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## Effects of ocean acidification on phenology and epiphytes of the seagrass *Posidonia oceanica* at two CO<sub>2</sub> vent systems of Ischia (Italy)

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### Abstract

Morphological features of the seagrass *Posidonia oceanica* (L.) Delile and its epiphyte community were studied in three acidified stations located in two CO<sub>2</sub> vents systems and one control station under normal pH conditions off the island of Ischia (Italy) to highlight the possible effects of ocean acidification. Plant phenology was analyzed every two months for one-year cycle (June 2016-April 2017), while epiphytes were studied in the period of highest development of both the leaf canopy and the epiphytic community (June, August, and October 2016). The shoot density of *Posidonia* beds in the acidified stations of the studied sites was significantly higher than that in the control area. Significant differences in the mean leaf length according to the pH condition, month, and the interaction of these two factors were observed (PERMANOVA); the mean leaf width differed also among pH conditions and months. We recorded lower leaf lengths and widths in the acidified stations in all the considered months, compared to those in the control station. These differences are consistent with the higher impact of grazing by the herbivorous fish *Sarpa salpa* observed on the leaves in the acidified stations. However, the overall leaf surface available for epiphytes was similar among stations because of the higher shoot density under ocean acidification conditions. Overall, the composition and structure of the epiphytic community on the *Posidonia* leaves showed significant differences in relation to acidification: in both acidified sites, all the calcareous forms, both encrusting red algae (Corallinales) and animals (bryozoans, foraminiferans, and spirorbid), disappeared or were strongly reduced, in favor of encrusting or erect fleshy algae, and non-calcifying invertebrates (hydrozoans, tunicates) which dominated the assemblage. Coralline algae are early species in the epiphytic colonization of *P. oceanica* and therefore their absence can further modify the pattern of leaf colonization by other species. Therefore, the changes found in the epiphyte community in low pH areas could have potential cascading effects on the seagrass trophic network and the functioning of the system.

**Keywords:** Ocean acidification; climate change; *Posidonia oceanica*; seagrasses; morphological features; epiphytic community; temporal trend; Mediterranean Sea.

### Introduction

Ocean acidification (OA), characterized by decreasing pH, increasing  $p\text{CO}_2$ , and the alteration of carbonate chemistry, combined with other types of natural and/or anthropogenic stresses, can cause marked changes in the structure and functioning of benthic communities (Kroeker *et al.*, 2010; 2012). The use of natural sites in which, for various reasons (CO<sub>2</sub> emissions, fresh water input, upwelling, etc.), natural acidification of the marine waters occurs, has increased in these last years, respect to laboratory or mesocosm experiments (Foo *et al.*, 2018; Gonzalez-Delgado & Hernandez, 2018; Rastrick *et al.*, 2018). In fact, these special systems contribute to formulate hypotheses of possible and more realistic future environmental scenarios on marine organisms in relation to

the lowering and/or variability of pH. The CO<sub>2</sub> emissions generated by volcanic vents correspond to less than 0.5% of anthropogenic emissions if compared to the global carbon balance; such systems can locally alter the chemistry of the oceans and can therefore be used as natural laboratories to understand the effects of oceanic acidification at the ecosystem level (see Foo *et al.*, 2018; Gonzalez-Delgado & Hernandez, 2018; Rastrick *et al.*, 2018 for recent reviews on worldwide vent systems).

On the island of Ischia (Tyrrhenian Sea, Italy) there are several easily accessible sites characterized by intense CO<sub>2</sub> emissions, located mainly on the north-eastern side of the island, ranging from 0.5 to 48 m depth (Tedesco, 1996; Hall-Spencer *et al.*, 2008; Gambi, 2014; Foo *et al.*, 2018; Gambi *et al.*, 2019). In particular, the shallow vent system off the Castello Aragonese is currently the most

investigated among the known shallow CO<sub>2</sub> systems (Foo *et al.*, 2018). Although these vent systems are not direct analogs of OA, because of the high pH variation and the close proximity of the acidified zones with areas with normal pH, the natural CO<sub>2</sub> vents may be useful in examining the long-term response of the community to OA (Kroeker *et al.*, 2011), especially in complex coastal systems, such as seagrass meadows.

The CO<sub>2</sub> vents off the Castello Aragonese include relatively large portions of *Posidonia oceanica* (L.) Delile beds (Garrard *et al.*, 2014), as well as sublittoral rocky substrata (Hall-Spencer *et al.*, 2008; Kroeker *et al.*, 2011). Thus, this is a model site to investigate how communities of benthic organisms associated with seagrass (epiphytes and vagile fauna) respond to OA. In addition, other vent sites around Ischia include *Posidonia* beds, such as the recently reported shallow 'Vullatura' system (3–6 m depth) (Gambi, 2014; Gambi *et al.*, 2019).

The effects of CO<sub>2</sub> surplus on the *P. oceanica* habitat near the Castello vents have been investigated in studies that considered different aspects of the distribution of the plant and the composition of epiphytes and the associated motile community (summarized in Foo *et al.*, 2018). As for shoot density, the highest values are associated with the highest pCO<sub>2</sub>–extreme low pH conditions (with almost 1,000 shoots/m<sup>2</sup>) if compared to those at the control stations (<500 shoots/m<sup>2</sup>) (Hall-Spencer *et al.*, 2008; Donnarumma *et al.*, 2014; Garrard *et al.*, 2014). OA influences the content of nitrogen (N) in the plants from acidified stations: *Posidonia* shows a lower carbon/nitrogen (C:N) ratio and has a higher nutritional value for herbivores (Ricevuto *et al.*, 2015; Scartazza *et al.*, 2017).

The reduction or disappearance of calcareous forms was reported in natural leaves (Martin *et al.*, 2008) as well as in artificial *Posidonia* mimics (Donnarumma *et al.*, 2014): calcareous invertebrates (e.g., barnacles and bryozoans) and coralline algae were abundant at normal pH levels in the control areas (both south and north of the Castello), but progressively reduced at intermediate pH conditions (N2, S2, at 7.7–7.8) and were not present at the high emission sites with extreme low pH levels (S3: mean pH 7.33, N3: 7.39). In contrast, at low pH levels, epiphytes were mainly non-calcareous taxa (e.g., hydroids and tunicates) and filamentous algae (Donnarumma *et al.*, 2014).

The communities of mobile invertebrates associated with *P. oceanica* meadow at the vents also change according to the pH levels (Garrard *et al.*, 2014). Several invertebrate taxa (e.g., gastropods, amphipods, bivalves, isopods, and polychaetes) tolerated pH < 7.8; twice as many individuals were recorded under low pH levels than under normal conditions. This could be due to the indirect effects of the low pH, which favor a higher density of *P. oceanica* and therefore offers a greater protection from predation (Pages *et al.*, 2012), as well as increased living space. Furthermore, the possible buffering of low pH levels, due to the production of O<sub>2</sub> by the plant during the photosynthesis process, must be considered.

