiment. Upper panel represents the treatment "Control" (no cage, grazing allowed); lower left panel represents the treatment "Exclusion" (cage present, no grazing); lower right panel represents the treatment Procedural Control (open cage, grazing allowed).

Cages were brushed periodically (almost each week) in order to avoid fouling growth on the mesh. Structures representing the three experimental treatments were deployed in triplicate at 3 m in the three sites selected in Levante Bay.

Physicochemical variables (temperature, salinity, pH) of the bottom seawater were measured with a multiparametric probe (HANNA Instrument) along the entire duration of the experiment (every two weeks at least sites). The physicochemical characterization of the area is described in details in Chapter 2 (see tables 1 and 2, with site "Vent 2" corresponding to site "Vent" of this study).

All structures were collected simultaneously at the end of the experiment (57 days fig.3), except for one replicate belonging to the treatment Exclusion which was lost in the site Vent.

Once recovered, the structures were immediately sealed in plastic bags and refrigerated until being processed in the laboratory. Here, the panels were carefully removed from their holding structures; the biofilm grown on their surface was carefully scrubbed using sterilized blades, well homogenized, transferred to sterile containers and frozen at -80°C until being furtherly processed.

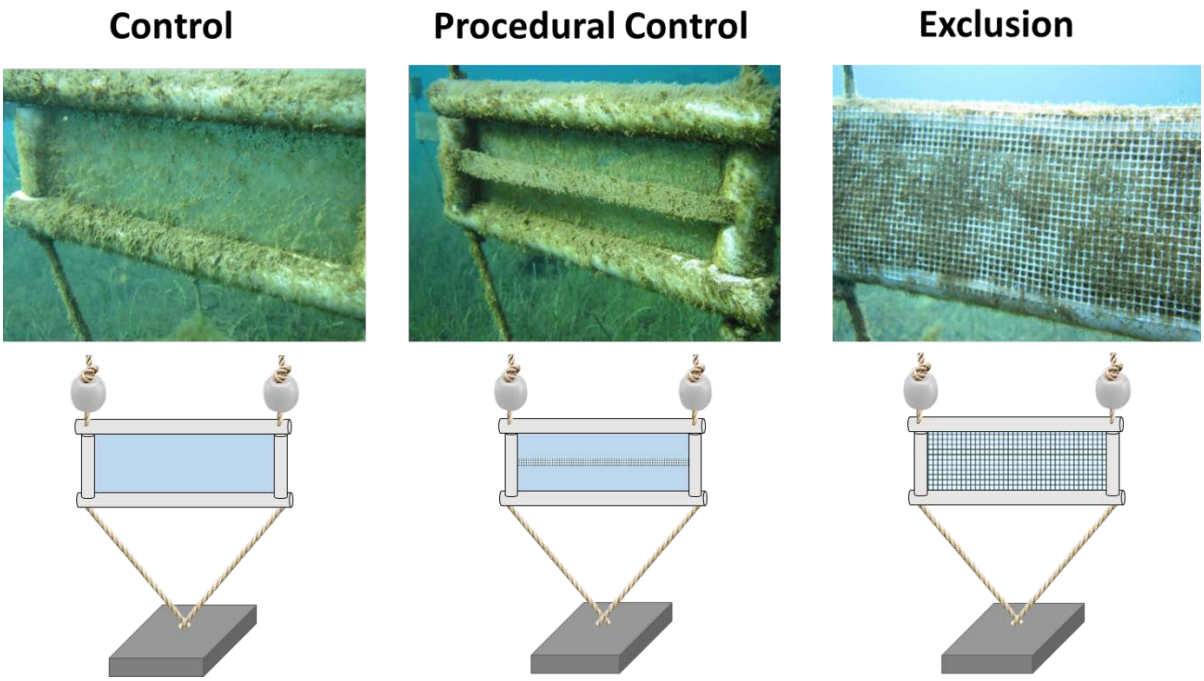


**Figure 3.** Photos of the biofilms collected at the end of the experiment in Levante Bay. Picture show the biofilm coverage on different panel representing the experimental treatments Control, Procedural Control and Exclusion respectively from left (see text for a detailed description of the structures).

### 3.2.3 Analytical methods

A biochemical characterization of the 57 days-old biofilm collected at Levante Bay was performed analyzing the following parameters: biomass, carbonate content, abundance of photopigments (chlorophyll-*a* and phaeophytin), Carbon:Nitrogen ratio (C:N), and Biopolymeric Organic Carbon (BPC).

Biomass and carbonates were determined using the loss on ignition method (LOI), described by (Heiri, Lotter, and Lemcke 2001). Aliquots of biofilms were weigthed after 24-hour at 105°C in order to calculate biofilm biomass, which was expressed as µg dry weight/$cm^2$ (DW µg/$cm^2$).

Successively, samples were placed in a muffle furnace at 550°C for 4 hours, a temperature at which organic matter is combusted to ash and carbon dioxide. The LOI was then determined using the following equation:

$$LOI550 = ((DW105 – DW550)/DW105)*100 \quad (1)$$

where LOI550 represents LOI at 550°C (as a percentage), DW105 represents the dry weight of the sample before combustion and DW550 the dry weight of the sample after heating to 550°C.

In a second step, samples were combusted at 1000°C for 2 hours during which carbon dioxide is evolved from carbonate, leaving oxide. The LOI was calculated based on the equation:

$$LOI950 = ((DW550 – DW950)/DW105)*100$$

where LOI950 is the LOI at 950°C (as a percentage), DW550 is the dry weight of the sample after combustion of organic matter at 550°C, DW950 represents the dry weight of the sample after heating to 950°C, and DW105 is again the initial dry weight of the sample before the organic carbon combustion. Carbonates were normalized to biofilm dry weight (mg/mg DW).

Chlorophyll-a and phaeopigments were extracted following the method by Plante-Cuny (1974). Briefly, a few mg of $MgCO_3$ were added to frozen biofilm to avoid chlorophyll-a degradation. 90% acetone was added to the samples, which were successively sonicated and incubated in the dark at 4°C for 12 hours. After incubation, the samples were centrifuged to remove the biofilm and the concentration of the pigments in the supernatant was determined by spectrophotometer (750nm and 650nm) before and after acidification with HCl 0.1 N. The concentrations of each photopigment was calculated against a standard curve and normalized to biofilm dry weight (µg/g DW). Total phytopigments (CPE=Chloroplastic Pigments Equivalents) were obtained from the sum of chlorophyll-a and phaeopigments concentrations.

C:N ratio was used as indicator of the nutritional quality of the biofilms as reported elsewhere (see Chiu et al. 2005 and references therein). Biofilm samples were freeze-dried using a LIO 5P DGT lyophilizer for 24 hours, after which they were reduced to a fine powder with a pestle and mortar and packed into tin capsule. Carbon and nitrogen content (%) was determined using an elemental analyser (Thermo Scientific EA 1112).

Total proteins were extracted following the procedure described by Hartree (1972), using the Folin-Ciocalteau solution. Spectrophotometric readings were performed at 650 nm against a bovine serum albumin (BSA) standard.

Total carbohydrates abundance was determined according to Dubois et al. (1956). After being treated with phenol and $H_2SO_4$ (96%), samples were read at the spectrophotometer at 490nm using Glucose as standard.

Total lipids were extracted according to Bligh and Dyer (1959) and determined after carbonization (Marsh e Weinstein, 1966). Readings at the spectrophotometer were performed at 375nm, with Tripalmitine used as the standard.

Total carbohydrates, proteins and lipids were used to calculate the Biopolymeric fraction of Organic Carbon (BPC), which is defined as the sum of carbohydrates, proteins and lipids after their conversion using the factors 0.40, 0.49 and 0.75 respectively (Fabiano et al., 1995).

Fatty acids (FAs) composition in biofilms was determined in freeze-dried samples after extraction of total lipids in distilled water/methanol/chloroform solvent mixture, based on a modified version of the

Bligh and Dyer protocol (1959). A butylated hydroxyl toluene solution (BHT, 0.01%) was added to prevent lipid oxidation. Two aliquots of each sample were used for the total lipid extraction: 100 mg for lipid class analysis; 50 mg for fatty acids analysis. From the first aliquot, total lipids content was determined gravimetrically and expressed as percentage of biofilm dry weight. Lipid classes were then separated by SPE using silica cartridges. Neutral lipids (NL), glycolipids (GL) and phospholipids (PL) were eluted based on their different affinity with chloroform, acetone and methanol respectively. Each fraction was evaporated to dryness under gentle nitrogen stream, weighted and expressed as percentage of TL. Lipid extract from the second aliquot was subjected to acid-catalysed transesterification with methanolic hydrogen chloride. Fatty acids were then analysed as methyl esters (FAME) by gas chromatography (GC-2010, Shimadzu) equipped with a BPX-70 capillary column (30 m length; 0.25 mm ID; 0.25 µm film thickness) and with a flame ionisation detector (FID). Peaks were identified using retention times from mixed commercial standards (37FAME and BAME from Supelco; BR1 and QUALFISH from Larodan). For quantification, tricosanoic acid (C23:00) was used as internal standard. Individual fatty acids were expressed as percentage of total fatty acids.

### 3.2.4 Data elaboration and statistical analysis

The variation in the biochemical and community composition of marine biofilms was investigated along a $pCO_2$/pH gradient (sites Vent, REF 1 and REF 2) as well as in the presence (treatments Control and Procedural Control) and absence of predators (treatment Exclusion). Site (Vent, REF 1, REF 2) and Treatment (Control, Procedural Control, Exclusion) were set as fixed factors. Significant differences in the quantitative composition of biofilms were tested through permutational analysis of variance (PERMANOVA) at univariate as well as multivariate level. Principal Coordinate analysis (PCO) was conducted at multivariate level. Statistical analysis was conducted using PRIMER v6 with PERMANOVA software (Plymouth Marine Laboratory, UK).

### 3.3 Results

Biomass density of marine biofilms (fig. 4) was highly variable among different sites and treatments, ranging from 0.55 µg/cm$^2$ (site Vent, treatment Exclusion) to 6.05 µg/cm$^2$ (site REF 2, treatment Procedural Control). Significant differences were found at the level of factors Site and Treatment. Biomass was significantly lower in the reference site REF 1 compared to the other sites, as well as in the Exclusion treatment with respect to the other conditions (table 1A).

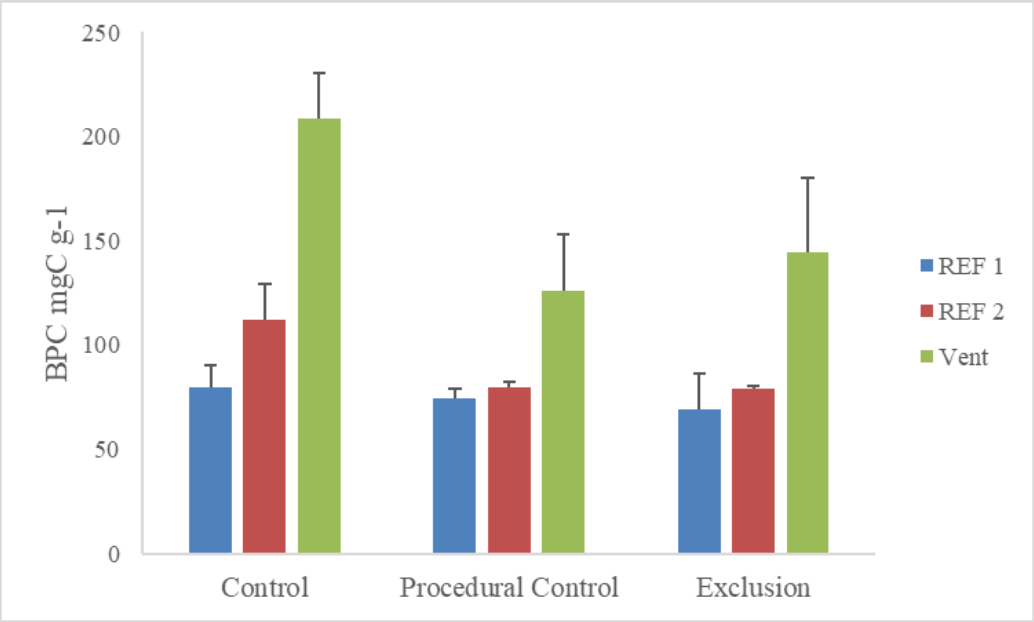


**Figure 4.** Concentration of biofilm biomass in different sites and experimental treatments. Values are reported as mean. Error bars represent standard deviations.

