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Temporal dynamics of benthic assemblages along a gradient of ocean acidification at a CO_2 vent's system of the Ischia Island

Tesi di laurea in
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Indice

1 INTRODUTION

1.1 Ocean Acidification (OA)

Recent studies indicate that impact of global climate changes is effecting also marine ecosystems (Fabry et al. 2008, Doney et al. 2012). The climate, at the present time, is changing extremely rapidly due to human activities, and since pre-industrial times, atmospheric composition has changed dramatically mainly due to the human use of fossil fuels. Rising atmospheric level of carbon dioxide (CO_2 one of the major greenhouse gases released by fossil fuel burning), is extremely critical because of its global effects irreversible on ecological timescales. The direct consequences of the increase of atmospheric CO_2 are ocean temperatures rise and acidification (Caldeira & Wickett 2003, Raven et al. 2005, IPCC 2013).

Ocean Acidification (OA) has been called the "evil twin of global warming" (Pelejero et al. 2010) and "the other CO_2 problem" (Doney et al. 2009) because of the threat it poses to the marine environment. In fact, due to anthropogenic CO_2 emissions, the carbonate marine system (regulated by pCO_2, pH, total alkalinity and saturation rate of calcium carbonate) is rapidly changing (Caldeira & Wickett 2003, Feely et al. 2004, Orr et al. 2005, IPCC 2013).

The term "Ocean Acidification" is used to describe the decrease in seawater pH due to ocean's absorption of CO_2 from the atmosphere. The average surface-ocean pH, which is currently around 8.1, has already fallen by 0.1 unit since the beginning of the industrial era, and it is likely to decline by another 0.2 to 0.4 unit by the end of this century (Caldeira & Wickett 2003, Sabine et al. 2004, IPCC 2013).

1.1.1 Carbonate in marine system

Because of gas-exchange between air and water, rising carbon dioxide partial pressure in the atmosphere causes an increase of CO_2 concentration in the sea.

When carbon dioxide dissolves in marine water, it is hydrated in carbonic acid (H_2CO_3). However, carbonic acid dissociates quickly to form bicarbonate (HCO_3^-)

and then carbonate (CO_3^{2-}) ions, releasing two hydrogen ions (H^+). The hydrolysis reaction of CO_2 and those that follow are reported in the equilibrium reactions (1), (2) and (3).

$$CO_2(g) \leftrightarrow CO_2(aq) \quad (1)$$

$$CO_2(aq) + H_2O(l) \leftrightarrow H^+(aq) + HCO_3^-(aq) \quad (2)$$

$$HCO_3^-(aq) \leftrightarrow H^+(aq) + CO_3^{2-}(aq) \quad (3)$$

Marine organisms use carbonate ions and calcium dissolved in seawater to produce calcium carbonate ($CaCO_3$) that is the main component of shellfish skeletons (4).

$$Ca^{2+}(aq) + CO_3^{2-}(aq) \leftrightarrow CaCO_3(s) \quad (4)$$

The H^+ ions released by the hydrolysis of CO_2 lower the water pH and bind with bicarbonate ions available, reducing the bioavailability of this compound for marine organisms and facilitating the dissolution of carbonate formations.

Therefore, the increase of carbon dioxide concentration in the atmosphere leads a rise of acidity of seawater and a decrease in the availability of carbonates (*Fig. 1*).

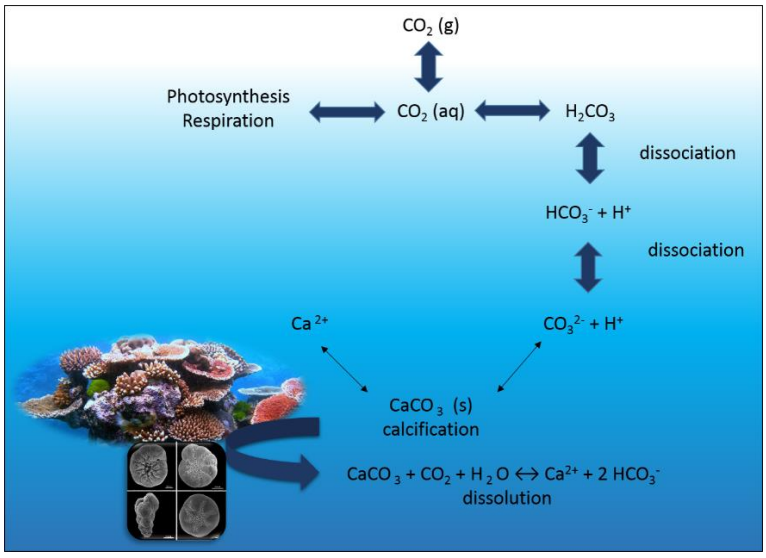

Fig. 1 Simplified diagram of carbonates in the marine system. Image modified from Cumani (2011).

The acidity of an aqueous solution is measured by the pH, which consists in the concentration of hydrogen ions inside the solution, equation (5).

$$pH = -log_{10}\,[H^+] \tag{5}$$

The pH takes values between 0 (strong acid) and 14 (strong base). The intermediate value 7 corresponds to the condition of neutrality, found in pure water at 25 ° C. Since the pH is based on a logarithmic scale, the lowering of one pH unit is equal to a 10-fold increase in acidity. The acidity of seawater changes over time in relation to seasonal variations in biological activity and geographical variations dependent on other factors, such as seawater temperature and depth (Sabine et al. 2004).
The presence of chemical species involved in the CO_2 carbonate chemistry in seawater affects and is affected by pH (*Fig.2*).

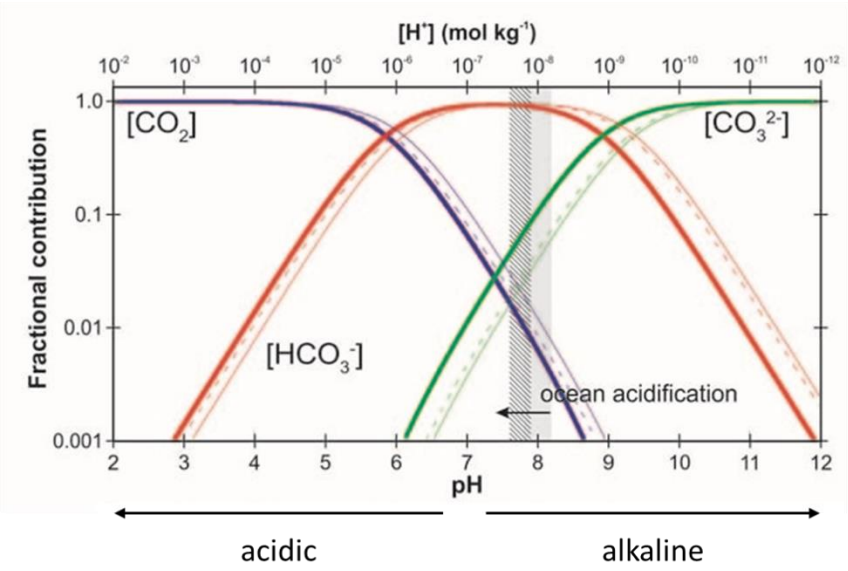

Fig. 2 Relation of ocean carbonate chemistry and pH. Picture modified from Barker and Ridgwell (2012).

The ability of the water to neutralize the acidic component is represented by total alkalinity (A_T), which is the sum of all the titratable bases from an acid. The alkalinity of a natural water having a pH less than 8.5 depends mainly on the content of bicarbonates, while at higher pH represents the content of bicarbonates, carbonates and hydroxides. Other bases, such as borates, phosphates, silicates,

ammonia, also contribute to the measure of alkalinity. A_T, usually expressed in mole per kilogram of solution, is independent of temperature and pressure of the water (Dickson 2010).

The A_T for the seawater can be approximated with the following formula:

$$A_T \approx [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] - [H^+] \quad (6)$$

Therefore, changes in marine chemistry due to ocean acidification (decrease of carbonates and increase of bicarbonate ions) affects total alkalinity.

The minerals that are primarily deposited in the marine environment are biogenic calcium carbonates, in particular aragonite (from corals and pteropods), calcite (from coccolitophorides and foraminifera) and magnesia calcites (from coralline algae) (Dickson 2010). They differ in the crystal structure and therefore for their solubility. At 25 °C, aragonite is about 1.5 times more soluble than calcite. Solubility of the different types of mineralization also depends on temperature and pressure. It increases at lower temperature and rises at higher pressure.

A useful parameter in studies on calcification and dissolution of calcium carbonates is the degree of saturation of seawater with respect to these minerals (Dickson 2010). This is defined as the product of the concentration of calcium and carbonates ions, divided by the stoichiometric solubility product (K_{sp}) for the in situ temperature, salinity and pressure, equation (7).

$$\Omega = \frac{[Ca^{2+}][CO_3^{2-}]}{K_{sp}} \quad (7)$$

In seawater, a natural horizontal boundary is formed as a result of these environmental factors and is known as the saturation horizon (Raven et al. 2005). Above this saturation horizon, Ω has a value greater than 1, seawater is over-saturated with respect to carbonates and these minerals do not readily dissolve. Below this depth, Ω has a value less than 1, and carbonates will dissolve (water under-saturated). When Ω=1, seawater is in equilibrium with the mineral phase. If carbonates production rate is high enough to offset dissolution, they can still occur

where Ω is less than 1. The carbonate compensation depth occurs at the depth in the ocean where production is exceeded by dissolution (Raven et al. 2005).
Rising of CO_2 leads to a decrease in carbonates concentration and consequent reduction of Ω value.

1.1.2 Past, present and future scenarios

Throughout history of Earth, concentration of atmospheric carbon dioxide $(CO_2)_{atm}$ has varied considerably (Kasting 1993). Generally $(CO_2)_{atm}$ decreased during Phanerozoic from over 5000 parts per million (ppm) to 1000 ppm for most of the Cenozoic (Berner 1990).
Up to middle Quaternary (650,000 years before Industrial Revolution), levels of $(CO_2)_{atm}$ were subjected to rhythmic oscillations, ranging from 180 ppm, during the glacial period, to 280 ppm, of the interglacial period (Siegenthaler et al. 2005).
Since Industrial Revolution (1750) atmospheric carbon dioxide concentration has been rapidly rising because of human activity, such as burning fossil fuels, deforestation, industrialization, production of cement, and other changes in land use (Guinotte & Fabry 2008). At present times values of CO_{2atm} are from 380 to 400 ppm (40% more than in pre-industrial era), these values represent as much as 50% of the increase occurring in the last three decades (Feely et al. 2009). These levels are going to rise by about 1,4 ppm per year (0,5%) during this century. This growth rate is almost 100 times higher than any other change recorded over the past 650,000 years (Raven et al. 2005, Siegenthaler et al. 2005, Guinotte & Fabry 2008, IPCC 2013).

The IPCC 5th assessment report, delineated four RCPs (Representative Concentration Pathways) scenarios based on the amount of greenhouse gases emitted (IPCC 2013) (*Fig. 3*).
In recent decades, only half of the anthropogenic CO_2 remains in the atmosphere, about 20% was absorbed by the terrestrial biosphere and the remaining 30% by the sea (Feely et al. 2004, Sabine et al. 2004). Increasing CO_2 concentration in the oceans leads to a decrease in pH of the seawater. In fact, since Industrial Revolution, in less of 250 years, pH of surface seawater decreased on average of 0.1 unity, and

are expected to further decreases by the end of this century (*Fig. 3*) (Caldeira & Wickett 2003, Sabine et al. 2004, Feely et al. 2009, IPCC 2013).

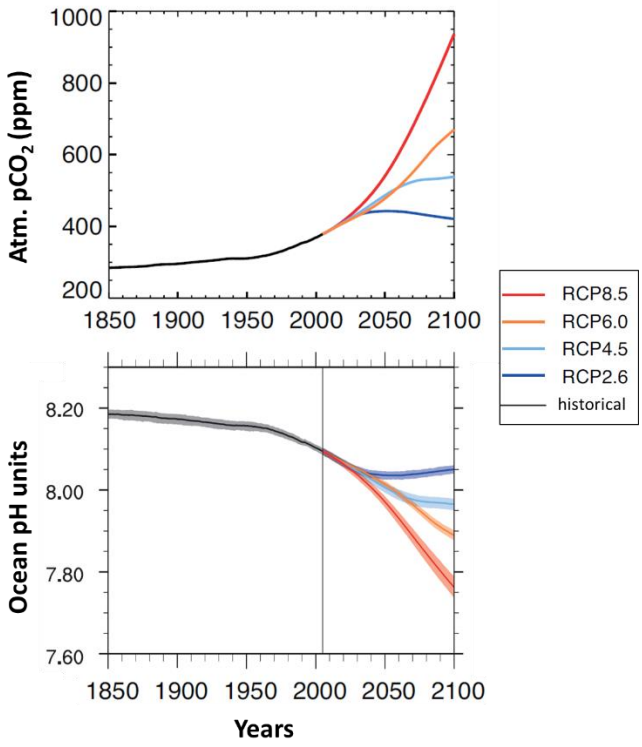

Fig. 3. RCPs scenarios of atmospheric CO2 levels (above) and Ocean pH units (under). Picture modified from Stocker et al. (2013).

