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Global and local stressors effects on community succession in the Mediterranean intertidal zone

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CHAPTER 1. ECOLOGICAL SUCCESSIONS IN INTERTIDAL HABITATS, AN OVERVIEW

1.1 DEFINING DISTURBANCE AND MODELS OF COMMUNITY DYNAMICS

Marine communities, as well as their terrestrial counterparts, can change over ecological and evolutionary time scale. Typically the main driver of community change is the disturbance, which includes both natural and/or anthropogenic stressors acting at global and local spatial scale. The disturbance is considered as any event that alters the composition and structure of a given community, the availability of resources or substrate, and the physical environment (Pickett and White, 1985). In a more general context, the disturbance can be considered as 'any process that causes the reduction or removal of living organisms in an area' (Farrell, 1989; 1991).

Disturbances may be classified as natural and anthropogenic (caused by humans), biotic (caused by living organisms) and abiotic (caused by non-living organisms). In relation to their frequency over time, the disturbances can be considered chronic (little intense and frequent disorder) or pulse (intense but rare disorder). Following the classification made by Pickett and White (1985), each disturbance event can be measured in space and time according to different characteristics, such as: their spatial distribution, frequency (mean number of events per time period), return interval (mean time between disturbances), rotation period (mean time needed to disturb an area), predictability (the inverse function of variance in the return interval), size of the disturbed area, their magnitude (intensity and severity of a given disturbance) and synergism (interacting effects on the co-occurrence of other disturbances).

A natural system can respond to disturbances in different ways; the ability of a natural system not to undergo the effects of a phenomenon of disturbance is known as resistance and its ability to return to conditions prior the disturbance phenomenon is known as resilience (Holling, 1973). Instead, the stability of a natural system is a concept that considers the ecological communities in a dynamic equilibrium, caused by different frequency of disturbances. To explain this concept, Connell (1978) hypothesized the intermediate disturbance theory. When disturbances are rare or small, top competitors eliminate the lower ones. Under a frequent or intense disturbance, the dominant competitors are reduced or eliminated and the pioneers' species (those typical of the

early stages) dominate the system. In both cases, the disturbance reduces the species richness of the whole system. Instead, under a disturbance regime of intermediate frequency and intensity, some of the resident species remain within the system together with the pioneers' species, which colonize the disturbed areas. In this way, the intermediate disturbance leads to the maximization of the biodiversity at a given spatio-temporal scale.

Disturbances affect community dynamics. The structure of any assemblage of populations of living organisms in a prescribed area or habitat and in a given temporal scale (i.e. the ecological community) is not static, but changes over an ecological time occur as a result of a single disturbance or the synergism between multiple disturbances that cause the new arrival or the loss of species. Therefore, community dynamics can be of three different types:

- Successions
- Patch dynamics
- Multiple stable states

The successions are unidirectional, non-seasonal and continuous changes in colonization and extinction patterns in an area that occur at community level in an ecological time (the topic will be dealt with in detail in the next section).

The patch dynamics are cyclic changes of species that occur in communities occupying spatially small patches (Watt, 1947b) and they repeat over time creating a mosaic of patches. The most important factors, which lead the cyclic changes in the community dynamics, are the internal (as the decline of perennial plant with the age) and external disturbance (as the dimension, form and frequency of gaps), the dispersion capacity, the recruitment and the growth of species. The mechanism of patch dynamics is common both in tropical terrestrial and intertidal environments, but studies on several examples of cyclic changes were carried out in the terrestrial vegetation. Watt (1947b) conducted an experiment on the British vegetation. *Calluna* heath is a dominant plant in Scotland and it covers vast areas. *Calluna* is pioneer in the establishment and growth in open patches and can achieves a maximum cover from 7 to 10 years. During senescence *Calluna* loss its canopy and the gaps are invaded by *Cladonia* lichens. When the lichen mat dies, leaves bare ground that is invaded by bearberry *Arctostaphylos*, which in turn is re-invaded by

Calluna (Fig. 1). These cycles of change lead a stable community to a constant state of fluctuations of phases over time and, in natural community, tend to operate on small spatial scales (Watt, 1947b).

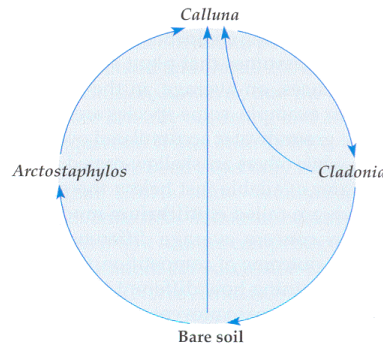


Fig. 1 - Cyclic phases of patch dynamics in the British vegetation. *Calluna* heath is a dominant plant in Scotland and is pioneer in the establishment and growth in open patches. During senescence *Calluna* loss its canopy and the gaps are invaded by *Cladonia* lichens. When the lichen mat dies, leaves bare ground that is invaded by bearberry *Arctostaphylos*, which in turn is re-invaded by *Calluna*.

When a community changes its composition over time and it does not observe a single stable configuration, the natural community can exist under multiple stable states. Communities may transition from one stable state to another when they are subjected to perturbations (Sutherland, 1990), and this process is known as phase shift. Because ecosystems are resistant to state shifts, significant perturbations are usually required to overcome ecological thresholds and cause shifts from one stable state to another (Holling, 1973). These alternative states are non-transitory and therefore considered stable over ecologically relevant timescales.

Connell and Sousa (1983) defined the criteria that a community exhibits under multiple stable states:

- The community has an equilibrium point at which it stays, or to which it comes back if the system is perturbed by a disturbance.
- If the system is enough disturbed, the community will shift to a second equilibrium point at which it will remain after the disturbance has terminated.
- The environment that sustains the community must be similar in diverse sites.
- The community must persist for more than one generation in all sites considered multiple stable states.

Multiple stable states may occur in communities affected by anthropic and natural disturbances, as the example of the Caribbean reef. The coral reefs of the Caribbean region suffered a dramatic shift from coral-dominated to communities dominated by photophile macroalgae, due a combination of natural (hurricanes and epidemics) and anthropic disturbances (overfishing and pollution) acting synergically on the reef leading to a phase shift (Hughes, 1994).

1.2 THE ECOLOGICAL SUCCESSIONS

In ecology a succession is defined as the gradual and orderly process of change in an ecosystem brought about by the progressive replacement of a community by another one (e.g. changes in species composition) over time that occurs after a disturbance takes place (Connell and Slatyer, 1977). Generally, ecologists identify two types of successions that differ for their starting point:

- The primary succession occurs in an uninhabited barren habitat or in an environment that is devoid of vegetation, and living organisms colonize the bare rock for the first time.
- The secondary succession occurs in a previously occupied area where a disturbance event kills much or all community, and a new re-colonization follows the disturbance.

During primary and secondary succession the sequential progression of species is not a random process, but often it involves from less stable communities with lower species diversity to more stable communities with higher species diversity. At the beginning of succession only a small number of pioneer species are capable to colonize and thrive in disturbed habitats. These early successional stages are characterized from small size, high growth rates and dispersion capacity. These species play a crucial role in determining the progression of an ecological succession. When the early successional stages settle on the substrate can modify the surrounding habitat making it less or more suitable for the settlement of later successional species. The latest species are characterized by slow growth rates, larger size, lower dispersion capacity and are more efficient competitors for limited resources than early successional stages. When the physical environment remains constant, the changes in specific composition become very slow or cease altogether. This

late stage of succession coincides with a state of equilibrium called “climax community”. The monoclimax theory was postulated by Clements (1916; 1936). Although the climax is considered as a state in which there is a constant specific composition, the relative abundance of species can vary in relation to little fluctuations in physical environmental conditions (Horn, 1974).

In absence of significant changes in abiotic conditions, Connell and Slatyer (1977) proposed three different models of successions for sessile organisms: facilitation, tolerance and inhibition (Fig. 2). The first step of all three models is the colonization of new available space from species with broad power of dispersal and rapid growth. The early successional stages are the only species able to recruit, settle and persist under adverse physical conditions that occur immediately after disturbance.

Connell and Slatyer (1977)

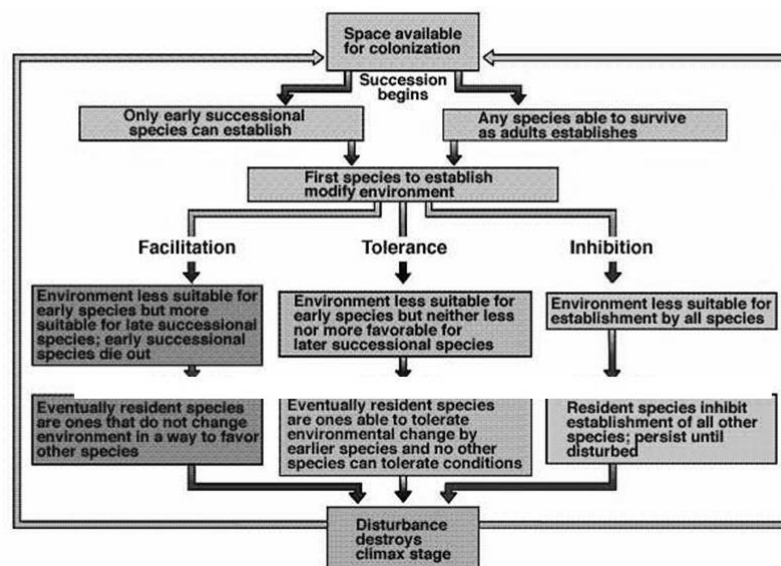


Fig. 2 - The Connell and Slatyer (1977) models of ecological successions for sessile organisms.

The three models differ in the mechanisms that determinate the replacement of species during the succession.

In the facilitation model the early successional stages modify the environment make it less suitable for the subsequent recruitment of individuals of their species, but facilitating the growth to maturity of later successional stages.

In the tolerance model the presence of early successional species does not influence the recruitment and growth rates of latest species. In fact, the early colonists modify the

environment make it less favorable for the individuals of the same species, but this modification can have little or no effects on the late successional stages, which are able to recruit and grow slowly also in presence of undamaged earlier species. Over time, species that appear later in the succession, being more efficient in the exploiting of resources, reduce available resources for early successional species (i.e. light or space). In this model, only the life history features of earlier and later colonists influence the succession of species.

In the inhibition model, the early successional species modify the environment make it less suitable for both early and late species' recruitment. The latest species live longer and gradually accumulate and replace earlier species, which are killed by local disturbance and not by competition with later species.

The Connell and Slatyer models were criticized by many authors (Dean and Hurd, 1980; Walker and Chapin, 1987) because do not considered many sources of variability naturally presents in a succession as temporal and spatial variation in recruitment, growth and mortality of species; changes in biotic interactions (e.g. grazing rates); direct and indirect effects of local and global stressors.

Most of the studies on successions were carried out in terrestrial communities where the progression of species takes place for a longer time than the course of ecological studies, instead few researches were performed in communities in which the time-course of succession is relatively short, such as the rocky intertidal shores, and the progression of succession is subjected to environmental physical disturbance, like local and global stressors, and biotic pressure, like grazing (Farrell, 1989; Benedetti-Cecchi and Cinelli, 1996; Kordas *et al.*, 2014; 2017).

Typically, the succession in the intertidal habitat, that is the zone comprised from the upper and the lower limit of a tidal range, occurs at different steps (Sousa, 1979b). In a primary succession, the early successional stages are typically represented by bacteria, cyanobacteria and diatoms and their settlement alters the substrate surface for the colonization of subsequent generalistic (cosmopolitan) or opportunistic algae, which have flagellate motile spores and usually colonize the marine rocky intertidal immediately after the first colonizers. These species, in most of cases filamentous algal turfs or foliose green algae (e.g. *Ulva* spp.), are able to settle and grow very fast and consequently occupy most of available space, forming dense mats. Successively, in the latest stages of intertidal succession more complex perennial algae (such as brown or red macroalgae) become

dominant, completely replacing the early successional species, and providing refuge and food to many intertidal invertebrate species (Fig. 3).

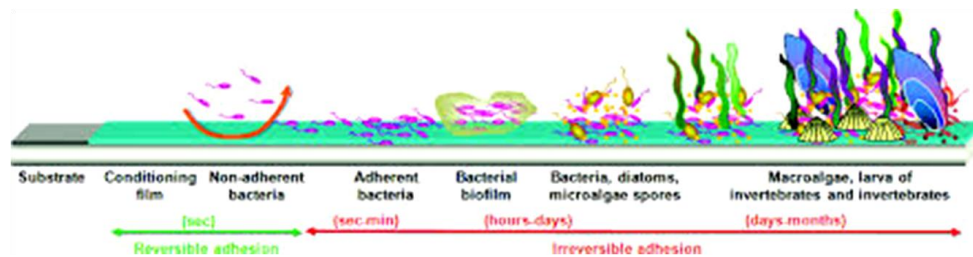


Fig. 3 - Example of a marine intertidal succession.

1.3 THE ROCKY INTERTIDAL SHORES

The intertidal rocky habitat is the zone of connection, through the influence of sea spray and waves (i.e. aerosol), between terrestrial and marine habitats and represents the interface between land and sea. Rocky shores are characterized by two different environmental gradients: vertical, between low and high shores, and horizontal, between exposed and sheltered zones (Foster, 1971). The vertical gradient is unidirectional and the desiccation stress and the temperature fluctuations experienced by intertidal organisms increase in the high shores, especially during the emersion period at low tide (Foster, 1971). The progression and species distribution is limited along the vertical gradient and is strictly dependent from the capacity of intertidal species to tolerate high desiccation rates and temperature variations (Connell, 1961a; Dring and Brown, 1982). The horizontal gradient is associated to the wave exposure and physical disturbance regimes such as wave energy and scouring by suspended sediments. The influence of wave exposure gradient is not unidirectional, but depends on the features of organisms to live in wave swept or sheltered conditions. For example suspension feeders, such as mussels, thrive in wave swept conditions (Moschella *et al.*, 2005; Vaselli *et al.*, 2008), instead other species prefer shelter conditions, as the intertidal canopy-forming macroalgae (Jonsson *et al.*, 2006).

Rocky intertidal shores sustain a great variety of marine flora and fauna that are subjected to changes in both marine conditions and atmospheric regimes, during the low tide (Hawkins *et al.*, 2016). The intertidal habitats are considered opened systems

because populations living on the rocky shores are often composed by larvae and propagules coming from outside sources. On the intertidal shores sessile organisms, such as algae and invertebrates, find available substrate to settle and grow. This habitat further provides spawning and nursery areas for marine mobile animals, refuge and food for intertidal grazers and fishes (Taylor and Schiel, 2010). Moreover, some communities inhabiting the rocky intertidal shores can play an important role in the regulation of environmental conditions and the provisioning of important ecosystem services. As an example, macrophytes and bivalves play a key role in seawater purification (Kohata *et al.*, 2003); instead, the macroalgal species have a fundamental role in the macronutrients removal (nitrogen and phosphorus) and in the carbon sequestration processes (Chung *et al.*, 2011). In addition, biogenic reef-forming species are important in the regulation of environmental conditions, e.g. attenuating storms and wave energy, and providing a natural coastal protection (Borsje *et al.*, 2011).

The particular position of the intertidal zone makes it subject to disturbances that act both at local and global scale, which may affect the successional patterns and the composition of species' assemblages inhabiting the rocky shores over time. At local scale, the succession and distribution of organisms on intertidal shores can be influenced by biological interactions such as grazing (Jenkins *et al.*, 2005; Taylor and Schiel, 2010). While the upper distributional limits of organisms are generally influenced by physical factors (e.g. desiccation rates and temperature), the lower distributional limit is controlled by grazing activity (Connell, 1972; Hawkins and Hartnoll, 1985).

The amount of impact on changes in community dynamics in intertidal assemblages is dependent by the grazers' density, but could be also related on the timing of recruitment of intertidal organisms like algae and other invertebrates (Underwood, 1983; Farrell, 1991; Benedetti-Cecchi, 2000). Some authors have demonstrated that the successional patterns of intertidal species are strongly affected by consumer presence, which can accelerate, slow down or not affect the progression of species' assemblages (Farrell, 1991).

In addition to biological interactions, also may physical factors acting at a local scale, as well as their frequency and intensity, can affect the successional patterns of intertidal species (i.e., influencing both the distribution and the dynamics of species). In a limited space, severe and chronic disturbances like storm waves could fragment the community

in a mosaic of different successional stages (Benedetti-Cecchi and Cinelli, 1996) and may lead to a start of a new succession.

The successional dynamics of assemblages living on rocky intertidal shores could be also affected by disturbances at global scale, such as ocean warming and acidification. In the last decades, growing evidence suggests that global stressors may have profound consequences for marine species, and the magnitude of disturbance effects could be exacerbated for intertidal organisms which are subjected to both terrestrial and marine stressors in this context. Disturbances acting at global scale may drive shifts in successional pattern, distribution, abundance and species diversity (Hughes, 2000; Zavaleta *et al.*, 2003) and may influence the ability of a given species to resist or to recover after a disturbance event.

The following paragraphs will discuss in a greater detail the main global stressors, i.e., ocean warming and acidification, and local stressors, grazing and storms waves acting on marine intertidal communities. This thesis will focus on rocky intertidal communities in the Mediterranean Sea. Despite these local and global stressors are relatively well understood as drivers of change in isolation, there is little knowledge about how the interaction of these disturbances can divert intertidal successions. Long-term manipulative studies in natural systems, combined with experimental investigations of multiple stressors effects on species and communities, are needed to better understand the local and global mechanisms that drive the succession of species in intertidal rocky systems.

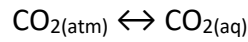
1.4 DESCRIPTION OF THE GLOBAL STRESSORS CONSIDERED IN THIS STUDY

(a) The ocean acidification effects in intertidal systems

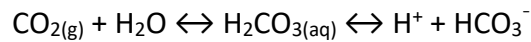
About one-quarter of anthropogenic CO₂ released in the atmosphere from the industrial era was absorbed by oceans (Sabine and Feely, 2007). Oceans represent an important 'sink' of carbon and contribute to the slowing down of CO₂ in the atmosphere, and currently absorb more than 25 million of tonnes per day of anthropogenic CO₂ (IPCC, 2007). The increase in CO₂ captured by the oceanic surface causes a consequent reduction in both pH and carbonate ions (CO₃²⁻) concentration in seawater (Zeebe and

Wolf-Gladrow, 2001). This phenomenon is commonly referred to as "oceans acidification" (OA) (Caldeira and Wickett, 2003).

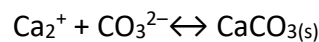
The first key reaction occurs when the gaseous CO₂ from the atmosphere dissolves in marine water:



This exchange is relatively fast, with a balance between ocean and atmosphere that persists for many months (Feely *et al.*, 2009). The CO₂ absorbed by oceanic water surface generates carbonic acid that increases the concentration of H⁺, HCO₃⁻ and dissolved CO₂, while there is a decrease in the carbonate concentration (CO₃²⁻) and oceanic pH (Caldeira and Wickett, 2003) in accordance with the following equation:



These reactions are very rapid, takes about ten seconds for CO₂ hydration and a few microseconds for the subsequent acid-base reactions (Zeebe and Wolf-Gladrow, 2001). A third key reaction that occurs in the marine environment leads to the formation or dissolution of calcium carbonate minerals:



The decrease in carbonate ions increases the rate of dissolution of CaCO₃ minerals and leads to a reduction in the saturation state of CaCO₃, which has a significant impact on calcification of marine organisms (Guinotte and Fabry, 2008). The saturation state (Ω) is the degree of CaCO₃ saturation in the seawater:

$$\Omega = \{[\text{Ca}^{2+}] \times [\text{CO}_3^{2-}]\} / K_{\text{ps}}$$

where [Ca²⁺] and [CO₃²⁻] are respectively calcium and carbonate ions *in situ*, and K_{sp} is the solubility of CaCO₃ (when the concentrations are in balance there is neither dissolution nor formation of CaCO₃). The K_{sp} values depend on the crystalline form of CaCO₃ and may also vary with temperature and pressure (Williamson and Turley, 2012). Highly saturated environments are potentially more suitable for calcifying organisms (algae and animals that form shells and skeletons of CaCO₃), considering that high Ω values reduce the energy required for bio-calcification, implying an active intracellular regulation of Ca²⁺, H⁺ and inorganic carbon, and also helping to maintain the integrity of calcium carbonate

after its formation. Conversely a low saturation state, and thus a reduction in the availability of carbonate ions, will make the calcification more difficult for marine organisms and more energy will be needed for the formation of biogenic CaCO_3 (Guinotte and Fabry, 2008). Calcium carbonate produced by marine calcifying organisms is mainly found in form of calcite and aragonite. These two different crystalline structures have a different degree of stability. Calcite is thermodynamically more stable, while aragonite is less stable. The tendency to dissolve a crystalline structure is strongly influenced by the saturation state (Ω) of the mineral, which in turn is related to the concentration of calcium and carbonate ions in seawater (Pelejero *et al.*, 2010). The depth of calcite and aragonite saturation horizon is important to calcification in marine organisms because it determines the limit in which the precipitation of biogenic calcium carbonate or dissolution occurs.

Due to continuous CO_2 emissions in the atmosphere from 1900 to today, a 30% increase in the concentration of H^+ ions in surface ocean waters occurred, leading to a decrease in the average oceanic pH units from 8.21 to 8.10 (Royal Society, 2005). Because the pH scale is logarithmic, small numerical variations represent significant effects. If this trend persists by the end of 2100, a decrease in seawater pH of up to 0.5 units will occur, resulting in an increase of 100-150% of $[\text{H}^+]$ (Caldeira and Wickett, 2003). Continued CO_2 emissions over the next centuries could produce higher oceanic pH changes than those recorded in the last 300 million years (Honisch *et al.*, 2012).

Intertidal organisms have exhibited mixed responses to ocean acidification, suggesting that changes in competition strength, dominance, and successional patterns of these organisms may occur (Connell and Russell, 2010). Many laboratory and field studies demonstrated that some marine algae can benefit from highest levels of CO_2 by increasing their growth and their ability to calcify (Iglesias-Rodriguez *et al.*, 2008), while other algal species showed different responses. Indeed, in a study conducted off Vulcano island (Italy) and Papua New Guinea, an increase in photosynthetic capacity was observed in *Padina pavonica* (Linnaeus) J.V. Lamouroux and *Padina australis* Hauck, associated with higher concentrations of CO_2 , while assisting to a loss of fronds' calcification (Johnson *et al.*, 2012). In general, even seagrasses are able to thrive under high CO_2 concentrations also due to a decrease in the coverage of calcifying epiphytes associated, which in normal conditions might reduce the photosynthetic surface (Martin *et al.*, 2008). More recently, it was demonstrated that marine and estuary phanerogams exposed to high CO_2

conditions undergo a reduction in the production capacity of tannins and phenolic compounds, which are photo-protective, antimicrobial and deterrent against grazing (Arnold *et al.*, 2012). Coralline algae are likely the most sensitive group of algae to ocean acidification as their skeleton mineralogy may dissolve at low saturation levels of calcium carbonate (Martin and Gattuso, 2009).

The global phenomenon of ocean acidification is known also to affect early and late successional species in marine habitats. In a research carried out along a natural pH gradient it was observed that the successional patterns of early species colonization under acidified or not acidified conditions. Diatoms are the first colonizing species during the spring when the cover of erect forms is reduced. After, the recruitment and growth of algal turf appears to be facilitated by exposure to low pH conditions, recruiting rapidly (Porzio *et al.*, 2013). Turf algae, generally considered opportunistic species, appear to be resilient to ocean acidification and the increase of algal turf biomass in low pH conditions was considered a threat to other benthic species (Russell *et al.*, 2009; Connell and Russell, 2010). Generally in marine benthic communities, successions starts with the adhesion of particulate material to the surface, followed by the aggregation of bacteria and microalgae and the formation of a biofilm layer which provides the basis for subsequent settlements of crustose, filamentous (turf) and erect algal forms (Ceccarelli *et al.*, 2011). In the study conducted by Porzio *et al.* (2013) crustose, filamentous and erect algae were found in succession over the experiment, but the colonization of the first morphological groups was strongly pH-dependent. In particular, calcifying forms were dominant in ambient pH, while in the most acidified sites they were replaced by small-sized turfing-form algae which recruited quickly. In another experiment, Porzio *et al.* (2011) monitored the successional patterns of late stages species under normal and acidified conditions. The experiment suggested that most of the later successional species can tolerate the CO₂ levels predicted to occur at the end of this century. Even if calcified species continue to grow under acidified conditions, there was a simplification of the macroalgal community and an increase in dominance by a few species. Both erect and crustose calcified species were largely replaced by non-calcified algae because may be better adapted to elevated CO₂ levels. The competition between calcifying species, turf and erect algae is common in marine ecosystems (Connell and Russell, 2010), and this shift between algal communities often led to a decline in biodiversity and alteration of ecosystems (Hughes *et al.*,

2007), suggesting that acidification of the oceans could have profound repercussions on marine successions.

(b) Ocean warming effects in intertidal systems

It has been estimated that ocean temperatures (from 0 to 700 meters depth) increased considerably between the 1971 and 2010 (Levitus *et al.*, 2012). Most of this heat was accumulated close to the surface of the oceans, causing a warm of about 0.7° C over the last hundred years on a global scale (Trenberth *et al.*, 2007). The rate of oceans warming over this period exceeds 0.1° C per decade above -75 meters, while it is decreasing by 0.015° C per decade under -700 meters (IPCC, 2013). The magnitude of the ocean surface warming during this century will be mainly determined by the amount of greenhouse gases that will be emitted into the atmosphere over the next decade (Meehl *et al.*, 2007). By contrast, heat exchanges with ocean depths were minimal until now, in fact the ocean temperature average, considering the entire water column, is only increased by 0.04° C. This differential heating of the water masses caused the expansion of the density's gradient between the surface and the ocean depths, increasing its stratification. Stronger stratification tends to reduce transport and mixing in the upper layer of the water column, which is constantly exchanging with the atmosphere and ocean depths. Heating and high stratification of water masses could also limit the absorption of greenhouse gases, such as CO₂ and CFCs, because transporting these gases from surface to ocean depths is the main limiting step (Gruber, 2011). The ocean surface temperature will have a slightly slower rate than the average global air temperature, and an increase between 2.5° C and 4° C in the next hundred years is expected (IPCC, 2007). At global level, the surface temperature of the oceans is not uniform, and there is higher warming to the tropics and high latitudes in the northern hemisphere. It was predicted that higher heating will occur in regions where marine ice will melt, as the shift from a higher reflective surface (high albedo) to a lower reflective surface (low albedo) will lead to increased absorption of low radiation wavelength from the sun; while a small warming of the oceanic surface is expected in the southern hemisphere, as the amount of ice on the water surface will remain almost unaltered. The increase in ocean temperatures will bring at least two types of biogeochemical effects and the functioning of ocean ecosystems. First, direct effects will occur due to changes in temperature-dependent biological and

biogeochemical processes. Secondly, a series of indirect effects will be caused by ocean warming, stratification and other changes in ocean water mixing and transport (Gruber, 2011).

As it affects almost all biological processes and plays a key role in affecting the physiological performances of intertidal ectotherms, the air-sea temperature has long been considered one of the most important environmental factor controlling growth, reproduction, development rates, recruitment dynamics of both algal and invertebrates species, along with their global distribution (O'Connor *et al.*, 2007).

A given species can tolerate thermal variations within a specific range of temperature; within this thermal range, organisms can reach a thermal optimum condition where fitness achieves an ideal state. Beyond certain limits, upper or lower the optimal eco-physiological fitness, the species enters in a "stress zone", resulting in a reduced performance (e.g. with decreasing fertility, productivity, and growth). Therefore, if the lower or the upper limit of tolerance is exceeded, organisms may die. From a molecular perspective, it is now well established that thermal stress experienced by organisms may induce oxidative damage, protein denaturation and death (Pörtner, 2010). In addition to this, studies on embryogenesis, swim performance, oxygen consumption, and planktonic larval duration have shown that temperature is the main environmental factor controlling the development of invertebrates and is also a key factor determining the distribution patterns of other marine species and recruitment dynamics. During high tide, the temperature increase in intertidal habitats can promote a greater foraging activity by mobile grazers (Thompson *et al.*, 2004) and lead to faster growth and productivity by algae (Oh and Koh, 1996). On the contrary, during the low tide the organisms can experience very stressful temperatures, which may cause a slower metabolism activity, and impaired acquisition of food, growth, and in extremes cases death (Somero, 2002).

In the literature there are mixed opinions about the influence of temperature on intertidal species. Some authors argue that in the thermo-tolerant species major developmental phases may not be compromised by future ocean warming because they are particularly adapted to temperature increase (Putnam *et al.*, 2010), other authors instead argue that intertidal organisms can be particularly sensitive to both the direct and indirect effects of environmental temperature variation (Somero, 2002), as these seem to already experience ambient temperatures close to their lethal limits (Stillman and Somero, 2000). For example, many coral reef-building living under temperatures close to

their upper thermal tolerance limits are subjected to a widespread bleaching phenomenon, caused by increasingly frequent heating episodes, which at the extreme limit can lead to a high mortality rate of corals (Hughes *et al.*, 2003; McWilliams *et al.*, 2005).

Temperatures can also represent a disturbance for the community succession, causing changes in composition and species variation. A recent laboratory experiment conducted by Cartaxana *et al.* (2015) showed that high temperatures had a significant effect on early stages of microalgal community, causing a change in the relative abundance of the major microphytobenthic groups. Diatoms were dominant in all experimental conditions of temperature (18° C and 24° C) but an increase in relative abundance of cyanobacteria was observed in high temperature conditions.

Temperature is also an important factor in determining the growth rates of primary marine producers (Raven and Geider, 1988). Generally, the temperature leads to an increase in growth rates of algal species, if this does not exceed the optimum growth temperature, while an excessive increase may be deleterious. In a recent study by Ras *et al.* (2013) it was observed that the temperature can modify the growth and photosynthetic rates of microalgal species, which represent the initial stages of an ecological succession. Specifically, the growth rates of these species above the optimal temperature were lower when compared with the low temperatures. Photosynthesis was also strongly influenced by temperature, with a decrease in photosynthetic rates of microalgal species and consequent decrease of biomass at high temperatures.

It was observed that the temperature may also affect the mature stages of an algal succession. Harley (2003) shown that the upper limit of intertidal algae is related to thermal stress and drying at low tide, and a long-term increase in atmospheric temperature pushing some intertidal macroalgal species to lower their upper limit. Field experiments showed significant responses in the intertidal community shifts. Kordas *et al.* (2014) manipulated the temperature at three different tide heights by building white and black frames, demonstrating that an increase of ~2° C of temperature can cause changes in the animal and algal community of the intertidal zone. In particular, it was observed that the abundance of herbivores decreased at the increasing of thermal stress to which they are subjected. While the total percentage of algal coverage was not affected by temperature treatments, the phenology of ephemeral green algae was undergone a delay at high temperatures. These results suggest that the species richness may diminish and

the composition in this species may move to the thermally tolerant ones at high temperatures.

These different responses suggest that ocean warming may affect intertidal communities in unpredictable ways and may lead changes in reproduction rates, and consequently affect successional dynamics and community composition on rocky intertidal shores.

1.5 DESCRIPTION OF THE LOCAL STRESSORS CONSIDERED IN THIS STUDY

(a) Grazing

Grazing is considered fundamental biotic process acting on rocky intertidal communities. Invertebrate grazers living in the intertidal zone can have significant influence on the ecological succession of marine benthic environments. Grazers can differentially damage or remove early, late successional species or both, but in disturbed habitats, grazing activity may affect the re-colonization patterns of intertidal species by accelerating, slowing or having little or no effects on successions (Sousa and Connell, 1992).

Previous studies demonstrated that under the inhibition model, in the absence of consumers, the early-stage species prevent or delay the establishment of the later successional species (Sousa, 1979b; Lubchenco, 1983). Instead the presence of grazers, which preferentially feed on early successional species, increases the replacement of species favoring the later colonists (Farrell, 1991).

Grazing can slow down or even stop an ecological succession. Following the facilitation model, barnacles can increase the rate of establishment of later successional species, such as perennial brown and red algae, and can provide protection from limpets (Farrell, 1991). In presence of grazers, consumers selectively damage or remove the later successional species slowing or stopping the succession compared with successions in absence of grazers.

Furthermore, grazing can have little or no effects on successional patterns when grazers equally negatively affect both early and later stages. Consumers can have little or no impact on algal population because the grazers' density could be too low, due to predation or competition with other sessile species, including algae (Underwood and Jernakoff, 1981), or the size of grazers is too small. In addition, the algal productivity may be important, because with a high algal productivity grazing rates are not significant.

The intensity of consumption by grazers suggests that grazing activity will affect the magnitude of successional rates. If consumers have great influence on species consequently they will have great effects on successional patterns. Thus, these evidences might suggest that grazers' density and their pressure on algal species can vary in space and time, and consequently may interact with other environmental factors modifying an ecological succession occurring in rocky intertidal shores.

(b) Storm waves

Marine waves are formed by the wind that, flowing over the surface of the sea, transmit a portion of its kinetic energy to the superficial layer of the seawater. When waves are closer to the coastline they dissipate part of their energy by frictioning with the sea bottom, and hence they decrease their speed and increase their amplitude. As soon as waves become higher, they start to break and all their energy is discharged onto the coastline. The energy exerted by this phenomenon may lead to the detachment of rock portions or to the transport of boulders from the backdrops, which by striking intertidal rocky environments, can create patches where the substrate is deprived of biological concretions. On one hand wave impact may lead intertidal habitat heterogeneity to increase, on the other hand can create a bare substratum where a new community succession may locally start. Generally, the re-colonization of free available space starts soon after a disturbance. The mechanisms that regulate the successional patterns and changes in species composition on patches of bare substratum are mainly the propagules availability of surrounding adult population, the size of cleared space (Sousa, 1979b) and the season in which the disturbance occurs (Benedetti-Cecchi and Cinelli, 1996). For example, in rocky intertidal systems the successional patterns are influenced by the dimension of cleared space. In small patches the species of surrounding area re-grow vegetatively and cover the free space becoming dominant during the favorable period, because develop much more rapidly due to their initial advantage, but during winter algae defoliate allowing to later successional stages to colonize. In large areas, instead, the early successional stages inhibit the subsequent colonists and the replace of one species with another is due to the interaction with biological disturbance, such as grazing (Sousa, 1979b). Also the size of bare substrate can affect the successional patterns of species, especially in highly stressful habitats as salt marshes. In small patches, were

physical conditions are less hard, the succession is led by competitive interactions between organisms; instead in large areas the succession is driven by mechanisms of facilitation (Shumaway and Bertness, 1994).

