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Running page head: *In situ* CO_2 enrichment on epiphytes

ABSTRACT: Alterations in seagrass epiphytic communities are expected under future ocean acidification conditions, yet this hypothesis has been little tested *in situ*. A Free Ocean Carbon Dioxide Enrichment (FOCE) system was used to lower pH by a ~ 0.3 unit offset within a partially enclosed portion (1.7 m^3) of a *Posidonia oceanica* meadow (11 m depth) between 21 June and 3 November 2014. Leaf epiphytic community composition (% cover) and bulk epiphytic mineralogy were compared every four weeks within three treatments, located in the same meadow: a pH-manipulated (experimental enclosure) and a control enclosure, as well as a nearby ambient area. Percent coverage of invertebrate calcifiers and crustose coralline algae (CCA) did not appear to be affected by the lowered pH. Furthermore, fleshy algae did not proliferate at lowered pH. Only Foraminifera, which covered less than 3% of leaf surfaces, declined in manner consistent with ocean acidification predictions. Bulk epiphytic magnesium carbonate composition was similar between treatments and percentage of magnesium appeared

to increase from summer to autumn. CCA did not exhibit any visible skeleton dissolution or mineral alteration at lowered pH and carbonate saturation state. Negative impacts from ocean acidification on *P. oceanica* epiphytic communities were smaller than expected. Epiphytic calcifiers were possibly protected from the pH treatment due to host plant photosynthesis inside the enclosure where water flow is slowed. The more positive outcome than expected suggests that calcareous members of epiphytic communities may find refuge in some conditions and be resilient to environmentally-relevant changes in carbonate chemistry.

KEY WORDS: ocean acidification, seagrass–epiphyte interactions, calcifiers, magnesium carbonate, coralline algae, Bryozoa, pH, remineralisation

Introduction

Seagrass leaves and rhizomes are colonized by taxonomically diverse animal and algal representatives referred to as epiphytes following the definition of Steel and Wilson (2003). Seagrass and epiphytes form meadows which are highly valued for the services they provide (Hemminga and Duarte 2000). For example, they play a fundamental role in maintaining populations of exploited fisheries (Jackson et al. 2015). In the Mediterranean Sea, the seagrass *Posidonia oceanica* L. (Delile) covers 23% of shallow water substratum (< 50 m, Pasqualini et al. 1998) and leaf epiphytes can constitute ~30% of the canopy biomass (Prado et al. 2008). Seagrass leaf epiphytes include coralline and filamentous algae, polychaetes, foraminiferans, and bryozoans (Borowitzka et al. 2006). Among these groups are several calcifiers (e.g. coralline algae, foraminiferans, serpulid polychaetes and some bryozoans) which contribute to carbonate cycling (Frankovich and Zieman 1994; Perry and Beavington-Penney 2005). Moreover, *P.*

oceanica epiphytes can contribute 20 and 60% to meadow primary production and nutrient uptake (Borowitzka et al. 2006; Lepoint et al. 2007). Most herbivores feed on algal epiphytes and several grazers feed on the epiphytic invertebrates (Lepoint et al. 2000). As evidence of their importance as a food source, epiphyte abundance and herbivore dynamics are tightly coupled (Tomas et al. 2005).

Through the process of ocean acidification, the pH in the ocean is being lowered with a subsequent decline in the proportion of carbonate ions (CO_3^{2-}) and an increase in the proportions of bicarbonate ions (HCO_3^-) and dissolved carbon dioxide (CO_2). Surface ocean pH decreased by 0.1 units since the pre-industrial era and an additional 0.07 to 0.33 units decrease is expected by 2100 (Gattuso et al. 2015). The decline in the CO_3^{2-} concentration is projected to affect the ability of calcifying organisms to maintain their skeletons (Feely 2004; Kroeker et al. 2013). Macroalgal species can also respond differently to the increased carbon available for photosynthesis and many, without calcified surfaces, are thought to be better competitors under future ocean acidification conditions (Beer and Koch 1996; Koch et al. 2013). The concern is changes in competitive abilities may cause shifts in composition at the community level (Fabry et al. 2008; Kroeker et al. 2012; Gaylord et al. 2015; Sunday et al. 2017).

Seagrass epiphytic coverage and composition examined under lower pH conditions near CO_2 vents and in the laboratory generally support future ocean predictions based upon physiology and mineralogy. The epiphytic calcified community, which is often dominated by crustose coralline algae (CCA), is less abundant at lowered pH. Furthermore, epiphytic invertebrates with lower Mg content and those organisms that lack calcified skeletons, such as filamentous algae, often persist at lowest pH conditions (Martin et al. 2008; Campbell and Fourqurean 2014; Donnarumma et al. 2014; Cox et al. 2015). The predicted loss of CCA at

lowered pH is a global concern for ecosystem function. Species of CCA occur in temperate and tropical seagrass beds and in a variety of other habitats (< 295 m depth) where they serve key ecological roles (Littler and Littler 2013). They are known to be a food source, cement and stabilize reefs, facilitate recruitment, and add significantly to sediments (Land 1970; Nelsen and Ginsburg 1986; Littler and Littler 2013; Gischler et al. 2013). Although vent systems predict their loss and shifts in the community under ocean acidification, they are not perfect predictors of future ocean ecology owing to the large variability of pH in space and time (Hall-Spencer et al. 2008; Kerrison et al. 2011). Furthermore, laboratory experiments have difficulties accounting for the many environmental variations and species interactions that can alter predicted outcomes (e.g. Burnell et al. 2014). Therefore, predictions on the fate of *P. oceanica* meadows could benefit from additional information provided by the manipulation of pH *in situ* on entire communities for an extended period of time (months to years).

Alteration in the abundance of *P. oceanica* epiphytes will likely have repercussions to meadow carbon cycling and feeding capacity. Therefore, the aim of the present study was to test the hypothesis that *P. oceanica* epiphytic community will be impacted by ocean acidification. We tested this hypothesis *in situ* with a Free Ocean Carbon Dioxide Enrichment (FOCE) system (see Gattuso et al. 2014). This system allows pH to be manipulated continuously, in an enclosure, at a fixed offset from ambient levels. The offset takes into account natural pH fluctuations that may alter organismal responses. During a 4 month-experimental period, epiphytic coverage as well as carbonate mass was quantified on *Posidonia* leaves. Lastly, some minerals in epiphytic calcified structures are more susceptible to dissolution and mineral changes at elevated partial pressure of CO_2 (pCO_2) are not well understood. Therefore, we analyzed epiphytic mineralogy throughout the duration of the study.

Methods

Experimental setup and system function

This study used the European FOCE (eFOCE) system which allows for the *in situ* manipulation of pH in benthic enclosures as an offset from ambient pH (Gattuso et al. 2014). The system was deployed in the Bay of Villefranche, approximately 300 m from the Laboratoire d'Océanographie de Villefranche (NW Mediterranean Sea, France; 43°40.73′N, 07°19.39′E).

The study design consisted of two clear, 1.7 m^3 (2 m long x 1 m width x 0.85 m tall) perspex enclosures that enclosed a portion of a *P. oceanica* meadow. The enclosures were located at 11 m depth and were placed approximately 1.5 m apart. The pH in one enclosure, referred to as the experimental enclosure, was lowered by ~0.3 units as an offset from ambient pH as measured on the total scale. This offset was based upon the business-as-usual representative concentration pathway RCP8.5 following Ciais et al. (2013) and corresponded to a mean (± SD) pH_T of 7.75 ± 0.13 and pCO_2 of 971 ± 323 μatm. In the second enclosure, the pH was not manipulated and it served as a control. A third treatment consisted of an open fiberglass frame of the same dimensions as the enclosure footprint (2 m^2). It was placed nearby (3 m of the experimental enclosure) and in the same meadow. It is referred to as a reference plot and was used to account for any effects generated by the enclosure structure. True replication was not logistically feasible. Replication was sacrificed to 1) control pH precisely within enclosures of a large enough size to contain *P. oceanica* and 2) sense pH and other aspects of the environment continuously in the three treatment locations.