Animal and algal epiphytes on seagrasses play a pivotal role in the food web and functioning of the ecosys-

tem (Mazzella *et al.*, 1992). Epiphyte dynamics and their role in food webs are strongly influenced by the seasonal phenology of the host plant (number and length of leaves, shoot leaf surface, shoot density, etc.) (Casola *et al.*, 1997; Lepoint *et al.*, 1999). Additionally, epiphytes significantly contribute to ecosystem primary production (Mazzella *et al.*, 1992), nutrient cycling, and sediment production (Libes & Boudouresque, 1987; Perry & Beavington-Penney, 2005), and they can regulate light availability at the seagrass leaf surface (Nelson & Waaland, 1997; Dalla Via *et al.*, 1998). Moreover, they represent an important food resource for small mesograzing invertebrates and sea urchins (Mazzella *et al.*, 1992; Tomas *et al.*, 2005).

Epiphytic assemblages of *P. oceanica* leaves are characterized by short-living organisms that respond quickly to environmental alterations. Therefore, changes in their biomass, spatial heterogeneity, and species composition have been used to detect environmental and human-related disturbances (Piazzi *et al.*, 2004; Leoni *et al.*, 2006; Balata *et al.*, 2008; Giovannetti *et al.*, 2010). Because of their ecological role and sensitivity, epiphytes can be used as bioindicators to assess the effects of global change (in particular OA).

Although previous studies have analyzed the phenology and variation of the epiphytic community along the acidification gradient at CO<sub>2</sub> vents (Martin *et al.*, 2008; Donnarumma *et al.*, 2014; Garrard *et al.*, 2014; Nogueira *et al.*, 2017), the data they provide are limited to single observations on short periods of investigation (a single month, e.g. Martin *et al.*, 2008; Nogueira *et al.*, 2017), or are related to artificial *Posidonia* mimics (e.g., Donnarumma *et al.*, 2014). Other studies that have focused on *Posidonia* and its epiphytes in acidified areas outside the Ischia vents include those performed at the CO<sub>2</sub> vents surrounding the island of Panarea (Aeolian Islands, Sicily) (Guilini *et al.*, 2017; Gaglioti *et al.*, 2019), and those using the Free Ocean CO<sub>2</sub> Enrichment (FOCE) systems and mesocosm experiments (Cox *et al.*, 2016 and references herein). However, these studies have also considered the analysis of epiphyte and plant phenology for short periods of time, generally a single month, except for the research of Donnarumma *et al.* (2014), which followed the colonization dynamic on artificial substrates (i.e., mimics). Longer time frames of study under the specific condition of vent systems would allow us to highlight changes in the whole ecosystem dynamic, both for *P. oceanica* and the epiphytic community, affected by OA over the leaf life cycle.

The aim of this study is to highlight the effects of the reduction and/or variability of pH on the *P. oceanica* phenology in two CO<sub>2</sub> vents systems and one control station, on a year-long temporal basis. For the analysis of the composition and structure of the epiphytic community, we considered the months corresponding to the period of highest epiphyte development on the leaves (early summer to early autumn).

## Materials and Methods

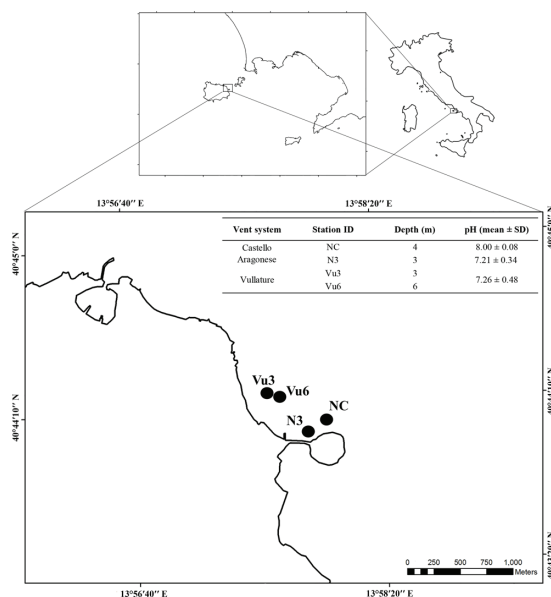
### Study area

The study was carried out in three acidified stations (one on the north side of the Castello Aragonese systems, and two in another CO<sub>2</sub> vent system, the “Vullatura”). A single station with normal pH conditions was also used for the control station (Fig. 1).

Carbon dioxide bubble emissions characterize the shallower waters around Castello Aragonese site (40.7322° N, 13.9660° E); chemical analyses have revealed that the vents are composed mostly of CO<sub>2</sub> (90.1–95.3%), there are no toxic sulfur compounds, and the temperature and salinity are the same as those of the surrounding waters (Hall-Spencer *et al.*, 2008; Foo *et al.*, 2018; Gambi *et al.*, 2019). The vents covers an area of 5,000 m<sup>2</sup> both at the north and south-western sides of the Castello Aragonese and causes a decrease of the seawater pH that ranges from 8.10 (normal condition, 150 m from denser emissions) down to 6.50 (Hall-Spencer *et al.*, 2008; Kroeker *et al.*, 2011); however, the pH reduction shows variable patterns, being characterized by large daily value fluctuations (Kerrison *et al.*, 2011; Kroeker *et al.*, 2011).

In the Castello vent system, two stations were selected, both located on the north side (Fig. 1). The control station (NC; 40.7344° N, 13.9658° E), at 4 m depth, is about 150 m from the high venting area and has a normal pH of 8.00 ± 0.08 (Kroeker *et al.*, 2011, see Fig. S1). In this area, the *P. oceanica* meadow extends over the sandy bottom with a density of 438 ± 88 shoots/m<sup>2</sup> (Donnarumma *et al.*, 2014). The other station in this area, N3 (40.7335° N, 13.9638° E), is where the highest bubble emissions occur; it is located at 3 m depth and is characterized by seagrass meadows and clearings of coarse sands mixed with stones and pebbles. The *Posidonia* bed develops on the edge of the highest emissions, with a mat height of about 50–60 cm. The recorded average pH is 7.21 ± 0.34 (Kroeker *et al.*, 2011; Ricevuto *et al.*, 2014; Foo *et al.*, 2018) (see Fig. S1). In this station, the meadow is quite dense, at 858 ± 85 shoots/m<sup>2</sup> (Donnarumma *et al.*, 2014).

The other vent system is in the area known by local fisherman as Vullatura, where two stations, Vu3 and Vu6, were selected at 3 and 6 m depth, respectively (40.7363° N, 13.9620° E). The area is located about 150 m from the shore and about 500 m from the north-west side of the Castello Aragonese (Fig. 1). The bubble emissions cover an approximate area of 30 m by 40 m, with a depth ranging from 3 to 6 m and the gas is composed 95.9% by CO<sub>2</sub> (Gambi, 2014; Gambi *et al.*, 2019). The difference in depth is due to the fact that the *Posidonia* meadow forms small patches at 5–6 m depth and rises with high pinnacles and ridges of mat (in some places are over 2.5–3 m high) up to 3 m depth. The *Posidonia* meadow has about 60% coverage of the area. Emissions are more intense and denser (more than 20 m<sup>2</sup> of bubbles) in clear sandy areas surrounded by *Posidonia*, while bubbling inside the meadow is reduced and relatively modest (Gambi, 2014). The pH *in situ*, recorded at 6 m depth with a SeaFet in April 2016, showed a relatively high acidification charac-



**Fig. 1:** Map of the study area (Ischia, Italy) showing the locations of stations (N3, Vu3, Vu6, and the control). The table reports on the right corner which of the vent systems the station belongs to, the station's identification code (ID), the depth, and the mean ± SD of pH measured.

terized by large daily fluctuation, with a minimum value of 6.20, and with 90% of the measurements below the normal value (8.1) (See Fig. S1) (Gambi *et al.*, 2019).

