Effects of in situ CO 2 enrichment on epibiont settlement on artificial substrata within a Posidonia oceanica meadow

T.E. Cox, V. Díaz-Castañeda, Sophie Martin, S. Alliouane, P. Mahacek, A. Le Fur, Jean-Pierre Gattuso, Frédéric Gazeau

Effects of *in situ* CO_2 enrichment on epibiont settlement on artificial substrata within a *Posidonia oceanica* meadow

T.E. Cox[a1*], V. Díaz-Castañeda[b], S. Martin[c,d], S. Alliouane[a], P. Mahacek[a], A. Le Fur[a], J.-P. Gattuso[a,e], F. Gazeau[a]

[a] Sorbonne Universités, UPMC Univ Paris 06, CNRS-INSU, Laboratoire d'Océanographie de Villefranche, 181 chemin du Lazaret, F-06230 Villefranche-sur-mer, France
[b] Department of Ecology, Center for Scientific Research and Higher Education of Ensenada, Postal 22 860, Ensenada, Mexico
[c] CNRS, UMR 7144, Station Biologique de Roscoff, Place Georges Teissier, Roscoff Cedex 29688, France
[d] Laboratoire Adaptation et Diversité en Milieu Marin, Sorbonne Universités, UPMC Univ. Paris 6, Station Biologique de Roscoff, Place Georges Teissier, Roscoff Cedex 29688, France
[e] Institute for Sustainable Development and International Relations, Sciences Po, 27 rue Saint Guillaume, F-75007 Paris, France

[1] Present address: Dauphin Island Sea Lab, 101 Bienville Boulevard, Dauphin Island, Alabama 36528, USA
*erincox@hawaii.edu

ABSTRACT

Alterations to colonization or early post-settlement stages may cause the reorganization of communities under future ocean acidification conditions. Yet, this hypothesis has been little tested by *in situ* pH manipulation. 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* seagrass meadow (11 m depth) between 21 June and 3 November 2014. Epibiont colonization and early post settlement stages were assessed within the FOCE setup, as part of the larger community-level study, to better understand the outcome for a multispecies assemblage and the ecological processes that result in reported community shifts under altered carbonate chemistry. Two types of artificial collectors (tiles and scourers) were placed within three treatments: a pH-manipulated enclosure, an un-manipulated control enclosure, and an open plot in the ambient meadow. Tiles and scourers were collected after one to four months.

Additionally, to see whether the outcome differed for communities in a later successional stage, previously settled scourer-collectors were also placed in the same three treatments. Enclosures acted to reduce settlement and migrant colonization. Scourers deployed for one to four months within the open-plot contained a community assemblage that could be distinguished from the assemblages within the enclosures. However, a comparison of enclosure assemblages on tiles showed evidence of a pH effect. There was lowered coverage of crustose coralline algae and fewer calcareous tube-forming polychaetes (*Spirorbis* sp. and *Spirobranchus* sp.) on tiles placed in the pH-manipulated enclosure compared to the un-manipulated enclosure. For assemblages in scourer collectors, shared and common taxa, in all treatments, were invertebrate polychaetes *Psamathe fusca*, *Sphaerosyllis* sp., *Chrysopetalum* sp., arthropods Harpacticoida, and Amphipoda, and the juvenile bivalve *Lyonsia* sp. Similar organism composition and abundance, as well as taxonomic richness and evenness, were found in scourers from both enclosures. Pre-settled scourers contained greater numbers of individuals and more calcified members, but the assemblage, as well as the growth rate of a juvenile bivalve *Lyonsia* sp., appeared unaffected by a two-month exposure to lowered pH and calcium carbonate saturation state. Results from this case study support the hypothesis that early stages of specific calcifiers (crustose coralline algae and calcareous tube-forming polychaetes) are sensitive to near future ocean acidification conditions yet suggest that negative effects on sessile micro-invertebrate assemblages will be minimal.

Keywords: Calcifiers; colonization; epiphytic community; early life history stages; ocean acidification, pH

1. Introduction

Because of the anthropogenic driven increase in atmospheric carbon dioxide (CO_2) and its dissolution in the ocean (25.2% of emissions in the decade 2006-2015; Le Quéré et al., 2016), the ocean is undergoing increased rate of change in carbonate chemistry. In the process of ocean acidification, the pH in the ocean declines, resulting in the decline in the concentrations of carbonate ions (CO_3^{2-}) and the increase in the concentrations of bicarbonate ions (HCO_3^-) and dissolved carbon dioxide (CO_2). Surface ocean average pH has 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). These changes in ocean carbonate chemistry are predicted to affect marine organisms (Kroeker et al., 2013) as well as populations and ecosystems (Gaylord et al., 2015).

Alterations in carbonate chemistry can have taxon-specific consequences on organism growth, reproduction, and survival (see reviews, Gattuso and Hansson, 2011; Kroeker et al., 2013). A decline in [CO_3^{2-}] has been shown to differentially affect the ability of calcifying organisms to precipitate calcium carbonate ($CaCO_3$) and has lead to subsequent increases in skeletal dissolution rates (Feely, 2004). Marine autotrophs that lack calcification, in contrast, may benefit from the increased CO_2 availability for photosynthesis (Koch et al., 2013). The concern is that by affecting energy allocation and mortality rates, ocean acidification impacts on the individual or population will also scale to shifts in the community (Kroeker et al., 2011).

Ocean acidification impacts on early life history stages may also lead to the reorganization of communities (Dupont et al., 2010; Kurihara, 2008). Many benthic species have a planktonic larval stage with a different morphology and ecology than the adult stage. The early life history stage of invertebrates and calcified algae is often found to be vulnerable to ocean acidification conditions (Jokiel et al., 2008; Kurihara, 2008). Ocean acidification impact can also vary with life-history stage within a species and affect the life-cycles of organisms in different

ways (Kurihara, 2008). For example, mollusc larvae and juveniles are often found to have a pronounced sensitivity to lowered pH (Gazeau et al., 2013). Lowered pH and $CaCO_3$ saturation state have been shown to impact rates of hatching, metamorphosis, larval and juvenile growth, and early survival from predation (Gazeau et al., 2010; Gazeau et al., 2013; Kurihara, 2008; Talmage et al., 2009). Furthermore, the sensitivity of echinoderms and molluscs in their earlier stages may be greater than other taxa (Kroeker et al., 2013). However, effects on early life-history stages in mixed populations are poorly understood because of the difficulty of culturing and rearing larvae.

Seagrass meadows are a model benthic system to examine effects on multispecies assemblages and ecological processes. They are composed of diverse taxa and are highly valued for the ecosystem services they provide (Hemminga and Duarte, 2000). For example, seagrass meadows play a fundamental role in maintaining populations of exploited fisheries (Jackson et al., 2015) and are global contributors to carbon sinks, with a net primary production (NPP) of 490 Tg C yr^{-1} (Mateo et al., 2006).

In the Mediterranean Sea, *Posidonia oceanica* (L.) Delile forms dense monospecific meadows that cover 23% of shallow water substrate (< 50 m; Pasqualini et al., 1998). *Posidonia* leaves are settlement substrate for a variety of sessile and sedentary colonizers with pelagic larvae such as algae, bryozoans, and serpulid polychaetes. Vagile invertebrates with both brooding and pelagic life-history stages such as polychaetes, gastropods, amphipods, tanaids, and copepods tend to colonize the leaf stratum (Gambi et al., 1992; Gobert et al., 2006). Among these floral and faunal groups are several calcifiers (crustose coralline algae or CCA, several species of polychaetes that form calcified tubes, molluscs, and bryozoans) that contribute to carbonate cycling (Frankovich and Zieman, 1994; Perry and Beavington-Penney, 2005). Epiphytes are

major contributors to meadow production and nutrient cycling (Borowitzka et al., 2006; Romero et al., 2006) and invertebrate fauna transfer energy to higher trophic levels (Lepoint et al., 2000). In turn, algal and invertebrate abundances can be dependent upon seagrass density and its seasonal growth (Alcoverro et al., 1997; Mazzella et al., 1989). The tight coupling between organism abundances, the seasonal environment, and the diverse species and life-history stages make it difficult to predict future meadow ecology from studies on single to few species conducted in the laboratory.

Observations and conclusions drawn from naturally acidified ecosystems (volcanic CO_2 vents, spring inputs, and upwelling areas) predict changes in benthic community composition and abundances under ocean acidification (e.g. Hall-Spencer et al., 2008) although such shifts have been attributed to a variety of ecological processes. Most observations examining community assembly or abundances on artificial substrata support a reorganization of communities as a direct result of taxa sensitivity, tolerance, or benefit (Allen et al., 2016; Cigliano et. al., 2010; Crook et al., 2016; Donnarumma et al., 2014). A few studies indicate shifts in benthic abundances related to indirect effects of lowered pH, such as loss of habitat complexity or changes in competitors and prey (Garrard et al., 2014; Kroeker et al., 2012). In one study, species interactions in later successional stages amplified direct effects (Kroeker et al., 2012) to result in an altered community assembly at lowered pH from ambient. While these observations at naturally acidified ecosystems capture diverse species interactions and acclimation, they are not perfect predictors of future ocean ecology. Conclusions are often hindered by large temporal pH variability (Kerrison et al., 2011) and outcomes can also be confounded by other environmental conditions that vary across the pH gradient. Therefore, similar investigations on intact assemblages where only pH is manipulated could help to clarify

126 community change and the role of early life stages on community development under ocean
127 acidification.

128 In the present study, the impact of ocean acidification on epibiont colonization and
129 recruitment was assessed on artificial substrata in the framework of an *in situ* pH-manipulation
130 experiment (European Free Ocean Carbon Dioxide Enrichment; eFOCE). This community-level
131 experiment was designed to assess the impacts of ocean acidification on a *P. oceanica*
132 community in the Northwestern Mediterranean Sea (Cox et al., 2016). The specific study
133 presented here aimed at testing for the effects of ocean acidification on the micro-invertebrate
134 assemblage colonizing artificial surfaces within a *P. oceanica* meadow and it tested the
135 prediction that the sensitivity of early life history stages alters community formation. Artificial
136 substrata were used to standardize for differences in substratum, collect small invertebrates that
137 surround the leaves, and to follow community development to identify ecological mechanisms
138 that result in shifts. Because organisms have different preferences for settlement surfaces, two
139 types of artificial collectors (tiles and scourers) were placed within the experimental design of
140 the FOCE system (see Gattuso et al., 2014). This system is unique because it allows pH to be
141 manipulated continuously *in situ*, in an enclosure, at a fixed offset from ambient levels. This
142 FOCE design also consisted of an un-manipulated control enclosure and a plot, fully open to the
143 ambient environment. All tile collectors were placed in the three treatments of the FOCE setup at
144 the start of the pH manipulation. A set was collected each month to gauge effects on early
145 recruits and to follow assemblage development through time. Scourer collectors which often
146 target the collection of micro-invertebrates that surround the seagrass leaves were placed in the
147 FOCE setup and collected after one to four months to gauge impacts to recruitment and to test
148 for longer effects on the assemblage development. A second set of scourers were pre-settled

149 within the ambient meadow, collected, and then placed within the FOCE setup for two and four
150 months to test whether the response of an assemblage, exposed in a later successional stage when
151 ecological interactions such as competition and the physiology of adult forms, differs from the
152 response of early recruits. At each collection interval, the community composition and organism
153 abundances were compared. Additionally, the growth of a juvenile bivalve was compared to test
154 whether the development of early calcifiers was impacted by lowered pH. Finally, because
155 species diversity can indicate ecosystem function and it is predicted to be affected by ocean
156 acidification, for scourer assemblages that were more complex, taxonomic richness and evenness
157 were compared. Study results are discussed in the broader context of ocean acidification effects
158 and published FOCE outcomes for epibionts found on natural leaves, which were collected
159 simultaneously with tiles and for which negative impacts of lowered pH could not be detected.
160 This planned comparison between settled artificial substrata and natural leaves was intended to
161 test our working hypothesis that, in contrast to epibionts found on leaves, organism abundances
162 and community formation on artificial substrata are not protected by the modification of
163 carbonate chemistry at the proximity of leaves due to seagrass photosynthesis and are therefore
164 more sensitive to any change in pH of the surrounding water.

165 **2. Materials and methods**

166 *2.1. Experimental setup and system function*

167 This study used the European FOCE (eFOCE) system which allows for the *in situ*
168 manipulation of pH in benthic enclosures as an offset from ambient pH (Cox et al., 2016). It was
169 deployed in the Bay of Villefranche, approximately 300 m from the Laboratoire
170 d'Océanographie de Villefranche (NW Mediterranean Sea, France; 43°40.73′N, 07°19.39′E).

171 The study design consisted of two clear, 1.7 m^3 (2 m long x 1 m width x 0.85 m tall)
172 perspex enclosures at 11 m depth that were open on the bottom to partially enclose a portion of
173 the *P. oceanica* meadow. The enclosures were placed end-to-end approximately 1.5 m apart. The
174 pH in one enclosure, referred to as the pH-manipulated enclosure, was lowered by ~0.3 units as
175 an offset from ambient pH as measured on the total scale (pH_T). This offset was based upon the
176 business-as-usual representative concentration pathway RCP8.5 following Ciais et al. (2013) and
177 led to a decrease of pH_T in the pH-manipulated enclosure to an average (± SD) pH_T of 7.75 ±
178 0.13 and an increase in pCO_2 to 971 ± 323 μatm. pH was not manipulated in the second
179 enclosure and it served as a control enclosure. A third treatment consisted of an open fiberglass
180 frame of the same dimensions as the enclosure footprint (2 m^2) placed nearby (3 m from the pH-
181 manipulated enclosure). It is referred to as an open plot and was used to account for any effects
182 that could be generated by the enclosure structure. True replication (at the enclosure level) was
183 not logistically feasible. It was sacrificed to control pH precisely within enclosures of a large
184 enough size to contain *P. oceanica*. For further discussion on the study design of FOCE systems,
185 the reader is encouraged to consult Gattuso et al. (2014).