The details of the eFOCE system function and maintenance are described in Cox et al. (2016) and a schematic can be found in Supplemental Figure 1 (Fig. S1). Briefly, the pH in the

experimental enclosure was altered using surface supplied seawater pumped into a mixing tank, which was located on a surface platform. Pure CO_2 was bubbled into the mixing tank and the resulting low pH seawater was pumped (flow rate up to 0.12 L min^{-1}), via tubing, underwater to the proximity of the benthic enclosures. Prior to entering the enclosures, low pH (pH_T ~ 5.5) and ambient seawater were mixed in an underwater tube and a set (x3) of centrifugal pumps (6.7 L min^{-1} each) injected ambient seawater in the control enclosure and lowered-pH seawater in the experimental enclosure. Seawater pH was measured before entering the enclosures enabling the automated adjustment of the low pH seawater injection to maintain the desired pH offset. Seawater inside enclosures was circulated by a set of centrifugal pumps (4 per chamber; 6.7 L min^{-1} each) and exited through two openings (12 cm diameter). The renewal time of seawater in each enclosure was ca. 1.5 h. The system contained a number of sensors: 4 potentiometric Seabird 18-S pH sensors located inside each enclosure and in each mixing tube and three Seabird 37 SMP-ODO CTD with SBE 63 O_2 optodes and three LI-COR-192 PAR (photosynthetic active radiation) sensors located in each enclosure and one nearby the enclosures (in ambient). The carbonate chemistry within each treatment was determined at high frequency using average total alkalinity together with sensed temperature, salinity and pH_T, in the R package, seacarb (see Cox et al. 2016 for more details).

Timeline

The experiment comprised three periods in 2014: (1) the pre-acidification period, before pH was manipulated, from 15 May to 11 June, (2) the transition period from 12 to 21 June, when pH in the experimental enclosure was slowly lowered by no more than 0.05 units per day until an offset of approximately -0.3 units was reached and (3) the experimental period from 22 June to 3 November when pH in the experimental enclosure was maintained at a offset of ~ -0.3 units.

Collection of seagrass leaves

Six to ten oldest leaf blades were collected from separate *P. oceanica* shoots growing within the reference plot and enclosures. Oldest leaf blades, or the outer most leaf in the bundle, were selected because these blades have more developed epiphytic communities (Cebrián et al. 1999). Divers collected intact leaves spaced evenly throughout the plot or enclosure at approximately four week intervals during the acidification period in 2014, at time (T) 1 to 4: T1 occurred on 31 July, 39 d after acidification, T2 occurred on 4 September, 74 d after acidification, T3 on 9 October after 109 d of acidification and T4 occurred on 10 November after 135 d of acidification. It should be noted that the acidification of the experimental enclosure ended on 4 November 2014 but due to logistical constraints the final collection of blades were made six days later. A set of ten leaves was also collected immediately after the transition period, on 26 June 2014 (referred to as sampling interval, T0). These leaves were collected in a 2 x 1 m area in the meadow, located ~2 m from the enclosures. They were collected outside the enclosures and the reference plot to limit destructive sampling within the experimental setup but still obtain a baseline measure. All leaves were collected above the sheath, placed into separate plastic bags, transferred into a darkened cooler, and transported to the laboratory.

Determination of epiphytic coverage and composition

Leaves used for determination of epiphytic coverage and composition were kept in a temperature controlled (20 to 22 ºC), darkened room for less than 24 h until scanning was completed. A high-resolution scanner *ZooScan* (Hydroptic, France; Gorsky et al. 2010) produced colour images (2400 dpi) of leaves and their epiphytes. Five to seven images of leaves were used to represent the assemblage within the reference plot and enclosures at each interval, except at T1 when error resulted in three to four scanned leaf images being used per treatment.

The cell counter plug-in, in ImageJ, generated a grid (0.1 cm x 0.1 cm) superimposed on the scanned image of the leaf. Organisms that occurred directly underneath each intersection of the grid or point (231 to 1244 depending upon leaf length) were identified to the lowest possible taxonomic or functional unit and counted. Fifteen lowest possible taxonomic or functional groups were identified. These 15 were lumped into 11 groups that shared functional or taxonomic similarity (see Table S3). The 11 groups were as follows: CCA (pigmented if pink in coloration or bleached if thallus appeared white), non-calcified algae, Bryozoa, serpulid polychaetes, Foraminifera, Hydrozoa, Porifera, unidentified, biofilm and ascidians. Biofilm was defined as a group of microscopic organisms that formed a visible film across the surface of the leaf. SEM images indicated this group is likely composed of diatoms and bacterial films and rod forms. Percent cover by organism (or unit) was determined for each leaf by dividing the organism intersections by the total number of intersections analysed and multiplying by 100.

Calcium carbonate mass

After scanning, the mass of $CaCO_3$ contained in the epiphytes was assessed using the weight loss after acidification method (Bosence 1989; Perry and Beavington-Penney 2005). Leaves with epiphytes were dried at 60 °C for 12 h, weighed (*A*, 0.01 mg), acidified with 5% HCl, rinsed twice with deionised water, dried again at 60 °C for 12 h and re-weighed (*B*). Ten young leaves without epiphytes were treated in the same manner (*C*). The weight of the epiphytic calcareous mass was then determined from the following equation, $A - [B / (1 - C)]$.

Determination of mineralogy

After each leaf collection, three leaves from each treatment were set aside to air-dry (~22 ºC) at room temperature. Dried epiphytes were gently scraped from separate leaves and ground into a fine powder for X-ray diffraction (XRD, N = 3 per treatment). Additionally, to obtain a

baseline mineral profile the XRD analysis was performed on separate Bryozoa and CCA sampled at T0 and carefully removed from the leaves and ground. Scanning electron microscopy-energy dispersive spectroscopy (SEM-EDS) was further used to understand how the minerals identified by XRD, were present on the leaf surface. Using SEM-EDS we compared the skeletal structure of CCA between treatments.

XRD was carried out using a SIEMENS D501 Bragg-Brentano diffractometer equipped with a graphite monochromator and scintillation detector, using CuKα radiation. Settings were a step size of 0.02° and a scan speed of 1° per minute. Precision for determination of Mg-content of Mg-calcite was ~0.5%. Scan interpretation followed procedures described in Nash et al. (2014). SEM-EDS was done using a Zeiss UltraPlus field emission scanning electron microscope, equipped with an Oxford Inca EDS. For EDS measurements, the Zeiss was set to 15.0 kV, 15 mm working distance with a beam interaction volume ca. 3 µm. Imaging was at 3 mm working distance and 3 kV. Samples were platinum coated. A sample was embedded in crystal bond and polished for precise SEM-EDS measurements or mounted intact and attached using carbon tape. Aragonite was quantified using the area under the curve method (Diaz-Pulido et al. 2014). Many Mg-calcite peaks had minor asymmetry on the lower 2-theta side indicating the presence of small amounts of calcite. Comparisons of relative calcite asymmetry were made using the principles of peak asymmetry developed in Nash et al. (2014).

Statistical analyses

The approach used was to monitor the epiphytic community at three sites: control enclosure, experimental enclosure, and a reference plot. This study, similar to many natural experiments, lacks true replication (Hurlbert 1984). In unreplicated designs in ecology, the emphasis is on the estimation of effect size and the unique ecological perspective provided

(Hurlbert 1984; Stewart-Oaten et al. 1986; Oksanen 2001; Davies and Gray 2015). Inferential statistics were avoided. Furthermore, a large effect size was expected based upon previous study results (e.g. Martin et al. 2008).

Multivariate analyses were used to compare the leaf epiphytic communities. Prior to analyses, the epiphytic coverage at each interval was averaged. Thus, there was one value for each of the three treatments (reference, control enclosure, and the experimental enclosure) at each interval (T1-T4). A square root transformation was applied and a Bray-Curtis resemblance matrix created between each interval-treatment assemblage (4 intervals x 3 treatments = 12 leaf assemblages). Dissimilarities were visualized with an nMDS (non-metric multi-dimensional scaling) plot. A two-way (treatment x interval) Analysis of Similarity (ANOSIM) without replication and 999 permutations was used to examine for differences. This is a valid approach in ecological monitoring when there is pseudoreplication (Clarke 1993). The global R from an ANOSIM indicates effect size. It ranges from -1 to 1 and is analogous to a correlation coefficient; a value close to zero indicates no or little distinction between a prior groups. The ANOSIM was followed by two separate (treatment and interval) similarity percentage analyses (SIMPER) to identify the amount each taxonomic or functional group contributed to dissimilarity.

Data from leaves collected at T0 (before the perturbation) were not used in multivariate analyses because they were only collected at one instance and outside the experimental setup. Similarly, organisms that occurred on one to three leaves out of 70 were removed prior to analyses to eliminate their inflated influence on dissimilarities.

The abundance (mean, median, and range) of specific taxa, $CaCO_3$ mass, and epiphytic mineral composition were compared qualitatively through time between the three treatments;

paying careful attention to any directional deviations observed on leaves from the experimental enclosure.

Results

Environmental and experimental conditions

Environmental and experimental conditions as well as seagrass growth are fully described in Cox et al. (2016). There were 150 to 175 shoots m^{-2} of *P. oceanica* inside each plot and enclosure and few other macrophytes (< 11% coverage). Leaf biometrics were not affected by the lowered pH. Average shoot height increased from 40.6 cm in April to 73.4 cm in August then declined to 24.8 cm in November.