We selected the vents and the control station to all be located on the north side of the Castello for comparison with the Vullatura system, and to maintain environmental conditions other than pH (light and hydrodynamic exposure to prevailing winds, etc.) as homogeneous as possible between the two locations. We decided to reduce the sampling effort to only one control station because of the abundance of studies dealing with *P. oceanica* phenology and epiphytic assemblages under normal pH conditions (Casola *et al.*, 1997; Mazzella *et al.*, 1989), including a recent review (Piazzi *et al.*, 2016).

### Sampling and data collection

Every two months, from June 2016 to April 2017 (June 2016, August 2016, October 2016, December 2016, February 2017 and April 2017), 15 orthotropic (vertically oriented) *P. oceanica* shoots were randomly collected at each of the four stations, and transported (by cooler box) to the laboratory inside small plastic bags filled with seawater. Shoots were fixed in 4% formalin solution. Morphometric analyses were performed on the 15 shoots: the number of leaves (divided into adults, intermediates, and juveniles), leaf biometry (mean leaf length, mean leaf width, percentage of green and brown tissues), coefficient *A* (percentage of leaves having lost their apices), and type of grazing pressure were measured. The

latter parameter was assessed by distinguishing among the bite marks of the fish *Sarpa salpa* (Linnaeus, 1758), sea urchins (*Paracentrotus lividus* [Lamarck, 1816]) and crustaceans (mainly isopods of the genus *Idotea*) (Buia *et al.*, 2004). Furthermore, at the Vu3 and Vu6 stations, shoot density measurements (40 × 40 cm square; five replicates) were carried out in June 2016, whereas the shoot densities at N3 (16 replicates) and NC (12 replicates) were measured in 2011 (Donnarumma *et al.*, 2014; Garrard *et al.*, 2014). We relied on these measures because the *Posidonia* shoot density in the area has been proven to be quite stable (Giraud *et al.*, 1979; Buia *et al.*, 2003), and no anthropogenic disturbances have occurred in the area since 2011. Shoot collection, leaf analyses, and density measurements allowed the calculation of the leaf area index (LAI) for each station and period, as the mean leaf surface multiplied for the shoot density per unit surface (1 m<sup>2</sup>) (Buia *et al.*, 2004). This measure represents the surface area provided by the *Posidonia* leaf canopy over 1 m<sup>2</sup> of substrate, and potentially available for epiphyte settlement.

For the study of epiphytic assemblages, we selected the external side of the two outermost adult leaves of each shoot (generally the oldest and therefore the most colonized), and took photos of randomly selected spots of 1 × 2 cm to have at least seven to nine spots per leaf. Epiphyte identification to the lowest possible taxonomic level was performed using a stereomicroscope, although we grouped organisms into seven taxonomical categories for image analysis: encrusting brown algae, encrusting red algae (mainly Corallinales), erect algae (both brown and green algae), bryozoans, hydrozoans, tunicates, spirorbid worms, and foraminifers. The percentage coverage of each category of colonial organisms was calculated using the image analyzing program Vidana 1.1; non-colonial forms, such as spirorbid worms and foraminifers, were counted as the number of specimens. The analyses of the epiphytic community were performed for the shoots collected in June, August, and October 2016; these months represent the maximum development period of the epiphytic community in *Posidonia* leaves in terms of coverage and diversification (Casola *et al.*, 1997; Piazzini *et al.*, 2016). Visual checks of the shoots during winter and early spring (December, February, and April) revealed the almost total absence of epiphyte colonization. Table S1 (Supplementary Materials) summarizes all of the parameters considered for this study.

### Data analysis

One-way analysis of variance (ANOVA) was used to verify *P. oceanica* shoot density variations between pH conditions (control vs. acidified); then, multiple comparisons between stations were conducted using Tukey's honestly significant difference (HSD) test. Shoot density data were previously log-transformed [ $\log(x + 1)$ ] to respect the assumption of normality and homogeneity of variances. Univariate permutational analysis of variance (PERMANOVA) based on Euclidean distance was performed

to assess changes in the morphometric features (number of leaves, mean leaf length, and mean leaf width) of the *P. oceanica* shoots under different pH conditions and according to the sampling period. The analysis consisted of one fixed factor and one random factor: pH condition (two levels) and month (six levels). The variability of leaf apex erosion (percentages of intact, or mechanically, and biologically eroded leaves) was tested through multivariate PERMANOVA based on Bray–Curtis dissimilarity with pH condition and month as the two random factors.

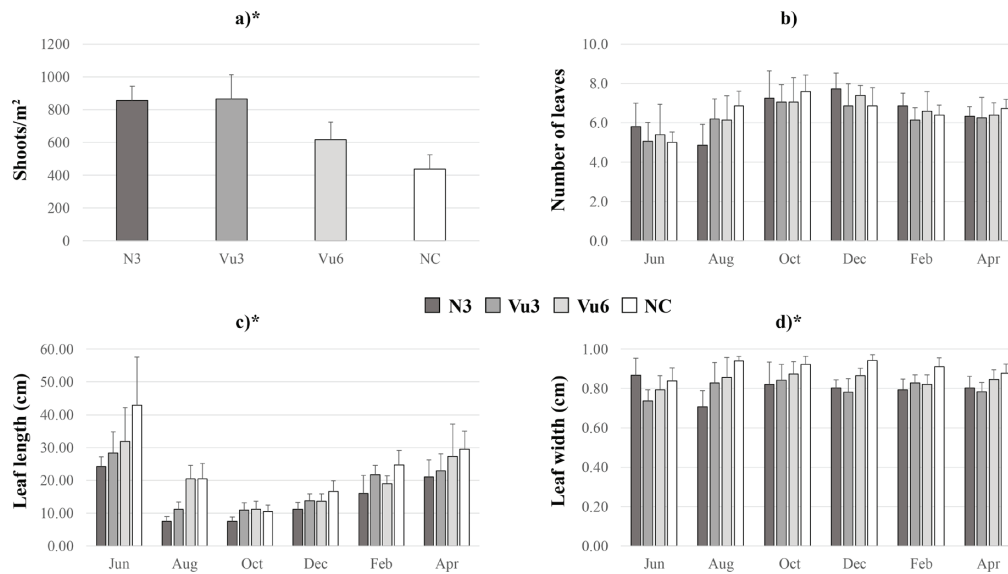
Data of the epiphytic assemblages (taxa-categories/stations) were square-root transformed and the differences in terms of composition and abundance of categories were tested through multivariate PERMANOVA with the same design used for the *P. oceanica* morphometric features. Furthermore, a Tukey's HSD pairwise test was used to discriminate among stations. A similarity percentage–species contribution analysis (SIMPER) was carried out to identify the taxonomical categories that mostly contributed to the dissimilarity among stations. The distance matrix was calculated using Gower's distance, which allows the treatment of mixed data (i.e., coverage percentages and abundances); to assess the differences in epiphytic assemblages at the study stations according to the pH conditions (stations) and period of sampling, non-metric multidimensional scaling (nMDS) was applied. All tests were performed with the Past 3.25 program.