186 The eFOCE system is fully described in Cox et al. (2016). Briefly, the pH in the pH-
187 manipulated enclosure was altered using subsurface (3 m depth) supplied seawater pumped into
188 a mixing tank, which was located on a surface platform. Pure CO_2 was bubbled into the mixing
189 tank and the resulting low pH seawater was pumped, via tubing, underwater to the proximity of
190 the benthic enclosures. Prior to entering the enclosures, low pH (pH_T ~ 5.5) and ambient
191 seawater were mixed in an underwater tube and a set (x3) of centrifugal pumps (6.7 L min^{-1}
192 each) injected ambient seawater in the control enclosure and lowered-pH seawater in the
193 manipulated enclosure. Seawater inside enclosures was circulated by another set of centrifugal

194 pumps (four per chamber; 6.7 L min^{-1} each) and exited through two openings (12 cm diameter).
195 Renewal time of seawater in each enclosure was ca. 1.5 h. The system contained a number of
196 sensors: four potentiometric Seabird 18-S pH sensors located inside each enclosure and in each
197 mixing tube and three Seabird 37 SMP-ODO CTD with SBE 63 O_2 optodes and three LI-COR-
198 192 PAR (photosynthetic active radiation) sensors located in each enclosure and one nearby the
199 enclosures (in ambient, close proximity to the open plot). Carbonate chemistry was determined
200 from an average of total alkalinity and sensed temperature, salinity and pH_T, in the R package,
201 seacarb (Gattuso et al., 2015). Average alkalinity used in each calculation was determined from
202 discrete water samples collected from within the enclosures and plot throughout the study (A_T
203 mean ± SD, pH-manipulated enclosure, n =12, 2545.5 ± 8.0 μmol kg^{-1}; control enclosure, n = 11,
204 2541.7 ± 12.2 μmol kg^{-1}; open plot (or ambient), 2556 μmol kg^{-1}, see Cox et al., 2016 for more
205 details)

206 *2.2. Experimental and environmental conditions*

207 The study area, where enclosures and plots were located, can be described as a
208 monospecific meadow of *P. oceanica* in a soft sediment bottom (see Cox et al., 2016). The pH_T
209 in the meadow (ambient) ranged from a mean of 7.98 (± 0.06 SD) in September to 8.11 (± 0.04
210 SD) in June (Fig. 1). The mean saturation states of aragonite (Ω_a) and calcite (Ω_c) ranged from
211 3.1 to 3.6 and 4.9 to 5.4 from June to September, respectively. The diel pH_T change differed
212 among months from 0.04 to 0.12. It corresponded to the daily change in CO_2 concentration
213 driven by community primary production, respiration and calcification.

214 The greatest difference between ambient open plot and control enclosure in monthly
215 mean pH_T values was 0.06 units; the control enclosure being more acidic. The diel change in pH_T
216 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 pH-manipulated enclosure was maintained at a mean -0.26 unit offset (monthly mean values ranged from -0.22 to -0.29 pH units) from the control enclosure (Table S1). In the pH-manipulated enclosure, the monthly mean values of saturation state with respect to aragonite (Ω_a) ranged from as low as 2.0 (± 0.05 SD) in October to a high of 2.5 (± 0.06 SD) in August and saturation state with respect to calcite (Ω_c) ranged from 3.0 (± 0.07 to 0.008 SD) in September and October to 3.8 (± 0.09 SD) in August. The median diel pH range in the pH-manipulated enclosure was two to three times larger than the control (monthly median ranged from 0.09 to 0.29 pH units).

Monthly differences were evident, particularly for temperature (mean monthly range: 17.7 to 24.2 °C) and PAR (mean monthly range: 1.3 to 7.3 mol photons m^{-2} d^{-1}, Table S2) but these variables were similar in the ambient, control and pH-manipulated enclosures.

2.3. eFOCE 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 manipulated 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 pH-manipulated period from 22 June to 3 November during which pH in the pH-manipulated enclosure was maintained at a constant offset of ~ -0.3 units and settlement was monitored.

2.4. Artificial collectors

Tiles and scourers (or scouring pads) were used as artificial collectors to assess settlement and benthic assemblage development. Both collectors are commonly used to collect

adult or early life history stages of benthic organisms (e.g. Cigliano et al., 2010; Kroeker et al., 2012).

Tiles were 5 x 5 cm squares cut from 0.5 cm thick sheets of polyvinyl plastic. Surfaces were sanded with steel wool and tiles fixed, via Velcro, to 15 polyvinyl plastic black boards connected to a fiberglass stake. Seven tiles were arranged on each board (only 3 per board were used) in a longitudinal row with 0.5 cm between them. Scourers were rounded, 8 cm in diameter, and composed of enrolled coarse nylon mesh attached by a plastic tie to a fiberglass rod. Tiles and sets of scourers were handled differently and collected at different sampling intervals for specific examinations. The methods for each collector type are described (in order of occurence) and an experimental outline can be seen in Fig. 2.

2.5. Scourer pre-settlement and calcein staining for growth measurements

Prior to the eFOCE experimentation, on 19 September 2013, many scourers were placed within the ambient meadow at 11 to 12 m depth to allow for settlement and community development. The fiberglass rod was staked into the substrate and the scourer sat at the surface of the seagrass canopy at the time of deployment. Three scourers were relocated on 24 June 2014 and used in the experiment.

Thirteen other scourers were placed in a similar manner within a 5 m^2 area on 6 February 2014 and collected after ~4 months (19 June 2014). These settled scourers were transported to the Laboratoire d'Océanographie de Villefranche, held in circulated seawater from the bay for 72 h under ca. 150 μmol photons m^2 s^{-1} (13:11 h light:dark cycle) and maintained at seawater temperature conditions (22-23 °C). After 24 h, the free-flowing addition of Bay water to the holding container was stopped, calcein (50 mg L^{-1}) was added, and organisms were fed *ad libitum* with the microalga *Isochrysis galbana* Parke. The staining lasted for 48 h then scourers

were removed, placed in separate seawater filled plastic bags, and taken by divers to the eFOCE setup.

2.6. Collector placement, collection intervals, and study design

On 23 June 2014, at the start of the acidification period (Time 0, T0) scourers and tiles on boards were placed into the plot and both enclosures (Fig. 2). Five boards (total of 15 boards) with tiles were staked by pushing the fiberglass rod into the soft substrate on the northern side of each plot and enclosure to face South. Seventeen scourers for each enclosure and 15 for the open plot (for a total of 49) were also placed on the northern side of the plot and each enclosure. Scourers were either attached to individual fiberglass rods and staked into soft sediment or were attached to a single fiberglass rod that was held in place on each side of the enclosure. All scourers sat at or above the seagrass canopy (0.5 to 0.7 m above the substrate) with at least 2 cm distance between them.

Collection of tiles and scourers occurred at one to four specified time-points, spaced apart by approximately four weeks, referred to as time (T) 1 to 4: T1 occurred on 30 July, T2 occurred on 3 September, T3 on 6 October, and T4 occurred on 10 November after 135 d of acidified conditions. It should be noted that the acidification of the pH-manipulated enclosure ended on 4 November 2014 while collections were made six days later.

Five tiles (one from each board) from each treatment location were collected at T1, T2, T3, and T4. In this manner, assemblage development was examined through time.

Out of the 17 scourers placed in each enclosure at T0 (15 for the open plot): (1) six were fresh (i.e. not pre-settled) scourers that were collected and replaced with six other fresh scourers at T1, T2, and T3 to assess monthly differences in settlement under ocean acidification, (2) five were fresh scourers that were left in place from T0 until T4 to assess lowered pH impacts on

assemblage development, (3) five (three for the open plot) were pre-settled for four months, stained scourers that were collected after two months (T2) and lastly (4) one scourer was pre-settled for nine months, and was collected at the end of the study (T4) in order to investigate lowered pH impacts on developed assemblages (and for #3, juvenile calcifier growth).

At each collection interval, divers removed each artificial collector, placed them into separate labeled plastic bags and brought them immediately to the Laboratoire d'Océanographie de Villefranche.

2.7. Organism identification and quantification

The 15 settlement tiles were kept in a temperature controlled (20 – 22 ºC) dark room for less than 24 h until scanning could be completed. A ZooScan (Hydroptic, France; Gorsky et al., 2010) was used to produce high resolution colour images (2400 dpi) of settled tiles. There were no canopy-forming macroalgae or large sessile macrofauna on tiles, thus "layering" did not occur and images could be directly analyzed.

Scans of settlement tiles were analyzed using ImageJ (imagej.net). There were few settlers at the first collection time point and these tiles were discarded from the analysis. The remaining scanned images were visually searched for fauna and, when encountered, individuals were identified and enumerated. Algae were also identified and the area occupied per tile was directly measured by adjusting the colour-threshold, followed by adjustments of the threshold on a converted 8-bit image. Adjustments were done to outline the algae and fill the space they occupied and surface area covered was estimated.

Each scourer was carefully un-rolled and washed onto a 0.35 mm mesh size. Retained organisms were transferred to 70% ethanol. Organisms were placed in Petri dishes and examined, sorted, identified, and enumerated under a microscope (Leica Wild M10).

Identification was conducted at the lowest possible taxonomic unit or, operational taxonomic unit (OTU). Organisms settled on tiles and analysed using the zooscan were mostly identified to broad functional categories. At the end of this process, each tile had a count of invertebrates, and an area determined for algae. For scourers, it was not possible to identify some young organisms to genus or species because identifiable structures were not yet visible. Polychaetes, gastropods, and bivalves were often identified to family and genus. Arthropods tended to be identified to Order. Echinoderms were mostly identified to Class. At the end of the process, for each scourer, there was a total count of individuals by OTU.

2.8. Growth rates

The bivalve *Lyonsia* sp. (Family Lionsiidae) within pre-settled, stained scourers was the only organism where a calcein mark was visible. Individuals were carefully placed in a horizontal position (hinge horizontal), in visible proximity to a scale bar, and photographed directly from above, through the binocular microscope. This resulted in 15, 5, and 8 imaged individual *Lysonia* sp. from 5, 2, and 3 scourers collected from the pH-manipulated enclosure, the control enclosure and the open plot, respectively. Images were analyzed using ImageJ. The complete bivalve shell and new growth were outlined by tracing the perimeter and their surface areas were estimated with the measure tool. Growth (area in mm^2) was provided as a proportion of new growth to total size (as area) and expressed as a rate (per day).

2.9. Statistical analyses

Tile and scourer data collected from T1 to T4 from the same plot or enclosure were considered replicates and values reported are mean ± standard deviation, SD. In addition, all photographed individuals were considered replicates and used to compare bivalve growth.

Two-way ANOVAs were used to test for differences in the abundances of functional groups (CCA, Serpulid polychaetes, and Other taxa) on tiles. Intervals (T2, T3, T4) and treatments (open plot, pH-manipulated and control enclosures) and their interaction (interval x treatment) were terms in the models. Prior to testing, CCA coverage (cm^2) failed to meet parametric requirements and a rank transformation was applied. Tukey's or a Dunn's post-hoc multiple comparison tests were used to identify pairwise differences ($P < 0.05$).

To test for differences in impacts from month to month, early assemblage development, and on previously settled assemblages, data from scourers were distributed into three groups as noted on Fig. 2 (1) scourers settled for a one-month duration collected at T1, T2, T3, and T4 (dataset #1), (2) scourers settled for four months; placed at T0 and collected at T4 (dataset #2), (3) scourers pre-settled for four months then placed at T0 and collected at T2 (dataset #3). Data from the scourers pre-settled for nine months, deployed at T0 and collected at T4 (n = 1 per treatment) were graphically presented but not statistically tested. Due to scourer loss in field, the number of replicate scourers may vary slightly from initial numbers placed.

All analyses presented for scourer data were done with OTUs. However, outcomes were similar when analyses were performed at the family, order, class, or phyla. Furthermore, for all statistical testing with scourer data, terms included were treatment (3 levels: pH-manipulated and control enclosures and open plot) and for dataset #1, the collection interval (4 levels: T1-T4) and their interaction.

A multivariate approach following the recommendations outlined in Anderson and Willis (2003) was taken to analyze scourer assemblages. A $\log_{10}$ (X+1) transformation was applied and the Bray-Curtis Index was used to create a resemblance matrix. A two-way permutational-MANOVA (PERMANOVA) was run on each matrix with 999 permutations and type III sum of

squares, followed by a Monte-Carlo simulation. Post-hoc pairwise differences were tested when a main effect was observed followed by a canonical analysis of principal coordinates (CAP). CAP uses ordination to find the appropriate subset of axes (m) in principal coordinate space that is best at discriminating among defined groups. The maximum leave-one-out allocation success was used to determine group distinctness. Spearman rank correlation of organism abundances with the axes was used to indicate which taxa were most responsible for separation.