Changes in marine pH is altering carbonates chemistry with a serious impact on marine ecosystems (Guinotte & Fabry 2008). The aragonite and calcite saturation horizons of the world's oceans are moving to shallower depths (Broecker & Takahashi 1977, Feely & Chen 1982, Chen et al. 1988, Kleypas et al. 1999, Broecker 2003, Caldeira & Wickett 2003, Feely et al. 2004, Orr et al. 2005).

Many of the areas where shoaling is predicted to occur within the century, are highly productive and home for many of the world's most important commercial species (Guinotte & Fabry 2008).

1.2 Effects of ocean acidification (OA) on marine organisms

Marine organisms vary broadly in their responses to OA, because of the wide variety of processes affected (e.g. dissolution and calcification rates, growth rates, development and survival) (Kroeker et al. 2010).

The rate of change in chemistry of marine carbonates will have significant consequences for marine taxa, particularly those that have calcareous structures (e.g. skeletons, shells, sclerites). These taxa may not be able to acclimate because of the higher energy consumption required for the formation of calcium carbonate under OA scenarios (Guinotte & Fabry 2008).

Many marine organisms form skeleton/shells of calcium carbonate, including foraminifera, corals, bryozoans, mollusks, echinoderms, and calcareous macroalgae. Several experiments in laboratories and mesocosms were carried out to study the effects of OA on calcifying organisms (Kroeker et al. 2010). Most of the organisms studied where affected by changes in the state of saturation of carbonates, showing a decrease in calcification rates (Broecker & Takahashi 1966, Gattuso et al. 1998, Langdon et al. 2000, Riebesell et al. 2000, Marubini et al. 2003, Ohde & Mozaffar Hossain 2004, Kleypas & Langdon 2006, Kuffner et al. 2008, Gazeau et al. 2010, Manno et al. 2012). Benthic invertebrates, such as crustaceans, annelids, brachiopods and tunicates have $CaCO_3$ skeletal elements, which are also affected by OA (Fabry et al. 2008). These organisms may use shell dissolution for the acid-base regulation at high internal pCO_2, as has been observed in mussels (Michaelidis et al. 2005). Moreover, other physiological indices such as survival, growth rate, development, metabolism and pH balance of organisms have been studied under elevated pCO_2 levels (Fabry et al. 2008).

Since OA affects all the marine biota, even non-calcifying organisms show physiological responses to this phenomenon. In fact, primary producers, such as marine algae and seagrasses, use CO_2 or HCO_3^- dissolved in seawater and release O_2, to form organic matter, essential for their livelihood. Photosynthesis general reaction in equation (8).

$$6CO_2 + 6H_2O + Light \rightarrow C_6H_{12}O_6 + 6O_2 \quad (8)$$

Experimental studies on phytoplanktonic and macroalgal species have shown that their photosynthesis and growth rates are likely to be enhanced by increasing CO_2 concentration in sea water (Gao et al. 1991, Gao et al. 1993, Riebesell et al. 1993, Hein & Sand-Jensen 1997, Beardall & Raven 2004, Schippers et al. 2004, Zou & Gao 2005). However, the responses are species-specific, depending on the physiological characteristics of the species (Rost et al. 2003, Yang & Gao 2003, Collins et al. 2006).

Physiological responses of individual species to acidification could indirectly lead to an ecosystem changes in an elevate pCO_2 ocean (Kroeker et al. 2013b). Understanding how OA may affect marine ecosystems is very limited as most experiments have been done in aquaria/mesocosms, and tested short-term, rapid perturbation on isolated elements of the ecosystem (Raven et al. 2005). In the last years, observational studies in naturally acidified systems have shown how OA leads to a decrease in community variability, diversity, biomass and trophic complexity (Hall-Spencer et al. 2008, Kroeker et al. 2010, Kroeker et al. 2011, Kroeker et al. 2013a, Kroeker et al. 2013b).

1.3 The Ischia CO_2 vent's system at Castello Aragonese: a naturally acidified laboratory at sea

Ischia is a volcanic island located at the North limit of the Gulf of Naples (Tyrrhenian Sea). It is the largest of the Phlaegrean islands and the third most populated island in Italy, after Sicily and Sardinia. Ischia is well known for its natural and cultural resources, and beautiful landscapes and seascapes (Gambi et al. 2003), that led in 2008 to the establishment of the marine protected area (MPA) 'Regno di Nettuno'. In addition, several marine and terrestrial geosites characterize the island (Monti 2011) and recently Ischia has been candidate to be include in the UNESCO World Heritage Sites of natural and cultural relevance to humanity (Leone & Greco 2014).

The island is subject to intense volcanic activity through a high hydrothermal venting, gas emission and CO_2 vents in various coastal parts (Pecoraino et al. 2005).

These emissions are characterized by the complete absence of sulfur and the presence at 90.1-95.3% CO_2, N_2 3.2-6.6%, 0.6-0.8% O_2, A_r 0.08-0.1% and 0.2-0.8% CH_4 (Hall-Spencer et al. 2008). Five shallow coastal sites of emissions in the North-east and east side of Ischia have been reported by Tedesco (1996) (*Fig. 4*). More CO_2 submerged emission sites, including some at greater depths, have been described in recent years by Gambi (2014a) (*Fig. 4*).

This study was carried out on the North and South sides of Castello Aragonese, where near shore, volcanic CO_2 vents occur (*Fig. A1*). These CO_2 vents cause local acidification of seawater by as much as 1.5 pH units below the average ocean pH of 8.1-8.2 (Kroeker et al. 2011). Previous studies at the Castello Aragonese high-CO_2 sites have shown community alteration and ecosystem simplification of the mature assemblages on natural substrata and of the early stages of recruitment (Kroeker et al. 2013a, Kroeker et al. 2013b). Several studies highlighted that a slight lowering of pH (mean pH 7.6) increases the local shoot density of *Posidonia oceanica* seagrass systems and substantially reduces the abundances of calcifying organisms such as coralline algae, sea urchins and gastropods (Hall-Spencer et al. 2008, Porzio et al. 2011, Kroeker et al. 2013b). Extremely low values of pH however, can lead to a large decline in species richness, with dominance of few species resilient to these conditions (Porzio et al. 2011).

The study area is a primary example of a naturally acidified marine ecosystem and allow to study adaptation of individual species, assemblages and ecosystems to OA (Gambi 2014a, b).

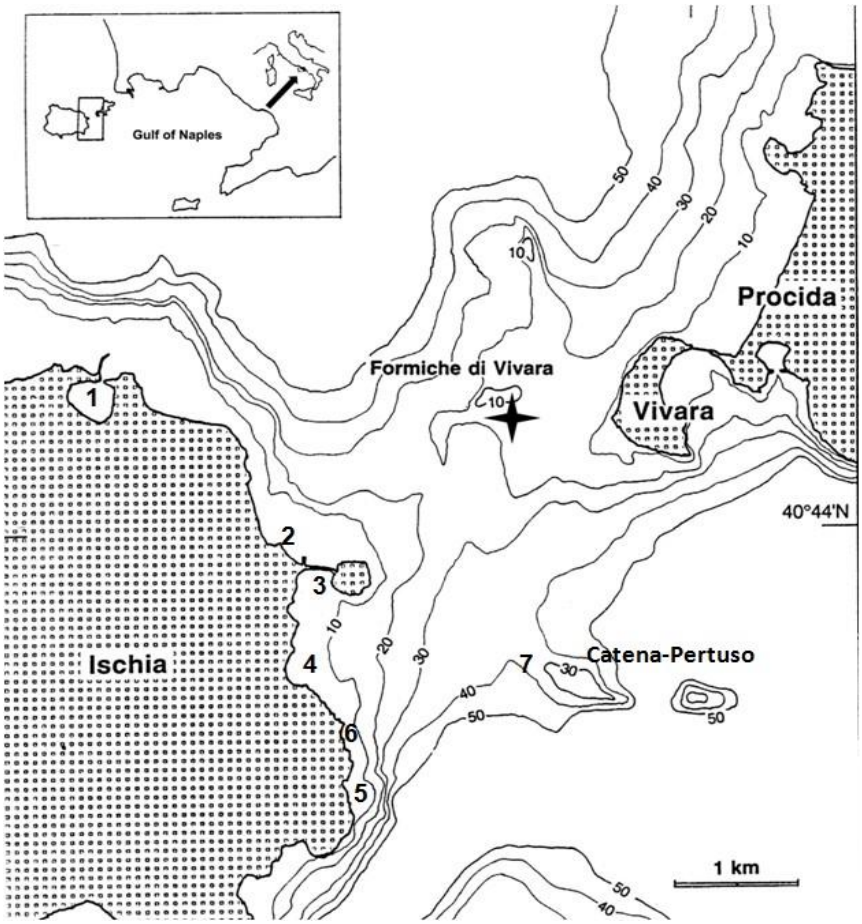

Fig. 4. Map of Ischia island, submerged CO_2 emissions are reported (from 1 to 5 by Tedesco, 1996). 1= porto d'Ischia; 2=" a vullatura" in front of Mandra beach (S. Antonio Harbour in Tedesco 1996); 3= Castello Aragonese; 4= Carta Romana; 5= Grotta del Mago; 6= chiane del Lume; 7= the Madonnina rocky shoal. Figure from Gambi (2014a).

1.4 Previous studies in the area

This thesis is the continuation of previous studies carried out in the Castello Aragonese islet. In particular the work of Kroeker et al. (2013b) that investigated the early colonization stages of volcanic rock tiles in three different acidification conditions (extreme low, low and ambient pH) during 14 months after the deployment. In extreme low pH assemblages were poor in species and in an early successional stage during the 14 months of the study. Whereas in low and ambient

pH, the assemblages continued to gain species through time, reaching a higher diversity and showing more complex successional trajectories. Calcareous species in ambient pH increased their abundance during the all study, while in low pH their abundance dropped after 3.5 months, when fleshy algae increased. The observed pattern was consistent in both North and South side. These results suggested that calcareous species that are able to withstand the direct effect of acidification might suffer reduction in abundance if they are in competition with fleshy algae under the predicted near-future acidification condition. Moreover, these results were in contrast with laboratory studies that have suggested that calcareous species will be limited by recruitment under future levels of acidification (Kuffner et al. 2008, Russell et al. 2009).

1.5 Objectives of the thesis

The main aim of this thesis is to study changes in shallow sublittoral benthic assemblage temporal dynamics along a pH gradient. For this purpose, we analysed the composition of mature assemblages established on artificial substrates (volcanic tiles) over a 3 yr- period. We estimated the frequency of benthic taxa and functional groups on tiles placed along a pH gradient on the South and North sides of the Castello Aragonese islet. Photographs were taken at different times and analysed with a software for image analysis. The specific objectives of this study are the followings:

1) To study over a 3-yr period the composition of the main benthic taxa and functional groups along a pH gradient in the North and South sides of the study area.
2) To study the patterns of diversity and dynamic in the three pH zones over the 3-yr period.
3) To compare the structure of the assemblages on recruitment substrates (tiles) *versus* natural substrates.

4) To study variability patterns of the “mature community” between 2013 (1-year-old community) and 2015 (3-years- old community).
5) To compare visual census versus image analyses methods to evaluate their efficacy in the identification of benthic taxa and functional groups.

2 MATERIALS AND METHODS

2.1 Study area

At the Castello Aragonese vents occur at 0.5-3 m depth on the North and South sides of the small islet, adjacent to sloping rocky reefs (*Fig. 5*).

These CO_2 vents create gradients in pH and carbonates chemistry by releasing predominantly CO_2 with very small amounts of other gases at ambient temperatures and salinities (Hall-Spencer et al. 2008).

At each side (North and South), three pH zones were identified: ambient (S1 and N1), low (S2 and N2) and extreme low pH (S3 and N3) (*Fig. 5*).

Fig. 5. View of the study area. The numbers represents different acidification conditions where the tiles were placed for up to 36 months. Ambient pH, (S1, N1); Low pH (S2N2); Extreme Low pH (S3, N3).

The conditions (*Table 1*) in the extreme-low pH zones (with values of pH: S3=6.6 ± 0.5 , N3=7.2 ± 0.4) correspond to scenarios expected in approximately 300 years, whereas those in the low pH zones (with values of pH: S2=7.8 ± 0.3 , N2=7.8 ± 0.2) are comparable to scenarios expected at the end of this century (Caldeira & Wickett 2003). The ambient pH zones are comparable to current conditions in the surface waters in the Mediterranean Sea (*Table 1*).

The values of environmental and biogeochemical parameters are based on measurements published in the study of Kroeker et al. (2011). They quantified the seawater carbonate chemistry in the experimental zones using *in situ* modified Honeywell Durafet pH sensor and temperature sensors for continuous data recording (every hour). The sensors were deployed in each pH zone on the southern side from 12 May to 14 June 2010, and in the northern side from 13 September to 8 October 2010, coupled with discrete water samples (Kroeker et al. 2011, Kroeker et al. 2013a).