The temporal and spatial shifts in the intertidal assemblage structure are recurrent under conditions of disturbance by waves (Stephenson and Stephenson, 1949; Dayton, 1971; Sousa, 1979b), but the recent increasing of intensity and frequency of storms may lead to changes in patterns of distribution, abundance and species' diversity (Barry *et al.*, 1995). The ongoing climate change is leading to an increase in intensity and frequency of storm waves, both in tropical environments where high tidal ranges are present (Walsh *et al.*, 2016), and in the Mediterranean where these are reduced (Marcos *et al.*, 2011).

Despite these evidences, to date there is few knowledge about the way in which local stressors, storm waves, may affect the successional patterns of intertidal organisms.

1.6 INTERACTIONS BETWEEN MULTIPLE STRESSORS

Forecasts for potential future changes in coastal systems are mainly based on impacts related not only on independent responses of stressors, but also on the possibility local and on global stressors can interact synergistically or antagonistically (Crain *et al.*, 2008; Darling and Côté, 2008). A synergistic effect generally has harmful consequences for ecological systems and amplifies negative effects by pushing ecosystems to alternative states. Conversely, antagonistic interactions could mitigate the effects and probably be less harmful to the biodiversity of a given system (Griffith *et al.*, 2012). Therefore, changes in marine assemblages depend primarily on the interactions of these multiple stressors (Poore *et al.*, 2013) and how they produce combined impacts on biodiversity and the functioning of ecosystems (Schindler, 2001).

A stressor factor is defined as an abiotic or biotic variable that exceeds the normal range of variability, negatively affecting individual physiology or performance of the population in a statistically significant way (Auerbach, 1981). To develop a better predictive understanding of how organisms respond to multiple stressors, it would be necessary to describe the mechanisms by which a first stressor modulates the physiological responses of the second stressor. At the organism level, a first factor of stress could either increase tolerance to a second factor (cross-tolerance) or cause it to be more susceptible (cross-susceptibility) (Todgham and Stillman, 2013). Considering the spatial scale at which act

the stressor, scientists are recognizing that environmental changes in ecosystems at global scale will be partially determined by the local conditions (Helmuth *et al.*, 2010), thus is really important identify “the scale of origin of stressors (global) and the scale at which it impacts (local)” (Russell and Connell, 2012). In turn, shifts in physical conditions at small scale can lead to unforeseeable biological responses (Pandolfi *et al.*, 2011).

An increasing body of literature suggested that the combination of global and local stressors can modulate responses of intertidal rocky shore communities. Local stressors may either ameliorate or exacerbate the global stressors’ effects. For example, on intertidal rocky shores the mortality of organisms due to global warming can be reduced by local environmental conditions as wave splash and tidal height (Helmuth *et al.*, 2006a). Also, the increase in consumption rate of grazers may ameliorate the effects global stressors as suggested by Connell and Russell (2010) in an experiment conducted in Australia, where the phase-shift from kelp forest to turf-forming algae due to warming was mediated by grazers, which consuming preferentially turf algae, favored the proliferation of kelp forest. On the contrary, in a study conducted by Lefebvre *et al.* (2011) the local nutrient pollution in open sea exasperated the effects of ocean acidification driving to a further reduction of calcification rates of the coccolithophore *Emiliana huxleyi* under low pH conditions. The interaction between local and global stressors is already having significant negative impacts on rocky intertidal communities, by pushing the decline of canopy-forming algae and promoting the increasing of fast-growing algal turf (Airoidi, 2003; Airoidi and Bulleri, 2011). The decreasing of algal canopies can increase the stress of the understory algae and invertebrates, leading to shifts at community and ecosystem level.

The direct and indirect impacts of global changes may be worsening by interaction with local impacts in intertidal rocky habitats (Hawkins *et al.*, 2016). While some organisms may be resilient to independent stressors, their sensitivity is often altered when multiple stressors act together (Przeslawski *et al.*, 2005). In the light of what was said, is highly presumable that the interaction of global and local stressors will reduce the resilience of intertidal systems and will lead to shifts in marine successional patterns suggesting that there is a need to discriminate and identify the responses of intertidal organisms, not only to a single stress factor, but to the interaction of local and global disturbances.

1.7 MAIN GOALS OF THE RESEARCH DISSERTATION

Under climate change scenario, local and global stressors will have significant effects on marine communities. Several studies showed that, among the different global stressors, temperature and pH/pCO₂ are considered two of the greatest physic-chemical factors that can affect ecological successions and the biodiversity of marine ecosystems. Instead, there are few evidences about how local stressors effects and interspecific interactions, acting in combination with global stressors, can influence the early and the late stages of succession in the intertidal successions. Considering this theoretical framework, the aim of the present thesis is:

to investigate whether and how global stressors (ocean acidification and warming), local stressors (physical disturbance, e.g. storm waves) and species interactions (biofilm/grazer interaction) can interact each other (synergistically or antagonistically) and affect early and late stages of community successions in the Mediterranean intertidal systems.

Hence, the three specific goals of my PhD thesis were:

- 1.To test the effects of multiple stressors (ocean acidification, ocean warming, grazing pressure) on early successional stages and to investigate whether and how temperature and pH/pCO₂ affect microphytobenthos/grazer interaction via cumulative, interactive or antagonist effects on intertidal early stage assemblages.
- 2.To assess the composition and structure of algal intertidal communities at late-stage of succession in relation to different pH conditions and to test whether the alteration of substrate carbonate chemistry and alkalinity in the small-scale can mitigate ocean acidification effects by influencing late intertidal successions.
- 3.To evaluate whether the long-term exposition at different timing and intensity of local physical disturbances, as storm surges, can influence the successional patterns and recovery trajectories of reef-associated benthic communities and to test if the availability of free substrate may trigger competitive interaction between *D. cristatum* larvae and intertidal algae.

To achieve these specific goals I set up both manipulative and observational experiments in the field, and coupled them with laboratory analyses. The fieldwork was carried out between May 2014 and July 2017 in different locations around Northern Sicily.

More specifically, the Goal #1, which is presented in the Chapter 2 of this dissertation, was approached during the first year of my PhD. To observe changes in the successional patterns of early stages of intertidal community under different $p\text{CO}_2$ levels, I carried out a short-term *in situ* experiment along the intertidal CO_2 natural gradient off Levante bay in Vulcano Island. To test the ocean warming effects on early successional stages I manipulated the temperature by building white and black frames around the recruiting surfaces, and to assess the grazing pressure on early intertidal succession I created open and closed cages to exclude or provide the access to herbivorous. The molecular analyses on larval stages of microzoobenthos, associated to early successional stages, were performed in collaboration with the School of Biological and Marine Sciences (University of Plymouth, UK) and the Marine Biological Association (MBA). The Goal #2, which is presented in the Chapter 3 of this dissertation, was approached in between the first and second year of my PhD. To observe changes in the successional patterns of late stages of intertidal community under different $p\text{CO}_2$ levels I carried out a long-term *in situ* experiment along a CO_2 natural gradient off Levante bay in Vulcano Island. To assess if differences in substrate composition can lead shifts in algal intertidal communities at late-stage succession, I used substrates with different chemical composition (olivine, sand, aragonite and PVC), some of which (e.g. olivine) can alter the carbonates chemistry and alkalinity at small-scale. This second experiment was carried out in collaboration with the School of Biological and Marine Sciences (University of Plymouth, UK). The Goal #3, which is presented in the Chapter 4 of this dissertation, was performed during the second and the third year of my PhD. To evaluate if the intensity and timing of local physical disturbances, i.e., storm waves, can affect the successional patterns of reef-associated benthic communities and the competition strength between algae and vermetid larvae, I carried out a long-term *in situ* experiment along pristine vermetid reefs in NW Sicily, where the main reef builder species is *Dendropoma cristatum* (Biondi 1857). The impact of storm waves was simulated by carrying out three different substrate removals mimicking different wave intensity effects (0%, 50% and 100% removal treatments). Two

different timings of disturbance (i.e., different seasons in which the disturbance took place) were considered (May and December 2016).

1.9 REFERENCES

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CHAPTER 2. ARE EARLY SUCCESSIONAL STAGES IN THE ROCKY INTERTIDAL SUBJECTED TO OA AND WARMING MEDIATED BY SPECIES INTERACTIONS?

Abstract: The co-occurring increase in seawater temperature and $p\text{CO}_2$ could interact each other to cause changes in species interactions and community shifts. To understand how these stressors may affect early successional stages of an intertidal epilithic microphytobenthos (EMPB) in the Mediterranean, I set-up an *in situ* experiment, assessing potential changes in grazer density and EMPB composition, by manipulating temperature on 10x10cm bare (cleared) rock substrata in two locations along a natural $p\text{CO}_2$ gradient off Vulcano Island. Thirty-six black and white-framed substrata were positioned and randomly assigned to closed cages (exclusion), open cages (artefact) and no cages (control) treatments. ‘Robo-limpets’ sensors revealed that black plots were on average +0.26°C warmer and reached a maximum temperature +2°C higher than white plots. No differences in grazing effects were recorded between $p\text{CO}_2$, temperature, and $p\text{CO}_2 \times \text{T}$ combinations. The early-stages of EMPB were affected by temperature changes. The microzoobenthic assemblages, associated to EMPB, were affected by increasing levels of $p\text{CO}_2$, and differences in community composition were mediated by species-specific responses to warming. No interactive interactions between abiotic and biotic stressors affected the EMPB. The present study suggests that warming might affect the composition EMPB by altering the stability of ecosystems and leading shifts in the successional patterns of intertidal communities.

Keywords: Early stages of intertidal succession – Ocean acidification – Global warming – Grazing – Global stressors – Mediterranean Sea.

2.1 INTRODUCTION

Carbon dioxide (CO_2) has a key role in essential life processes such as photosynthesis and respiration, at the same time it is considered the main greenhouse gas in the Earth’s atmosphere causing ongoing climate change (Hoegh-Guldberg *et al.*, 2007). Since the industrial revolution the concentration of anthropogenic CO_2 in the atmosphere dramatically increased (Solomon *et al.*, 2007), leading to warming of the oceans and to an

unprecedented alteration of their carbonate chemistry, a process known as ocean acidification (OA).

On a global scale, the ocean warming is largest near the surface, with the upper 75 m warming up by 0.11°C [from 0.09 to 0.13° C] per decade in the last few decades (IPCC, 2014). Warming is taking the form of both increasing average seawater temperatures and elevated frequency and severity of thermal extremes (Trenberth, 2012; IPCC, 2013). Future scenario models have estimated a further ocean temperature increase of 2-5°C by the end of this century (IPCC, 2013). Such increase in average ocean temperature is occurring concurrently with ocean acidification (Caldeira and Wickett, 2003). Since 1970 about one quarter of anthropogenic CO₂ has been absorbed by ocean systems (Sabine *et al.*, 2004) causing a reduction of 0.1 pH units from the pre-industrial era and an alteration of seawater chemistry (Caldeira and Wickett, 2003; 2005; Orr *et al.*, 2005; Doney *et al.*, 2009). Future projections made by the IPCC suggest that average pH could further drop up to 0.3-0.5 units by the end of 2100 (Meinshausen *et al.*, 2011).

Ocean warming and acidification are already affecting marine ecosystems worldwide (Pörtner *et al.*, 2014), shifting the range of species, affecting both calcifying and non-calcifying organisms, changing the strength of species interactions and ultimately promoting community reshuffling (Kroeker *et al.*, 2012; 2013; Kordas *et al.*, 2017). The combination of these stressors reduced the calcification, growth and survival rates of heterotrophs and, conversely, positively affected the growth of autotrophs, likely due to the effect of temperature on metabolic rates and the increase in CO₂ on photosynthesis, which likely indirectly promoted growth (Harvey *et al.*, 2013).

In rocky intertidal ecosystems, the main autotrophic component of early successional stages is represented by the biofilm (Williams *et al.*, 2000). The biofilm of epilithic microphytobenthos (EMPB) consists of microscopic eukaryotic algae (e.g. diatoms), photosynthetic bacteria (e.g. cyanobacteria) and propagules of macroalgae (MacIntyre *et al.*, 1996) that colonize both natural (Thompson *et al.*, 2004) and artificial (Russell *et al.*, 2013) substrata. The marine microbial film sustains a significant portion of primary production in intertidal rocky systems (Ortega-Morales *et al.*, 2005) and provides an important food resource for intertidal marine invertebrates (Thompson *et al.*, 2004). In addition, EMPB provides a suitable substrate, cueing the settlement of macroalgal propagules and of invertebrates' larvae (Lau *et al.*, 2005). At present, available empirical data on temperature and pH/pCO₂ interacting effects on marine biofilm are increasing in

number, despite researchers mainly focused their attention on a single factor in the recent past. As an example, in a 7-day experiment Hicks *et al.* (2011) found a detrimental effect of higher temperatures on biofilm biomass. A short-term exposition to higher temperature levels led to increased photosynthesis and productivity of incubated diatoms and natural biofilm communities and to a transition from photo- to heterotrophic benthic community has been previously reported for the intertidal zone (Cartaxana *et al.*, 2015). Similarly, changes in other environmental stressors, such as $p\text{CO}_2$, may drive significant ecological responses (Hall-Spencer and Rodolfo-Metalpa, 2008). In Mediterranean CO_2 seeps systems, natural analogues which are increasingly used to assess responses and interactions of organisms in the wild (e.g. Hall-Spencer *et al.*, 2008), Johnson *et al.* (2013) showed no differences in the percentage cover of cyanobacteria settling on artificial substrata along a $p\text{CO}_2$ gradient, whilst lowered pH conditions enhanced diatom productivity. More recently, finer sequencing analyses at the same sites revealed altered composition of intertidal biofilm communities inhabiting natural substrata along a CO_2 gradient off Vulcano Island, with significantly higher relative abundance of cyanobacteria with decreased pH (Taylor *et al.*, 2014). Another recent experiment by Johnson *et al.* (2015) suggested that biofilm associated to sediments along the Vulcano CO_2 gradient, increased their photosynthetic rates with increasing $p\text{CO}_2$ levels. In particular, the abundance and shifts in community composition of diatoms increased sensibly with the rise in $p\text{CO}_2$ levels, instead cyanobacterial abundance was elevated only at the extremely high levels of $p\text{CO}_2$ ($>1400 \mu\text{atm}$), demonstrating that the marine benthic microalgae thrive under ocean acidification.

Recent evidences suggest that the ocean warming and acidification may directly affect the survival, calcification, growth, development and reproduction of invertebrate grazers (Kroeker *et al.*, 2013; Kordas *et al.*, 2017). In particular, both field and lab investigations demonstrated that the extreme low pH levels can drive a great decrease in abundance and species diversity of calcifying organisms as gastropods (Cigliano *et al.*, 2010; Kroeker *et al.*, 2011) or other molluscs, which appear to be a taxon particularly vulnerable to acidification (Parker *et al.*, 2013). In addition, ocean warming may induce stress responses on intertidal grazers, such as limpets, and recent studies demonstrated that the rising temperatures can potentially affect their growth, survival as well as raising their metabolic rates (Miller *et al.*, 2015). Interestingly, ocean acidification may indirectly and positively affect the abundance of calcifying grazers under increased primary productivity

in natural CO₂ vents providing both habitat and food for calcifying grazers (Connell *et al.* 2017).

Grazing pressure *per se* may affect the patterns of re-colonization on disturbed rocky shores, by enhancing, inhibiting or diverting biological successions (Sousa, 1979; Farrell, 1991; Benedetti-Cecchi, 2000). Some evidence suggested that in rocky intertidal habitats invertebrate grazers could affect algal assemblages influencing the patterns of distribution and abundance both by direct and indirect effects (Sousa *et al.*, 1981; Menge, 1995; Benedetti-Cecchi, 2000). In particular, limpets play a key role in regulating the distribution and abundance of algae on intertidal rocky shores (Hawkins *et al.*, 1992) and some studies suggest grazing activity as a great top-down control for microbial film in the marine environment (Benedetti-Cecchi and Cinelli, 1993; 1996). Grazing pressure maintains the biofilm at an early successional stage, while microalgal and then macroalgal biomass increase when grazing is reduced or removed (Hawkins and Hartnoll, 1983; Thompson *et al.*, 2000b). A recent experiment conducted by Kordas *et al.* (2017) showed that, under experimental warming, the presence of grazers (e.g. limpets) alleviated the warming effects and maintained natural trophic interactions. Instead, in absence of limpets, warming modified the stability of trophic interactions leading to an altered community succession.

Indeed, ongoing ocean warming and acidification can influence marine ecosystems via direct effects on individual organisms and indirect effects mediated by species interactions. However, at present little is known on how such changing environmental factors will interact each other affecting organisms' responses and will scale-up through higher level of organizations (i.e. communities and ecosystems).

To date there is increasing knowledge of how multiple environmental stressors, such as temperature and pH/pCO₂, can influence interactions within marine communities (Wernberg *et al.*, 2012a). Some experimental evidences demonstrated that the effects of such stressors may lead to either enhanced (synergistic) or reduced (antagonistic) effects, which respectively may be greater or less than the sum of individual stressors' effect (Folt *et al.*, 1999; Crain *et al.*, 2008; Darling and Côté, 2008). However, the direction and magnitude of responses of interacting species exposed to OA and warming are often contrasting, showing mixed responses (Alsterberg *et al.*, 2013; Russell *et al.*, 2013). In a laboratory experiment conducted by Alsterberg *et al.* (2013) benthic microalgae had different responses under ocean acidification and warming and in the presence/absence

of their consumers. In presence of grazers the climate change effects were not significant, but in absence of grazers the benthic microalgae were positively affected by acidification and warming. By contrast, Russell *et al.* (2013) showed that the combination of elevated temperatures and ocean acidification did not have significant effects on biofilm and grazers, instead individual factors in isolation showed significant effects as rising temperature increased gastropod grazing on biofilm, while higher levels of CO₂ reduced the grazing pressure. Another recent mesocosm experiment conducted by Sampaio *et al.* (2017) suggested that both warming and acidification negatively affected grazer's survival, inducing differences in grazing pressure on algal consumption rates under global change scenarios.

Given that, our ability to predict responses to altered temperature and $p\text{CO}_2$ relies on a coherent understanding of how warming and acidification affect levels of organization from individual to ecosystem level. Much is still unknown about the responses at community level given the expected reshuffling of assemblages (i.e. community shifts) and the changing strength of interspecific interactions. Indeed, field approaches may provide more information than experiments carried out under controlled conditions on the community-level responses, potential interspecific interactions between primary producers and their consumers.

Therefore, to have a better focus on how changing environmental conditions affect intertidal communities, here I focus on the early community succession of intertidal rocky shore communities under temperature and CO₂ rise in presence/absence of gastropod grazers.

More specifically, to assess how multiple stressors can affect the early successional stages and the interactions occurring between the intertidal species, an *in situ* manipulative experiment was set up along a coastal CO₂ gradient at shallow water seeps system off Vulcano Island (NE Sicily, Italy). The specific aim of this experiment was to investigate whether abiotic (i.e. temperature and pH/ $p\text{CO}_2$) and biotic (grazing) factors might have synergistic or antagonistic effects on the early stage assemblages of a Mediterranean intertidal community. To achieve this goal I combined *in situ* experimental warming with herbivore manipulations along a natural CO₂ gradient on a rocky intertidal community for two months.

Specifically, to investigate the primary productivity of the early microphytobenthic stages the analysis on chl-a content was performed. The total biomass of early MPB was

analyzed by wet weight and the coverage percentage of Encrusting Coralline Rhodophyta (ECR) was estimated by photo-sampling. The structure and composition of microphytobenthic assemblages was assessed by photo-sampling crossed with microscopy analysis. The structure and composition of microzoobenthic assemblages, associated to the EMPB, was investigated by DNA extraction and molecular analyses, as the Polymerase Chain Reaction (PCR), the electrophoresis and the Denaturing Gradient Gel Electrophoresis (DGGE). At the end, the grazing pressure on early MPB was assessed by the density of *Patella* ssp., the main herbivore living on the intertidal rocky shores of Vulcano Island.

2.2 MATERIALS AND METHODS

(a) Study area

The experiment was carried out from 3th August to 5th October 2015 along the rocky intertidal zone off Levante Bay (38°25' N, 14°57' E; Vulcano Island, NE Sicily, Italy). In this area an active shallow-water CO₂ seep, occurring at less than 1 m depth, creates a pH/*p*CO₂ gradient running parallel to the north-western side of the island. The pH at the emission site ranges from 5.2 to 5.5 units and along the gradient arrives to an ambient pH level of ~ 8.1 units, at a distance of about 500 meters from the intense CO₂ emissions site. This CO₂ gradient includes a large variety of intertidal and subtidal communities at <2 meters deep (Arnold *et al.*, 2012). In the study area, currents are mostly driven by dominant winds (Boatta *et al.*, 2013) and tidal range is minimal (<40 cm) (Day *et al.*, 1995). Sediments within the bay differ considerably in the concentration of trace elements. About half of the elements (Cd, Co, Cr, Cu, Fe, Mn, V) analyzed by Vizzini *et al.* (2013) have the highest values in the sites at about 100-150 meters from the seeps. The other elements (As, Ba, Hg, Mo, Ni, Pb, Zn) are more concentrated within the seep area, reducing their concentration by distance. The decrease of pH and Eh may affect the solubility and bioavailability of certain metals and metalloids. This mainly contributes to maintaining Fe and some elements (Cd, Co, Cr, Cu, Mn, V) in dissolved form at low pH sites, resulting in low enrichment in sediments (Vizzini *et al.*, 2013). The bay represents a relatively sheltered environment, with a trade-off with the open sea enough to maintain a fairly stable chemical-physical and environmental condition over time. Furthermore, the bay has an extension such as to allow a wide range of acidification levels, and this permits to study levels of ocean acidification to different future periods and which resume various atmospheric CO₂ scenarios (Boatta *et al.*, 2013).

(b) Experiment set-up

Two intertidal sites were chosen along the well-established natural pH/*p*CO₂ gradient off Vulcano Island: Site 1 (High *p*CO₂) which is located at pH/*p*CO₂ conditions expected by the next decades and Site 2 (Ambient *p*CO₂) exerting a control *p*CO₂ (Fig. 4).

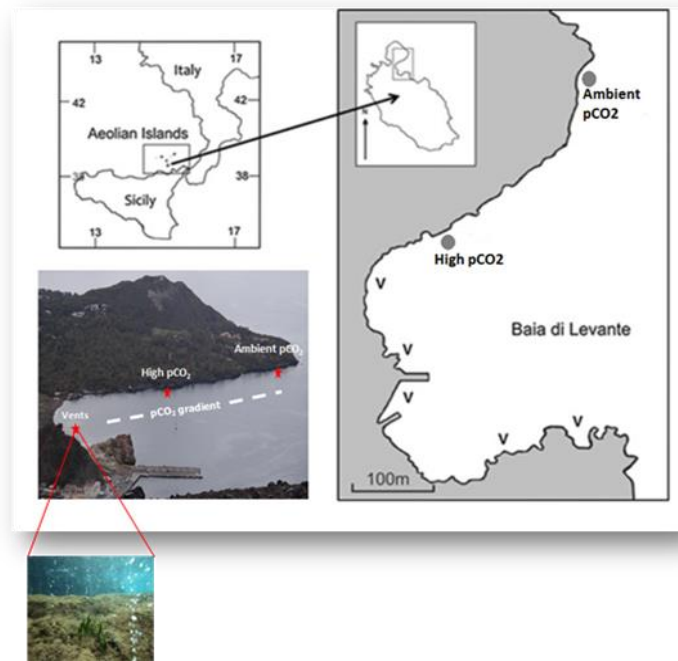


Fig. 4 – Map of the study area, Levante Bay (Vulcano Island). The stars indicate the sampling stations High and Ambient pCO_2 , and the CO_2 volcanic vents. The dashed line indicates the natural pH/ pCO_2 gradient off Vulcano Island.

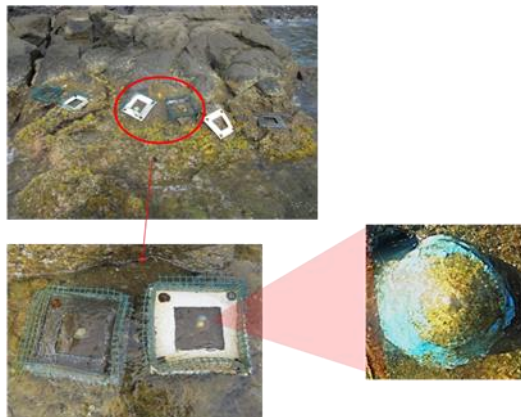


Fig. 5 – Manipulative treatments positioned on intertidal rocky shores of Vulcano Island during the experimental period.

Thirty-six 10 x 10 cm plots were randomly positioned in two sites along the natural pH/ pCO_2 gradient and cleaned to remove preexistent community. To manipulate the temperature of the settlement substrates (i.e. natural bare volcanic rock) I used white (normal T) and black (elevated T) forex frames (20 x 20 x 0.3 cm) with a centered 10 x 10 cm area holed for the species recruitment.

For each Temperature treatment and within each Site, three substrata were enclosed within cages to remove herbivores (Exclusion treatment), three substrata had open cages, providing access to herbivores (Artefact treatment), three substrata were without cage and used as Control treatment (Fig. 5). Temperatures were recorded by the iButton devices inserted within shell of limpets (“robo-limpets”), to assess temperature experienced by the main grazer species off Vulcano intertidal zone. IButton devices were set to record temperature at regular intervals (1 hour), during the



Fig. 6 – IButton devices inserted within the shells of limpets, “robo-limpets”.

hours of maximum irradiance (10 a.m – 16 p.m), across the experiment duration (2-months) (Fig. 6). IButtons devices are microchips enclosed within 16 millimeters thick stainless steel "buttons". An iButton device uses its external enclosure as an electronic communication interface. The iButton must be set up with the 1-Wire® protocol. Information can be transferred between the iButton device and a PC through a 1-Wire adapter for USB, serial, or parallel ports. IButtons devices have an edge in terms of durability and longevity. The stainless steel casing has a high ability to survive in a temperature range between -40° C and + 70° C and in extreme environments (such as salt water exposure or long exposure to physical impacts).

Tiles were placed at a 15°-90° angle to the seafloor to approximate the orientation of the natural substrate. This experiment was carried out in the mesolittoral area and so it was necessary to retrieve tidal activity data on the Vulcano Island. I used the WXTide application to acquire data on tidal cycles off Vulcano Island and to know the trends of high tide and low tide, as well as the height at which the tides will arrive. It is an interesting application that allows you to know the tidal activities in most parts of the planet. The application can collect information since 1970 and make predictions until 2037, performing forecasts based on data obtained from the 9500 measurement points existing in coastal locations around the world.

(c) Seawater carbonate chemistry

At each field site, the seawater carbonate system was characterized multiple times during the experiments. A 556 MPS YSI (Yellow Springs, USA) probe was used to measure salinity and pH. The pH sensor was calibrated using NBS scale standards buffers and then soaked in seawater for one hour. Hobo Onset loggers were also deployed to monitor seawater temperatures (°C) at 15-min interval for the whole duration of the experiment. For each site, average pH was calculated from hydrogen ion concentrations before reconverting back to pH values. Water samples for total alkalinity (TA) were filtered through 0.2 µm pore size filters, poisoned with 0.05 ml of 50% HgCl₂ to avoid biological alteration, and then stored in the dark at 4° C. Three replicate sub-samples were analyzed at 25° C using a titration system (Mettler Toledo, Inc.). The pH was measured at 0.02 ml increments of 0.1 N HCl. Total alkalinity was calculated from the Gran function applied to pH variations from 4.2 to 3.0, as µEq Kg⁻¹ from the slope of the curve HCl volume versus pH. The *p*CO₂ and the saturation state of aragonite were calculated from pH_{NBS}, TA, temperature and

salinity with the free-access CO₂ SYS package, using the constants of Roy *et al.* (1993) and Dickson (1990).

(d) Sampling techniques and lab analyses

To ensure the independence in space and time of experimental substrata, it must be sure that the replicates do not influence between them over time. Generally, representativeness is guaranteed by the collection of random samples. Random sampling helps to reduce the possible sources of error, generated either by the operator or by the type of response variable we want to consider.

Randomly selected experimental substrata were cleaned using the clearing method (Sousa, 1979). The rock surface was scraped with metal brushes to remove all organisms, including encrusting algae, and leaving only bare rock as the starting condition for a community succession. The removal of plants and animals to expose bare rock in experimental substrata is a useful simulation of severe physical disturbance on rocky reefs (Airoldi, 2000; Benedetti-Cecchi and Cinelli, 1994). At the end of experiment, a standard photo-sampling technique was employed (Dethier *et al.*, 1993) to assess epilithic microphytobenthic assemblages (EMPB). Specifically close-up pictures of each substratum were taken for subsequent community analyses (i.e. to assess species identity). In addition to this, individuals of *Patella sp.* were counted within each experimental substratum to estimate density on 100 cm⁻² surface. To cross-check species identification by photo-sampling and to estimate the total biomass of the epilithic community, at the end of the experiment each experimental substratum was removed at low tide by a standard scraping technique using hammer and chisel and immediately stored in separated plastic bags at -18°C. In addition, chips of colonized rocks were collected for successive molecular analyses and chlorophyll-a extraction (see description below). Microzoobenthic assemblages, associated to EMPB, mostly consisting of larval and juvenile stages of benthic invertebrates unidentifiable at species level, were identified by DNA extraction and DGGE analyses (see DNA extraction and molecular analyses). To assess assemblages' composition, laboratory analyses were performed by coupling photo-identification at the computer screen and observations under an optic microscope (Nikon) of the different EMPB taxa. Algal biomass (wet weight, mg) was assessed by an

analytical balance (brand Sartorius, for weighing up to 520 g maximum with 0.1 or 0.01 mg resolution), after removing the excess of water from each sample by absorbent paper. Chlorophyll-a content of the epilithic benthic assemblages retrieved from each experimental substratum was examined. Each rock chip was placed in a jar and weighed. Then, 10 ml of 90% ethanol was added to each jar for the sample extraction and left in a cool, dry and dark area for 12 hours. Successively the jar was weighed again to assess the tare. Subsequently for each sample extracted, the solution was pipetted in a vial and its chlorophyll-a content was analyzed by a standard method by Thompson *et al.* (1999). Varian Cary 100 Bio UV visible spectrophotometer was used to measure the absorbance of each sample at Å665 and Å750. The concentration of the total Chl-a in each sample ($\mu\text{g mm}^{-2}$) was calculated using the following algorithm: $(12.2 \times \text{Å}_{\text{net}} \times V) / (d \times a)$ (Thompson *et al.*, 1999), where 12.2 is a constant for ethanol; Å_{net} is the net absorbance of each chlorophyll solution ($\text{Å}_{\text{net}} = \text{Å}_{665} - \text{Å}_{750}$); V is the final volume of the solvent; d is the path length of cell (10 mm); a is the surface area of the sample.

(e) DNA extraction and Denaturing Gradient Gel Electrophoresis analyses

The EMPB recruited on rock chips was carefully scraped with sterile razor blade and collected into a 2 ml micro-tube. Then EMPB DNA was extracted using the PowerSoil DNA isolation kit (MO-BIO Laboratories, USA) according a standard protocol available in the handbook of instructions (Instruction Manual, MO-BIO Laboratories, Inc.; Catalog No. 12888-50, 12888-100).

Following DNA extraction, PCR amplification of 18S rRNA was carried out. For PCR, a total volume of 50 μl was used containing 10 μl of 5X PCR buffer, 25 mM MgCl_2 , 2.5 mM dNTPs, 10 pmol of each primer, 0.25 μl (1.25 U) Go *Taq* DNA polymerase (Promega Corporation), 30.75 μl sterile Milli-Q water and 2 μl DNA. For eukaryotic DNA the primers used to amplify the region of 18S rDNA, a fragment approximately 210 bp long, were UNI1392R (5'-ACGGGCGGTGTGTRC-3') and EUK1209F-GC (5'-CGCGCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCGCCCCGAGGTCTGTGATGCCC-3'), according the methodology of Díez *et al.* (2001). PCR products were amplified using following conditions: initial denaturation at 94°C for 1 min and 10 touchdown cycles of denaturation at 94°C for 1 min. Subsequently, annealing at 65°C (with the temperature

decreasing 1°C for each cycle) for 1 min and extension at 72°C for 3 min, followed by 20 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. During the last cycle, the length of the final elongation step was at 72°C for 7 min. An aliquot of all amplification PCR products were analyzed by electrophoresis in a 3% agarose gel, stained with ethidium bromide and quantified with Hyper Ladder at 25 and 100 bp, for 50 min at 100 V.

DGGE (Denaturing Gradient Gel Electrophoresis) was used to study the structure and composition of the microzoobenthic assemblages on the different experimental substrates. DGGE was carried out with a Dcode gel system (Biorad, Hercules, CA, USA) with some changes of method of Cunliffe and Kertesz (2006a). Acrylamide denaturing gradient gel of 30-70%, with a 0% stacking gel, was used to allow the run of PCR products of each sample. Gels were run in 150 ml 50x TAE buffer for 16 h at a constant voltage of 63 V and temperature of 60°C. At the end of the run, gels were stained with SYBR Gold nucleic acid gel stain (Invitrogen, UK) for the visualization at UV trans-illuminator. DGGE bands were removed from gel, DNA was amplified by PCR, using the same primers as before without GC clamps, cleaned with Gen-Elute PCR Clean-Up kit (Sigma-Aldrich) and quantified spectrophotometrically. Subsequently, the PCR amplification products from 18S rRNA genes were sequenced.

(f) Statistical analyses

Temperature data were analysed using the univariate PERMANOVA (PERMutational ANalysis Of Variance) using a one way design to compare efficacy of the black and white frames: Temperature ("Te") treated as fixed with two levels (White and Black). For a better comparison of temperature data, one pairs of black and white treatments were chosen into one site (in this case, the High $p\text{CO}_2$ site). These data were compared in pairs and were plotted in a graph in relation to tidal cycles.

Three-way designs were used to assess the density of the herbivore *Patella* ssp., the total biomass of the early microphytobenthic communities and its Chl-a content, the structure and composition of early microzoobenthic community and the coverage percentage of Encrusting Coralline Rhodophyta (ECR %) with pH fixed with 2 levels (High and Ambient $p\text{CO}_2$), Temperature ("Te") fixed and orthogonal with two levels (White and Black) and Cage ("Ca") fixed and orthogonal with three levels (Exclusion, Artefact and Control).

To graphically evaluate differences in structure and composition of microphytobenthic community and associated microzoobenthic community, at different temperature conditions, two separately nMDS graphs were performed.

In an univariate context, PERMANOVA was based on Euclidean distance matrix with 9999 permutations and was chosen because this method does not assume a normal distribution of errors allows for factorial designs and accounts for interaction effects (Anderson *et al.*, 2008). The Monte Carlo approach, was used only when few permutations explained the results. This method is based on a specified number of randomly drawn permutations to provide efficient implementations of hypothesis tests. In a multivariate context, the structure and composition of microphytobenthic community and early stages of associated microbenthic assemblages was based on Bray-Curtis similarity and were analysed by multivariate PERMANOVA again with 9999 permutations.