### Results

The temperature, measured at 5 m depth in a long-term temperature–depth transect along a rocky cliff at Ischia, 3.5 km far from the Castello (Moschella *et al.*, 2010), ranged during the sampling period (July 2016–April 2017) from a maximum of 27.38 °C in August 2016 to a minimum of 14.23 °C in March 2017 (T-MedNet: [www.t-mednet.org/t-sites/](http://www.t-mednet.org/t-sites/)). The pH values, collected from previous measurements, reported severe acidification at the Vullatura stations at 6 m depth (mean pH of  $7.26 \pm 0.48$ , with low values up to 6.20, 90% of the values below the normal conditions of 8.10, and large daily fluctuations) (Gambi *et al.*, 2019) and at N3 ( $7.21 \pm 0.34$ ), compared to conditions at NC ( $8.00 \pm 0.08$ ) (Kroeker *et al.*, 2011; Ricevuto *et al.*, 2014; see Fig. S1). We did not have direct pH measurements for station Vu3; however, it is reasonable to hypothesize values and fluctuations that are comparable to the Vu6 station.

### Phenology of *Posidonia oceanica*

The *Posidonia oceanica* shoot density showed higher values for both the Vullatura (mean ± SD: Vu3 =  $866 \pm 148$  shoots/m<sup>2</sup>; Vu6 =  $619 \pm 106$  shoots/m<sup>2</sup>) and Castello Aragonese (N3 =  $667 \pm 155$  shoots/m<sup>2</sup>) acidified stations (Fig. 2a) than that at the control site (NC =  $391 \pm 95$  shoots/m<sup>2</sup>). These differences were significant by ANOVA ( $F = 64.70$ ,  $p$ -value < 0.001); Tukey's HSD test



**Fig. 2:** Temporal variation in *Posidonia oceanica* morphological features at the studied stations: mean shoot density (a), mean number of leaves per shoot (b), and mean leaf length (c) and width (d). Bars represent the standard deviation. Gray colors indicate low pH conditions: N3 (pH  $7.21 \pm 0.34$ ), Vu3, and Vu6 (pH  $7.26 \pm 0.48$ ); and white indicates the control station (NC; pH  $8.00 \pm 0.08$ ). Asterisks highlight features that show significant differences according to pH conditions.

reported significantly different  $p$ -values between the control and acidified stations (NC–N3:  $p$  adj < 0.001, NC–Vu3:  $p$  adj < 0.001, and NC–Vu6:  $p$  adj < 0.001), and between N3–Vu6 ( $p$  adj = 0.03). Comparisons between N3–Vu3 and Vu3–Vu6 did not display significant differences. The number of leaves (adult, intermediate, and juveniles pooled together) significantly varied among months, with the same pattern in both the acidified and control stations (Fig 2b). Maximum values were reported in October and December, whereas the minimum values were found during the summer months (June and August). The mean number of leaves per shoot did not differ among pH conditions (Table 1). Conversely, the mean leaf length was higher in June, decreasing up to the minimum reached in October (Fig. 2c). The mean leaf width (Fig. 2d) showed minimum values in June for NC, Vu3, and Vu6, and in August for N3. The maximum mean width was reported in August, October, and December for all the studied stations.

Significant differences in the mean leaf length according to the pH condition, month, and the interaction between these two factors were observed (Table 1, PERMANOVA test). The mean leaf width also differed among pH condition and month (Table 1). In the acidified stations we recorded lower leaf length and width in all the considered months, respect to the control station.

The leaf area index (LAI =  $m^2$  of leaf surface/ $m^2$  of sea bottom) ranged between 0.6 to 2.3 and was lower at all stations in October and higher in July (Supplementary Materials, Fig. S2). The LAI did not vary among stations/pH, since in the acidified stations, the lower leaf lengths, and therefore lower leaf surface areas, are compensated by higher shoot densities compared to those at the control station.

**Table 1.** Summary of permutational multivariate analysis of variance (PERMANOVA) results for *Posidonia oceanica* phenological features: number of leaves per shoot, leaf length, and leaf width.

Number of Leaves				
Source	df	M.S.	Pseudo-F	P
pH	1	1.79	1.38	0.18
Month	5	32.43	24.96	<b>0.001</b>
pH x Month	5	-20.54	-15.81	0.95
Residual	348	1.29		
Mean leaf length				
Source	df	M.S.	Pseudo-F	P
pH	1	2707.2	65.8	<b>0.001</b>
Month	5	3,935.10	95.6	<b>0.001</b>
pH x Month	5	-135.30	-3.3	<b>0.001</b>
Residual	348	41.17		
Mean leaf width				
Source	df	M.S.	Pseudo-F	P
pH	1	0.55	73.37	<b>0.001</b>
Month	5	0.02	2.80	<b>0.005</b>
pH x Month	5	-0.13	-18.06	1.00
Residual	348	0.007		

The leaf apex erosion (coefficient *A*) showed greater percentages of eroded leaves occurring at all the stations during the summer months (Fig. 3). Grazing (biological erosion) was the main source of leaf damage, especially in the acidified stations N3, Vu3, and Vu6, whereas mechanical erosion was less represented. Differences in the type of apex erosion were detected among pH conditions, months, and the interaction between these two factors (PERMANOVA, see Table 2). Leaves grazed by the herbivore fish *S. salpa* were the most common and dominant in the acidified stations, with sea urchin and crustacean (mainly isopods) bites never exceeding 35% of the analyzed leaves (Fig. 4). On the contrary, grazing by crustaceans and sea urchins was higher in the control station, where *S. salpa* bites were less frequent.

### Epiphyte assemblages

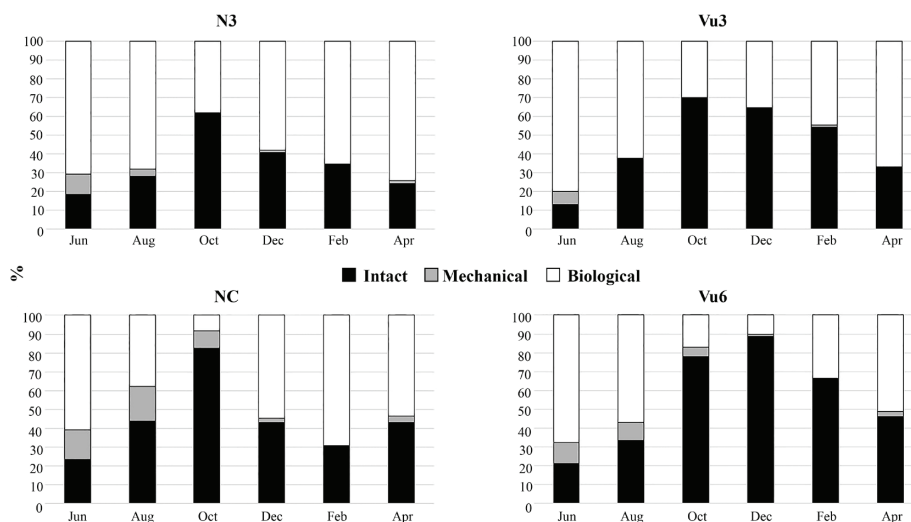
The study of the epiphyte assemblages was limited to the months of June, August, and October, corresponding to the maximum development of the leaf canopy and associated epiphytic organism colonization. A total of 23 epiphytic taxa were identified (Table 3). The development of the epiphytic assemblages showed differences

**Table 2.** Summary of permutational multivariate analysis of variance (PERMANOVA) results for type of *Posidonia oceanica* leaf erosion.

