Mediterranean Marine Science

Vol 15, No 3 (2014)

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doi: 10.12681/mms.677

To cite this article:

DONNARUMMA, L., LOMBARDI, C., COCITO, S., & GAMBI, M. (2014). Settlement pattern of Posidonia oceanica epibionts along a gradient of ocean acidification: an approach with mimics. *Mediterranean Marine Science*, *15*(3), 498–509. https://doi.org/10.12681/mms.677

Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
Doi: http://dx.doi.org/10.12681/mms.677

Settlement pattern of *Posidonia oceanica* epibionts along a gradient of ocean acidification: an approach with mimics

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Handling Editor: Argyro Zenetos

Received: 29 October 2013; Accepted: 4 February 2014; Published on line: 28 February 2014

Abstract

Effects of ocean acidification (OA on the colonization/settlement pattern of the epibiont community of the leaves and rhizomes of the Mediterranean seagrass, *Posidonia oceanica*, have been studied at volcanic CO_2 vents off Ischia (Italy), using "mimics" as artificial substrates. The experiments were conducted in shallow *Posidonia* stands (2-3 m depth), in three stations on the north and three on the south sides of the study area, distributed along a pH gradient. At each station, 4 rhizome mimics and 6 artificial leaves were collected every three months (Sept 2009-Sept 2010). The epibionts on both leaf and rhizome mimics showed clear changes along the pH gradient; coralline algae and calcareous invertebrates (bryozoans, serpulid polychaetes and barnacles) were dominant at control stations but progressively disappeared at the most acidified stations. In these extremely low pH sites the assemblage was dominated by filamentous algae and non calcareous taxa such as hydroids and tunicates. Settlement pattern on the artificial leaves and rhizome mimics over time showed a consistent distribution pattern along the pH gradient and highlighted the peak of recruitment of the various organisms in different periods according to their life history. *Posidonia* mimics at the acidified station showed a poor and very simplified assemblage where calcifying epibionts seemed less competitive for space. This profound difference in epiphyte communities in low pH conditions suggests cascading effects on the food web of the meadow and, consequently, on the functioning of the system.

Keywords: Seagrass, epiphytes, mimics, CO_2 vents, colonization pattern, recruitment, biodiversity, Mediterranean Sea.

Introduction

The amount of CO_2 in the atmosphere has steadily increased since the industrial period. According to estimates, if greenhouse gas emissions continue to rise at current rates, the atmospheric CO_2 concentration levels will be 500 ppm by 2050 and 800 ppm by 2100 (IPCC, 2007). The surface ocean pH level may therefore fall to 7.7 or 7.8 with an increase in acidity of 150 % compared to pre-industrial values (Hardt & Safina, 2010). This decrease in the ocean pH level, as a result of atmospheric CO_2 dissolution in the surface waters of the oceans, is termed "ocean acidification" (OA) (Caldeira & Wickett, 2003) or "the other CO_2 problem" (Doney *et al.,* 2009). In particular, OA is the result of carbonic acid formation (H_2CO_3), which dissociates to bicarbonate (HCO_3^-), carbonate ions (CO_3^{2-}) and protons (H^+).

Acidification affects mainly the process of calcification of organisms such as corals, molluscs, and many other organisms with skeletons and shells composed of calcium carbonate (Royal Society, 2005; Doney *et al.,* 2009; Lombardi *et al.,* 2011a, b). In addition, when the seawater is combined with other kinds of natural and/or anthropogenic stress factors (e.g. warming, pollution and overfishing), it may cause sensible changes in the benthic community structure (Kroeker *et al.,* 2010; Rodolfo-Metalpa *et al.,* 2011), impacting most fundamental biological and geochemical processes (Kleypas *et al.,* 2006; Fabry *et al.,* 2008). Many laboratory studies have shown that the early life stages of several organisms are also negatively influenced by acidified seawater (Kroeker *et al.,* 2010) and this has been observed also in *in situ* experiments at CO_2 vents (Cigliano *et al.,* 2010; Ricevuto *et al.,* 2012). However, the response of multispecies assemblages to OA at naturally acidified water is still poorly documented, especially regarding highly complex systems built by structuring, habitat-forming species, such as seagrass meadows (Martin *et al.,* 2008).

Posidonia oceanica (L.) Delile is the endemic and dominant seagrass in the Mediterranean Sea. It forms extensive meadows from the surface down to a maximum of about 40 m depth (Procaccini *et al.,* 2003). Studies on the fauna associated to *P. oceanica* revealed high diversity of species settled both on leaves and rhizomes (Mazzella *et al.,* 1992; Cocito *et al.,* 2012) and belonging to different taxonomic groups, which includes many calcifying taxa such as coralline algae, crustaceans, molluscs, serpulid polychaetes, bryozoans (Mazzella *et al.,* 1992; Buia *et al.,* 2000; Borg *et al.,* 2006; Balata *et al.,* 2007; Nesti *et al.,* 2009).

Within the *Posidonia* complex system, the epiphytes settled on the leaves and the rhizomes play an important role in the food web and functioning of the meadows (Mazzella *et al.,* 1992). The epiphytic algal component represents the main item for energy transfer within the "grazing chain" of the seagrass food-web, from primary producers to higher trophic levels through the consumption due to small mesoherbivores, a guild of grazers that not only fed on the system but also found shelter and permanent habitat in the meadow (Brawley, 1992). The animal component of the epiphytes is also consumed by other specialized invertebrates (Gambi & Morri, 2008) and shows interesting examples of adaptation to the special local conditions of the leaf canopy, with several characteristic and/or exclusive species (e.g. the bryozoan *Electra posidoniae*).

In addition, the epiphytes represent sensitive indicators of natural or anthropogenic impacts (e.g. eutrophication) (Morri, 1991), and are able to record the environmental alterations resulting from climate change (e.g. seawater acidification) (Martin *et al.,* 2008). In fact, the epiphytic community of *P. oceanica*, and especially that found on the leaves, mainly consists of short-lived species which show a highly seasonal development, following the cycle of leaf growth (Mazzella *et al.,* 1989), as well as a high degree of small scale spatial variability (Nesti *et al.,* 2009 and literature herein).

Up to date the only study dealing with seagrass epiphytes in relation to OA is that carried out by Martin *et al.* (2008) along an OA gradient at natural CO_2 vents off Ischia. These authors highlighted big differences in the composition and structure of the calcareous epiphytic community of *P. oceanica* leaves in acidified seawaters, where coralline algae and other calcifiers were strongly reduced or absent. However, Martin *et al.* (2008) limited their study to a single spatial transect (on the south side of the study area) and on a single observation period (April), and did not consider the community settled on the rhizomes.