The carbonate chemistry is summarized in Table 1 and the diel variability is provided in Table S1. The pH_T in the meadow (ambient) ranged from a monthly mean of 7.98 (± 0.06 SD) to 8.11 (± 0.04 SD, Table 1). The mean saturation states of aragonite (Ω_a) and calcite (Ω_c) ranged from 3.1 to 3.6 and 4.9 to 5.4 from June to September, respectively. The diel pH_T change differed among months from 0.04 to 0.12. It corresponded to the daily change in CO_2 concentration driven by community primary production, respiration and calcification.

The carbonate chemistry in the control enclosure and the ambient environment were similar (monthly mean differed $\leq$ 0.06 units). The diel change in pH_T within the control enclosure was slightly greater than in ambient and was consistent in the pre- and during acidification period (median ± median absolute deviation 0.14 ± 0.06 and 0.14 ± 0.06).

During the acidification period, the pH in the experimental enclosure was maintained at a mean -0.26 unit offset (monthly mean values from -0.22 to -0.29 pH units) from the control enclosure (Table S1). Monthly mean values of saturation state with respect to aragonite (Ω_a) ranged from 2.0 (± 0.05 SD) in October to a high of 2.5 (± 0.06 SD) in August and saturation

263 state with respect to calcite (Ω_c) ranged from 3.0 (± 0.07 to 0.008 SD) in September and October
264 to 3.8 (± 0.09 SD) in August. Median diel pH range in the experimental enclosure was two to
265 three times larger than the control (monthly ranged from 0.09 to 0.29 pH units) and had greater
266 variability. Variation was attributed to lowered buffering capacity of seawater with lowered pH.
267 Monthly differences as summarized in Cox et al. (2016) were evident, particularly for
268 temperature (mean monthly range: 17.7 to 24.2°C) and PAR (mean monthly range: 1.3 to 7.3
269 mol photons m^{-2} d^{-1}, Table S2) but were similar in the ambient, control and experimental
270 enclosures.

271 **Leaf epiphytic community description**

272 Overall, CCA were the most dominant epiphyte occurring on all leaves at coverages
273 between 0.8 to 58.8%, followed by the lesser abundant biofilm (0 to 22.0%) and Bryozoa (0 to
274 20.8 %). Hydroids and sponges were found on 3 of the 70 leaves (< 2%). An ascidian occurred at
275 12% on one leaf collected from the reference plot.
276 SEM images confirmed the presence of CCA, Bryozoa, Foraminifera, serpulid
277 polychaetes and biofilm. At this increased SEM resolution, bacterial films, rod structures and
278 diatoms were visually distinguishable. These organismal groups were likely undetected or
279 grouped to 'biofilm' in the quantification of macroepiphytes. Unidentified rod structures of 1-2
280 µm in length were commonly found on the epiphytes but not directly on the leaves (Fig. S2).
281 Diatoms were observed both on epiphytes and leaf surfaces.

282 **Spatial and temporal patterns in epiphytic community**

283 There was little distinction in epiphytic composition and coverage found on leaves from
284 the enclosures and reference plot yet, clear differences were observed between T1-T4 intervals

285 (ANOSIM: treatment, global R = 0.25, p-value = 0.28; interval, global R = 0.56, p -value =
286 0.003; Fig. 1)

287 Indeed, SIMPER routine indentified dissimilarities (TS4) between communities in the
288 plot and enclosures to be small (ranged from 19.2 to 26.7%). Differences in abundances of
289 biofilm and pigmented CCA contributed most (Table S4, 19.1 to 33.3%) to treatment
290 dissimilarities. Leaves from the reference plot had an overall (across all sampling intervals, n =
291 18 to 20 leaves) greater coverage of pigmented CCA (mean ± SD, reference = 27.9 ± 15.3,
292 control = 19.5 ± 9.4 and experimental = 23.4 ± 10.7%) and leaves from the control had a greater
293 cover of biofilm (mean ± SD, reference = 0.8 ± 0.9, control = 7.2 ± 5.3, experimental = 2.8 ±
294 2.5%). It was also noted that there was a greater coverage of biofilm in enclosure communities,
295 with percentages more similar to those observed at T0 in leaves from the ambient. In SEM
296 images, relatively greater numbers of diatoms were observed on leaves collected in the
297 enclosures than on leaves collected at T0 and in the reference plot.

298 Dissimilarity in communities increased with increasing duration between sampling
299 intervals. For example, the overall (combined treatments) community at T1 was most dissimilar
300 from communities at T3 and T4 (20.9 to 30.2% dissimilar, respectively) and least dissimilar from
301 the community at T2 (14.1%). Also, the community at T2 was more similar to the community at
302 T3 than the community at T4 (Table S5, T2 and T3 were 18.2% dissimilar, T2 and T4 were
303 26.7% dissimilar).

304 In enclosures and in the reference plot, there was a decline in the abundance of CCA
305 (bleached and pigmented, separate groups in analyses) from July (mean ± SD, T1 31.4 ± 8.3%)
306 to November (mean ± SD, T4 11.7 ± 6.2%). Bleached and healthy appearing CCA showed
307 similar trends in time. Together, they accounted for 33 to 55% of the dissimilarity between

intervals. Other epiphytic groups also declined from T1 to T4 and contributed to interval dissimilarities (each contributed between 4.1 to 13.7%), these included non-calcified algae (mean ± SD, T1 2.2 ± 2.7% to T4 0.3 ± 0.4%) and Bryozoa (mean ± SD, T1 2.1 ± 1.4% to T4 0.5 ± 0.6%).

The abundances of epiphytes found on the T0 leaves (n = 7, collected from nearby enclosures before the pertubation), highlight the large spatial or temporal variability in abundance of some groups, such as Bryozoa and Foraminifera (Fig 2).

Trends in organismal coverage to evaluate predicted pH effects

Overall (pooled across sampling intervals), leaves from the experimental enclosure had a slightly greater mean coverage of pigmented CCA than those from the control enclosure (Fig. 2 A) and the range of coverage often overlapped. The coverage of non-calcified algae (Fig. 2D, mostly *Dictyota* sp.) declined in all treatments and the overall mean (± SD) was slightly lower in the experimental than in the control enclosure (1.2 ± 1.8% *vs* 0.7 ± 0.7%). It contributed 4.7% to enclosure differences. Leaves from the experimental enclosure also tended to have a relatively greater coverage of invertebrate calcifiers (Fig. 2E, F; mean ± SD, control *versus* experimental, Bryozoa: 0.6 ± 0.8% *vs* 1.0 ± 0.9%; serpulid polychaetes: 0.1 ± 0.1%, *vs* 0.4 ± 0.4%). These abundances contributed 8% each to differences between enclosures. Only, leaf epiphytic Foraminifera (Fig. 2G) had a directional change in abundance distinct from the change in abundances on leaves from the control enclosure and reference plot. Foraminifera coverage was greatest on leaves at T1 within the experimental enclosure (0 to 1%), they declined at T2 (0 to 0.1%) and disappeared from the collected leaves at T3 and T4. However, this taxon is rare (indicated by low coverage, <1%) and coverage between leaves can be highly variable (see T0). It contributes 7% to enclosure community differences (Table S4).

Calcium carbonate mass

$CaCO_3$ mass on leaves ranged from 8.6 to 24.7 mg cm^{-2} (Fig. 3). There were no clear consistent patterns that would indicate seasonal changes or lowered pH effect.

Mineralogy

The magnesium carbonate composition of leaf epiphytes ranged from 10.6 to 13.2 mol% $MgCO_3$ and there was no indication of a low pH effect (Fig. 4). The mean (± SD) mol % $MgCO_3$ was 11.9 ± 0.6 on leaves from the reference plot, 12.1 ± 0.9 on leaves from the control and 12.0 ± 0.7 on leaves from the experimental enclosure. Values obtained on samples collected at T0 confirmed that CCA and Bryozoa were, respectively, 11.3 to 11.7 and 8.3 to 8.8 mol% $MgCO_3$.

Changes in epiphytic mol% $MgCO_3$ by sampling interval appeared to be seasonal (Fig.4). The community mean (±SD) value tended to increase from T0 (June, 10.7 ± 0.1) to T1 (July, 11.1 ± 0.4) and maintained a similar composition between T2 and T4 (September to November, 12.2 ± 0.4 and 12.4 ± 0.6 mol% $MgCO_3$).