After the PERMANOVA, the post hoc Pairwise T test comparison, based on permutations of residuals under a reduced model, was performed.

All analyses were performed using the PRIMER 6.1.10 & PERMANOVA β 20 software package (PRIMER Ltd., Plymouth).

2.3 RESULTS

(a) Tide, temperature and seawater carbonate chemistry

The experimental substrates, positioned along the Vulcano Island CO₂ gradient, were exposed to a typical tidal cycle for the Mediterranean region with a maximum and minimum height recorded during the 2-month experimental period of +0.17 and -0.25 m, respectively (Fig. 7). Throughout the experiment, the temperature (°C) recorded during the hours of maximum irradiance (10:00-16:00 h) and at low tide showed coherent trends with tidal ranges (Fig. 7). On average temperatures recorded on the black-framed substrates were higher (i.e. by about 0.26° C) and more variable than white-framed substrates (PERMANOVA, Pseudo-F_{1,1190}:5.9085; $p=0.0162$), peaking between 34°C and 32°C respectively (Table 1).

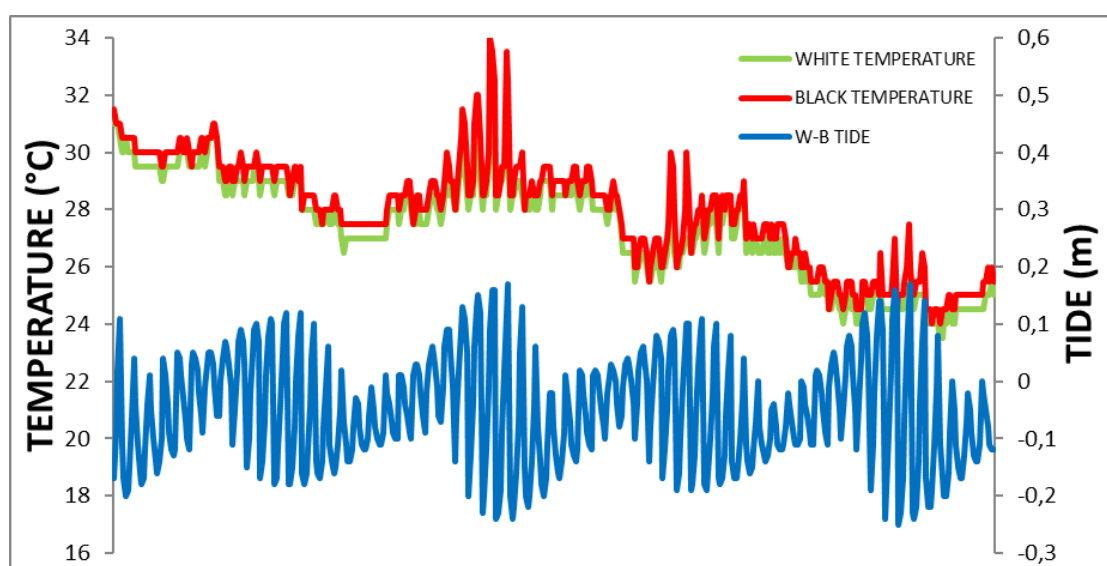


Fig. 7 – Trend of the temperature data recorded by “robo-limpets” positioned within a couple of white/black recruitment plots during the hours of maximum irradiance (from 10am to 4pm) in relation to tidal cycles. The green and red lines show the temperatures respectively recorded within white and black treatments, the blue line shows the trend of tidal cycles recorded in Vulcano Island during the experimental period.

Table 1 – Mean, Standard Deviation, Median, Minimum and Maximum values of temperatures (°C) recorded by “robo-limpets” positioned on white and black experimental plots during the low tide in Vulcano Island.

Temperature treatments (°C)	Mean	S.D.	Median	Min	Max
White	27.77852	1.800684	28	23.5	<u>32</u>
Black	28.03859	1.892094	28.5	22.5	<u>34</u>

The carbonate chemistry recorded during the experimental period is reported in Table 2. Throughout the experiment mean seawater pH_{NBS} sensibly decreased towards the CO_2 seep zone with on average 8.13 ± 0.03 in the Ambient pCO_2 site and 7.94 ± 0.09 pH_{NBS} units at the High pCO_2 site. Seawater temperature ($^{\circ}\text{C}$), salinity and total alkalinity (TA, $\mu\text{mol kg}^{-1}$) remained relatively constant between sites (Table 2). As expected, the average pCO_2 values were recorded at the High pCO_2 site (834 ± 188 $\mu\text{atm pCO}_2$) than the Ambient pCO_2 site (493 ± 39 $\mu\text{atm pCO}_2$).

Table 2 - Carbonate chemistry recorded at the two pH/pCO_2 sites off Vulcano Island (High pCO_2 and Ambient pCO_2). pH , Salinity and Temperature (T) were measured on different days between July and October 2015, using a multiparametric YSI probe ($n=10$). For each site, average pH was calculated from hydrogen ion concentrations before reconvertig back to pH values. TA was measured from three replicated $0.2\mu\text{m}$ filtered water samples collected in each CO_2 sites and data were averaged for subsequent calculations. Calculated pCO_2 was obtained from CO_2SYS .

Site	pH_{NBS}	TA ($\mu\text{mol kg}^{-1}$)	Salinity	T($^{\circ}\text{C}$)	Calculated pCO_2 (μatm)
Ambient pCO_2	8.13 (± 0.03)	2507	38.2 (± 0.06)	27.2 (± 0.3)	493 (± 39)
High pCO_2	7.94 (± 0.09)	2484	38.2 (± 0.09)	27.4 (± 0.2)	834 (± 188)

(b) Macrograzers' density

At the end of the experiment, the density of the macrograzer *Patella* spp. did not differ among pH/pCO_2 and temperature treatments whilst, as expected, significantly differed among Cage treatments (PERMANOVA, Pseudo- $F_{2,35}$: 7.4; $p=0.0032$), being lower in Exclusion than Artefact (Pairwise T test; $p=0.0027$) and Control plots (Pairwise T test; $p=0.00917$) respectively; which in turn did not differ each other (Pairwise T test; $p=0.1948$) (Fig. 8).

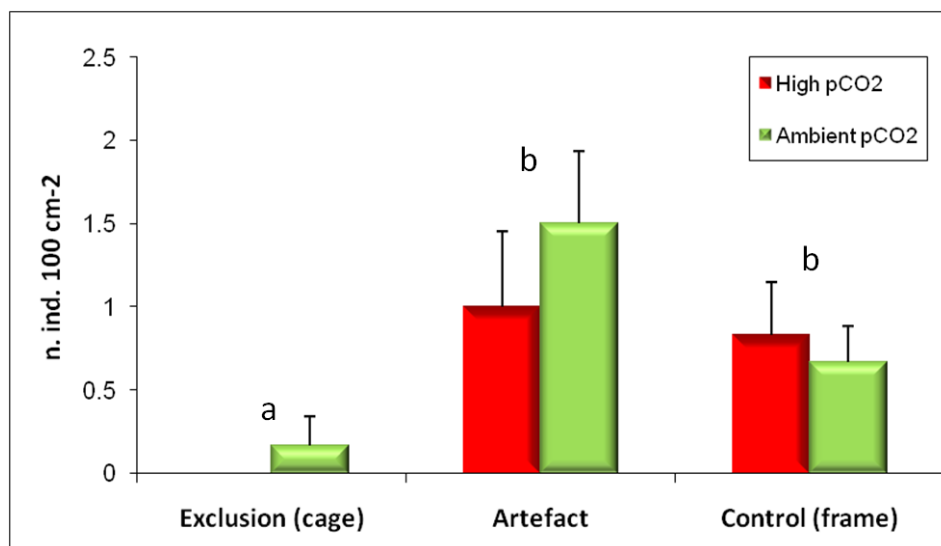


Fig. 8 – Density of macro-grazers (mean \pm S.E.; n=36) found on Exclusion, Artefact and Control treatments in High (red bars) and Ambient (green bars) pCO₂ sites.

(c) Changes in the composition of the early microphytobenthic assemblages

After a 2-month exposure to different pCO₂ conditions the overall early-stage epilithic microphytobenthos (EMPB) mainly consisted of turf-forming algae, biofilm taxa and macroalgal propagules. On average frequency % of turf forming algae was 24.3% in High pCO₂ and 31.3% in Ambient pCO₂ with *Lophosiphonia* sp. (Fr%= 55.6% in High pCO₂ and 72.2% in Ambient pCO₂), *Polysiphonia* sp. (55.6% and 61.1%, respectively) and *Cladophora* sp. (16.7% and 38.9%, respectively) being the most frequent taxa. Biofilm taxa mostly were Cyanobacteria and benthic diatoms with on average a frequency of 50% in High pCO₂ and 19.4% in Ambient pCO₂. The most frequent macroalgal taxa, which were mostly in the form of propagules and germlings were *Laurencia* sp., *Anadyomene stellata*, and Ectocarpales in High pCO₂ plots and *Laurencia* sp., *Anadyomene stellata*, and *Dyctiota linearis* in Ambient pCO₂ conditions. Interestingly, the Encrusting Coralline Rhodophyta exerted high frequency % in both pCO₂ level conditions (Table 3).

Table 3 - Average frequency % of the early-stage epilithic microphytobenthos (EMPB) taxa identified on the experimental plots positioned in High and Ambient $p\text{CO}_2$ sites along the pH/ $p\text{CO}_2$ gradient off Vulcano Island. * Calcifying taxa.

	High $p\text{CO}_2$	Ambient $p\text{CO}_2$
<i>Stilomena alsidii</i>	11.1	0.0
<i>Sphacelaria</i> sp.	27.8	44.4
<i>Lophosiphonia</i> sp.	55.6	72.2
<i>Polysiphonia</i> sp.	55.6	61.1
<i>Spermothamnion</i> sp.	11.1	11.1
Ceramiales	16.7	11.1
<i>Cladophora</i> sp.	16.7	38.9
<i>Hydrolithon</i> sp.	0.0	11.1
Turf-forming	24.3	31.3
Cyanobacteria	55.6	38.9
Diatomea	44.4	0.0
Biofilm	50.0	19.4
<i>Laurencia</i> sp.	61.1	38.9
<i>Anadyomene stellata</i>	22.2	27.8
Ectocarpales	16.7	5.6
<i>Halopteris filicina</i>	0.0	11.1
<i>Jania rubens</i> *	0.0	11.1
<i>Padina pavonica</i> *	0.0	11.1
<i>Cystoseira</i> sp.	0.0	5.6
<i>Dyctiota linearis</i>	0.0	16.7
Macroalgae	12.5	16.0
Encrusting Coralline Rhodophyta*	55.6	66.7

Quantitative analyses on %cover of Encrusting Coralline Rhodophyta (ECR) revealed that this group of calcifying algae was more abundant in Ambient than High $p\text{CO}_2$ conditions with on average 7.19 ± 2.49 % and 0.69 ± 0.3 % cover respectively (Fig. 9). PERMANOVA supported significant differences between pH levels (Pseudo- $F_{1,35}$: 7.1183; $p=0.0135$), but no differences were detected for Temperature and Herbivory levels (i.e. Cage treatments) and the other factors' interactions (Table 4).

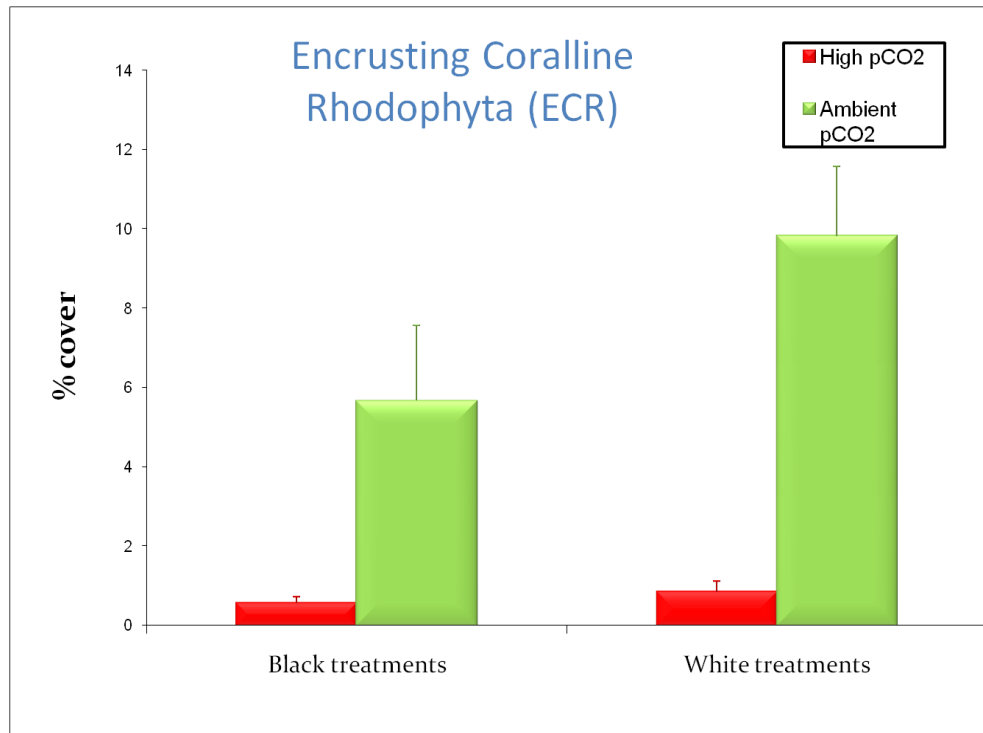


Fig. 9 – Coverage percentage of ECR (mean \pm S.E.; n=36) recruited on black and white treatments in High (red bars) and Ambient (green bars) pCO₂ sites.

Table 4 – Multivariate PERMANOVA analysis on the coverage percentage of ECR. The analysis includes three factors: pCO₂ (High pCO₂ vs Ambient pCO₂), “Te”, Temperature (White vs Black) and “Ca”, Cage (Exclusion, Artefact, Control). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
pCO ₂	1	15.558	15.558	7.1183	<u>0.0135</u>	9860
Te	1	1.1039	1.1039	0.50508	0.4846	9833
Ca	2	8.1301	4.0651	1.8599	0.1797	9957
pCO ₂ xTe	1	0.6163	0.6163	0.28198	0.5931	9815
pCO ₂ xCa	2	6.5361	3.2681	1.4952	0.2439	9953
TexCa	2	2.1943	1.0971	0.50197	0.6171	9952
pCO ₂ xTexCa	2	2.8376	1.4188	0.64913	0.5314	9958
Res	24	52.456	2.1857			
Total	35	89.432				

The two-dimensional nMDS shows a clear separation between the epilithic microphytobenthos (EMPB) assemblages recruited on white-framed and black-framed experimental substrata, suggesting an evident early EMPB community shift controlled by temperature changes rather than the other factors considered (Fig. 10).

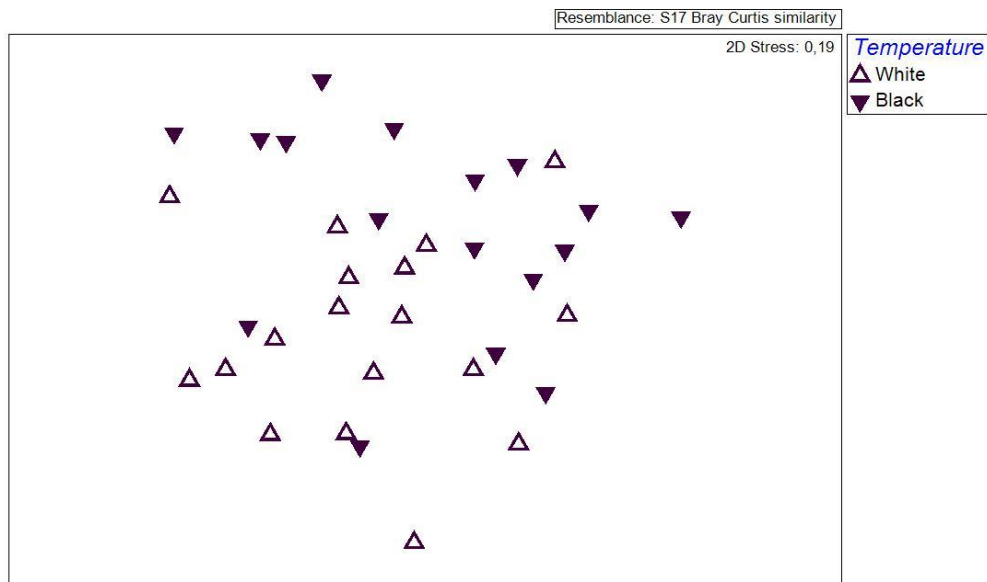


Fig. 10 – nMDS plot of the early microphytobenthic community composition recruited on white (empty triangle) and black (full triangle) experimental substrata along a natural gradient of pH/pCO₂ in Vulcano Island.

PERMANOVA revealed that the presence/absence data of epilithic microphytobenthos assemblages showed a significant Temperature effect (Pseudo-F_{1,34}: 3.1937; $p=0.02$), whilst no significant differences for pH, Cage and the factors' interactions (Table 5).

Table 5 - Multivariate PERMANOVA analysis on the presence/absence data of epilithic microphytobenthos. The analysis includes three factors: pCO₂ (High pCO₂ vs Ambient pCO₂), "Te", Temperature (White vs Black) and "Ca", Cage (Exclusion, Artefact, Control). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
pCO ₂	1	4171.9	4171.9	2.4605	0.0602	9956
Te	1	5415.1	5415.1	3.1937	<u>0.02</u>	9946
Ca	2	5724.1	2862	1.688	0.1363	9947
pCO ₂ xTe	1	70.385	70.385	4.1511E-2	0.9324	9949
pCO ₂ xCa	2	4482.4	2241.2	1.3218	0.2717	9961
TexCa	2	6538.4	3269.2	1.9281	0.0839	9948
pCO ₂ xTexCa	2	410.13	205.06	0.12094	0.9677	9930
Res	23	38998	1695.6			
Total	34	65996				

(d) Patterns of biomass and Chl-a of the early microphytobenthic assemblages

The overall biomass ($\text{mg } 100 \text{ cm}^{-2}$) of the early microphytobenthic assemblages was on average higher in the Ambient $p\text{CO}_2$ ($253.37 \pm 3.03 \text{ mg } 100 \text{ cm}^{-2}$) than the High $p\text{CO}_2$ ($231.96 \pm 9.75 \text{ mg } 100 \text{ cm}^{-2}$) on experimental substrata (Pseudo- $F_{1,35}$: 5.536; $p=0.0154$) (Fig. 10). Microphytobenthic biomass significantly differed in the interaction term of all factors considered in the experimental design (Pseudo- $F_{1,34}$: 4.2741; $p=0.0139$) (Table 6). The Pair-Wise tests on such interaction highlighted that in presence (Control) and in absence (Exclusion) of grazers there are not significant differences on EMPB biomass between $p\text{CO}_2$ and Temperature levels (Fig. 11).

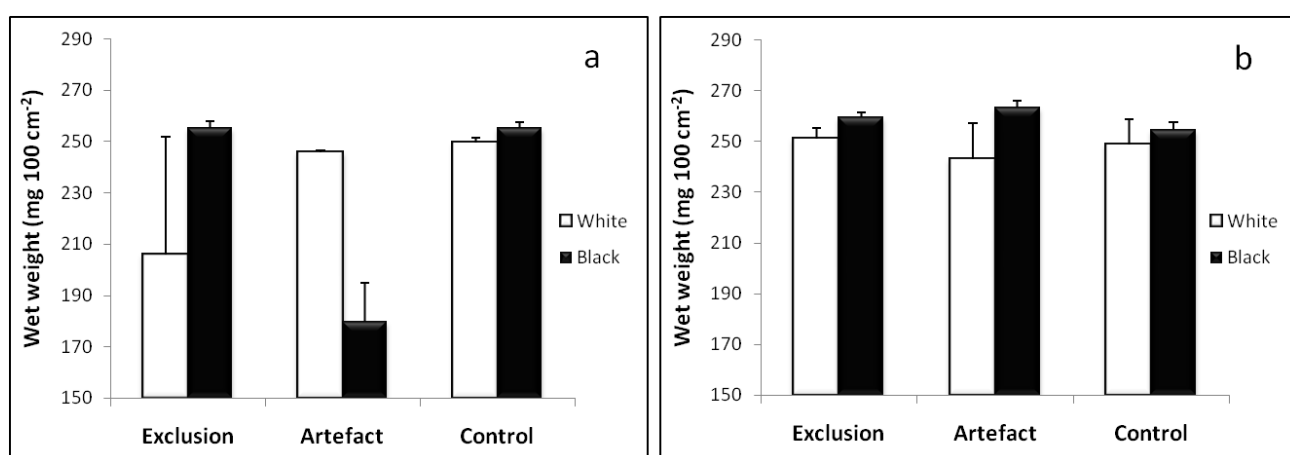


Fig. 11 – Wet weight (mean \pm S.E.; $n=36$) of the early microphytobenthic successional stages recruited on Exclusion, Artefact and Control treatments in High $p\text{CO}_2$ (a) and in Ambient $p\text{CO}_2$ (b) sites.

Table 6 - Multivariate PERMANOVA analysis on the epilithic microphytobenthos biomass. The analysis includes three factors: $p\text{CO}_2$ (High $p\text{CO}_2$ vs Ambient $p\text{CO}_2$), "Te", Temperature (White vs Black) and "Ca", Cage (Exclusion, Artefact, Control). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
$p\text{CO}_2$	1	5.1049	5.1049	5.536	<u>0.0154</u>	9857
Te	1	0.15724	0.15724	0.17052	0.7251	9871
Ca	2	2.6044	1.3022	1.4122	0.2769	9956
$p\text{CO}_2 \times \text{Te}$	1	0.4823	0.4823	0.52303	0.5087	9849
$p\text{CO}_2 \times \text{Ca}$	2	3.032	1.516	1.644	0.215	9962
Te \times Ca	2	5.2668	2.6334	2.8558	0.0523	9962
$p\text{CO}_2 \times \text{Te} \times \text{Ca}$	2	7.8825	3.9413	4.2741	<u>0.0139</u>	9949
Res	24	22.131	0.92212			
Total	35	46.661				

Chlorophyll-a content of the microphytobenthic assemblages was on average significantly higher in High $p\text{CO}_2$ ($2.58 \pm 0.19 \mu\text{g mm}^{-2}$) than Ambient $p\text{CO}_2$ ($1.72 \pm 0.2 \mu\text{g mm}^{-2}$) experimental substrata (Pseudo- $F_{1,35}$: 10.05, $p=0.0049$) with the interaction between $p\text{CO}_2$ and Cage treatments (Pseudo- $F_{1,35}$: 4.132, $p=0.0301$) (Table7). No differences in the Chl-a concentration were recorded between Temperature treatments. Pair-Wise test revealed that the observed differences of chl-a concentration were recorded on the Exclusion (Pairwise T test; $p=0.0078$) and Artefact (Pairwise T test; $p=0.0056$) treatments between High and Ambient $p\text{CO}_2$ (Fig. 12). Moreover, the chl-a content was significantly higher in Control than Artefact plots in Ambient $p\text{CO}_2$ (Pairwise T test; $p=0.0312$) (Fig. 12).

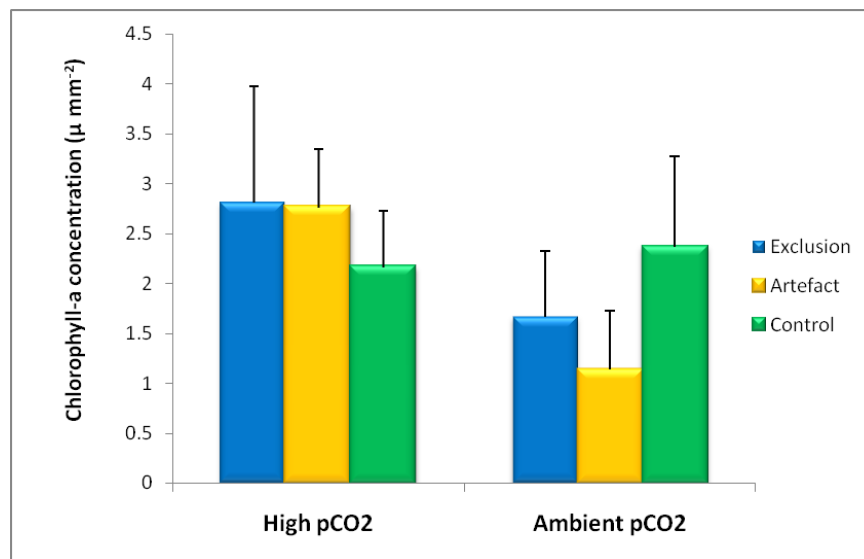


Fig. 12 – Chlorophyll-a concentration (mean \pm S.D.; $n=36$) of microphytobenthic assemblages recruited on experimental plots (Exclusion, Artefact and Control) in High and Ambient $p\text{CO}_2$ sites.

Table 7 - Multivariate PERMANOVA analysis on the Chl-a content of epilithic microphytobenthic assemblages. The analysis includes three factors: $p\text{CO}_2$ (High $p\text{CO}_2$ vs Ambient $p\text{CO}_2$), "Te", Temperature (White vs Black) and "Ca", Cage (Exclusion, Artefact, Control). The significant values are underlined.

	df	SS	MS	Pseudo-F	P(perm)	Unique perms
$p\text{CO}_2$	1	6.5621	6.5621	10.05	<u>0.0049</u>	9856
Te	1	0.42903	0.42903	0.65705	0.4241	9868
Ca	2	0.73687	0.36843	0.56425	0.579	9962
$p\text{CO}_2 \times \text{Te}$	1	1.1556	1.1556	1.7698	0.1874	9827
$p\text{CO}_2 \times \text{Ca}$	2	5.396	2.698	4.132	<u>0.0301</u>	9944
TexCa	2	0.35387	0.17693	0.27097	0.7562	9959
$p\text{CO}_2 \times \text{TexCa}$	2	0.4254	0.2127	0.32575	0.7279	9952
Res	24	15.671	0.65296			
Total	35	30.73				

(e) Patterns of microzoobenthos associated to EMPB

The DGGE sequencing analysis employed on the microzoobenthos associated to the EMPB confirmed such pattern of response. The identified microzoobenthos belonged to larval and early life stages mostly of Crustaceans, Cnidarians and Nematoda whose frequency % did not differ between High and Ambient $p\text{CO}_2$ plots, except for the copepod *Harpacticus* sp. which doubled its Frequency % in High $p\text{CO}_2$ (Fr%: 100%) compared with Ambient conditions (Table 8).

Table 8 - Average frequency % of the early life stage microzoobenthic taxa identified by molecular analyses on the experimental plots positioned in High and Ambient $p\text{CO}_2$ sites along the pH/ $p\text{CO}_2$ gradient off Vulcano Island.

	High $p\text{CO}_2$	Ambient $p\text{CO}_2$
<i>Chthamalus antennatus</i>	5.6	11.1
<i>Xestoleberis</i> sp.	27.8	33.3
<i>Harpacticus</i> sp.	100.0	50.0
<i>Parastenhelia</i> sp.	11.1	27.8
Arthropoda - Class Crustacea	36.1	30.6
<i>Rhombognathus levigatoides</i>	22.2	27.8
Arthropoda - Class Arachnida	22.2	27.8
<i>Acritopus lucens</i>	0.0	5.6
Arthropoda - Class Insecta	0.0	5.6
<i>Bartholomea annulata</i>	11.1	38.9
Cnidaria	11.1	38.9
<i>Brachyrhynchoides triplostylis</i>	0.0	5.6
Platelminta	0.0	5.6
<i>Mopsechiniscus franciscae</i>	27.8	33.3
Tardigrada	27.8	33.3
<i>Pareurystomina</i> sp.	38.9	33.3
Nematoda	38.9	33.3

The nMDS on the presence/absence data showed a clear separation of the micro-faunal assemblages recruiting on white-framed and black-framed substrata (Fig. 13).

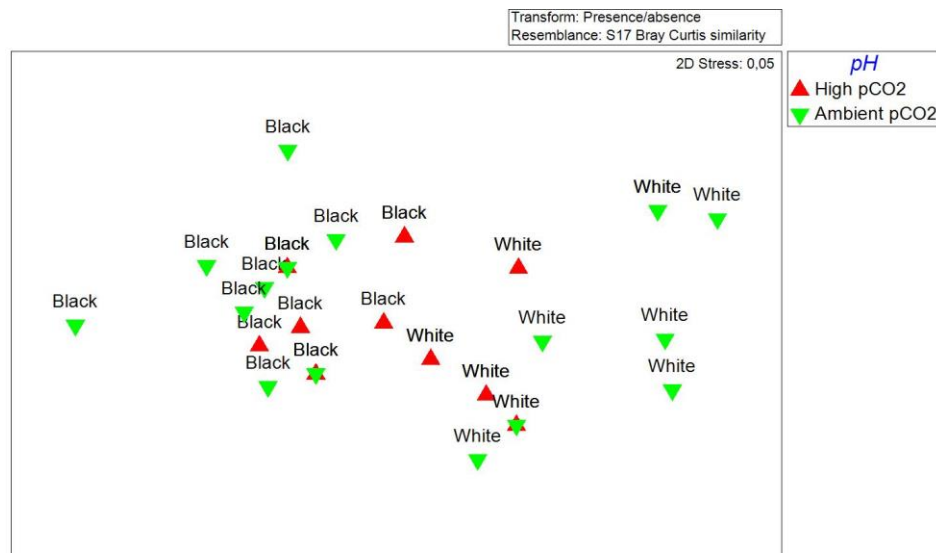


Fig. 13 – nMDS plot of the early stage microzoobenthic assemblages composition, associated to EMPB communities, recruited on white and black treatments in High (red triangle) and Ambient (green triangle) $p\text{CO}_2$ sites.

More specifically, PERMANOVA revealed that the composition of the microzoobenthic assemblages associated with EMPB was affected both by the $\text{pH} \times \text{Temperature}$ (Pseudo- $F_{1,35}$: 3.7034; $p=0.0332$) and the $\text{Temperature} \times \text{Cage}$ (Pseudo- $F_{2,35}$: 2.7872; $p=0.0358$) interactions (Table 9). The Pairwise T test showed that microzoobenthic communities recruited on white substrata differed significantly between High and Ambient $p\text{CO}_2$ (Pairwise T test; $p=0.0024$), but such differences were not detected in black-framed substrata (Pairwise T test; $p=0.3064$). Such analyses also supported a composition shift of the microzoobenthos community between white-framed and black-framed substrata both in High (Pairwise T test; $p=0.0002$) and Ambient $p\text{CO}_2$ (Pairwise T test; $p=0.0001$). The faunal communities found on white-framed and black-framed substrata differed significantly both in Exclusion (Pairwise T test; $p=0.0031$), Artefact (Pairwise T test; $p=0.0021$) and Control (Pairwise T test; $p=0.0023$) treatments.

Table 9 - Multivariate PERMANOVA analysis on the microzoobenthic assemblages associated to the EMPB. The analysis includes three factors: $p\text{CO}_2$ (High $p\text{CO}_2$ vs Ambient $p\text{CO}_2$), “Te”, Temperature (White vs Black) and “Ca”, Cage (Exclusion, Artefact, Control). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
$p\text{CO}_2$	1	11837	11837	13.065	<u>0.0002</u>	9964
Te	1	33713	33713	37.21	<u>0.0001</u>	9959
Ca	2	4060.3	2030.1	2.2407	0.0851	9962
$p\text{CO}_2 \times \text{Te}$	1	3355.3	3355.3	3.7034	<u>0.0332</u>	9968
$p\text{CO}_2 \times \text{Ca}$	2	938.44	469.22	0.5179	0.73	9957
Te \times Ca	2	5050.5	2525.3	2.7872	<u>0.0358</u>	9960
$p\text{CO}_2 \times \text{Te} \times \text{Ca}$	2	3867.6	1933.8	2.1344	0.0932	9966
Res	24	21744	906.01			
Total	35	84566				

2.4 DISCUSSION

This research represents one of the first *in situ* experiments which has concomitantly manipulated both biotic (acidification and warming) and abiotic (grazers) predictive variables in the intertidal system. The results of this research highlighted that ocean warming levels predicted to occur within this century will affect specie's composition of the early stages of microphytobenthic successions in the intertidal community on natural substratum, with $p\text{CO}_2$ treatments and grazing rates having little or no influences on the assemblages considered. Conversely, the structure and composition of the microzoobenthic assemblages associated to EMPB was significantly influenced by the ocean acidification, but the specie-specific responses were mediated by temperature changes.

The structure and composition of the early successional stages of microphytobenthic community was mainly influenced by temperature changes, but interactions with ocean acidification and grazing were not highlighted. An evident community shift leaded by temperature was detected on my white and black experimental recruiting substrata, suggesting that warming may affect community dynamics via altering the recruitment of the early successional microphytobenthic communities. According with my results, only a limited number of in-field experiments showed that the community composition changes with warming (Schiel *et al.*, 2004; Wernberg *et al.*, 2012b). For example, Kordas *et al.* (2014) demonstrated that the intertidal community was affected by the increasing of temperatures. Results of this in field experiment suggested that the abundance of ephemeral algae and the species richness of intertidal community declined, due to thermally stressful conditions. Also Schiel *et al.* (2004) showed that the increased temperatures may lead to a rapid decline in abundance of algal species. Instead, Wernberg *et al.* (2012b) highlighted a reduction in structure complexity of subtidal community with the rise in thermal stress. Contrary to my results, some studies revealed interactive effects of warming and acidification, as a meta-analysis study conducted by Harvey *et al.* (2013) which demonstrated that the combination of the two stressors caused significant negative effects on calcification, reproduction and survival, and a significant positive effect on the photosynthetic rates. Instead, in another meta-analysis study conducted by Kroeker *et al.* (2013) they did not find differences due to the interaction between acidification and temperature in the biomass and primary

production of structured microalgal populations, but was recorded a lower trend of survival, growth and development of these species at high temperatures.

Conversely to my in-field experiment, some studies carried out in mesocosm assessed the responses of early phytobenthic successional stages, under ocean acidification and warming in presence/absence of consumers, revealing that the presence of grazers can mitigate (Alsterberg *et al.*, 2013) or have no effects (Russell *et al.*, 2013) on early stages of microalgae. In my experiment I found that the presence of *Patella* ssp. consumer did not have significant effects on the EMPB community composition and its density did not change in function of $p\text{CO}_2$ levels and temperature treatments, even though is a calcifying organism. The capacity of limpets, living close to the vent area, to maintains their density constant despite the increasing of $p\text{CO}_2$ is, probably, due to their ability in the regulation of calcification rates. As demonstrated by Rodolfo-Metalpa *et al.* (2011), the limpets living in the low pH area had higher calcification rates and, therefore, were able to contrast the higher shell dissolution rates than the individuals living outside the vent sites. The likely abundance of algal turf, food for limpets, in acidified areas may sustain the high energetic costs required to support the higher calcification rates (Hall-Spencer *et al.*, 2008; Porzio *et al.*, 2011; Connell *et al.*, 2017).