C:N ratio (fig. 5) ranged from 7.13 (site Vent, treatment Exclusion) to 14.98 (site REF 2, treatment Control). C:N ratio decreased significantly in the Vent site compared to REF 1, as well as in the Exclusion treatment with respect to the other experimental conditions where predators were allowed to graze on biofilms (treatments Control and Procedural Control) (table 1A).

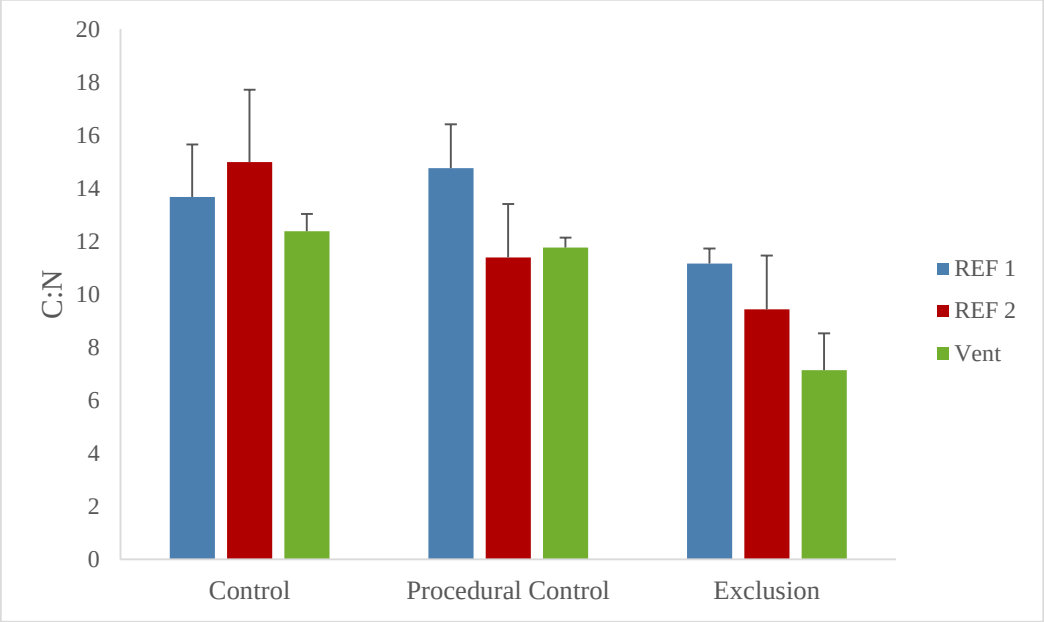


**Figure 5.** C:N ratio of biofilms in different sites and experimental treatments. Values are reported as mean. Error bars represent standard deviations.

The concentration of Biopolymeric Organic Carbon (BPC, fig. 6) ranged from 69.23 mgC $g^{-1}$ (site REF 1, treatment Exclusion) to 208.67 mgC $g^{-1}$ (site Vent, treatment Control). Significant differences were found according to the factor Site, as well as Treatment (table 1A). BPC concentration increased significantly from reference site REF 1 to reference site REF 2, however their abundance in both REF sites was highly significantly lower compare to site Vent. BPC concentration were overall significantly higher when the cage was not present (treatment Control).

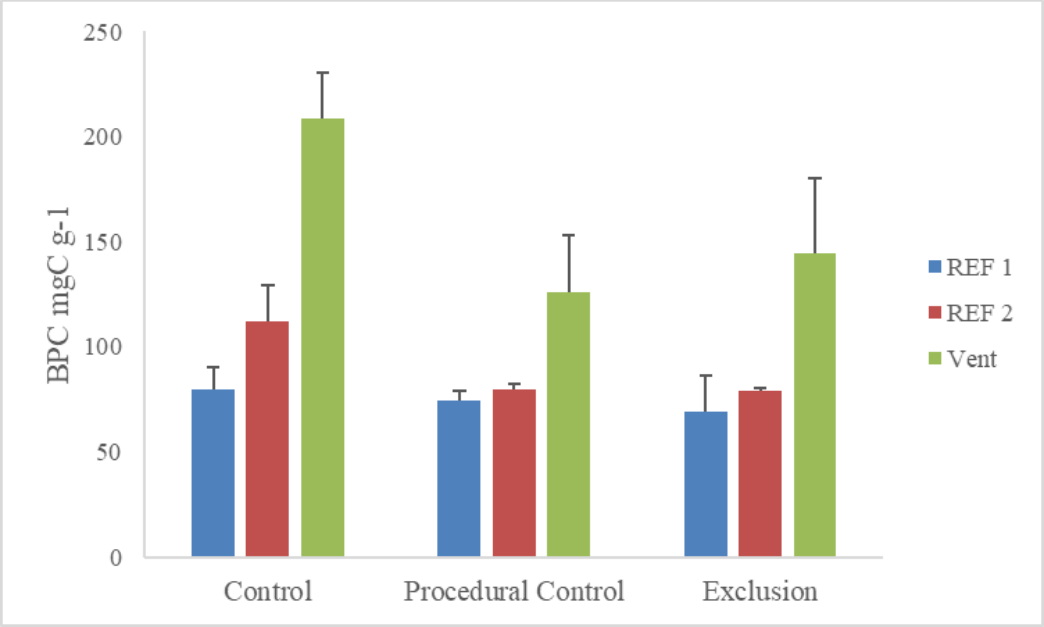


**Figure 6.** Biopolymeric Organic Carbon concentration of biofilms in different sites and experimental treatments. Values are reported as mean. Error bars represent standard deviations.

Carbonate content (fig. 8) ranged from 0.53 mg/mg DW to 0.81 mg/mg DW (site Vent, treatment Exclusion). Carbonate content was significantly lower in the treatment Control compared to the other experimental conditions (table 1A).

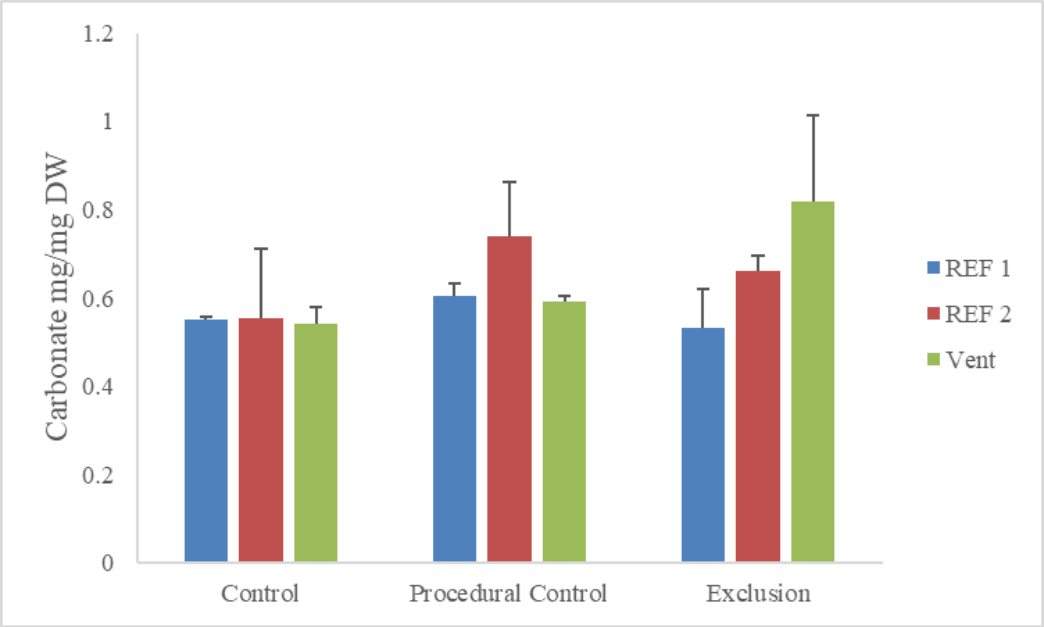


**Figure 8.** Carbonate content of biofilms in different sites and experimental treatments. Values are reported as mean. Error bars represent standard deviations.

The concentration of Chlorophyll-*a* ranged from 2.17 µg/g DW to 13.42 µg/g DW (fig. 7a); Pheophytin concentration ranged from 5.35 µg/g DW to 27.79 µg/g DW (fig. 7b). In both cases the lowest values were detected in the reference site REF 1 (treatment Control), while the highest were found in the site Vent (treatment Procedural Control). Significant differences were found for the interaction of the two factors Site and Treatment for both photopigments (table 1B). Within the reference site REF 1, the concentration of both photopigments was significantly higher when predators were excluded (treatment Exclusion) compared to the other experimental conditions; in the same site, significant differences were found between the treatments Control and Procedural Control. The concentration of photopigments did not show significant variation among the experimental treatments within the sites Vent and REF 2. When predators were allowed to graze (treatments Control and Procedural Control), the concentration of photopigments was significantly lower in the reference sites compared to the vent site.