Table 1. Environmental and biogeochemical parameters in experimental sites, table by Kroeker et al. (2013a).

pH zone	Temp, C	pH_T	TA	pCO_2	HCO_3^-	CO_3^{2-}	Ωcalcite
Ambient, N1	23,4 ± 0,7	8,0 ± 0,1	2,563 ± 3	568 ± 100	2,059 ± 54	202 ± 22	4,8 ± 0,5
Ambient, S1	19,6 ± 1,5	8,1 ± 0,1	2,563 ± 2	440 ± 192	2,016 ± 71	219 ± 29	5,1 ± 0,7
Low, N2	23,8 ± 0,7	7,8 ± 0,2	2,560 ± 7	1,075 ± 942	2,180 ± 115	150 ± 47	3,5 ± 1,1
Low, S2	17,5 ± 2,8	7,8 ± 0,3	2,560 ± 7	1,581 ± 2,711	2,232 ± 136	128 ± 56	3,0 ± 1,3
Extreme low, N3	23,4 ± 0,7	7,2 ± 0,4	2,559 ± 13	6,590 ± 21,352	2,428 ± 85	54 ± 35	1,3 ± 0,8
Extreme low, S3	17,5 ± 2,8	6,6 ± 0,5	2,563 ± 13	23,989 ± 16,638	2,508 ± 100	21 ± 41	0,5 ± 0,9

The pH and temperature data were based on hourly measurements from in situ pH meters taken during separate deployments for N and S sides [N = 604 (North) – 3162 (south)]. Total alkalinity (TA) and salinity estimates (mean 38 psu) were measured in water samples collected during sensor deployments (n = 3 – 10), and all other carbonate parameters were estimated by assuming constant TA and salinity and applying the mean TA and salinity values to pH and temperature values measured by in situ sensors. Differences in temperature between South and North sides were due to deployment times: early summer (North) and early fall (south). Values are means ± SD. TA, HCO_3^-, CO_3^{2-} are reported in $\mu mol \cdot kg^{-1}$.

2.2 Sampling design

From 15 to 17 volcanic rock tiles, size 15x15 cm, were bolted to the substrate on May 27th-28th 2012 at about 1.5 m depth in each pH zone (ambient, low and extreme low pH) at both sides (North and South). Tiles were hung at 95°-115° to approximate the orientation of the natural substrate. Settlement tiles were chosen because they allow to study a definite area, can be easily identified during the photographic sampling, and can be removed avoiding impacts on natural community. The tiles were placed on the same spots used during a previous study (Kroeker et al. 2013b). So that the results of this study are comparable with previous observations.

Tiles were photographed *in situ* at 13 month (16th June 2013), 17 (24th October 2013), 22 (21st March 2014), 33 (20th February 2015) and 36 (27th May 2015) months after the deployment in May 2012. A sampling with visual quadrats was done in July 2015.

2.3 Field work

2.3.1 Photo sampling

Photography is a useful technique in studying marine benthic communities, providing a non-destructive sample of the assemblages, with high resolution over large spatial and temporal scales. Moreover, photography reduces the time spent underwater and facilitates safety diving conditions. There are several methods for image analysis allowing to calculate the cover of benthic species and/or different ecologically relevant categories (Magorrian & Service 1998, Teixido et al. 2011). However, these methods involve time-consuming processing.

The photographic sampling of hard bottom assemblages consists in taking photographs of a defined area, usually delimited by a frame. In this study, the surface of the tiles was the sampling area.

Because of the long period covered by the study, sampling was done using different cameras and by different scientist. During the first three periods, June 2013, October 2013 and March 2014, sampling was done using a Nikon Coolpix S2006 with a dedicated underwater cage. In the last two periods, February 2015 and May 2015, sampling was done using a Nikon D70S digital SLR camera fitted with a Nikkor 20 mm DX lens (3000 * 2000 pixel resolutions) and housed in a Subal D70S. Lighting was achieved by two electronic strobes fitted with diffusers.
Pictures were taken at about 50 cm from the subject and perpendicular to the tiles surface. Examples of photos are reported in *Fig. A2*.

2.3.2 Sampling with visual quadrats

Visual sampling using quadrats is one of the non-destructive methods, for the study of sessile benthos, most commonly used in the Mediterranean Sea (Bianchi et al. 2004). Quadrats are frames enclosing a standard area of the substrate (e.g. 50x50 cm or 100x100 cm) (Leujak & Ormond 2007). For this study, we compare the number of taxa identified using photographs with the taxa observed using visual sampling. The surface area of the quadrats was 15x15 cm, the same surface of the tiles. Surveys were conducted on the 27th May 2015. In each pH zone and on both sides, we have chosen the same tiles used for temporal analysis to obtain data comparable to those of photo sampling.

2.4 Data collecting

Both methods, photos and visual quadrats, were used to evaluate the biotic frequency of taxa and functional groups, in order to provide a quantitative estimation of the assemblages. Usually in photo surveys it is estimated the percentage cover of the organisms, i.e. the portion of substrate covered by every species individually (Boudouresque 1971). We have chosen to detect frequency because it is faster than cover-estimation and the two techniques yield similar results (Parravicini et al. 2010). Moreover, shallow subtidal rocky Mediterranean communities are characterized by high algae coverage with different layers of

species (Piazzi et al. 2007). Frequency combines rapidity and precision of quantitative estimates; in fact, the number of quadrats where a species appears is counted independently from the abundance with which it is present in every quadrat (Bianchi et al. 2004).

A grid of 36 squares cell with 6,25 cm^2 unit area (225cm^2 tot) was projected over the tiles in both photos and visual quadrats. The percentage of subquadrats in which species appeared was recorded and used as the unit of measure. A highly abundant species that occurred in all subquadrats would produce a frequency of 100% presence, whereas absence of species would produce a presence of 0%.

2.4.1 Taxa identification

Practice in taxa identification was done during a preliminary diving and by a literature check using the following web sites (http://www.algaebase.org/, http://doris.ffessm.fr/) and guide books Riedl (1991), Prieto et al. (2013). For the identification of taxa that were not recognized in field, we collected small samples and identified them in the laboratory with the stereomicroscope using the current identification guides: Riedl (1991), Prieto et al. (2013).

Taxa were identified to the lowest taxonomic resolution possible and then grouped into seven functional groups, plus a category for bare tile (*Table.2*).

2.4.2 Photo analysis

Temporal analyses: for this objective, 5 tiles in every condition of pH (ambient, low and extreme low) and in both sides (North and South) were chosen. These tiles were selected for their best quality of resolution, and because they were observed in each sampling period. In this way, the 30 tiles were "followed" over time, during the five sampling periods. Therefore, temporal analysis was based on the analysis of 30 images in each sampling period (total 150).

Tiles vs natural substrates: the assemblage structure based on functional groups frequency on the tiles exposed for 36 months was compared with assemblages on natural substrates. The natural substrates were of two types: "disturbed", representing 20x20 cm plot cleared and followed through time for 36 months; and

"undisturbed", representing natural undisturbed 20x20 cm substrate plot. Both type of natural substrates were analyzed by photographic samples (estimating % cover of different taxa, then grouped in to functional groups), and results have been summarized by Kroeker et al. (2013a).

Community variability: 4 tiles were randomly chosen on both sides, in each pH zone. We analysed the differences in community between two sampling periods: 13 and 36 months after installation. Thus, 24 pictures were analysed in June 2013 and May 2015 respectively (48 overall).

Image software

PhotoQuad software was used system for the analysis of all the photographic samples. The photoQuad software is an image-processing tool created for ecological information contained in digital or digitized photographs, with particular focus on quadrat samples (Trygonis & Sini 2012). We used this system because it operates in a layer-based environment that allows multiple analyses to be performed simultaneously on the same source image. The advantages of working in layers are the reduction of analysis time, the simplification of data management, and the minimization of data redundancy; results can be reproduced using only the original input image and photoQuad's native layer file, which can be saved, loaded or modified during processing (Trygonis & Sini 2012).

In each image the tile boundary was outlined to define the effective sampling area and the image was calibrated to provide a pixel to real-distance calibration factor (pixels x cm^{-1}), that allowed image scaling to metric units and the estimation of actual species area or related descriptors (*Fig. 6*). We projected a 6x6 square cell grid over the images to collect frequency data.

Fig. 6. Screenshot of the photoQuad software. Cells that include Corallina elongata *are highlighted*; *the numbers of small quadrats where the species occurs represent its frequency in the tile.*

2.5 Data analyses

2.5.1 Diversity indices

A diversity index is a mathematical measure of species diversity in a community. Diversity indices provide more information about community composition than simply species richness. Species Richness is given as the total number of species (S), which is dependent on sample size (the bigger the sample, the more species there are likely to be). Different diversity indices emphasize the species richness or equitability components of diversity to varying degrees. The most commonly used diversity measure is the Shannon-Wiener diversity index:

$$H' = -\sum_i p_i ln(p_i)$$

Where p_i is the proportion of the total count (biomass ecc.) arising from the *i*th species (Shannon 1997).

2.5.2 Statistical analysis

Spearman-rank correlation was used to test the relationship between functional groups and taxa datasets. Spearman correlation coefficient (ρ) measures the strength of association between two ranked variables (McDonald 2009). It assesses how well the relationship between two variables can be described using a monotonic function. For a sample of size n, the n raw scores X_i, Y_i are converted to ranks x_i, y_i and ρ is computed from:

$$\rho = 1 - \frac{6\sum d_i^2}{n(n^2 - 1)}$$

Where $d_i = x_i - y_i$, is the difference between ranks (Spearman 1904).

To test variances of individual taxa and functional groups univariate permutational analyses based on matrices of Euclidean distances of square root transformed data were done. PERMANOVA univariate analyses were run also on Species Richness and Shannon-Wiener indices starting on Euclidean distances matrices of untransformed data.

Matrices of frequency data of taxa and functional groups were analysed through different multivariate techniques. Separate permutational multivariate analyses of variance (PERMANOVA) were done to test different hypotheses. The first two analyses tested the differences of the same tile over time, considering separately the datasets derived from species and functional groups. We tested the null hypothesis of no difference in each of the selected tiles among the sampling times (13, 17, 24, 33 and 36 months). For these analyses, the experimental design consisted of three factors: side (two levels, fixed), pH (3 levels, fixed) and time (5 levels, random).

The third and the fourth analysis were performed to detect differences between the first (13 months 2013) and the last (36 months 2015) sampling periods in frequency data of taxa and functional groups. Tiles were chosen randomly. The experimental design for these analysis consisted of three factors: side (two levels, fixed), pH (3 levels, fixed) and time (two levels, fixed).

The last analysis tested differences in taxa identification derived from analyses of photos *vs* visual quadrats, with the null hypothesis of no differences between the two analytical methods. The dataset derived from taxa colonizing the tiles and the photographs after 36 months. The experimental design consisted of three factors:

side (two levels, fixed), pH (three levels, fixed) and method (two levels, fixed). Significant interactions among main factors were investigated by post-hoc pair-wise tests. Multivariate patterns of possible differences between periods and between methods were visualized for taxa and functional groups dataset by non-metric multidimensional scaling ordination (nMDS) derived from the Bray–Curtis similarity matrix on square-roots transformed data. Multivariate multiple regression analyses (DISTLM) tested the significance of species contributions by fitting a linear model based on Bray-Curtis similarities using 9999 permutations and the R^2 and step-wise selection criteria. All analyses of the data were performed using the PRIMER v6 software package (Clarke & Gorley 2006), including the PERMANOVA+ add-on package (Anderson et al. 2008). Finally, the graphs of community structure, based on functional groups were elaborated with Excel software.

3 RESULTS

3.1 Taxa and functional groups found

3.1.1 Identification

A total of 41 macrobenthic taxa were identified in species assemblages along the pH gradient at the Castello Aragonese, 31 were classified to species level and 10 classified into higher taxonomic ranks (*Table.2*). These taxa comprised 30 macroalgae, 5 sponges, 1 bryozoan, 1 polychaete, 1 crustacean and 2 tunicates. Six taxa were recognized only by visual census, while 5 only in photographs and 30 in both visual census and photographic samples. Taxa were grouped into 7 functional groups plus one category for bare tile (not colonized) (*Table.2*).

Table.2 List of taxa and functional groups found along the pH gradient at the Castello Aragonese. Asterisks (*) specify taxa recognized only with visual quadrats. Hildenbrandia sp. was often mixed with Biofilm. Thus, we called this mixture complex as "Biofilm red" and classified in Biofilm/Filamentous algae/Encrusting Fleshy algae.