In my study, as expected, the biofilm increased sensibly its frequency % in the high $p\text{CO}_2$ sites, whilst the early stages of calcifying macroalgae as *Padina pavonica*, *Jania rubens* and the encrusting coralline rhodophyta decreased their frequency % under acidified conditions.

In accordance with the results obtained in my study, Johnson *et al.* (2011) conducted an experiment on the early microalgal stages of intertidal community in the Vulcano Island and observed a significant increase in biofilm percentage coverage in relation to the increase in $p\text{CO}_2$. Porzio *et al.* (2013) demonstrated that cyanobacteria and diatoms represent the first colonizing forms and are dominant especially in low pH stations. Concordant results were also obtained from Kroeker *et al.* (2012) where biofilm and filamentous algae dominated at low pH stations, while a decrease in $p\text{CO}_2$ led to an increase in algal diversity. Lidbury *et al.* (2012) achieved similar results by working on subtidal communities in Vulcano Island. In accordance with the following study conducted in the intertidal zone, Lidbury *et al.* (2012) observed that biofilm formation significantly increases in natural high $p\text{CO}_2$ conditions even in the subtidal zone.

The early stages of calcifying algae (*J. rubens*, *P. pavonica*, encrusting coralline rhodophyta) decreased their frequency % with the increasing in $p\text{CO}_2$. In my experiment the ECR showed a more lower coverage percentage in acidified conditions than ambient $p\text{CO}_2$, despite they had an elevated frequency % also in the high $p\text{CO}_2$ site. Similar results to my experiment were obtained by Kamenos *et al.* (2016) in a study conducted in a natural $p\text{CO}_2$ gradient, which found that the crustose coralline algae maintained their growth and skeletal structure in low pH conditions although reduced their abundance, probably by their interactions with other species at high $p\text{CO}_2$ levels. Instead, at extreme low pH the coralline algae reduced their size and altered the mineralogy of skeletons and therefore played a reduced ecological role under these conditions.

The $p\text{CO}_2$ plays a crucial role in the early stages of coralline algae altering their development, as suggested by Ordoñez *et al.* (2017) in a laboratory experiment. They highlighted that elevated $p\text{CO}_2$ had strong negative effects on the coralline alga *Porolithon* cf. *onkodes*, and led to the decreasing on spore germination success, generating abnormal cells and hindering the growth rates. In addition, Ordoñez *et al.* (2017) found that other concurrent factors, as irradiance and temperature, could intensify the $p\text{CO}_2$ effects.

The encrusting coralline algae are amongst the first colonizers of bare rocks in marine environment (Dethier, 1994) and are known to facilitate the recruitment and settlement of later colonists. ECR, increasing surface rugosity, provide a suitable substrate for algal propagules and invertebrate larvae in comparison to bare rocks (Arenas *et al.*, 2006; Bulleri *et al.*, 2009). Coralline algae appear to be the most photoautotroph sensitive organisms to pH reduction due to the mineralogy of their skeletons that easily dissolve to the lower saturation levels of CaCO_3 (Gao *et al.*, 1993; Martin and Gattuso, 2009). The negative effects of $p\text{CO}_2$ on calcifying algae were well documented and included a reduction in growth rates (Hofmann *et al.*, 2012), calcification (Gao and Zheng, 2010), distribution and abundance (Kuffner *et al.*, 2008; Hall-Spencer *et al.*, 2008; Porzio *et al.*, 2011). The rate of growth of ECR is greatly inhibited under high $p\text{CO}_2$ conditions, suggesting that no physiological tolerance of the early microphytobenthic stages could represent a driver of change in community's succession due to the impact of ocean acidification. Both in mesocosm and *in situ* experiments confirm the trend of ECR showed in my experiment, the ECR cover was significantly lowered under acidified conditions

(Gao *et al.*, 1993; Hall-Spencer and Rodolfo-Metalpa, 2008; Martin and Gattuso, 2009; Kroeker *et al.*, 2013).

In this study, the early stages of macroalgae and turf-forming algae had a little difference in the frequency % between ambient and high $p\text{CO}_2$ conditions probably because this study considered the early stages of algal succession. Instead a lot of studies, which considered the latest successional stages, suggested that turf algae are positively affected (Kroeker *et al.*, 2010; Connell *et al.*, 2013) and macroalgae became resilient under acidified conditions (Porzio *et al.*, 2011; Kroeker *et al.*, 2012).

In my experiment, the primary productivity of early EMPB assemblages increased significantly in High $p\text{CO}_2$ and decreased in Ambient $p\text{CO}_2$. According to Johnson *et al.* (2011), their data indicate that the effects of $p\text{CO}_2$ enrichment on benthic communities stimulate primary productivity. Similarly, Lidbury *et al.* (2012) observed that under natural high $p\text{CO}_2$ conditions the biofilm production significantly increased also in the subtidal. This suggests that communities inhabiting naturally acidified habitats are adapted to constant stress conditions (Hall-Spencer and Rodolfo-Metalpa, 2008) and could take advantage of the enrichment in CO_2 to increase the primary productivity.

The biomass of early microphytobenthic successional stages decreased in high $p\text{CO}_2$ conditions. My results are in accordance with experiments in which the microphytobenthic species could have small (Tortell *et al.*, 1997; 2000) or negative responses in biomass (Torstensson *et al.*, 2011) to ocean pH changes. Disagreeing with my experiment, some studies revealed the opposite trend, the biomass of biofilm increased under acidified conditions (Johnson *et al.*, 2011; Johnson *et al.*, 2015). Instead, Porzio *et al.* (2013) found that changes in pH did not affect the abundance of early stages of algal succession. The reason why in my experiment the biomass decreased with the increasing in $p\text{CO}_2$ is probably due to the short time of experiment, hence the macroalgal species and the calcifying rhodophyta, prevailing in ambient $p\text{CO}_2$ site, had a major contribution in terms of biomass than the biofilm, dominant under acidified conditions.

The results of my study revealed that the microzoobenthic assemblages, associated to the early EMPB, were affected by $p\text{CO}_2$ changes. The experiment showed a significant increasing in frequency % of copepods, which appeared in each sample, and a decreasing in frequency % of barnacles in high $p\text{CO}_2$ site. Furthermore, despite the direct effects of ocean acidification were evident in my experiment, differences in the composition of

early microzoobenthic stages were mediated by species-specific responses to temperature.

Barnacles are an ecologically important group of calcified marine invertebrates and their reduction in high $p\text{CO}_2$ conditions, as evidenced in my study, was documented (Kroeker *et al.*, 2012), even if they may survive and grow at very low mean pH 6.6 as showed by Hall-Spencer *et al.* (2008). In accordance with my results, an experiment conducted by Findlay *et al.* (2010) in the intertidal zone suggested that acidified conditions may have a great influence on the early life stages of barnacles, altering the number of settling larvae and affecting individual fitness, but in turn these responses could be mediated by the rise in sea surface warming resulting in a significant reduction in the population abundance. In opposition to my results, McDonald *et al.* (2009) revealed that the chronic exposure to elevated CO_2 seawater may do not affect the larval development of barnacles, but negative effects were found on the subsequent post-settled juveniles.

In accordance with my results, previous studies suggested that copepods have high buffering capacity against the ocean acidification (Kurihara and Ishimatsu, 2008; Weydmann *et al.*, 2012; Vehmaa *et al.*, 2013) suggesting that they are able to survive, grow, develop and reproduce in lower pH (Reusch, 2014). Probably, the high tolerance of some crustaceans to high $p\text{CO}_2$ is expected for their low degree of calcification and for their capacity to internal acid-base regulation (Ries *et al.*, 2009; Melzner *et al.*, 2009). Furthermore, the rise in frequency % of copepods under acidified conditions can be explained by density compensation responses (Kroeker *et al.*, 2011) of microzoobenthic assemblages. A compensatory increase in abundance of copepods in high $p\text{CO}_2$ may be caused by the decrease in abundance of other microzoobenthic groups found in my experiment. In disagree with my study, there are works showing negative impacts in moderate $p\text{CO}_2$ conditions (Fitzer *et al.*, 2012) and more negative impacts of copepods abundance in extreme low $p\text{CO}_2$ (Kurihara *et al.*, 2004; Weydmann *et al.*, 2012).

Although a hypothesis of this study is a possible interactive effect between biotic and abiotic factors, as seen in other mesocosm studies (Alsterberg *et al.*, 2013; Russell *et al.*, 2013; Sampaio *et al.*, 2017), this experiment reveals that the temperature controls the initial stages of microphytobenthic assemblages and that the primary producers, at least in early phase of successional stages, are not particularly affected by the variation in $p\text{CO}_2$. Hence, I exclude interactive effects between these factors on the early microphytobenthic stages of intertidal successions. Studies of multiple stressors (Griffith

et al., 2012; Todgham *et al.*, 2013) observed complex and often non-linear interactions suggesting that investigations of a single stressor may create misleading inferences about physiological responses in a multivariate natural environment. Same authors have noted that warming and acidification can act in synergy by showing interactive effects on already structured communities. For example, Byrne and Przeslawski, (2013) presented a review of the combined impact of $p\text{CO}_2$ and temperature on the early life stages of invertebrates. Their analyses showed that in 16 of the 23 species studied so far, warming and acidification had interactive effects on the organisms' responses. Negative additive effects were the most common since heating increased the impacts due to acidification; however, there have also been some antagonistic interactions where, instead, warming reduced the negative impacts due to ocean acidification. A study conducted in Australia showed that acidification and warming had interactive effects: the exposure to lower pH conditions led to the decreasing of the survival and development rates of some marine organisms under moderate temperature, but these effects were alleviated under higher temperatures (Davis *et al.*, 2013). In another experiment, Rodolfo-Metalpa *et al.* (2011) examined the effects of ocean acidification in relation to warming on some species of corals and mollusks along the CO_2 gradient on the island of Ischia. They observed that at low pH levels the *M. galloprovincialis* and *C. caespitosa* species were still able to calcify, thanks to the presence of external coatings that act as protection for the shell. In contrast, the ability of these organisms to calcify under low pH conditions has decreased dramatically when acidification was combined with high temperatures, indicating a probable synergistic effect between these stressors.

In summary, the present study showed that the early successional stages of microphytobenthic intertidal community were affected by temperature changes. Instead, the microzoobenthic assemblages associated to the early EMPB were affected by ocean acidification, and differences in community composition were mediated by species-specific responses to warming. In addition, we did not find that interactive interactions between abiotic (acidification and warming) and biotic (grazing pressure) stressors affected the early successional stages of EMPB. These results highlighted that the temperature is the main driver of community change in early stages of microphytobenthic assemblages and they provide a support to suggest that the ongoing ocean warming may modify the composition of species at early stages of succession, by altering the stability of ecosystems and leading shifts in the successional patterns of intertidal species.

In conclusion, investigating the role of composition and structure of early MPB and associated microzoobenthic community can be highlighted the mechanisms underlying the interactions between species and between them and the abiotic environments. The data presented in this chapter provide one of the first contributions for a further detailed analysis of the role that major ocean-change climate drivers may have on the structure and temporal variability in the short-term of early intertidal communities. Actually, there are few examples of ecological processes under different temperature regimes to understand the effects that climate change can have on early successions in intertidal communities. Longer-term studies will have to be conducted to know whether and how differences in climate changes may also affect subsequent successional stages, especially in mature communities, in the near future.

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CHAPTER 3. DO DIFFERENCES IN OCEAN PH AND SETTLEMENT SUBSTRATE WITH DIFFERENT CHEMISTRY AFFECT THE LATE STAGES OF A ROCKY INTERTIDAL COMMUNITY?

Abstract: Many macroalgal species can take advantage from the increasing in CO₂ by enhancing growth and productivity, but the settlement, abundance, calcification and growth of calcified algae can be negatively affected at lower carbonate saturation states. Ocean acidification effects could be mediated by substrata with different chemical composition, as some of these may alter carbonates chemistry and alkalinity at small-scale, likely playing a role in affecting the algal succession and the composition and structure of the associated invertebrate community. Here I assessed the intertidal community late stage of succession and the responses of the benthic fauna on different substrata (olivine, sand, aragonite, PVC) after a 17-month exposure to altered pH conditions (Low, Mid, Control). Our results showed evident community shifts (from highly complex macroalgae to low profile turfs) at decreasing pH levels and despite the substratum characteristics. The structure and composition of the algal-associated benthic community varied significantly between different pH levels and were also affected by the higher coverage of macroalgae in Control treatment than Mid and Low pH, where in turn turf forms dominated. As expected, the number of calcifying organisms was higher in Mid and Control sites than the heavily acidified site. In summary, ocean acidification may affect the invertebrate fauna both in a direct (via pH reduction) and in an indirect (via decreased community complexity) way, therefore pushing intertidal communities to change.

Keyword: Late stages of intertidal succession – Ocean acidification – Global stressors – Olivine – Chemical composition of recruiting surfaces – Diffusion Boundary Layer.

3.1 INTRODUCTION

Oceans have acidified by 0.1 pH units since the pre-industrial time (Caldeira and Wickett, 2003, 2005; Orr *et al.*, 2005; Doney *et al.*, 2009) are expected to fall a further 0.3-0.4 pH units by 2100 (Meinshausen *et al.*, 2011). Macroalgal species are ubiquitous of intertidal

communities worldwide but are sensitive to many different kinds of disturbance (Ballesteros *et al.*, 2007), as changes in ocean chemistry.

When subjected to global change stressors, such as ocean acidification, the benthic primary producers which under some circumstances may dominate the late-stage of succession in intertidal communities (i.e. macroalgal species) could be affected in different ways exhibiting contrasting responses with the decreasing in pH (Connell and Russell, 2010).

Late stages of algal succession are typically formed from large perennial brown and red algae that become established replacing the earlier colonists (Sousa, 1979b). These perennial species have a seasonal recruitment and reproduction and are considered ecosystem engineers and consequently can regulate the community structure positively (i.e. providing food, refuges and shelter from extreme temperatures, solar radiation, desiccation, wave action) (McCook and Chapman, 1991; Bertness *et al.*, 1999) or negatively (limiting space availability and light) (Kennelly, 1989).

Many macroalgal species can take advantage from the increasing in CO₂ (Connell and Russell, 2010; Fabricius *et al.*, 2011; Porzio *et al.*, 2011), by enhancing growth and productivity (Connell and Russell, 2010). In a study conducted along a natural CO₂ gradient in Vulcano Island, Johnson *et al.* (2012) showed that calcifying macroalgal canopies (i.e. *P. pavonica*) can increase in abundance under acidified conditions despite the reduction in CaCO₃ content with CO₂ enrichment. On the other hand, a lot of studies demonstrated that settlement, abundance, calcification and growth of calcified algae can be negatively affected at lower carbonate saturation states (Martin *et al.*, 2008; Martin and Gattuso, 2009; Russell *et al.*, 2009). In particular, the most sensitive calcifying photoautotrophs under acidified conditions seem to be the Crustose Coralline Algae (CCA) (Gao *et al.*, 1993; Martin and Gattuso, 2009). CCA are ubiquitous species belonging to benthic communities. This algal group has a fundamental ecological importance, both in temperate and tropical near-shore systems, because provides food, a settlement substratum for other species of seaweed and invertebrates (Morse *et al.*, 1988; Vermeij and Sandin, 2008) and can contribute to carbonate enrichment in their habitat (Steneck and Adey, 1976). The normal conditions of seawater pH favor the precipitation of CaCO₃ by CCA during the biogenic calcification process for growth and production, but the decreasing in seawater pH induces a decreasing in the saturation states of carbonate minerals consequently affecting the normal calcification rate (Hoegh-Guldberg *et al.*,

2007). A lot of laboratory and in field studies demonstrated that crustose coralline algae are negatively affected by ocean acidification showing reduction in recruitment (Russell *et al.*, 2009), growth (Anthony *et al.*, 2008; Russell *et al.*, 2009), and calcification rates (Martin *et al.*, 2013) with the decreasing in pH. Opposite to CCA, filamentous turf algae, that are fleshy seaweeds which form extensive mats in benthic communities, are positively affected from ocean acidification.

Turf algae may enhance rates of photosynthesis and growth with the increase in accessible carbon, under ocean chemistry changes (Kroeker *et al.*, 2010; Connell *et al.*, 2013). In the later stages of succession, under normal environmental conditions, crustose coralline algae can coexist with turf algae or recruit understory to canopies (Bulleri, 2006). Instead under acidified conditions, the differential susceptibility of these algal groups to changes in pH suggests that the ocean acidification could lead to potential shifts in community structure (Hall-Spencer *et al.*, 2008; Porzio *et al.*, 2011). While CCA are affected negatively from ocean acidification, the growth and metabolism of turf algae may increase under low pH conditions (Kuffner *et al.*, 2008; Levitan *et al.*, 2007).

A study conducted by Short *et al.* (2014) highlighted that the calcification of crustose coralline algae under elevated CO₂ conditions could be facilitate by the creation of a particular microenvironment called diffusive boundary layer (DBL). Kuzmin and Sobolev (2003) showed that the olivine, a mineral component of some silicate rocks, is able to form a boundary layer around the crystal as a consequence of slow diffusion of mainstream. Weathering reactions of olivine are often alkalizing. Literature evidences showed that enhanced weathering of olivine in marine environment may mitigate the rising in atmospheric CO₂ concentrations and also mitigate the associated CO₂ problem linked to ocean acidification (Griffioen, 2016). In another study conducted by Taylor *et al.* (2015) demonstrated that the weathering of olivine could cause significant drop of atmospheric CO₂ and ameliorate forecasted ocean acidification scenario by 2100. Cornwall *et al.* (2013) found that the diffusive boundary layer creates a buffering zone within which could be mitigate the effects of ocean acidification overall for calcifying species. In a next experiment, Cornwall *et al.* (2014) demonstrated also the importance of the seawater flow (fast or slow) on thickness of DBL in acidified conditions. At 7.65 pH units, under slow flows with thick DBL, coralline algae maintained a net growth and calcification; whilst a net dissolution was detected under higher flows with thin DBL. Hurd *et al.* (2011) showed that calcifying organisms recorded a different pH gradient between

their surface and mainstream seawater, suggesting that the DBL could be useful for countering the pH changes. Until now, these studies focused their attention on changes in chemical seawater due to olive weathering, but there is a lack of knowledge of how this geochemical process may affect biological successions and specie's interaction in marine environments.

The shift in algal community due to ocean acidification may sustain different benthic fauna communities. Evidences suggest that ocean acidification may have significant consequences for marine taxa, overall for organisms with skeletons and shells in calcium carbonate. Guinotte and Fabri (2008) examined the comportment of many marine taxa in relation to ocean acidification, showing that potential changes in species' distribution and abundance is dependent from the tolerability of species to acidified conditions. Kroeker *et al.* (2011b) demonstrated that the community structure of benthic fauna changed with different pH conditions. The invertebrate taxonomic richness decreased significantly with the decreasing pH because the heavily calcified organisms (i.e. mollusks and decapods) are absent under acidified conditions. Conversely, the less or no calcified taxa (i.e. amphipods, tanaids and some species of polychaetes) are found most abundant in extreme pH conditions. Also Hale *et al.* (2011) showed that not all the benthic fauna species investigated decreased in abundance in response to acidification. Some species, such as arthropods and annelids, resulted is less vulnerable to acidified conditions, whilst echinoderms and mollusks were the most negatively impacted by pH reduction. The high vulnerability of mollusks to ocean acidification is showed also by Rodolfo-Metalpa *et al.* (2011) in a transplanting experiment conducted along a pH gradient near volcanic CO₂ vents off Ischia (Italy) demonstrating that mollusks exposed to low pH conditions dissolved their shell faster. Generally the low pH can cause the reduction in infaunal diversity, as showed by Widdicombe *et al.* (2009a) in a mesocosm experiment, and the loss of calcifying species such as echinoderms and mollusks in a *in situ* experiment (Hall-Spencer *et al.*, 2008). Also Cigliano *et al.* (2010) found a clear decrease in species diversity under acidified conditions due to a strong reduction of many calcifying organisms, such as Foraminifera, Bivalvia and Gastropoda. These groups of organisms, which have need the production of calcified shells for the survivor, are particularly vulnerable to lowering pH. In fact, many studies demonstrated that the increasing of *p*CO₂ lead a decreasing of calcification rates in the calcifying organisms (Gattuso *et al.*, 1998; Kleypas *et al.*, 2006; Rodolfo-Metalpa *et al.*, 2010a). Probably, shifts in structure and composition of benthic

fauna under different pH conditions could be driven by the interactions with macroalgae, but studies are needed to elucidate the indirect effect of different macroalgal communities on benthic fauna assemblages exposed to altered pH conditions.

In the light of above, I tested whether substrata with different chemical composition (olivine, sand, aragonite, PVC), some of which may alter carbonates chemistry and alkalinity at small-scale (i.e. olivine), could play a role in determining the composition and structure of algal intertidal communities at late-stage succession, and associated benthic phytal fauna community, in relation to different pH conditions. Furthermore, to have a better understand about the importance of substratum's nature on the late stages of algal succession I examined differences in algal coverage between natural and artificial substrata.

3.2 MATERIALS AND METHODS

(a) Study area

This experiment was carried out along the natural pH/ $p\text{CO}_2$ gradient within the Levante Bay ($38^\circ 25' \text{ N}$, $14^\circ 57' \text{ E}$; Vulcano Island, NE Sicily, Italy). The experiment was conducted from May 2014 to October 2015. The sites are well characterized by a geochemical point of view (Boatta *et al.*, 2013) and previously used for other experiments on ocean acidification.

Three sites were selected along the pH/ $p\text{CO}_2$ gradient, having the following GPS coordinates: the Low pH site (Low pH) Latitude $38^\circ 25.184' \text{ N}$ and Longitude $14^\circ 57.696' \text{ E}$; the Mid pH site (Mid pH) Latitude $38^\circ 25.193' \text{ N}$ and Longitude $14^\circ 57.763' \text{ E}$; the Ambient pH site (Control) Latitude $38^\circ 25.248' \text{ N}$ and Longitude $14^\circ 57.853' \text{ E}$ (Fig. 14).

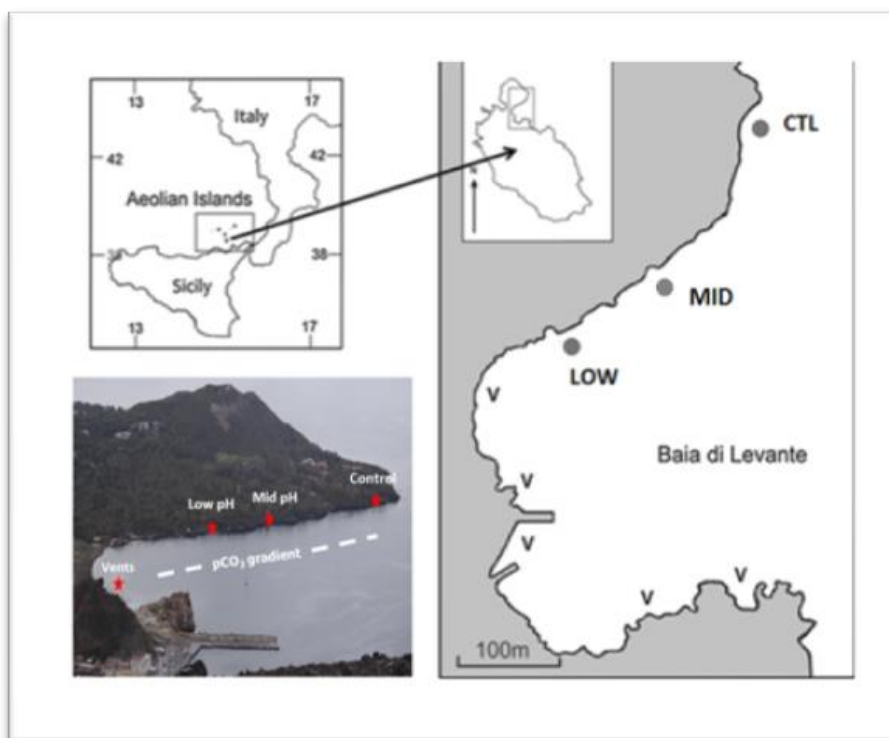


Fig. 14 – Study site, Levante Bay (Vulcano Island). The red stars indicate the sampling stations (Low pH, Mid pH and Control) and the position of the natural volcanic CO_2 vents. The dashed line indicates the natural pH/ $p\text{CO}_2$ gradient off Vulcano Island.

The Low pH site records 7.61 ± 0.13 pH units, the Mid pH site has 8.12 ± 0.04 pH units representing the conditions forecasted for the middle of this century (Nakicenovic and Swart, 2000) and the control site records a normal pH of 8.21 ± 0.01 pH units.

At each site, 6.5 × 6.5 cm PVC tiles were attached to the rock surface (n = 40) at the low tide level (Fig. 15).



Fig. 15 – Example of experimental recruitment tiles attached to intertidal rocky shores on Vulcano Island, Italy.

Tiles were grouped within 1 of 4 treatments (n = 10) based on the surface coating applied to the tile, namely: (i) olivine, (ii) sand, (iii) aragonite, or (iv) no coating (PVC) (Fig. 16). Coatings were ground particles of olivine and aragonite, and locally-sourced marine sand (particle sizes (ϕ) ranged between 100-300 μ m), that were attached to the PVC surface using epoxy resin (Gorilla Glue).

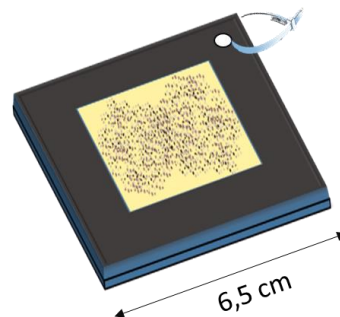
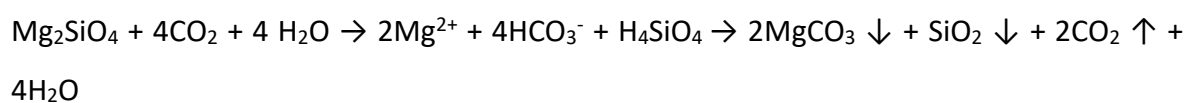


Fig. 16 – Prototype of a recruiting tile.

(b) Type of substrata

Olivine is a silicate mineral whose chemical formula is Mg_2SiO_4 . Olivine reacts with CO_2 according to this reaction:



(Educs → ions and silica in solution, secondary minerals → precipitation reactions in the ocean)

This formula suggests that 4 moles of CO₂ are sequestered by 1 mole of olivine, equivalent to 1.25 tonnes of CO₂ per ton of olivine. This process creates an alkaline environment.

The aragonite is a mineral composed from neutral calcium carbonate (CaCO₃).

The sand is formed from deposition of particles that were transported from areas adjacent by the sea currents. The two main types of materials that make up the sand are quartz (silica) and carbonate.

PVC, vinyl polychloride, is the material that forms the base of the different recruiting tiles. In order to distinguish the effects of the type of substratum on recruitment and succession from those of support were placed PVC tiles as control.

Different cable ties were applied at the recruiting surfaces to identify the different substrata during the experimental time. The yellow color indicated the olivine, green the sand, blue the aragonite and red the PVC (Fig. 17).

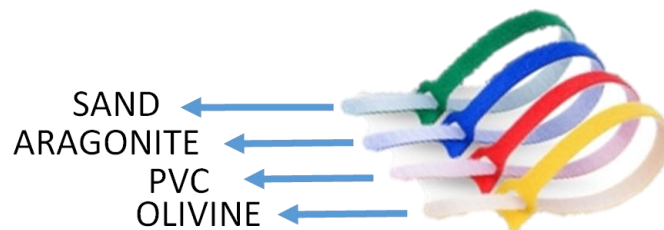


Fig. 17 – Cable ties applied at the recruiting tiles to identify the different substrata composition.

The large variability in community structure and composition caused by habitat heterogeneity makes difficult the experiments on natural community. The use of artificial substrata is a simple method to sample recruitment of natural communities using uniform experimental units (McGuinness and Underwood, 1986), but the settlement responses on artificial substrata could be unrepresentative of natural settlement substrata. To have a better understand of the importance of substratum's nature on late stages of algal community succession I examined differences in algal percentage coverage between

natural and artificial substrata. The algal coverage found in the experimental substrata along the three pH sites (Low, Mid and Control) were compared with another experiment conducted in the same pH sites on natural substrata where late stages of benthic assemblages were naturally present.

Benthic assemblages were assessed using a standard visual census method and samples were collected using 20×20 cm quadrates (Fraschetti *et al.*, 2005). In each pH zone, two sites (located tens of meters from each other) were haphazardly chosen and five replicate quadrates sampled for percentage cover of animals and plants. A frame with 25 of 4×4 cm squares was used to assess percentage cover (C%). Percentage coverage of benthic species was determined by assigning each taxon a score ranging from 0 to 4 within each square and summing the 25 estimates (Dethier *et al.*, 1993). Final values were expressed as percentages. Percentage coverage of the main algal groups (i.e. macroalgae, turf and CCA) on artificial and natural substrata under different pH regimes were compared using univariate PERMANOVA (Anderson, 2003) with two fixed and orthogonal factors namely pH, (three levels: Low, Mid and Control), and Substratum ('Su', two levels: Natural and Artificial).

(c) Sampling techniques and laboratory analyses

After 17 months some recruitment substrata were lost. Were found a total of 26 recruitment tiles both in the Low pH and Control site and 30 in the Mid pH site.

The substrata were photographed and stored in freezer at the temperature of -18° C. Subsequently, the various tiles were defrosted, rinsed with filtered seawater and after filtration the biological component was analyzed by microscope by dividing the animal and vegetable organisms. Specifically, animal organisms were subdivided and stored in 70% alcohol test tubes and vegetable organisms were stored in freezer at -18° C.

For animal organisms, the water containing the biological material extracted from each substratum was filtered with a sieve of 200 µm to collect small organisms belonging to the dimensional scale of meiobenthos (e.g. copepods and foraminifers) and macrozoobenthos (> 0.5 mm). The filtered material was transferred to sample orders containing a 70% seawater solution and alcohol for storage. Seawater was microfiltrated to avoid flocculation that can reduce visibility during the next stage sorting phase. Each sample order was labeled by marking the sampling date, station, pH level, and replicate

number (rep1, rep2, rep3). Once the extraction of the organisms was completed, the sorting technique was used to separate, identify and count the collected organisms. The method consists of gradually removing the biological material stored in alcohol in the sample holder and placing it in a Petri capsule by a pipette, adding sea water or 70% alcohol. The content of the Petri capsule was examined 40x magnification at the binocular microscope model Optika SZM-1. The sorting technique foresees that the sample inside the Petri capsule is observed starting from an angle and proceeding systematically vertically and horizontally with a defined scanning procedure. All organisms present within the sample were collected with ophthalmic stainless steel tweezers (for smaller and fragile organisms a pipette with a thin tip were used) and classified in Operational Taxonomic Units (OTUs). OTUs are taxonomic units identified by the scientist and do not necessarily represent a real group existing in nature. The species are grouped into groups that exhibit similar morpho-anatomical characteristics. Each tube was filled with 70% microfiltered alcohol and seawater for the preservation of organisms and labeled with the name of the taxon and the sample of origin. The different taxa of each sample were again observed at the microscope, counted and as far as possible identified at the taxonomic level more detail by using classification guides from the class level to the phylum level using specific texts (Riedl, 2005; Brusca *et al.*, 1996) and keys for identification (Ruffo, 1982; 1993). The raw data of abundance of each taxon obtained for each sample were reported in matrixes.

For vegetable organisms, the samples were defrosted, rinsed with seawater and analyzed with the binocular microscope model Optika SZM-1. The vegetable organisms were separated in OTUs with the sorting technique. Subsequently, the OTUs were identified to specie level as possible by using keys of classification (Maggs and Hommersand, 1993; Bressan and Babbini, 2003; Sfriso, 2010, 2011; Cormaci *et al.*, 2012). Each taxonomic group was preserved in tubes labeled with all the informations of the sample.

The algal biomass of each taxonomic group of every sample was analyzed by the dry weight. Each taxa identified was placed in a support and dried at 60° C for 24 hours. After 24 hours, the dry algae were weighted in a 4 decimal balance and the dry weight obtained for each taxa was reported in an excel matrix.

The seawater carbonate chemistry of the experimental sites was recorded with the same method described in the previous chapter.

(d) *Statistical analyses*

To graphically evaluate differences in structure and composition of late stages of algal succession and the associated benthic phytal fauna community three separate nMDS graphs were performed. To verify whether the differences observed graphically in the nMDS were significant, the PERMANOVA analyses were carried out.

To assess the structure and composition of late stages of algal succession recruited on different substrata under diverse pH conditions, a multivariate PERMANOVA analysis was performed on the algal biomass, expressed as dry weight (in g). The factors considered, all fixed and orthogonal, were: pH with three levels (Low, Mid and Control) and Substratum ('Su') with four levels (Olivine, Sand, Aragonite and PVC).

To estimate the patterns of the total algal biomass of the main taxonomic groups (macroalgae, turf algae and CCA) found on experimental tiles, in function of different pH levels, a two-way univariate PERMANOVA was performed. Each taxonomic group was analyzed in function of pH factor, fixed and orthogonal with three levels (Low, Mid and Control), and in function of Substratum ('Su'), fixed and orthogonal with four levels (Olivine, Sand, Aragonite and PVC).

The total number of individuals (N), species richness (S) and the structure and composition of benthic phytal fauna associated to late stages of algal communities was analyzed by the univariate PERMANOVA with the pH factor, fixed with three levels (Low, Mid and Control).

To evaluate the relation between benthic phytal fauna communities and algal biomass for each pH condition the Canonical Analysis of Principal coordinates was performed (CAP). CAP is a computer program that calculates a canonical analysis on the principal coordinates based on any symmetric distance matrix, including a test by permutation. CAP analysis was used to assess shifts in the benthic fauna abundance between the three pH levels (Low, Mid and Control) and to evaluate their correlation with the total biomass of macroalgae, turf algae and CCA.

In an univariate context, PERMANOVA was based on Euclidean distance matrix with 9999 permutations and was chosen because this method does not assume a normal distribution of errors allows for factorial designs and accounts for interaction effects (Anderson *et al.*, 2008). In a multivariate context, the structure and composition of microphytobenthic community and early stages of associated microbenthic assemblages

was based on Bray-Curtis similarity and were analysed by multivariate PERMANOVA again with 9999 permutations.

After the PERMANOVA, the post hoc Pairwise T test comparison, based on permutations of residuals under a reduced model, was performed.

All analyses were performed using the PRIMER 6.1.10 & PERMANOVA β 20 software package (PRIMER Ltd., Plymouth).