Leaf apex erosion				
Source	df	M.S.	Pseudo-F	P
pH	1	0.27	4.29	<b>0.015</b>
Month	5	2.16	33.99	<b>0.001</b>
pH x Month	5	-0.59	-9.30	<b>0.001</b>
Residual	348	0.06		

between the acidified and control stations (Fig. 5). The dominant taxonomical categories found at N3, Vu3, and Vu6 were encrusting brown algae and erect algae: the former strongly increased from June to October, whereas the latter showed a peak in August and then decreased in October. Bryozoans showed a constant coverage percentage at N3, whereas the coverage increased over time at both Vu3 and Vu6. Hydrozoans shared the same pattern at all acidified stations: increasing over time and peaking in October. On the contrary, tunicates were sporadically present at low pH levels. *Posidonia* leaves showed an absence of encrusting coralline algae at acidified stations in all months, except for in October at N3. Other calcified organisms, such as foraminifers and spirorbid polychaetes, were rarely found at all the stations, and no more than a single specimen per sampling spot was counted at the acidified stations. On the contrary, the control station was characterized by a dominance of coralline algae, which also increased over time to maximum coverage in October. Encrusting brown algae were also present at normal pH conditions but showed lower coverage percentages than at the acidified stations. Furthermore, they did not display an increasing pattern from June to October. Both erect algae and bryozoans were present throughout the sampling period at the NC station, but with low abundance. Tunicates were present only in August at the NC station. On the contrary, calcareous foraminifers and spirorbid polychaetes were more abundant at NC if compared to the acidified stations, especially in October. The observed differences in epiphyte composition and relative dominance were significant according to the pH condition, month, and pH × month interaction (PERMANOVA analysis, Table 4); pairwise testing highlighted that these differences were between the acidified and control stations. The taxonomical categories responsible for the 80% dissimilarity between stations (SIMPER analyses) are shown in Table S2 (Supplementary Materials).

The nMDS ordination plot of the epiphyte assemblage



**Fig. 3.** Percentage of leaves showing different types of leaf apex erosion (intact, mechanical, and biological) over time at the studied stations. At least 30 of the oldest leaves were examined at each month and station.

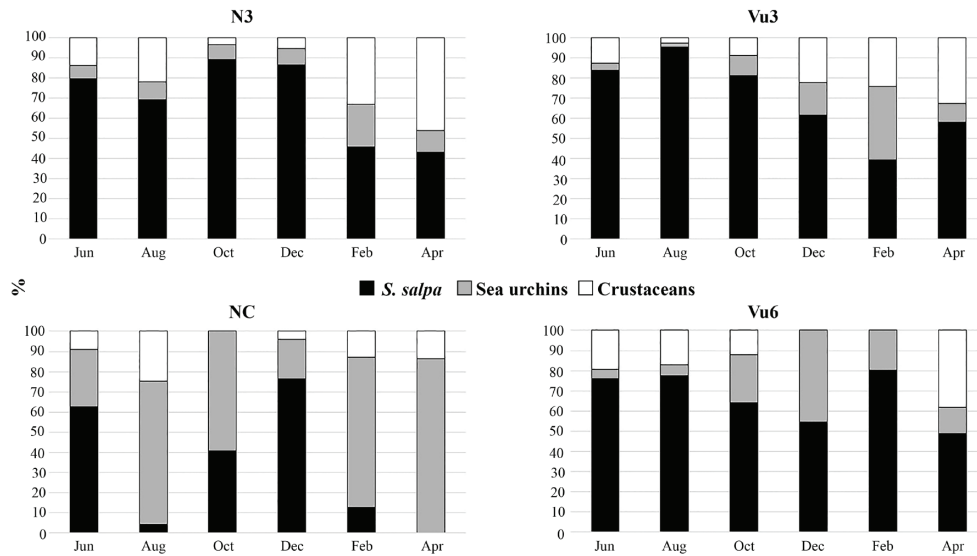


Fig. 4: Percentage of different types of biological apex erosion (*Sarpa salpa*, sea urchins, and crustaceans) during time at the studied stations and considering only the leaves showing biological erosion.

Table 3. List of the epiphytic taxa found in the samples.

#### Encrusting Brown Algae

*Myrionema orbiculare* J. Agardh, 1848

#### Erect Brown Algae

*Giraudia* spp. Derbès & Solier, 1851

*Dictyota* spp. J.V.Lamouroux, 1809

*Sphacelaria cirrosa* (Roth) C. Agardh, 1824

*Halopteris filicina* (Grateloup) Kützing, 1843

#### Encrusting Red Algae

*Pneophyllum* spp. Kützing, 1843

*Hydrolithon* spp. (Foslie) Foslie, 1909

#### Erect Green Algae

*Cladophora* spp. Kützing, 1843

*Pseudochlorodesmis* spp. Børgesen, 1925

#### Foraminifera

Foraminifera spp.

#### Hydrozoans

*Aglophenia harpago* Schenck, 1965

*Pachycordyle pusilla* (Motz-Kossowska, 1905)

*Sertularia perpusilla* Stechow, 1919

#### Spirorbids

*Janua heterostropha* (Montagu, 1803)

*Spirorbis marioni* Caullery & Mesnil, 1897

*Spirorbis spirorbis* (Linnaeus, 1758)

#### Bryozoans

*Electra posidoniae* Gautier, 1954

*Chorizopora brongniartii* (Audouin, 1826)

*Microporella ciliata* (Pallas, 1766)

*Disporella hispida* (Fleming, 1828)

*Tubulipora plumosa* (Thompson in Harmer, 1898)

#### Tunicates

*Botryllus schlosseri* (Pallas, 1766)