There were two other mineral phases present in the epiphyte community in addition to magnesium calcite, calcite and aragonite. Aragonite was present on all 24 leaves examined from the enclosures but was not present on leaves collected from the reference plot, nor from T0 leaves from the ambient environment (16 leaves in total). The proportion of aragonite in bulk epiphytes between enclosures was similar at each interval from T1 to T3. At T4, it was greater in two of the three epiphyte samples collected from the control enclosure and in three of three samples collected from the experimental enclosure (Fig. 4).

Calcite was predominantly present in epiphytes collected from the reference plot and at T0 in ambient epiphytes (Fig. 4). Epiphytes from the ambient environment at T0 and T1 to T3 in the reference plot had minor calcite amounts present as indicated by slight asymmetry of the Mg-

calcite peak. There were separate peaks for calcite and Mg-calcite for bulk epiphytes at T4, indicating substantial amounts present, but they were not quantified. The asymmetry method is not appropriate when the peaks are entirely separate, as for the T4 samples. In this case the value of (-) 6 was given, being the approximate difference between the value for calcite (which contains ~3-4 mol% $MgCO_3$) and Mg-calcite (9-10 mol% $MgCO_3$).

SEM-EDS was used to visualize the surfaces of leaf epiphytes and examine the location of mineral phases. Imaging was undertaken on subsamples from three leaves collected at T0 from the ambient environment and from both enclosures and reference plot at T1 and T4. Loose grains of Mg-calcite were present on the seagrass surface (Fig. S3). These appeared to be remnant grains after the surficial CCA had broken off, possibly during sample preparation. Calcification features of the CCA from the enclosures and reference plot appeared similar in structure (Fig. 5). There were not any structural indications of dissolution from the lowered pH. EDS measurements also confirmed that the CCA were Mg-calcite.

All the imaged CCA had areas of alteration where their cellular structure was no longer intact (Fig. 6). EDS measurements showed that alteration areas were responsible for the calcite or aragonite identified by XRD. Altered surfaces of epiphytic CCA revealed different mineral phases related to a "structural effect" from the enclosures. On leaves from the ambient environment (reference plot as well as T0), the altered CCA surfaces appeared rough and were composed of calcite with no micro-endoliths visible. In contrast, the altered surfaces of CCA on leaves from the enclosures were aragonitic. The aragonite-altered CCA showed areas that appeared similar to the calcitic altered areas on CCA from the ambient, with the exception that there were also partially eroded cells that had altered to aragonite (Fig. 6, Fig. S4). Crystal morphology of the aragonite varied from blocky to typical aragonite needle shape. Particular

attention was paid to the November samples from the reference plot to determine whether there were other epiphytes or changes that could be responsible for the substantially greater amount of calcite observed relative to the amount observed at T0 and T1 in the epiphytes grown in the ambient environment. The only calcite detected was in alteration areas that appeared similar to that observed previously at T0 and T1. Diatoms and bacterial films were observed on the surfaces of leaves and CCA, often in close proximity to the altered algal surfaces (Fig. S4).

Discussion

The lack of a pH effect on *P. oceanica* epiphytic community is in contrast with findings from previous laboratory manipulations and observations of communities conducted near CO_2 vents where the pH is naturally lower (Hall-Spencer et al. 2008; Martin et al. 2008; Donnarumma et al. 2014; Cox et al. 2015). Martin et al. (2008) showed a complete disappearance of epiphytic coralline algae and the persistence of bryozoans at an average pH_T of 7.7, but with large temporal variations from < 7.0 to > 8.1. Donnarumma et al. (2014), at the same CO_2 seep, showed that the calcifying species tend to be less competitive as pH_T decreases (8.1 to 6.7) and that leaves were dominated by filamentous algae, hydroids and tunicates at the lowest pH_T (mean 6.7). In the laboratory, Cox et al. (2015) exposed *P. oceanica* shoots with their associated epiphytes to three constant pH levels (pH_T 8.1 ambient, 7.7 and 7.3) for four weeks. Under both low pH treatments, there was a reduction of CCA and reduced calcification rates. Similar shifts in community composition have been noted on other seagrass species as a consequence of lowered pH (Burnell et al. 2014; Campbell and Fourqurean 2014; Martínez-Crego et al. 2014).

In the present study, the epiphytic community was largely composed of CCA and Bryozoa. A similar proportion of epiflora to epifauna composition on *P. oceanica* has been

described in other investigations (Lepoint et al. 1999; Martin et al. 2008; Prado et al. 2008; Cox et al. 2015) and coverages were similar to those reported by Cox et al. (2015) and Prado et al. (2008). The decline in coverage at T4 coincides with the period of known decline of seagrass biomass and leaf turnover after storm events in the autumn (Alcoverro et al. 1995).

CCA are often identified as having a large susceptibility to ocean acidification (Nelson 2009; Koch et al. 2013; Hofmann and Bischof 2014; McCoy and Kamenos 2015). CCA epiphytes on *P. oceanica* have exhibited lowered calcification rates and coverage near and below the pH_T of 7.7 (Martin et al. 2008; Cox et al. 2015). Martin et al. (2008) has also demonstrated their vulnerability to dissolution at pH_T of 7.0 with strong undersaturation of carbonate. Although some species are able to reduce carbonate demands by altering their structural thickness (McCoy and Ragazzola 2014), we did not observe any visible or quantifiable alteration in CCA skeletons related to pH manipulation, even after four months of exposure.

Bryozoa have also been studied in the vicinity of CO_2 vents as well as in the laboratory (Rodolfo-Metalpa et al. 2010; Lombardi et al. 2011b; Lombardi et al. 2011a; Smith 2014). Many have an outer cuticle beneath which the mineralized skeleton forms. The protective cuticle barrier and low Mg-calcite composition or ability to alter mineral composition has been used to explain their persistence on leaves at volcanic CO_2 seeps with a pH_T as low as 6.98 (Martin et al. 2008; Rodolfo-Metalpa et al. 2010; Donnarumma et al. 2014). Transplant experiments, however, indicate that some group members can be negatively affected (decreased thickness and signs of dissolution) by ocean acidification particularly in warmer months (Rodolfo-Metalpa et al. 2010; Lombardi et al. 2011b; Lombardi et al. 2011a). Therefore, it appears that the pH environment in the experimental enclosure, even during the warmer months, was not detrimental to calcification for the bulk of the community.

423 It should be noted that despite rarity, Foraminifera did decline in the experimental
424 enclosure in a pattern consistent with a response to ocean acidification. This observation is in
425 agreement with 20 out of 26 studies reviewed on Foraminifera under elevated pCO_2 that have
426 reported negative responses to lowered pH (Keul et al. 2013).
427 Recently, there have been several studies with outcomes which conflict or fail to support
428 widely-held ocean acidification projections. For example, Martin and Gattuso (2009), and
429 Egilsdottir et al. (2013) describe no clear effect of minimally lowered pH on calcifiers. Even for
430 epiphytes, Apostolaki et al. (2014) and Saderne and Wahl (2013) did not find a loss in calcified
431 coverage or reduced calcification rates at lowered pH. The present study outcome adds to the
432 growing literature which suggests that calcified communities in their natural settings can be little
433 affected by minimal changes in surrounding carbonate chemistry.
434 There have been many speculations on the conditions that result in conflicting outcomes
435 for calcifiers under lowered pH. Discrepancies are attributed to species specifity, other
436 environmental conditions that stress or limit organismal physiology (e.g. differences in light,
437 temperature, or combined stressors), or limitations and differences in study design (e.g. treatment
438 levels used, Kroeker et al. 2010; Koch et al. 2013; Gazeau et al. 2013).
439 Some macroalgae and benthic invertebrates are known to respond differently to pH
440 fluctuations than they do to a mean pH difference (Britton et al. 2016; Small et al. 2016). The
441 median diel pH variation was 0.1 in the ambient meadow. The median diel pH range in the
442 experimental enclosure was two to three times larger than the control (0.09 to 0.29 pH units).
443 This difference could not be explained by O_2 fluxes alone and instead was attributed to lowered
444 buffering capacity of seawater with lowered pH (Cox et al. 2016). Because of natural pH
445 fluctuations, it is hypothesized that organisms in seagrass meadows may already experience and

446 be pre-adapted to pH levels projected into the next century (Hendriks et al. 2014a). In the present
447 study, the 0.1 diel pH variability in the meadow would not support possible acclimation to a pH_T
448 of 7.7. Productivity of coastal macrophytes can buffer the impacts of ocean acidification by
449 providing a daily window of maximum $CaCO_3$ saturation where calcification can be more
450 efficient (Anthony et al. 2011; Anthony et al. 2013; Hendriks et al. 2014b; Cornwall et al. 2015).
451 It is possible that the pH offset used could have allowed for buffering at the leaf blade surface
452 and prevented CCA loss. To limit an impact, however, buffering in daylight must offset the
453 lowered pH that surrounds the community in the absence of photosynthesis at night. For
454 sensitive taxa the benefit of pH fluctuations appears limited (Cornwall et al. 2014; Johnson et al.
455 2014; Roleda et al. 2015). For instance, in the laboratory recruits of coralline algae had relatively
456 lowered growth rates when pH fluctuated as opposed to when pH was constant and lowest
457 growth occurred when pH fluctuations were altered to mimic a future ocean acidification
458 scenario (-0.4 difference from ambient, Roleda et al. 2015). Adult coralline algae, often more
459 tolerant to ocean acidification conditions, were unaffected (Roleda et al. 2015). Congruent with
460 the reduced numbers observed in the present study with exposure to lower pH, algal surfaces did
461 not provide refugia for foraminifera assemblages along a gradient of overlying seawater
462 acidification in Levante Bay, Italy (Pettit et al. 2015).