Similar results, showing a reduced colonization of calcareous macroalgae and calcifying benthic invertebrates colonizing the hard rocky reefs, adjacent to the *Posidonia* stands in the same study area, were shown by other authors (Hall-Spencer *et al.,* 2008; Kroeker *et al.,* 2011; Porzio *et al.,* 2011). Some of the patterns observed in the adult populations of various species of the rocky substrate were due to selective pressure on the larval and juvenile stages (Cigliano *et al.,* 2010; Ricevuto *et al.,* 2012).

The overall aim of this study is to highlight the effects of ocean acidification on the colonization and settlement pattern of the *Posidonia* epibiont community of the leaves and rhizomes along a gradient of pH reduction, and increased pCO_2, using a new experimental approach with "mimics" (see Methods) (Gambi *et al.,* 2011; Cocito *et al.,* 2012). The plant mimics were used to reduce the impact of experimental studies on the natural *Posidonia* meadow within the study area. Moreover, mimics are essential for having an un-colonized substrate to examine the seasonal cycles of epiphyte re-colonization at a given time interval and to compare possible quantitative and qualitative differences in species between artificial and natural substrates (i.e. *P. oceanica* tissue). In detail, our goals are i) to characterize the *Posidonia* stands where *Posidonia* mimics were placed; ii) to report colonization pattern on the first three months of exposure along the gradients for both the leaves and rhizome mimics; iii) to study the settlement pattern through time of the main epibionts (limited to the south side only).

Materials and Methods

Study site

The study area is adjacent to Castello Aragonese, a volcanic dome and islet located at the north-eastern side of Ischia island (Gulf of Naples, Italy) (40°43.84'N 13°57.08'E) (Rittmann & Gottini, 1981). Previous and recent gas analyses (Tedesco, 1996; Hall-Spencer *et al.*, 2008) showed that the seawater is acidified by gas comprising 90.1-95.3 % CO_2, 3.2-6.6 % N_2, 0.6-0.8 % O_2, 0.08-0.1 % Ar and 0.2-0.8 % CH_4 (no sulphur present, while both water temperature and salinity do not change respect to normal conditions) (Hall-Spencer *et al.,* 2008; Kerrison *et al.,* 2011; Kroeker *et al.,* 2011). The seawater pH varies from the normal value of 8.17 to as low as 6.57 along a continuous gradient occurring both at the north and south-western sides of the Castello (Kroeker *et al.,* 2011; Lombardi *et al.,* 2011b). On both sides of the Castello islet a shallow *Posidonia oceanica* meadow subjected to CO_2 emission is present (Buia *et al.,* 2003; Hall-Spencer *et al.,* 2008). On the south side, the most intense venting activity include also *Posidonia* meadow, here in some restricted areas *Posidonia* can reach very shallow depth (0.5-1 m), and forms a sort of reef. This side of the Castello is also more sheltered to water movement respect to the north side which is exposed to the dominant north-western winds. At the north side, *Posidonia* meadow is very close to the active venting area, but direct gas bubbling is very limited or absent inside the meadow. In addition, in this area, anecdotal observations of one of us (MCG) as well as other researchers, testify that venting was absent in the early '80 (Russo G.F., Boudouresque C-F., Cinelli F., Ott J., Pronzato R., personal communication), so that water acidification is a relatively recent phenomenon on the north side of the Castello. The experiments are conducted in shallow *Posidonia* stands at 2.5-3.5 m depth, in three stations located on the north side (N1, N2, N3) and three on the south side (SC, S2, S3) distributed along a gradient of pH. The stations have been selected based on previous studies in relation to proximity to CO_2 emissions and mean pH values recorded (see Cigliano *et al.,* 2010 for a map of the area, Lombardi *et al.,* 2011b). The S3 and N3 stations, acidified sites with very low pH, are located in an area with high bubbling and dense emissions (<10 bubbles emissions to m^2). Mean pH values are approximately 7.2 at the northern and 6.6 at the southern sites, near the rocky reef (Kroeker *et al.,* 2011). In these stations, the *P. oceanica* meadow is very dense (over 900 shoots/m^2) (Buia *et al.,* 2003) with the short leaves due to the frequent grazing by the herbivorous *Sarpa salpa*, which is the most abundant fish species in the area (Guidetti & Bussotti, 1998; Bussotti & Guidetti, 1999). The S2 and N2 stations, low-intermediate pH conditions, are located approximately 60 m far from S3 and N3. S2 and N2 have mean pH values around 7.7-7.8 (Hall-Spencer *et al.,* 2008; Lombardi *et al.,* 2011b), but it has also a considerable variability in time, also at daily scale (Kerrison *et al.,* 2011; Kroeker *et al.,* 2011), and the bubbles emissions are reduced compared to the acidified area (>5 bubbles emissions to m^2). The SC and N1 are control stations, located approximately 80 m from the S2-N2, where the CO_2 emissions are almost absent and the pH values are those of normal sea waters (8.1). The south side station (SC) has been used as a control site for previous transplant experiments (Lombardi *et al.,* 2010, 2011a,b; Rodolfo-Metalpa *et al.,* 2010), and it does not coincide with the south control station of other studies (e.g., Kroeker *et al.,* 2011, 2013a).