3.3 RESULTS

(a) Seawater carbonate analyses

In the sampling sites, the pH values and the saturation rates of Calcite and Aragonite are strongly influenced by the presence of seeps. Conversely, the temperature, salinity and total alkalinity are relatively constant among the sites. During the experimental time the substrates were exposed to the mean pH 7.77 (± 0.19) for the 2014 and 7.68 (± 0.16) for the 2015 in the Low pH site. In the Mid pH site the mean pH was of 8.01 (± 0.11) for the 2014 and of 8.03 (± 0.01) for the 2015; while in the Control site the mean values of pH were of 8.08 (± 0.02) for the 2014 and 8.09 (± 0.03) for the 2015. The highest mean values for $p\text{CO}_2$ were recorded during the 2015 in the Low pH site, that consequently recorded the lower mean values of calcite (Ω_{cal} : 2.42 ± 0.69) and aragonite (Ω_{ara} : 1.58 ± 0.45) saturation (Table 10).

Table 10 - Carbonate chemistry recorded at the three pH sites off Vulcano Island (Low pH, Mid pH and Control). pH, Salinity and Temperature (T) were measured on different days between May 2014 and October 2015, using a multiparametric YSI probe (n=10). TA was measured from three replicated 0.2 μm filtered water samples collected in each CO_2 sites and data were averaged for subsequent calculations. Calculated pH was obtained from CO_2SYS .

2014	Low	Mid	Control
Salinity (ppm)	Mean \pm E.S.	37.2(± 0.1)	37.5(± 0.1)
	Range	36.3-38.3	36.3-38.3
	Median	37.1	37.7
Temperature ($^{\circ}\text{C}$)	Mean \pm E.S.	21.4(± 0.5)	21.4(± 0.5)
	Range	13.7-29.2	13.9-28.6
	Median	20.5	20.3
pH	Mean \pm E.S.	7.77 (± 0.19)	8.01 (± 0.11)
	Range	7.50-7.84	7.93-8.05
	Median	7.81	7.99
$p\text{CO}_2$ (μatm)	Mean \pm E.S.	1122 (± 592)	653 (± 282)
	Range	752 – 1805	412 – 805
	Median	810	613
Total Alkalinity (mmol kg^{-1})	Mean	2527	2538
Ω Calcite	Mean \pm E.S.	2.60 (± 0.93)	3.71 (± 0.43)
	Range	1.54 – 3.26	3.26 – 4.68
	Median	3.01	3.71
Ω Aragonite	Mean \pm E.S.	1.69 (± 0.61)	2.69 (± 0.31)
	Range	1.00 – 2.12	2.18 – 3.01
	Median	1.95	2.55

2015	Low	Mid	Control
Salinity (ppm)	Mean	38	38
Temperature	Mean \pm E.S.	21.6 (\pm 0.3)	21.4(\pm 0.5)
(°C)	Range	23.9 – 28.24	23.9-28.6
	Median	20.5	20.3
pH	Mean \pm E.S.	7.68 (\pm 0.16)	8.03 (\pm 0.01)
	Range	7.5 – 7.79	7.52-8.24
	Median	7.77	8.07
$p\text{CO}_2$ (μatm)	Mean \pm E.S.	1176 (\pm 500)	633 (\pm 28)
	Range	866 – 1753	353 – 1224
	Median	909	671
Total Alkalinity	Mean	2501	2514
(mmol kg ⁻¹)			2520
Ω Calcite	Mean \pm E.S.	2.42 (\pm 0.69)	3.90 (\pm 0.08)
	Range	1.63 – 2.91	2.76 – 4.86
	Median	2.73	3.89
Ω Aragonite	Mean \pm E.S.	1.58 (\pm 0.45)	2.77 (\pm 0.27)
	Range	1.06 – 1.90	2.08 – 3.12
	Median	1.77	2.59

(b) *The late stages of algal community*

In my experiment 24 algal taxonomic groups were found: *Acetabularia acetabulum*, *Anadyomene stellata*, *Antithamnion* spp., unidentified Biofilm, *Calothrix* spp., *Chaetomorpha* spp., *Chondria capillaris*, *Cladophora* spp., Encrusting Coralline Rodophyta, *Cystoseira compressa*, *Derbesia* spp., *Dictyota* spp., *Ectocarpus* spp., *Gelidiella* spp., *Herposiphonia* spp., *Jania rubens*, *Laurencia* complex, *Padina pavonica*, *Polysiphonia* spp., *Rivularia* spp., *Sphacelaria* spp., *Stypocaulon scoparium*, *Ulothrix flacca*, *Ulva* spp.

The two-dimensional nMDS shows a clear separation between the late stages of algal community recruited along three pH conditions (Low, Mid and Control), suggesting an evident community shift controlled by pH changes rather than the chemical composition of substrata, or their interaction (Fig. 18).

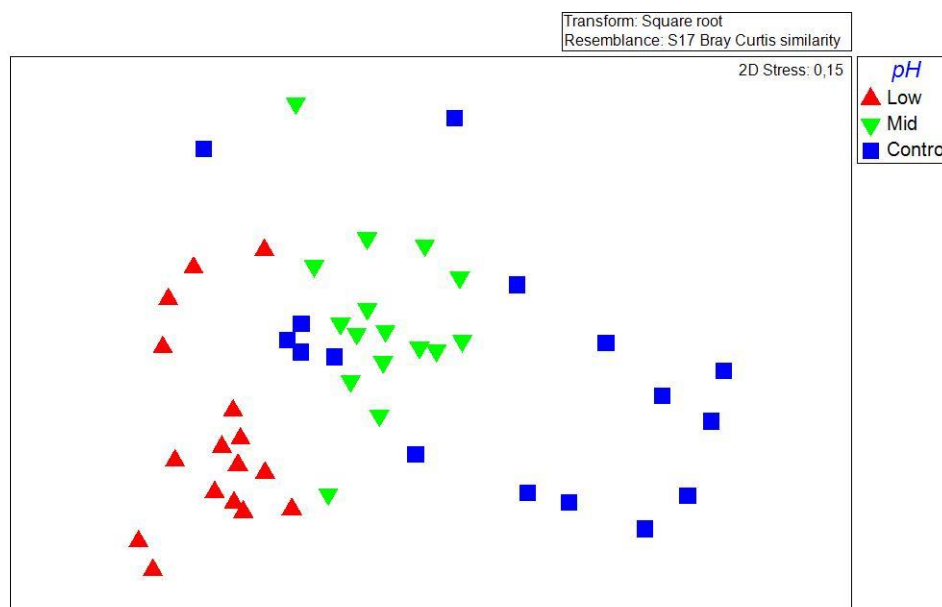


Fig. 18 - nMDS plot of the late stages of algal community composition recruited under three pH levels (Low pH, Mid pH and Control) on Vulcano Island.

The multivariate PERMANOVA revealed that the structure and composition of late stages of algal community varied significantly as a function of pH (PERMANOVA, $p=0.0001$), but there were not significant differences among the Substratum treatments and their interaction (Table 11). The Pairwise T test on the pH levels (Low, Mid and Control) showed highly significant differences (PERMANOVA, $p=0.0001$) among all the pH levels considered.

Table 11 - Multivariate PERMANOVA analysis on the structure and composition of late stages of algal community. The analysis includes two factors: pH (Low pH, Mid pH and Control) and "Su", Substrate (Olivine, Sand, Aragonite and PVC). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
pH	2	15607	7803.5	12.297	<u>0.0001</u>	9936
Su	3	2951.2	983.74	1.5503	0.0922	9922
pHxSu	6	1992.5	332.08	0.52332	0.9872	9883
Res	36	22844	634.57			
Total	47	43395				

(c) Total biomass of the main algal taxonomic groups

The macroalgal biomass changed significantly as a function of the different pH levels considered (PERMANOVA, $p=0.0003$), but not for the different substrata or their interaction. At the Control site there is a significant increase in macroalgal biomass, which instead decreased in Low pH conditions (Fig. 19).

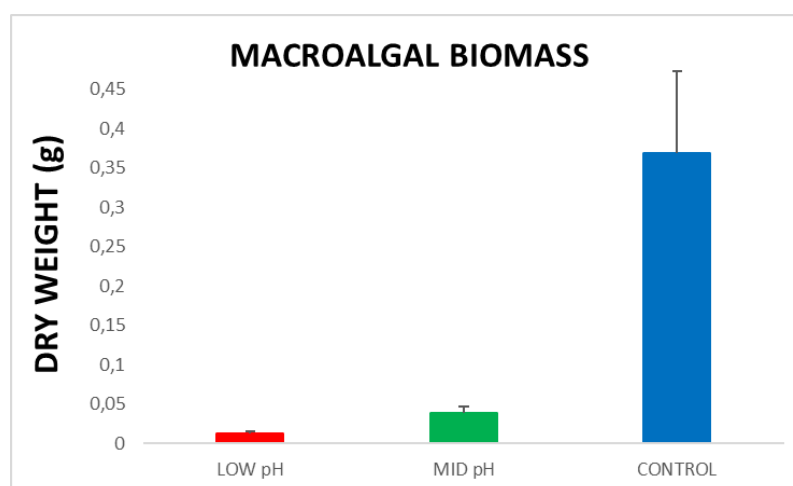


Fig. 19 – Total biomass (mean \pm E.S.; $n=48$) of the late stages of macroalgal community under three different pH conditions (Low pH, Mid pH and Control).

The turf biomass was affected by the pH levels (PERMANOVA, $p=0.0001$) and by the substrate chemistry (PERMANOVA, $p=0.0375$), but not from their interaction. The fig. 20 shows the turf biomass increases with a decrease in pH. The Pairwise T test showed the turf biomass recruited in Low pH site differed significantly from Mid pH ($p=0.0001$) and Control ($p=0.0002$). The Pairwise T test on chemical composition of substrata revealed that only the PVC treatment differed significantly from the other substrata treatments.

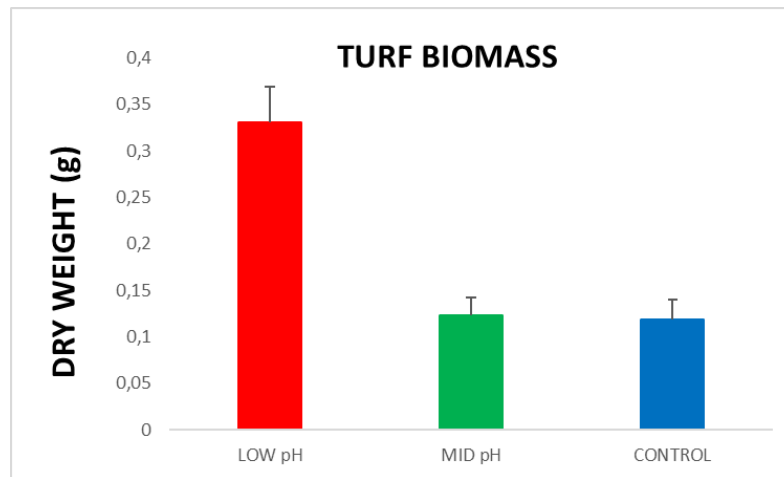


Fig. 20 – Total biomass (mean \pm E.S.; $n=48$) of the late stages of turf community under three different pH conditions (Low pH, Mid pH and Control).

The Crustose Coralline Algae (CCA) biomass was affected by the pH levels (PERMANOVA, $p=0.0002$) and by the substratum with different chemistry (PERMANOVA, $p=0.0357$), but not from their interaction. The Pairwise T test on the substrata with different chemical composition showed that only the PVC treatment differed significantly from the other substrata treatments. CCA biomass was higher in Mid pH, decreased in Control site and declined in Low pH (Fig. 21).

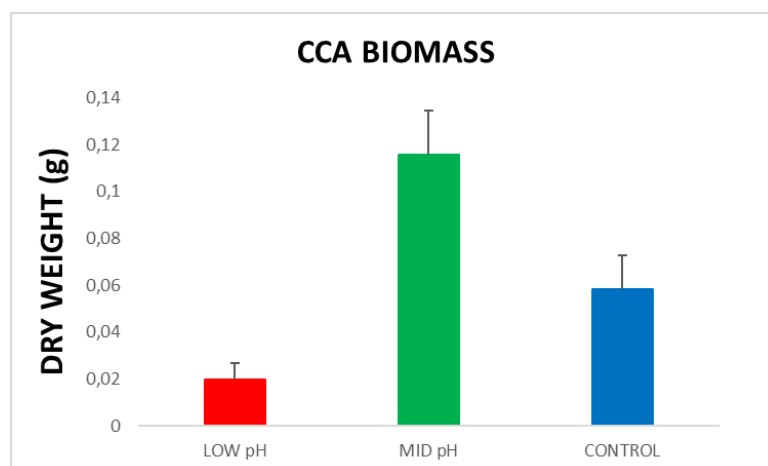


Fig. 21 –Total biomass (mean \pm E.S.; $n=48$) of the late stages of CCA community under three different pH conditions (Low pH, Mid pH and Control).

(d) Natural vs artificial substrata

To have a better understand of the importance of substratum's nature on late stages of algal community succession I examined differences in algal percentage coverage between natural and artificial substrata. The percentage coverage of macroalgae varied significantly as a function of pH levels (PERMANOVA, $p=0.0001$), different nature of substrata (natural vs. artificial) (PERMANOVA, $p=0.0001$) and the interaction between these factors (PERMANOVA, $p=0.0009$) (Table 12). The Pairwise T test showed that differences in the macroalgal percentage coverage between natural and artificial substrata were detected in Low ($p=0.0001$) and Mid pH ($p=0.0001$), but not in the Control site ($p=0.1378$).

Table 12 - Multivariate PERMANOVA analysis on the coverage percentage of late stages of macroalgae recruited on natural and artificial substrates under three pH conditions. The analysis includes two factors: pH (Low pH, Mid pH and Control) and "Su", Substrate (Olivine, Sand, Aragonite and PVC). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
pH	2	82.17	41.085	15.719	<u>0.0001</u>	9959
Su	1	208.95	208.95	79.947	<u>0.0001</u>	9853
pHxSu	2	39.144	19.572	7.4883	<u>0.0009</u>	9945
Res	76	198.64	2.6137			
Total	81	548.3				

The fig. 22 shows a clear distinction between the macroalgal % coverage that is higher in natural than artificial substrata. In addition, while in the artificial substrates the macroalgal coverage increases with increasing pH, on natural substrata the coverage is higher at Mid pH than Low and Control sites.

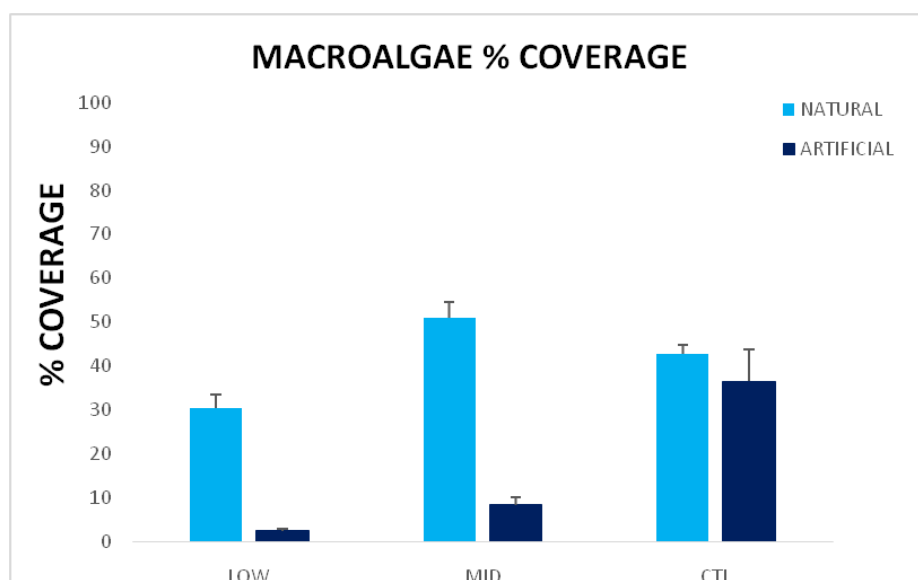


Fig. 22 – Percentage coverage (mean \pm S.E.; $n=82$) of the late stages of macroalgae recruited on natural and artificial substrates under three different pH conditions (Low pH, Mid pH and Control).

The percentage coverage of turf algae recruited along pH gradient on natural and artificial substrata is significantly affected by pH (PERMANOVA, $p=0.0001$) and the different nature of substrata (PERMANOVA, $p=0.0015$) (Table 13). The Pairwise T test showed highly significant differences of % coverage in the turf between low and control ($p=0.0001$) and low and mid pH ($p=0.0001$), but were not detected differences between mid and control ($p=0.2623$). Furthermore, the Pairwise T test on substrates showed a significant difference in % coverage between natural and artificial substrata ($p=0.0013$).

Table 13 - Multivariate PERMANOVA analysis on the coverage percentage of late stages of turf algae recruited on natural and artificial substrates under three pH conditions. The analysis includes two factors: pH (Low pH, Mid pH and Control) and “Su”, Substrate (Olivine, Sand, Aragonite and PVC). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
pH	2	27691	13846	82.257	<u>0.0001</u>	9954
Su	1	1843.1	1843.1	10.95	<u>0.0015</u>	9822
pHxSu	2	389.64	194.82	1.1574	0.3173	9949
Res	76	12792	168.32			
Total	81	43981				

The percentage coverage of turf had a similar trend both in natural and artificial substrates, but was higher in the artificial than natural substrates (Fig. 23).

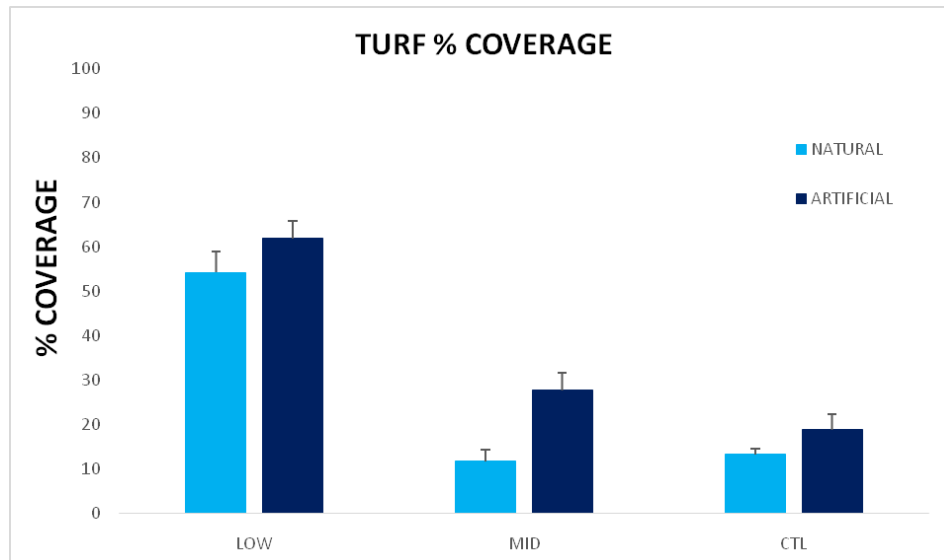


Fig. 23 -Percentage coverage (mean \pm S.E.; $n=82$) of the late stages of turf algae recruited on natural and artificial substrates under three different pH conditions (Low pH, Mid pH and Control).

The percentage coverage of Crustose Coralline Algae varied significantly in function of pH (PERMANOVA, $p=0.0001$) and in their interaction (PERMANOVA, $p=0.0237$) (Table 14). The Pairwise T test showed that the % coverage of CCA varied significantly between natural and artificial substrata in control site ($p=0.0219$), but does not vary in low ($p=0.7354$) and mid pH ($p=0.2429$). Furthermore, the coverage of CCA recruited on natural substrata showed highly significant differences between low and mid ($p=0.0001$) and between low and control sites ($p=0.0001$), whilst the coverage of CCA recruited on artificial substrata varied significantly between low and mid ($p=0.0001$) and mid and control sites ($p=0.0068$).

Table 14 - Multivariate PERMANOVA analysis on the coverage percentage of late stages of CCA recruited on natural and artificial substrates under three pH conditions. The analysis includes two factors: pH (Low pH, Mid pH and Control) and "Su", Substrate (Olivine, Sand, Aragonite and PVC). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
pH	2	95.851	47.925	22.819	<u>0.0001</u>	9949
Su	1	1.7823	1.7823	0.84862	0.3589	9829
pHxSu	2	15.762	7.8809	3.7524	<u>0.0237</u>	9962
Res	76	159.62	2.1002			
Total	81	285.6				

The percentage coverage of CCA resulted be similar in low pH conditions between natural and artificial substrata, instead was lower in mid pH and increased in control site on natural than artificial substrata (Fig. 24).

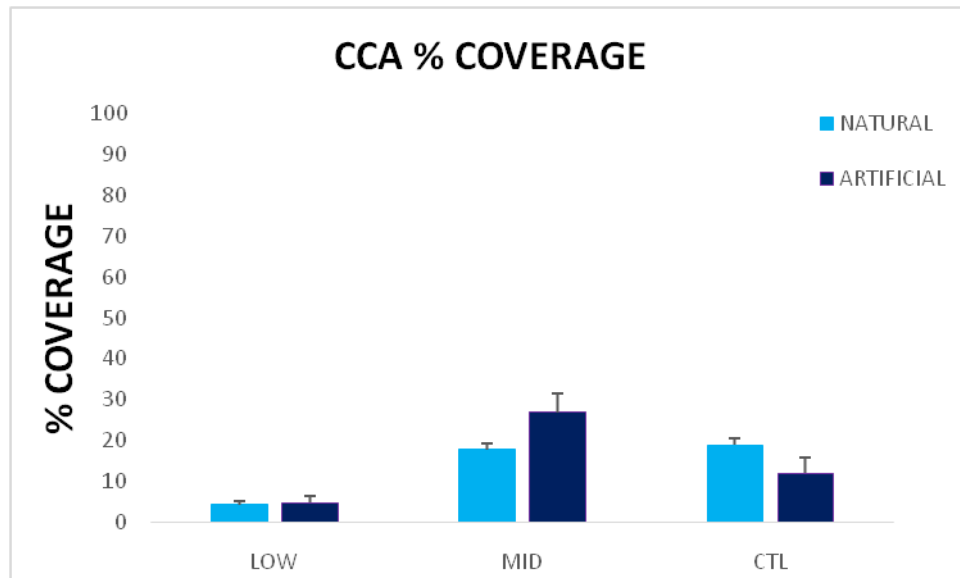


Fig. 24 -Percentage coverage (mean \pm S.E.; n=82) of the late stages of CCA recruited on natural and artificial substrates under three pH conditions (Low pH, Mid pH and Control).

(e) Benthic fauna associated to late stages of algal communities

Thirteen OTUs of benthic fauna were identified: Foraminifera, Gasteropoda, Bivalvia, Serpulida, Cirripedia, Ostracoda, Copepoda, Amphipoda, Tanaidacea, Isopoda, Acarina, Polichaeta and Nematoda. There were clear differences in composition and structure of benthic fauna, associated with late stages of algal community, between the three pH levels. The samples collected at Control and Mid pH sites clearly shows that the composition and structure of benthic fauna community varies slightly under these conditions, obviously keeping a minimum overlap between the two pH conditions. Conversely, the samples collected in the Low site are more spaced apart, thus indicating less similarity in composition and structure in this condition (Fig. 25).

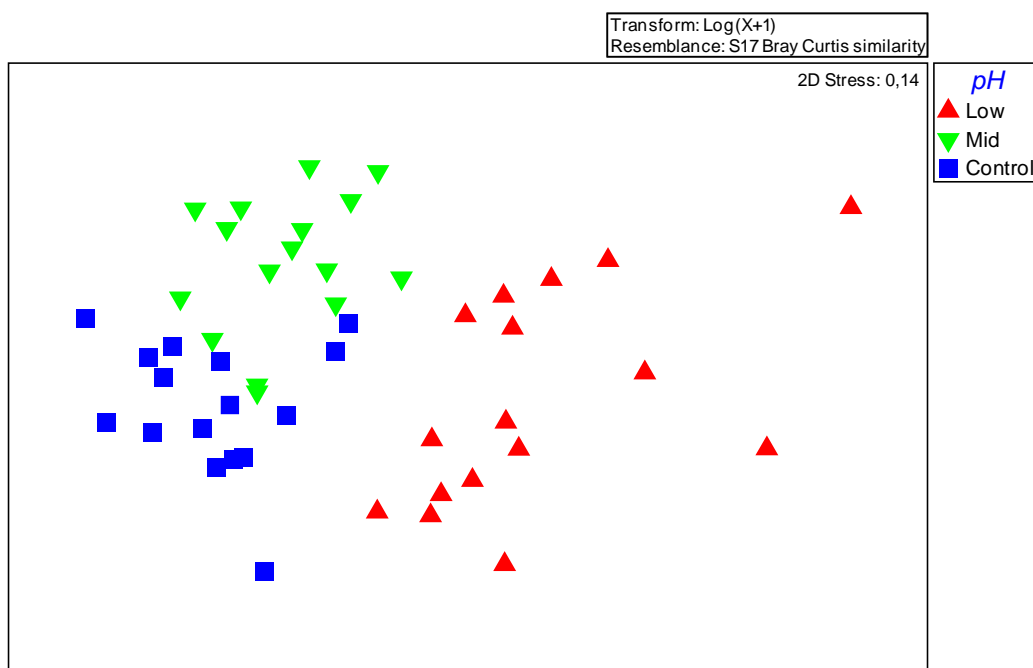


Fig. 25 - nMDS plot of the structure and composition of benthic fauna, associated with late stages of algal community, recruited under three different pH levels (Low pH, Mid pH and Control) on Vulcano Island.

The PERMANOVA analysis showed that the structure and composition of the benthic fauna collected on the experimental plots varies significantly with pH levels (PERMANOVA, $p=0.0001$) (Table 15). The Pairwise T test on the pH factor levels shows highly significant differences between all the pH conditions.

Table 15 - PERMANOVA analysis on the benthic fauna, associated with the late stages of algal community, recruited under three different pH conditions. The analysis includes one factor: pH (Low pH, Mid pH and Control). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
pH	2	8138.7	4069.3	18.111	<u>0.0001</u>	9940
Res	45	10111	224.69			
Total	47	18250				

(f) Number of individuals (N)

The total number of individuals varied considerably in function of the pH factor (PERMANOVA, $p=0.0001$) (Table 16).

Table 16 - PERMANOVA analysis on the total number of individuals (N), associated with late stage of algal community, recruited under three pH conditions. The analysis includes one factor: pH (Low pH, Mid pH and Control). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
pH	2	37.675	18.838	46.224	<u>0.0001</u>	9941
Res	74	30.157	0.40753			
Total	76	67.832				

The Pairwise T test showed that the number of individuals was significantly lower in Low pH site than in Mid ($p=0.0001$) and Control sites ($p=0.0001$), which did not show significant differences between them (Fig. 26).

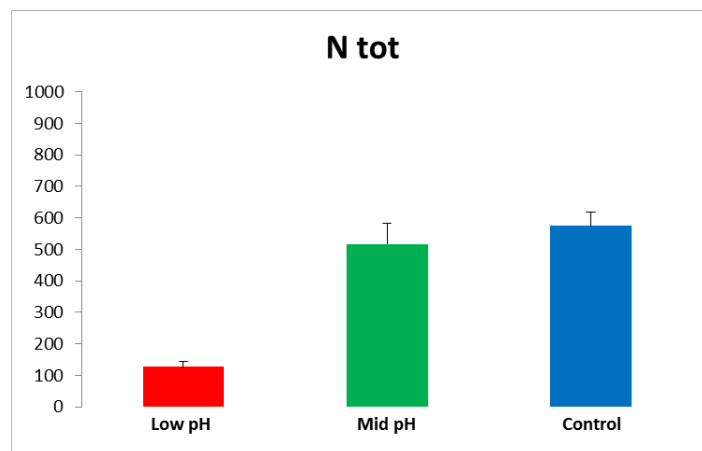


Fig.26 – Total number of individuals (mean \pm S.E.; $n=76$) of benthic fauna, associated with late stage of algal community, recruited under three different pH conditions (Low pH, Mid pH and Control).

(g) Species richness (S)

Similarly, the comparison of species richness of benthic fauna between the pH levels showed significant differences (PERMANOVA, $p=0.0001$) (Table 17) and the recruiting surfaces collected in the Low pH showed a lower number of species than the Mid ($p=0.0003$) and Control sites ($p=0.0001$) which did not vary among them (Fig. 27).

Table 17 - PERMANOVA analysis on the species richness (S) of benthic fauna, associated with late stages of algal community, recruited under three pH conditions. The analysis includes one factor: pH (Low pH, Mid pH and Control). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
pH	2	1.8867	0.94336	17.384	<u>0.0001</u>	9944
Res	74	4.0157	5.4266E-2			
Total	76	5.9024				

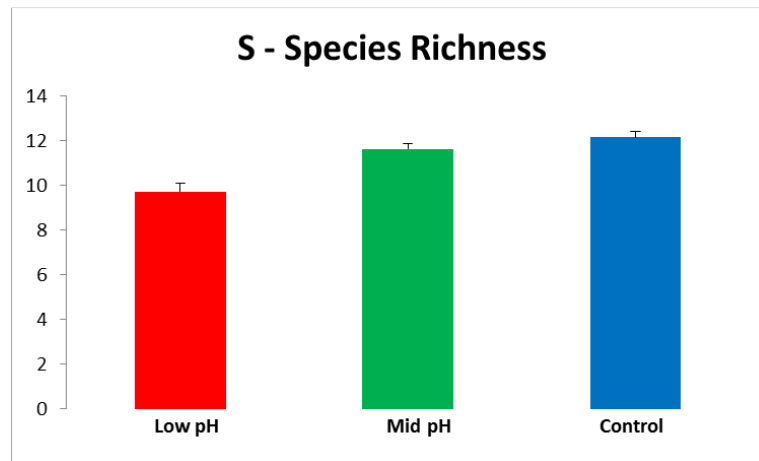


Fig. 27 – Species richness of benthic fauna (mean \pm S.E.; $n=76$), associated with late stages of algal community, under three different pH conditions (Low pH, Mid pH and Control).

(h) Indirect effect of ocean acidification on benthic phytal fauna community

The bi-dimensional graph shows the disposition in the space of benthic phytal fauna community, associated to different algal groups, in function of the different pH conditions. The abundance of different algal taxonomic groups recruited on experimental plots sustains different benthic phytal fauna communities. In addition, the increased macroalgal and CCA biomass on mid and control sites supported a different benthic fauna community than the turf biomass, which increased its biomass under low pH conditions. Consequently, the structure and composition of benthic fauna communities recruited in Mid and Control sites are more similar in structure and composition than communities recruited in the Low pH site (Fig 28).

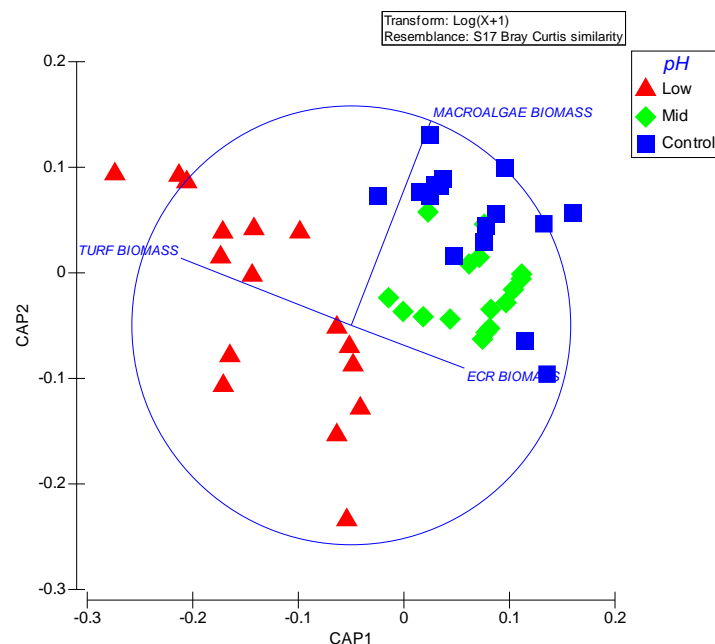


Fig. 28 – Composition and structure of benthic phytal fauna, associated to different algal groups, in function of the different pH conditions (Low pH, Mid pH and Control).

(i) Direct effects of ocean acidification on benthic phytal fauna community

The density of individuals recruited on recruiting substrata varied in function of pH levels for almost all the most abundant taxonomic groups considered. The pH changes were highly significant for the most taxonomic groups, significant for Serpulidae and Tanaidacea and not significant for Amphipoda and Acarina (Fig. 29). The abundance of the almost all taxonomic groups, especially the heavily calcified, decreases in the Low pH site, instead in Mid pH and Control there are mixed responses of abundance.

In detail, the density of gastropods found on recruitment substrata was lower in the Low pH than Mid ($p=0.0001$) and Control ($p=0.0001$) sites, which did not vary between them. Instead, the density of bivalves differed significantly between Low and Mid pH ($p=0.0002$) and between Mid and Control pH ($p=0.0021$), increasing significantly to intermediate pH.