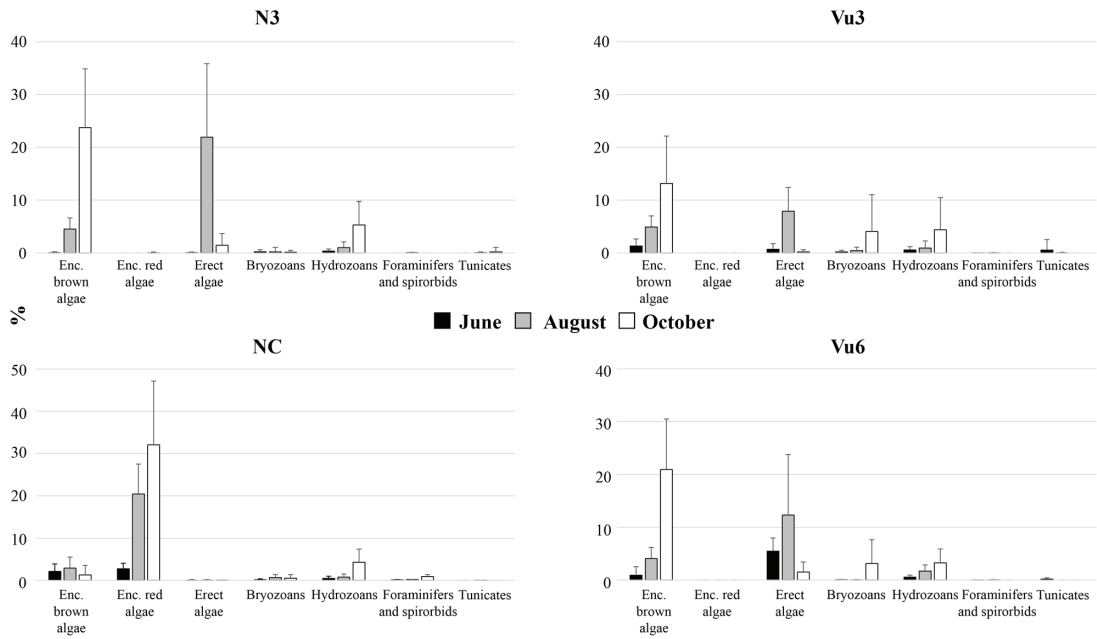
*Trididemnum cereum* (Giard, 1872)

structure produced two main clusters: one encompassing the acidified stations, and the other including the control one (Fig. 6a–c). These two clusters remained well separated in each of the three separately analyzed months. Furthermore, the separation between the acidified stations was more appreciable in the June ordination (representing an early stage of epiphyte colonization) (Fig. 6a), and less pronounced in the August and October plots (Fig. 6b–c), representing a more mature assemblage. As a general pattern, the acidified stations of N3, Vu3, and Vu6 showed a lower dispersion, which indicated less variability between individual shoots compared to that at the NC station; this indicated that at normal pH levels, the epiphytic assemblage was more variable and diversified

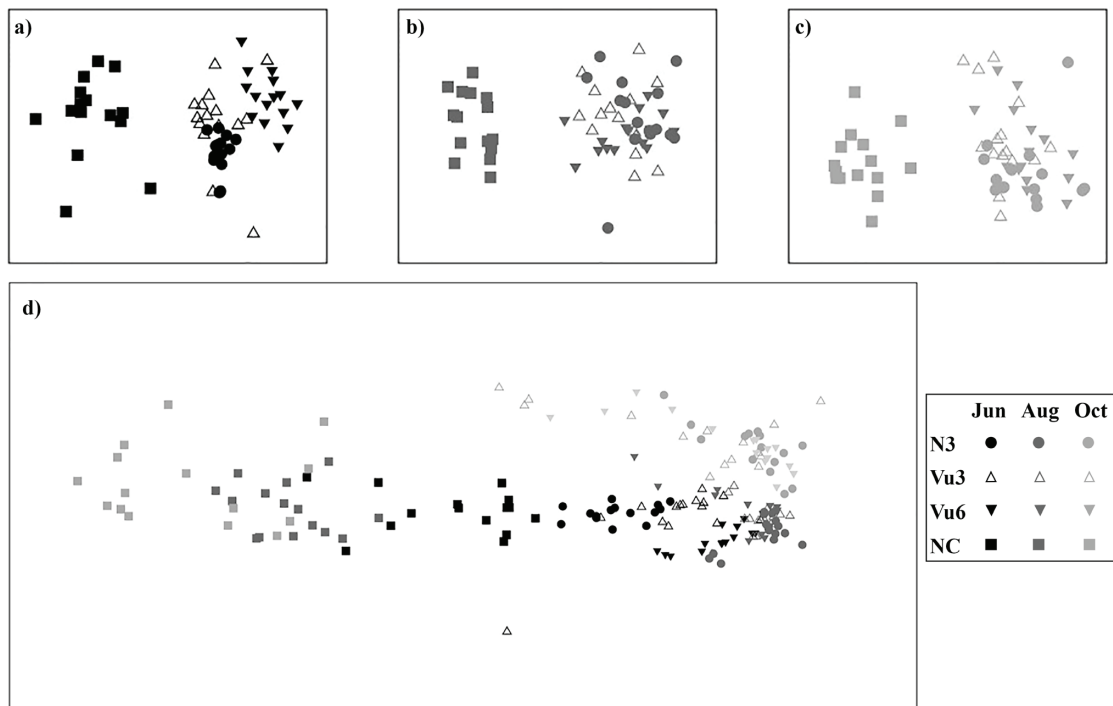
with greater patchiness.

The time evolution of the epiphytic assemblages in the different stations is shown in the nMDS plot in Fig. 6d to better highlight the time differences according to acidification. The epiphytic assemblages at the NC station was more abundant, diversified, and characterized by a higher patchiness and inter-shoot variability compared to those at the three acidified stations. The NC assemblage was widely distributed along the first axis according to sampling month, while the assemblages of the three acidified station showed a more limited distribution along the same axis, indicating that the assemblages under OA conditions do not evolve into a more mature community, but remain in a relatively early colonization stage with a





**Fig. 5:** Temporal evolution of the coverage percentage of the epiphytic assemblages (June, August, and October) at the studied stations.



**Fig. 6:** Non-metric multidimensional scaling (nMDS) plot of the epiphytic assemblages: a) June (stress = 0.16); b) August (stress = 0.15); c) October (stress = 0.12); d) three months plotted together (stress = 0.25) to highlight the evolution of the community over time. Circles indicate N3, empty triangles indicate Vu3; inverse filled triangles indicate Vu6; quadrats identify the control (NC). Colors indicate sampling month: black = June; dark gray = August; and light gray = October.

**Table 4.** Results of permutational multivariate analysis of variance (PERMANOVA) and pairwise test on epiphytic assemblages found on *Posidonia oceanica* leaves. Significant values are highlighted in bold.

<b>Epiphytic assemblages</b>				
Source	df	M.S.	Pseudo-F	P
pH	1	1.61	172.56	<b>0.001</b>
Month	2	0.34	37.01	<b>0.001</b>
pH x Month	2	0.06	6.57	<b>0.001</b>
Residual	174	0.01		

<b>Pairwise test comparison (Station)</b>		
Pairs	p value	adj. p value
N3 - NC	<0.001	<b>&lt;0.001</b>
N3 - Vu3	0.15	0.95
N3 - Vu6	0.34	1
NC - Vu3	<0.001	<b>&lt;0.001</b>
NC - Vu6	<0.001	<b>&lt;0.006</b>
Vu3 - Vu6	0.015	0.09

lower diversity and coverage.

## Discussion

This study provides information on the effects of OA on *P. oceanica* meadows, considering plant phenology, associated epiphytic assemblage, and grazing pressure. The results indicated that plant structure will change under future scenarios, and animal and algal communities will be altered in parallel by those modifications.