Sampling methods and data analysis

Artificial structures mimicking the plant morphology, both rhizome and leaves have been developed *ad hoc* for this study. This methodology, implying the use of artificial substrates, is analogous to the use of panels and volcanic tiles to study the fouling, community re-colonization and succession pattern in hard bottom environments (Relini & Faimali, 2004; Kroeker *et al.,* 2013a). Moreover, artificial structures mimicking the physical structure and morphology of a seagrass have already been used by other authors (Pinkney & Micheli, 1998), especially to study the re-colonization of associated flora and fauna (Bologna & Heck, 1999; Lee *et al.,* 2001; Cocito *et al.,* 2012) and to detect the specificity of the epiphytes for the seagrass substrate (Mazzella *et al.,* 1981). Although seagrass mimics are not exact surrogates of the plant (Pinkney & Micheli, 1998), if appropriately designed, they can simulate plant architecture and structure. So they have the merit to reduce variability of plant features, respect to the natural context. Mimics also allow to test the simple influence of plant architecture and physical structure on the epibiont community colonization *vs* the biological and chemical effect. In addition, in the specific context of the natural CO_2 vents, the use of mimics reduces the impact on the *Posidonia* system caused by shoot collection in the relatively limited extension of the *Posidonia* meadows here available. Mimics (Fig. 1) of *Posidonia* rhizomes consist of hollow cylinders made of earthenware with rough and finely grooved surface to reproduce the roughness of the natural rhizomes as best as possible. The length of the cylinders, set according to the rhizome mean height from literature data, is 13 cm, with a diameter of approximately 3 cm (Gambi *et al.,* 2011; Cocito *et al.,* 2012). Mimics of *Posidonia* leaves are made with non toxic, dark green flexible PVC (IDROEVA ®, Pati s.p.a.); the material is cut in strips 1 cm wide and 36 cm long. Four PVC strips (artificial leaves) are attached together with plastic straps to a hooked iron stake (Fig. 1). The stakes with artificial leaves are then inserted in the hollow of the cylinder and hammered on the bottom among the *Posidonia* natural rhizomes, producing a minimal impact on the meadow. At each of the 6 stations, *Posidonia* mimics were inserted into the meadow, in an area of approximately 3 x 6 m, where the condition of CO_2 emissions and the pH value variability could be considered relatively homogeneous in time according to previous measurements on the same spots (Cigliano *et al.,* 2010; Lombardi *et al.,* 2011b). At each station, 16 mimics were placed in September 2009. Every three months (December 2009, March, June and September 2010), 4 mimics of the rhizome and 6 artificial leaves were randomly sampled. After the sampling, mimics were transported in the laboratory inside of small plastic bags and within a cool box. Wet structures were firstly photographed (Camera: Nikon Coolpix 5700, resolution: 5 megapixel) then fixed in 4 % formol and transferred after 48 hours to 70 % ethanol for species preservation. On the leaves, we randomly selected spots of 1 x 2 cm on the external side of the artificial leaf (generally the most colonized) as to have at least 6-7 spots per artificial leaf available. All the rhizome mimics were photographed. In order to study the pattern of settlement of

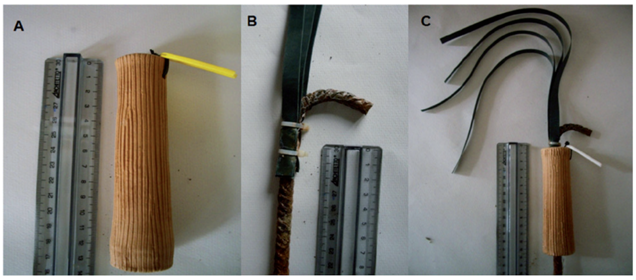

Fig. 1: Artificial structures mimicking the *P. oceanica* rhizome and leaves used to study colonization and settlement patterns of the epibiont populations. A) rhizome mimic; B) the metal hooked stick with artificial leaves tied; C) a whole view of a mimic *Posidonia* shoot.

epibionts over time in the south side stations (SC, S2, S3), 3 additional rhizome mimics and 6 artificial leaves were taken, while 3 new mimics and 6 artificial leaves were reinserted at three month time intervals from September 2009 (December 2009, March, June and September 2010). The south side was selected to reduce the sampling effort, since here the pH gradient is stronger than to the north side (Kroeker et al., 2011). At each station, *Posidonia* shoot density was measured in September 2010, and samplings of *Posidonia* natural shoots (6-10 shoots) for the morphometric analysis were done in five periods through the year in order to characterize the features of the *Posidonia* shoots in the plot used for mimic's deployment (Buia *et al.,* 2004). On rhizome mimics and artificial leaves, the epibiont identification at the lowest taxonomic level possible was performed using a stereo microscope. The percentage of epibiont coverage and the abundance of the main epibiont algal and animal taxa were calculated using the image-analyzing program Vidana 1.1. Data related to cover, as well as those on shoot morphometric measurements were subjected to statistical analysis using ANOVA (one and two ways) to test differences among stations (pH factor), side and sampling periods. Tukey HSD post-hoc analysis was performed to further highlight differences among stations. Data was checked for homogeneity of variance using a Cochran C test ($p > 0.05$). Where data was found to be heterogeneous, data was $\sqrt{(X + 1)}$ transformed (Underwood, 1997). The analyses were performed with the program STATISTICA 7. Only for the community settled on the rhizome mimics, a matrix was produced (taxa/station) for a multivariate analysis. The distance matrix was calculated using the Bray-Curtis algorithm to obtain the ordination model (MDS) and the ANOSIM test was applied to verify the significance of pHs (stations) and side (north *vs* south). These analyses were performed with the program PRIMER+PERMANOVA v.6 (Warwick & Clarke, 1991).

Results

Shoot density and morphometric analysis of Posidonia along the pH gradient

Posidonia oceanica shoot density showed, both at the north and south sides, a significant decrease from the most acidic stations (N3 and S3) to the control ones (N1 and SC) (two-way ANOVA, F = 14.67, p = 0.000, d.f. 5), although the depth did not vary much between the stations (2-3.5 m). Indeed, the shoot density values were the highest at 2-2.5 m depth at the acidified stations (N3 = mean 858 ± 85 (s.d.) shoot m^2; S3 = 1014 ± 73; N2 = 708 ± 98; S2 = 726 ± 123; N1 = 438 ± 88; SC = 494 ± 125). The mean leaf length and mean shoot surface (pooling the 5 sampling periods) were higher on the south side respect to the north (two-way ANOVA, leaf length F = 5.25; p = 0.02, d.f. 1; leaf surface F = 7.01; p = 0.013, d.f. 1). The mean leaf length was also significantly different among stations, with values in N2, N3 and S3 lower than all the others (two-way ANOVA, F = 2.87; p = 0.03, d.f. 5, Tukey post-hoc comparisons). Leaf surface varied significantly only in S2 which showed much higher values (two-way ANOVA, F = 3.97; p = 0.009, d.f. 5, Tukey post hoc comparisons) (Fig. 2A, B). The analysis of the apex erosion coefficient (Fig. 2C) reveals that biological erosion, due to various grazers, is the main source of leaf damaging. The biological erosion was significantly higher in N3 and