Taxonomic richness (as number of OTUs), Pielou's evenness index, and total number of individuals per scourer was determined, followed by statistical comparisons using either a two-way ANOVA (for dataset #1), Kruskal-Wallis, or one-way ANOVA. A rank transformation was applied to values of evenness to meet parametric requirements. One-way ANOVA was also used to test for differences in *Lyonsia* sp. growth. Tukey's or Dunn's post-hoc multiple comparison tests were used to identify pairwise differences.

3. Results

3.1. Settlement on tiles

Organisms found on tiles and grouped to a category referred to as "Other taxa" were brown colored crusts of algae (non-calcaerous species), cyanobacteria, and red filamentous algae. These taxa were grouped because of the low occurrence of filamentous algae and difficulty distinguishing brown colored crusts of algae from cyanobacterial mats in scans. CCA and two calcareous tube worms, *Spirorbis* sp. and *Spirobranchus* sp. (only two individuals of genus *Spirobranchus*) from the family Serpulidae were identified. Overall 0.3 to 18.6% of tile surfaces were colonized by organisms and there were between 0 and 16 calcareous tube worms per tile (n = 45 tiles).

CCA coverage significantly differed between treatments and sampling intervals (Table 1: Fig. 3A). On open tiles, the coverage of CCA was greatest and it gradually increased from T2 to T4 (Fig. 3A). The coverage at intervals T2 and T4 statistically differed. On control tiles, there was relatively lower coverage of CCA and the coverage also increased from T2 to T4. In contrast, CCA coverage on pH-manipulated tiles was never above 0.1 cm^2 and the change in coverage tended to be minimal (from 0.01 ± 0.00 at T2 to 0.00 ± 0.00 cm^{-2} at T4).

There was a significantly greater coverage of "Other taxa" on tiles from the open plot and control enclosure than on tiles from the pH-manipulated enclosure (Table 1, Fig. 3B). Differences were largely driven by the lower coverage observed at T2 on pH-manipulated tiles (0.23 vs 0.54 to 1.10 cm^2). For all treatments, the coverage of "Other taxa" increased from September (T2) to October (T3) and declined again in November (T4).

Calcareous tube-forming polychaetes (serpulids) occurred in significantly greater numbers on tiles from the open plot (monthly means ranged from 4 to 7 individuals) and the control enclosure (monthly means were about 4 individuals) than in the pH-manipulated enclosure (monthly means ranged from 0 to 2 individuals, Table 1, Fig. 3C). There was no statistical indication of a change in abundance with time.

3.2. Overall description of scourer assemblages

Out of a combined 106 scourers, 7,220 individuals from eight phyla were found and identified to 106 different OTUs. At least 77 families were identified with 33 identifications (31%) performed at the level of genus or species. The most abundant group of organisms were annelids (2,891 individuals) closely followed by arthropods (2,646 individuals), then by molluscs (1,569 individuals), echinoderms (28 individuals), cnidarians (6 individuals), ascidians (6 individuals), nematodes (5 individuals), nemerteans (4 individuals), and platyhelminthes (1

399 individual). Specifically, the most abundant taxa were the polychaetes *Psamathe fusca* (Johnston,
400 1836), *Sphaerosyllis* sp., *Chrysopetalum* sp., the arthropod orders Harpacticoida, and
401 Amphipoda, and the bivalve *Lyonsia* sp. Taxonomic richness appeared to be the greatest within
402 the phylum Annelida (44 OTUs), followed by Mollusca (33 OTUs), and Arthropoda (13 OTUs).
403 All other identified phyla were composed of three or less OTUs.

404 *3.3. Settlement on fresh scourers deployed for one to four months*

405 *3.3.1. Monthly availability of benthic colonizers*

406 The organisms found in monthly deployed scourers differed between collection intervals.
407 The composition and abundance of organisms at T2 and T3 were similar and statistically
408 different from the composition and abundance at T1 and T4 (Table 2; Fig. 4). The assemblages
409 were weakly discriminated within three CAP axes (n = 69, choice of m = 7, $\delta^2_1 = 0.36$, $\delta^2_2 =$
410 0.20, $\delta^2_3 = 0.09$) with low overall classification success (44.9%). Assemblages at T1 were most
411 distinctive. Mis-classification increased for scourer assemblages at T2 and T3 and they were
412 often confused (Table 2). The change in the abundances of two polychaete species and
413 Gastropoda appeared to be driving these allocations (abundances have > 60% correlation).
414 Specifically, *Chrysopetalum* sp. and *Polyophthalmus pictus* (Dujardin, 1839) were more
415 numerous at T1 and T4 than T2 and T3 (mean individuals ± SD, respectively: T1-T4, 7.0 ± 10,
416 0.5 ± 2.4, 0.4 ± 1.1, 2.7 ± 4.1 and T1-T4: 7.0 ± 0.9, 0.6 ± 1.1, 1.0 ± 1.5, 2.2 ± 2.9). Also clearly
417 noticeable was the decline of gastropod colonizers from T1 to T4 (Fig. 4). Taxonomic richness
418 and total number of individuals also varied by interval but evenness did not (Fig. 5; Table 2).

419 *3.3.2. Enclosure effect on settlement*

420 Data were most congruent with an effect caused from enclosures which acted to reduce
421 settlement and migrant colonization (Figs 4, 5; Tables 2, 3). Assemblages settled on fresh

scourers (one month and four month deployments) from the open plot statistically differed from the assemblages found in the pH-manipulated and control enclosures (Table 2). Furthermore, nMDS (not shown) and CAP analyses (Fig. 6) showed clear discrimination of enclosure assemblages from the open assemblages (Table 3). To determine whether enclosure or open plot differences could be driven by random chance sampling, an iterative permutation procedure (SIMPROF, 1000 mean permutations, 999 simulations) was used on the monthly collected scourer data. Assuming same species abundance distributions (common species list) and random community assembly (by reshuffling), the Bray-Curtis similarity values for enclosures and open plot assemblages were greater than the 99% confidence envelope (8 -12 % resemblance) around the similarity profile predicted by the model. Therefore, there is genuine multivariate structure not likely due to chance but rather driven by the enclosure conditions. Polychaete and crustacean abundances were more numerous in settled scourers from the open plot (Fig. 4). The specific OTUs which had correlation values greater than 70% with discriminating axes can be observed on Fig. 6. In addition, taxonomic richness and total number of individuals were greater in the scourers collected from the open plot (Fig. 5, Table 2).

Many taxa were common in all treatments (Tables S3, S4). The SIMPER routine was performed on the dataset from monthly collected scourers. It indicated dissimilarities between enclosures and the open plot assemblages (80-82 % dissimilar) were based largely (85-86 % of cumulative contribution) upon the density of the shared common species and less upon unique species to enclosures or open plots. Nevertheless, out of 83 taxa found in monthly collected scourers, 18 were exclusively found in enclosures (total individuals per OTU < 7, combined) and 11 were exclusively found in the open plot (total individuals per OTU < 2, except 19 individuals of *Musculus costulatus* (Risso, 1826) and 15 individuals of Family Carditidae). Out of a

445 combined 49 taxa found in the scourers placed at T0 and collected at T4, 16 were exclusively
446 found in the open plot (at < 7 total individuals per OTU, except 14 molluscs of the Family
447 Carditidae, and 18 polychaetes *Spirobranchus* sp.) and 11 of the 49 were exclusively found in
448 the enclosures at < 4 total individuals per OTU combined.

449 In addition, for scourers that were not pre-settled, the most abundant taxa were common
450 at all locations (Tables S3, S4). In the monthly collected scourers, the polychaetes *Psamathe*
451 *fusca, Chrysopetalum* sp., and the peracarid crustaceans Amphipoda, Isopoda, and Ostracoda
452 were more numerous in the open plot but, were also in the 15 most abundant taxa in the
453 enclosure assemblages. In the scourers placed at T0 and collected at T4, the biggest differences
454 in terms of most abundant taxa was for *Spirobranchus* sp., it was in the 15 most numerous OTUs
455 in the open and absent in the enclosures. When combined *Nerilla* sp. was in the 15 most
456 abundant taxa in the enclosures (14 individuals) and only two individuals were found in the open
457 plot.

458 *3.3.3. Enclosure comparison - settlement within one month*

459 Despite changes in monthly availability of colonizers, acidified enclosure assemblages
460 were similar to control exclosure assemblages (Table 2). Overall the collection intervals, the five
461 most numerous taxa, in combined order of abundance, were: Harpacticoida*, Psamathe fusca*,
462 *Lyonsia* sp., Copepoda, and Trochidae (Table S3). CAP analyses had low to moderate success at
463 discriminating between enclosures (Table 3). On average 6 to 9 and 5 to 9 taxa were found per
464 scourer collected monthly from the pH-manipulated and control enclosure, respectively.
465 Acidified enclosure assemblages had similar total number of individuals per scourer than control
466 enclosure assemblages (Fig. 5, monthly mean range: 13 to 24 vs 16 to 26 for the pH-manipulated
467 and control enclosure, respectively) and the assemblages were similar to each other in evenness

(monthly mean range: 0.95 to 0.96 vs 0.94 to 0.97 for the pH-manipulated and control enclosure, respectively).

3.3.4. Enclosure comparison - settlement within four months

After four months of deployment, the assemblage in scourers collected from the acidified enclosure was similar to the assemblage found within the control exclosure (Table 2). The five most numerous taxa settled over the four-month period, in combined order of abundance, were: Harpacticoida*, Psamathe fusca*, Amphipoda, Tanaidae, *Nerilla* sp. and *Polyophthalmus pictus* (Table S4). The CAP analyses had moderate to low success discriminating between assemblages within the control and the acidified enclosures (Fig. 6; Table 3), however, there was some visual separation (Fig. 6). Scaphopoda abundance and the polychaetes *Fauvelia* sp. and *Nerilla* sp. abundances most correlated to the axes (65, 65, 80%, respectively) that discriminated between enclosures, yet the patterns were not indicative of typical cited ocean acidification impacts. Scaphopoda, a calcifying group, and *Fauvelia* sp., a syllid polychaete, occurred in the scourers collected from the pH-manipulated enclosure (mean 2.5 ± 0.5 individuals for both taxa) and were absent in the control and in the open scourers; whereas individuals of the polychaete *Nerilla* sp. tended to be more numerous in the control enclosure (mean 2.6 ± 1.6 *vs.* 0.25 ± 0.5 and 0.5 ± 1.0 individuals in the open plot). Other taxa abundances that correlated in the direction of enclosure separation had lower correlation values (35 to 50%), occurred in one scourer at < 3 individuals, and their location of occurrence also does not support ocean acidification predictions for calcifiers. The calcifying groups identified to Order Ophiurida (brittle stars) and juveniles from the Family Serpulidae (calcareous tube worms) occurred in the pH-manipulated enclosure and the polychaete *Sphaerodorum* sp. (does not form calcified tubes) occurred in the control enclosure.

A total of 17 to 22 and 17 to 23 taxa were found per scourer at T4 collected from the pH-manipulated and control enclosure, respectively. They also had a statistically similar total number of individuals per scourer (Fig. 5; range: 17 to 45 vs 31 to 59 for the pH-manipulated and control enclosure, respectively) and had similar evenness (range: 0.94 to 0.97 vs 0.94 to 0.98 for the pH-manipulated and control enclosure, respectively).

3.4. Pre-settled scourers

The pre-settled scourers had 26 to 44 different taxa and 73 to 461 total individuals per scourer. The total number of individuals tended to be greater within scourers settled and deployed for longer duration. For example, the pre-settled scourers collected at T4 had between 366 to 461 (mean ± SD, 400 ± 92) individuals per scourer while the pre-settled scourers collected at T2 had between 73 to 414 (mean ± SD, 190 ± 85) individuals per scourer. In addition to polychaetes and arthropods the calcifiers *Spirobanchus* sp. and molluscs (*Lyonsia* sp., Gastropoda juveniles, class Bivalvia, family Trochidae, and *Musculus costulatus*) tended to be abundant (Table S5, S6).

There was no indication of an enclosure or pH effect on pre-settled scourers collected after two months of exposure (Fig. 7). Pre-settled assemblages did not statistically differ among treatments for all considered parameters (Table 2). The most numerous OTUs in the pre-settled scourers at T2 were the bivalve *Lyonsia* sp., the polychaetes *Psamathe fusca*, *Sphaerosyllis* sp., and Copepoda (an arthropod; Table S5). Calcareous members of the assemblage (calcareous tube-forming polychaetes, gastropods, and bivalves) that were predicted to decline with lowered pH, actually tended to be more numerous in the pH-manipulated enclosure than in the control enclosure at T2 (Fig. 7).

513 Trends in organism density and in richness in pre-settled scourers collected at T4,
514 however, tended to support an effect of lowered pH on assemblages and also showed indications
515 of an enclosure effect (Figs. 5, 7). There was a greater taxonomic richness within the open plot,
516 followed by a lowered richness in the control enclosure, with the least richness in the pH-
517 manipulated enclosure (Fig. 5). The two enclosures were similar in terms of mean total number
518 of individuals, and it was greatest in the open plot. The density of serpulid polychaetes
519 (*Spirobranchus* sp.) and gastropods were considerably lower in the pH-manipulated enclosure
520 (Fig. 7). In addition, there were 29 individuals of *Syllis* sp. (a non-calcifying polychaete) in the
521 pH-manipulated enclosure but only four in the open plot and control enclosure. Evenness did not
522 support any trend.