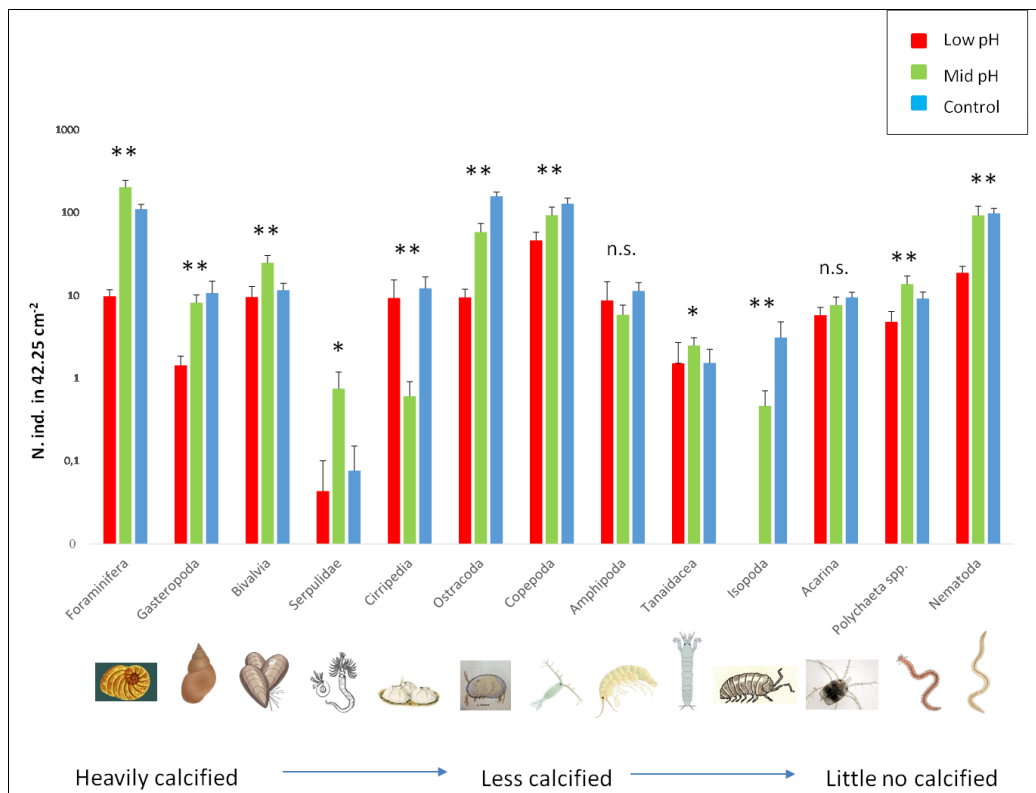


Fig. 29 – Density of the main taxonomic groups of benthic phytoplankton (mean \pm S.E.), associated with the late stages of algal community, under different pH conditions (Low pH, Mid pH and Control). Highly significant, (); significant, (*); not significant, (n.s.).**

3.4 DISCUSSION

This study is one of the first attempts to check the effects of ocean acidification in the long term on the late-stages of the algal succession and associated benthic phytal fauna communities in the intertidal system. After 16 months the assemblages recruited on the experimental tiles were visually indistinguishable from the surrounding benthos and differed significantly among pH zones. Remarkably, algal communities on different artificial substrates shifted in composition and structure exclusively as a result of different pH conditions and not according to the chemical composition of substratum. In fact, my results suggest that the succession evolved into assemblages with a similar composition and structure on the four recruiting substrata used (Olivine, Sand, Aragonite, PVC). The biomass of macroalgae, turf and CCA showed different responses to ocean acidification. The biomass of turf algae increased becoming dominant under low pH conditions, but the ocean acidification caused a reduction in macroalgal biomass which increased sensibly with the rise in pH, instead understory crustose coralline algae showed an increasing in mid pH site and a little decrease in control site. Accordingly to the present findings, many studies demonstrated that turf algae are positively affected (Kroeker *et al.*, 2010; Connell *et al.*, 2013) by ocean acidification and CCA are negatively affected (Gao *et al.*, 1993; Martin and Gattuso, 2009). Instead, the macroalgal canopies responses are contrasting with the other literature evidences that showed the resilience of macroalgae under acidified conditions (Porzio *et al.*, 2011; Kroeker *et al.*, 2012) despite a decrease of calcification rates (Johnson *et al.*, 2012). Turf forms are fast-growing and can be an important competitor for the space in intertidal ecosystems (Connell *et al.*, 2014), overall in disturbed environments. The morphology of filamentous turf allows them to occupy the available space and subsequently inhibit the recruitment of macroalgal canopies (Airoidi, 1998) and CCA (Sebens, 1986), which are considered lower competitors for space by previous studies (Dethier, 1994; Steneck and Dethier, 1994). On the contrary, under normal environmental conditions when the macroalgal canopies were established, the shading effect by the macroalgal canopy species may have inhibited the growth of turf and favored the recruitment of understory CCA (Melville and Connell, 2001).

Contrary to expectations the different substrates, some of which (i.e. olivine) potentially altering carbonates chemistry and alkalinity at small-scale and hence favoring the recruitment of calcifying organisms, did not lead to algal community shifts, and only the PVC treatment showed little significant responses than other substrata. The reason why

weathering of olivine did not create environmental conditions via diffusive boundary layer for the recruitment of CCA may be related to the high seawater flow encountered in field conditions, which may prevent the formation the boundary layer. Therefore it is likely that larger substrate tile may be needed to correctly understand the effects of silicate rocks weathering on marine communities impacted by oceans acidification.

The PVC led to small changing responses of the algal assemblages. Despite some studies suggested the potential importance of type of substrate for the settlement of marine organisms, a few field studies on intertidal rocky shores considered its effects (Crisp, 1974). Both in natural and artificial substrata macroalgae, turf and CCA had similar trend under the different pH conditions: turf algae dominated under acidified conditions and decreased pH, instead macroalgae and CCA had a lower coverage in acidified conditions whilst increased with pH levels. Despite the % coverage of algae recruited on natural and artificial substrata had a similar trend along the natural pH/pCO₂ gradient according with the previous results commented above, the macroalgal coverage was higher on natural than artificial substrata, conversely, the filamentous turf was more higher on artificial than natural substrata. Instead, the coverage of CCA increased in mid and control sites. These differences suggest that the filamentous turf settles faster on the artificial substrata than macroalgae and CCA. Probably, the faster overgrowth of filamentous turf on bare substrata may lead to reduction in the light available for the CCA and a reduction in space for the settlement of macroalgae. Instead in the natural substrata, where the succession went on for a much longer period of time, macroalgae increased their abundance. In support of this, a study conducted by McGuinness (1989) demonstrated differences in number of species settling and/or surviving, the total area occupied and the abundances of some species between natural and artificial substrata, suggesting a community shift leaded by the nature of substratum.

As well as the algal community investigated, the benthic fauna associated to the late stages of algal succession collected on the experimental substrata shifted significantly as a function of pH levels. A reduction in pH clearly influenced the structure and composition of the benthic fauna, which was different from mid pH and control pH assemblages. A similar experiment on natural substrata was conducted by Kroeker *et al.* (2012) where volcanic rocks recruiting on basaltic surfaces were used along a natural pH gradient. According to the present study, the results showed that after 14 months of exposure, the shift in benthic phytal fauna community driven by the pH changes observed in this

experiment followed similar patterns to that of previous studies carried along a natural pH gradients (Cigliano *et al.*, 2010; Kroeker *et al.*, 2011). In addition to this, species richness (S) and the total number of individuals (N) were negatively affected by low pH, with mid and control sites hosting a more abundant and speciose assemblages. Most of the taxa found in ambient pH site were also present in low pH, but the major contribution of benthic community shift under acidified conditions was given by the total absence of Isopoda and a strong reduction of calcifying organisms, such as the gastropods. This last pattern seems clearly connected to direct negative effect of oceans acidification on calcifying organisms (Kroeker *et al.*, 2010). The decrease ocean pH affects the capacity of marine calcified groups to secrete their CaCO_3 structures (Feely *et al.*, 2004; Orr *et al.*, 2005) as the carbonate saturation states lowering may dissolve their shells. According with my results, Kroeker *et al.* (2011) showed that the abundance of gastropods had a great reduction at low pH. Furthermore, Cigliano *et al.* (2010) found a few gastropod species in low pH, but the diversity increased significantly to ambient pH. The density of bivalves exhibited mixed responses to acidification showing a similar abundance in low pH and control sites, and peak of abundance at intermediate pH. The shift in density between gastropods and bivalves under acidified conditions may be due to the composition of the outer shell layer. Probably bivalves were more competitive because their periostracum protected the shell. This protective organic layer is secreted by many marine calcifiers and seems to be an important defense against the oceans acidification effects (Rodolfo-Metalpa *et al.*, 2010a). The trend in abundance of bivalves also suggests that the variability in sensitivity to oceans acidification within this taxonomic group can be mediated by other factors. For example, Melzner *et al.* (2011) assessed that the reduction in calcification and growth in mussels under acidified conditions may be compensated by the increasing in food availability. Furthermore, whereas some taxonomic groups may be able to acclimate to ocean acidification over long time (Form and Riesell, 2011), probably the long duration of my experiment influenced the responses of bivalves. In addition to this, the relative high abundance of bivalves in low pH site indicates perhaps a compensatory biological response to ocean acidification. Density compensation occurs when the decrease in species competition leads to an increasing of abundance of other competitive species. In agreement with my experiment, Kroeker *et al.* (2011) demonstrated compensatory responses of the juveniles of bivalves that were very abundant in extreme low pH conditions off Ischia seeps site. On the contrary, Cigliano *et*

al. (2010) found a highly significant reduction in bivalves' recruitment in acidified conditions in the same site. Another possible explanation of this uncommon response may be the high variability in carbonate chemistry variables in the natural cold vents systems that maybe could have driven higher tolerances organism's at lower pH levels (Kroeker *et al.*, 2011). Isopoda was the only taxon totally absent in low pH site. Probably the response of these crustaceans was indirectly led by the interaction with the algal community.

My results also highlighted that ocean acidification indirectly affected the structure and composition of the associated benthic phytal fauna. In particular, under acidified conditions the dominance of turf-forming forms likely supported a lower recruitment of the benthic phytal fauna community. Conversely, in ambient pH conditions macroalgal canopies, which form more structurally complex systems, and CCA assemblages hosted a greater number of taxa. In other words, I propose that in addition to direct effects on marine invertebrates, ocean acidification led to algal community shifts that in turn sustained different benthic phytal fauna assemblages. This evidence suggests that the indirect effects may be as important as the known direct effects, but further researches are needed to confirm this finding (Sunday *et al.*, 2017).

In summary, the results of this experiment demonstrated that the structure and composition of the latest stages of algal succession shifted in responses to different pH levels, but the different substrates did not exert any influence on the late-stage of the algal community succession. Ocean acidification favored the recruitment of simplified and homogeneous communities (i.e. turf algae), whilst ambient pH conditions supported more complex and structured algal communities. Despite similar trends in algal coverage were detected both in natural and in artificial substrata, the macroalgal coverage was higher in natural substrata while the opportunistic turf species risen their coverage in artificial substrata. This study demonstrates also that OA influenced the benthic fauna communities associated to late stages of algal communities directly, driving shifts in structure and composition of community, richness and density of organisms, and indirectly, acting on shifts in algal communities that favored the recruitment of different benthic phytal fauna communities.

In conclusion, this study provides an example of how complex dynamic of community could shift in a simplified ecosystem under projected future changes in rates of ocean acidification. In particular, this study suggests that the ocean acidification can act via both

direct and indirect way on late stages of algal succession and associated benthic phytoplankton fauna in Mediterranean intertidal rocky shores. These evidences highlighted that the ocean acidification represents a bottleneck for many calcifying organisms, but probably the long exposure to low pH conditions can lead to acclimatization and adaptation of some taxa that were thought to be very vulnerable and susceptible to expected ocean acidification levels.

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CHAPTER 4. DO LOCAL STRESSORS AND TIMING OF DISTURBANCE ALTER SUCCESSIONAL PATTERNS AND RECOVERY TRAJECTORIES IN MEDITERRANEAN VERMETID REEFS?

Abstract: The vermetid gastropod *Dendropoma cristatum* is a reef-building species characterized by low growth, larval dispersal and resilience, and strongly interacting with macroalgae along intertidal rocky shores. Intensity and timing of physical disturbances (i.e. storm waves) may affect such competition strength representing a threat for the whole vermetid reef community and potentially leading to an algal dominated habitat. By manipulating the substrate heterogeneity of the inner edge of reef habitat (i.e., 0%, 50%, 100% removal treatments) at two different times of the year (May vs December), here I assessed the response of the vermetid reef and their associated benthic community over 14 months, in three sites.

The timing of disturbance combined with the disturbance intensity, affected the recovery trajectories of the benthic assemblages associated to vermetid reefs. Furthermore, *D. cristatum* settlement success was dependent upon adult density (i.e. intra-specific interaction) in the short-term, whereas inter-specific interactions with intertidal algae shaped the vermetid reef community in the mid-term. The results of this study suggested that an increase of intensity of extreme meteorological events, such as storms are potentially able to affect the recovery patterns of the vermetid community triggering intra- and inter-specific interactions at a small spatial scale.

Keywords: Patches dynamics – *D. cristatum* – Vermetid reef – Recovery dynamics – Local disturbance – Storm waves.

4.1 INTRODUCTION

Marine ecosystems are affected by large environmental variability and disturbance levels over different spatial and temporal scales (Menge and Olson, 1990; Denny *et al.*, 2009). Ongoing climate change is causing an increase of intensity and frequency of physical disturbances (e.g. extreme storms) on many shallow and coastal ecosystems worldwide (Marcos *et al.*, 2011; Walsh *et al.*, 2016; Oey and Chou, 2016), with documented abnormal waves and related high energy impacting a variety of shallow habitat types,

such as coastal lagoons, mudflats, rocky shores and coral reefs (Costanza *et al.*, 2008; Dodet *et al.*, 2013; Roig-Munar *et al.*, 2017; Vila-Concejo and Kench, 2017). Intertidal ecosystems, in particular, are vulnerable to such types of disturbance as low atmospheric pressure favors the upward expansion of the ocean water, while extreme winds contribute to impact them with high energy waves (Denny *et al.*, 2009), often leading to the removal of biotic communities at local scale (Farrell, 1989). In addition to this, the wave transport of boulders and rocks from the backdrops can concur to create patches, where the substratum becomes locally deprived of biological concretions (i.e., the algae and the sessile invertebrate assemblages).

Severe storms provide an unpredictable source of mortality for benthic organisms and may strongly influence intertidal communities. When an event of disturbance creates a cleared patch many species are able to immediately colonize it if a source of spores and propagules is immediately available (Sousa, 1979). For the majority of marine organisms the choice of the settlement substratum is not a random process, but various factors operating at different spatial and temporal scales may drive this dynamics. For instance, after a disturbance event has occurred, the subsequent space occupation process is dependent upon the interaction between settlers supply (i.e. larval supply from adults and/or the transport of algal propagules), life-history traits of settler organisms (Underwood *et al.*, 1983; Benedetti-Cecchi and Cinelli, 1993; Tapia and Navarrate, 2010), intra- and inter-specific interactions (Benedetti-Cecchi and Cinelli, 1996), substratum heterogeneity (Bers and Wahl, 2004), along with local variations in abiotic conditions (Dayton, 1975; Eckman *et al.*, 1990; Foster *et al.*, 2003).

Indeed, storm waves contribute to shape marine communities particularly in the intertidal zone, promoting novel free space and increased habitat heterogeneity, as well as changing the strength of inter-specific interactions (e.g. leading highly efficient competitors for space to successfully colonize new bare substrates) (Connell *et al.*, 1997) and a novel ecological succession to start (Sousa, 1979b). The clonality and growth plasticity of many macroalgae have important implications for their ability to occupy and spread across a given bare substratum (Yñiguez, 2007). Storm waves impacting the intertidal zone can determine the patterns of space occupation by algal species, as strong physical disturbance levels might promote an increased fragmentation of some macroalgal species, a consequent rise in spatial cover and a competitive displacement potentially leading to community reshuffling (Yñiguez *et al.*, 2015). Overall, the resulting

effects are that composition and structure, ecological succession and recovery patterns of benthic assemblages of disturbed intertidal ecosystems do change (Sousa, 1979b; Farrell, 1989; Benedetti-Cecchi and Cinelli, 1993; 1996).

Seasonal variation in physical disturbance is an important selective force for intertidal benthic habitats. Since biological processes like reproduction, recruitment/settlement and growth of species occur seasonally, the course of the community succession may be affected by the time at which a patch is created (Sousa, 1979b; Turner, 1983). In addition to this, the intensity of storm waves' disturbance may lead to different patch sizes, which in turn may affect species abundance and identity among disturbed patches (Sousa, 1979b; Farrell, 1989).

Indeed, in regions with very low tidal ranges such as the Mediterranean Sea, the physical space represents a key limiting factor for intertidal species. Under optimal conditions of temperature (Chemello *et al.*, 2000), absence of pollution (Di Franco *et al.*, 2011) and suitable characteristics of the geological substratum (Schiaparelli *et al.*, 2003; Chemello, 2009; Chemello and Silenzi, 2011) a particular bioformation called vermetid reef develops along the Mediterranean intertidal zone.

Mediterranean vermetid reefs –a biogenic habitat built by the sessile shelled snails *Dendropoma* spp. and the encrusting red algae *Neogoniolithon brassica-florida* and *Lithophyllum byssoides*– are important ecosystem engineers, which alter the local physical environment (i.e., by affecting hydrodynamism and sedimentation of a given area), modify the shore profile, and generate highly complex biological substrates, that are home to very speciose intertidal benthic communities (Milazzo *et al.*, 2017). *Dendropoma cristatum* (Biondi, 1859), the main reef building species in the Central Mediterranean, is a slow-growing sessile snail with low dispersion ability and population connectivity which make them vulnerable to both environmental and anthropogenic disturbances (Di Franco *et al.*, 2011; Milazzo *et al.*, 2017). *D. cristatum* larvae are brood inside the maternal shells, and the crawling hatchlings are able to settle on the substratum in their close proximity (Calvo *et al.*, 1998), potentially giving the reef-building species an advantage over other intertidal competitors (Barash and Zenziper, 1985), particularly macroalgae (Safriel, 1975). The algal components living on the reef, especially the canopy-forming species, can attenuate wave energy through their structure and mediate the physical impact on the vermetid reef performing an additional hydrodynamic

control. In turn high macroalgal cover prevents vermetid recovery from disturbance (Di Franco *et al.*, 2011; Milazzo *et al.*, 2017).

A few seminal studies assessed the relationship between changes in intensity and timing of storm waves (e.g. Dayton, 1971; Alvarado *et al.*, 2001) and the recovery of benthic assemblages in disturbed and undisturbed areas (Underwood, 1989). However their effects on vulnerable slow-growing reef building species have been assessed only very recently. Vila-Concejo and Kench (2017) demonstrated that storm waves may affect coral reefs both in a destructive and a constructive way. The destructive effects caused the different degree of coral breakage, instead the constructive effects were subsequent transport the coral fragments to form rubble islands.

The aim of the present experiment was to assess whether the intensity (different removal patch size) and the timing of disturbance (May vs December) on portions of the inner edge of the Mediterranean vermetid reefs may affect the recovery patterns of the reef-building species *D. cristatum* and to verify if the availability of bare substrate may trigger competitive displacement between vermetid larvae and intertidal algae, therefore diverting community succession.

More specifically, to understand the responses of pristine vermetid reefs at diverse physical disturbance levels and to assess the direction of community succession and the recovery trajectories, I evaluated the recruitment rates of the reef building species *D. cristatum* and the patterns of response of their associated benthic assemblages by performing two consecutive clearing experiments (i.e. two different timings of disturbance: (1) May: when wave storms are infrequent, vermetid settlement occurs and algal biomass is low; (2) December: when wave storms are more frequent and intense, vermetid settlement does not occur and algal biomass peaks) at three different disturbance levels (0%, 50% and 100% removal). As to produce a consistent disturbance, the clearing technique was chosen to mimic wave effects on vermetid reefs.

4.2 MATERIALS AND METHODS

(a) Study area

This study was carried out off Punta Raisi (NW Sicily), approximately 30 Km west from Palermo. During Summer the area is lowly frequented by fishers and tourists. During the winter the coast is subjected to intense coastal storms and high-energy wave action being exposed to dominant winds from the Northern sectors. Despite the anthropogenic presence and the consistent storms during the Winter, the assemblages living within this area seem not affected in their physical structure, which results to be patchy only in much localized zones. These features make the vermetid reefs of Punta Raisi suitable to perform manipulative experiments, mimicking storm disturbances and hence assessing the responses of benthic assemblages in the long-term.

From a geological point of view, the stratigraphy of the area is constituted by a carbonate platform of the Tyrrhenian age, with quaternary lithological complex underneath and the base formation consisting of limestones and limestones dolomites. On the top of the carbonate platform the vermetid reef takes place developing all along the rocky coast between the lower mesolittoral and the upper infralittoral.

(b) The vermetid reef

The Mediterranean vermetid reefs are the product of the synergistic building activity of the gregarious vermetid gastropods *Dendropoma* spp. (Calvo *et al.*, 2009) and the encrusting red algae *Neogoniolithon brassica-florida*. These gastropod species are well adapted to live in the intertidal zone due to the presence of a thick operculum which hermetically seals the opening of its tubular shell, allowing the animal to survive during the emersion periods at a 6-h tidal interval. Inside the Mediterranean basin, the vermetid reefs distribution is predominantly between average surface temperatures of 14° C during the Winter and 24° C during the Summer.

Four vermetid species have been recently described. The species living in the central Mediterranean (i.e. the Tyrrhenian Sea, previously known under the name *Dendropoma petraeum* (Monterosato, 1892), was recently reclassified as *Dendropoma cristatum* (Biondi, 1859) (Templado *et al.*, 2016). The *D. cristatum* males produce pelagic spermatophores, which are captured in the water column and stored in the mantel cavity by females until gonadic maturity occurring in late Spring (April-May). The species

recruitment periods peaks in Summer during the warmest months (mostly July in NW Sicily) (Calvo *et al.*, 2009; Franzitta *et al.*, 2016).

From the description of the Sicilian reefs and other literature evidence, it is possible to define a typical structural model of the Mediterranean vermetid reefs (Fig. 30) from the coastline towards the open sea (Chemello *et al.*, 2000):

1. A proximal frame, few centimeters thick and considered the upper limit of the vermetid reef, is formed by the two encrusting algae *Neogoniolithon brassica-florida* and *Lithophyllum byssoides*.
2. An inner edge, built by a calcified complex of *D. cristatum* snails and the encrusting red alga *Neogoniolithon brassica-florida* that contributes to the consolidation of the reef cementing the tubular shells of *D. cristatum*. This rim is a few centimeters thick usually ranging from 5 to 50 cm depending on local wave exposure. The inner edge is exposed to high desiccation stress and long emersion periods in the warmer months during low tide.
3. The cuvettes, from 0.5 to up 6 m long and 0.3-1 m deep tidal pools generally colonized by subtidal benthic species.
4. An outer edge, consisting of a 10-60 cm thick encrustation of *D. cristatum* often 30-40 cm wide. The outer edge is reach in crevices and represents the active portion of reef with horizontal and upward growth.
5. An upper infralittoral belt generally made by the brown alga *Cystoseira amentacea* var. *stricta*, located just below the outer margin of the vermetid platform.

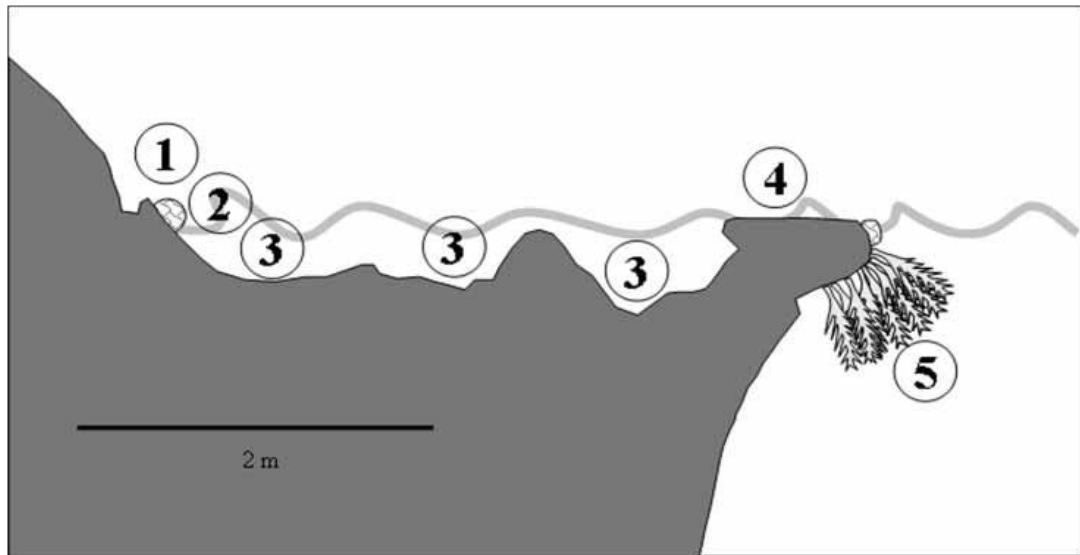


Fig. 30 – A typical structural model of the Mediterranean vermetid reefs from the coastline towards the open sea. 1 – Proximal frame; 2 – Inner edge; 3 – Cuvettes; 4 – Outer edge; 5 – Upper infralittoral belt made by *Cystoseira amentacea* var. *stricta*.

(c) Experiment set-up

This study was conducted along the inner edge of pristine vermetid reefs located off Punta Raisi coast (Palermo, Italy). The two clearing experiments were carried on the inner edges of these reefs with different timings and duration since the disturbance event.

- The first timing experiment lasted 14 months, starting on May 2016 and ending on July 2017. The experimental plots, subjected to different removal treatments, were carried out 1-2 months before the first recruiting event of *D. cristatum* larvae which occurred on July-August 2016. In this period algal cover was minimal as biomass starts to decline due to elevated temperatures and increasing of desiccation rates. Three sites were randomly chosen along the inner edge of vermetid platform: site 1 extends from Lat 38°11.483'N Long 13°7.26'E to Lat 38°11.486'N Long 13°7.238'E, site 2 extends from Lat 38°11.492'N Long 13°7.193'E to Lat 38°11.5'N Long 13°7.173'E, and site 3 extends from Lat 38°11.506'N Long 13°7.146'E to Lat 38°11.496'N Long 13°7.128'E (Fig. 31). For each site, fifteen 10x10 cm squares of vermetid reef were randomly chosen. The plots were positioned on average 12 cm high from the mean sea level and with an orientation North/North-West. To identify the experimental plots for the entire duration of experiment, two stainless steel screws were put in the opposite corners of plots. To simulate the consequences of storm waves on the vermetid

community, three different removal treatments of substratum were carried out. In five replicated plots the vermetid encrustation was totally removed using hammer and chisel, and biofilm and algal propagules were scraped off by steel brushes leaving only the underlying carbonate rock (100% removal treatment). In five plots was removed only half of substratum (50% removal treatment), by creating two different types of recruitment substrata (half vermetid reef and half carbonate rock). Five plots were untreated, representing the control conditions without disturbance (0% removal treatment) (Fig. 32). The settlement of *D. cristatum* and the evolution of community succession associated to vermetid reef on the different experimental plots were then followed for 14 months at average intervals of two months (T0-T6). On such plots a second recruitment event occurred at the end of July 2017, hence 14 months after the disturbance event.

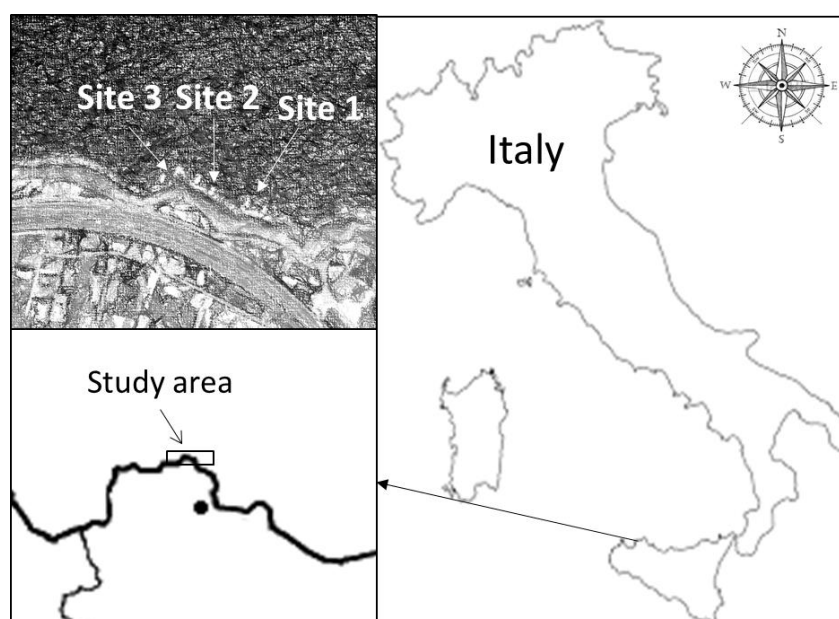


Fig. 31 – Map of the study area, Punta Raisi locality (PA), and position of the sampling sites.

- The second timing experiment, followed an identical experimental set-up of the first timing experiment, and lasted 7 months starting on December 2016 and ending on July 2017. Removal treatments were carried out at site 1 and site 2 and experimental plots were interspersed to prevent potential spatial segregation. The community succession and the settlement of *D. cristatum* larvae were

monitored at average intervals of two months over experimental time (T0-T3). In this case the disturbance occurred 7 months before the recruiting event of *D. cristatum* settlers, and algal biomass peaked three months after the disturbance occurred, during early Spring.

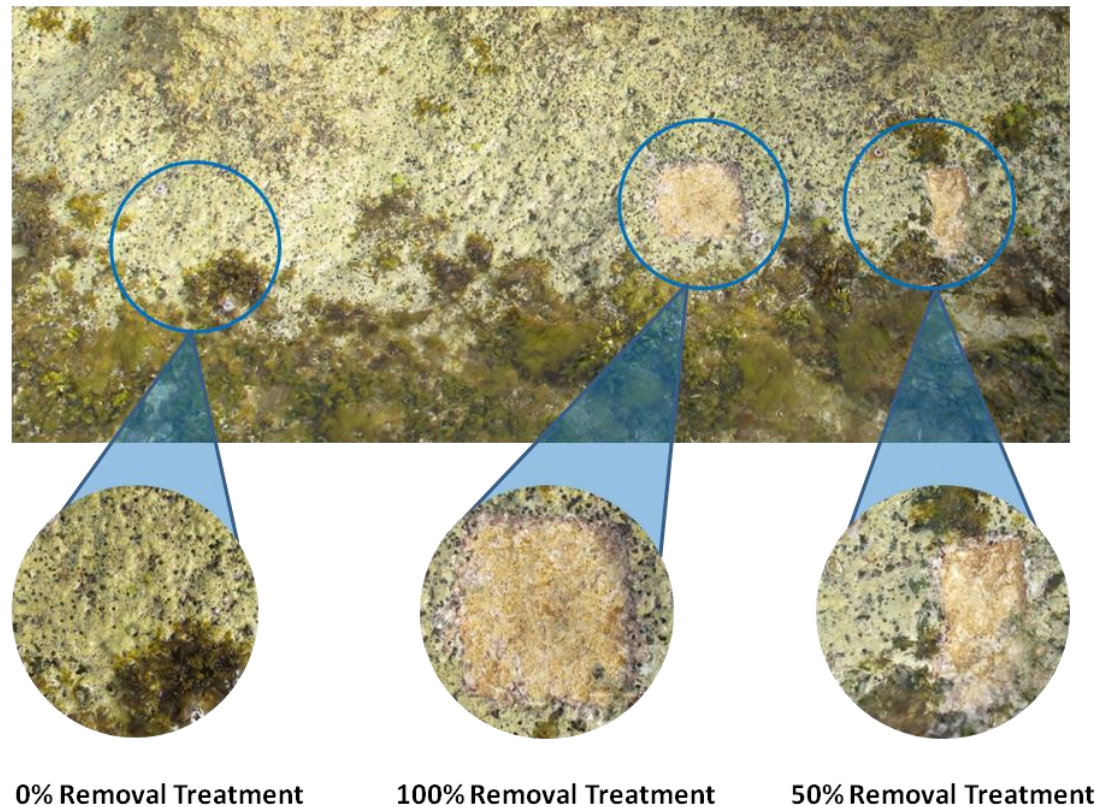


Fig. 32 – Example of 0%, 100% and 50% removal treatments of substrate along the inner edge of vermetid reef.

(d) Sampling techniques

A standard photo-sampling technique was used to assess the recruitment rate of *D. cristatum* settlers (Franzitta *et al.*, 2016), the number of *D. cristatum* adults, the density of herbivores (mostly *Patella* spp.), the coverage percentage (%C) of the vermetids and the main algal species on each experimental plot in both timings. More specifically the experimental 10x10 cm plots were monitored on May 2016 (T0), July 2016 (T1), August 2016 (T2), December 2016 (T3), March 2017 (T4), May 2017 (T5) and July 2017 (T6) for the first experiment and on December 2016 (T0), March 2017 (T1), May 2017 (T2) and July 2017 (T3).

Each replicated photograph was analyzed by the ImageJ open access software. ImageJ is a public domain Java-based digital image processing program, developed by the National Institutes of Health of USA, which provides the possibility of having extensions through small sub-programs "Java plugin". The sub-program ObjectJ allows images to be superimposed with graphical objects that appear as colored and non-destructive markers. The markers were used to separately estimate the number of herbivores and *D. cristatum* settlers and adults on each photograph. Every individual was identified with a colored marker and it was counted by an automatic counter, which returned an output with the total number of counted individuals.

For the calculation of percentage coverage, each photograph was divided into twenty-five squares, and in turn each square was further divided into four square areas for a total of one hundred sections. Each section represents the 1% of total percentage coverage (Fig. 33). If the cover within a square is less than 1%, it is assigned 0.5 (presence). The sum of the partial estimates of the various species provides us the total percentage coverage. The raw data of percentage coverage of each taxon recognized for each sample were reported in an excel matrix.

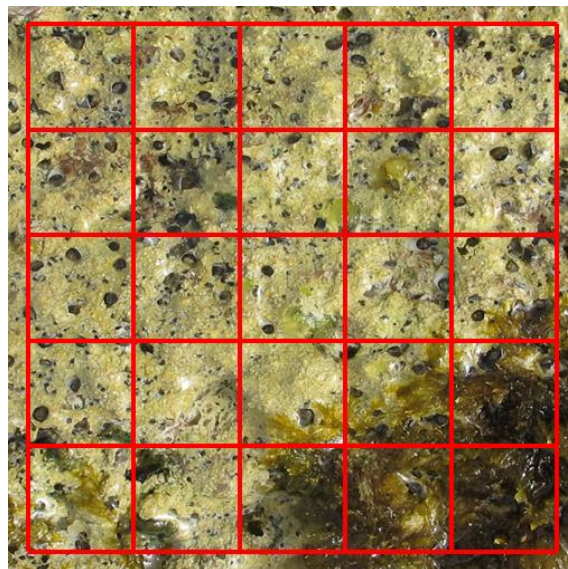


Fig. 33 – Division of the recruitment area for the calculation of benthic assemblage percentage coverage. Every square represents the 4% of the total coverage.

(e) Experimental design and statistical analyses

Linear regression analyses were performed to estimate potential density-dependent effects on the settlement dynamics of the vermetid reef by assessing the relationship between the density of *D. cristatum* adults and their settlers (i.e. crawling larvae) density at 2 and 14 months (for the first timing experimental plots) and at 7 months (for the second timing experimental plots) after their respective disturbance events (i.e. removal treatments) occurred. The simple linear regression analysis is a technique that allows analyze the linear relationship between a dependent variable (or response variable) and one independent variable (or predictor) and is based on the hypothesis of the existence of a cause-effect relationship between the independent and the dependent variable. It is assumed that the independent variable "influences" or "predicts" the dependent variable. The proportion of total variation of the dependent variable explained by the independent variable is given by the determination coefficient R^2 . In my experiment R^2 value was used to measure "how much" of the dependent variable Y, density of *D. cristatum* larvae, is predicted by independent variable X, density of *D. cristatum* adults. The value of R^2 is comprised between 0 and 1, with $R^2=1$ when the trend of Y variable is totally explained by X variable.