463 The outcome for epiphytes growing in host plant boundary layers may also depend upon
464 the pH scenario used. For example, recruitment and growth of calcifying serpulids and bryozoan
465 on the alga *Fucus serratus* were weakly to not affected at $pH_T = 7.7$ but were reduced at $pH_T =$
466 7.3 (Saderne and Wahl 2013). The ocean acidification scenario used (mean pH_T of 7.75) could
467 also explain the outcome in the present study. Maintaining a calcified skeleton presumably
468 becomes more difficult and costly as seawater gets closer to undersaturation (Kleypas et al.

1999). Seawater in the pH manipulated enclosure was lower than ambient yet, it remained saturated with respect to both calcite and aragonite (3.6 and 2.2). CCA have the ability to raise the pH within their boundary layer to limit the potential negative impacts of decreased ambient pH when seawater is not undersaturated (Hofmann et al. 2016). In contrast, at CO_2 seeps, pH near the vents can be highly variable and organisms can be exposed to pH levels substantially lower than projections for the next century (Kerrison et al. 2011). In addition, organismal physiological responses can be confounded by biological conditions facilitated by venting, not related to lowered pH (Vizzini et al. 2013).

Enclosures, while circulated and partly open, likely slowed water motion and could possibly account for the relative increase of diatoms and provided a better refuge for calcifiers. Diatoms are ubiquitous members of seagrass microepiphytic communities (Borowitzka et al. 2006; Mabrouk et al. 2014). Pinckeny and Fiorenza (1998) reported increased prevalence of diatoms on leaves in slower flows in the Atlantic. Microalgae were also more prominent on *P. oceanica* which has a greater structural canopy that can slow water movement than on *Cymodocea nodosa* (Mabrouk et al. 2014). Slowed water flows, like those that occur in dense canopies, increase boundary layer thickness surrounding plants and potentially allow for greater buffering capacity in daylight (Hurd 2015) and lower buffering capacity at night. Therefore, in the eFOCE experiment, the structural barrier of the enclosure may have affected the response both positively and negatively. The lack of effect on epiphytes suggest that the combined response was balanced.

pH effects on communities are known to be altered by seasonal factors (Burnell et al. 2014; Baggini et al. 2014; Martínez-Crego et al. 2014), yet in a year-long study, at an area with volcanic CO_2 seeps, epiphytic calcifier abundance on *P. oceanica* leaves was negatively

492 correlated with pH (Donnarumma et al. 2014). In contrast, the pH pertubation in the present
493 study occurred during a period with large seasonal environmental change (July to November)
494 and a climax epiphytic community, and no pH effects were observed. We also observed, in all
495 treatments, what appeared to be a recruitment event of filamentous algae and both CCA recruits
496 (small patches) and adults with reproductive conceptacles. Thus, we surmise that if the eFOCE
497 experiment was extended for a full year the outcome would be the same. Even though this
498 experiment was conducted in a period of biomass decline, we do not think it masked an impact.
499 In other studies, a decline in CCA calcification rates and coverage has occurred rapidly (weeks to
500 months), a time frame well within the duration of study and the sampling frequency. A repeated
501 experiment with extended experimental duration is needed to clarify long-term effects and to
502 include the period of peak faunal recruitment not captured in the current study.
503 To the best of our knowledge, this is the only study to concurrently investigate temporal
504 changes and pH effects on bulk epiphytic mineralogy. The only identifiable trend for Mg content
505 was over time. The increase in $MgCO_3$ after August (T2) could be explained by the seasonal
506 reduction in abundance of invertebrate calcifiers often composed of lower Mg-calcite.
507 Alternatively, or in combination, the incorporation of more Mg may be due the 2 to 6 °C increase
508 (e.g. Chave and Wheeler 1965; Diaz-Pulido et al. 2014) that occurred from June to August (T0 to
509 ~T2). The presence of calcite in the epiphytes from the reference plot, compared to its absence in
510 the epiphytes sampled in the enclosures, which instead had aragonite, is at this time without
511 explanation. Similarly, the increase in calcite and aragonite that occurred at T4 is without
512 explanation. There are many reports of aragonite in CCA (Nash et al. 2011; Smith et al. 2012;
513 Diaz-Pulido et al. 2014; Krayesky-Self et al. 2016). We are not aware of any report of calcite in
514 live CCA and thus this is the first documented alteration to calcite for CCA.

Accurate ecological projections of future oceans should arise as a consensus from combined study approaches: observational, controlled laboratory, modeling, and *in situ* experimentation. This study addressed a need for *in situ* pH manipulation to account for the complexity in community response to ocean acidification. Additionally, the study design accounted for natural pH variation that is often ignored when pH is manipulated *in situ*. While large scale unreplicated experiments, like eFOCE, can provide valuable ecological information they do have drawbacks (Hurlbert 1984; Oksanen 2001; Davies and Gray 2015). Replicated enclosures were not feasible at this stage. Alternative hypotheses that we cannot robustly exclude include (1) there were small pH effects difficult to quantify (2) that the conflicting outcome is due to some 'lurking' variable. However, several recommended steps (Oksanen 2001; Davies and Gray 2015) were taken to try to reduce erroneous conclusions that may occur including: (1) care was taken to select study locations that were similar in depth and seagrass density to reduce confounding variables (2) the environment was continuously monitored to ensure they were similar to those in ambient, (3) repeated measurements were made at the same location through time and compared to 'before' measurements when possible, (4) comparisons from the pH manipulated enclosure were made to two different spatial locations and (5) statistics used did not require replication.

The use of a FOCE system to study the epiphytic community on *P. oceanica* leaves provides a more positive outlook on the future of meadows than the projections based largely on observations near CO_2 vents. This conclusion should be tempered until more assessments are conducted with greater replication under a variety of conditions found in meadows. Nevertheless, results add to the growing evidence that calcareous members of macrophyte dominated communities may be more resilient to minimal changes in carbonate chemistry.

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Ethical Statement

The authors declare that they have no conflict of interests and all applicable guidelines for the use of animals were followed.

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Figure captions

Fig. 1 nMDS based upon the Bray-Curtis Index of dissimilarity for the epiphytic community within the reference plot, control and experimental enclosure (represented by symbols) at each sampling interval, labeled 1 to 4 for T1 to T4 which corresponds to 0, 39, 74, 109, 135 d after the pH manipulation in the months of June, July, September, October and November respectively

Fig. 2 A - G are boxplots showing the minimum, maximum, mean (dotted line) and median (solid line) coverage (%) of each epiphytic taxonomic or functional unit as occurred on leaves (N = 3 to 7) collected from the ambient (sampled 1x, T0), reference plot (ambient environment, T1 to T4) and two enclosures (control and experimental) at sampling intervals T0 to T4 (intervals correspond to 0, 39, 74, 109, 135 d after the pH manipulation in the months of June, July, September, October and November, respectively)