523 *3.4.1. Bivalve growth*

524 *Lyonsia sp.* were between 1.4 to 7.0 mm in length (surface area of 67 to 156 mm^2). Rates
525 of growth, as a proportion of their initial total area, were found to be statistically similar between
526 the enclosures (0.26 ± 0.13 and 0.25 ± 0.12 in 72 days for control and pH-manipulated
527 enclosures, respectively) and differed between bivalves growing in the open plot (0.36 ± 0.13 in
528 72 days) and in the pH-manipulated enclosure (df = 2, MS = 0.04, F = 3.5, P = 0.047, open plot
529 *vs*. pH-manipulated enclosure q = 3.7, P = 0.04).

530 **4. Discussion**

531 *4.1. CCA and serpulids on tiles*

532 CCA and serpulids found on tiles are reported members of *Posidonia* meadows
533 (Borowitzka et al., 2006) and both taxa were clearly reduced in abundance by lowered pH. Other
534 studies have found reduced recruitment of serpulid tube worms (Cigliano et al., 2010; Kroeker et
535 al., 2012; Rodolfo-Metalpa et al., 2010) and CCA (e.g. Doropoulos et al., 2012; Donnarumma et

al., 2014; Jokiel et al., 2008; Kuffner et al., 2008) at a $pH_T < 7.8$. Although the spores and larvae lack calcified structures, settled CCA and tubes of *Spirorbis* are susceptible to dissolution because they are composed of calcite and/or aragonite (Ippolitov & Rzhavsky, 2015) or magnesian calcite; 11.3 to 11.7 mol% magnesium in carbonate ($MgCO_3$) for CCA (Cox et al., in press) and calcite with ~15% $MgCO_3$ reported for spirorbid tubes (Bornhold and Milliman, 1973). Several studies have shown dissolution, reduced calcification, or a loss of coverage for CCA under lowered pH (reviewed by Hofmann and Bischof, 2014; Koch et al., 2013; McCoy and Kamenos, 2015; Nelson, 2009).

In studies on multispecies assemblages in natural settings, the process attributed to observed shifts in benthic communities under lowered pH seems to vary, particularly for algal dominated communities. Indeed, some studies have attributed loss of calcifiers to direct sensitivity (Hall-Spencer et al., 2008; Jokiel et al., 2008; Martin et al., 2008; Price et al., 2012) while others have indicated losses through competition (Kroeker et al., 2012) or both (Donnarumma et al., 2014; Porzio et al., 2011). In the present study, reduced abundances of calcifiers on acidified tiles were seen at early stages of recruitment. Differences in abundance appeared to be driven by taxa sensitivity and not post-settlement competition because other taxa were also reduced by the lowered pH condition and there was still bare space available for colonization.

The *in situ* control of pH (present study) could explain any contrasting conclusions drawn from CO_2 vent observations which are relied upon extensively to predict future ocean ecology. For example, meadow ecology has been studied along CO_2 vents in *Posidonia* meadows in Ischia, Italy where the pH_T varies in a gradient from 8.1 to 6.6 nearest the CO_2 source (e.g. Hall-Spencer et al., 2008). Kroeker et al. (2012) noted that there were fewer recruits of CCA on tiles

559 placed at lowest pH locations (mean pH_T ~ 6.7); whereas recruits at locations with a mean pH_T
560 of ~ 7.7 and ~ 8.0 were similar in terms of size and coverage. Then, after four months,
561 competition favored filamentous algae and resulted in loss of calcifiers (both serpulids and
562 algae). Yet, the conclusions drawn from observations along CO_2 vents are hampered by the large
563 temporal pH variability that results from venting activity (Kerrison et al., 2011). In the cited
564 example, sensitive calcifiers may have recruited during periods of more favorable carbonate
565 chemistry.

566 Grazing could also have reduced non-calcified algae and masked competition in the
567 present study. There are a number of mesograzer invertebrates in seagrass meadows (Lepoint et
568 al., 2000; Michel et al., 2015) and invertebrate density and diversity, unlike in other studies
569 (Allen et al., 2016; Cigliano et al., 2010; Garrard et al., 2014; Hall-Spencer et al., 2008; Kroeker
570 et al., 2011), were not obviously affected by lowered pH conditions.

571 Interactions with the seagrass host may limit a negative outcome. Epiphytic calcifier
572 coverage on *P. oceanica* leaves collected from the eFOCE system during the same period did not
573 appear to be altered (Cox et al., in press). The use of an *in situ* system, which accounts for diel
574 pH fluctuations, may have allowed for the host plant to alter carbonate chemistry at the
575 proximity of the leaves and buffered potential effects of ocean acidification. Alternatively,
576 because response can vary with species or morphological thickness (Doropoulos et al., 2012;
577 McCoy and Ragazzola, 2014), algae that colonized tiles could have been more sensitive to
578 lowered pH conditions than those found on leaves. Nevertheless, this does not explain the
579 different responses observed for *Spirorbis* sp. that appeared to be the same species on tiles (this
580 study) and leaf surfaces (Cox et al., 2017). Consistent with the conclusion that host plants can
581 buffer potential negative effects of ocean acidification, *Spirorbis spirorbis*, maintained under a

582 moderate ocean acidification scenario, recruited on the macroalga *Fucus serratus* and their
583 calcification rates were greater in daylight when algal photosynthesis occurs (Saderne and Wahl,
584 2013). However, they did not compare recruitment on algae to recruitment on artificial surfaces
585 which would have shed light on the extent of the ability of algae to buffer impacts on epibiont
586 early life stages.

587 *4.2. Invertebrates within scourers*

588 Many of the taxonomic groups observed on scourers were juveniles or adult stages of
589 invertebrates and are reported members of the *Posidonia* epifaunal community (Cigliano et al.,
590 2010; Gambi et al., 1992; Michel et al., 2015). Some such as crustaceans, gastropods, and
591 echinoderms are mobile and have the ability to migrate or move to more favorable areas. In the
592 present study, their recruitment/colonization appeared relatively resilient to lowered pH
593 conditions as projected for the end of the century. In contrast, Cigliano et al. (2010), using
594 scourers placed for one month along the CO_2 vent near Ischia (Italy), found a decreasing gradient
595 of calcifier (foraminiferans, serpulid polychaetes, gastropods and bivalves) density from high to
596 low pH_T (~ 8.0 to 7.0). Few taxa (which lack calcified tubes) appeared more prevalent either at
597 the mid (*Amphiglena mediterranea, Leptochelia dubia, Caprella acanthifera*) or low pH
598 locations (*Syllis prolifera*). The different outcome to the present study could be explained by the
599 greater range of pH_T (~ 8.0 to 7.0) and, at least partially, by differences in community
600 composition. Calcifying Foraminifera and serpulid polychaetes were common recruits in
601 meadow locations away from vents in Ischia, Italy (Cigliano et al., 2010; Kroeker et al., 2011)
602 and tended to be absent or much reduced in numbers on fresh scourers within the enclosures in
603 the present study. When serpulids were observed in the present study, they did appear to be
604 sensitive to lowered pH in early life history stages (on tiles) and perhaps in later adult stages with

prolonged exposure. It is also important to keep in mind that vent studies can be hampered by the presence of trace elements and are observational; making it difficult to imply causation (Kerrison et al., 2011; Vizzini et al., 2013).

Other studies focused on invertebrate adult stages (Kroeker et al., 2011) or seasonal influence (over similar months as the present study, Garrard et al., 2014) at the Ischia CO_2 vents mirror the observations of Cigliano et al. (2010). However, these authors attribute increases in particular arthropods or polychaetes to indirect effects, such as release of invertebrate predators or interactions with macrophytes. Predation has long been thought to be a structuring factor of invertebrate assemblages in seagrass meadows (Edgar, 1990; Heck and Thoman, 1981; Heck and Orth, 2006). In *P. oceanica* meadows, Gambi et al. (1998) attributed seasonal differences in polychaetes to differences in fish predation and Stuaro et al. (2016) found total density and biomass of amphipods to be greater in ambient than within fish inclusion plots. In the present study, indirect effects from fishes cannot be addressed because they were excluded from enclosures. Furthermore, ocean acidification effects on fish predatory behavior have largely been ignored. However, predator foraging ability, as well as invertebrate density, are often related to seagrass density (Heck and Thoman, 1981; Orth et al., 1984). Elevated seagrass density and canopy structure has been suggested to facilitate habitat heterogeneity and lower predation ability (Garrard et al., 2014; Orth et al., 1984). In the present study, seagrass density was similar between enclosures and *Posidonia* leaf biometrics and photosynthesis unaffected by lowered pH (Cox et al., 2016).

Results from the present study indicate that gastropods and bivalves can settle under moderate ocean acidification scenarios. These groups also occurred at similar mean pH at the vents (Kroeker et al., 2011). Juvenile Mytilidae occurred at greater numbers at a vent location

628 with a mean pH_T of 6.7 than at a control site (pH_T of 8.1; Garrard et al., 2014; Kroeker et al.,
629 2011). The similar growth rates of *Lyonsia* sp. found in the two enclosures would also suggest
630 that at least one calcifier was able to maintain growth. Therefore, it appears that in seagrass
631 meadows, mollusc density and diversity is somewhat resilient to near future pH conditions.
632 However, predation and other shell characteristics (hardness and elasticity) which may facilitate
633 predation were not measured. Arthropods, particularly peracarids, and polychaetes (except
634 Serpulids) appeared tolerant in the present study to lowered pH and also dominated assemblages
635 at the lowest pH levels in all CO_2 vent studies; although there were species specific differences
636 in distribution patterns (Allen et al., 2016; Cigliano et al., 2010; Garrard et al., 2014; Kroeker et
637 al., 2011). Reduced or lack of calcification and internal acid-base regulation may account for
638 their increased tolerance (Melzner et al., 2009).
639 Biodiversity can be an indication of ecosystem function. A loss or change is used to
640 indicate ecosystem health (Hooper et al., 2005). In general, species richness is often greater in
641 habitats with greater structure (Sunday et al., 2016) and seagrass meadows in the Mediterranean
642 Sea provide refuge for numerous species and tend to be highly diverse (Borowitz et al., 2006).
643 Indeed, the epibiont assemblage in the present study was taxonomically diverse and varied. Taxa
644 richness in monthly collected scourers was lower on average and within range (5-9 enclosures,
645 15-20 in reference) of the richness in invertebrate taxa settled in one month (range 10-35) in a
646 *Posidonia* meadow at the Ischia (Italy) volcanic CO_2 vent yet, the evenness in the present study
647 had a higher range (0.91 - 0.99 this study, 0.6 - 0.9 in Cigliano et al., 2010). The concern is that
648 ocean acidification will alter diversity both directly via loss of calcified species and indirectly by
649 changing habitat structural complexity or density (Sunday et al., 2016). There are several
650 predicted scenarios for effects on local diversity in seagrass or fleshy algal meadows under

elevated pCO_2 which are dependent upon whether the producer density or complexity is increased, replaced, or reduced. However, at low pH near volcanic CO_2 vents in the Mediterranean Sea, elevated seagrass density was not correlated with increases in local diversity (Sunday et al., 2016). The results from the present study do not support changes in local diversity via indirect effects with changes in the habitat complexity. Seagrass physiology and abundance as well as algal abundance were not affected by the pH manipulation in eFOCE (Cox et al., 2016). The reduction of calcifiers on tiles, however, would be congruent with a loss of local diversity through direct effects. Yet, the only decline in taxonomic richness associated with the lowered pH treatment was in the epibont assemblage found within the unreplicated, pre-settled scourers left in lowered pH for the duration of study.

It is not clear from the results of the present study how invertebrate assemblages will be affected in later development stages or with more prolonged lowered pH exposure. The similar invertebrate density and diversity between enclosures within pre-settled scourers after two months of exposure suggests species were able to survive, recruit, or were able to do both at the lowered pH and that outcomes were not affected by competition, predation, nor sensitivity at later life-history stage. In comparison, the pre-settled scourers in later stages of development, and left in acidified conditions for four months revealed a pattern congruent with ocean acidification predictions. The interpretation of the latter pre-settled outcome is limited by scourer replication. In addition, the study did not address differences in biomass, metabolism, or reproductive demands that could influence species interactions or larval production over a longer time scale.

4.3. Caveats

Large-scale unreplicated experiments, such as eFOCE, because of their unique qualities (e.g. pH manipulated at an offset in an intact community), size, and temporal scope can provide

674 valuable ecological information that may bolster the conclusions of laboratory or natural studies.
675 Alternatively, they can provide a different hypothesis to pursue more thoroughly. The eFOCE
676 study addressed a need for *in situ* manipulation to increase ability to predict future ecology.
677 Additionally, the study design accounted for natural pH variation that is often ignored when pH
678 is manipulated *in situ*. While large scale unreplicated experiments can provide valuable
679 ecological information, they do have drawbacks (Davies and Gray, 2015; Hurlbert, 1984;
680 Oksanen, 2001). Replicated enclosures were not feasible at this stage (see discussion in Gattuso
681 et al., 2014). Therefore, alternative hypotheses that we cannot robustly exclude include (1) there
682 were other small pH effects difficult to quantify (2) that the conflicting outcome is due to some
683 'lurking' variable. Yet, several recommended steps (Davies and Gray, 2015; Oksanen, 2001)
684 were taken to try to reduce erroneous conclusions that may occur including: (1) care was taken to
685 select study locations that were similar in depth and seagrass density to reduce confounding
686 variables, (2) the environment was continuously monitored to ensure they were similar to those
687 in ambient, (3) repeated measurements were made at the same location through time, and (4)
688 comparisons from the pH-manipulated enclosure were made to two different spatial locations.
689 The enclosure structure did inhibit colonization possibly by reducing water flow or general
690 movement, yet assemblages had many of the same taxonomic members and dominant taxa were
691 common in all locations.