FUNCTIONAL GROUP	TAXA IN PHOTO	TAXA IN VISUAL QUADRATS
Erect Fleshy Algae (EFA)	*Dictyota dichotoma,* *Dictyota dichotoma var.intricata,* *Dictyota fasciola* *Halopteris scoparia* *Flabellia petiolata* *Chaetomorpha linum* *Acetabularia acetabulum* *Parvocaulis parvulum* *Falkenbergia hildenbrandii*	*Dictyota dichotoma,* *Dictyota dichotoma var.intricata,* *Dictyota fasciola* *Halopteris scoparia* *Flabellia petiolata* *Chaetomorpha linum* *Acetabularia acetabulum* *Parvocaulis parvulum* *Sphacelaria cirrosa** *Caulerpa cylindracea** *Anadyomene stellata**
Encrusting Calcareous Algae (EnCA)	*Hydrolithon farinosum,* *Neogoniolithon brassica-flora* *Peyssonnelia rosa-marina*	*Hydrolithon farinosum,* *Neogoniolithon brassica-flora* *Peyssonnelia rosa-marina* *Peyssonnelia squamaria**
Erect Calcareous Algae (ErCA)	*Amphiroa rigida* *Amphiroa rubra* *Corallina elongata* *Jania longifurca* *Jania rubens* *Padina pavonica*	*Amphiroa rigida* *Amphiroa rubra* *Corallina elongata* *Jania rubens* *Padina pavonica*
Fleshy Turf Algae (FTA)	*Cladophora coelotrix* *Cladophora prolifera* Turf Turf+sediment	*Cladophora coelotrix* *Cladophora prolifera* Turf Turf+sediment
Fleshy Filter Feeders (FFF)	*Chondrosia reniformis* *Crambe crambe* *Haliclona mediterranea* *Ircina spp,* *Cystodytes dellechiajei* *Diplosoma spongiforme*	*Crambe crambe* *Haliclona mediterranea* *Ircina spp,* *Sycon**
Calcified Filter Feeders (CFF)	*Balanus perforatus* Spirorbinae spp. Bryozoa encrusting orange	*Balanus perforatus* Spirorbinae spp. *Bryozoa encrusting orange* *Anomia ephippium**
Biofilm/Filamentous Algae/Encrusting Fleshy Algae (BEF)	Biofilm Biofim red Biofilm+sediment Hildenbrandia sp.	Biofilm Biofim red Biofilm+sediment Hildenbrandia sp.
Tile	Tile	Tile

3.1.2 Species assemblages on tiles in the different pH zones

Community composition of both taxa and functional groups changed among the three pH zones. To estimate abundances, data from the North and from the South sides and from all the sampling periods were pooled. Abundances, expressed as perecent frequency, are showed in *Fig. 7*.

In the extreme low pH zones, only four functional groups were found, with Biofilm/Filamentous algae/Encrusting Fleshy algae (BEF) as the most abundant group (73,13%). Within this functional group the dominant taxa were Biofilm red (29,45%) and Biofilm (29,34%). Biofilm red was found just in extreme low pH zones.

In low and ambient pH zones, all the eight functional groups were found, with a marked dominance of calcyfying invertebrates (39,86% in low pH and 30,88% in ambient pH) rapresented by *Balanus perforatus* (18,79% low pH, 15,01% ambient pH) and *Spirorbinae spp*. (19.34% low pH, 13,16% ambient pH). However, there were some differences in composition between low and ambient pH.

Erect Fleshy Algae (EFA) were more frequent in low pH (35,51%) than in ambient pH (20,25%); especially *Dictyota dichotoma* (16,06% low pH, 9,45% ambient pH) and *Halopteris scoparia* (15,65% low pH, 5,08% ambient pH). Instead, there was a couspicuos presence of Encrusting Calcareous Algae (EnCA) (18,99%) in ambient pH, represented by *Neogoniolithon brassica-flora* (10,30%) and *Hydrolithon farinosum* (8,47%).

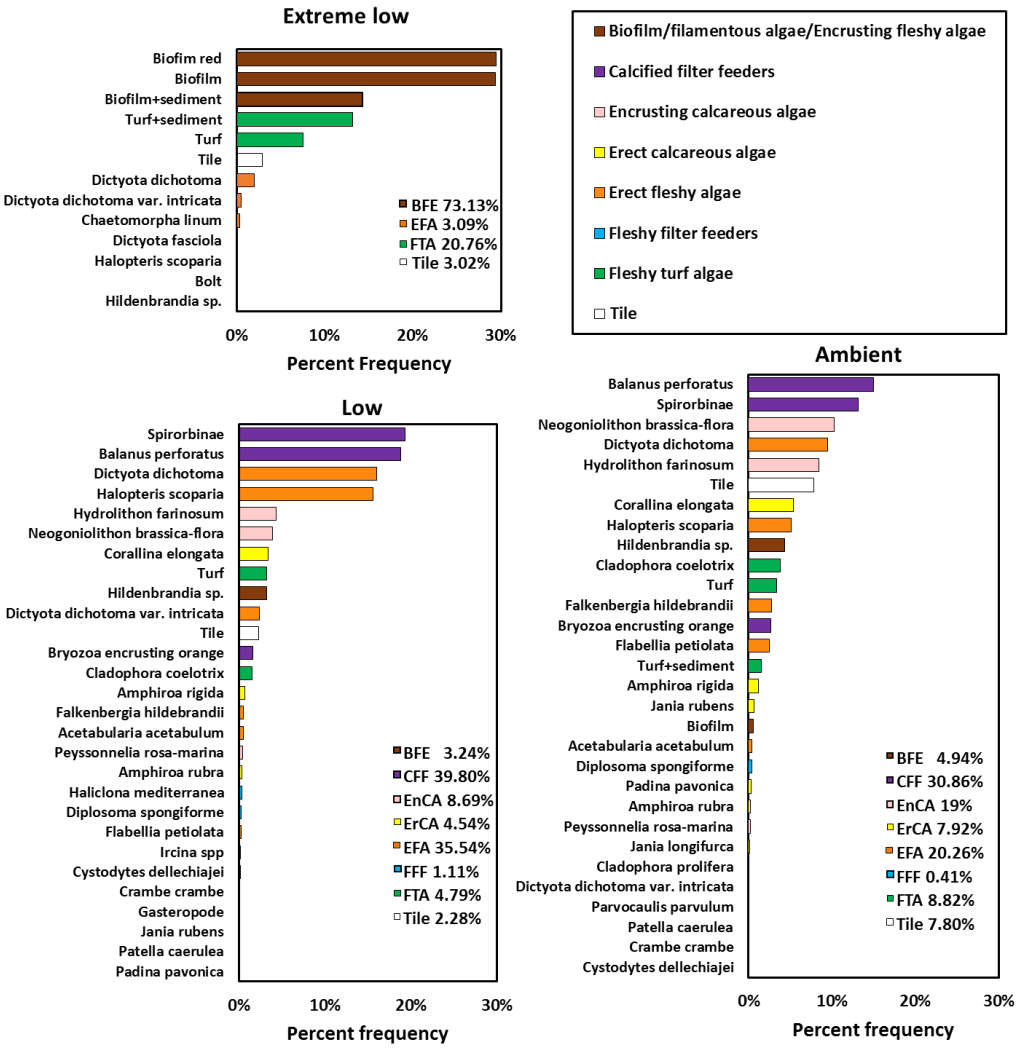

Fig. 7. Taxa found in each pH conditions, ordered in function of their abundances.

3.2 Temporal analyses

3.2.1 Functional groups analyses

We analysed the abundance of the functional groups along the pH gradient in the five sampling periods (June 2013, October 2013, March 2014, February 2015, May

2015). Reported data represents the average of percentage frequencies of each of those groups.

Functional groups found in both North and South sides and in all the 3 pH zones were almost equal for each sampling time (*Fig. 9*). At extreme low pH the same functional groups were found during all the five studied periods. An exclusive dominance of Biofilm/Filamentous algae/Encrusting Fleshy algae (BEF) was observed both at the North and South sides but with higher values (between 95% and 100 %) in the South station (S3). On the North side (N3), Fleshy Turf Algae (FTA) were also abundant especially after 33 months of exposure (February 2015) (56%).

In low pH, the most abundant functional groups were Calcifying Filter Feeders (CFF) and Erect Fleshy Algae (EFA), which alternate their dominance during the 5 sampling dates. On the North side (N2) there was a majority of EFA at the 13th, 22nd and 36th month after the deployment. At the South side (S2), CFF was the most abundant group in all the sampling periods, except in February 2015 (30%). Fleshy Turf Algae (FTA) was more abundant on the North than on the South side in low pH conditions, where sometimes this group was absent. Interestingly, Fleshy Filter Feeders (FFF) occurred only on the North side.

At the ambient pH on the North side (N1), all functional groups showed a homogenous distribution already at month 13. On the contrary, on the South side (S1), there was a dominance of Calcified Filter Feeders (CFF) at the first sampling time (78%). Overall, Calcareous groups (CFF, EnCA, ErCA) were the most abundant in ambient pH zones in all the five periods for both North and South sides, except for May 2015. In this period, Erect Fleshy Algae (EFA) (52%) and Fleshy Turf Algae (FTA) (32%) showed the highest frequencies on the North side.

Fleshy filter feeders (FFF) group was rare in all pH zones and appeared only on the North side after 33 months in the ambient pH.

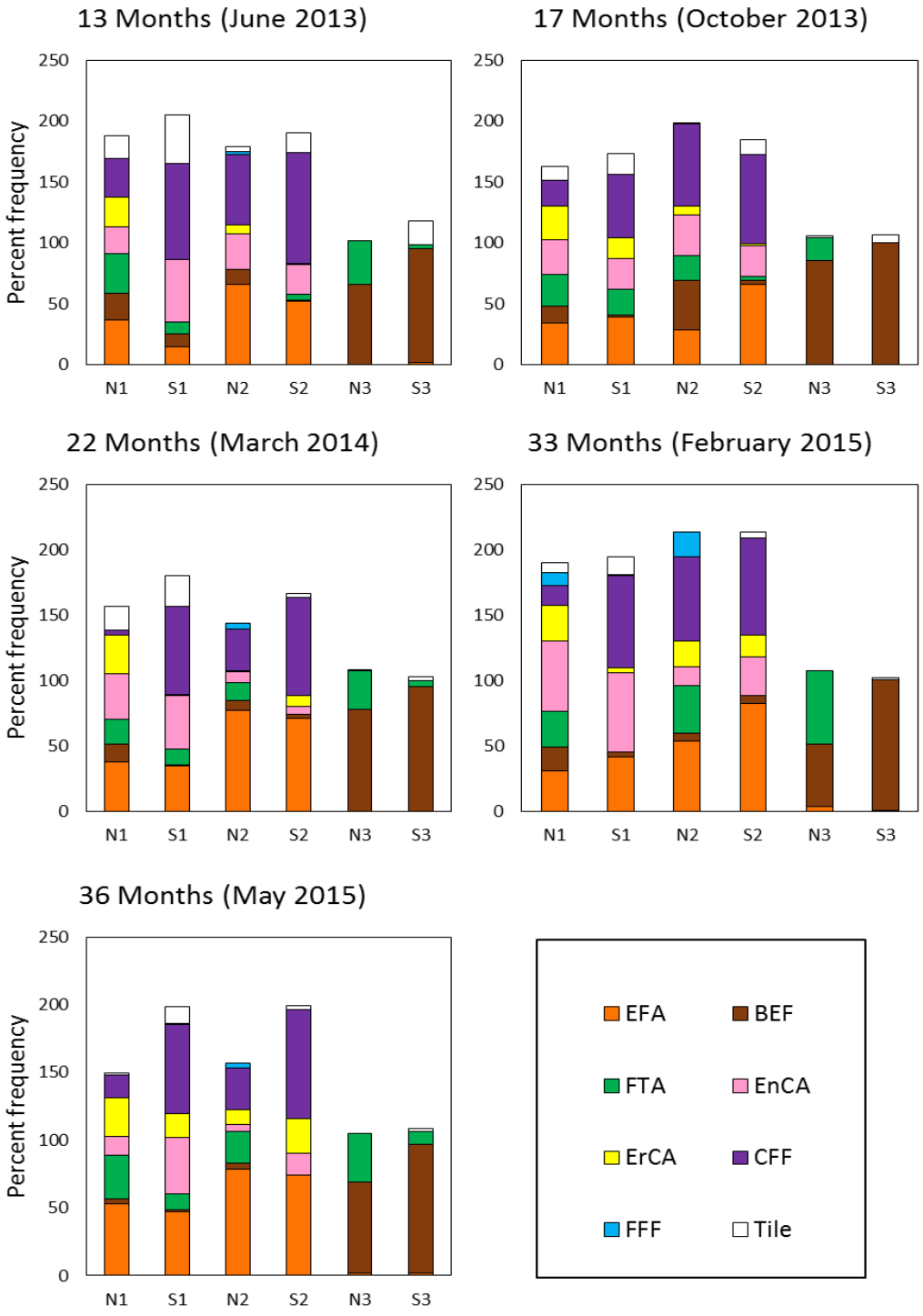

Fig. 9. Community structure (based on functional groups) trough time. Each bar represents the mean of percent frequencies of the functional groups that occured in each station in the respective period. Functional groups: FFF: Fleshy Filter Feeders; CFF: Calcified Filter Feeders; EnCA: Encrusting Calcareous Algae; ErCA: Erect Calcareous Algae; BEF: Biofilm/Filamentous algae/Encrusting Fleshy algae; FTA: Fleshy Turf Algae; EFA: Erect Fleshy Algae.

Multivariate analyses based on functional groups gave an overall picture of the community distribution along the pH gradient, and their variability among sampling periods.