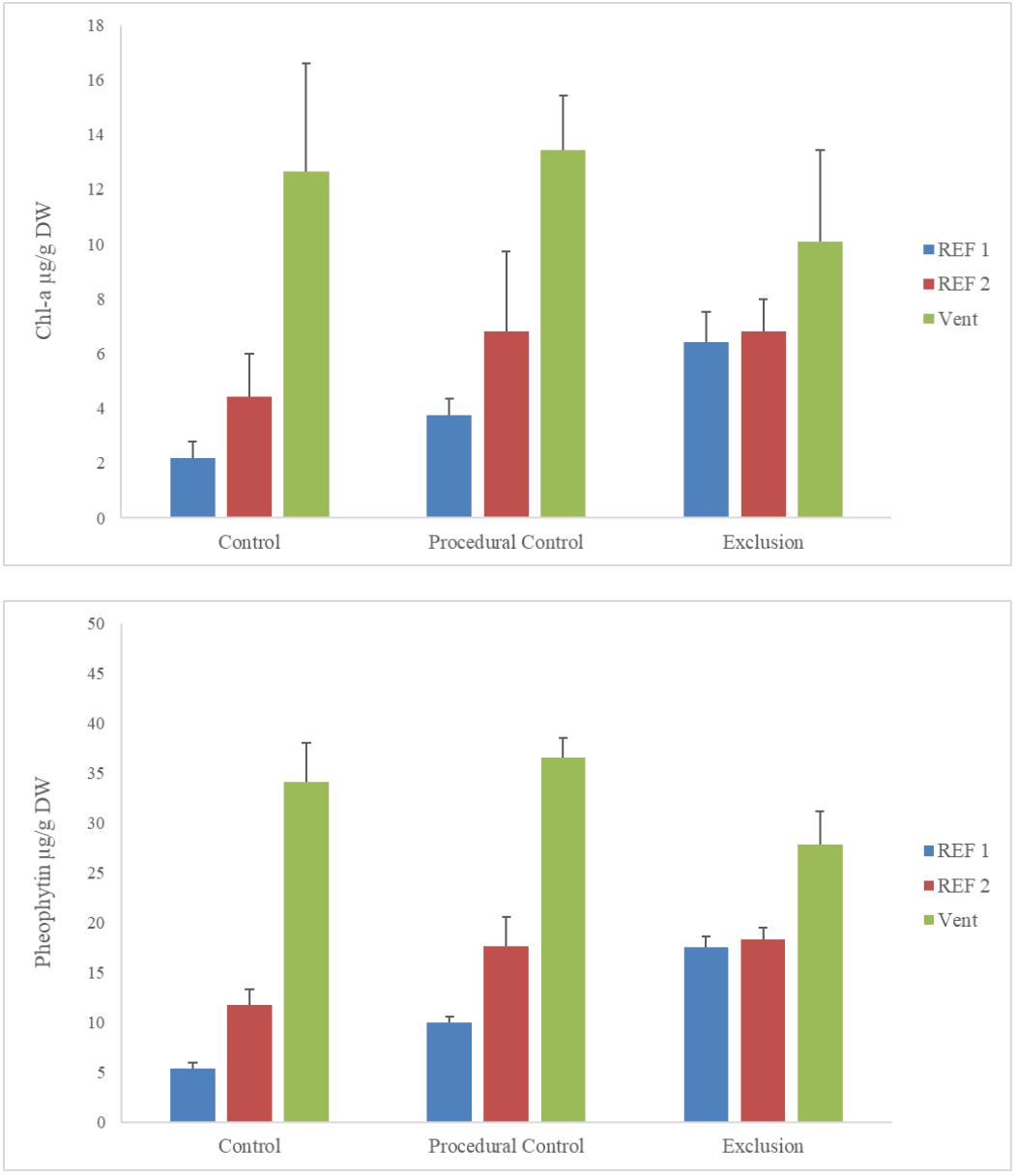


**Figure 7.** Concentration of Chlorophyll-*a* and Pheophytin of biofilms in different sites and experimental treatments. Values are reported as mean. Error bars represent standard deviations.

**Table 1A.** Permanova analysis and Pair-wise tests at univariate level on the biochemical composition of biofilms. in different sites (Vent, REF 1 and REF 2) and experimental treatments (Ctrl = Control; Pr. Ctrl = Procedural Control; Excl = Exclusion). Significant results are highlighted.

| | | Biomass DW µg/cm$^2$ | | | | C:N ratio | | | | BPC mgC g$^{-1}$ | | | | Carbonate mg/mg DW | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** |
| **Site** | 2 | 0.57186 | 5.1594 | **0.019** | 998 | 0.3634 | 6.266 | **0.008** | 999 | 34.632 | 53.161 | **0.001** | 999 | 0.008364 | 2.6332 | 0.092 | 999 |
| **Treatment** | 2 | 1.3415 | 12.103 | **0.005** | 999 | 1.0047 | 17.324 | **0.001** | 998 | 8.0796 | 12.402 | **0.001** | 999 | 0.014003 | 4.4083 | **0.023** | 999 |
| **Site x Treatment** | 4 | 0.16543 | 1.4925 | 0.253 | 998 | 0.1106 | 1.907 | 0.162 | 999 | 1.7243 | 2.6467 | 0.07 | 999 | 0.00901 | 2.8365 | 0.052 | 999 |
| **Residuals** | 17 | | | | | | | | | | | | | | | | |
| ***Pair-wise tests*** | **Groups** | | **t** | **P(perm)** | **Unique perms** | | **t** | **P(perm)** | **Unique perms** | | **t** | **P(perm)** | **Unique perms** | | **t** | **P(perm)** | **Unique perms** |
| Site | REF 1, REF 2 | | 2.8962 | **0.019** | 998 | | 1.5018 | 0.167 | 998 | | 2.9767 | **0.018** | 996 | | | | |
| | REF 1, Vent | | 2.3977 | **0.044** | 999 | | 4.8668 | **0.001** | 999 | | 8.5535 | **0.001** | 997 | | | | |
| | REF 2, Vent | | 1.3619 | 0.21 | 995 | | 1.7684 | 0.107 | 996 | | 7.3029 | **0.001** | 998 | | | | |
| Treatment | Ctrl, Excl | | 3.1403 | **0.015** | 996 | | 5.3719 | **0.001** | 995 | | 3.8623 | **0.003** | 998 | | 2.4357 | **0.035** | 995 |
| | Ctrl, Pr. Ctrl | | 1.7229 | 0.112 | 999 | | 1.2775 | 0.225 | 998 | | 4.8606 | **0.002** | 995 | | 2.5083 | **0.032** | 994 |
| | Excl, Pr. Ctrl | | 5.3644 | **0.002** | 996 | | 4.6838 | **0.003** | 998 | | 0.33515 | 0.747 | 998 | | 0.47008 | 0.623 | 996 |

**Table 1B.** Permanova analysis and Pair-wise tests at univariate level on the biochemical composition of biofilms in different sites (Vent, REF 1 and REF 2) and experimental treatments (Ctrl = Control; Pr. Ctrl = Procedural Control; Excl = Exclusion). Significant results are highlighted.

| | | Chl-a µg/g DW | | | | Pheopytin µg/g DW | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Source** | **df** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** |
| **Site** | 2 | 4.6509 | 35.874 | **0.001** | 998 | 13.298 | 38.882 | **0.001** | 999 |
| **Treatment** | 2 | 0.4247 | 3.2758 | **0.064** | 999 | 1.3334 | 3.8987 | **0.04** | 998 |
| **Site x Treatment** | 4 | 0.3759 | 2.8994 | **0.043** | 999 | 1.0597 | 3.0983 | **0.034** | 998 |
| **Residuals** | 17 | | | | | | | | |
| ***Pair-wise tests*** | | | | | | | | | |
| **Within level:** | **Groups** | **t** | **P(perm)** | **Unique perms** | **P(MC)** | **t** | **P(perm)** | **Unique perms** | **P(MC)** |
| Control | REF 1, REF 2 | 2.6179 | 0.097 | 7 | 0.06 | 2.6716 | 0.098 | 10 | 0.068 |
| | REF 1, Vent | 6.1128 | 0.084 | 10 | **0.004** | 6.8299 | 0.103 | 10 | **0.001** |
| | REF 2, Vent | 3.855 | 0.104 | 7 | **0.02** | 4.1148 | 0.102 | 10 | **0.018** |
| Procedural | REF 1, REF 2 | 1.9482 | 0.187 | 7 | 0.123 | 1.7789 | 0.172 | 10 | 0.175 |
| Control | REF 1, Vent | 9.5369 | 0.093 | 10 | **0.001** | 10.821 | 0.117 | 10 | **0.002** |
| | REF 2, Vent | 3.0853 | 0.115 | 10 | **0.036** | 3.2519 | 0.1 | 10 | **0.035** |
| Exclusion | REF 1, REF 2 | 0.42023 | 0.776 | 7 | 0.716 | 0.32711 | 1 | 7 | 0.758 |
| | REF 1, Vent | 1.9285 | 0.196 | 7 | 0.124 | 1.9112 | 0.319 | 10 | 0.153 |
| | REF 2, Vent | 1.6711 | 0.309 | 10 | 0.188 | 1.7861 | 0.213 | 10 | 0.157 |
| REF 1 | Ctrl, Excl | 5.9904 | 0.095 | 10 | **0.004** | 6.0671 | 0.098 | 10 | **0.004** |
| | Ctrl, Pr. Ctrl | 3.028 | 0.096 | 10 | **0.041** | 3.427 | 0.085 | 10 | **0.031** |
| | Excl, Pr. Ctrl | 3.8452 | 0.096 | 10 | **0.019** | 3.9429 | 0.093 | 10 | **0.024** |
| REF 2 | Ctrl, Excl | 2.1305 | 0.199 | 7 | 0.101 | 2.176 | 0.192 | 10 | 0.074 |
| | Ctrl, Pr. Ctrl | 1.2898 | 0.281 | 7 | 0.275 | 1.1532 | 0.298 | 10 | 0.327 |
| | Excl, Pr. Ctrl | 9.28E-02 | 1 | 10 | 0.926 | 2.47E-01 | 0.811 | 10 | 0.807 |
| Vent | Ctrl, Excl | 0.76439 | 0.619 | 10 | 0.496 | 0.73831 | 0.576 | 10 | 0.533 |
| | Ctrl, Pr. Ctrl | 0.3724 | 0.72 | 10 | 0.734 | 0.45025 | 0.638 | 10 | 0.688 |
| | Excl, Pr. Ctrl | 1.4661 | 0.289 | 10 | 0.246 | 1.4308 | 0.308 | 10 | 0.242 |

Principal Coordinates Analysis conducted on all the variables (fig.9) showed a clear separation between samples of Vent (on the right) and reference sites (on the left) along the first axes explaining for the 36.8% of the total variation (PCO1). Regarding the factor Treatment, clustering of samples was different according to the site: in acidified conditions (Vent), samples where predators were excluded (treatment Exclusion) were separated from the others (treatments Control and Procedural Control), while in the reference sites the separation was less pronounced. The over-imposition of the vectors showed that BPC and phytopigments (CPE) were more abundant in biofilms in acidified conditions (Vent) when grazers were allowed (treatments Control and Procedural Control). Carbonates and biomass where higher in biofilms from the reference site REF 2, while the C:N ratio was higher in biofilms from the reference site REF 1. The total variation explained by the first two axes was 65.1%.

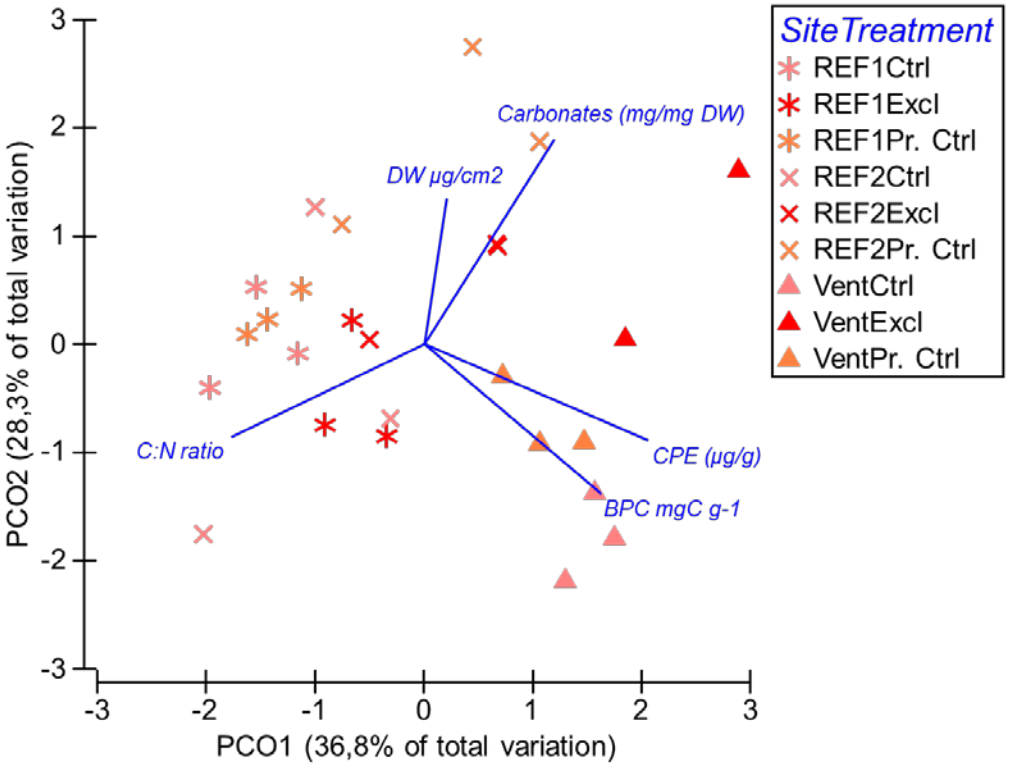


**Figure 9.** Principal coordinates analysis on the biochemical composition of biofilms in different sites (Vent, REF 1, REF 2) and experimental conditions (Ctrl=Control; Pr. Ctrl=Procedural Control; Excl=Exclusion).