692 **5. Conclusions**

693 It appears from results that specific calcifiers, such as CCA and serpulid polychaetes, are
694 highly sensitive to ocean acidification and that small peracarid crustaceans and polychaetes
695 without calcified tubes appear robust. In contrast to vent observations that tend to support shifts
696 resulting from competitive interactions or physiological limits across a wider pH range, there

was evidence that near future pH conditions (pH_T 7.75) can impact the early life-history stages of the dominant calcifiers. Contrasting results within eFOCE on tiles and leaves also stresses the need to identify the extent of host plant buffering and take action to alleviate threats to host plants to minimize ocean acidification impacts on the community. Lastly, conclusion should be tempered or put into the context of results from other studies until more *in situ* pH manipulation studies are done that address the limitations of eFOCE and occur in variety of conditions that persist throughout the Mediterranean Sea.

Acknowledgements

We would like to acknowledge the following people who assisted in the laboratory, in the field, or with system engineering or maintenance: E. Beck Acain, J. Acain, J. Delille, L. van der Heijden, M. Maillot, F. Moullec, M. Necoechea, S. Schenone, L. Urbini, K. Walzyńska. We also thank J.-J. Pangrazi, R. Patrix, and E. Tanguy for aide in construction of the enclosures. Éric Béraud, G. de Liege, D. Luquet, L. Mangialajo, S. Reynaud, and D. Robin kindly assisted in diving activities. We also thank B. Kirkwood at Monterey Bay Aquarium Research Institute who advised in system design. We thank the Service d'Observation Rade de Villefranche and the Service d'Observation en Milieu Littoral for their kind permission to use Point B data. We also thank the Service National d'Analyse des Paramètres Océaniques du CO_2 for performing the determination of A_T at Point B. This work was funded by the 'European Free Ocean Carbon Enrichment' (eFOCE; BNP Paribas Foundation), the European Commission through the project 'Mediterranean Sea Acidification in a changing climate' (MedSeA; grant agreement 265103) and the MISTRALS-MERMEX (INSU, CNRS) program.

References

719 Allen R., Foggo, A., Fabricius, K., Balistreri, A., Hall-Spencer, J.M., 2016. Tropical CO_2 seeps
720 reveal the impact of ocean acidification on coral reef invertebrate recruitment. Mar. Poll.
721 Bull. doi: 10.1016/j.marpolbul.2016.12.031

722 Alcoverro, T., Duarte, C., Romero, J., 1997. The influence of herbivores on *Posidonia oceanica*
723 epiphytes. Aquat. Bot. 56, 93–104.

724 Anderson, M.J., Willis, T.J., 2003. Canonical analysis of principal coordinates: a useful method
725 of constrained ordination for ecology. Ecology 84, 511–525. doi: 10.1890/0012-
726 9658(2003)084[0511:CAOPCA]2.0.CO;2

727 Bornhold, B.D., Milliman, J.D., 1973. Generic and environmental control of carbonate
728 mineralogy in serpulid (polychaete) tubes. J. Geol. 81, 363–373.

729 Borowitzka, M.A., Lavery, P.S., van Keulen, M. 2006., Seagrasses: Biology, Ecology and
730 Conservation. In: Larkum, A.W.D., Orth, R.J., Duarte, C.M. (Eds.) Epiphytes of
731 seagrasses. Springer, Dordrecht, The Netherlands, pp 441–461.

732 Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., Chhabra, A., DeFries, R.,
733 Galloway, J., Heimann, M., Jones, C., Le Quéré, C., Myneni, R.B., Piao, S., Thornton, P.,
734 2013. Carbon and other biogeochemical cycles. Cambridge University Press, Cambridge,
735 United Kingdom and New York, NY, USA

736 Cigliano, M., Gambi, M.C., Rodolfo-Metalpa, R., Patti, F.P., Hall-Spencer, J.M., 2010. Effects
737 of ocean acidification on invertebrate settlement at volcanic CO_2 vents. Mar. Biol. 157,
738 2489–2502. doi: 10.1007/s00227-010-1513-6

739 Cox, T.E., Gazeau, F., Alliouane, S., Hendriks, I.E., Mahacek, P., Le Fur, A., Gattuso, J.-P.,
740 2016. Effects of *in situ* CO_2 enrichment on structural characteristics, photosynthesis, and
741 growth of the Mediterranean seagrass *Posidonia oceanica*. Biogeosciences 13, 2179–
742 2194. doi: 10.5194/bg-13-2179-2016

743 Cox, T.E., Nash, M., Gazeau, F., Déniel, M., Legrand, E., Alliouane, S., Mahacek, P., Le Fur, A.,
744 Gattuso, J.-P., Martin, S., 2017. Effects of *in situ* CO_2 enrichment on *Posidonia oceanica*
745 epiphytic community composition and mineralogy. Mar. Biol. 164, 103. doi:
746 10.1007/s00227-017-3136-7

747 Crook, E.D., Kroeker, K.J., Potts, D.C., Rebolledo-Vieyra, M., Hernandez-Terrones, L.M.,
748 Paytan, A., 2016. Recruitment and succession in a tropical benthic community in
749 response to *in-situ* Ocean Acidification. PLoS ONE 11, e0146707.
750 doi:10.1371/journal.pone.0146707

751 Davies, G.M., Gray, A., 2015. Don't let spurious accusations of pseudoreplication limit our
752 ability to learn from natural experiments (and other messy kinds of ecological
753 monitoring). Ecol. Evol. 5, 5295–5304. doi: 10.1002/ece3.1782

754 Donnarumma, L., Lombardi, C., Cocito, S., Gambi, M.C., 2014. Settlement pattern of *Posidonia*
755 *oceanica* epibionts along a gradient of ocean acidification: an approach with mimics.
756 Mediterr. Mar. Sci. 15, 498–509. doi: 10.12681/mms.677

757 Doropoulos, C., Ward, S., Diaz-Pulido, G., Hoegh-Guldberg, O., Mumby, P.J., 2012. Ocean
758 acidification reduces coral recruitment by disrupting intimate larval-algal settlement

759 interactions: elevated CO_2 alters CCA-larval interactions. Ecol. Lett. 15, 338–346. doi:
760 10.1111/j.1461-0248.2012.01743.x

761 Dupont, S., Dorey, N., Thorndyke, M., 2010. What meta-analysis can tell us about vulnerability
762 of marine biodiversity to ocean acidification? Estuar. Coast. Shelf Sci. 89, 182–185. doi:
763 10.1016/j.ecss.2010.06.013

764 Edgar, G.J., 1990. Population regulation, population dynamics and competition amongst mobile
765 epifauna associated with seagrass. J. Exp. Mar. Biol. Ecol. 144, 205–234.
766 doi:10.1016/0022-0981(90)90029-C

767 Feely, R.A., 2004. Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science
768 305, 362–366. doi: 10.1126/science.1097329

769 Frankovich, T.A., Zieman, J.C., 1994. Total epiphyte and epiphytic carbonate production on
770 *Thalassia testudinum* across Florida Bay. Bull. Mar. Sci. 54, 679–695.

771 Gambi, M.C., Conti, G., Bremec, C.S., 1998. Polychaete distribution, diversity and seasonality
772 related to seagrass cover in shallow soft bottoms of the Tyrrhenian Sea (Italy). Sci. Mar.
773 62, 1–17.

774 Gambi, M.C., Lorenti, M., Russo, G.F., Scipione, M.B., Zupo, V., 1992. Depth and seasonal
775 distribution of some groups of the vagile fauna of the *Posidonia oceanica* leaf stratum:
776 structural and trophic analyses. Mar. Ecol. 13, 17–39. doi: 10.1111/j.1439-
777 0485.1992.tb00337.x

778 Garrard, S.L., Gambi, M.C., Scipione, M.B., Patti, F.P., Lorenti, M., Zupo, V., Paterson, D.M.,
779 Buia, M.C., 2014. Indirect effects may buffer negative responses of seagrass invertebrate
780 communities to ocean acidification. J. Exp. Mar. Biol. Ecol. 461, 31–38. doi:
781 10.1016/j.jembe.2014.07.011

782 Gattuso, J.-P., Epitalon, J.-M., Lavigne, H., 2015. Seacarb: seawater carbonate chemistry. R
783 package version 3.0.6. http://CRAN.R-project.org/package=seacarb

784 Gattuso, J.-P., Hansson, L., (Eds.) 2011. Ocean acidification. Oxford University Press, Oxford;
785 New York

786 Gattuso, J.-P., Kirkwood, W., Barry, J.P., Cox, T.E., Gazeau, F., Hansson, L., Hendriks, I.,
787 Kline, D.I., Mahacek, P., Martin, S., McElhany, P., Peltzer, E.T., Reeve, J., Roberts, D.,
788 Saderne, V., Tait, K., Widdicombe, S., Brewer, P.G., 2014. Free-ocean CO_2 enrichment
789 (FOCE) systems: present status and future developments. Biogeosciences 11, 4057–4075.

790 Gaylord, B., Kroeker, K.J., Sunday, J.M., Anderson, K.M., Barry, J.P., Brown, N.E., Connell,
791 S.D., Dupont, S., Fabricius, K.M., Hall-Spencer, J.M., 2015. Ocean acidification through
792 the lens of ecological theory. Ecology 96, 3-15.

793 Gazeau, F., Gattuso, J.-P., Dawber, C., Pronker, A.E., Peene, F., Peene, J., Heip, C.H.R.,
794 Middelburg, J.J., 2010. Effect of ocean acidification on the early life stages of the blue
795 mussel *Mytilus edulis*. Biogeosciences 7, 2051–2060. doi: 10.5194/bg-7-2051-2010

796 Gazeau, F., Parker, L.M., Comeau, S., Gattuso, J.-P., O'Connor, W.A., Martin, S., Pörtner, H.-
797 O., Ross, P.M., 2013. Impacts of ocean acidification on marine shelled molluscs. Mar.
798 Biol. 160, 2207–2245. doi: 10.1007/s00227-013-2219-3

799 Gobert, S., Cambridge, M.L., Velimirov, B., Pergent, G., Lepoint, G., Bouquegneau, J.M.,
800 Duaby, P., Pergent-Martini, C., Walker, D.I., Larkum, A.W.D., Orth, R.J., Duarte, C.M.,
801 2006. Biology of *Posidonia*. In: Larkum, A.W.D., Orth, R.J., Duarte, C.M. (Eds.)
802 Seagrasses: Biology, Ecology and Conservation. Springer, Dordrecht, The Netherlands,
803 pp 387–408.

804 Gorsky, G., Ohman, M.D., Picheral, M., Gasparini, S., Stemmann, L., Romagnan, J.B., Cawood,
805 A., Pesant, S., Garcia-Comas, C., Prejger, F., 2010. Digital zooplankton image analysis
806 using the ZooScan integrated system. J. Plankton Res. 32, 285–303.

807 Hall-Spencer, J.M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S.M.,
808 Rowley, S.J., Tedesco, D., Buia, M.C., 2008. Volcanic carbon dioxide vents show
809 ecosystem effects of ocean acidification. Nature 454, 96–99.

810 Heck, K.L., Orth, R.J., 2006. Predation in seagrass beds, in: Seagrasses: Biology, Ecology and
811 Conservation. Springer, Dordrecht, The Netherlands, pp. 537–550.

812 Heck, K.L., Thoman, T.A., 1981. Experiments on predator-prey interactions in vegetated aquatic
813 habitats. J. Exp. Mar. Biol. Ecol. 53,125–134. doi:10.1016/0022-0981(81)90014-9

814 Hemminga, M.A., Duarte, C.M., 2000. Seagrass Ecology. University of Cambridge, Cambridge,
815 United Kingdom

816 Hofmann, L.C., Bischof, K., 2014. Ocean acidification effects on calcifying macroalgae. Aquat.
817 Biol. 22, 261–279.

818 Hooper, D.U., Chapin, F.S., Ewel, J.J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J.H.,
819 Lodge, D.M., Loreau, M., Naeem, S., Schmid, B., Setälä, H., Symstad, A.J., Vandermeer,
820 J., Wardle, D.A., 2005. Effects of biodiversity on ecosystem functioning: a consensus of
821 current knowledge. Ecol. Monogr. 75, 3–35. doi:10.1890/04-0922

822 Hurlbert, S., 1984. Pseudoreplication and the design of ecological field experiments. Ecol.
823 Monogr. 54, 187–211.

824 Ippolitov A. & A.V. Rzhavsky., 2015. Tube morphology, ultrastructures and mineralogy in
825 recent Spirorbinae (Annelida: Polychaeta: Serpulidae). II. Tribe Spirorbini. Invert. Zool.
826 12, 61–92.

827 Jackson, E.L., Rees, S.E., Wilding, C., Attrill, M.J., 2015. Use of a seagrass residency index to
828 apportion commercial fishery landing values and recreation fisheries expenditure to
829 seagrass habitat service: Seagrass contribution to fishery value. Conserv. Biol. 29, 899–
830 909. doi: 10.1111/cobi.12436

831 Jokiel, P.L., Rodgers, K.S., Kuffner, I.B., Andersson, A.J., Cox, E.F., Mackenzie, F.T., 2008.
832 Ocean acidification and calcifying reef organisms: A mesocosm investigation. Coral
833 Reefs 27, 473–483. doi: 10.1007/s00338-008-0380-9

834 Kerrison, P., Hall-Spencer, J.M., Suggett, D.J., Hepburn, L.J., Steinke, M., 2011. Assessment of
835 pH variability at a coastal CO_2 vent for ocean acidification studies. Estuar. Coast. Shelf.
836 Sci. 94, 129–137.