MDS ordination revealed separate clusters corresponding to different pH conditions and sides while the sampling periods appeared scattered and without a clear pattern (*Fig.* 10).

PERMANOVA analysis showed significant community variations among: pH zones, sides and interaction between these two factors, but not related to sampling times (*Table 3*).

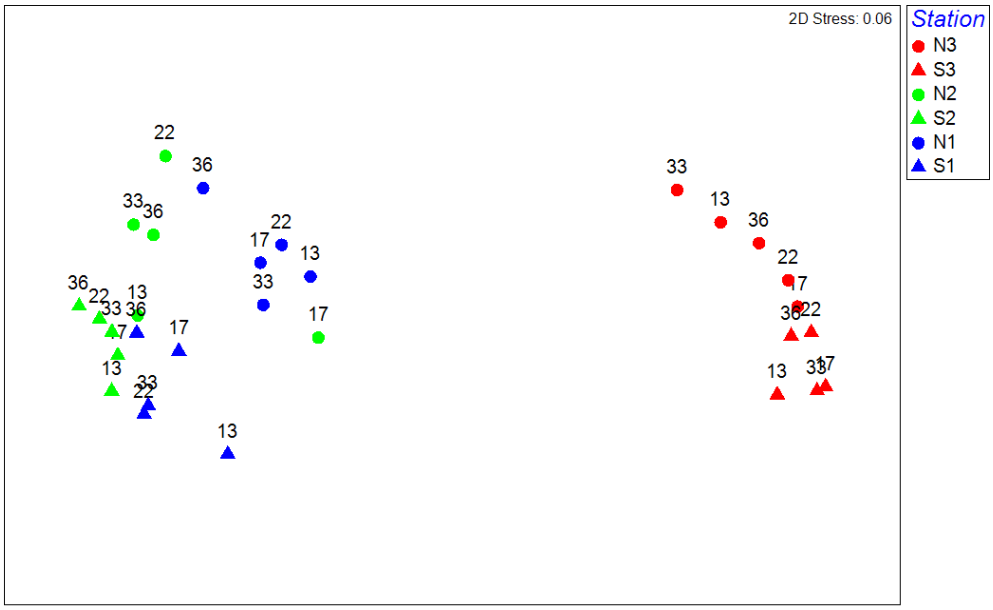

Fig. 10. MDS of distances among centroids of community structure. Based on percent frequency of functional groups in each station and time. Numbers represents months after installation of the tiles (13, 17, 22, 33 and 36). Circles, North side; triangles, South side. Red, extreme low pH; green, low pH; blue, ambient pH.

Table 3. PERMANOVA analysis on functional groups in five sampling times.

Source of variation	df	SS	MS	Pseudo-F	P(perm)
pH	2	1.9113E5	95565	90.937	0.0002
Si	1	15567	15567	17.67	0.0024
Ti	4	5059.4	1264.8	1.3717	0.1947
pHxSi	2	8532.7	4266.4	9.4566	0.0027
pHxTi	8	8407.2	1050.9	1.1397	0.3054
SixTi	4	3523.8	880.95	0.95538	0.4665
pHxSixTi	8	3609.2	451.15	0.48926	0.9672
Residual	120	1.1065E5	922.1		
Total	149	3.4648E5			

A stepwise DistLM analysis showed the functional groups that most contributed to the total variance. These were BFE (Pseudo-F=173.08, *pperm*=0.0001, Prop.=0.53905) and FTA (Pseudo-F= 159.91, *pperm*=0.0001, Prop.=0.24017). The percent frequency of these functional groups over time is showed in *Fig. 11*.

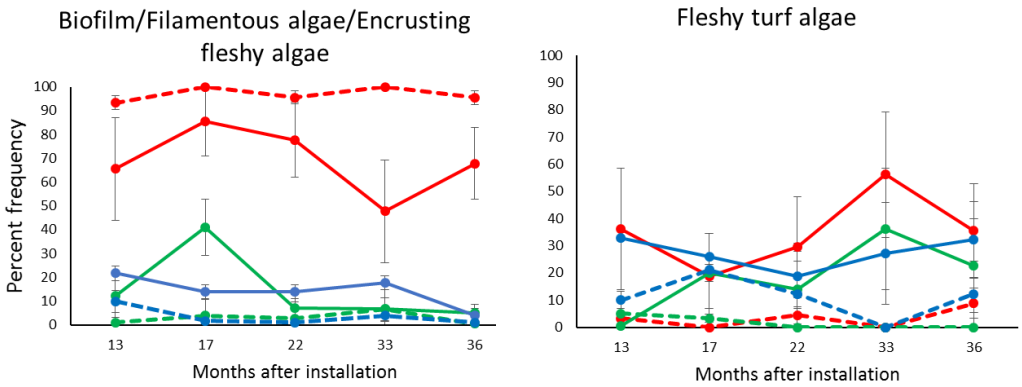

Fig. 11. Mean percent frequency of Biofilm/Filamentous Algae/ Encrusting Fleshy Algae (BEF) and Fleshy Turf Algae (FTA) through time, in each station ± standard error (n=5). Red, extreme low pH; green, low pH; blue ambient pH. Continuous line, North side; dotted line, South side.

The abundances of Biofilm/Filamentous Algae/Encrusting Fleshy Algae (BEF) differed significantly between sides and among pH zones (PERMANOVA pH x Si: pseudo-$F_{2,8}$=17.044, *p*= 0.0018) but not over time (Pseudo-$F_{4,120}$ = 2.0459, *p*=0.0952). Particularly, BEF frequencies in extreme low pH were significantly higher (about 97% on the South side and 68% on the North side) compare to the other two pH zones (in low pH 3% on the South side and 14% on the North side; in ambient pH 14% on the South side and 3.5% on the North side; *Fig. 11*).

Fleshy Turf Algae showed significant differences both according to pH conditions and sides (PERMANOVA pH: pseudo-$F_{2,8}$= 5.3571, *p*=0.0379; Si: pseudo-$F_{1,4}$=17.101, *p*=0.0218). This functional group appeared significantly more abundant on the North than on the South sides (*p*=0.0233). In particular, pairwise test showed that in low pH condition the abundances were significantly lower respect to both ambient and extreme low pH.

3.2.2 Analyses of taxa

The analyses between matrices based on functional groups and taxa showed a high correlation (Spearman index $\rho = 0{,}824$). Species richness (*S*) was clearly lower (from 1 to 2.4 taxa) in extreme low pH than in the other pH zones (from 4.4 to 8.8 taxa at low pH and from 5 to 6.6 taxa at ambient pH) in all the sampling times *(Fig. 12)* (PERMANOVA pH x Ti: Pseudo-$F_{8,120}$= 2.9982, *p*=0.004). Pairwise tests showed that species richness of the extreme low pH was significantly different from low and ambient pH at the 13, 17, 22 and 33 months. In the last sampling period (36 months), assemblages in all the pH zones appeared significantly different one from the other; in fact, there were more taxa in low pH than in ambient pH (*Fig. 12*).

Analyses on Shannon-Wiener diversity indices (*H'*) highlighted that diversity was lower in extreme low pH (from 0 to 0.66) than in low (from 1.12 to 1.75) and ambient (from 1.26 to 1.62) *(Fig. 12).* This was verified in both sides and in all sampling times (*Fig. 12*) (PERMANOVA pH x Si: Pseudo-$F_{2,8}$= 5.7847, *p*=0.0293).

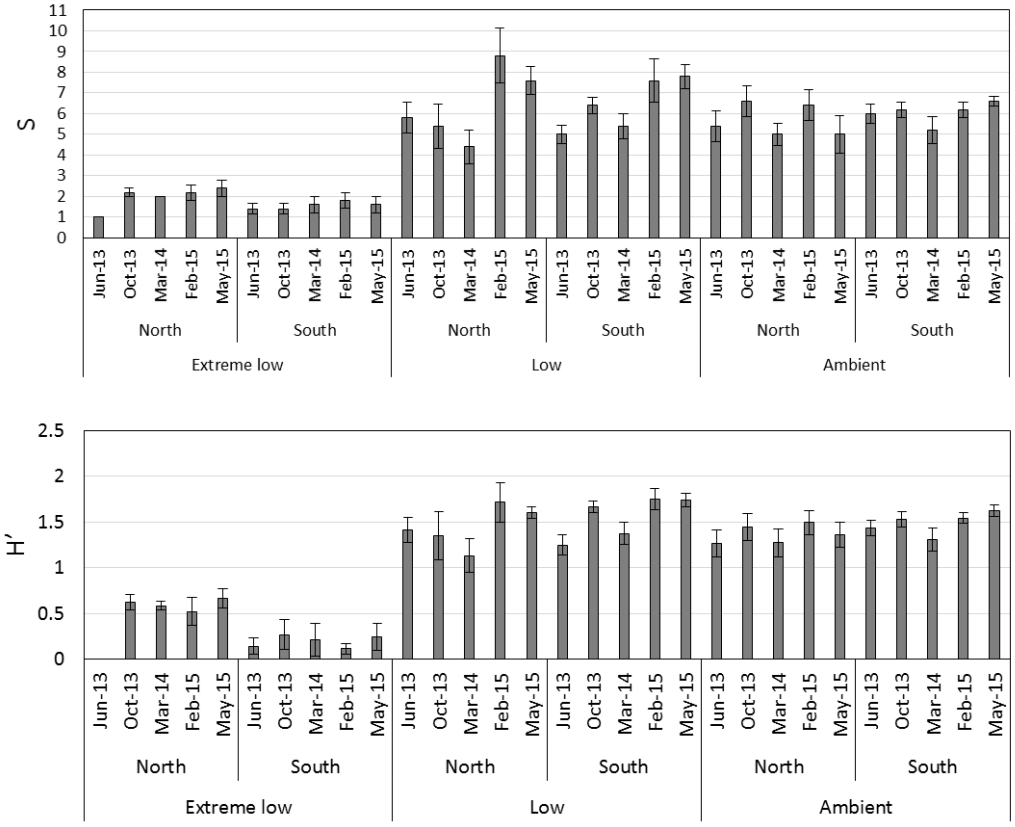

Fig. 12. Species richness (S) and Shannon-Wiener diversity index (H'). The data are means ± standard errors (n=5).

The MDS plot on taxa dataset showed a higher dispersion in very low pH conditions for both sides (*Fig. 13*). Data in low and ambient pH were closer and clustered together (*Fig. 13*).

Significant differences in frequency of taxa were found in relation to the interaction between pH and side and in time (*Table 4*). Pairwise tests showed that all the pH conditions were different one from the other on both sides of the islet and in all the sampling times. An exception was the 36th month samples, where low and ambient pH community became more similar. In extreme low pH, there was no significant difference in taxa composition between North (1-2.4 mean number of taxa) and South (1.4-1.8 mean number of taxa), whereas the two sides were different in the other pH zones.

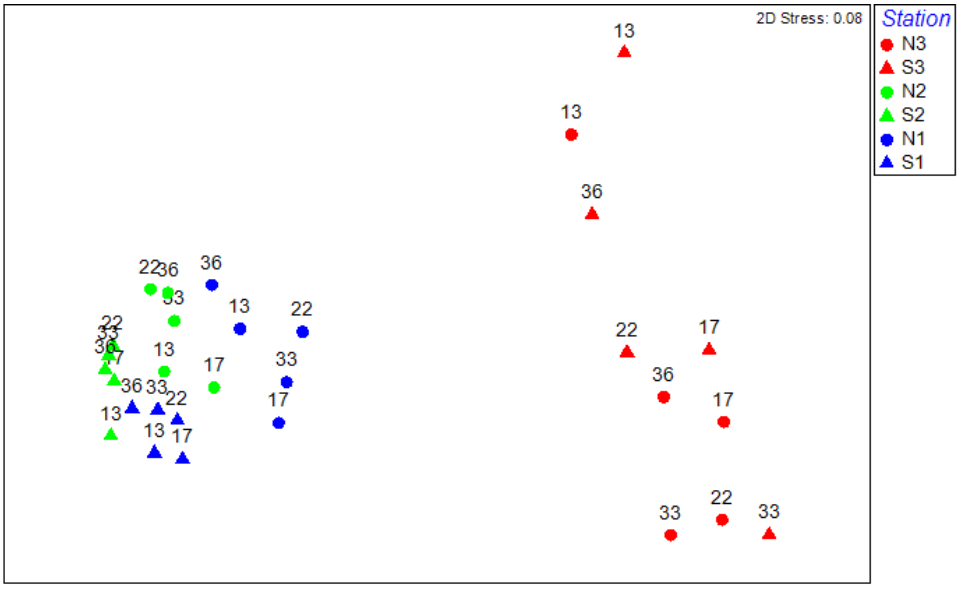

Fig. 13. MDS based on distances among centroids of taxa percent frequency. Numbers represents months after installation of the tiles (13, 17, 22, 33 and 36). Circles North side, triangles South side. Red, extreme low pH; green, low pH; blue, ambient pH.

Table 4. PERMANOVA on Taxa dataset in five sampling times.