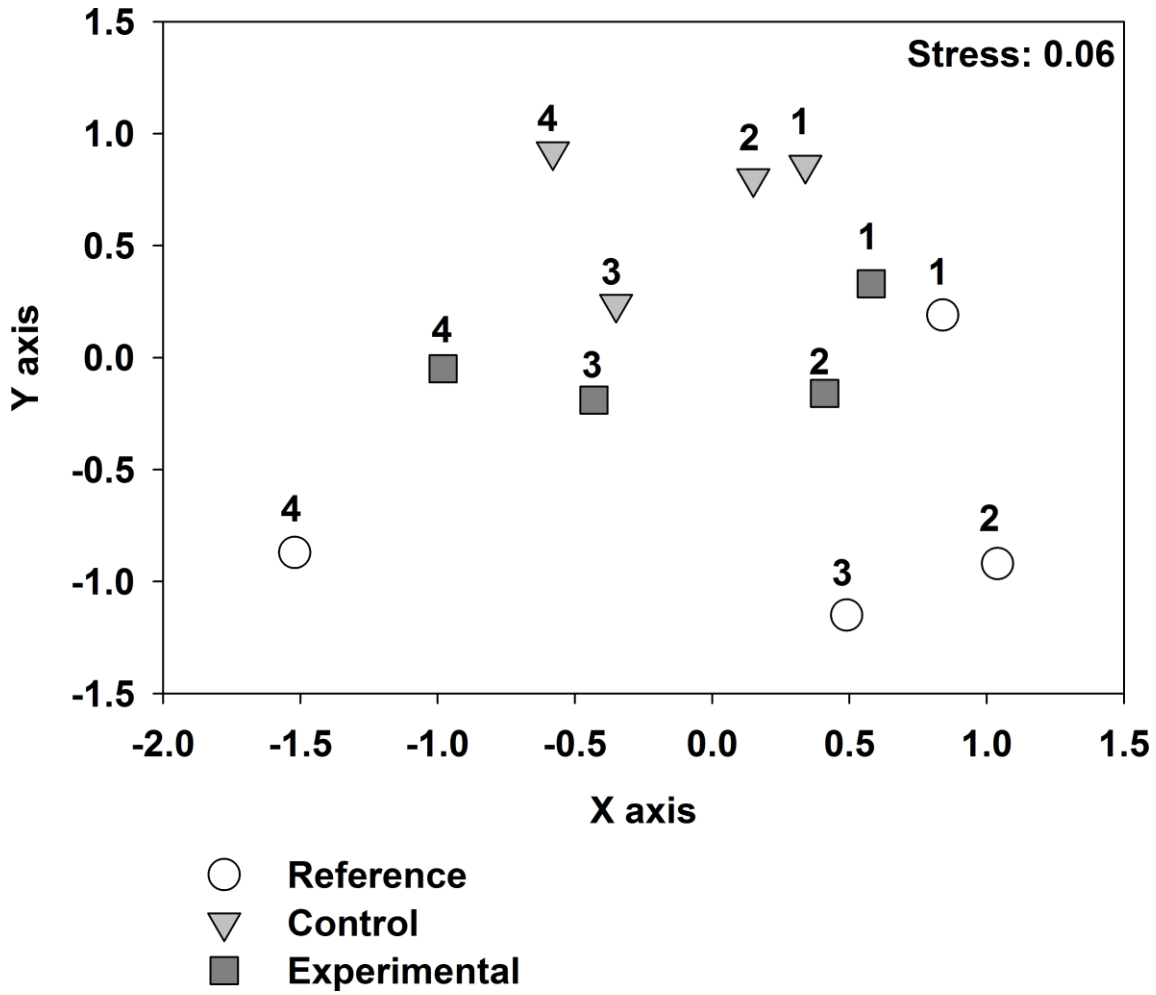
Fig. 3 Boxplots showing the minimum, maximum, mean (dotted line) and median (solid line) epiphytic total and calcareous organism coverage (%) as well as calcium carbonate ($CaCO_3$) mass (mg cm^{-2}, N = 3 to 7 leaves) in the ambient (sampled 1x, T0), reference plot (ambient environment, T1 to T4) and two enclosures (control and experimental) at sampling intervals T0 to T4 (See Fig.1 for additional details on intervals)

Fig. 4 Results from XRD with principles of peak asymmetry show, for each sample, the mol % magnesium in carbonate for the bulk epiphytes in the ambient (location sampled

1x, T0), reference plot (ambient environment, T1 to T4) and two enclosures (control and experimental) at sampling intervals T0 to T4 (N = 3 leaves, see Fig. 1 for additional details on intervals). The relative calcite asymmetry is shown in panel B; values were not quantified. Aragonite (C) was determined from the area under the curve

Fig. 5 SEM-EDS images of typical cell wall calcification of epiphytic crustose coralline algae (CCA) as observed in enclosures (control and pH-manipulated experimental) and ambient environment (T0 and reference plot) throughout the duration of study. Images A and B show crustose coralline algae on the leaf. B is a closer view of the outlined area where Mg-calcite (Mg-C) grains were observed. C and D show the crustose coralline cell walls and interfilaments engrained with Mg-calcite crystals