837 Koch, M., Bowes, G., Ross, C., Zhang, X.H., 2013. Climate change and ocean acidification
838 effects on seagrasses and marine macroalgae. Glob. Change Biol. 19, 103–132. doi:
839 10.1111/j.1365-2486.2012.02791.x

840 Kroeker, K.J., Kordas, R.L., Crim, R., Hendriks, I.E., Ramajo, L., Singh, G.S., Duarte, C.M.,
841 Gattuso, J.-P., 2013. Impacts of ocean acidification on marine organisms: quantifying
842 sensitivities and interaction with warming. Glob. Change Biol. 19,1884–1896. doi:
843 10.1111/gcb.12179

844 Kroeker, K.J., Micheli, F., Gambi, M.C., 2012. Ocean acidification causes ecosystem shifts via
845 altered competitive interactions. Nat. Clim. Change 3, 156–159. doi:
846 10.1038/nclimate1680

847 Kroeker, K.J., Micheli, F., Gambi, M.C., Martz, T.R., 2011. Divergent ecosystem responses
848 within a benthic marine community to ocean acidification. Proc. Natl. Acad. Sci. 108,
849 14515–14520. doi: 10.1073/pnas.1107789108

850 Kuffner, I.B., Andersson, A.J., Jokiel, P.L., Rodgers, K.S., Mackenzie, F.T., 2008. Decreased
851 abundance of crustose coralline algae due to ocean acidification. Nat. Geosci. 1, 114–
852 117. doi: 10.1038/ngeo100

853 Kurihara, H., 2008. Effects of CO_2-driven ocean acidification on the early developmental stages
854 of invertebrates. Mar. Ecol. Prog. Ser. 373, 275–284. doi: 10.3354/meps07802

855 Lepoint, G., Nyssen, F., Gobert, S., Dauby, P., Bouquegneau, J.M., 2000. Relative impact of a
856 seagrass bed and its adjacent epilithic algal community in consumer diets. Mar. Biol. 136,
857 513–518.

858 Le Quéré, C., Peters, G. P., Andres, R. J., Andrew, R. M., Boden, T. A., Ciais, P., Friedlingstein,
859 P., Houghton, R. A., Marland, G., Moriarty, R., Sitch, S., Tans, P., Arneth, A., Arvanitis,
860 A., Bakker, D. C. E., Bopp, L., Canadell, J. G., Chini, L. P., Doney, S. C., Harper, A.,
861 Harris, I., House, J. I., Jain, A. K., Jones, S. D., Kato, E., Keeling, R. F., Klein
862 Goldewijk, K., Körtzinger, A., Koven, C., Lefèvre, N., Maignan, F., Omar, A., Ono, T.,
863 Park, G. H., Pfeil, B., Poulter, B., Raupach, M. R., Regnier, P., Rödenbeck, C., Saito, S.,
864 Schwinger, J., Segschneider, J., Stocker, B. D., Takahashi, T., Tilbrook, B., van Heuven,
865 S., Viovy, N., Wanninkhof, R., Wiltshire, A. and Zaehle, S., 2013. Global carbon budget.
866 Earth Systems Sci. Data, 6, 235–263.

867 Martin, S., Rodolfo-Metalpa, R., Ransome, E., Rowley, S., Buia, M.C., Gattuso, J.-P., Hall-
868 Spencer, J., 2008. Effects of naturally acidified seawater on seagrass calcareous
869 epibionts. Biol. Lett. 4, 689–692. doi: 10.1098/rsbl.2008.0412

870 Mateo, M., Cebrián, J., Dunton, K., Mutchler, T., 2006. Carbon flux in seagrass ecosystems. In:
871 Larkum, A.W.D., Orth, R.J., Duarte, C.M. (Eds.) Seagrasses: Biology, Ecology and
872 Conservation. Springer, Dordrecht, The Netherlands, pp 159–192.

873 Mazzella, L., Scipione, M.B., Buia, M.C., 1989. Spatio-temporal distribution of algal and animal
874 communities in a *Posidonia oceanica* meadow. Mar. Ecol. 10, 107–129.

875 McCoy, S.J., Kamenos, N.A., 2015. Coralline algae (Rhodophyta) in a changing world:
876 integrating ecological, physiological, and geochemical responses to global change. J.
877 Phycol. 51, 6–24. doi: 10.1111/jpy.12262

878 McCoy, S.J., Ragazzola, F., 2014. Skeletal trade-offs in coralline algae in response to ocean
879 acidification. Nat. Clim. Change 4, 719–723. doi: 10.1038/nclimate2273

880 Melzner, F., Gutowska, M.A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M.C.,
881 Belich, M., Pörtner, H.-O., 2009. Physiological basis for high CO_2 tolerance in marine
882 ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6,
883 2313–2331.

884 Michel, L.N., Dauby P., Gobert S, Graeve, M., Nyssen, F. Thelen, N., Lepoint, G., 2015.
885 Dominant amphipods of *Posidonia oceanica* seagrass meadows display considerable
886 trophic diversity. Mar. Ecol. 36, 969–981. doi: 10.1111/maec.12194

887 Nelson, W., 2009. Calcified macroalgae–critical to coastal ecosystems and vulnerable to change:
888 a review. Mar. Freshw. Res. 60, 787–801. doi: 10.1071/MF08335

889 Orth, R.J., Heck, K.L., Vanmontfrans, J., 1984. Faunal communities in seagrass beds – a review
890 of the influence of plant structure and prey characteristics on predator-prey relationships.
891 Estuaries 7, 339–350.
892
893 Oksanen, L., 2001. Logic of experiments in ecology: is pseudoreplication a pseudoissue? Oikos
894 94, 27–38. doi: 10.1034/j.1600-0706.2001.11311.x

895 Pasqualini, V., Pergent-Martini, C., Clabaut, P., Pergent, G., 1998. Mapping of *Posidonia*
896 *oceanica* using aerial photographs and side scan sonar: application off the island of
897 Corsica (France). Estuar. Coast. Shelf Sci. 47, 359–367.

898 Perry, C.T., Beavington-Penney, S.J., 2005. Epiphytic calcium carbonate production and facies
899 development within sub-tropical seagrass beds, Inhaca Island, Mozambique. Sediment.
900 Geol. 174, 161–176. doi: 10.1016/j.sedgeo.2004.12.003

901 Porzio, L., Buia, M.C., Hall-Spencer, J.M., 2011. Effects of ocean acidification on macroalgal
902 communities. J. Exp. Mar. Biol. Ecol. 400, 278–287. doi: 10.1016/j.jembe.2011.02.011

903 Price, N.N., Martz, T.R., Brainard, R.E., Smith, J.E., 2012. Diel variability in seawater pH
904 relates to calcification and benthic community structure on coral reefs. PLoS ONE 7,
905 e43843. doi: 10.1371/journal.,pone.0043843

906 Romero, J., Lee, K., Marta, P., Mateo, M., Alcoverro, T., Larkum, A.W.D., Orth, R.J., Duarte,
907 C.M., 2006. Nutrient Dynamics in Seagrass Ecosystems. In: Larkum, A.W.D., Orth, R.J.,
908 Duarte, C.M. (Eds.) Seagrasses: Biology, Ecology and Conservation. Springer,
909 Dordrecht, The Netherlands, pp 227–254.

910 Saderne, V., Wahl, M., 2013. Differential responses of calcifying and non- calcifying epibionts
911 of a brown macroalga to present-day and future upwelling pCO_2. PLoS ONE 8, e70455.
912 doi: 10.1371/journal.,pone.0070455

913 Sturaro, N., Gobert, S., Pérez-Perera, A., Caut, S., Panzalis, P., Navone, A., Lepoint, G., 2016.
914 Effects of fish predation on *Posidonia oceanica* amphipod assemblages. Mar. Biol. 163,
915 58. doi:10.1007/s00227-016-2830-1

916 Sunday, J.M., Fabricius, K.E., Kroeker, K.J., Anderson, K.M., Brown, N.E., Barry, J.P., Connell,
917 S.D., Dupont, S., Gaylord, B., Hall-Spencer, J.M., Klinger, T., Milazzo, M., Munday,
918 P.L., Russell, B.D., Sanford, E., Thiyagarajan, V., Vaughan, M.L.H., Widdicombe, S.,

Harley, C.D.G., 2016. Ocean acidification can mediate biodiversity shifts by changing biogenic habitat. Nat. Clim. Change 7, 81–85

Talmage, S.C., Gobler, C.J., 2009. The effects of elevated carbon dioxide concentrations on the metamorphosis, size, and survival of larval hard clams (*Mercenaria mercenaria*), bay scallops (*Argopecten irradians*), and Eastern oysters (*Crassostrea virginica*). Limnol. Oceanogr. 54, 2072.

Vizzini, S., Di Leonardo, R., Costa, V., Tramati, C.D., Luzzu, F., Mazzola, A., 2013. Trace element bias in the use of CO_2 vents as analogues for low pH environments: implications for contamination levels in acidified oceans. Estuar. Coast. Shelf Sci. 134, 19–30. doi: 10.1016/j.ecss.2013.09.015

Figures

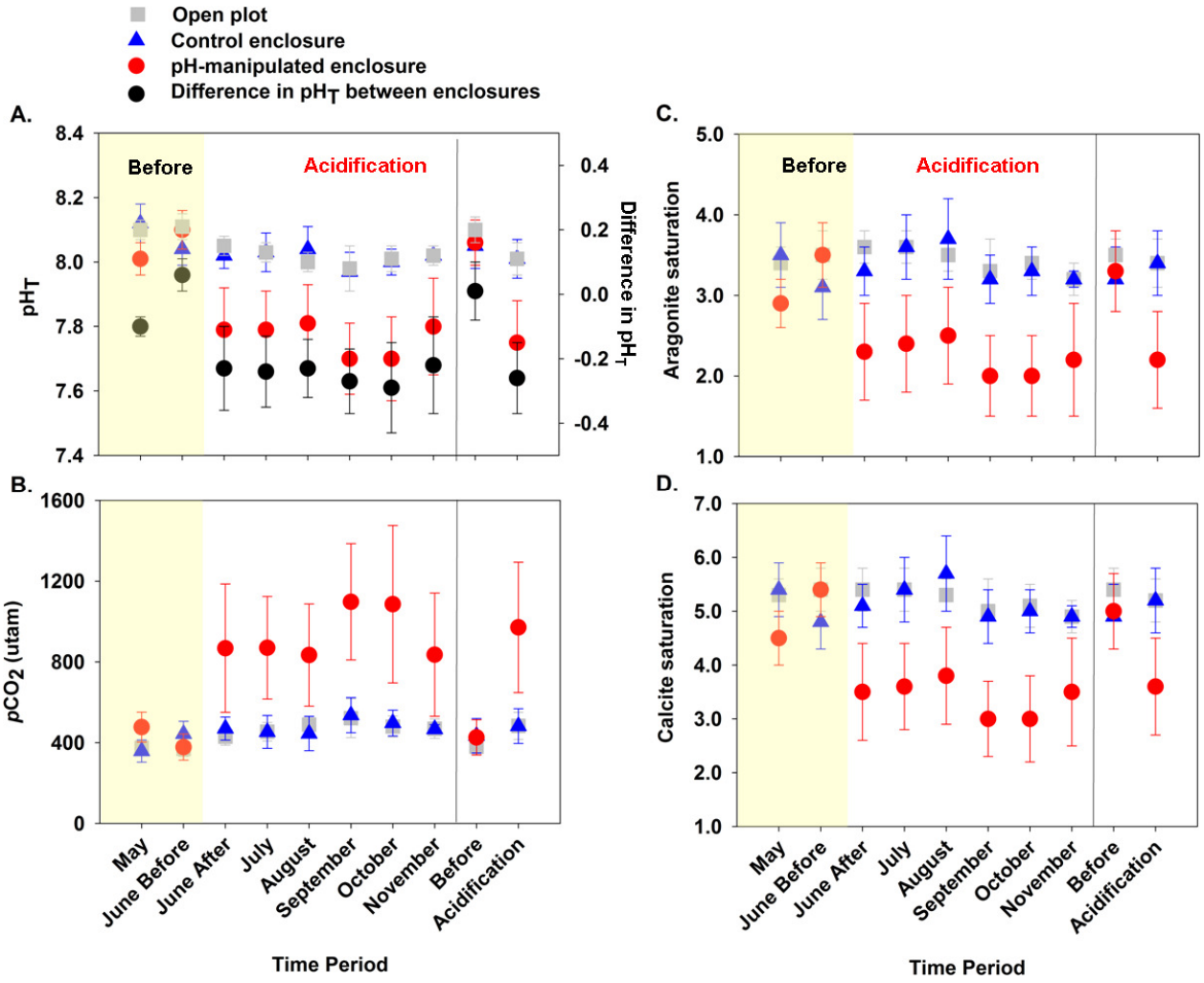
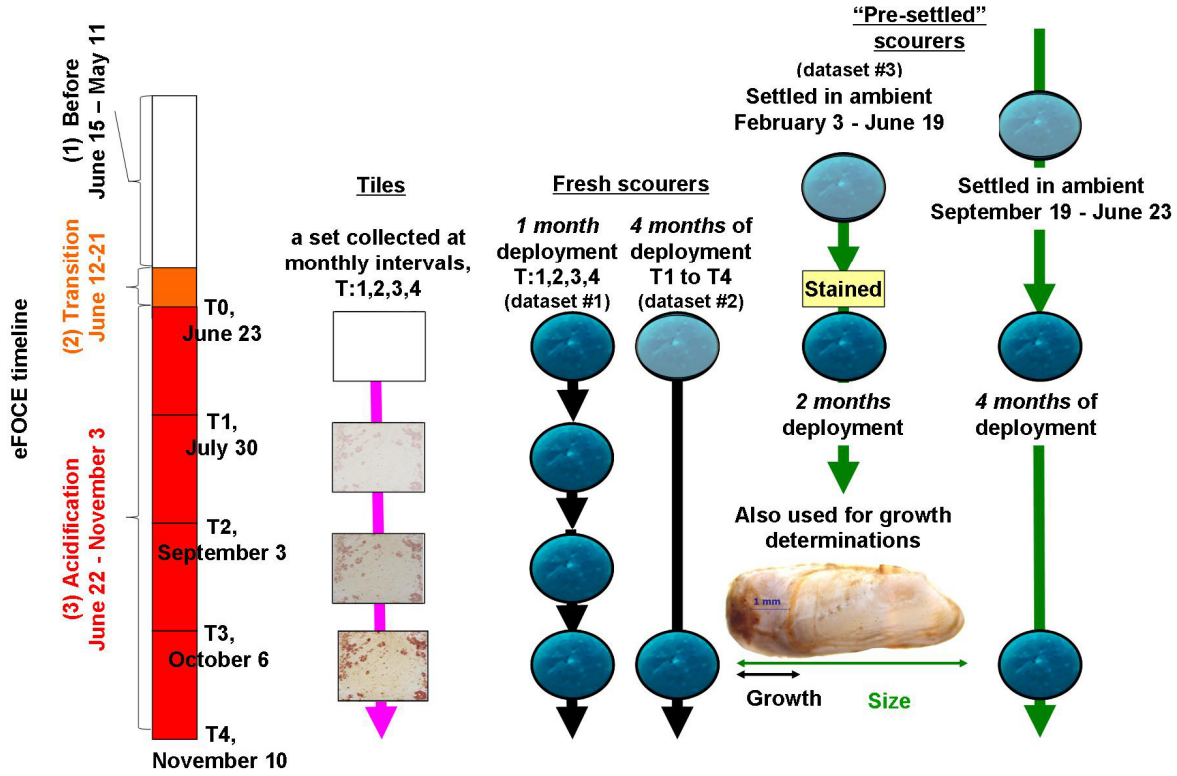