General Linear Models (GLMs) analyses were performed to determine whether *D. cristatum* settlement substrata and recruitment success were potentially affected by macroalgal % cover and to assess the potential differences between disturbance timings (14 vs. 7 months) and levels (0%, 50% and 100%). To estimate the total cover of macroalgae, the main algal species found on the experimental plots – *Cladophora* sp., *Rivularia* sp., *Dictyota* sp., *Anadyomene stellata*, *Polysiphonia* sp. – were grouped together.

GLMs are always composed of three components (e.g. Venables and Dichmont, 2004):

- The distribution of the response variables (i.e. the Number of settlers);
- a predictor functions (the specification of the covariates i.e. Timing 1 and 2, Removal treatments);
- the link between the predictor functions and the mean of the distribution.

Being the number of settlers a count, a common distribution used for this kind of data is the Poisson distribution with the *log-link* function. However, when the Poisson-GLM was fitted on the data over-dispersion was detected, therefore in order correct the standard errors, a quasi-Poisson GLM model was instead used. Quasi-Poisson GLM introduces a

dispersion parameter ϕ in the model which by multiplying it with the standard errors allows us to correct the significance of the parameters (Zuur *et al.*, 2009).

In a multivariate context, nMDS and PERMANOVA analyses were performed separately for the first and the second timing experiment to determine potential difference in composition and structure of the benthic community associated to the vermetid reef over time and at different physical disturbance levels.

To graphically evaluate differences in structure and composition of benthic community associated to vermetid reef at different degree of disturbance, after 7, 14 months and in July 2017 three separately nMDS graphs were performed. To verify whether the differences observed graphically in the nMDS were significant, the PERMANOVA analyses were carried out.

A three-way design was considered for the first timing experiment with Time (Ti), fixed with seven levels (T0,T1...T6), Removal treatment (Re), fixed and orthogonal (three levels: 0%, 50%, 100%), and Site (Si), fixed and orthogonal (three levels: site 1, site 2, site 3). In the second time experiment the factor considered were Time (Ti), fixed and orthogonal (four levels: T0, T1, T2, T3), Site (Si), fixed and orthogonal (two levels: site 1, site 2) and Removal treatment (Re), fixed and orthogonal (three levels: 0%, 50%, 100%). In these analyses was necessary to use the dummy variable with value=1 to bypass some anomalous observations given by the 0% removal treatments at Time 0, in which no species were present.

In addition, to compare potential differences in composition and structure of the benthic assemblages associated to the disturbed vermetid reefs after 7 and 14 months (i.e. on July 2017) a multivariate PERMANOVA analysis was performed. The three-way design consisted of the following factors: Timing (Ti), fixed with two levels (First vs Second Timing Experiment), Removal treatment (Re), fixed and orthogonal with three levels (0%, 50%, 100%), and Site (Si), fixed and orthogonal with two levels (site 1 and site 2).

More detailed patterns of %cover of the dominant taxa *Neogoniolithon-Dendropoma* complex, the brown macroalga *Dictyota* sp. and the cianobacteria *Rivularia* sp. and the *Patella* spp. density were evaluated by univariate PERMANOVA analyses both in the first and the second timing experiment, separately. The factors used were, Time (Ti), Removal treatment (Re) and Site (Si).

All PERMANOVA analyses were based on Euclidean distance matrices with 9999 permutations in an univariate context and on Bray-Curtis similarity distance in multivariate context.

After the PERMANOVA, the post hoc Pairwise T test comparison, based on permutations of residuals under a reduced model, was performed.

Such nMDS and PERMANOVAs were performed using the PRIMER 6.1.10 & PERMANOVA β 20 software package.

4.3 RESULTS

(a) Density-dependence responses of *D. cristatum*

Independently from removal treatments considered in this study, the density of *D. cristatum* adults and settlers on each experimental plot were related by simple linear regressions for both disturbance timings. In the first timing experiment, our analysis showed that 3 months after the first timing disturbance the higher the *D. cristatum* density the lower the number of larvae settling on the plots ($R=0.72$; $p<0.001$; $n=45$) during the first recruitment peak (August 2016), hence supporting a density-dependence response (Fig. 34).

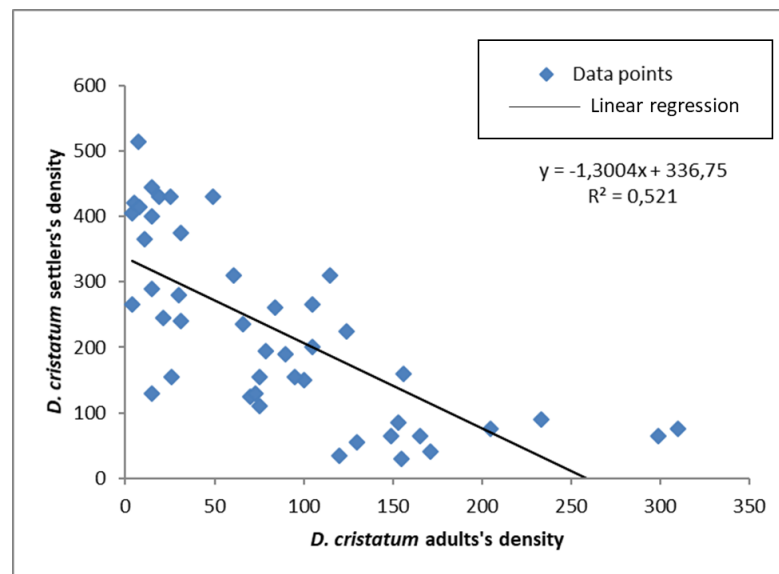


Fig. 34 – Simple linear regression between *D. cristatum* settlers (Y axis) and adults (X axis) density 3 months after the first timing disturbance.

However, such response pattern was sensibly reduced 14 months after the disturbance event, during the second *D. cristatum* recruitment peak (July 2017). Despite showing a slightly negative slope the relation between *D. cristatum* settlers and adults' density was not significant ($R=0.19$; $p=0.2$; $n=45$) (Fig. 35).

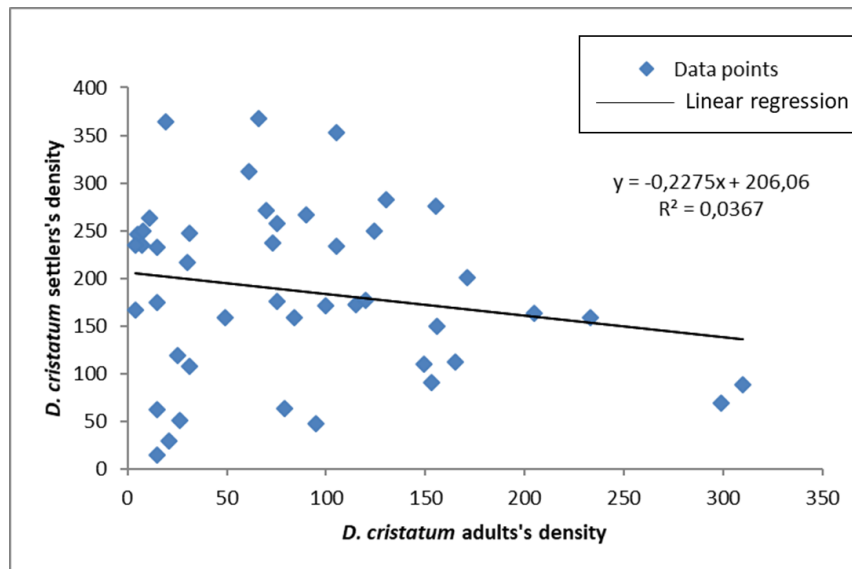


Fig. 35 – Simple linear regression between *D. cristatum* settlers (Y axis) and adults (X axis) density 14 months after the first timing disturbance.

In the second timing experiment 7 months after the winter disturbance event, a highly significant density-dependence response of *D. cristatum* settlers to adults presence was again evident ($R=0.79$; $p<0.001$; $n=30$) during the vermetid recruitment peak (July 2017) (Fig. 36).

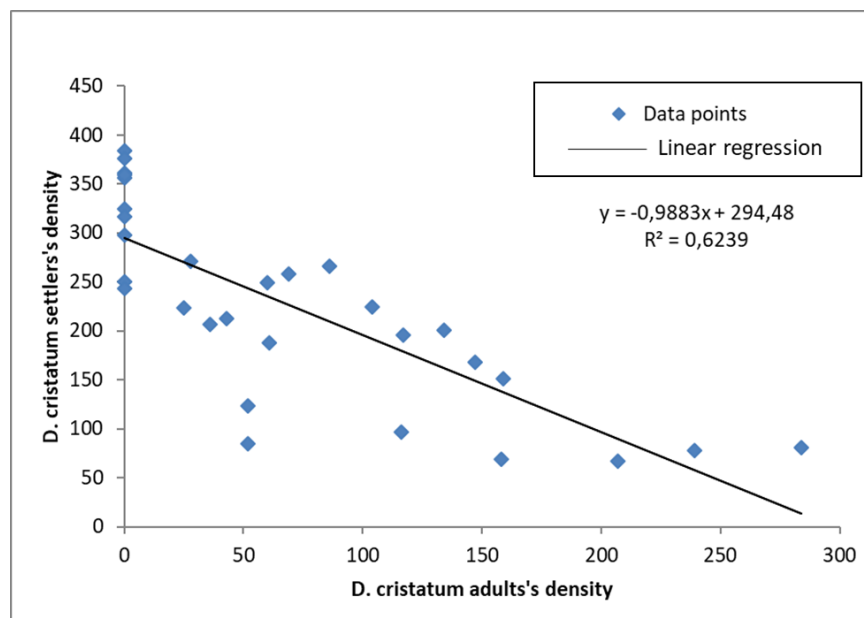


Fig. 36 - Simple linear regression between *D. cristatum* settlers (Y axis) and adults (X axis) density 7 months after the Winter disturbance event.

(b) Temporal variability of the benthic assemblages associated with the vermetid reefs in the two timing experiments

As expected, the benthic taxa that colonized the 0%, 50% and 100% vermetid reef removal treatments changed over time, following species-specific recruitment periods and seasonal patterns. Irrespectively the disturbance timing considered, average macroalgal %C was overall higher in early Spring than the Summer period, likely as a result of the rise in temperature levels and desiccation rates in this latter season. More specifically, the most dominant algal species were *Dictyota* sp. (29.35% vs. 26.55% in Spring and Summer respectively), *Laurencia* sp. (1.04% vs. 0.68%), *Jania rubens* (0.18% vs. 0.26%), *Anadyomene stellata* (1% vs 1.5%). Opposite patterns were observed for Cyanobacteria and turf-forming algae with higher average cover during the Summer than the Spring period. In this case, the most representative taxa were the cyanophicean *Rivularia* sp. (4.31% vs 5.43 in Spring and Summer respectively), and the turf-forming *Cladophora* sp. (0.58% vs 2.09%) and *Polysiphonia* sp. (0.24% vs 0.24%).

According to such findings, both in the first and second timing experiments, I focused the analyses on the temporal patterns over the course of the experiment on the most dominant groups which werethe macroalgae *Dictyota* sp. and the cyanobacteria *Rivularia* sp., along with the *Neogoniolithon-Dendropoma* complex.

The patterns of %cover of the *Neogoniolithon-Dendropoma* complex was site-specific, but similar trends over time were observed in all the sites considered (Fig. 37).

**%cover of the *Neogoniolithon-Dendropoma*
complex**

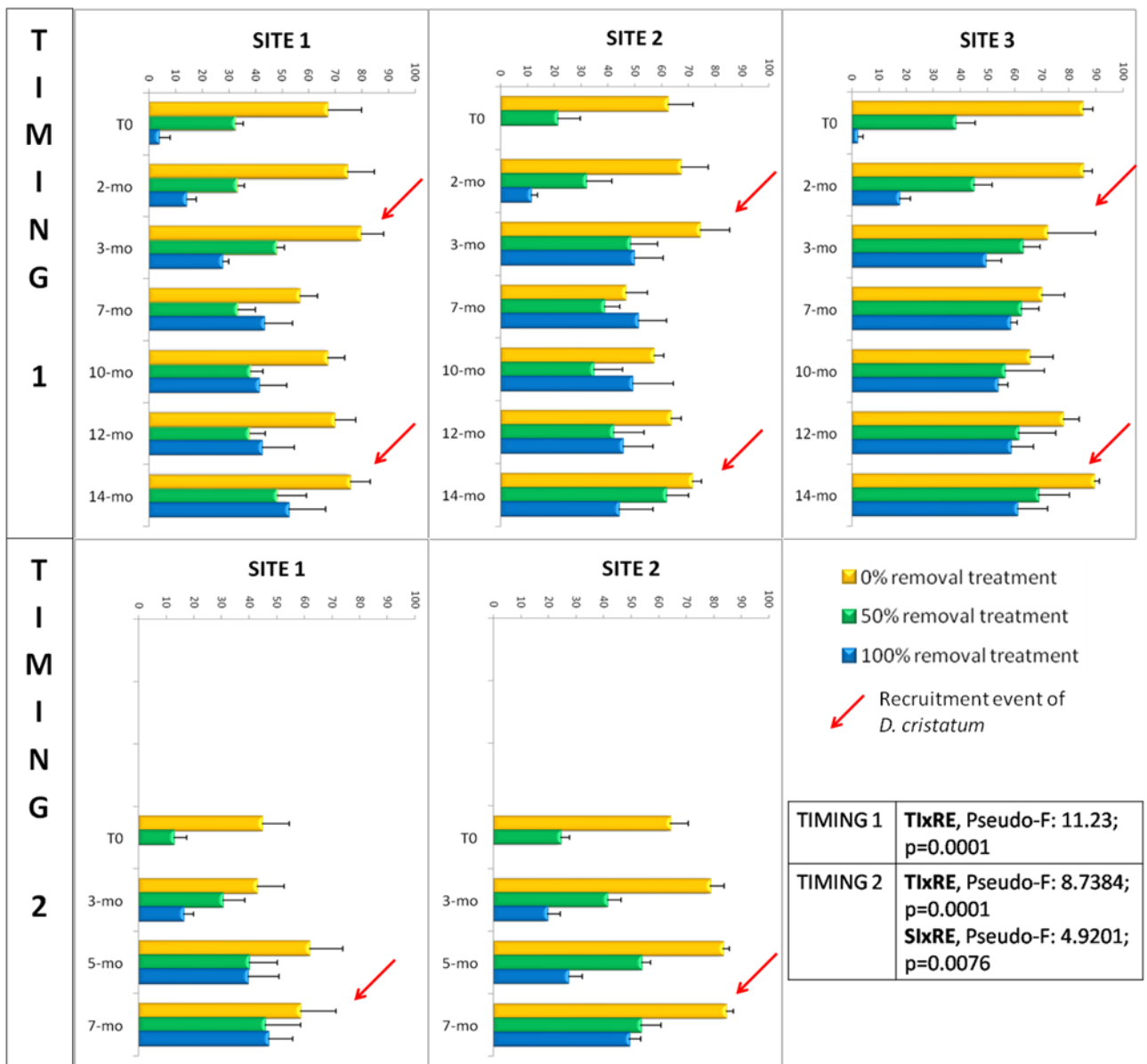


Fig. 37 - T0 represents the time in which the disturbance was carried out (May 2016 for the Timing 1 and December 2016 for the Timing 2). The 0% removal treatment (yellow bars) represents the natural trend of *Neogoniolithon-Dendropoma* complex. The green bars and the blue bars represent the 50% and 100% removal treatments, respectively. The peaks of *D. cristatum* larval recruitment were showed using the red arrows.

Both in the first and the second timing experiment, a seasonal trend of *Dictyota* sp. can be observed (Fig. 38) in the 0% removal treatments, with high % cover during the Spring which dropped during the Summer. When looking at 50% and 100% disturbed plots in the first timing experiment the seasonality of *Dictyota* sp. became less evident over time, with %C values exceeding the observed ‘natural’ trends. Such response however was not confirmed for the second timing experimental plots.

%cover of *Dictyota* sp.

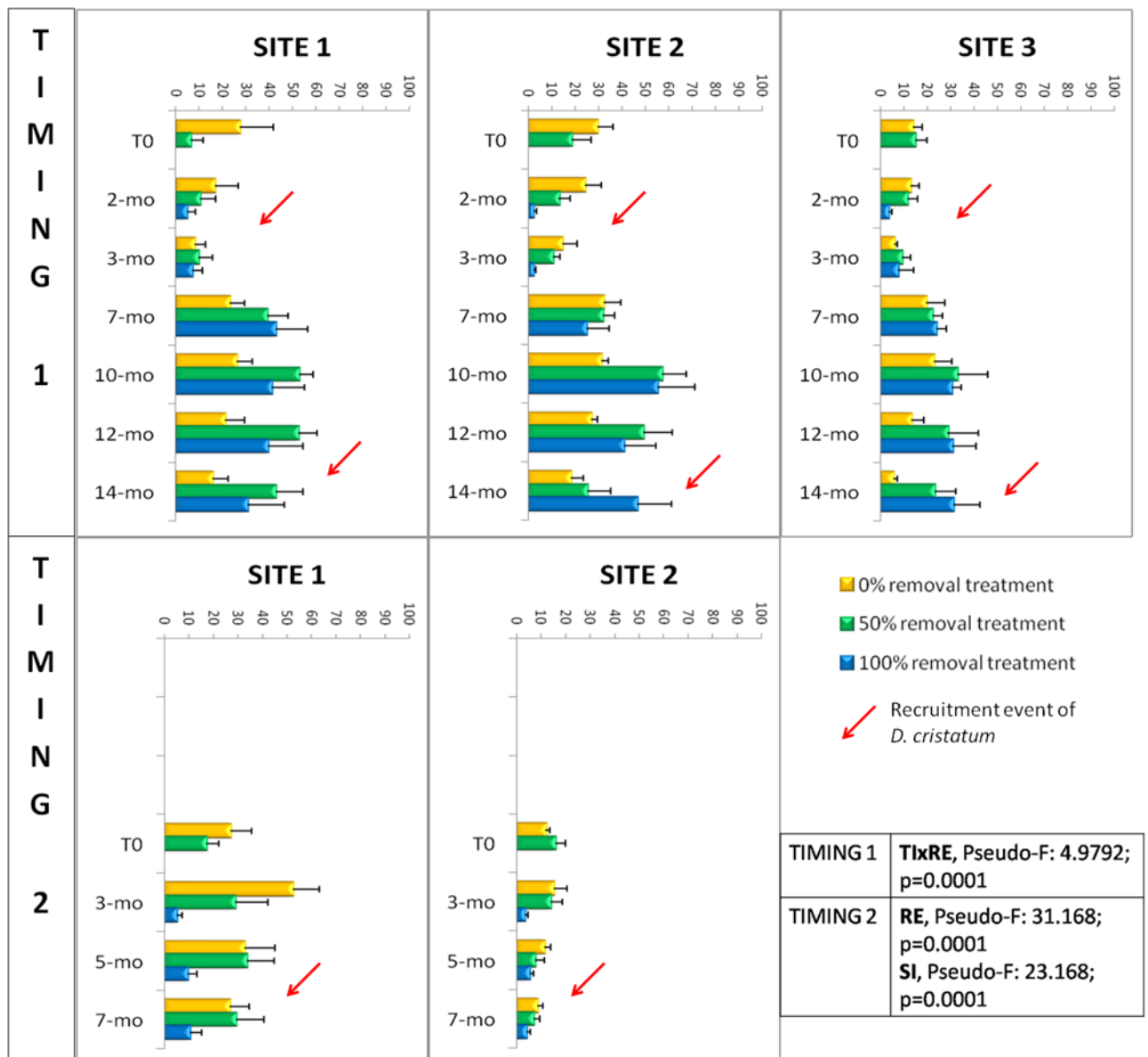


Fig. 38 - T0 represents the time in which the disturbance was carried out (May 2016 for the Timing 1 and December 2016 for the Timing 2). The 0% removal treatment (yellow bars) represents the natural trend of *Dictyota* sp. The green bars and the blue bars represent the 50% and 100% removal treatments, respectively. The peaks of *D. cristatum*' larval recruitment were showed using the red arrows.

The % cover of the colonial cyanobacteria *Rivularia* sp. was sensibly higher during the Summer than the other periods (Fig. 39). In both timing experiments, the temporal patterns of *Rivularia* sp. document an increasing %C in the Summer months (both July 2016 and 2017) particularly in the disturbed plots (50% and 100% removal treatments). This finding might suggest this species can be highly competitive for space occupation when the %C of macroalgae is low.

%cover of *Rivularia* sp.

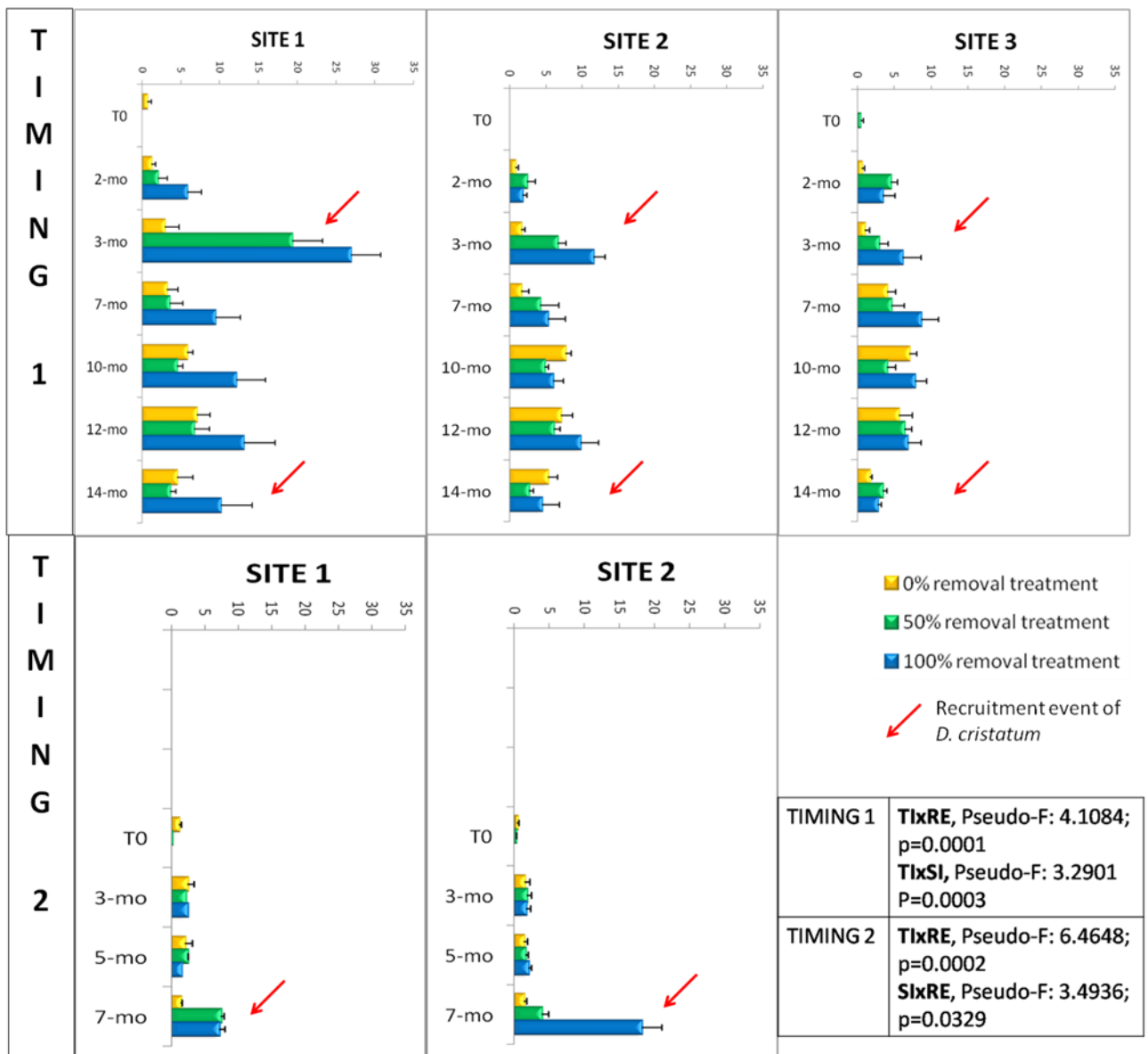


Fig. 39 - T0 represents the time in which the disturbance was carried out (May 2016 for the Timing 1 and December 2016 for the Timing 2). The 0% removal treatment (yellow bars) represents the natural trend of *Rivularia* sp. The green bars and the blue bars represent the 50% and 100% removal treatments, respectively. The peaks of *D. cristatum* larval recruitment were showed using the red arrows.

The density of *Patella* spp. was independent from the removal treatments both in the first and second timing experiment, but followed a temporal trend that might suggest a recruitment event occurring between December and March 2017, which in turn was not detected before (Fig. 40). Therefore, starting from the recruitment event of *Patella* spp., the benthic assemblages associated with vermetid reefs were exposed to a greater grazing pressure.

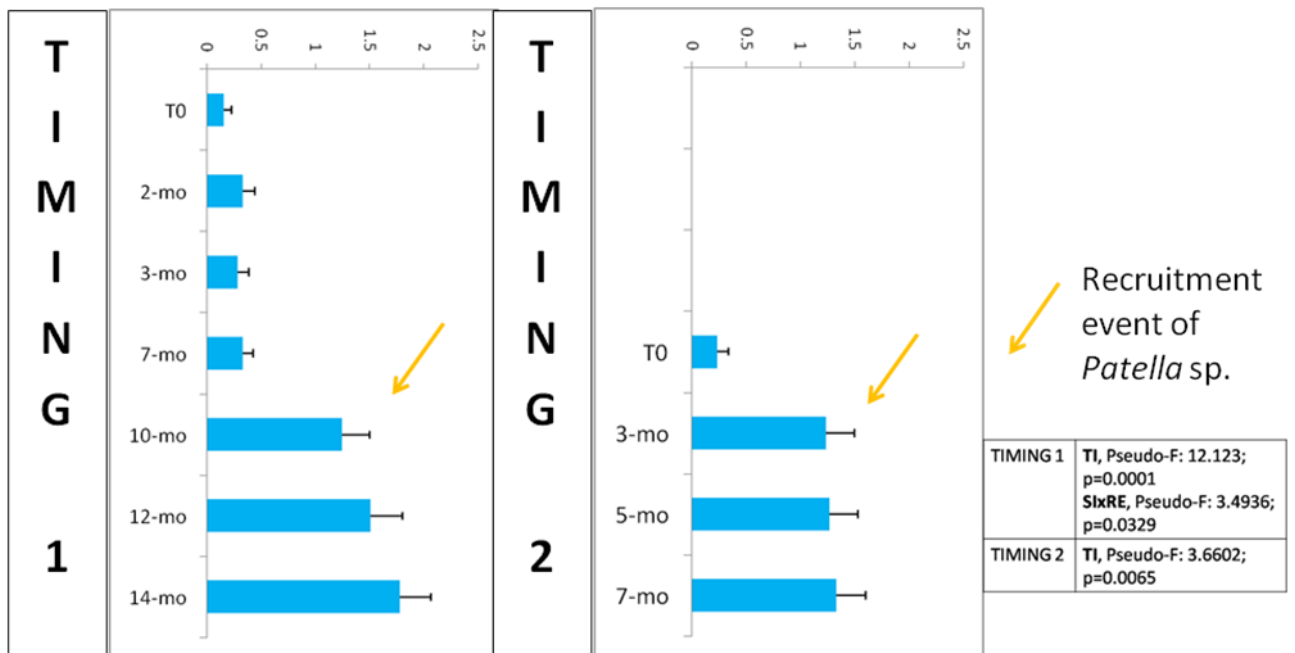


Fig. 40 – Density of *Patella* sp. in 100 cm². T0 represents the time in which the disturbance was carried out (May 2016 for the Timing 1 and December 2016 for the Timing 2). The orange arrows represent the recruitment event of *Patella* sp.

(c) Is *Dendropoma cristatum* recruitment success affected by competition with intertidal macroalgae?

GLM analysis revealed that settlers' density significantly decreased with the increasing of percentage coverage of algae in all the removal treatments considered, both in the first and in the second timing experiment. However, the number of settlers found on the 0%, 50% and 100% removal treatments significantly varied between timing (1) and (2) (Fig. 41 a,b,c,; Table 18).

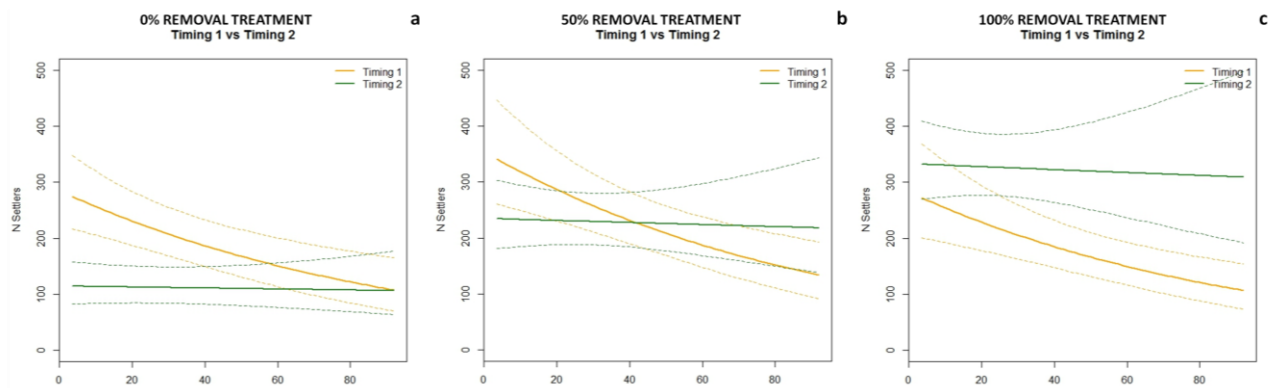


Fig. 41 – The X-axis represents the number of *D. cristatum* settlers. The Y-axis represents the %C of algae. The trend of *D. cristatum* in function of C% of algae is showed for the Timing 1 (orange line) and for the Timing 2 (green line) in 0% (a), 50% (b) and 100% (c) removal treatments.

Table 18 – Results of the Quasi Poisson Generalized Linear Model (GLM). Re= removal treatments; 0%= 0% removal treatment; 50%= 50% removal treatment; 100%= 100% removal treatment; C%= cover percentage of macroalgae. * = significant; * = highly significant.**

	Estimate	Std. Error	t value	Pr(> t)
Intercept (Re 0%, Timing 1)	5.651793	0.123408	45.798	<u>< 2e-16 ***</u>
C% MACROALGAE	-0.010602	0.002796	-3.791	<u>0.000391 ***</u>
Re 50%	0.217258	0.145798	1.490	0.142232
Re 100%	-0.008280	0.158536	-0.052	0.958547
C% MACROALGAE:Timing 2	0.009786	0.004276	2.289	<u>0.026188 *</u>
Timing 2	-0.911252	0.207676	-4.388	<u>5.62e-05 ***</u>
Timing 2:Re 50%	0.501691	0.227180	2.208	<u>0.031653 *</u>
Timing 2:Re 100%	1.075844	0.229446	4.689	<u>2.02e-05 ***</u>

The number of *D. cristatum* settlers was significantly higher in the Timing 1 than the Timing 2 experiment on the 0% removal treatments (Pairwise R comparison; $p = 0.01129$), did not vary in the 50% removal treatments (Pairwise R comparison; $p = 0.9879$) and was significantly lower in the 100% removal treatments (Pairwise R comparison; $p = 0.0113$) (Fig. 42).

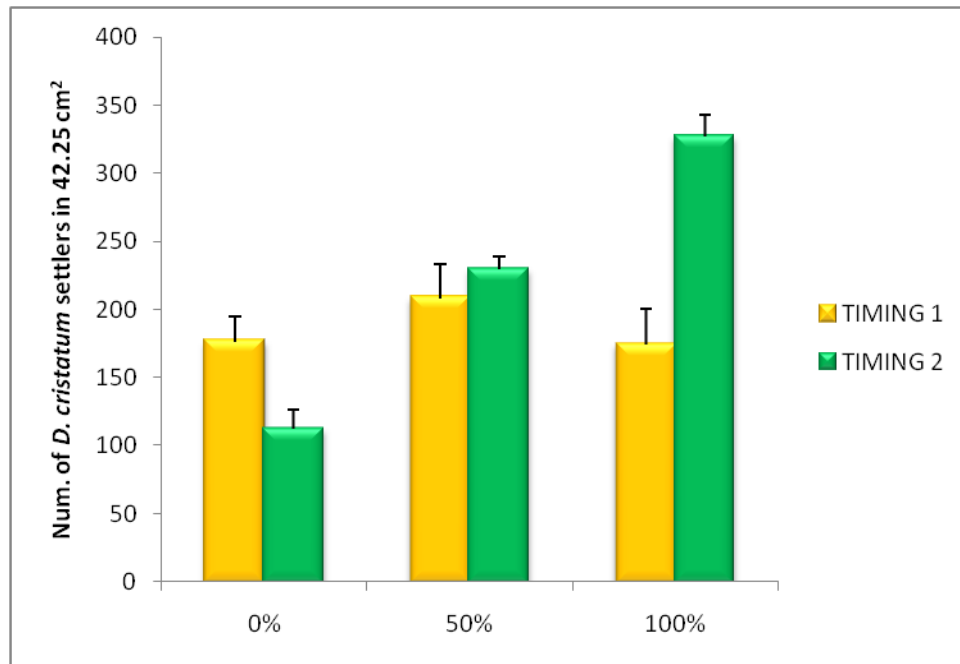


Fig. 42 - *D. cristatum* settlers' density as a function of 0%, 50% and 100% removal treatments, 7 (green bars) and 14 (yellow bars) months after the two disturbance events (i.e. the two timings).

In the first timing experiment (i.e., 14 months after the disturbance) the number of settlers did not significantly vary among the removal treatments (Fig. 41 a,b,c; Table 18). On the contrary, in the second timing experiment (7 months after the disturbance) the density of *D. cristatum* settlers significantly increased with intensity of removal treatments (i.e., from 0% to the 100% removal) (Fig. 41 a,b,c; Table 18). The GLM analysis also showed that *D. cristatum* settlers' density varied significantly between timing 1 (after 14 months from disturbance) and timing 2 (after 7 months from disturbance) in all the removal treatments considered, independently from macroalgal %cover (Fig. 41 a; Table 18).

(d) Recovery trajectories of the benthic assemblages associated with vermetid reefs: comparing responses after two timing of disturbance

In the first timing experiment the composition and structure of the benthic assemblages associated with vermetid reefs did change over time. As expected, when undisturbed (i.e., the 0% removal treatment) the benthic assemblages evolved in a similar way among different times (Fig.43). Instead in presence of a disturbance the composition and structure of the benthic assemblages significantly changed over time. At T0 (May 2016),

2-month (2-mo; July 2016) and 3-month (3-mo; August 2016) from the disturbance event, the composition and structure of the benthic assemblages on the 50% and 100% removal treatments were spatially separated each other as showed in the two-dimensional nMDS plot (Fig. 43), which interestingly also documented that from 7-month (7-mo; December 2016) on (i.e. 10-mo, 12-mo and 14-mo) the benthic assemblages associated to vermetid reefs became similar among the different removal treatments (0%, 50% and 100%).