Source of variation	df	SS	MS	Pseudo-F	P(perm)
pH	2	1.7524E5	87619	16.386	0.0001
Si	1	21401	21401	9.4323	0.0003
Ti	4	24702	6175.5	3.6455	0.0001
pHxSi	2	19866	9933.2	6.1687	0.0005
pHxTi	8	42777	5347.1	3.1565	0.0001
SixTi	4	9075.4	2268.9	1.3393	0.1308
pHxSixTi	8	12882	1610.3	0.95056	0.5498
Residual	120	2.0328E5	1694		
Total	149	5.0922E5			

DistLM analyses on the entire species dataset showed that Biofilm red (Pseudo-F=42.344 *pperm*=0.0001, Prop.=0.22246) and Biofilm (Pseudo-F=46.205, *pperm*=0.0001, Prop.=0.18595) were the taxa that mostly contributed to the

variance, as they occurred only in Extreme low pH (Biofilm red) very abundantly (Biofilm).

A succession trajectory of the different periods (based on taxa) illustrated that there was not a defined pattern of variation during time and among the pH zones (*Fig. 14*), except for those in ambient pH, which showed a more regular pattern (*Fig. 14*).

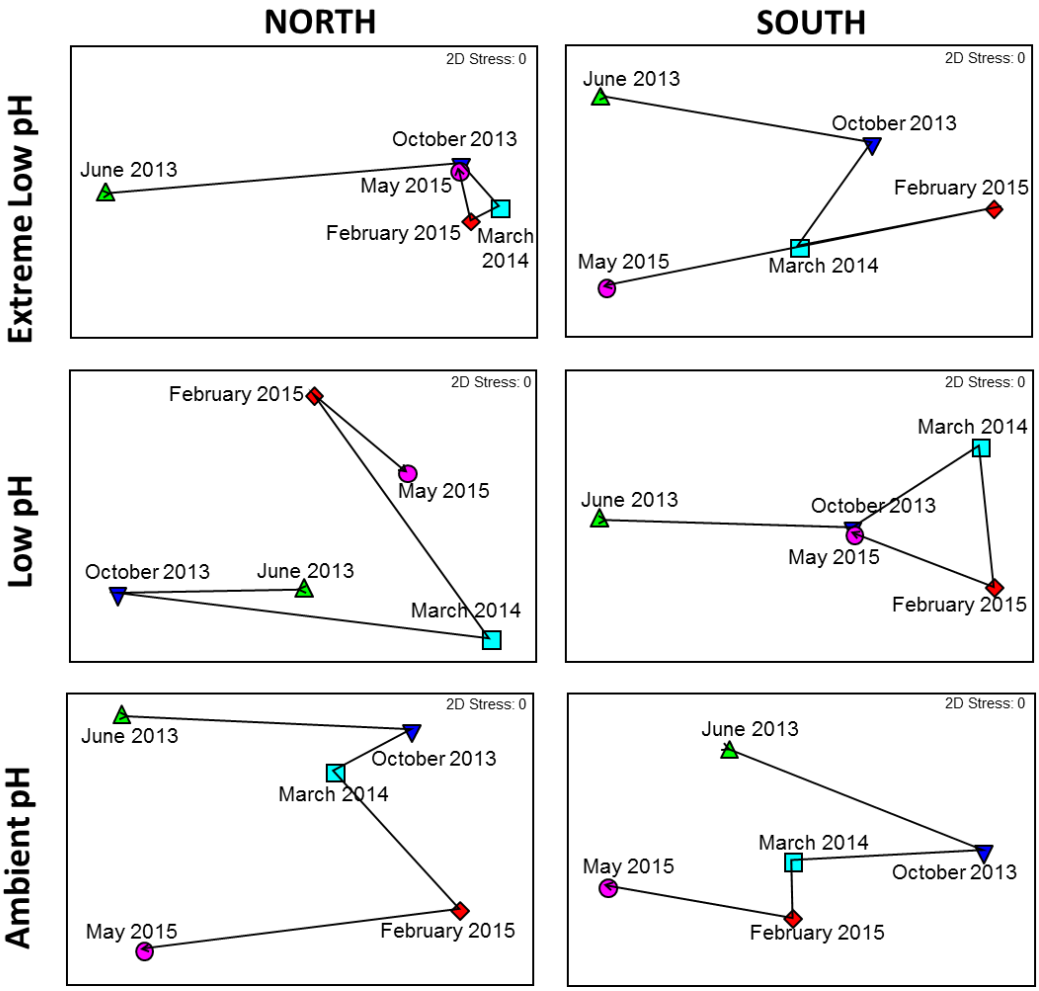

Fig. 14. Successional trajectories of community through time, based on distances among centroids of taxa percent frequency in each sampling time. Red triangle, June 2013; blue triangle, October 2013; blue square, March 2014; red diamond, February 2015, pink circle, May 2015.

3.3 Assemblage structure on tiles *vs* natural substrates

The structure of the species assemblages based on functional groups on the oldest tiles (36 months of exposure) was compared with natural "disturbed" and "undisturbed" substrates (*Fig. 15).*

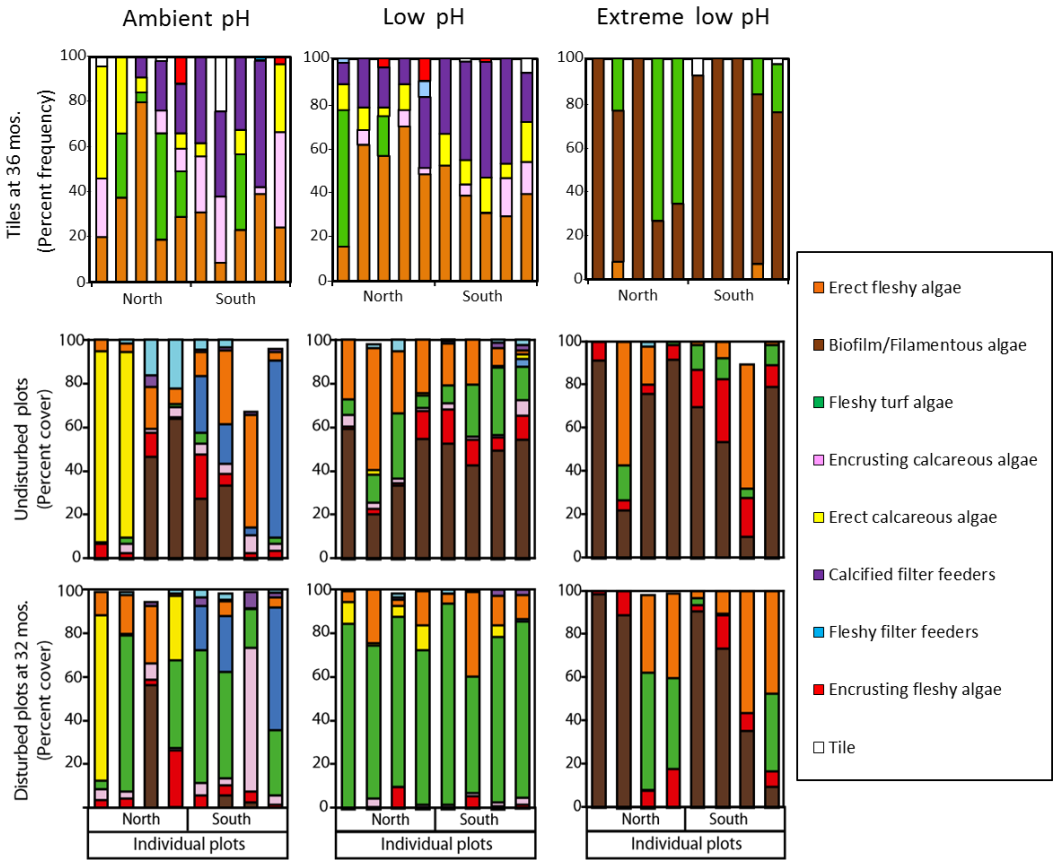

Fig. 15. Community structure based on functional groups, comparisons between tiles after 36 months (on top), and natural undisturbed substrate (middle) and disturbed natural plots (bottom) after 32 months (natural plots from Kroeker et al., 2013b).

In extreme low pH, there was homogeneity between all types of substrates. However, natural plots (both disturbed and undisturbed) showed much more Erect fleshy Algae (EFA) than tiles. Furthermore, the Encrusting Fleshy Algae group was represented mainly by *Hildenbrandia* sp. in natural substrate (Kroeker et al. 2013b). In our results, this species was included in the Biofilm (*Fig. 16*). Thus, we called this mixture complex as "Biofilm red" and classified in Biofilm/Filamentous algae/Encrusting Fleshy algae (BEF).

At low pH, differences between tiles and type of natural substrates were more clear *(Fig. 15).* The most abundant functional groups were Biofilm and Erect Fleshy Algae on undisturbed natural plots, Fleshy Turf Algae on disturbed plots and Erect Fleshy Algae (EFA) and Calcified Filter Feeders (CFF) on tiles. Moreover, in this pH zone, Calcified Filter Feeders (CFF) were very frequent only on tiles and absent in both types of natural substrates. Furthermore, Erect Calcareous Algae (ErCA) was totally absent in undisturbed natural plots, whereas it was present in almost all tiles and disturbed natural plots.

In ambient pH, community on tiles was very similar to both natural types of substratum as regards to group variability and patchiness (*Fig. 15*). Overall, there was a lack of the Calcified Turf Algae (CTA) on the tiles but this group was very abundant on natural plots. Biofilm was absent on tiles, but it was abundant on natural substrates, especially on those undisturbed. Erect Calcareous Algae (ErCA) and Encrusting Calcareous Algae (EnCA) were abundant on tiles and all the natural plots in ambient pH.

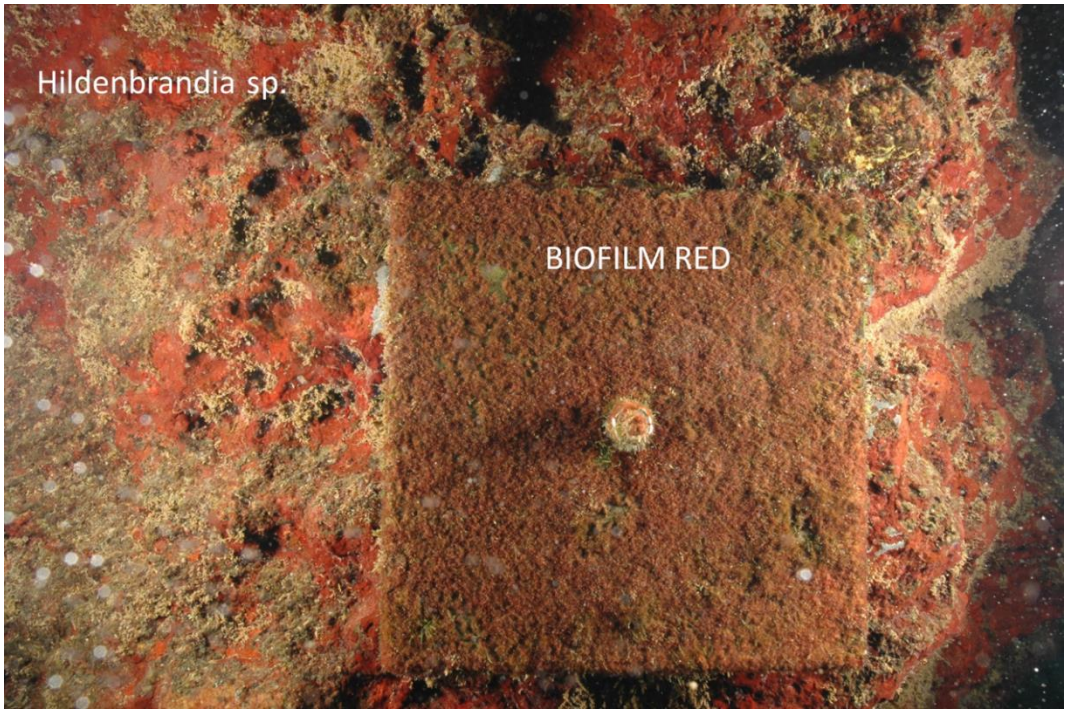

Fig. 16. Differences between Hildenbrandia sp. on natural substrate and Biofilm red (complex Hildenbrandia sp./Biofilm) that grows only on tiles rock.

3.4 Variability patterns between 2013 and 2015

3.4.1 Variability of functional groups

Differences in the abundances of functional groups between the first (13 months) and the last (36 months) sampling periods were investigated with multivariate analyses.

MDS plot showed that the samples were dispersed according to pH zones and sides, but there was no pattern through time (*Fig. 17*). Abundances of functional groups differed significantly between the pH zones and between sides, wile limited variation occurred over time (*Table 5*).