The *P. oceanica* shoot density, plant phenology, and epiphyte assemblages in the studied stations of the two vent systems showed significant differences among pH conditions. High shoot densities under OA have also been observed in the Castello vents in previous studies (Hall-Spencer *et al.*, 2008, Donnarumma *et al.*, 2014; Garrard *et al.*, 2014) and even prior to the high venting recorded in this area, especially on the south side of the CO<sub>2</sub> gradient (Giraud *et al.*, 1979; Buia *et al.*, 2003; Foo *et al.*, 2018). The densities recorded at both Vullatura stations confirmed this pattern; the Vu3 station at 3 m depth has an average of > 800 shoots/m<sup>2</sup>, which is comparable to that reported for the N3 station at a similar depth (3 m) (Donnarumma *et al.*, 2014). The density at the Vu6 station is also higher (> 600 shoots/m<sup>2</sup>) than that reported for the control station, with an average of approximately 400 shoots/m<sup>2</sup>, considering that the Vu6 station is at 6 m depth and the control station is at 4 m depth, and therefore should have higher shoot density according to Pergent-Martini & Pergent (1996). This result confirms that *Posidonia* has higher levels of shoot density where the highest emission of bubbles occurs, and the water shows higher acidification. Although it is difficult to discern the

role of depth (and therefore of the intensity of light radiation) compared to other factors (e.g., greater availability of CO<sub>2</sub> for photosynthesis), low pH conditions seem to have a positive influence on the total *Posidonia* shoot density (Invers, 2001; Hall-Spencer *et al.*, 2008).

The other morphological features considered, such as leaf length, width, and the coefficient *A* (% of apex erosion), show significant variations according to the pH conditions. In general, under normal pH conditions (NC station), the leaves tended to be longer and wider and thus have higher leaf surface values than those found at the three acidified stations in all the periods studied. For example, in June, when the maximum length of the leaf canopy was observed, in stations N3, Vu3, and Vu6, average lengths of less than 40 cm were recorded, compared to the average of 60 cm observed at the NC station. This trend can be attributed to a greater vulnerability of the seagrass to erosion under acidified conditions, especially biological erosion due to the heavy grazing of the herbivorous fish *S. salpa*. In fact, at the acidified stations the impact of grazing by *S. salpa* is significantly higher than at the NC station. This herbivorous fish has already been identified as one of the dominant and most frequent fish species in the Castello's shallow *Posidonia* meadow (Guidetti & Bussotti, 1998), and is frequently observed grazing in shoals of more than 50 individuals, especially at low pH stations (Garrard 2013; Gambi MC, personal observation). In addition, the phenological analysis of *P. oceanica* in a previous study at the Castello vents (Donnarumma *et al.*, 2014), highlighted the same trend, with the dominance of grazing marks of *S. salpa* both in the south (S3) and north (N3) stations, this latter coinciding with our study station.

Few or no coralline algae (which are of low nutritional value) in the epiphyte assemblages of acidified areas may make *Posidonia* more attractive for herbivorous fishes, thus increasing the grazing activity. Moreover, some studies have found a higher nitrogen content (lower ratio C:N) in *P. oceanica* leaves taken from sites where CO<sub>2</sub> emissions are present compared to those in leaves taken from sites under normal pH conditions (Vizzini *et al.*, 2010); some of these studies also include the N3 station (Ricevuto *et al.*, 2015; Scartazza *et al.*, 2017). This slight excess of N in the tissues of the plant translates into its greater nutritional value in acidified environments (Ricevuto *et al.*, 2015; Scartazza *et al.*, 2017), and therefore could explain the high levels of grazing by herbivorous fishes.

Another consideration that may support the increased palatability of *Posidonia* leaves under acidified conditions is related to the phenolic content of this seagrass that decreases in response to water acidification (Arnold *et al.*, 2012). The only data available on the phenol content in *Posidonia* in the acidified areas at the Castello, show a one-third reduction in the phenol content in plants settled in CO<sub>2</sub> emissions compared to those in control areas (Migliore *et al.*, 2012). Thus, this result seems to support the preference of herbivorous fishes for the shallow acidified *Posidonia* stands.

The most interesting of our results concerns the ef-

fects of acidification on the composition and structure of the epiphyte community, especially the effects on calcifying algae and invertebrate organisms and their evolution in time.

Encrusting red algae (Corallinales), which were the dominant group of *P. oceanica* epiphytic organisms under normal pH conditions (Casola *et al.*, 1987; Piazzini *et al.*, 2016), were not present at acidified stations except for October at N3. Numerous studies have confirmed that the Corallinales are highly vulnerable to acidification (Hall-Spencer *et al.*, 2008; Martin *et al.*, 2008; Kroeker *et al.*, 2011; Porzio *et al.*, 2011; Donnarumma *et al.*, 2014), because of the solubility of their carbonate skeletons, which have high magnesium contents (with variable concentrations of 3.5%–6% Mg; Milliman, 1974). The algal epiphytic assemblage on *Posidonia* leaves at low pH sites is mainly composed of fleshy algae (encrusting and erect brown and green algae) and non-calcareous invertebrates, such as hydroids and tunicates. This profound difference in the epiphyte assemblages in low pH areas certainly has cascading effects on the trophic web and functioning of the ecosystem. In these sites, the direct grazing on the leaves by *S. salpa* is dominant.

Specifically, the decrease/absence of the Corallinales cover could be another of the possible causes of greater *Posidonia* leaf palatability, as discussed above, and therefore the greater impact of grazing in acidified environments; and reduced leaf lengths all over the year.

Other calcifying taxa, such as calcareous foraminifers and spirorbid polychaetes, show a reduction in acidified stations. In fact, the sensitivity of these taxa at low pH values is dependent on the carbonate composition and phenotypic plasticity of the species, although with a few exceptions with regard to some spirorbid species (Lucey *et al.*, 2018). In addition, coralline algae act as pioneer species in the colonization of *Posidonia* leaves (Casola *et al.*, 1997), and once established, allow colonization by other groups, including foraminifers and polychaetes (Corlett & Jones, 2007). The disappearance of the Corallinales cover could therefore influence many other epiphytic groups and, consequently, cause a series of changes in the entire trophic web associated with the leaf canopy (Martin *et al.*, 2008).

A different pattern has been observed for the bryozoans, which, although being calcifying organisms, do not seem to be so negatively influenced by low pH conditions. Their ability to withstand low pH values can be explained by the presence of an organic tissue that, by covering the skeleton of the zooids, gives protection against the acidity of the water (Rodolfo-Metalpa *et al.*, 2010). This tolerance to low pH conditions could also be explained by the mineralogical composition of Bryozoa. The encrusting bryozoans are characterized by a skeleton composed of about 60% calcite and 40% aragonite (Poluzzi & Sartori, 1974), with a modest amount of magnesium (0.1%–3% Mg versus 3.5%–6% Mg of coralline algae, Milliman, 1974) which make these organisms more resistant to chemical dissolution in acidified environments (Martin *et al.*, 2008).