.

PERMANOVA at the multivariate level revealed significant differences for the interaction SitexTreatment (table 3). Pair-wise test at the level of the factor “Site” revealed significant differences in the treatments Control and Procedural Control between the vent and the reference sites, while the comparison of the treatment Exclusion resulted significantly different only between the sites Vent and REF 1. At the level of the factor “Treatment”, significant differences were found between biofilms exposed to predators (treatments Control and Procedural Control) vs biofilms that did not experience grazing (treatment Exclusion) in the reference site REF 1 (table 2). In the site Vent, biofilms from treatment Control resulted significantly different compared to the other experimental treatments (Exclusion and Procedural Control).

**Table 3.** Permanova analysis and Pair-wise tests at multivariate level on the biochemical composition of biofilms in different sites (Vent, REF 1 and REF 2) and experimental treatments (Ctrl = Control; Pr. Ctrl = Procedural Control; Excl = Exclusion). Significant results are highlighted.

| Source | df | MS | Pseudo-F | P(perm) | Unique perms |
|---|---|---|---|---|---|
| **Site** | 2 | 21.892 | 11.349 | **0.001** | 997 |
| **Treatment** | 2 | 15.204 | 7.8814 | **0.001** | 999 |
| **Site x Treatment** | 4 | 4.3825 | 2.2718 | **0.008** | 999 |
| **Residuals** | 17 | 1.929 | | | |

| ***Pair-wise tests*** **Within level:** | Groups | t | P(perm) | Unique perms | P(MC) |
|---|---|---|---|---|---|
| Control | REF 1, REF 2 | 0.86747 | 0.687 | 10 | 0.536 |
| | REF 1, Vent | 4.3024 | 0.111 | 10 | **0.002** |
| | REF 2, Vent | 2.1884 | 0.099 | 10 | **0.036** |
| Procedural | REF 1, REF 2 | 1.935 | 0.088 | 10 | 0.079 |
| Control | REF 1, Vent | 4.4736 | 0.116 | 10 | **0.004** |
| | REF 2, Vent | 2.2174 | 0.1 | 10 | **0.049** |
| Exclusion | REF 1, REF 2 | 1.8574 | 0.113 | 10 | 0.089 |
| | REF 1, Vent | 2.672 | 0.094 | 10 | **0.041** |
| | REF 2, Vent | 1.701 | 0.114 | 10 | 0.15 |
| REF 1 | Ctrl, Excl | 2.2978 | 0.089 | 10 | **0.022** |
| | Ctrl, Pr. Ctrl | 1.1963 | 0.328 | 10 | 0.275 |
| | Excl, Pr. Ctrl | 2.9904 | 0.109 | 10 | **0.009** |
| REF 2 | Ctrl, Excl | 1.9504 | 0.101 | 10 | 0.08 |
| | Ctrl, Pr. Ctrl | 1.6202 | 0.189 | 10 | 0.138 |
| | Excl, Pr. Ctrl | 1.8709 | 0.103 | 10 | 0.071 |
| Vent | Ctrl, Excl | 2.4242 | 0.111 | 10 | **0.042** |
| | Ctrl, Pr. Ctrl | 2.2158 | 0.112 | 10 | **0.044** |
| | Excl, Pr. Ctrl | 2.2416 | 0.089 | 10 | 0.08 |

A total of twenty-eight individual FAs were detected in the biofilms collected from Levante Bay (table 4). With the exception of few cases (*19:0, 14:1 n5, 20:1 n9, 24:1 n9, 20:3 n3*), the same FA were present in all sites and treatments, however their relative abundance varied among the different experimental conditions. The most abundant FAs across all samples were Palmitic (16:0), Palmitoleic (16:1 n7), Stearidonic (18:4 n3) and Timnodic (20:5 n3). Palmitic and Timnodic showed similar abundance across the experimental treatments in the reference sites, while in the vent site their relative abundance varied across treatments: Palmitic decreased in the treatment Exclusion compared to the two control treatments, while Timnodic showed the opposite trend. In the Vent site, Palmitoleic and Vaccenic (18:1 n7) increased in the treatment Exclusion with respect to the two control treatments, reaching concentrations similar to biofilms from the reference sites, where these FAs showed less variations in their abundance across the different experimental conditions.

Principal Coordinates Analysis on FAs composition (fig.10) showed overall a clear separation along the first axes (PCO1) between control treatments (on the right side) and predator exclusion (on the left side) of samples from site Vent. Clustering of samples from the two reference sites was less evident, with samples from control treatments clustering toward the centre, and sample from the treatment of predator exclusion clustering on the left bottom, together with samples from the vent site. 63.5% of the total variation of the fatty acids composition in the biofilms was explained by the first axes (PCO1), while the second axes explained for the 17.8%. PERMANOVA at the multivariate level revealed significant differences for the interaction Site x Treatment (table 5). FAs composition within treatment Control was significantly different for the comparison of all sites. Significant differences were also found within the treatment Procedural Control between vent and reference sites. Within site REF 1, FAs composition was significantly different only between treatment Control and the Exclusion. In the Vent, significant differences in the composition of fatty acids in biofilms were found between the treatment Exclusion and the two controls (Control and Procedural Control).

**Table 4.** Relative abundance of FAs expressed as percentage on total FAs of biofilms from different sites (Vent, REF 1, REF 2) and experimental treatments (Control, Procedural Control, Exclusion). LCFA=Long Chain FAs; SFA=Saturated FAs; MUFA=Monounsaturated FAs; PUFA=Polyunsaturated FAs; CY=Cyclic FAs; OH=Hydroxy FAs. Biomarker assignment were derived from Alfaro et al. (2006), Bachok et al. (2003), Jaschinski et al. (2011), Kharlamenko et al. (2001) and references therein.