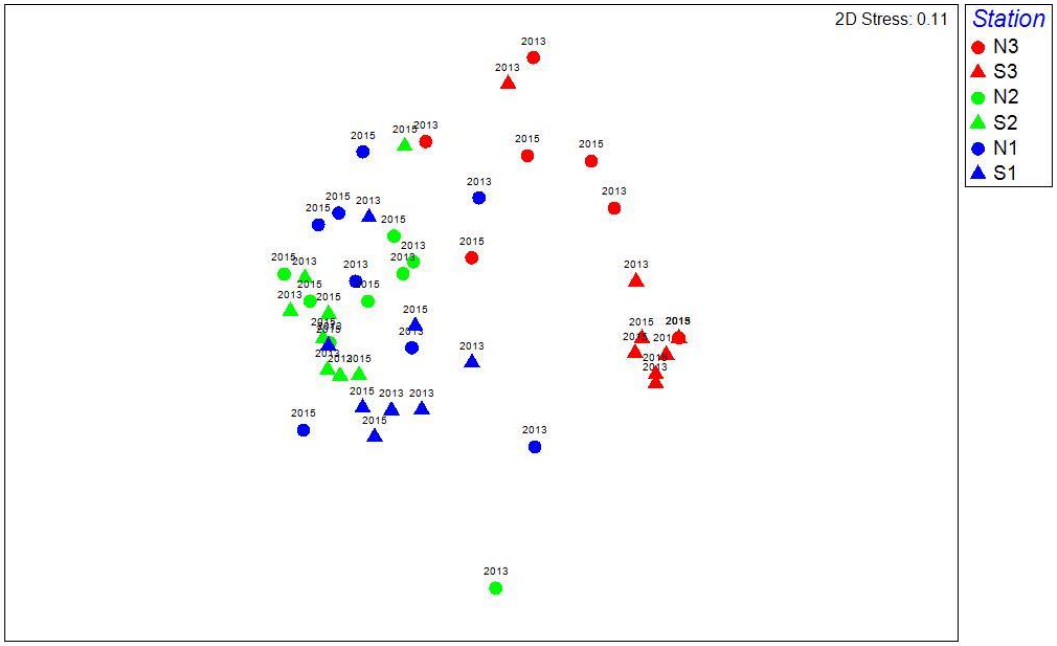

Fig. 17. MDS community structure of functional groups. Triangles, North; circles, South. Red, extreme low pH; green, low pH; blue, ambient pH.

Table 5. PERMANOVA on Functional groups in 2013 and 2015.

Source of variation	df	SS	MS	Pseudo-F	P(perm)
pH	2	46009	23005	18.771	0.0001
Si	1	5445.7	5445.7	4.4435	0.0099
Ye	2	2665	2665	2.1745	0.1024
pHxSi	2	4807	2403.5	1.9611	0.0925
pHxYe	2	3969.4	1984.712	1.6194	0.1532
SixYe	1	1247.4	47.4	1.0178	0.3718
pHxSixYe	2	880.26	440.13	0.35912	0.8807
Residual	36	44120	1225.6		
Total	47	1.0914E5			

3.4.2 Variability and diversity of taxa

The analysis of the two periods (2013, 2015) considering taxa, showed significant differences in species richness (*S*) between years and among pH zones (PERMANOVA pH: Pseudo-$F_{2,36}$= 45.272, p=0.0001; Year: Pseudo-$F_{1,36}$=5.4444, p=0.0282). In particular, there was a significantly lower number of taxa (from 1.75 to 3 on average) in extreme low pH than in the other two conditions (on average from 4.75 to 7.25 in low pH and from 4.5 to 7.5 in ambient pH).

PERMANOVA on Shannon-Wiener (*H'*) diversity showed that there were not significant differences related to the year of sampling, while the interaction of pH zones and side was significant (pH x Si: Pseudo-$F_{2,36}$=5.051, p=0.0124). Pairwise tests indicated that species diversity in extreme low pH was significantly lower (on average 0.36-0.65 between 2013 and 2015) respect to the other two conditions (1.28-1.55 low pH and 1.02-1.41 ambient pH), at the North side. On the other hand, on the South side, diversity was lower (1.19-1.6 on average between 2013 and 2015) in low pH respect to ambient (1.55-1.77 on average between 2013 and 2015). Furthermore, differences between sides were not significant, except for ambient pH conditions.

MDS ordination on frequencies of taxa showed that samples were distributed according to pH and sides (*Fig. 18*).

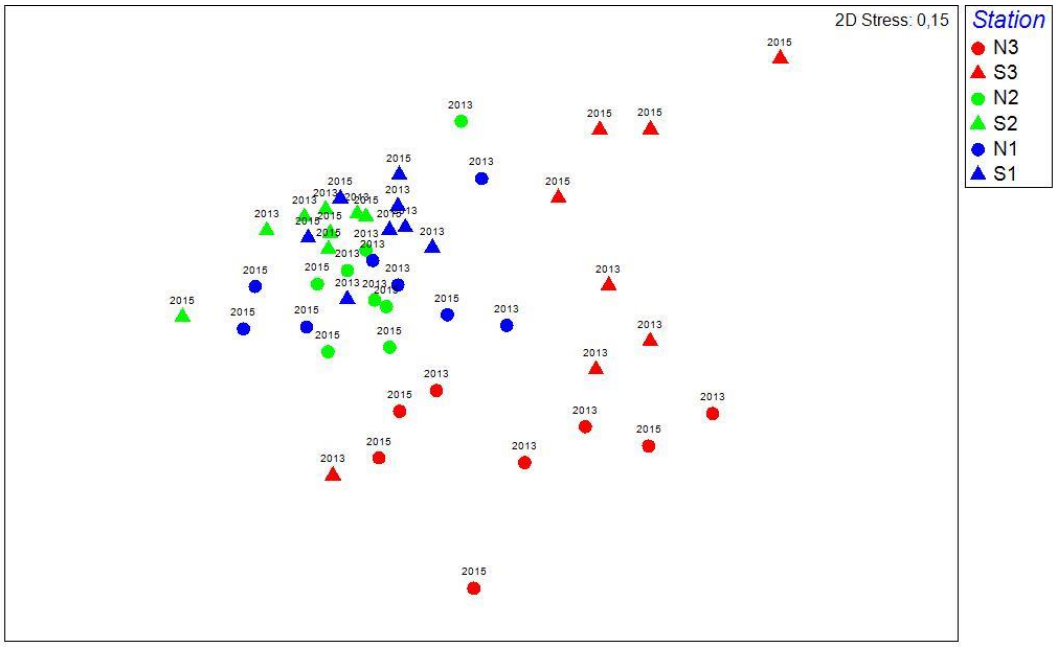

Fig. 18. MDS community structure of taxa in the first (2013) and last (2015) sampling period. Triangles, North side; circles, South side. Red, extreme low pH; green, low pH; blue, ambient pH.

PERMANOVA showed significant differences in the interaction between pH, side and year (*Table 6*). Post hoc test indicated that community variability in Extreme low pH was significantly different from the other two conditions. Moreover, there were not significantly differences between North and South in extreme low and ambient pH in 2013. Finally, differences between 2013 and 2015 were recorded for extreme low pH in both sides and for ambient pH in the North side.

Table 6. PERMANOVA on taxa data at 13 (2013) and 36 (2015) months after the installation.

Source of variation	df	SS	MS	Pseudo-F	P(perm)
pH	2	38621	19310	11.301	0.0001
Si	1	9967.6	9967.6	5.8334	0.0002
Ye	1	8119.1	8119.1	4.7516	0.0003
pHxSi	2	11923	5961.5	3.4889	0.0001
pHxYe	2	4371.7	4371.762	2.5585	0.0021
SixYe	1	6298.6	98.6	3.6862	0.0021
pHxSixYe	2	9293	4646.5	2.7193	0.0011
Residual	36	61513	1708.7		
Total	47	1.5448E5			

3.5 Comparing methods: visual census *vs* image analyses

Visual census analysis allowed to recognize a higher number of taxa than photo analysis. We found 10 taxa analysing photos and 11-13 taxa using the visual census at low pH, and 8 taxa in photos and 9-11 taxa by visual census in ambient pH zones (*Fig. 19*). In extreme low pH, results were identical with both methods (3-4 taxa). Therefore, we excluded the Extreme low pH condition in the comparison of the methods using multivariate analysis.

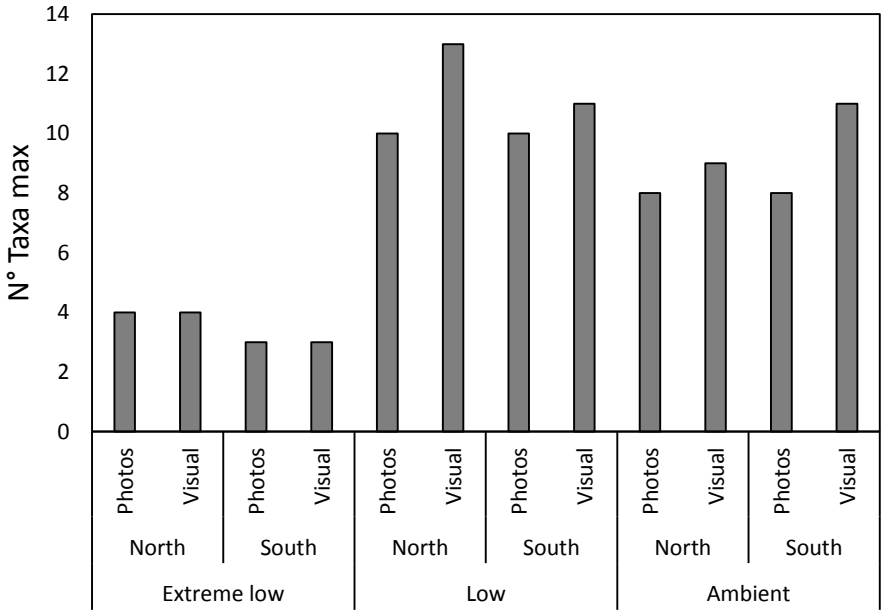

Fig. 19. Total number of taxa recorded in each station (pH zone). Photos, identification form photographs; Visual, identification based on visual census.

MDS ordination showed no differences in the ordination of the sampling points according to the methods (*Fig. 20*). Thereby, the method used to estimate the abundance of taxa did not affect results of the analyses. The main source of variability in the data set was related to different pH zone and the sides (*Table 7*).

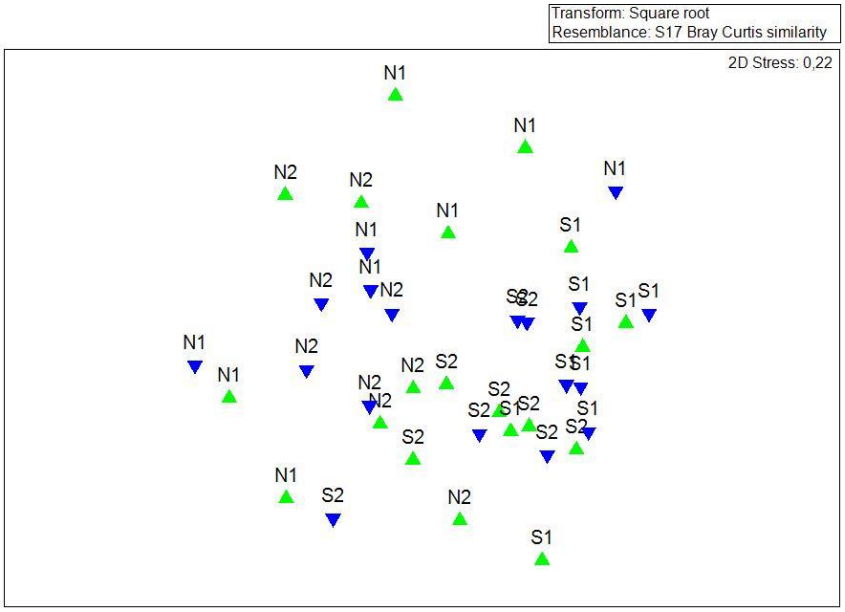

Fig. 20. MDS based on taxa frequency on tiles. Green triangles, photos; blue triangles visual census. We excluded extreme low pH data from the analysis because of their identical results obtained with both methods.

Table 7. PERMANOVA analysis on taxa frequencies data obtained with photos and visual census techniques.

Source of variation	df	SS	MS	Pseudo-F	P(perm)
pH	1	3582,2	3582,2	2,6765	0,0117
Si	1	11874	11874	8,872	0,0001
Me	1	1207,1	1207,1	0,90193	0,5109
pHxSi	1	2633	2633	1,9673	0,0669
pHxMe	1	598,23	598,23	0,44698	0,8615
SixMe	1	1475,5	1475,5	1,1025	0,3688
pHxSixMe	1	460,97	460,97	0,34442	0,917
Residual	30	40152	1338,4		
Total	37	62781			

4 DISCUSSIONS

Coastal ecosystems present high diversity and represent one of the most productive marine habitats. They provide many goods and services to society (e.g. fisheries, aquaculture, protection, commercial, and cultural services) and thus are critically important with respect to future impacts from environmental changes and ocean acidification (OA) (Cooley et al. 2009). Shallow rocky shore habitats represent nursery areas and home for many fish and crustacean species, provide shelter, are a resource of food, and contribute to the stabilization of inshore sediments. Thus, alteration in these communities can bring negative consequences in coastal lands, affecting human wellbeing and economy.