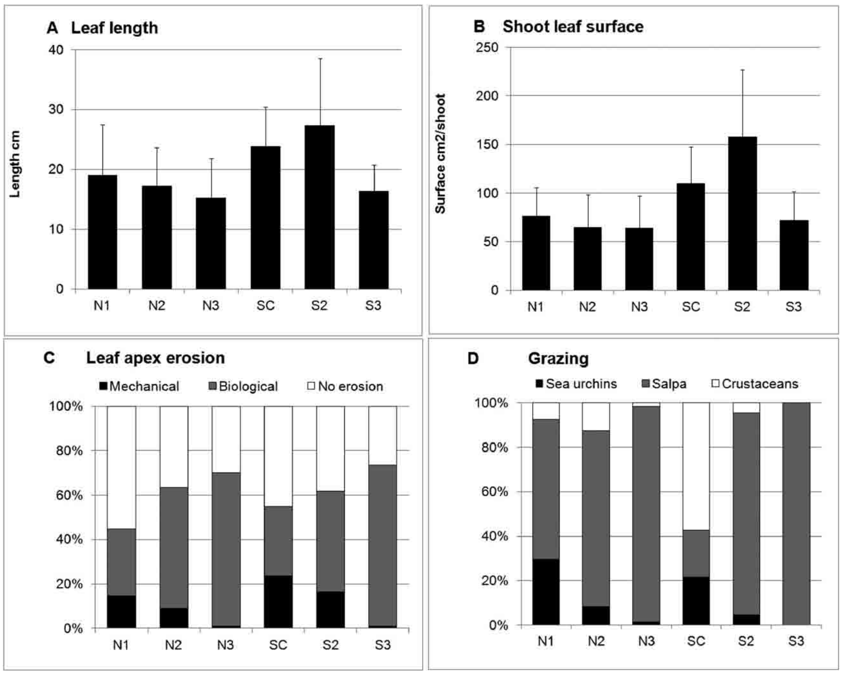

Fig. 2: Trends of *Posidonia oceanica* morphological features measured in the mimic's plots along the pH gradient (each station is the mean of 5 sampling periods between Sept 2009 - Sept 2010). Bars represent standard deviations. A) mean leaf length; B) mean surface of the shoot (cm^2); C) percentage of types of leaf apex erosion; D) percentage of types of biological apex erosion.

S3 stations (two-way ANOVA, F = 5.52; p = 0.001, d.f. 5). Among the type of biological damaging, *Sarpa salpa* grazing (Fig. 2D) resulted the most common and the dominant type in N3 and S3 stations (two-way ANOVA, F = 16.55; p = 0.000, d.f. 5). Grazing by crustaceans (manly isopods) resulted significantly higher in SC (two-way ANOVA, F = 3.69; p = 0.012, d.f. 5, Tukey post-hoc comparisons). So overall, the acidified *Posidonia* stands are characterized by extremely high shoots density, short leaf length and consequently low leaf surfaces, mainly due to intense grazing by *Sarpa salpa* fish.

Colonization of mimics along the pH gradient

For the first three months of exposure (Sept-Dec 2009) (Fig. 3), the algal cover on the artificial leaves at both south and north sides showed a large presence of coralline algae (genera *Hydrolithon/Pneophyllum* and *Titanoderma*), found under control or intermediate pH conditions (N1, N2, SC) and significantly reduced or absent at the acidified stations (N3, S3) (Fig. 3) (one-way ANOVA, Table 1). The genus *Titanoderma* was present only at the stations on the north side. On the contrary, the filamentous macroalgae, belonging to Chlorophyceae and Phaeophyceae (Chloro/Phaeo in Fig. 3), were abundant at the most acidified stations (ANOVA, Table 1). The sessile fauna was represented only by Hydrozoa and only at the control station N1. Grazing traces, due to radular marks of large gastropods (e.g. *Gibbula* spp.) on epiphytic biofilm and slime on artificial leaves, were visible at the acidified stations (N3, S3) and in S2, where Chloro/Phaeo algal cover dominated. Rhizome mimics (Fig. 4) showed a large presence of encrusting coralline algae at the control and intermediate stations of the north side (N1, N2) and at the control station of the south side (SC). These coralline algae were absent at the acidified station S3, while they were reduced at N3 and S2 (one-way ANOVA, Table 2). On the contrary, at the acidified stations green and brown filamentous algae (Chloro/Phaeo) increased, and non-calcifying red algae (other Rhodophyceae) were also present (Fig. 4A). The abundance of calcifying sessile fauna, including Cirripedia (*Balanus* spp.), Bryozoa, and Serpulidae (which include Serpulinae and Spirorbinae) polychaetes, showed a significant decrease from control stations to the acidified ones where all these groups were absent (Fig. 4B). Spirorbinae polychaetes, characterized by spiral calcareous tubes, showed high abundances at control stations and a trend consistent with the other calcifying groups:

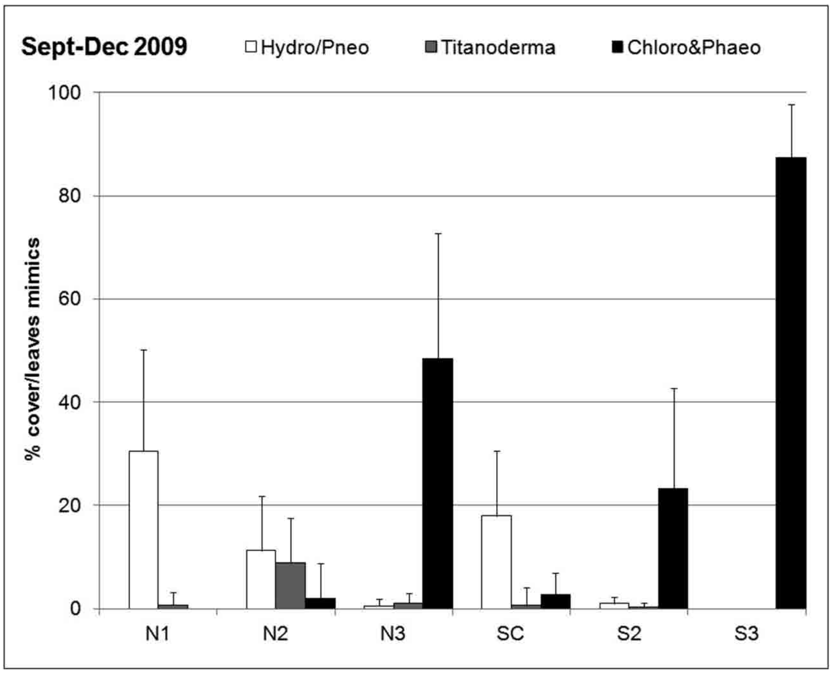

Fig. 3: Trend of cover (%) of the main macroalgal taxa in the artificial leaves of *P. oceanica* after the first 3 months of exposure (Sept. - Dec. 2009) at the north and south sides of Castello Aragonese. Bars represent standard deviations.