| Site | | | Vent | | | | | | REF1 | | | | | | REF2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | | | Control | | Procedural Control | | Exclusion | | Control | | Procedural Control | | Exclusion | | Control | | Procedural Control | | Exclusion | |
| **Fatty acids** | **Class** | **Biomarker** | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. | Mean | St. Dev. |
| 12:0 | SFA | | 0.42 | 0.04 | 0.54 | 0.10 | 1.31 | 0.12 | 0.96 | 0.20 | 1.24 | 0.33 | 1.78 | 0.44 | 1.74 | 0.62 | 1.07 | 0.86 | 0.16 | 0.09 |
| 14:0 | SFA | bacteria | 7.92 | 0.73 | 8.10 | 0.65 | 7.41 | 0.75 | 6.74 | 0.32 | 6.75 | 0.20 | 6.93 | 0.52 | 7.02 | 0.19 | 6.93 | 0.21 | 7.27 | 0.37 |
| 15:0 | SFA | | 0.55 | 0.05 | 0.62 | 0.06 | 0.91 | 0.02 | 0.78 | 0.16 | 0.84 | 0.16 | 1.15 | 0.21 | 1.00 | 0.23 | 0.95 | 0.10 | 1.16 | 0.14 |
| 16:0 | SFA | diatoms, bacteria | 25.13 | 2.43 | 23.57 | 1.63 | 14.05 | 2.13 | 22.83 | 0.39 | 19.87 | 0.78 | 15.72 | 4.14 | 18.69 | 2.47 | 17.43 | 0.61 | 16.74 | 2.32 |
| 17:0 | SFA | bacteria | 0.07 | 0.01 | 0.08 | 0.01 | 0.13 | 0.04 | 0.35 | 0.07 | 0.27 | 0.03 | 0.33 | 0.15 | 0.26 | 0.16 | 0.29 | 0.05 | 0.39 | 0.13 |
| 18:0 | SFA | | 1.68 | 0.22 | 1.57 | 0.27 | 2.63 | 0.68 | 2.24 | 0.13 | 2.56 | 0.86 | 3.14 | 1.31 | 2.10 | 0.82 | 1.58 | 0.47 | 2.49 | 0.58 |
| 19:0 | SFA | bacteria | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.67 | 0.20 | 0.86 | 0.24 | 1.38 | 0.48 | 0.25 | 0.43 | 0.71 | 0.63 | 0.97 | 0.84 |
| 20:0 | SFA | terrestrial organic matter | 0.48 | 0.02 | 0.58 | 0.07 | 1.13 | 0.09 | 0.86 | 0.19 | 1.07 | 0.25 | 1.56 | 0.53 | 1.45 | 0.50 | 1.28 | 0.07 | 1.67 | 0.15 |
| **LCFA (>22:0)** | | terrestrial organic matter | 0.85 | 0.04 | 1.20 | 0.14 | 2.11 | 0.14 | 1.56 | 0.40 | 1.69 | 0.21 | 2.14 | 0.40 | 2.07 | 0.64 | 1.90 | 0.23 | 2.27 | 0.20 |
| **∑SFA** | | | 36.26 | 3.37 | 35.06 | 1.98 | 27.58 | 3.71 | 35.44 | 0.31 | 33.46 | 0.80 | 31.99 | 3.94 | 32.50 | 2.50 | 30.25 | 0.92 | 30.84 | 2.48 |
| 14:1 n5 | MUFA | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.77 | 0.06 |
| 16:1 n7 | MUFA | diatoms, chemoautotroph bacteria | 10.38 | 1.28 | 11.25 | 1.41 | 14.52 | 0.14 | 16.30 | 0.88 | 14.89 | 0.28 | 11.28 | 2.82 | 11.93 | 0.95 | 12.92 | 0.15 | 10.02 | 0.83 |
| 18:1 n7 | MUFA | chemioautotrophic bacteria | 1.56 | 0.05 | 2.12 | 0.23 | 4.48 | 0.24 | 4.43 | 0.43 | 4.21 | 0.40 | 5.22 | 0.46 | 5.13 | 0.93 | 4.47 | 0.19 | 5.84 | 0.82 |
| 18:1 n9 | MUFA | phytoplankton | 7.73 | 0.48 | 6.81 | 0.42 | 3.32 | 0.14 | 2.84 | 0.13 | 3.07 | 0.02 | 3.47 | 0.46 | 3.77 | 0.04 | 3.22 | 0.37 | 3.46 | 0.44 |
| 20:1 n9 | MUFA | zooplankton | 0.17 | 0.01 | 0.23 | 0.03 | 0.23 | 0.32 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 22:1 n9 | MUFA | zooplankton | 0.23 | 0.07 | 0.26 | 0.07 | 0.49 | 0.04 | 0.43 | 0.12 | 0.53 | 0.17 | 0.68 | 0.20 | 0.68 | 0.24 | 0.59 | 0.04 | 0.72 | 0.03 |
| 24:1 n9 | MUFA | | 0.16 | 0.01 | 0.20 | 0.03 | 0.00 | 0.00 | 0.35 | 0.10 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **∑MUFA** | | | 20.23 | 0.73 | 20.88 | 1.63 | 23.03 | 0.04 | 24.36 | 0.84 | 22.70 | 0.54 | 20.64 | 1.94 | 21.51 | 0.81 | 21.20 | 0.55 | 20.03 | 0.48 |
| 18:2 n6c | PUFA | seagrasses | 4.16 | 0.64 | 3.79 | 0.62 | 2.22 | 0.15 | 1.95 | 0.23 | 2.05 | 0.17 | 2.05 | 0.14 | 2.38 | 0.22 | 2.19 | 0.01 | 2.17 | 0.08 |
| 18:3 n3 | PUFA | seagrasses | 2.50 | 0.70 | 2.35 | 0.76 | 0.98 | 0.03 | 1.93 | 0.45 | 1.58 | 0.30 | 1.77 | 0.45 | 2.16 | 0.32 | 1.99 | 0.40 | 2.27 | 0.43 |
| 18:3 n6 | PUFA | green algae | 1.50 | 0.19 | 1.62 | 0.15 | 1.70 | 0.07 | 1.87 | 0.13 | 1.82 | 0.13 | 1.77 | 0.16 | 1.70 | 0.36 | 1.80 | 0.09 | 1.73 | 0.08 |
| 18:4 n3 | PUFA | dinoflagellates, seagrasses | 13.24 | 1.45 | 11.53 | 0.93 | 4.56 | 0.23 | 5.00 | 0.74 | 5.57 | 1.29 | 4.60 | 1.35 | 5.54 | 1.40 | 6.23 | 1.70 | 6.25 | 1.17 |
| 20:2 n6 | PUFA | | 0.41 | 0.00 | 0.52 | 0.07 | 1.08 | 0.14 | 0.74 | 0.19 | 1.02 | 0.30 | 1.48 | 0.49 | 1.39 | 0.49 | 1.22 | 0.07 | 1.06 | 0.93 |
| 20:3 n3 | PUFA | | 0.16 | 0.02 | 0.15 | 0.13 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 20:3 n6 | PUFA | | 0.51 | 0.08 | 0.51 | 0.07 | 0.66 | 0.13 | 0.59 | 0.06 | 0.71 | 0.06 | 0.81 | 0.14 | 0.85 | 0.16 | 0.83 | 0.04 | 0.90 | 0.12 |
| 20:4 n3 | PUFA | macroalgae | 0.75 | 0.10 | 0.82 | 0.13 | 1.00 | 0.17 | 0.87 | 0.19 | 1.08 | 0.10 | 1.28 | 0.18 | 1.29 | 0.42 | 1.23 | 0.04 | 1.30 | 0.09 |
| 20:4 n6 | PUFA | red macroalgae | 4.54 | 1.23 | 4.51 | 1.21 | 5.32 | 0.41 | 3.77 | 0.23 | 4.15 | 0.69 | 4.97 | 1.32 | 4.79 | 0.71 | 5.17 | 0.51 | 5.38 | 1.29 |
| 20:5 n3 | PUFA | diatoms, dinoflagellates | 9.97 | 1.47 | 10.97 | 1.47 | 16.48 | 4.04 | 11.67 | 0.61 | 12.25 | 1.39 | 10.75 | 0.70 | 12.01 | 1.20 | 12.88 | 0.98 | 11.30 | 0.91 |
| 22:4 n6 | PUFA | | 0.25 | 0.05 | 0.31 | 0.06 | 0.83 | 0.16 | 0.55 | 0.11 | 0.69 | 0.16 | 1.23 | 0.21 | 0.97 | 0.26 | 0.89 | 0.10 | 0.70 | 0.62 |
| 22:5 n3 | PUFA | | 0.67 | 0.03 | 0.82 | 0.11 | 1.69 | 0.28 | 1.28 | 0.21 | 1.61 | 0.40 | 2.17 | 0.50 | 2.12 | 0.75 | 1.89 | 0.13 | 2.36 | 0.18 |
| 22:6 n3 | PUFA | dinoflagellates | 1.33 | 0.06 | 1.56 | 0.05 | 2.60 | 0.14 | 3.19 | 0.18 | 3.30 | 0.31 | 4.76 | 2.55 | 3.35 | 0.84 | 3.66 | 0.36 | 4.33 | 0.65 |
| **∑PUFA** | | | 39.98 | 3.14 | 39.45 | 3.86 | 39.13 | 4.14 | 33.41 | 1.51 | 35.83 | 2.36 | 37.63 | 6.06 | 38.57 | 2.95 | 39.99 | 2.19 | 39.74 | 1.21 |
| **∑Branched FA** | | bacteria | 0.84 | 0.20 | 1.02 | 0.19 | 2.85 | 0.18 | 1.87 | 0.40 | 2.28 | 0.81 | 3.58 | 0.68 | 2.55 | 0.92 | 2.60 | 0.51 | 2.66 | 0.30 |
| **∑CY** | | bacteria | 0.21 | 0.18 | 0.41 | 0.07 | 1.09 | 0.20 | 0.44 | 0.39 | 0.98 | 0.28 | 0.80 | 0.72 | 0.00 | 0.00 | 0.00 | 0.00 | 0.58 | 1.01 |
| **∑OH** | | bacteria | 1.64 | 0.20 | 1.98 | 0.30 | 4.20 | 0.22 | 2.92 | 0.35 | 3.06 | 0.45 | 3.22 | 0.33 | 2.81 | 0.47 | 4.06 | 0.22 | 3.10 | 0.14 |
| **Lipids mg/g** | | | 18.53 | 1.10 | 17.89 | 2.84 | 14.18 | 1.22 | 7.18 | 0.36 | 12.75 | 2.70 | 11.63 | 0.79 | 17.05 | 2.42 | 11.59 | 2.36 | 13.25 | 5.30 |

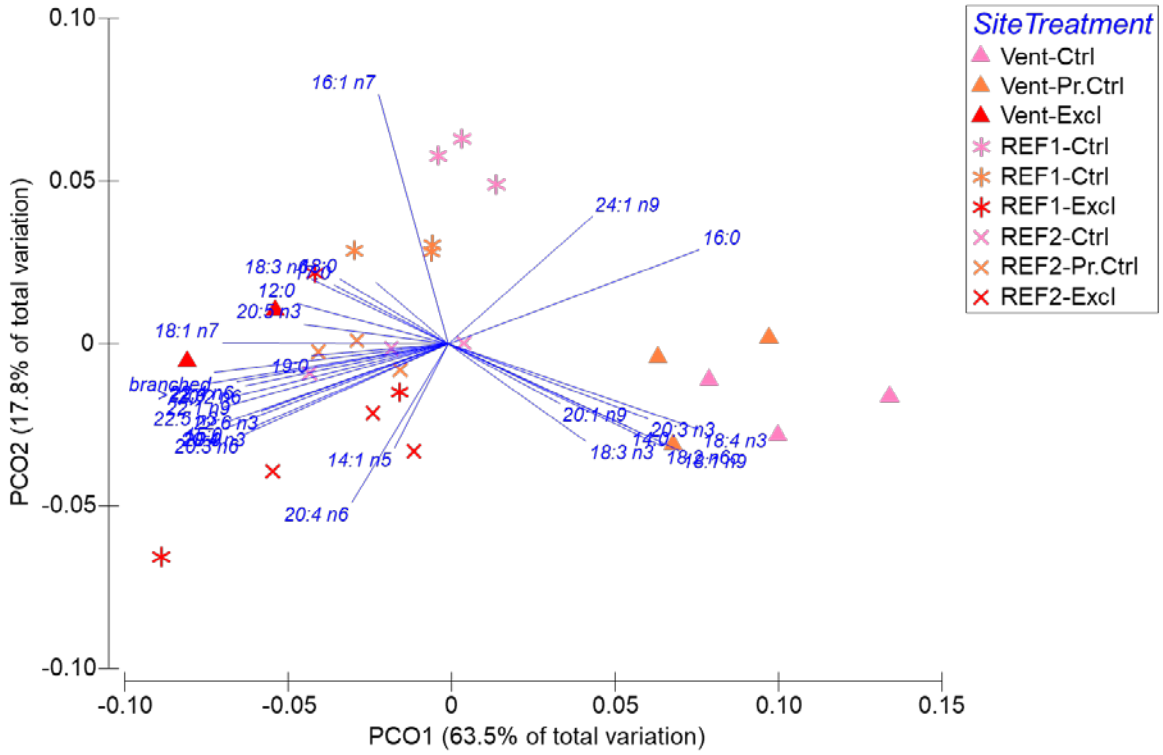


**Figure 10.** Principal coordinates analysis on the fatty acids profile of biofilms in different sites (Vent, REF 1, REF 2) and experimental conditions (Ctrl=Control; Pr. Ctrl=Procedural Control; Excl=Exclusion).

**Table 5.** Permanova analysis and Pair-wise tests at multivariate level on the Fatty Acids (FAs) profile of biofilms in different sites (Vent, REF 1 and REF 2) and experimental treatments (Ctrl = Control; Pr. Ctrl = Procedural Control; Excl = Exclusion). Significant results are highlighted.

| **Multivariate FAs** | | | | | |
|---|---|---|---|---|---|
| **Source** | **df** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** |
| **Site** | 2 | 1.61E-02 | 10.463 | **0.001** | 998 |
| **Treatment** | 2 | 1.61E-02 | 10.467 | **0.001** | 999 |
| **Site x Treatment** | 4 | 8.12E-03 | 5.2921 | **0.001** | 999 |
| **Residuals** | 17 | | | | |
| ***Pair-wise tests*** | | | | | |
| **Within level:** | **Groups** | **t** | **P(perm)** | **Unique perms** | **P(MC)** |
| Control | REF 1, REF 2 | 2.5418 | 0.099 | 10 | **0.025** |
| | REF 1, Vent | 5.0234 | 0.092 | 10 | **0.004** |
| | REF 2, Vent | 3.8219 | 0.103 | 10 | **0.007** |
| Procedural Control | REF 1, REF 2 | 1.8175 | 0.105 | 10 | 0.071 |
| | REF 1, Vent | 3.973 | 0.1 | 10 | **0.006** |
| | REF 2, Vent | 4.3234 | 0.105 | 10 | **0.006** |
| Exclusion | REF 1, REF 2 | 0.79842 | 0.792 | 10 | 0.597 |
| | REF 1, Vent | 1.4062 | 0.115 | 10 | 0.187 |
| | REF 2, Vent | 2.1687 | 0.094 | 10 | 0.064 |
| REF 1 | Ctrl, Pr. Ctrl | 1.91 | 0.091 | 10 | 0.055 |
| | Ctrl, Excl | 2.4837 | 0.101 | 10 | **0.043** |
| | Excl, Pr. Ctrl | 1.6098 | 0.121 | 10 | 0.129 |
| REF 2 | Ctrl, Pr. Ctrl | 0.97773 | 0.597 | 10 | 0.407 |
| | Ctrl, Excl | 1.1863 | 0.404 | 10 | 0.299 |
| | Excl, Pr. Ctrl | 1.5797 | 0.106 | 10 | 0.103 |
| Vent | Ctrl, Pr. Ctrl | 1.01 | 0.505 | 10 | 0.389 |
| | Ctrl, Excl | 4.5793 | 0.094 | 10 | **0.008** |
| | Excl, Pr. Ctrl | 4.1769 | 0.104 | 10 | **0.012** |

## 3.4 Discussion

In this study, biofilm biomass was differentially affected by bottom-up and top-down forces. Biomass was highly variable overall, and differences in patterns between vent and reference sites could not be detected clearly. Unlike laboratory investigations, field experiments are inevitably subjected to a certain degree of natural variability going beyond the control of the operators. This variability could help explaining the pattern observed in this study for the biomass of biofilms. Contrary to expectations, biofilm biomass decreased significantly when predators were excluded. This was evident in both vent and reference sites, and suggest that top-down control was not only relevant in all sites, but constituted the predominant factor in controlling biofilm biomass. It is likely that grazing, when occurred, determined a moderate disturbance that allowed the biofilms to modify its assemblage, resulting in the higher biomass observed (Kaehler and Froneman 2002). Indeed, biofilms are dynamic communities of microorganisms embedded in a gelatinous matrix whose composition often changes with time and physical disturbances. Predation on biofilms is a selective process with different grazers actively preying on specific components of the community; the predatory selectivity depends on both the anatomy of the predator feeding structures, as well as the prey's nutritional quality, physical characteristics and toxicity (Kaehler and Froneman 2002; Matz et al. 2008; Nagarkar et al. 2004).