With regard to the structure of the epiphyte commu-

nity, we observed a reduction in taxonomic diversity due to the absence of calcareous species in favor of a community dominated by fleshy algae, from normal pH to acidified environments. In contrast, the animal component was mostly represented by the taxa that, having no calcareous skeleton, are less affected by water acidification, such as hydrozoans and tunicates. Overall, the community of acidified areas is impoverished and more homogeneous, and seems to not evolve with time after the initial colonization stage, remaining in a kind of “forever young” state. This is suggested by the lower dispersion of replicates of the acidified stations of N3, Vu3, and Vu6 in multivariate ordination models, which indicate low variability between individual shoots; in contrast, in the NC station, the community is more variable and diversified, with greater patchiness and community evolution over time. The Vu6 station had a higher degree of dispersion than the two shallower acidified stations, likely due to the greater depth and more diverse community.

The natural leaf assemblage described here is very similar to those found by previous studies on the epiphytic component of *P. oceanica* in the Castello area (Martin *et al.*, 2008; Nogueira *et al.*, 2017) and studies on mimics of artificial leaves of *Posidonia* (Donnarumma *et al.*, 2014). In particular, the artificial leaves (mimics) showed, albeit with some differences, a rather similar composition of the dominant forms, and a very similar pattern of colonization along the gradient. In both types of substrate (natural and artificial), there is clearly a reduction, to a total absence, of all calcareous forms in the highly acidified stations, which are instead replaced by filamentous forms, with only the sporadic presence of some calcified and low pH-resistant species (e.g., some bryozoans, spirorbids).

In a study by Nogueira *et al.* (2017), *Posidonia* epiphytic communities were found to vary significantly in terms of composition and abundance at two naturally acidified sites (the southern S2 and northern N2 low pH areas at the Castello) and at a control site far from the vents (Lacco Ameno, Ischia). These differences resulted in a greater abundance of non-calcareous encrusting brown algae and a decline in spirorbid polychaetes in both acidified sites. A further reduction in coralline algae was observed only in the south side of the vents, suggesting that OA may alter the epiphytic groups in different ways because of its interaction with local factors, such as the different exposures to hydrodynamics and the different levels of grazing by fish.

Phenology and epiphytic communities have also been studied in *Posidonia* meadows under the influence of CO<sub>2</sub> emissions off the island of Panarea (Guilini *et al.*, 2017). Here, the shoot density and the LAI are not affected by the relatively low levels of OA, although the authors still note shorter leaves at the site of CO<sub>2</sub> emission, suggesting a greater susceptibility to erosion, potentially by herbivores, as we found in our study. The sessile epiphyte community differs in composition and is characterized by a greater species richness in the weakly acidified site, although the net concentration of epiphyte calcium carbonate was similar. These authors suggested the presence

of a greater ecosystem complexity at the emission site, which may have favored the higher diversity of benthic copepods, compared to the rest of the mobile epiphyte community, which remains otherwise unaltered between the acidified site and the control site. The differences between our results and those observed at Panarea (Guilini *et al.*, 2017) seem to be due to the different degree of acidification between the reference areas of the two studies, being certainly that the acidified areas at N3 and Vullatura were subjected to a mean pH and range of variability much greater than those at Panarea island, where a difference of only 0.1 unit of pH was observed between sites.

CO<sub>2</sub> enrichment through the FOCE system did not reveal the same changes in *Posidonia* shoot density and structural characteristic of the leaf (Cox *et al.*, 2016). The short time exposure (6 months) to low pH conditions of FOCE experiments is not likely sufficient to produce the difference highlighted in our natural systems, where the *Posidonia* shoots have been exposed to OA for several decades. In contrast, the effects on the epiphytic community assessed through the laboratory study are consistent with our results (Cox *et al.*, 2015). In fact, a decrease in taxonomical diversity and lack of coralline algae were observed after enrichment of CO<sub>2</sub> and low pH levels.

There is a final consideration regarding the possible interactions between the phenology, its variation over time, and the structure of the epiphytic community. It has already been pointed out how intense grazing activity, especially by herbivorous fishes, causes a significant modification of the leaf canopy, which in the acidified environments has much shorter and narrower leaves compared to that in the normal pH zones. This is reflected in a lower average leaf surface area of the plants, and therefore a smaller space potentially available for epiphyte colonization. However, the high shoot density registered in the acidified areas contributed to maintaining the LAI high. Therefore, the overall available space offered by the plant for epiphyte colonization is comparable between the acidified and control stations; the differences found in the epiphyte components are therefore not attributable to possible limitations or competition for space, but appear to be influenced by the microenvironmental conditions of the leaf layer, which include the pH of the site, as well as biotic interactions.

Similar shifts in the composition and richness of epiphytic assemblages have been detected in disturbed seagrass meadows. In particular, the effect of nutrient enrichment can produce an increase in algal biomass; both encrusting coralline algae and filament/erect (not crustose) algae are favored by nutrient increase (Cambridge *et al.*, 2007; Balata *et al.*, 2008). Although the former take up nutrients very quickly, the latter are highly tolerant to environmental changes and become extremely diffused in polluted areas, resulting in them being advantaged when competition occurs (Giovannetti *et al.*, 2010). In the vent systems of the Castello at Ischia, a similar competitive interaction between coralline and fleshy algae has been demonstrated on hard substrates (Kroeker *et al.*, 2012).

The recent study on the role of nutrient enrichment

and its synergistic effect with OA has been highlighted by Ravaglioli *et al.* (2017) at the vents on the Castello south side. These authors showed that the structure of the epiphytic community on *P. oceanica* leaves was influenced by both the pH and nutrient levels, but there were no effects of their interaction. There were less epiphytes (especially coralline algae) under low pH exposure; however, all epiphytes (including coralline algae) showed higher cover under both moderate and high nutrient enrichment. Finally, there were no differences in epiphyte dispersion for both pH and nutrient conditions, indicating that nutrient addition did not cause changes in the spatial heterogeneity of the epiphytic assemblage (Ravaglioli *et al.*, 2017).

The synergetic actions of several factors of global change (OA and human disturbances, such as nutrient increase) could lead to a rapid acceleration of the whole process, causing winners and losers among species in marine benthic communities. The changes in phenology and epiphyte communities that have been here confirmed can be considered important aspects of the effects of climate change, and could represent the drastic modifications that seagrass systems are going to face by the end of the century.

The alteration of epiphytic assemblage, especially in a complex ecosystem such as the *P. oceanica* meadows, can not only open the way to possible extinction of species exclusive on its leaves, but also produce a series of cascading effects on the associated communities, as well as on the entire trophic web and functioning of one of the most important ecosystems in coastal Mediterranean areas.

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The following supplementary information is available online for the article:

*Fig. S1:* Trend in time of the pH (measured with hourly acquisition of SeaFet instruments, see Kroeker et al., 2011 for technical details) in the studied stations. At N3 and NC boxplots were measured in May 2010 and graphically modified from Kroeker et al. (2011). At Vu (twenty-three days SeaFet deployment) data were acquired in April 2016 at 6 m depth (Vu6) and partially integrated with observation in Gambi et al. (2019).

*Fig. S2:* Leaf Area Index (LAI) calculated at the four sampling stations from June 2016 to April 2017.

Table S1. Summary of all the parameters taken into account in the present study; date indicate when measurements were acquired.

Table S2. Results of the SIMPER analyses, showing dominant taxonomical categories mostly responsible for the dissimilarity among stations.