Table 1A. One-way ANOVA of the main macroalgal forms, on the artificial leaves after the first 3 months of exposure (Sept - Dec 09), among stations (SC - N3) and between sides (North vs. South) of Castello Aragonese.
df: degree of freedom, p: probability with ns (p>0.05), * (p<0.05), ** (p<0.01); *** *P* < 0.001.

	Stations	**SC - N3**		**Side**	**S - N**	
	***F* values**	**df**	**p**	***F* values**	**df**	**p**
Hydro/Pneo	55.46	5	***	15.16	1	***
Titanoderma	30.06	5	***	27.58	1	***
Chloro/Phaeo	275.82	5	***	26.22	1	***

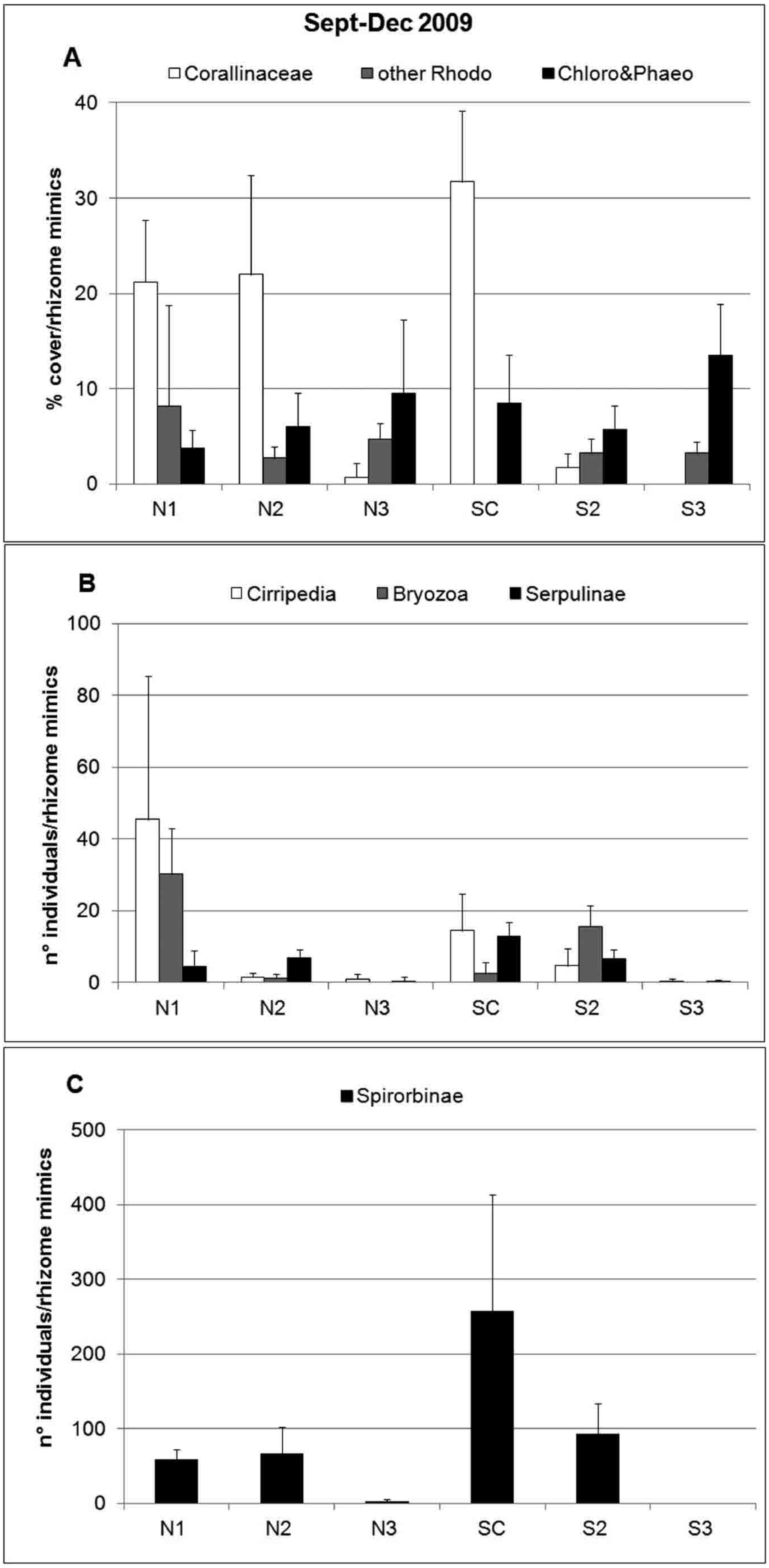

Fig. 4: Trend of cover (%) of the main macroalgal taxa and abundance of the main sessile invertebrates on the rhizome mimics of *P. oceanica* after the first 3 months of exposure (Sept. - Dec. 2009), at the north and south sides of Castello Aragonese. Bars represent standard deviations.

Table 1B. One-way ANOVA of epibiont on the rhizome mimics after the first 3 months of exposure (Sept - Dec 09), among stations (SC - N3) and between sides (North vs. South) of Castello Aragonese.
df: degree of freedom, p: probability with ns ($p>0.05$), * ($p<0.05$), ** ($p<0.01$); *** $P < 0.001$.

	Stations SC - N3			Side S - N		
	F **values**	**df**	**p**	***F*** **values**	**df**	**p**
Corallinaceae	22.09	5	***	0.47	1	n.s.
Rhodophyceae	1.46	5	n.s.	3.23	1	n.s.
Chloro/Phaeophyceae	9.04	5	***	4.45	1	**
Cirripedia	4.29	5	**	1.08	1	n.s.
Bryozoa	17.46	5	***	0.72	1	n.s.
Serpulinae	6.85	5	***	0.66	1	n.s.
Spirorbinae	8.03	5	***	3.17	1	n.s.

absent at the acidified stations (N3, S3) (Fig. 4C). The multivariate analysis (nMDS model, graph not shown) showed a clear separation between mimics at the acidified S3 and N3, and all other stations, with mimics of the control station SC forming a relatively compact subgroup. The ANOSIM test highlighted a global R = 0.71, P 0.1 % for the station factor (pH), while the side factor (north *vs* south) was not significant.