Fig. 6 SEM-EDS images of alteration of mineral composition in epiphytic crustose coralline algae as occurred on leaves from the ambient environment (T0 and reference plot) and two enclosures (control and pH-manipulated experimental). A-C (left) demonstrate mineral alteration between Mg-calcite (Mg-C) and calcite (abbreviated as C) which occurred in the ambient environment. A shows the location of calcite (white outline) and Mg-calcite. In closer view of the altered area (B and C) calcite grains can be observed. D-E demonstrates alteration from Mg-calcite to aragonite which occurred in the enclosures. D is an image of crustose coralline algae on the leaf surface showing altered patches where aragonite occurred; in closer view (E and F) the altered surfaces lacked cell wall features and clear crystal shape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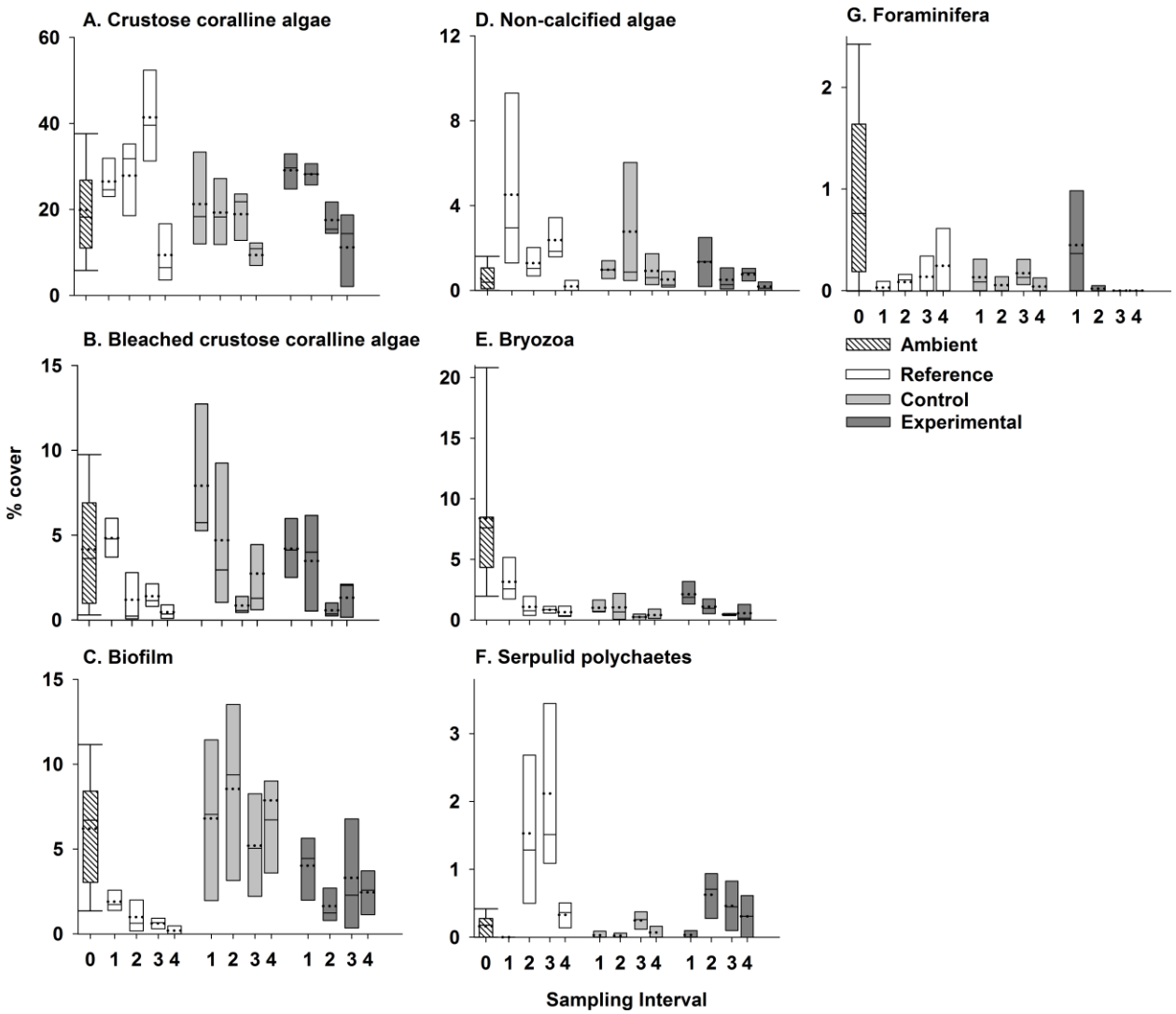
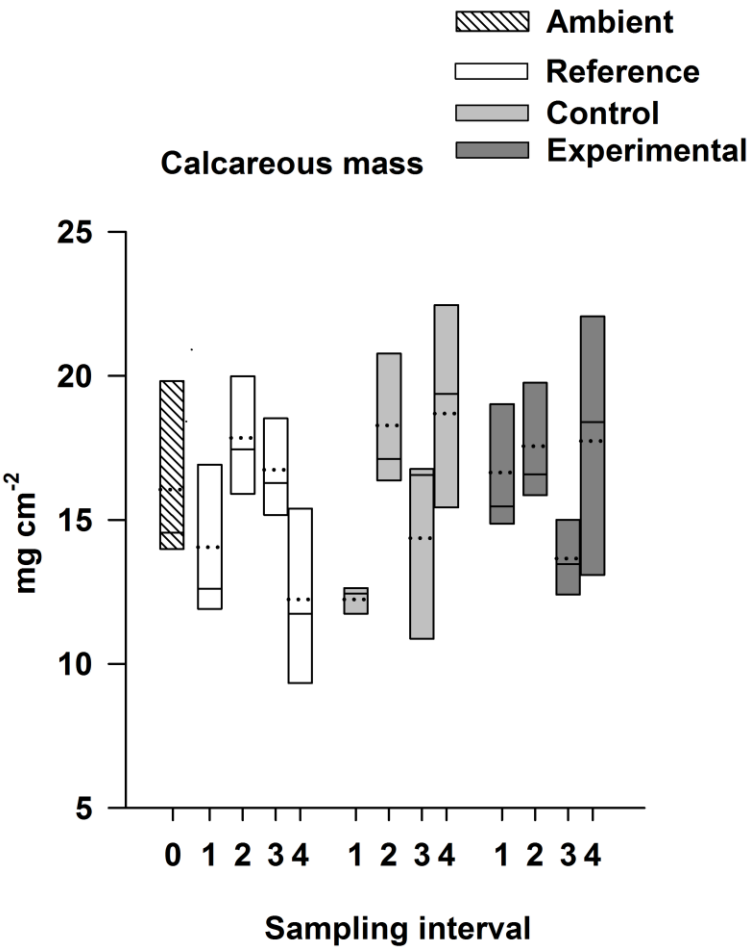
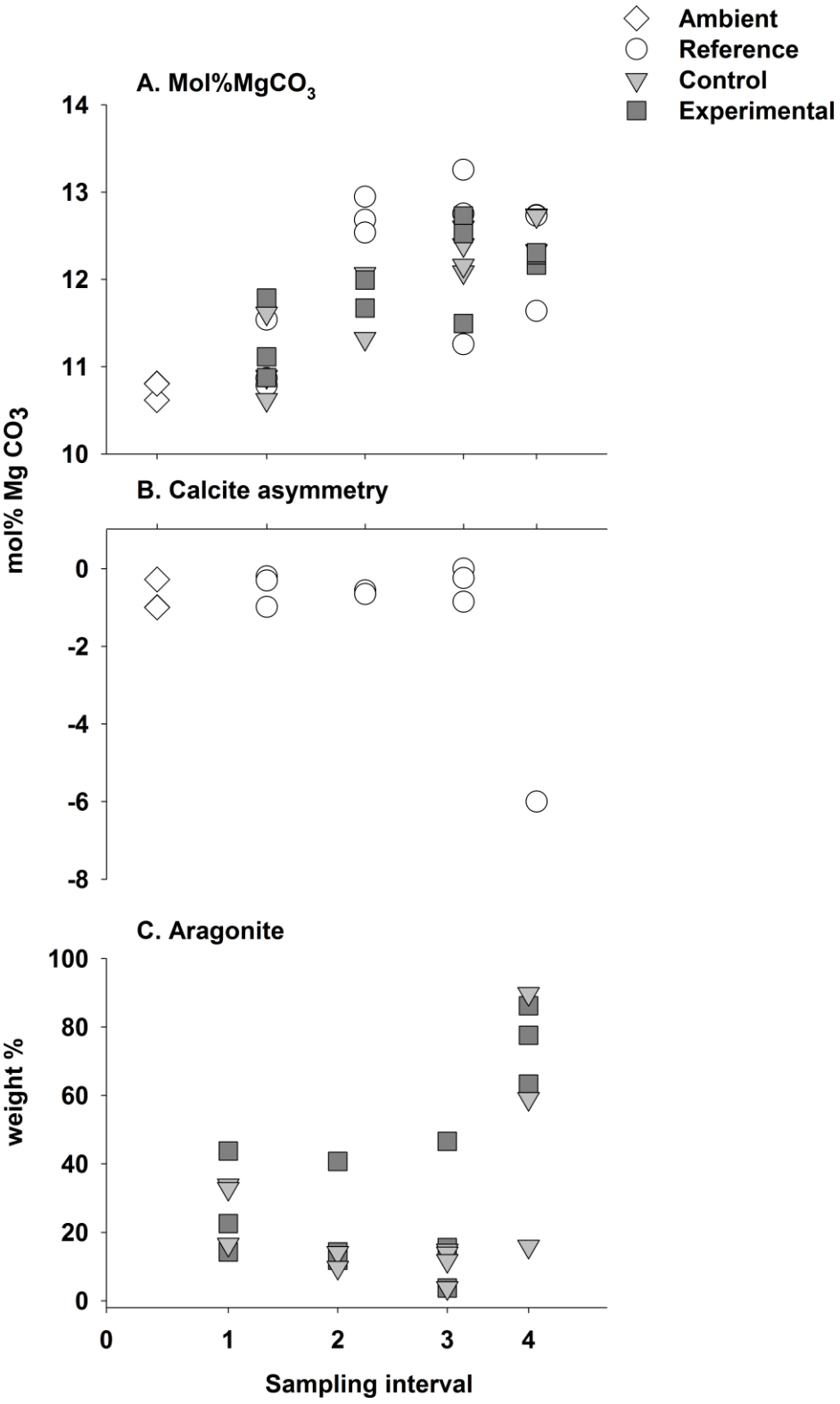
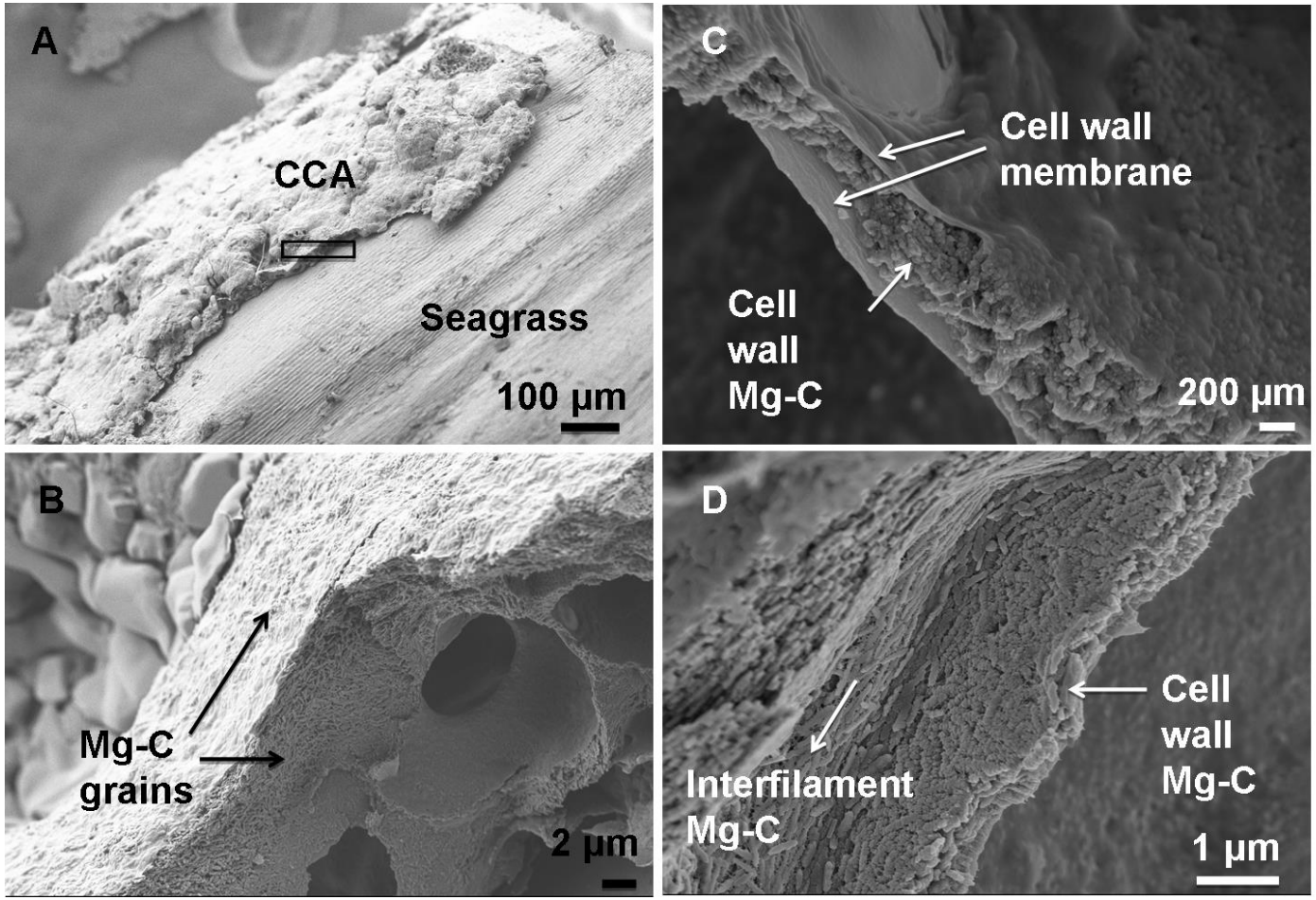
Y axis
X axis
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Reference
Control
Experimental