Biofilm nutritional quality in this study was influenced by both acidification as well as grazing pressure. C:N ratio was significantly lower in biofilms from the vent site compared to biofilms from the reference site REF 1, indicating that the nutritional value of biofilms is enhanced by ocean acidification. However, the elevated natural variability detected in this experiment could not support this hypothesis statistically. The lower C:N ratio in biofilms that were not exposed to grazers (treatment Exclusion) compared to biofilms that experienced predation (treatments Control and Procedural Control) support the hypothesis that top-down was more relevant than bottom-up forces for this parameter, in this investigation.

The nutritional value of biofilms is not only related to the elemental ratio, but it also depends on the quality of the exopolysaccharides (EPS) forming the gelatinous matrix in which microorganisms are embedded. In this experiment, the composition of the organic matter of the entire biofilms (microbial cells as well as the EPS gelatinous matrix), varied according to the site and the treatment. Indeed, the Biopolymeric Organic Carbon content resulted overall higher in biofilms from the vent site, indicating

that these biofilms were richer in labile organic matter compared to biofilms from reference sites. This evidence would confirm previous findings about the enhanced content of uronic acids, a key component of the EPS matrix, in biofilms exposed to acidification in the same area (Lidbury et al. 2012). These changes in organic matter composition could be related to the sensitivity of enzyme activity to pH variations. Grossart et al. (2006) found that the activities of leucine aminopeptidase (LAPase), α-glucosidase (AGase), and β-glucosidase (BGase) increased at pH 7.8 corresponding to 2100 climate projections. Piontek et al. (2010) observed that the degradation of polysaccharides by bacterial extracellular enzymes AGase and BGase was significantly enhanced during experimental simulation of ocean acidification. Contrary to these results, Yamada and Suzumura (2010) reported that the activity of LAPase and lipase (L-ase) decreased with acidification, while the activities of phosphatase (P-ase) and BGase were not appreciably influenced by pH variation corresponding to a realistic scenario of ocean acidification, although the response of P-ase differed between coastal and semipelagic samples. Indeed, P-ase optimum activitiy typically varies in different organisms and different environments. For instance, in freshwater algae the optimum pH for P-ase acitivity was found to be 4.5, while the same enzyme exhibited maximum hydrolysis at pH 9.5 in marine bacteria (see Yamada and Suzumura 2010 and refereces therein). Although the effects of ocean acidification on enzyme activity seems to vary according to enzyme type and location, it is likely that acidification will affect the cycling of organic matter in marine ecosystems. This hypothesis would be furtherly supported by the evidences from the study here conducted. The influence of predators on the content of Biopolymeric Organic Carbon was not clear, as the presence of the cage itself affected this parameter. Based on this result, it seems that in this case bottom-up forces were prevalent compared to top-down.

Photosynthetic pigments are conventionally used as ecological indicators of the abundance as well as the photosynthetic rates of primary producers. In this study, the concentrations of photopigments were significantly lower in the reference sites compared to the vent site, but only when predators were allowed to graze (treatments Control and Procedural Control). When predators were removed (treatment Exclusion), the concentration of photopigments was similar among sites, suggesting that the resource effect of elevated $pCO_2$ on primary producers within the biofilm was not compensated by predation in acidified conditions.

In this study the carbonate content detected in the biofilms did not vary with acidification. Although there has been an intense research effort in reporting negative effects of ocean acidification on biological calcification, the response is not linear as for some organisms calcification rates in low pH conditions are actually enhanced (Ries, Cohen, and McCorkle 2009).

FAs profile of biofilms collected at the end of the experiment showed some analogies between samples from treatment Exclusion across the different sites: these samples were characterized by higher concentrations of FAs biomarkers for bacteria and diatoms with respect to control treatments, suggesting that grazing targeted especially these components of the biofilms. On the other side, based on FAs profiles, the two control treatments were more diverse and characterized by elevated abundance of microalgae and seagrasses.

Looking at the whole biochemical composition of biofilms in this experiment, it seems likely that both bottom-up and top-down forces had a major effect on the photoautotrophic members of the community. The elevated concentration of $pCO_2$ stimulated primary producers and resulted in biofilms characterized by higher nutritional values. It is possible that grazing on such biofilms decreased because grazers were able to meet their metabolic demand with less quantity of higher quality food. Top-down control was also important in regulating the composition and structure of the biofilms, affecting especially its biomass and carbonate content. However, when bottom-up and top-down controls interacted, predation could not compensate for the increase of primary producers in acidified conditions.

The results of this investigation support evidences that elevated $pCO_2$ could act as a resource on primary producers, and that compensatory processes such as predation might not be successful in buffering this effect. If in the future acidified ocean grazers will not be able to consume the exceeding biomass or photosynthetic activity of primary producers in biofilms, the structure of benthic communities sustained by these microorganisms will be likely affected.

## General Conclusions

The absorption of anthropogenic $CO_2$ emissions from the atmosphere into the oceans is causing alteration in the seawater chemistry, impacting marine ecosystems and their functioning (Hoegh-Guldberg and Bruno 2010).

This thesis aimed at investigating how elevated $pCO_2$ concentration and the consequent ocean acidification can affect two different marine communities, i.e. *Posidonia oceanica* meadows and marine microbial biofilms, which deliver essential services and products to the environment as well as human beings.

The hypothesis of the study was that ocean acidification could represent a resource for primary producers such as seagrasses and microbial biofilms ($CO_2$ enrichment, bottom-up effect), and that this resource effect could be compensated by interspecific interactions such as predation (top-down control). The results of the experiment, however, provided that the response of primary producers and their consumers is not linear. The finding of lower density and biomass in *P. oceanica* meadows, along with a reduced number of leaves that were narrower and shorter close to the Panarea vents, showed that the long-term exposure to acidification impaired the seagrass. The impact of elevated $CO_2$ concentration was evident also on the seagrass epiphytes, whose biomass decreased in acidified conditions. At the same time, the predation on leaves with less epiphytes decreased as well, suggesting that negative effects of acidification might propagate through the trophic web. As for *P. oceanica*, marine biofilms were found to be affected by ocean acidification too. Differences in the community composition of biofilms along the $pCO_2$/pH gradient in Levante Bay confirmed hypothesis on the sensitivity of these communities to these environmental changes (see Taylor et al. 2014 and references therein). Although the response was taxa-specific, the overall community assemblage changed significantly in acidified conditions. Based on this investigation, Gammaproteobacteria could be identified as potential "winners" in the future high-$CO_2$ world, while other taxa such as Alphaproteobacteria and Bacteroidetes resulted as potential "looser". Further investigations are required in order to better define the pattern of the community, as natural environments - shallow-water vents in particular - are characterized by elevated variability potentially impacting the outcomes of the investigations. This was particularly evident when

the effects of bottom-up (acidification) and top-down (grazing) controls on the biochemical composition of biofilms were investigated. Results did not allow to define clear patterns in the response of biofilms to ocean acidification and predation. It seems that bottom-up forces mainly affected the nutritional quality of the biofilms, while top-down control was more relevant in determining the biomass of biofilms. The increase in photopigments concentration in acidified conditions may suggest that $CO_2$ acted as resource (bottom-up control), and that predation (top-down control) was not able to compensate for this change. Perhaps, the physical disturbance caused by grazers changed the composition of biofilms toward one dominated by more productive taxa.

Predicting the effects of elevated $pCO_2$ concentration on marine communities is challenging, depending on both the degree of tolerance of each species as well on the effects of the interspecific interactions.

For some organisms such as *Posidonia oceanica*, ocean acidification might induce a physiological stress that propagate trough the meadow and reduce its ecological role. For other primary producers such as phototrophs within biofilms, elevated $pCO_2$ concentrations could represent a resource which could affect either the population density or their photosynthetic rates. It is likely that predators cannot not able to compensate the resource effect of elevated $pCO_2$ on primary producers because higher quality food in acidified conditions requires less consumption. Therefore, trophic interactions might fail at stabilizing the ecological changes determined by ocean acidification.

# Bibliography


Alcoverro, Teresa, Marta Pérez, and Javier Romero. 2004. “Importance of Within-Shoot Epiphyte Distribution for the Carbon Budget of Seagrasses: The Example of Posidonia Oceanica.” *Botanica Marina* 47(4): 307–12.

Aliani, Stefano, Giovanni Bortoluzzi, Giorgio Caramanna, and Francesco Raffa. 2010. “Seawater Dynamics and Environmental Settings after November 2002 Gas Eruption off Bottaro (Panarea, Aeolian Islands, Mediterranean Sea).” *Continental Shelf Research* 30(12): 1338–48.

Apostolaki, Eugenia T., Salvatrice Vizzini, Iris E. Hendriks, and Ylva S. Olsen. 2014a. “Seagrass Ecosystem Response to Long-Term High CO2 in a Mediterranean Volcanic Vent.” *Marine Environmental Research* 99(December): 9–15.

Bao, Wei Yang, Cyril Glenn Satuito, Jin Long Yang, and Hitoshi Kitamura. 2007. “Larval Settlement and Metamorphosis of the Mussel Mytilus Galloprovincialis in Response to Biofilms.” *Marine Biology* 150(4): 565–74.

Bhaskar, P V, and N B Bhosle. 2005. “Microbial Extracellular Polymeric Substances In\rmarine Biogeochemical Processes.” *Current Science* 88(1).

Bligh, E. G., and W. J. Dyer. 1959. “A Rapid Method of Total Lipid Extraction and Purification.” *Canadian Journal of Biochemistry and Physiology* 37(8).

Boatta, F. et al. 2013. “Geochemical Survey of Levante Bay, Vulcano Island (Italy), a Natural Laboratory for the Study of Ocean Acidification.” *Marine Pollution Bulletin* 73(2): 485–94.

Bopp, L. et al. 2013. “Multiple Stressors of Ocean Ecosystems in the 21st Century: Projections with CMIP5 Models.” *Biogeosciences* 10(10): 6225–45.

Bowman, John P. 2007. “Bioactive Compound Synthetic Capacity and Ecological Significance of Marine Bacterial Genus Pseudoalteromonas.” : 220–41.