Settlement pattern through time on mimics

The study of the settlement pattern over time was conducted only on the south side (replacement of 3 mimics and 6 artificial leaves every three months). The analysis of succession on artificial leaves (Fig. 5) showed that coralline algae were absent in S3 in all periods of exposure, and always low in S2. Settlement at control station occurred mainly in June-Sept 2010 with mean cover significantly higher than in the other periods (ANOVA, Table 2A). The filamentous macroalgae (Chloro/Phaeo) were present in all stations, but with a greater abundance at the acidified station S3 with a peak in Sept-Dec 2009, and in S2 in Jun-Sept 2010 (ANOVA, Table 3). The sessile fauna showed a scarce occurrence (not shown) of a few taxa represented by both calcifying (Spirorbinae, Bryozoa) and non-calcifying organisms (Hydrozoa, Ascidiacea), without any trend according to the pH or time.

Rhizome mimics (Fig. 6) showed absence of coralline algae in S3 in all periods and a strong reduction in S2, consistently with pattern observed on the artificial leaves (ANOVA, Table 2B). At the control station coralline settlement peaked in Sept-Dec 2009, although they are present throughout the year. On the contrary, the green and brown filamentous macroalgae (Chloro/Phaeo) prevailed in S3 and S2, although scarcer in Dec-Mar 2010, and increased in Mar-June 2010 especially at the acidified station S3 (ANOVA, Table 2B). Also the sessile fauna showed different settlement pattern along the pH gradient and in time (Fig. 7). The Cirripedia (represented by two *Balanus* species), always scarce at the acidified station and absent in June-Sept 2010, settled on the other

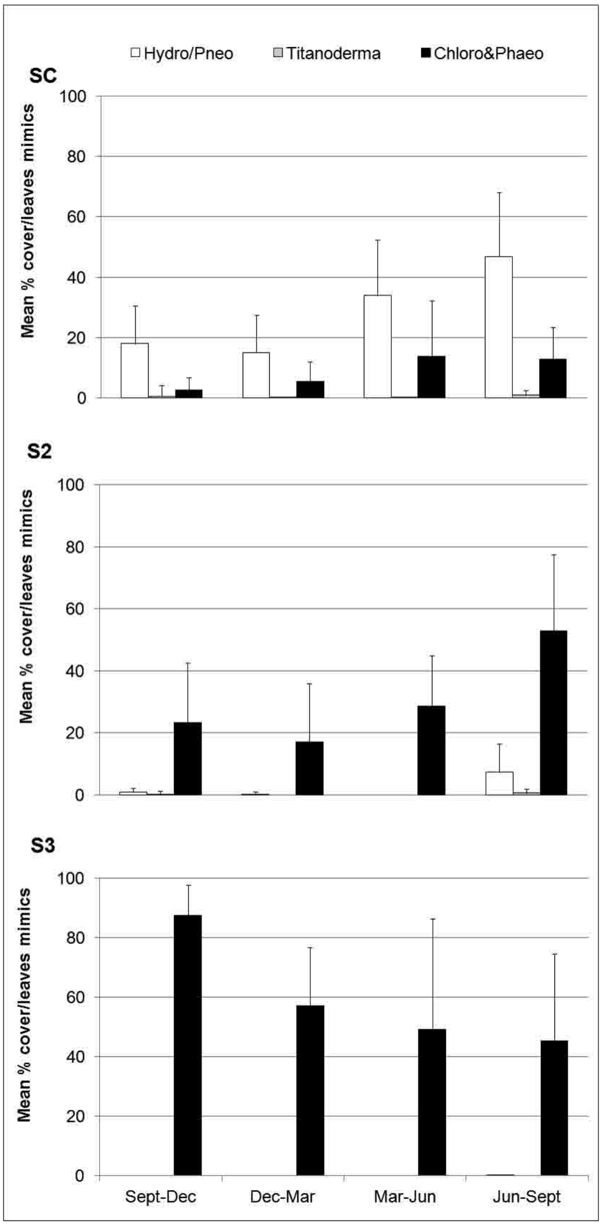

Fig. 5: Trend of settlement (algal cover) of the main macroalgal taxa in the artificial leaves of *P. oceanica* in the period Sept. 2009 - Sept. 2010 along the gradient, in the south side of Castello Aragonese. Bars represent standard deviations.

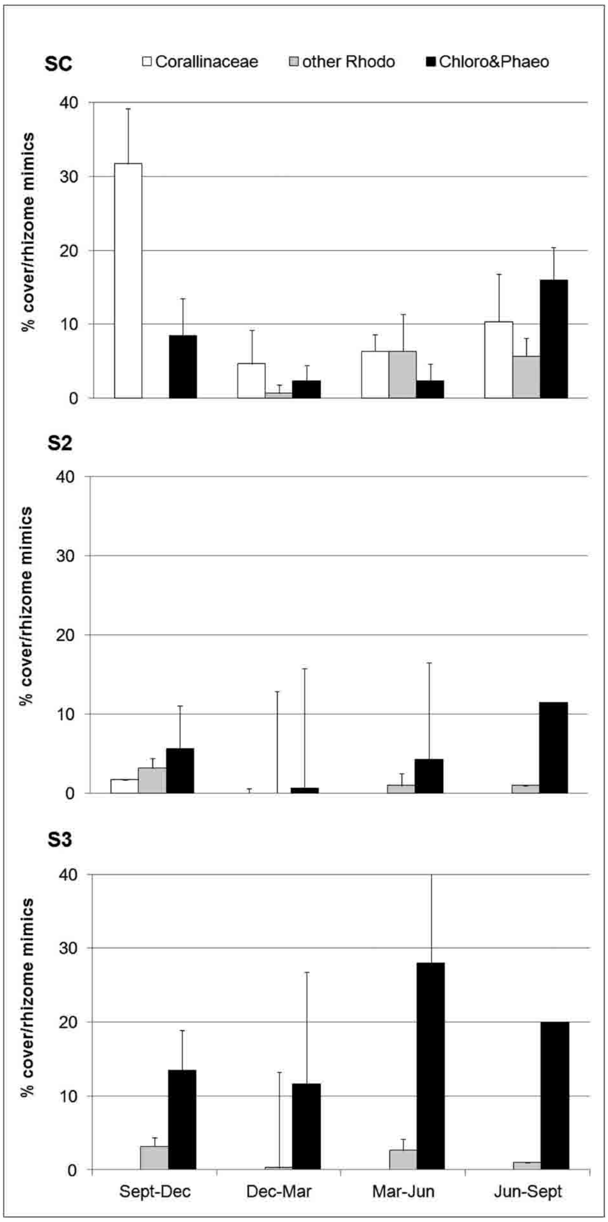

Fig. 6: Trend of cover (%) of the main macroalgal forms in the rhizome mimics of *P. oceanica* in the period from Sept. 2009 - Sept. 2010 along the gradient, in the south side of Castello Aragonese. Bars represent standard deviations.