A. Crustose coralline algae
D. Non-calcified algae
G. Foraminifera
B. Bleached crustose coralline algae
E. Bryozoa
C. Biofilm
F. Serpulid polychaetes
% cover
Sampling Interval
0 1 2 3 4 1 2 3 4 1 2 3 4
Ambient
Reference
Control
Experimental


Ambient
Reference
Control
Experimental
Calcareous mass
25
20
15
10
5
mg cm-2
0 1 2 3 4 1 2 3 4 1 2 3 4
Sampling interval


Ambient
Reference
Control
Experimental
A. Mol%MgCO3
B. Calcite asymmetry
C. Aragonite
mol% Mg CO3
weight %
Sampling interval
14
13
12
11
10
0
-2
-4
-6
-8
100
80
60
40
20
0
0
1
2
3
4


A
CCA
Seagrass
100 µm
C
Cell wall
membrane
Cell
wall
Mg-C
200 µm
B
Mg-C
grains
2 µm
D
Interfilament
Mg-C
Cell
wall
Mg-C
1 µm

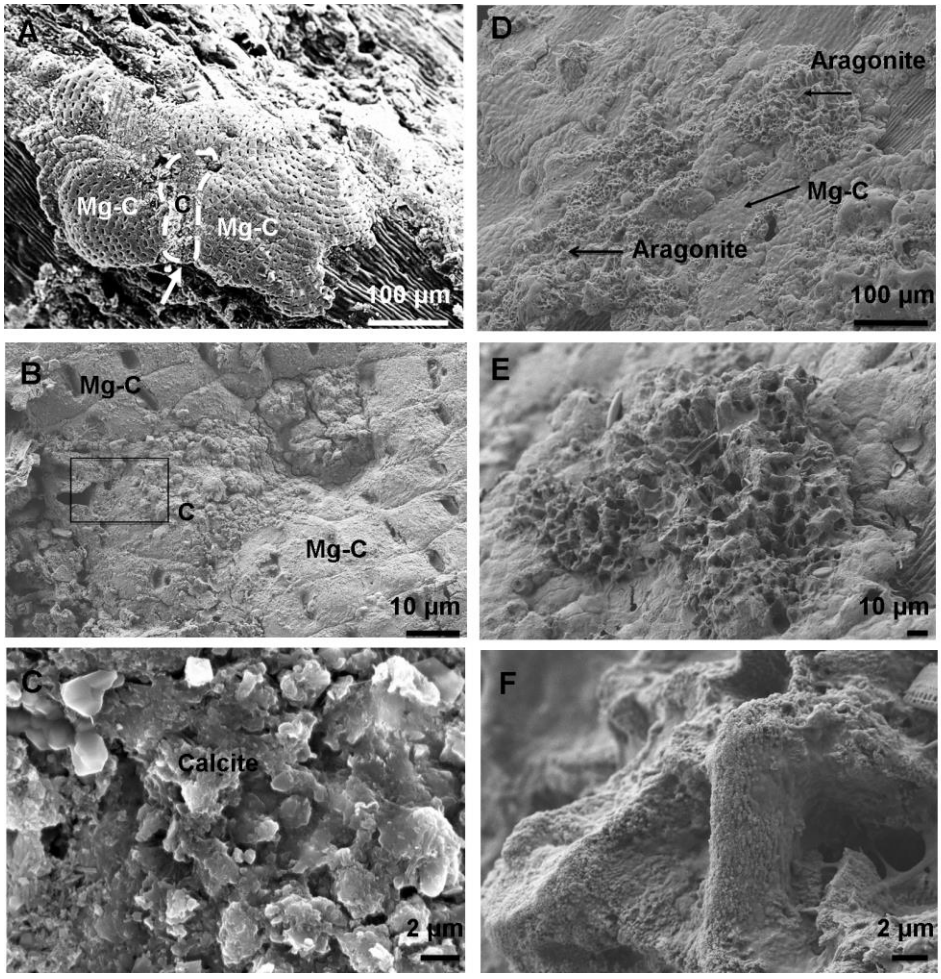

A
Mg-C
C
Mg-C
100 µm
D
Aragonite
Mg-C
Aragonite
100 µm
B
Mg-C
C
Mg-C
10 µm
E
10 µm
C
Calcite
2 µm
F
2 µm

Table 1. Carbonate chemistry within ambient and enclosures: mean (± standard deviation, SD) pH (on the total scale; pH_T), partial pressure of carbon dioxide (pCO_2) and saturation states with respect to aragonite (Ω_a) and calcite (Ω_a) for each month and the period before and during acidification. The difference in pH_T between the experimental and the control enclosure is also shown (Diff)

		pH_T								**pCO_2** (μatm)						**Ω_a**						**Ω_c**					
	N	Ambient		Control		Experimental		Diff		Ambient		Control		Experimental		Ambient		Control		Experimental		Ambient		Control		Experimental	
Period Months	Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Before																											
May	11840	8.10	0.03	8.12	0.06	8.01	0.05	-0.10	0.03	374	30	358	55	477	74	3.4	0.2	3.5	0.4	2.9	0.3	5.3	0.3	5.4	0.5	4.5	0.5
June	8119	8.11	0.04	8.04	0.05	8.10	0.06	0.06	0.05	369	38	443	63	378	65	3.5	0.3	3.1	0.4	3.5	0.4	5.4	0.4	4.8	0.5	5.4	0.5
Acidification																											
June	6226	8.05	0.03	8.02	0.04	7.79	0.13	-0.23	0.13	430	42	470	57	868	318	3.6	0.2	3.3	0.3	2.3	0.6	5.4	0.4	5.1	0.4	3.5	0.9
July	21007	8.03	0.03	8.03	0.06	7.79	0.12	-0.24	0.11	454	46	453	81	870	254	3.6	0.2	3.6	0.4	2.4	0.6	5.4	0.4	5.4	0.6	3.6	0.8
August	22682	8.00	0.03	8.04	0.07	7.81	0.12	-0.23	0.09	489	42	445	85	834	253	3.5	0.2	3.7	0.5	2.5	0.6	5.3	0.3	5.7	0.7	3.8	0.9
September	21854	7.98	0.07	7.97	0.06	7.70	0.11	-0.27	0.10	521	96	536	87	1098	288	3.3	0.4	3.2	0.3	2.0	0.5	5.0	0.6	4.9	0.5	3.0	0.7
October	22420	8.01	0.04	8.00	0.04	7.70	0.13	-0.29	0.14	480	52	497	64	1086	390	3.4	0.2	3.3	0.3	2.0	0.5	5.1	0.4	5.0	0.4	3.0	0.8
November	5377	8.02	0.03	8.02	0.02	7.80	0.15	-0.22	0.15	469	48	467	22	836	305	3.2	0.2	3.2	0.1	2.2	0.7	4.9	0.3	4.9	0.2	3.5	1.0
Before	24334	8.10	0.04	8.05	0.07	8.06	0.07	0.01	0.09	380	39	434	85	426	87	3.5	0.2	3.2	0.4	3.3	0.5	5.4	0.4	4.9	0.6	5.0	0.7
Acidification	95711	8.01	0.05	8.01	0.06	7.75	0.13	-0.26	0.11	483	67	482	86	971	323	3.4	0.3	3.4	0.4	2.2	0.6	5.2	0.4	5.2	0.6	3.6	0.9