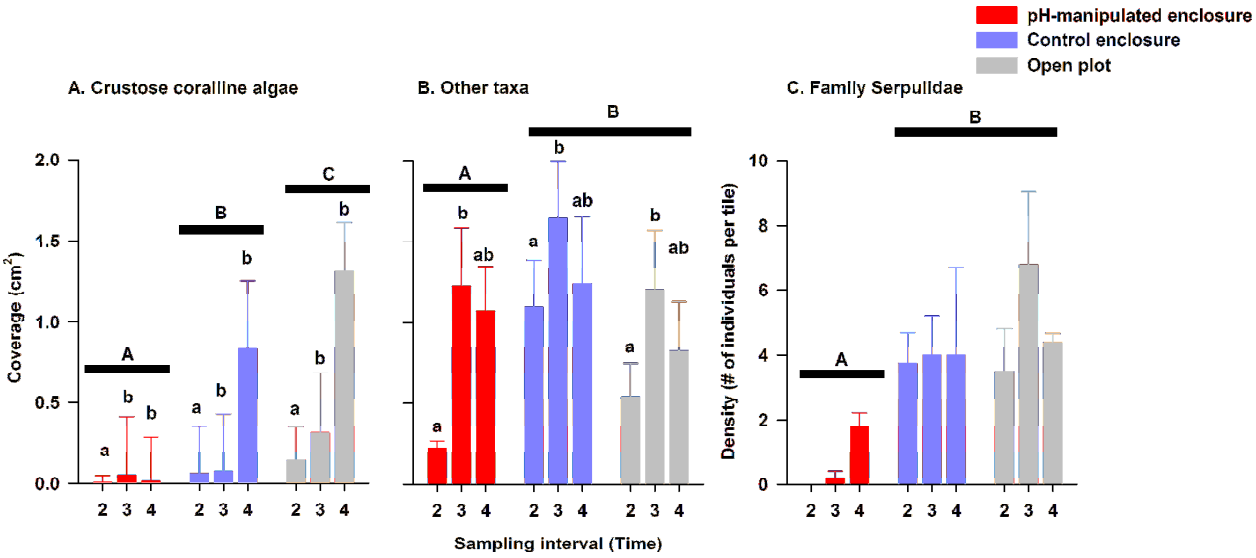
Fig. 1. Carbonate chemistry within ambient (open plot) and enclosures: averaged (± standard deviation, SD): Panel A, pH (on the total scale; pH_T), Panel B, partial pressure of carbon dioxide (pCO_2) and Panel C and D, saturation states with respect to aragonite (Ω_a) and calcite (Ω_c) for each month and for the period before and during acidification. The difference in pH_T between the pH-manipulated and the control enclosure is also shown in Panel A.



936

937 **Fig. 2.** Artificial collectors used and collection intervals. Tiles were placed in the three eFOCE
938 treatments (control and pH-manipulated enclosures and open plot) at the start of the acidification
939 period (T0) and a set of three to five tiles from each treatment were collected every four weeks at
940 sampling intervals T1 to T4 to examine assemblage development. Fresh scourers were also
941 placed in three treatments at T0 to target the collection of sessile micro-invertebrates. These
942 scourers were collected and replaced at monthly sampling intervals T1, T2, T3, T4 and collected
943 after four months (placed at T0 left until T4) to examine the effects of ocean acidification on the
944 micro-invertebrate assemblage over short and longer durations. Pre-settled scourers were settled
945 in ambient for four to nine months. One set was stained for 48 h in calcien and deployed in the
946 enclosures and open plot for two months. The other set of pre-settled scoures (n = 1) were
947 deployed in each treatment and left for the duration of the study (four months). Pre-settled
948 scourers were used to test whether the response of an assemblage exposed in a later successional

949 stage differs from assemblages developed under ocean acidification. Invertebrates in pre-settled
950 scourers stained with calcein were used to measure calcifier growth.



951

952 **Fig. 3.** Mean abundance (+ SD) of operational taxonomic units (OTU) found on tiles placed
953 initially during the pH manipulation and collected (n = 5) every four weeks (sampling intervals
954 T2 to T4) from the pH-manipulated and control enclosure and the open plot. Letter groups and
955 horizontal bars above the vertical bars are the significant ($P < 0.05$) results of pairwise tests (see
956 Table 1). Other taxa category includes cyanobacteria, brown colored algal crusts, and
957 filamentous algae.

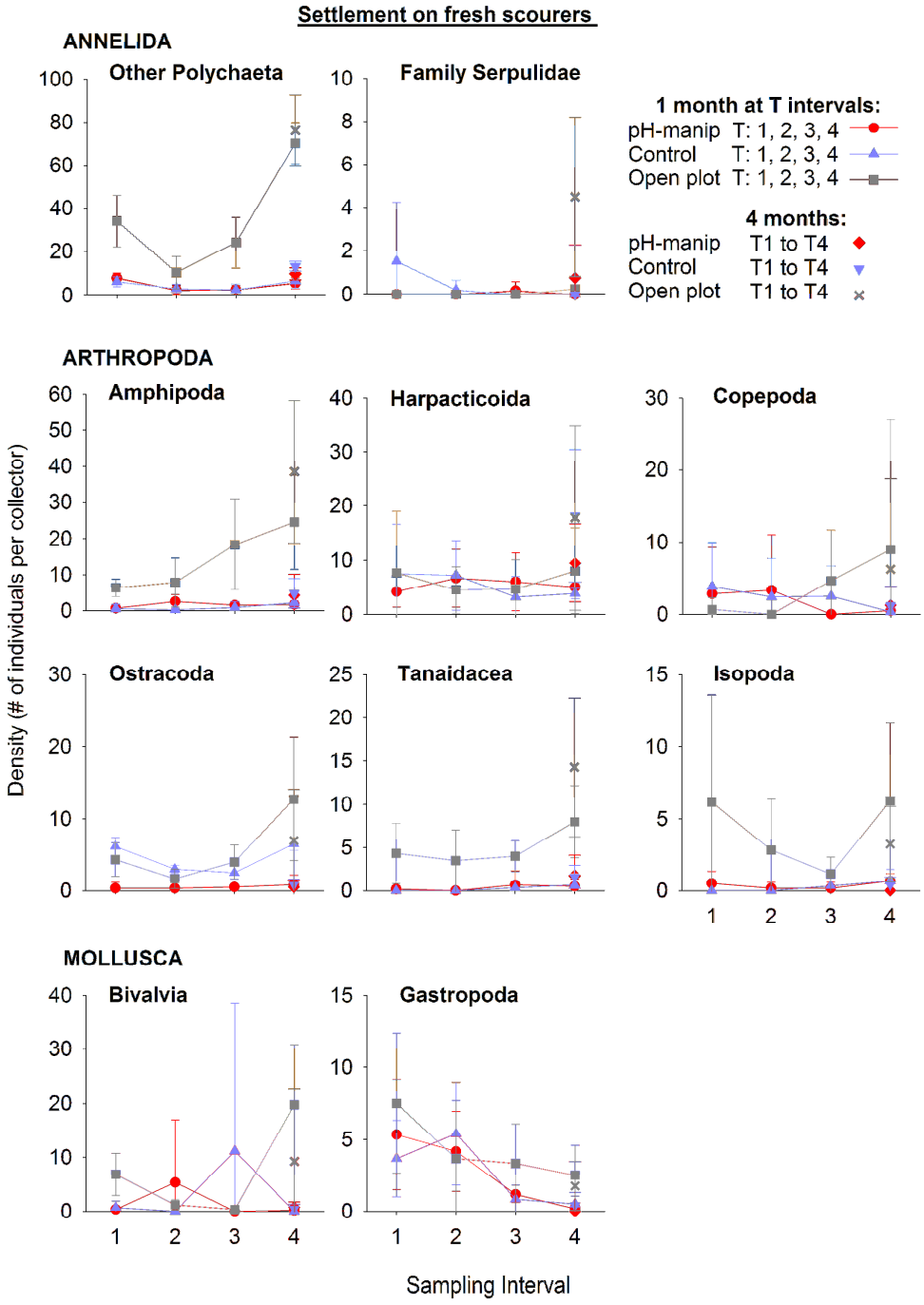
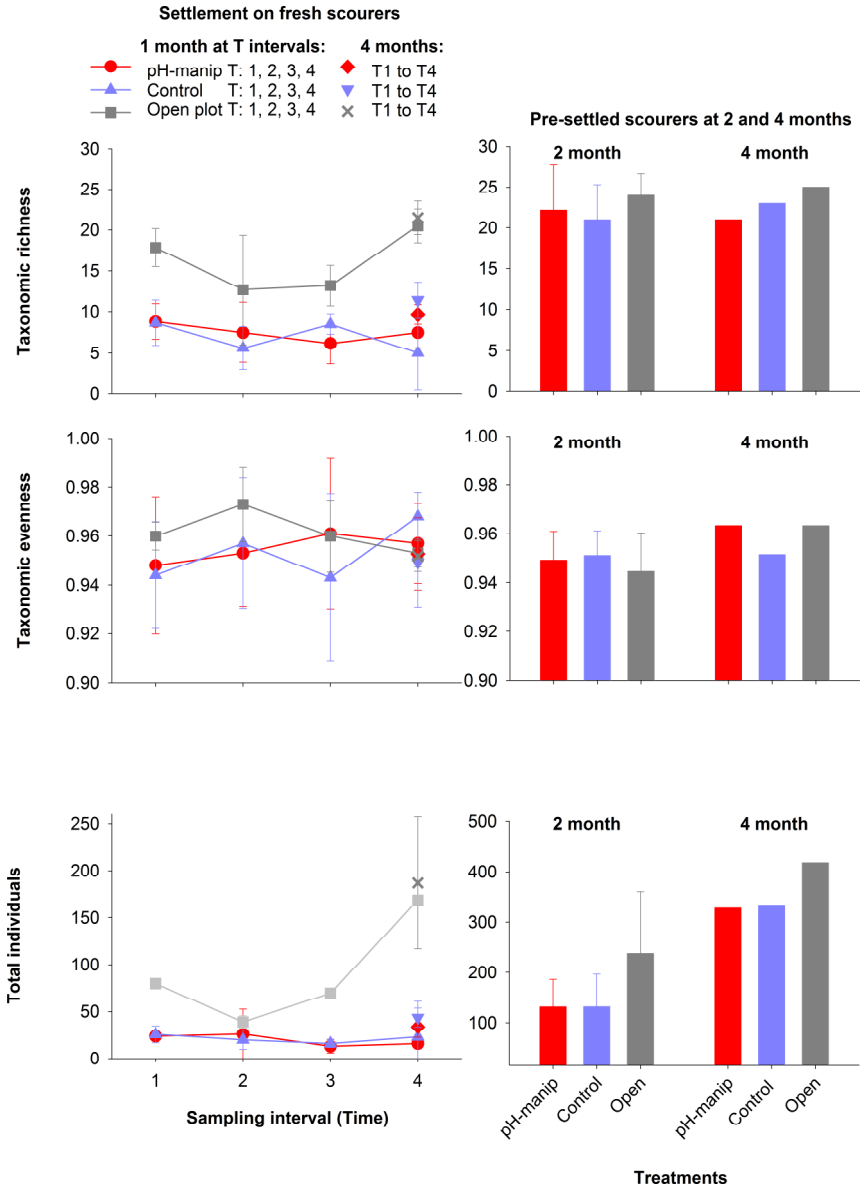


Fig. 4. Mean density (individuals per scourer, ± SD) of taxonomic groups on scourers collected and replaced at sampling intervals T1, T2, T3, T4 (symbols connected by line) and collected after four months (placed at T0 left until T4, symbols only) from the pH-manipulated , control enclosures, and open plot. Plots are arranged by phylum and overall decreasing densities.