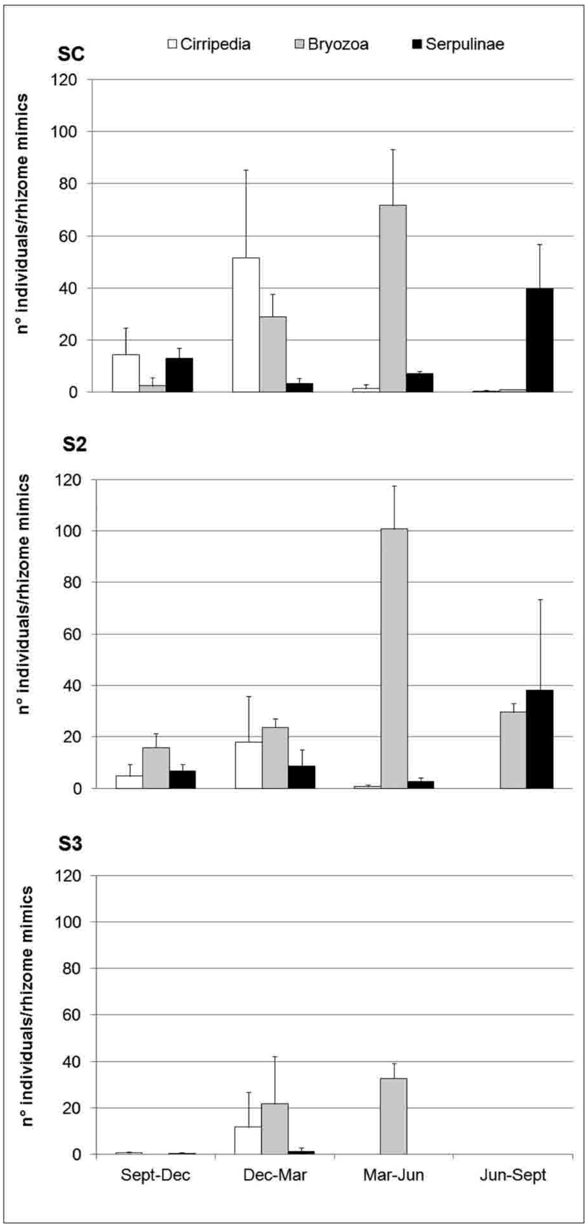

Fig. 7: Trend of the abundance of the main sessile invertebrates in the rhizome mimics of *P. oceanica* in the period from Sept. 2009 - Sept. 2010 along the gradient, in the south side of Castello Aragonese. Bars represent standard deviations.

Table 2A. One-way ANOVA of the main macroalgal forms in the artificial leaves, among stations (SC - S3), every three months (December 2009, March, June and September 2010), and in the period (Sept 09 - Sept 10).
df: degree of freedom, p: probability with ns (p>0.05), * (p<0.05), ** (p<0.01); *** *P* < 0.001.

Factors	Hydro/Pneo			Titanoderma			Chloro/Phaeo		
	F values	df	p	*F* values	df	p	*F* values	df	p
Stations SC - S3									
Sept - Dec 09	74.11	2	***	0.85	2	n.s.	523.16	2	**
Dec 09 - Mar 10	35.03	2	***	0.57	2	n.s.	104.63	2	**
Mar - June 10	131.27	2	***	0.03	2	n.s.	20.23	2	***
June - Sept 10	149.90	2	***	6.47	2	**	36.30	2	***
Period									
Sept 09 - Sept 10	10.59	3	***	4.08	3	**	4.63	3	**
Post Hoc Test	June - Sept 10			June - Sept 10			Dec 09 - Mar 10		

Table 2B. One-way ANOVA of epibiont on the rhizome mimics among stations (SC - S3), every three months (December 2009, March, June and September 2010), and in the period (Sept 09 - Sept 10).

Factors	**Corallinaceae**			**Rhodo**			**Chloro/Phaeo**			**Cirripedia**			**Bryozoa**			**Serpulinae**			**Spirorbinae**		
	***F* values**	**df**	**p**	***F* values**	**df**	**p**	***F* values**	**df**	**p**	***F* values**	**df**	**p**	***F* values**	**df**	**p**	***F* values**	**df**	**p**	***F* values**	**df**	**p**
Stations SC-S3																					
Sept - Dec 09	74.33	2	***	11.64	2	**	13.57	2	**	4.72	2	*	20.18	2	***	19.03	2	***	8.11	2	**
Dec 09 - Mar 10	21.77	2	n.s.	0.60	2	n.s.	1.83	2	n.s.	2.48	2	n.s.	0.25	2	n.s.	4.38	2	n.s.	31.97	2	***
Mar - June 10	22.56	2	**	2.18	2	n.s.	11.48	2	**	1.50	2	n.s.	13.16	2	**	48.14	2	***	20.61	2	**
June - Sept 10	2.83	2	n.s.	3.86	2	n.s.	1.00	2	n.s.	0.37	2	n.s.	10.03	2	*	1.04	2	n.s.	2.79	2	n.s.
Period																					
Sept 09 - Sept 10	2.15	3	n.s.	2.61	3	n.s.	1.60	3	n.s.	6,32	3	**	21,53	3	***	11,63	3	***	9,16	3	***
Post Hoc Test										Dec 09 - Mar 10			Mar - June 10			June - Sept 10			Mar - Sept 10		

stations mainly in Dec-Mar 2010 (ANOVA, Table 2B); the Bryozoa were scarce in S3 in all periods, and showed a clear peak in Mar-June 2010 in intermediate and control stations. The bryozoans represent the most diverse group of epibionts found on the mimics, with at least eleven species some of which have been found also in low pH; Serpulinae, always absent in S3 in all periods, showed in the other stations the greater settlement in June-Sept 2010. Finally, Spirorbinae (graph not shown) almost absent in S3 in all periods, showed in the other stations a long settlement period, although wth strong time fluctuations, with minima in Dec-Mar (mean number of individuals: 79 per mimics in SC and 35 in S2) and maxima in Jun-Sept (mean number of individuals: 669 per mimic in SC and 637 in S2) (ANOVA, Table 2B).