Boyd, Philip W., and David A. Hutchins. 2012. “Understanding the Responses of Ocean Biota to a

Complex Matrix of Cumulative Anthropogenic Change." *Marine Ecology Progress Series* 470: 125–35.

Buia, Maria Cristina, Maria Cristina Gambi, and M Dappiano. 2004. "Seagrass Systems." *Biologia Marina Mediterranea* 11: 133–83.

Caldeira, K., and M.E. Wickett. 2005. "Ocean Model Predictions of Chemistry Changes from Carbon Dioxide Emissions to the Atmosphere and Ocean." *Journal of Geophysical Research* 110: C09S04.

Caldeira, Ken, and Michael E Wickett. 2003. "Anthropogenic Carbon and Ocean PH." *Nature* 425(6956): 365.

Caliro, Stefano et al. 2004. "Evidence of a Recent Input of Magmatic Gases into the Quiescent Volcanic Edifice of Panarea, Aeolian Islands, Italy." *Geophysical Research Letters* 31(7): L07619.

Campagne, Carole Sylvie, Jean Michel Salles, Pierre Boissery, and Julie Deter. 2014. "The Seagrass Posidonia Oceanica: Ecosystem Services Identification and Economic Evaluation of Goods and Benefits." *Marine Pollution Bulletin* 97(1–2): 391–400.

Capaccioni, B., F. Tassi, and O. Vaselli. 2001. "Organic and Inorganic Geochemistry of Low Temperature Gas Discharges at the Baia Di Levante Beach, Vulcano Island, Italy." *Journal of Volcanology and Geothermal Research* 108(1–4): 173–85.

Capaccioni, Bruno et al. 2005. "The November 2002 Degassing Event at Panarea Island (Italy): Five Months of Geochemical Monitoring." *Annals of Geophysics* 48(October).

Caporaso, J Gregory et al. 2010. "QIIME Allows Analysis of High-Throughput Community Sequencing Data." *Nat Methods.* 7(5): 335–36.

Caracausi, A. et al. 2005. "Massive Submarine Gas Output during the Volcanic Unrest off Panarea Island (Aeolian Arc, Italy): Inferences for Explosive Conditions." *Geochemical Journal* 39(5): 459–67.

Carapezza, M. L. et al. 2011. “Diffuse CO2soil Degassing and CO2and H2S Concentrations in Air and Related Hazards at Vulcano Island (Aeolian Arc, Italy).” *Journal of Volcanology and Geothermal Research* 207(3–4): 130–44.

Cebrián, J. et al. 1999. “Epiphyte Accrual on Posidonia Oceanica (L.) Delile Leaves: Implications for Light Absorption.” *Botanica Marina* 42(2): 123–28.

Chefaoui, Rosa M., Carlos M. Duarte, and Ester A. Serrão. 2018. “Dramatic Loss of Seagrass Habitat under Projected Climate Change in the Mediterranean Sea.” *Global Change Biology* (July).

Chiu, J. M. Y., V. Thiyagarajan, M. M. Y. Tsoi, and P. Y. Qian. 2005. “Qualitative and Quantitative Changes in Marine Biofilms as a Function of Temperature and Salinity in Summer and Winter.” *Biofilms* 2(03): 183.

Collier, C. J., and M. Waycott. 2014. “Temperature extremes reduce seagrass growth and induce mortality". *Marine Pollution Bulletin* 83(2):483-490

Connell, Sean D et al. 2013. “The Other Ocean Acidification Problem: CO 2 as a Resource among Competitors for Ecosystem Dominance.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 368(1627): 20120442.

Costa, Monya M. et al. 2015. “Epiphytes Modulate Posidonia Oceanica Photosynthetic Production, Energetic Balance, Antioxidant Mechanisms, and Oxidative Damage.” *Frontiers in Marine Science* 2(December): 1–10.

Cox, T. Erin et al. 2015. “Effects of Ocean Acidification on Posidonia Oceanica Epiphytic Community and Shoot Productivity.” *Journal of Ecology* 103(6): 1594–1609.

Dahms, Hans Uwe et al. 2018. “Marine Hydrothermal Vents as Templates for Global Change Scenarios.” *Hydrobiologia* 818(1).

Das, Surajit, and Neelam Mangwani. 2015. “Ocean Acidification and Marine Microorganisms: Responses and Consequences.” *Oceanologia* 57(4): 349–61.

Dattolo, E. et al. 2014. “Response of the Seagrass Posidonia Oceanica to Different Light

Environments: Insights from a Combined Molecular and Photo-Physiological Study." *Marine Environmental Research* 101(1): 225–36.

Davey, Mary Ellen, a O George, and George a O Toole. 2000. "Microbial Biofilms : From Ecology to Molecular Genetics." *Microbiology and Molecular Biology Reviews* 64(4): 847–67.

Donlan, Rodney M. 2002. "Biofilms: Microbial Life on Surfaces." *Emerging Infectious Diseases* 8(9): 881–90.

Donnarumma, L., C. Lombardi, S. Cocito, and M. C. Gambi. 2014. "Settlement Pattern of Posidonia Oceanica Epibionts along a Gradient of Ocean Acidification: An Approach with Mimics." *Mediterranean Marine Science* 15(3): 498–509.

Duarte, Carlos M. 2002. "The Future of Seagrass Meadows." *Environmental Conservation* 29(2): 192–206.

Dubois, Michel et al. 1956. "Colorimetric Method for Determination of Sugars and Related Substances." *Analytical Chemistry* 28(3): 350–56.

Engel, Anja et al. 2014. "Impact of CO2enrichment on Organic Matter Dynamics during Nutrient Induced Coastal Phytoplankton Blooms." *Journal of Plankton Research* 36(3): 641–57.

Esposito, A, Gianmarco Giordani, and M Anzidei. 2006. "The 2002-2003 Submarine Gas Eruption at Panarea Volcano (Aeolian Islands, Italy): Volcanology of the Seafloor and Implications for the Hazard Scenario." *Marine geology* 227(1–2): 119–34.

Fabiano, M, R Danovarot, and S Fraschettit. 1995. "A Three-Year Time Series of Elemental and Biochemical Composition of Organic Matter in Subtidal Sandy Sediments of the Ligurian Sea ( Northwestern Mediterranean )." 15(11): 1453–69.

Garrett, Trevor Roger, Manmohan Bhakoo, and Zhibing Zhang. 2008. "Bacterial Adhesion and Biofilms on Surfaces." *Progress in Natural Science* 18(9): 1049–56.

Gaylord, Brian et al. 2015. "Ocean Acidification through the Lens of Ecological Theory." *Ecology* 96(1): 3–15.

Ghedini, Giulia, and Sean D. Connell. 2017. “Moving Ocean Acidification Research beyond a Simple Science: Investigating Ecological Change and Their Stabilizers.” *Food Webs*: 1–7.

Giovannelli, Donato et al. 2013. “Diversity and Phylogenetic Analyses of Bacteria from a Shallow-Water Hydrothermal Vent in Milos Island (Greece).” *Frontiers in Microbiology* 4(JUL): 1–13.

Grossart, Hans Peter, Martin Allgaier, Uta Passow, and Ulf Riebesell. 2006. “Testing the Effect of CO2concentration on the Dynamics of Marine Heterotrophic Bacterioplankton.” *Limnology and Oceanography* 51(1 I): 1–11.

Gugliandolo, Concetta, Francesco Italiano, and Teresa Maugeri. 2006. “The Submarine Hydrothermal System of Panarea (Southern Italy): Biogeochemical Processes at the Thermal Fluids-Sea Bottom Interface.” *Annals of Geophysics* 49(2/3): 783–92.

Guilini, Katja et al. 2017. “Response of Posidonia Oceanica Seagrass and Its Epibiont Communities to Ocean Acidification.” *PloS one* 12(8): e0181531.

Guinotte, John M., and Victoria J. Fabry. 2008. “Ocean Acidification and Its Potential Effects on Marine Ecosystems.” *Annals of the New York Academy of Sciences* 1134(September): 320–42.

Haas, Brian J et al. 2011. “Chimeric 16S RRNA Sequence Formation and Detection in Sanger and 454-Pyrosequenced PCR Amplicons.” *Genome Research* 21: 494–504.

Hadfield, Michael G. 2011. “Biofilms and Marine Invertebrate Larvae: What Bacteria Produce That Larvae Use to Choose Settlement Sites.” *Annual review of marine science* 3: 453–70.

Hall-Spencer, Jason M et al. 2008. “Volcanic Carbon Dioxide Vents Show Ecosystem Effects of Ocean Acidification.” *Nature* 454(7200): 96–99.

Hassenrück, Christiane, Halina E. Tegetmeyer, Alban Ramette, and Katharina E. Fabricius. 2017. “Minor Impacts of Reduced PH on Bacterial Biofilms on Settlement Tiles along Natural PH Gradients at Two $CO_2$ Seeps in Papua New Guinea.” *ICES Journal of Marine Science: Journal du Conseil* 74(March).

Heiri, O., A. F. Lotter, and G. Lemcke. 2001. “Loss on Ignition as a Method for Estimating Organic

and Carbonate Content in Sediments: Reproducibility and Comparability of Results Oliver." *Journal of Paleolimnology* 25: 101–10.

Hemminga, Marten A., and Carlos M. Duarte. 2000. "Seagrass Ecology." In *Cambridge University Press,*.

Hendriks, I. E. et al. 2014. "Photosynthetic Activity Buffers Ocean Acidification in Seagrass Meadows." *Biogeosciences* 11(2): 333–46.

Hoegh-Guldberg, Ove, and John F. Bruno. 2010. "The Impact of Climate Change on the World's Marine Ecosystems." 328(June): 1523–29.

Inguaggiato, Salvatore et al. 2012. "Total CO<inf>2</Inf> Output from Vulcano Island (Aeolian Islands, Italy)." *Geochemistry, Geophysics, Geosystems* 13(2): 1–19.

Invers, Olga et al. 2001. "Inorganic Carbon Sources for Seagrass Photosynthesis: An Experimental Evaluation of Bicarbonate Use in Species Inhabiting Temperate Waters." *Journal of Experimental Marine Biology and Ecology* 265(2): 203–17.

Italiano, F., and P. M. Nuccio. 1991. "Geochemical Investigations of Submarine Volcanic Exhalations to the East of Panarea, Aeolian Islands, Italy." *Journal of Volcanology and Geothermal Research* 46(1–2): 125–41.

Jenkins, S. R. et al. 2001. "European-Scale Analysis of Seasonal Variability in Limpet Grazing Activity and Microalgal Abundance." *Marine Ecology Progress Series* 211: 193–203.

Jiang, Zhi Jian, Xiao-Pin Huang, and Jing-Ping Zhang. 2010. "Effects of CO(2) Enrichment on Photosynthesis, Growth, and Biochemical Composition of Seagrass Thalassia Hemprichii (Ehrenb.) Aschers." *Journal of integrative plant biology* 52(10): 904–13.

Johnson, V. R. et al. 2013. "Responses of Marine Benthic Microalgae to Elevated CO2." *Marine Biology* 160(8): 1813–24.

Johnson, Vivienne, Colin Brownlee, Marco Milazzo, and Jason Hall-Spencer. 2015. "Marine Microphytobenthic Assemblage Shift along a Natural Shallow-Water CO2 Gradient Subjected to

Multiple Environmental Stressors." *Journal of Marine Science and Engineering* 3(4): 1425–47.