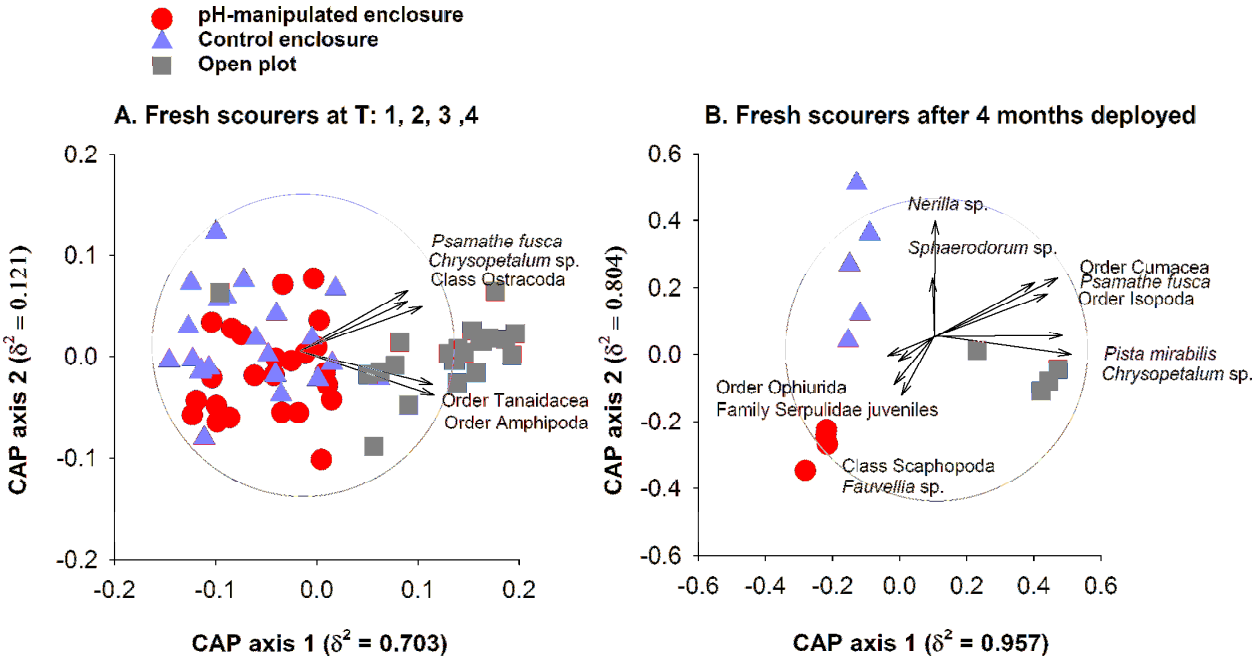
963



964

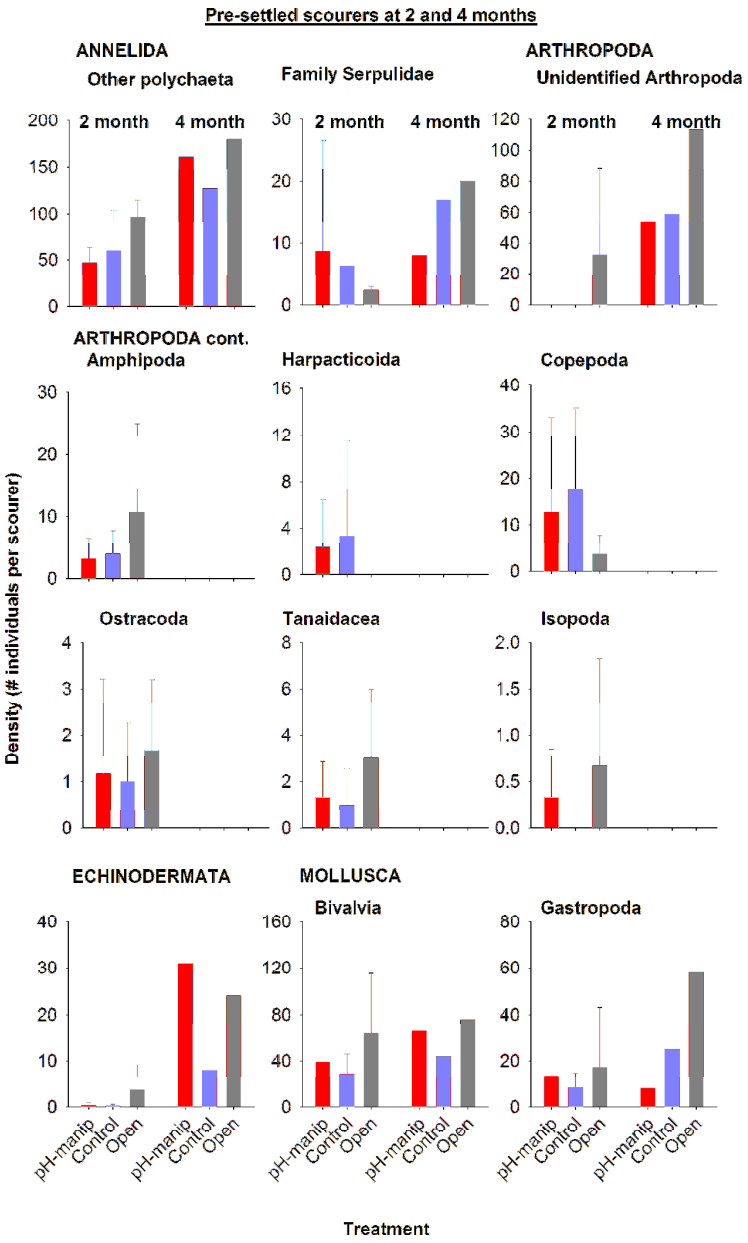
965 **Fig. 5.** Mean species richness, taxonomic evenness, and total number of individuals (± SD)
966 found within fresh scourers (left) and pre-settled scourers (right) deployed within the pH-
967 manipulated, control enclosures and open plot for one to four months. Fresh scourers were
968 collected and replaced monthly at intervals T1, T2, T3, and T4 and are represented by symbols
969 with lines while fresh scourers placed at T0 and collected at T4 are represented by symbols (no
970 line). Pre-settled scourers were collected after two and four months of exposure.

971



972

973 **Fig. 6.** Discrimination of assemblages in fresh scourers collected and replaced at sampling
974 intervals T2, T3, T4 (left) and collected after four months (placed at T0 left until T4, right) from
975 the pH-manipulated, control enclosures, and open plot. Canonical analysis of principal
976 coordinates (CAP) ordination plots based upon Bray-Curtis dissimilarities presented when a
977 PERMANOVA indicated statistical differences (pairwise open plot ≠ enclosures, P <0.05). The
978 plot shows the canonical axes that best discriminate assemblages within the three locations (pH-
979 manipulated and control enclosures and open plot). Overlaid on the plot are correlations of
980 original taxa variables with canonical axes, the circle aides in the visualization of the center of
981 the biplot and the arrow indicates direction and strength (as length) of the correlation. A cut-off
982 of > 70% in the biplot direction that separates open plot assemblage is shown and a cut off of >
983 35% in the biplot direction that separates enclosure assemblages is shown.



.

Fig. 7. Mean density (individuals per scourer, + SD when n > 1) of common taxonomic groups found in pre-settled scourers placed within the pH-manipulated, control enclosures and open plot for two and four months. Plots are arranged by phylum and overall decreasing abundances.

989

Table 1. Statistical results on abundance of operational taxonomic units (OTU) found on tile surfaces. Terms included treatment (pH-manipulated and control enclosure, and open plot) and sampling interval (T2, T3, T4) and their interactions. Pairwise results are found as letter groups on Fig. 1. Other taxa category includes cyanobacteria, brown colored algal crusts, and filamentous algae.

Terms tested	DF	MS	F	*P*
CCA coverage				
Treatment	2	1373.07	18.26	< 0.001
Sampling interval	2	738.15	9.82	< 0.001
Interaction	4	163.87	2.18	0.091
Other taxa coverage				
Treatment	2	0.34	4.00	0.027
Sampling interval	2	0.64	7.56	0.002
Interaction	4	0.09	1.02	0.413
Family Serpulidae				
Treatment	2	71.74	8.32	0.001
Sampling interval	2	10.95	1.27	0.293
Interaction	4	5.98	0.69	0.602

990

Table 2. Statistical results from comparisons of scourer assemblages (datasets #1-3). Terms tested included: treatment = pH-manipulated and control enclosures and the open plot and sampling interval = time (T) 1 to 4.

	Assemblage structure				Taxonomic richness			Taxonomic evenness			Total # of individuals		
Source	*Df*	MS	*F*	*P*	MS	F or H	*P*	MS	F or H	*P*	MS	F or H	*P*
Settlement within one month at T1-T4 (dataset #1)													
Treatment	2	14868.0	7.9	0.001	6864.2	37.11	< 0.001	0.0004	0.90	0.41	6152.6	30.59	< 0.001
Interval	3	4476.7	2.4	0.001	979.9	5.30	0.003	0.0004	0.81	0.50	588.3	2.93	0.04
Treatment x interval	6	2371.0	1.2	0.08	121.6	0.66	0.68	0.0005	0.99	0.44	406.5	2.02	0.08
Residuals	57	1875.7			185.0			0.0005			201.1		
Settlement within four months (dataset #2)													
Treatment	2	3845.7	2.9	0.002		8.54	0.005		2.79	0.26		8.63	0.005
Residuals	10	1336.3											
Pre-settled scourers collected at T2 (dataset #3)													
Treatment	2	1967.7	1.6	0.1		0.42	0.67	0.00004	0.30	0.74	13504.5	2.46	0.13
Residuals	12	1228.2						0.0001			5497.6		
Post-hoc pairwise comparisons													
	T	*P*			*Q*	*P*		*q*	*P*		*q*	*P*	
Settlement within one month at T1-T4 (dataset #1)													
pH manipulated, Control	1.1	0.2			0.86	0.82		N/A	N/A		0.22	0.9	
Open, Control	3.5	0.001			11.0	< 0.001		N/A	N/A		9.5	0.001	
Open, pH manipulated	3.5	0.001			10.3	< 0.001		N/A	N/A		9.8	0.001	
T1,T2	1.7	0.003			3.9	0.04		N/A	N/A		3.2	0.1	
T2,T3	0.9	0.4			0.5	0.99		N/A	N/A		0.5	0.9	
T3,T4	1.4	0.01			3.9	0.04		N/A	N/A		2.1	0.5	
T4,T1	1.5	0.01			0.4	0.99		N/A	N/A		1.5	0.7	
T1,T3	1.8	0.003			4.5	0.01		N/A	N/A		3.8	0.046	
T2,T4	1.7	0.001			3.4	0.09		N/A	N/A		1.6	0.7	
Settlement within four months (dataset #2)					*Q*	*P*					*Q*	*P*	
pH manipulated,	0.9	0.6			-0.7	0.24		N/A	N/A		-0.9	0.17	

Control								
Open, Control	2.1	0.01	2.2	0.01	N/A	N/A	2.0	0.02
Open, pH manipulated	2.0	0.02	2.8	0.003	N/A	N/A	2.9	0.002

Table 3. Results of leave-one-out allocation success from the canonical analysis of principal coordinates (CAP) done on fresh scourer assemblages. The analyses were done using m (see below) principal coordinate axes based on the Bray-Curtis dissimilarities on log10(X + 1) transformed data. The percent classified correctly to either pH-manipulated and control enclosures and open plot are presented for fresh scourer assemblages (dataset#1 and #2).

Settlement within one month at T1-T4 (dataset #1)

Sampling interval classification, m = 7

Classified	T1	T2	T3	T4	Total	% Correct
Original						
T1	13	2	1	2	18	72.2
T2	3	2	1	2	17	23.5
T3	2	4	6	6	18	33.3
T4	4	1	3	8	16	50.0
Total correct %	44.9					

Treatment classification, m = 5

Classified	pH-manipulated	Control	Open	Total	% Correct
Original					
pH-manipulated	17	7	0	24	70.8
Control	9	12	2	23	52.2
Open	0	1	21	22	95.5
Total correct %	72.5				

Settlement within four months (dataset #2)

Treatment classification, m = 6

Classified	pH-manipulated	Control	Open	Total	% Correct
Original					
pH-manipulated	2	2	0	4	50.0
Control	1	4	0	5	80.0
Open	0	0	4	4	100.0
Total correct %	76.9				

991