Discussion

In the present study *Posidonia* mimics were exposed to a natural acidification gradient due to volcanic CO_2 emissions. The results show that mimics highlight well the differences between leaves and rhizomes in epibiont colonization which occur in the natural shoots, although we have not yet compared the mimic epibiont composition of both rhizome and artificial leaves with the natural epiphytic communities. In natural *Posidonia* shoots, leaves always show reduced epiphyte diversity represented by more specialized taxa, respect to rhizomes assemblages (Chimenz *et al.,* 1989; Balata *et al.,* 2008; Gambi & Morri, 2008). The single study related to natural leaf epiphytes along the pH gradient at the Castello south side (Martin *et al.,* 2008) highlighted the dominance of coralline algae and some bryozoans at the control stations, and a strong reduction of all the calcareous organisms at the acidified sites. So our results related to artificial leaves are very consistent with those occurring in natural shoots.

The artificial leaves show a clear reduction of all encrusting calcareous Corallinaceae and animal taxa at the acidified stations both on the north and south sides (N3, S3) and, on the contrary, an increase of the filamentous algae (Chloro/Phaeo). These results are consistent with data reported in previous studies which highlight the sensitivity of coralline algae to the low pH level (Jokiel *et al.,* 2008; Martin *et al.,* 2008; Porzio *et al.,* 2011). Filamentous algae and thick biofilm occurring at acidified stations can explain the grazing traces visible on some artificial leaves due to radular scraping by gastropods (e.g. *Gibbula* spp.) (Mazzella & Russo, 1989), given the fact that artificial leaves lack the phenolic compound that has been shown to prevent or limit grazing (Agostini *et al.,* 1998; Dumay *et al.,* 2004).

The rhizome mimics' analysis shows a community with a higher diversity of organisms, compared to the artificial leaves. Indeed, different serpulid and spirobid species, two barnacle species, and eleven bryozoan species, in addition to the algal species, are present on the mimics. The distribution of both plant and sessile animals on the rhizome mimics along the pH gradient is consistent with a significant reduction of the calcareous organisms on the leaf mimics at the stations with very low pH, and an increase of the filamentous algae. This trend is detectable at both north and south side stations, and the pH is the factor that influences the similarity of the community at the stations as a whole, as summarized by the multivariate analysis model nMDS and ANOSIM test.

The colonization and settlement on rhizome mimics in time shows temporal trends diversified among the different sessile organisms. According to Cocito *et al.* (2012) who studied bryozoan settlement on mimics in a deep *Posidonia* meadow off Ischia, bryozoan settlement mainly occurred in spring time, thus confirming observations on bryozoans' recruitment peak on mimics. It is worth to note that the obliged epiphytic species of *Posidonia*, *Electra posidoniae*, is present only on natural leaves and never on the artificial leaves, a pattern observed also in a different study using artificial leaves (Michel, 2011). This fact supports once more that natural leaves are not a simple substrate for epiphytes but exert an attractive or repulsive action due to specific compounds. For both serpulids and spirorbids recruitment peaks in summer are consistent with the actual knowledge on the reproductive biology, at least for the Mediterranean species (Bianchi, 1981).

Overall, every analyses of leaves' and rhizomes' mimics show a similar trend represented by a reduction or total disappearance of calcifying forms in the low pH stations. This is consistent with what observed in studies on benthic plant components (Porzio *et al.,* 2011) and animals of hard substrata studied in the same vent area (Hall-Spencer *et al.,* 2008; Kroeker *et al.,* 2011). These findings confirm that most calcifying organisms are particularly sensitive to increased seawater acidification, and may be less competitive for space than non-calcifying organisms in areas with very low pH levels. However, some of them may still persist in low pH due to their mineralogy and calcification features. In fact, cellular wall in coralline algae is impregnated with deposits of calcium carbonate in the form of calcite, but with a variable amount of magnesium in the crystal lattice (with variable concentrations 3.5-6% Mg; Milliman, 1974). Similarly, the ability of some bryozoans to persist at least for short period in low pH could be explained by the diverse skeletal mineralogies. Most bryozoan species are calcitic, but some have aragonitic or bimineralic skeletons, and the complex mineralogies can vary among species, within a single colonies and sometimes transition from one mineralogy to another can be observed within the same modular unit (Smith *et al.,* 2006; Taylor *et al.,* 2008, 2009). Recent experiments conducted on bryozoans transplanted in the same volcanic vent area off Ischia where this study was conducted, revealed different responses depending on organic components and mineralogy of the species, with possible reallocation of energy resources within the colonies when exposed to unfavourable conditions such as low pHs (Lombardi *et al.,* 2011a, b). The presence of calcifying bryozoans species along a pH gradient, as those observed on the rhizome mimics, could be explained by the possibility of the larvae to settle and tolerate low pH at least for few weeks or species could be potentially able to 'adapt' to the changing chemistry (pH) conditions. The Cyclostome *Patinella radiata,* for example, has been found on natural *Posidonia* leaves in Ischia growing along a pH gradient revealing its potential to settle, live and reproduce in below normal pH environments (Lombardi C., personal observation).

Overall community simplification and altered succession dynamics have been highlighted on hard bottoms off the Castello area, using artificial substrates (volcanic tiles) by Kroeker *et al.* (2013a), and on natural rocky substrates (Kroeker *et al.,* 2013b). As for the *Posidonia* system, considering that mimics resulted a good proxy of the natural epiphytic community, this profound difference in epibiont communities in areas with low and very low pH levels, showed also by the natural leaf community (Martin *et al.,* 2008), has certainly cascading effects on the food web of the meadow, and must influence the functioning of the system. Studies on both epiphytes and motile fauna of these acidified *Posidonia* stands are in progress to highlight the effects of acidification on the whole community associated to this important ecosystem of the Mediterranean Sea.

Acknowledgements

We wish to thank the staff of the Stazione Zoologica Anton Dohrn at Villa Dohrn, in particular Capt. V. Rando, for assistance during samplings; B. Iacono and V. Zupo, for assistance in the acquisition of mimic's photos. We also wish to thank Prof. G. Relini for identification of barnacles, and L. Porzio for identification of *Titanoderma*; K.J. Kroeker for help in the use of the image analysis program Vidana 1.1; and the ceramist C. Mattera (Ischia) for the construction of *Posidonia* mimics. Rosanna Messina kindly checked the English. This work has been finalized in the framework of the Flagship RITMARE - The Italian Research for the Sea - coordinated by the Italian National Research Council and funded by the Italian Ministry of Education, University and Research within the National Research Program 2011-2013.

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