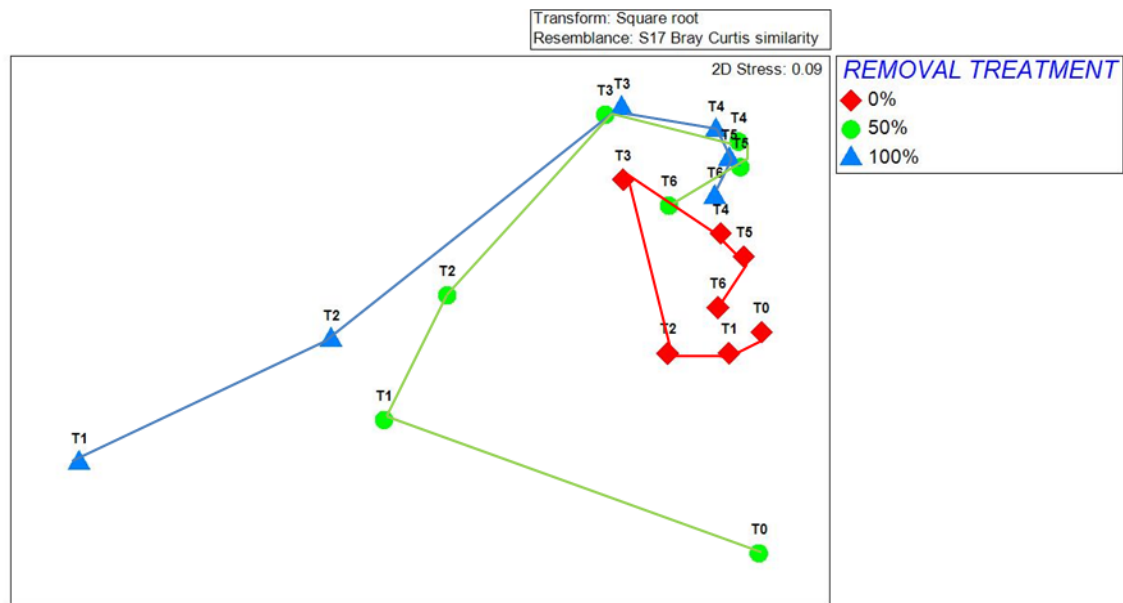


Fig. 43 -nMDS plot of recovery trajectories of benthic assemblages, associated with vermetid reef, over 14 months (first timing experiment). Each point on the nMDS plot represents the average community composition of the 0% (red line), 50% (green line) and 100% (blue line) removal treatments at that time. T0 represents the start of the experiment (May 2016). In the figure, the point T0 of 100% removal treatments is not showed because an outlier, having been deprived of all species.

The PERMANOVA analysis supported such observed responses as benthic assemblages significantly differed in the combination of TimexRemoval factors (PERMANOVA, Pseudo- $F_{12,252}=13.929$; $p=0.0001$), despite a site-dependent response was also documented (Table 19). Consistently, the Pairwise T tests on the significant TimexRemoval interaction variation over time occurred mostly from T0 (May 2016) to 3-mo (August 2016) in 0%, 50% and 100% removal treatments, mean while such differences between experimental treatments were less evident later in the succession trajectories, supporting a resilience interval of 7 month after the first timing.

Table 19 - Multivariate PERMANOVA analysis on the recovery trajectories of benthic assemblages, associated with vermetid reefs, over 14 months (first timing experiment). The analysis includes three factors: "TI", Time (T0, T1, T2,...T6), "RE", Removal treatments (0%, 50%, 100%) and "SI", Site (Site 1, Site 2, Site 3). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Perms
TI	6	96305	16051	39.812	<u>0.0001</u>	9901
RE	2	29824	14912	36.987	<u>0.0001</u>	9934
SI	2	7780.8	3890.4	9.6496	<u>0.0001</u>	9958
TIxRE	12	67390	5615.9	13.929	<u>0.0001</u>	9858
TIxSI	12	4803.3	400.27	0.99283	0.4793	9895
SIxRE	4	4031.8	1007.9	2.5001	<u>0.0058</u>	9934
TIxSIxRE	24	3572.3	148.85	0.36919	1	9846
Res	252	1.016E5	403.16			
Total	314	3.153E5				

In the second timing experiment, the composition and structure of the benthic assemblages associated with vermetid reefs did change over time among the 0%, 50% and 100% removal treatments (Fig. 44), varying more in the 100% removal treatments than the 0% and 50% removal treatments.

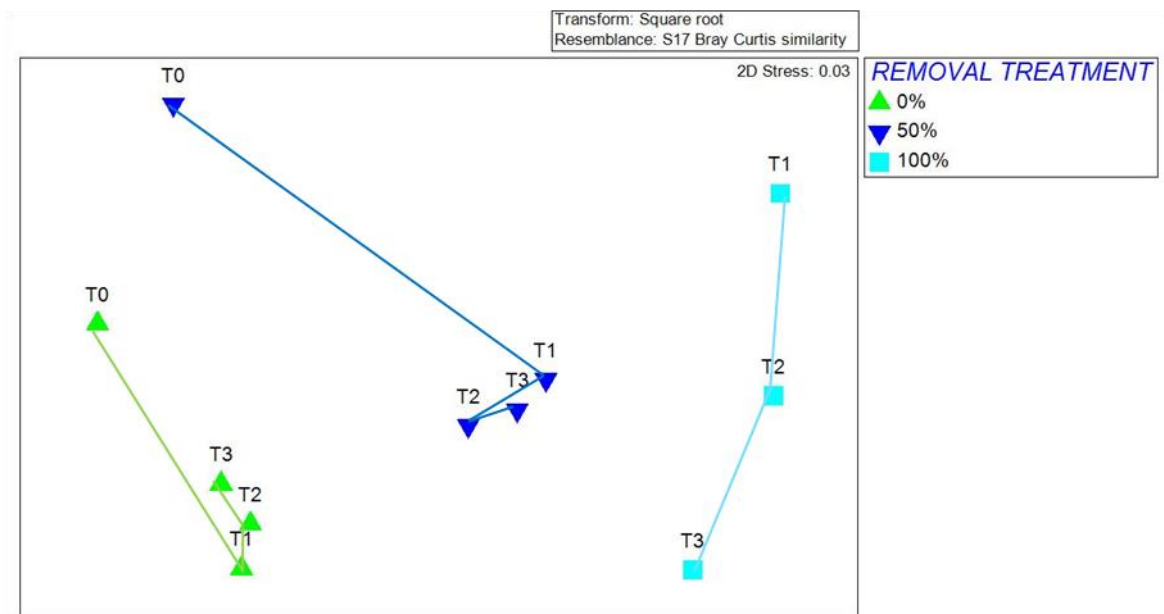


Fig. 44 - nMDS plot of recovery trajectories of benthic assemblages, associated with vermetid reef, over 7 months (second timing experiment). Each point on the nMDS plot represents the average community composition of the 0% (green line), 50% (blue line) and 100% (azure line) removal treatments at that time. T0 represents the start of the experiment (December 2016). In the figure, the T0 of 100% removal treatments is not showed because an outlier, having been deprived of all species.

Consistently to the Timing 1, the three-way PERMANOVA documented that the benthic assemblages significantly differed in the combination of TimexRemoval factors (PERMANOVA, Pseudo-F_{6,119}=18.195; $p=0.0001$), a showed a site-dependent response

(Table 20). Pairwise T tests showed that the composition and structure of benthic assemblages recruiting on the different removal treatments varied over time, significantly differing from T0 (December 2016) to 5-mo (5-month; May 2017) in all the removal treatments considered, while at 7-mo (July 2017) only 0% vs. 100%, and 0% vs. 50% differences were detected, with 50% and 100% removal treatments not differing each other. Such findings support that after the winter disturbance (timing 2 experiment) the full recovery of the benthic assemblages did not occur after 7 months, as observed for the spring disturbance event (timing 1 experiment).

Table 20 - Multivariate PERMANOVA analysis on the benthic assemblages associated with vermetid reefs over 7 months (second timing experiment). The analysis includes three factors: "TI", Time (T0, T1, T2, T3), "RE", Removal treatments (0%, 50%, 100%) and "SI", Site (Site 1, Site 2). The significant values are underlined.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms
TI	3	30105	10035	29.202	<u>0.0001</u>	9925
RE	2	30756	15378	44.751	<u>0.0001</u>	9936
SI	1	4748.3	4748.3	13.818	<u>0.0001</u>	9950
TIxRE	6	37516	6252.7	18.195	<u>0.0001</u>	9903
TIxSI	3	2136.3	712.11	2.0722	<u>0.0268</u>	9936
SIxRE	2	2479.8	1239.9	3.6081	<u>0.001</u>	9945
TIxSIxRE	6	1678.4	279.73	0.81402	0.6874	9920
Res	96	32989	343.64			
Total	119	1.4241E5				

Additional multivariate analyses were performed to compare composition and structure of the benthic assemblages associated with the vermetid reefs on July 2017, 7 months and 14 months after the two timings disturbance events, respectively. The nMDS plot showed that in the 0% removal treatments composition and structure of the different experimental plots did not vary between timings, while different disturbance timings led to divergent successions of the benthic assemblages, as the 50% and 100% removal treatments were spatially separated between them both after 7 (timing 2) and 14 (timing 1) months (Fig. 45).

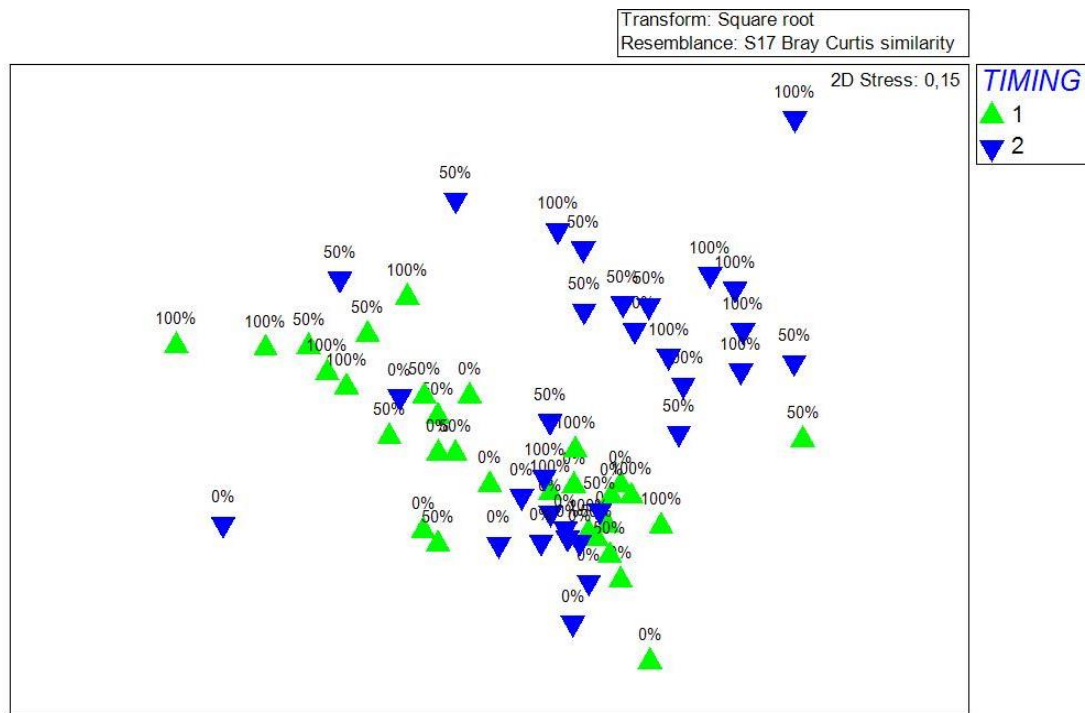


Fig. 45 - nMDS plot on the comparison of the composition and structure of benthic assemblages, associated with vermetid reef, 7 (timing2 experiment) and 14 months (timing 1 experiment) after the disturbance event (July 2017). The labels 0%, 50% and 100% represent, respectively, the 0%, 50% and 100% removal treatments of substrate.

More specifically the interaction Timing \times Removal was significant (PERMANOVA, Pseudo- $F_{2,60}=3.544$; $p=0.0044$) and the related Pairwise T tests confirmed that benthic assemblages differed in the 50% removal treatments ($p=0.0184$) and in the 100% removal treatments ($p=0.0002$) after 7 and 14 months from disturbance (Table 21); whilst no differences were detected in the 0% removal treatments ($p=0.1767$). Interestingly, after 14 months from the first disturbance event the benthic assemblages recruiting on the 0%, 50% and 100% removal treatments did vary each other.

Table 21 - Multivariate PERMANOVA analysis on the benthic assemblages, associated with vermetid reef, 7 (second timing experiment) and 14 months (first timing experiment) after the disturbance event (July 2017). The analysis includes three factors: "TI", Timing (Timing 1 vs Timing 2), "RE", Removal treatments (0%, 50%, 100%) and "SI", Site (Site 1, Site 2). The significant values are underlined.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
TI	1	4019.2	4019.2	9.788	<u>0.0001</u>	9955
RE	2	3836.1	1918.1	4.6711	<u>0.0003</u>	9956
SI	1	944.17	944.17	2.2994	0.0796	9942
TI \times RE	2	2910.5	1455.3	3.5441	<u>0.0044</u>	9955
TI \times SI	1	982.08	982.08	2.3917	0.0732	9954
SI \times RE	2	818.94	409.47	0.9972	0.4193	9952
TI \times SI \times RE	2	747.84	373.92	0.91062	0.488	9950
Res	49	20121	410.62			
Total	60	34419				

4.4 DISCUSSION

The present manipulative experiment showed for the first time that both the intensity and the timing of physical disturbance may affect the recovery trajectories and hence the successional patterns of pristine vermetid reef communities at least at a local scale. In addition to this, this study documented that the recruitment patterns of the reef building species *D. cristatum* can be influenced both by the density of the vermetid adults and the % cover of the macroalgae recruiting on the experimental plots, therefore suggesting changes in the strength of intra- and interspecific interactions might occur after a physical disturbance event, mimicking storm waves, takes place.

In the first timing experiment, a strong intra-specific interaction between settlers and adults of *D. cristatum* was detected in the inner edge of the vermetid reef just three months after the disturbance, when *D. cristatum* recruitment peaked (August 2016) and the biomass of associated algae was low due to rising of Summer temperatures and increasing desiccation rates. When the same timing experiment was followed up to 14 months (on July 2017; i.e. the second recruitment event) such density-dependent responses were not evident anymore, as recruitment rates did not vary as a function of vermetid adults' density. Therefore, the higher is the availability of bare substrate, the higher is the ability of the vermetids larvae to colonize it. Such kind of response was also detected in the second timing experiment, suggesting that when a physical disturbance occurs with a low frequency and intensity, as in the present case, the recovery dynamics of the reef building species seems to be rather quick. This is indeed an intriguing finding as it has been suggested that such very peculiar settlement characteristics of the Mediterranean *Dendropoma* species, along with their low larval dispersal and population connectivity, make unlikely the recovery of vermetid reefs after a local mortality event occurs.

Historically, large vermetid reefs demises have been linked to natural disturbances and shifts in environmental conditions. Hurricanes and sharp changes in local conditions recorded in the last century have been linked the persistence of some vermetid reefs off the Florida coast (Shier, 1969). Similarly, in the West Indies the occurrence of dead vermetid reefs in low energy coastal environments was related to temperature changes and decreasing exposure of the coast occurred at the end of the Little Ice Age (1625-1740) (Jones and Hunter, 1995). Due to their peculiar developmental biology, Mediterranean *Dendropoma* species are considered at high risk under ongoing

environmental change (Annexes of the Berna Convention, Annex II of the Protocol for Specially Protected Areas in the Mediterranean (SPAMI Protocol of the Barcelona Convention) and Mediterranean Red Data Book). *Dendropoma cristatum* larvae metamorphose and hatch inside the mantle cavity of the female individuals (Calvo *et al.*, 1998). After hatching, crawling juveniles usually settle on a suitable substratum very close to the maternal shell (i.e. within a few meters) and in the short-term (i.e. within a few hours). Once selecting the suitable settlement substrate, they scrape it by the radula to remove biofilm and immediately calcify a flat base to firmly attach and start a sessile life. In the last decades, an increasing number of studies are documenting local extirpations and detrimental responses of Mediterranean *Dendropoma* reef building species as a consequence of coastal habitat degradation, increasing pollution, the spread of invasive species and projected ocean acidification levels (Di Franco *et al.*, 2011; Milazzo *et al.*, 2014; Milazzo *et al.*, 2017). Importantly, large vermetid reefs die off were documented in the Eastern Mediterranean, particularly along the Israeli coast (Galil, 2013, as *Dendropoma petraeum*). My experiment suggests that the ability of *D. cristatum* larvae to colonize new bare substrates is indeed important for the local persistence of this reef-building species after a disturbance event occurs. However at the same time the present findings do support the hypothesis that whatever the spatio-temporal patterns of disturbance, interspecific interactions encompassing recruiting macroalgae might play a role in the successful *D. cristatum* recruitment dynamics and more importantly in the recovery trajectories of the entire vermetid reef community. It has been long considered that algal growth is not lush when *Dendropoma* is healthy (Safriel, 1975). The present removal experiment supports the evidence that the density of vermetid settlers can decrease as a result of higher cover of algal species as, either 7 or 14 months after the disturbance events the recruitment pattern of *D. cristatum* was impaired by competitive interactions with macroalgae. This finding suggests that algal species associated to the vermetid reef may represent direct competitors for space for *D. cristatum* crawling larvae and hence for their settlement success. Similar changes in the recruitment patterns of intertidal species after a physical stress were also assessed by Leonard (2000) in a long-term study on barnacles off the New England intertidal shores. The experiment showed that the recruitment of barnacles was strongly influenced by a high cover of canopy algal species, hence supporting the hypothesis that survival and growth of one species can be strongly influenced by the colonization ability of another species.

The macroalga *Dictyota* sp. and the cyanophyta *Rivularia* sp. were the algal species showing the highest ability in recruiting on the experimental plots. Despite *Dictyota* sp. was recorded throughout the year, the different *habitus*, physiological tolerances and resistance to desiccation may explain the strong seasonal pattern in the ability of colonizing the different removal treatments. *Dictyota* sp. cover peaked from the late Autumn to the early Spring and was present as germling in the warmest months. By contrast, *Rivularia* sp. was more abundant in late Spring and Summer. Whatever the algal species covering the substrate, these two species were likely the most efficient in competing with the reef-building *D. cristatum* settlers, therefore contrast a full recovery of the community. Specifically, the analysis of temporal patterns might suggest that such algal-vermetid interaction was not in force shortly after the disturbance event, when intra-specific density-dependence processes likely regulated the recruitment rates of *D. cristatum*. However some months after disturbance event, both algae were successful in interacting with *D. cristatum* settlers, preventing the complete community recovery and affecting its resilience. Such assertion may be valid for both disturbance timings during which the two species played a different role. Regarding to this, the present findings suggest that *Dictyota* sp. mostly affected the recruitment rates of *D. cristatum* when the disturbance took place in Winter (i.e. the second timing experiment), whereas *Rivularia* sp. likely played a competitive role mostly in the first timing experiment, which was more evident 14 months after the disturbance took place. *Rivularia* sp. is a filamentous blue-green algae that lives in hemispherical or irregular gelatinous colonies attached to the substrate, usually preferring limestone habitats where can favor the precipitation of CaCO_3 around their filaments to fix itself on the substrate. When exposed to an environmental stress cyanobacteria differentiate via hormogonia, which are short chains of cells formed and asexually released from the apical parts of the trichomes. Hormogonia have an important function in the dispersal of the species when free substrate is available. In both timings, the coverage of *Rivularia* sp. increased in abundance during the warm season coinciding with the peak of *D. cristatum* recruitment, and the coverage of cyanobacteria was significantly higher in the disturbed than the undisturbed plots. Furthermore, *Rivularia* sp. had a very fast recovery capacity sensibly increasing in coverage just 3 months after the disturbance, mostly in the 100% removal treatments. The high recovery rate, its asexual reproduction during environmental stress

and its preference for the limestone substrate might suggest *Rivularia* sp. is an efficient competitor for *D. cristatum* settlers.

Therefore another interesting finding of this study may be that specific life-history characteristics (e.g. prompt availability of propagules via asexual reproduction, and germlings resistance to desiccation) of the algal species present in the inner edge of the vermetid reef may confer them a more efficient ability in colonizing the available bare space than the crawling vermetid larvae. This pattern of response seems not to be affected by the recruitment of the intertidal grazer *Patella* spp., even if the free substrate should allow an increase in the abundance of primary producers that represent a source of food for intertidal grazers.

Limpets are considered important species in determining the sequence of algal succession (Kim, 1997). Many studies have demonstrated that consumers either speed up the succession rates (Lubchenco and Menge, 1978; Sousa, 1979a; Lubchenco, 1983) or slow the intertidal successions by affecting the abundance of macroalgae, which colonize more slowly (Farrell, 1991). In communities where consumers have little or no effect on algal abundances they are unlikely to affect the rate of succession. For example, Turner (1983a) found herbivores had only a short-term influence on the abundance of early successional algae and did not affect the rate of succession. Underwood and Jernakoff (1981) supposed that consumers can have not impact on algal assemblage because the grazers' density could be too low, due to predation or competition with other sessile species, including algae. Algal productivity levels may be also important, as where the productivity is high, grazing rates effects are not significant (Turner, 1983b).

When assessing response at community level, the recovery trajectories after the different removal treatments of the *Neogoniolithon-Dendropoma* complex were strongly affected by the disturbance timing as the proximity with the vermetid recruitment event may affect the whole community resilience. In the second timing experiment the composition and structure of the benthic assemblages associated with the experimental plots at 50% and 100% removal was similar each other 7 month after the disturbance, but these were still different from the undisturbed community treatments. In the first timing experiment the whole community recovered just 7 months after the disturbance. These findings suggest that when the timing of a physical disturbance occurs in Winter, hence far from the recruitment event of the reef-building species, community recovery of the *Neogoniolithon-Dendropoma* complex is slower than Spring/Summer disturbance events.

In addition to this, the benthic assemblages recruiting on the 50% and 100% removal treatments strongly diverged in both timings whilst were similar in the undisturbed plots. This evidence support a high interannual 'stability' of the vermetid reef community which in turn may diverge with different disturbance timings. During the Winter the frequent storms and the impact of waves on the shores create large cleared patches, and can provide an available substrate for the increasing in abundance of macroalgae during the early Spring reducing the colonizable space for *D. cristatum* settlement; storms and high waves are less frequent in Spring and Summer where the high desiccation rates may lead to the decline of macroalgal species and potentially a new substrate is available for *D. cristatum* settlement.

In agreement with my results, previous studies highlighted that the intensity and the timing of disturbance could have significant effects, and may cause shifts on recovery patterns of marine benthic organisms (Sousa, 1984a; 1985; Benedetti-Cecchi and Cinelli, 1993; Underwood and Chapman, 2006; Jenkins and Martins, 2010; Kim *et al.*, 2014). The intensity of disturbance (intended as the size of patches) in highly stressful habitats, such as salt marshes, can affect the mechanism of succession. For example, Shumaway and Bertness (1994) reported that facilitatory mechanisms were responsible for successional changes in large patches, while succession was driven by competitive interactions in small areas where physical conditions were less hard. Also, previous studied carried out on rocky intertidal shores highlighted that the recovery patterns of benthic community structure were affected by different intensity and timing of disturbance. Sousa (1984a), manipulating the size of disturbed patches on the rocky intertidal shores, suggested that the surrounding community affected successional dynamics within the disturbed patch. Kim and DeWreede (1996) found that the medium patches were colonized faster than small and large patches and algal species responded differently to the seasonal effect of disturbance depending on their life histories and reproductive strategies. In another experiment conducted by Kim *et al.* (2014) on intertidal shores, differences in community structure were detected between disturbed vs undisturbed plots, with an increase in coverage percentage of sessile organisms on undisturbed plots, were the coverage of macroalgae was lower. Furthermore, the coverage of green algae was higher in plots where the disturbance occurred in Spring than Autumn, probably due to low spatial competition, as the disturbance occurred just before the green algal bloom. Over large spatial scale, the timing of disturbance was investigated by Martins *et al.* (2017) in an

experiment conducted on the rocky intertidal in three regions, with different latitude, by carrying out a manipulative disturbance at three distinct times of the year. This study revealed that, despite changes in species pools, productivity and environmental conditions among the three regions, the β -diversity was higher on the early stages of community but decreased on late stages of succession, suggesting that disturbed intertidal community can follow a successional convergence over time.

Generally, the recovery of a community from a localised disturbance event may depend on its original composition and the species pool present on a local scale, and evidences suggest that high and frequent intensity of disturbance reduced the ability of both adults and larvae of intertidal community to re-colonize the substrate (Cowie *et al.*, 2000). These findings demonstrated that the intensity and the timing of disturbance are two important factors in the recovery patterns of benthic organisms, suggesting that when the disturbance occurs just before a recruiting event of a species, it is able of more fast recovery than a disturbance event occurred far of recruiting event. Likely, the new free substrate created just before the recruitment peak of some species, as *D. cristatum*, may receive a great numbers of larvae which colonize the substrate accelerating the recovery patterns of benthic community associated with vermetid reef.

In summary, the timing of disturbance, representing the different season (May vs December) during which a storm wave disturbance was simulated combined with the disturbance intensity (i.e. the different removal treatments), affected the recovery trajectories of the benthic assemblages associated to vermetid reefs. Importantly this study highlights that different mechanisms may act over time, as *D. cristatum* settlement success was dependent upon adult density (i.e. intra-specific interaction) in the short-term, whereas inter-specific interactions with intertidal algae shaped the vermetid reef community in the mid-term.

An increase of intensity of extreme meteorological events, such as storms, as predicted by climate change models (Michener *et al.*, 1997) may lead to direct and indirect effects similar to my experimental disturbance. The occurrence and the effects of same disturbance events are rarely predictable, but the results of this study might suggest that these events are potentially able to affect the recovery patterns of the vermetid community triggering intra- and inter-specific interactions at a small spatial scale. The significant variation in the direction of successional trajectories of vermetid reef and associated benthic community observed in the present study highlights the importance of

additional long-term studies to better understand the recovery dynamics of benthic assemblages associated with vermetid reef after different intensities and timings of physical disturbances.

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CHAPTER 5. CONCLUDING REMARKS AND POTENTIAL RESEARCH PRIORITIES

This thesis contributed to expand the current knowledge on the understudied mechanisms of ecological succession occurring in the rocky intertidal zone with a special focus on the Mediterranean Sea. Overall, the main results of this thesis documented that the ocean warming rather than ocean acidification and grazing pressure may modify the intertidal community at early stages of succession (Fig. 46). By contrast, ocean acidification led to shifts in the composition and structure of the late stages of succession in the rocky intertidal zone, facilitating the recruitment of simplified algal forms and leading to community homogenization (Fig. 46). Another intriguing finding of the present thesis is related to the role of storm waves' intensity and timing in driving local-scale changes in the community dynamics and the recovery trajectories of a key Mediterranean biogenic habitat: the vermetid reef (Fig. 46). Overall, each of the short- and long-term experiments here presented suggest that community successions in the Mediterranean intertidal may exert combinations of species-specific responses to disturbance and context-dependent interaction strengths. Therefore, the present study might suggest that community successions should be investigated with a case by case approach, and that prediction on how intertidal successions may occur in the Mediterranean rocky intertidal as results of both global and local stressors are indeed challenging.

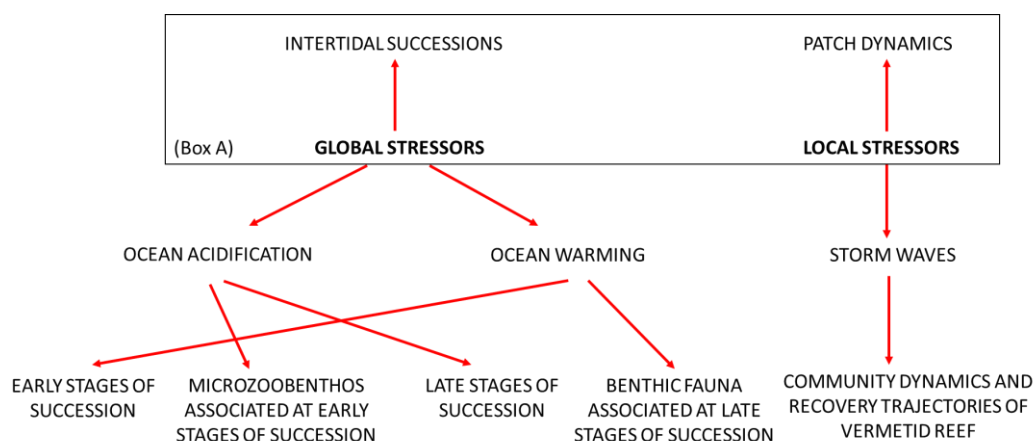


Fig. 46 – Effects of global and local stressors on benthic assemblages' succession in the intertidal zone of Sicily (Italy). **BOX A:** displays global stressors affecting the intertidal successions and local stressors affecting the patch dynamics. Among global stressors, ocean warming affected the early stages of microphytobenthic succession and the benthic fauna associated at late stages of succession; by contrast, ocean acidification affected the late stages of algal succession and the microzoobenthos associated at early stages of microphytobenthic succession. Among local stressors, storm waves affected the community dynamics and recovery trajectories of vermetid reef.

In detail, the early successional stages of microphytobenthic intertidal community were affected by temperature changes, with acidification and grazing rates having little or no influences. The microzoobenthic assemblages associated to the early EMPB were affected by ocean acidification, and differences in community composition were mediated by species-specific responses to warming. Contrary to expected, they were not found interactive interactions between abiotic (acidification and warming) and biotic (grazing pressure) stressors affecting the early successional stages of EMPB, as seen in other mesocosm studies (Alsterberg *et al.*, 2013; Russell *et al.*, 2013; Sampaio *et al.*, 2017). According with my results, only a limited number of in-field experiments showed that the benthic community composition changes with warming (Schiel *et al.*, 2004; Wernberg *et al.*, 2012b; Kordas *et al.*, 2014), suggesting that the temperature controls the initial stages of the primary producers, at least in the early phase of successional stages. These results sustain the hypothesis that a global disturbance such as the ocean warming is the main driver of community change in the early stages of intertidal succession. Furthermore, these findings provide support that changes in species composition at early stages of succession may lead to shifts in the successional patterns of intertidal species at later stages.

Despite ocean acidification affected the late stages of algal succession, they were able to evolve into assemblages with a similar composition and structure on the four recruiting surfaces with different chemical composition, suggesting that the acidification has a greater effect than any nature of substrates. According with my experiment, previous studies demonstrated that turf algae are positively (Kroeker *et al.*, 2010; Connell *et al.*, 2013) and CCA are negatively (Gao *et al.*, 1993; Martin and Gattuso, 2009) affected by ocean acidification. Instead, the macroalgae were negatively affected by acidification contrasting with the other literature evidences, which showed the resilience of macroalgal species under acidified conditions (Porzio *et al.*, 2011; Kroeker *et al.*, 2012) despite a decrease of calcification rates (Johnson *et al.*, 2012).

These findings provide evidence that ocean acidification effects on late stages of succession lead to simplified and homogeneous states (e.g. turf algae), whilst under ambient pH conditions more complex and structured algal communities were present (e.g. macroalgal species and CCA). Furthermore, the dominance of turf algae under acidified conditions inhibited the recruitment of macroalgal species, suggesting that the ocean acidification could be a driver of change for the late stages of succession via

pushing the succession in a simplified community and inhibiting the recruitment of more complex algal species (Connell and Slatyer, 1977). This study also suggests that ocean acidification driving shifts in structure and composition of algal assemblages, indirectly, might act affect the associated benthic faunal communities. This suggests that the complex dynamics of community at the late stages of succession could shift in a simplified ecosystem under projected future changes in rates of ocean acidification, but probably the long exposure to low pH conditions may lead to acclimatization and adaptation of some taxa that seems to be very vulnerable and susceptible to acidification.

Community dynamics and the recovery trajectories of benthic assemblages associated with the vermetid reef changed over successional time under different timing and intensity of disturbance. The recovery of community was faster when disturbance occurred just before the vermetid recruitment event, and the recruitment success of the reef-building larvae was density-dependent of *D. cristatum* adults. By contrast, the presence of algae slowed the vermetid recovery by triggering the inter-specific interactions with *D. cristatum* larvae. Furthermore, the density of *D. cristatum* settlers increased with the intensity of physical disturbance, showing the higher is the availability of free bare substratum, the higher is the ability of the vermetids larvae to colonize it.

In agreement with my results, previous studies highlighted that the intensity and the timing of disturbance could have significant effects, and may cause shifts on recovery patterns of marine benthic organisms (Sousa, 1984a; 1985; Benedetti-Cecchi and Cinelli, 1993; Underwood and Chapman, 2006; Jenkins and Martins, 2010; Kim *et al.*, 2014). The findings of this experiment also suggest that when the disturbance occurs just before a recruiting event of a species community recovery is faster. Likely, the new free substrate created just before the recruitment peak of some species, as *D. cristatum*, may receive a great numbers of larvae that colonize the substrate accelerating the recovery patterns of benthic community associated with vermetid reef. For a species with very peculiar biological characteristics such as the low larval dispersal and population connectivity, as the Mediterranean *Dendropoma* species, the recovery capacity after a low frequency and intensity disturbance is an important finding because make it able to recover after a local mortality event. Despite this local extinctions of *Dendropoma* species were recorded along the Israeli coast after unknown disturbances, likely acting in concert, have occurred a couple of decades ago (Galil, 2013).

In summary, I expect that the interaction between global and local stressors will reduce the resilience of some Mediterranean intertidal systems and will lead to shifts in marine successional patterns suggesting that there is a need to discriminate and identify the responses of intertidal organisms, not only to a single stress factor, but to the interaction of local and global disturbances.

The suite of studies presented here will probably contribute to the growing body of field research on the mechanisms involving the early and late stages of intertidal successions under global and local stressors. However, other mechanisms involved in the intertidal successions, not covered in this research, are recommended to be further investigated. First, manipulative experiments over large geographical scales would be of valuable importance to evaluate the responses of successional patterns of intertidal assemblages subjected to different environmental conditions, which could vary at different spatial scales, under global change scenarios. Secondly, analyses based on traits and life histories of organisms are increasingly recommended as a tool to better understand the species-specific responses and the successional patterns of intertidal organisms. Thirdly, what is likely really missing is the full understanding of interacting effects acting at the same time. An integration of detailed laboratory studies and *in situ* manipulation experiments, that consider all stressors acting together, are needed to better understand the successional dynamics of intertidal organisms.

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