Joint, Ian, Scott C Doney, and David M Karl. 2011. "Will Ocean Acidification Affect Marine Microbes?" *The ISME journal* 5(1): 1–7.

Kaehler, S., and P. W. Froneman. 2002. "Herbivore-Mediated Increase in the Photosynthetic Capacity of Marine Biofilms: Indirect Effects of Changing Microalgal Assemblage Composition." *Marine Ecology Progress Series* 234: 15–22.

Koch, Marguerite, George Bowes, Cliff Ross, and Xing Hai Zhang. 2013. "Climate Change and Ocean Acidification Effects on Seagrasses and Marine Macroalgae." *Global Change Biology* 19(1): 103–32.

Koopmans, Dirk et al. 2018. "The Response of Seagrass ( Posidonia Oceanica ) Meadow Metabolism to CO 2 Levels and Hydrodynamic Exchange Determined with Aquatic Eddy Covariance." (April): 1–23.

Kroeker, Kristy J., Rebecca L. Kordas, and Christopher D. G. Harley. 2017. "Embracing Interactions in Ocean Acidification Research: Confronting Multiple Stressor Scenarios and Context Dependence." *Biology Letters* 13(3): 20160802.

Kroeker, Kristy J, Maria Cristina Gambi, and Fiorenza Micheli. 2013. "Community Dynamics and Ecosystem Simplification in a High-CO2 Ocean." *Proceedings of the National Academy of Sciences of the United States of America* 110(31): 12721–26.

Lau, Winnie W Y. 2013. "Beyond Carbon: Conceptualizing Payments for Ecosystem Services in Blue Forests on Carbon and Other Marine and Coastal Ecosystem Services." *Ocean and Coastal Management* 83: 5–14.

Lauritano, C. et al. 2015. "Response of Key Stress-Related Genes of the Seagrass Posidonia Oceanica in the Vicinity of Submarine Volcanic Vents." *Biogeosciences* 12(13): 4185–95.

Lentini, Valeria et al. 2014. "Diversity of Prokaryotic Community at a Shallow Marine Hydrothermal Site Elucidated by Illumina Sequencing Technology." *Current Microbiology* 69(4): 457–66.

Lidbury, Ian et al. 2012. “Community-Level Response of Coastal Microbial Biofilms to Ocean Acidification in a Natural Carbon Dioxide Vent Ecosystem.” *Marine Pollution Bulletin* 64(5): 1063–66.

Mancuso Nichols, C. A., J. Guezennec, and J. P. Bowman. 2005. “Bacterial Exopolysaccharides from Extreme Marine Environments with Special Consideration of the Southern Ocean, Sea Ice, and Deep-Sea Hydrothermal Vents: A Review.” *Marine Biotechnology* 7(4): 253–71.

Manini, E. et al. 2008. “Prokaryote Diversity and Virus Abundance in Shallow Hydrothermal Vents of the Mediterranean Sea (Panarea Island) and the Pacific Ocean (North Sulawesi-Indonesia).” *Microbial Ecology* 55(4): 626–39.

Marbà, Núria, and Carlos M. Duarte. 2010. “Mediterranean Warming Triggers Seagrass (Posidonia Oceanica) Shoot Mortality.” *Global Change Biology* 16(8): 2366–75.

Marco-Méndez, Candela et al. 2015. “Epiphyte Presence and Seagrass Species Identity Influence Rates of Herbivory in Mediterranean Seagrass Meadows.” *Estuarine, Coastal and Shelf Science* 154: 94–101.

Martin, Sophie et al. 2008. “Effects of Naturally Acidified Seawater on Seagrass Calcareous Epibionts.” *Biology letters* 4(6): 689–92.

Matz, Carsten et al. 2008. “Marine Biofilm Bacteria Evade Eukaryotic Predation by Targeted Chemical Defense.” *PLoS ONE* 3(7): 1–7.

Munné-Bosch, S., and L. Alegre. 2002. “Plant Aging Increases Oxidative Stress in Chloroplasts.” *Planta* 214(4): 608–15.

Nagarkar, Sanjay, Gray A. Williams, G. Subramanian, and S. K. Saha. 2004. “Cyanobacteria-Dominated Biofilms: A High Quality Food Resource for Intertidal Grazers.” *Hydrobiologia* 512: 89–95.

Nogueira, Patricia et al. 2017. “Altered Epiphyte Community and Sea Urchin Diet in Posidonia Oceanica Meadows in the Vicinity of Volcanic CO2vents.” *Marine Environmental Research*

127: 102–11.

O'Brien, Charles E. et al. 2015. "Microbial Biofilms Associated with Fluid Chemistry and Megafaunal Colonization at Post-Eruptive Deep-Sea Hydrothermal Vents." *Deep-Sea Research Part II: Topical Studies in Oceanography* 121: 31–40.

Ondiviela, Barbara et al. 2014. "The Role of Seagrasses in Coastal Protection in a Changing Climate." *Coastal Engineering* 87: 158–68.

Ortega-Morales, Benjamn Otto, Manuel Jesùs Chan-Bacab, Susana del Carmen De la Rosa-Garcìa, and Juan Carlos Camacho-Chab. 2010. "Valuable Processes and Products from Marine Intertidal Microbial Communities." *Current Opinion in Biotechnology* 21(3): 346–52.

Orth, RJ et al. 2006. "A Global Crisis for Seagrass Ecosystems." *Biogeoscience* 56(12): 987–96.

Ott, Joerg A. 1980. "Growth and Production in Posidonia Oceanica (L.) Delile*." *Marine Ecology* 1(1): 47–64.

Piazzi, Luigi, David Balata, and Giulia Ceccherelli. 2016. "Epiphyte Assemblages of the Mediterranean Seagrass Posidonia Oceanica: An Overview." *Marine Ecology* 37(1): 3–41.

Qian, P. Y. et al. 2007. "Marine Biofilms as Mediators of Colonization by Marine Macroorganisms: Implications for Antifouling and Aquaculture." *Marine Biotechnology* 9(4): 399–410.

Raven, John A., Mario Giordano, John Beardall, and Stephen C. Maberly. 2012. "Algal Evolution in Relation to Atmospheric CO2: Carboxylases, Carbon-Concentrating Mechanisms and Carbon Oxidation Cycles." *Philosophical Transactions of the Royal Society B: Biological Sciences* 367(1588): 493–507.

Ries, Justin B., Anne L. Cohen, and Daniel C. McCorkle. 2009. "Marine Calcifiers Exhibit Mixed Responses to CO2-Induced Ocean Acidification." *Geology* 37(12): 1131–34.

Rosenberg, Eugene. 2013. The Prokaryotes: Alphaproteobacteria and Betaproteobacteria *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*.

Russell, Bayden D., Sean D. Connell, Sven Uthicke, et al. 2013. “Future Seagrass Beds: Can Increased Productivity Lead to Increased Carbon Storage?” *Marine Pollution Bulletin* 73(2): 463–69.

Russell, Bayden D., and Sean D. Connell. 2012. “Origins and Consequences of Global and Local Stressors: Incorporating Climatic and Non-Climatic Phenomena That Buffer or Accelerate Ecological Change.” *Marine Biology* 159(11): 2633–39.

Russell, Bayden D et al. 2012. “Predicting Ecosystem Shifts Requires New Approaches That Integrate the Effects of Climate Change across Entire Systems.” *Biology letters* 8(2): 164–66.

Russell, Bayden D, Sean D Connell, Helen S Findlay, et al. 2013. “Ocean Acidification and Rising Temperatures May Increase Biofilm Primary Productivity but Decrease Grazer Consumption.” *Phil Trans R Soc B* 368: 20120438.

Sabine, Christopher L et al. 2004. “The Oceanic Sink for Anthropogenic CO2.” *Science (New York, N.Y.)* 305(5682): 367–71. http://science.sciencemag.org/content/305/5682/367.abstract.

Salta, Maria et al. 2013. “Marine Biofilms on Artificial Surfaces: Structure and Dynamics.” *Environmental Microbiology* 15(11): 2879–93.

Sandrini, Giovanni et al. 2014. “Genetic Diversity of Inorganic Carbon Uptake Systems Causes Variation in CO2response of the Cyanobacterium Microcystis.” *ISME Journal* 8(3): 589–600.

Stocker, T.F. et al. 2013. *IPCC, 2013: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*.

Takahashi, M, S H C Noonan, Katharina E Fabricius, and Catherine J. Collier. 2015. “The Effects of Long-Term in Situ CO2 Enrichment on Tropical Seagrass Communities at Volcanic Vents.” *ICES Journal of Marine Science* 69(3): 1205–17.

Taylor, Joe D. et al. 2014. “Intertidal Epilithic Bacteria Diversity Changes along a Naturally Occurring Carbon Dioxide and PH Gradient.” *FEMS Microbiology Ecology* 89(3): 670–78.

Thomason, J., and S. Dürr. 2010. *Biofouling*.

Unsworth, Richard K F, Mike van Keulen, and Rob G. Coles. 2014. “Seagrass Meadows in a Globally Changing Environment.” *Marine Pollution Bulletin* 83(2): 383–86.

Vizzini, S. et al. 2013. “Trace Element Bias in the Use of CO2 Vents as Analogues for Low PH Environments: Implications for Contamination Levels in Acidified Oceans.” *Estuarine, Coastal and Shelf Science* 134: 19–30.

Vizzini, S., Martínez-Crego, B., Andolina, C., Massa-Gallucci, A., Connell, S. D., Gambi, M. C.. 2017. “Ocean Acidification as a Driver of Community Simplification via the Collapse of Higher-Order and Rise of Lower-Order Consumers.” *Scientific Reports* 7(1): 1–10.

Vizzini, S., G. Sarà, R. H. Michener, and A. Mazzola. 2002. “The Role and Contribution of the Seagrass Posidonia Oceanica (L.) Delile Organic Matter for Secondary Consumers as Revealed by Carbon and Nitrogen Stable Isotope Analysis.” *Acta Oecologica* 23(4): 277–85.

Vizzini, Salvatrice et al. 2010. “Effect of Explosive Shallow Hydrothermal Vents on δ13C and Growth Performance in the Seagrass Posidonia Oceanica.” *Journal of Ecology* 98(6): 1284–91.

Wang, Qiong, George M. Garrity, James M. Tiedje, and James R. Cole. 2007. “Naïve Bayesian Classifier for Rapid Assignment of RRNA Sequences into the New Bacterial Taxonomy.” *Applied and Environmental Microbiology* 73(16): 5261–67.

Waycott, M. et al. 2009. “Accelerating Loss of Seagrasses across the Globe Threatens Coastal Ecosystems.” *Proceedings of the National Academy of Sciences* 106(30): 12377–81.

Witt, Verena et al. 2011. “Effects of Ocean Acidification on Microbial Community Composition of, and Oxygen Fluxes through, Biofilms from the Great Barrier Reef.” *Environmental Microbiology* 13(11): 2976–89.

Yamada, Namiha, and Masahiro Suzumura. 2010. “Effects of Seawater Acidification on Hydrolytic Enzyme Activities.” *Journal of Oceanography* 66(2): 233–41.

Zimmerman, R. C., D. G. Kohrs, D. L. Steller, and R. S. Alberte. 1997. “Impacts of CO2 Enrichment on Productivity and Light Requirements of Eelgrass.” *Plant physiology* 115(2): 599–607.

Zimmermann, Petra, and Ulrike Zentgraf. 2005. “The Correlation between Oxidative Stress and Senescence during Plant Development.” *Cellular and molecular biology letters* 10(August): 515–34.