In this study, the effects of OA on Mediterranean rocky-shore communities over time was-studied using artificial tiles deployed along a natural gradient of OA and sampled at different times. In extreme low pH zones, at both North and South sides of Castello Aragonese, taxa and functional groups of the benthic assemblages were very simplified and showed a homogeneous structure (reduced patchiness) in comparison to low and ambient pH zones. According to previous studies (Kroeker et al. 2013a, Kroeker et al. 2013b), assemblages in extreme acidified condition remained in early-colonization status, showing few dominant functional groups (Biofilm/Filamentous Algae/Encrusting Fleshy Algae-BEF and Fleshy Turf Algae-FTA) and the lowest species diversity. This structure of the assemblage was consistent in all the sampling dates during the 3 years of the study. It is well known that loss of species diversity alters ecosystem functions affecting the efficiency of energy transfer among trophic levels (Barry et al. 2011).

Several abiotic components (e.g. irradiance, exposure to waves, wind effect) vary between the northern and the southern side of Castello Aragonese. Even pH values and vents activity differ between the two sides, especially in the extreme acidified zones, resulting in lower pH values in the South, respect to the North side (*Table 1*). Our results have shown, in extreme low pH zone of the northern side, a high abundance of FTA during most of the sampling dates. Our results agrees with previous short-term study of Porzio et al. (2013) showing that on tiles located in

lowest pH zones, turf algae recruit rapidly and thrive, and that southern stations recorded lower number of species compare to the North stations. Turf algae are resilient to ocean acidification (Russell et al. 2009) and their opportunistic behavior could limit the growth of other species in extremely acidified situations both in early stages of recruitment and in mature assemblages.

As pH, or other environmental condition shifts, organisms initially respond based on physiological and behavioral adaptations molded through their evolutionary history (Somero 2012). New conditions may be physiologically tolerable, allowing acclimatization (an adjustment of physiology within individuals) or adaptation (increased abundance and reproduction of tolerant genotypes over generations). If new conditions may not be tolerable possible responses are migration (of species or populations), change in phenology (timing of annual events), or death and local extinction if adaptation is not possible (Parmesan 2006). The overall lack of calcareous species on the tiles in extreme low pH, during the 3 years study, may be a result of physiological intolerance to acidified situation. Otherwise, the presence of calcareous groups in low pH, according with previous studies in natural acidified conditions (Hall-Spencer et al. 2008, Kroeker et al. 2013a, Kroeker et al. 2013b), could be due to acclimatization. Indeed, the natural variability in carbonate chemistry of vents system could facilitate the settlement of calcifying propagules/larvae of some taxa, including calcareous algae (Steneck 1986) and barnacles, found in low acidified condition. Furthermore, vents areas are surrounded by normal seawater conditions, and most propagules/spores may be supplied by population living in ambient conditions.

Although Encrusting Calcareous Algae (EnCA) and Erect Calcareous Algae (ErCA) were found in low pH, their abundance were reduced under this condition. Otherwise, we have found the highest frequency of Erect Fleshy Algae (EFA) in low pH. Many fleshy seaweeds can increase their photosynthetic and/or growth rates with the increase concentration of CO_2 or HCO_3^- associated with acidification (Hurd et al. 2009, Connell & Russell 2010). Therefore, we hypothesized that near-future acidification condition could alter the competitive interaction between EFA and calcareous algae, promoting the growth of the first group disadvantaging the

second. Moreover, higher frequencies of EFA may be due to the lack or the reduction of grazers (such as sea urchins or limpets) on artificial substrates.

The highest abundances of calcareous taxa (members of EnCA, ErCA and Calcified Filter Feeders-CFF) were found in ambient pH, with elevate values of diversity indices.

Although pH and orientation affected clearly the assemblages, functional groups did not show a temporal variation; therefore, tiles could actually host mature and complex assemblages at functional level, already after 13 months from the deployment. Otherwise, taxa composition revealed variations during sampling periods but we did not identify clear and defined dynamic pattern. Thus, functional groups of the shallow benthic assemblages were constant but species composition changed over time. This pattern was also found when analyzing the variation of composition of functional groups and taxa between the first (June 2013) and the last (May 2015) sampling dates. These results suggest that temporal variation is associated to species belonging to the same functional group, showing functional redundancy. Functional redundancy is based on the observation that some species perform similar roles in communities and ecosystems, and may therefore be interchangeable with little impact on ecosystem processes (Lawton & Brown 1994). Therefore, the analysis of functional groups, on which previous studies in this area have been based, keeps information on the functional structure, but loses information on species composition and diversity. Thus, further analyses are required to investigate functional relationship between species to help conservation decisions, in response to future OA, and provide a basis for anticipating the impact of different management scenarios on ecosystem function (Walker 1995).

Mediterranean phytobenthic communities shows seasonal patterns with peak of number of species cover/biomass in spring period (Ballesteros 1992). Thus, the variability not explained by pH and side, could be due to the effect of seasonality of the species masking the temporal factor. However, in the present study seasonality *sensu stricto* has not been considered. Therefore, further studies are required to investigate the seasonal variations under acidification conditions that could affect temporal dynamics of taxa.

Comparing community structure on tiles with natural substrates, our study showed the presence of the taxon "Biofilm red" only on artificial volcanic rock and in the extreme low pH zones. Indeed, we hypothesized that this taxa is the result of an interaction between *Hildenbrandia* sp. and Biofilm. Studies on natural substrates have shown that Encrusting Fleshy Algae (EFA), including *Hildenbrandia* sp., were better adapted than other algal forms at stress conditions (biotic and abiotic) (Dethier 1994). Accordingly, the natural substrate conditions showed an elevate frequency of this species in extreme low pH zones. The presence of Biofilm red on tiles may be due to differences in fine mineralogical composition between natural rocky substrate and artificial volcanic rock tiles.

Finally, advantages and disadvantages are associated with both photo analyses and visual census methods used in this study (Pech et al. 2004). Visual quadrats allowed identification of taxa through direct observation and recognition of organisms hidden by other species, commonly found in shallow benthic communities. On the other hand, this method required longer underwater time and divers trained in species identification. Photography reduces the time spent underwater but involves laboratory time for processing after sampling. Moreover, the post-sampling analyses greatly depend on the quality of the photos (Magorrian & Service 1998). In this study, the sampling unit observed trough the two techniques was the same (the surface of the tiles), photos and visual quadrats did not give different responses in the analysis of the assemblages. However, there were differences in the number of taxa identified in low and ambient pH zones. Indeed, visual quadrats allowed to identify species below the canopy layer of the Erect Fleshy Algae (EFA), whereas in photo analysis only a bi-dimensional layer were investigated.

5 CONCLUSION

Volcanic CO_2 vents systems are not perfect predictors or analogs of future ocean acidification (Hall-Spencer et al. 2008), however they acidify seawater on sufficiently large spatial and temporal scales to integrate ecosystem process. This study revealed how composition and dynamics of a shallow benthic community were altered and simplified in conditions representing future acidification scenarios. Extreme low conditions of pH (pH of about 6.9), affected strongly the assemblages both at the level of taxa and of functional groups. Patterns of temporal variation were observed in species composition but not in functional groups, suggesting that they were related to species belonging to the same functional group, and suggesting the occurrence of functional redundancy. Therefore, the analysis of functional groups kept information on the structure, but lost information on species dynamics.

Decreasing in ocean pH is only one of many future global changes that will occur at the end of this century (increase of ocean temperature, water level rise, eutrophication etc.). The interaction between these factors and OA could exacerbate the community and ecosystem effects found in this thesis.

6 APPENDIX

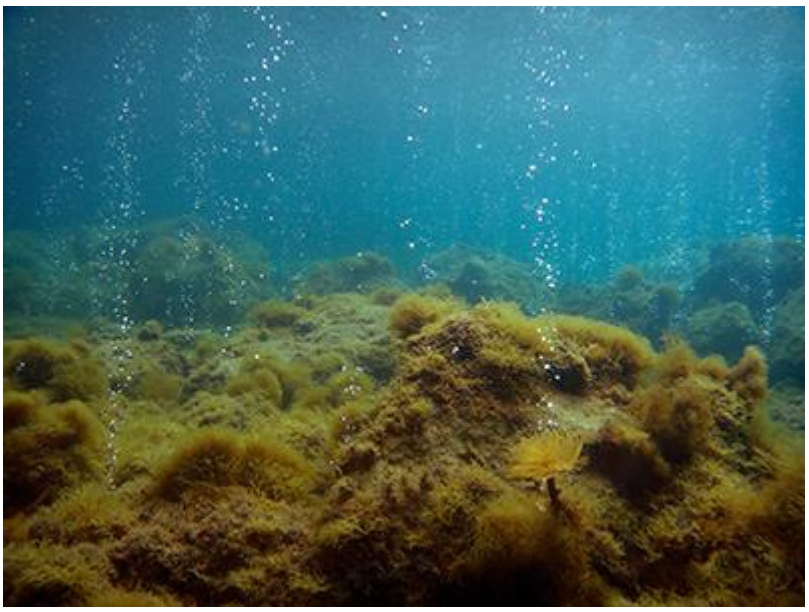

Fig. A1. Photo of vents activity in the extreme acidified condition of the southern side of Castello Aragonese. Photo of Enric Ballesteros.

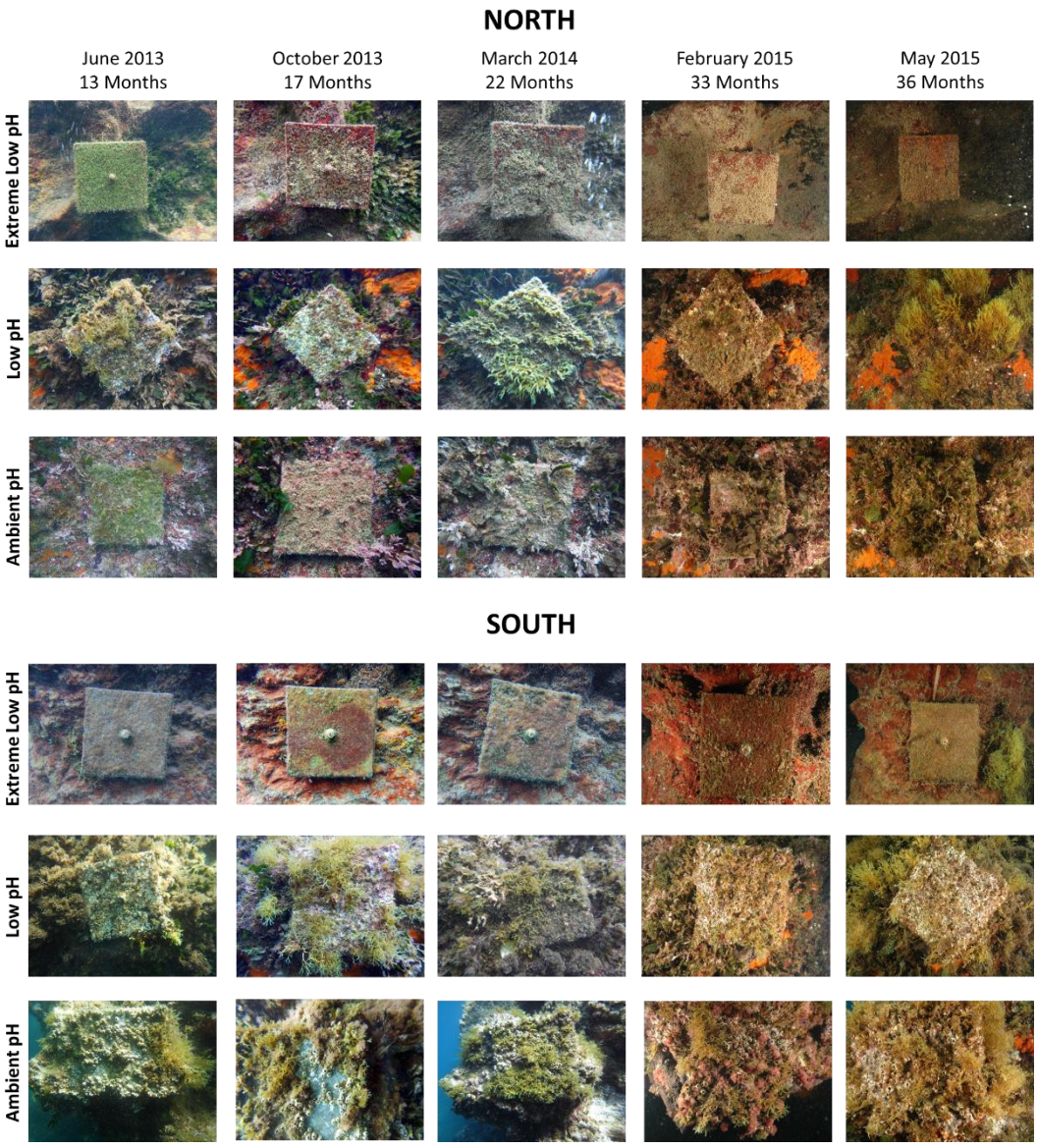

Fig. A2. Examples of the same tiles followed over time.

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