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1 **Effects of *in situ* CO₂ enrichment on epibiont settlement on artificial substrata within a**
2 ***Posidonia oceanica* meadow**

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22 **ABSTRACT**

23 Alterations to colonization or early post-settlement stages may cause the reorganization
24 of communities under future ocean acidification conditions. Yet, this hypothesis has been little
25 tested by *in situ* pH manipulation. A Free Ocean Carbon Dioxide Enrichment (FOCE) system
26 was used to lower pH by a ~ 0.3 unit offset within a partially enclosed portion (1.7 m³) of a
27 *Posidonia oceanica* seagrass meadow (11 m depth) between 21 June and 3 November 2014.
28 Epibiont colonization and early post settlement stages were assessed within the FOCE setup, as
29 part of the larger community-level study, to better understand the outcome for a multispecies
30 assemblage and the ecological processes that result in reported community shifts under altered
31 carbonate chemistry. Two types of artificial collectors (tiles and scourers) were placed within
32 three treatments: a pH-manipulated enclosure, an un-manipulated control enclosure, and an open
33 plot in the ambient meadow. Tiles and scourers were collected after one to four months.

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

53

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

56 **1. Introduction**

57 Because of the anthropogenic driven increase in atmospheric carbon dioxide (CO₂) and
58 its dissolution in the ocean (25.2% of emissions in the decade 2006-2015; Le Quéré et al., 2016),
59 the ocean is undergoing increased rate of change in carbonate chemistry. In the process of ocean
60 acidification, the pH in the ocean declines, resulting in the decline in the concentrations of
61 carbonate ions (CO₃²⁻) and the increase in the concentrations of bicarbonate ions (HCO₃⁻) and
62 dissolved carbon dioxide (CO₂). Surface ocean average pH has decreased by 0.1 units since the
63 pre-industrial era and an additional 0.07 to 0.33 units decrease is expected by 2100 (Gattuso et
64 al., 2015). These changes in ocean carbonate chemistry are predicted to affect marine organisms
65 (Kroeker et al., 2013) as well as populations and ecosystems (Gaylord et al., 2015).

66 Alterations in carbonate chemistry can have taxon-specific consequences on organism
67 growth, reproduction, and survival (see reviews, Gattuso and Hansson, 2011; Kroeker et al.,
68 2013). A decline in [CO₃²⁻] has been shown to differentially affect the ability of calcifying
69 organisms to precipitate calcium carbonate (CaCO₃) and has lead to subsequent increases in
70 skeletal dissolution rates (Feely, 2004). Marine autotrophs that lack calcification, in contrast,
71 may benefit from the increased CO₂ availability for photosynthesis (Koch et al., 2013). The
72 concern is that by affecting energy allocation and mortality rates, ocean acidification impacts on
73 the individual or population will also scale to shifts in the community (Kroeker et al., 2011).

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

80 ways (Kurihara, 2008). For example, mollusc larvae and juveniles are often found to have a
81 pronounced sensitivity to lowered pH (Gazeau et al., 2013). Lowered pH and CaCO₃ saturation
82 state have been shown to impact rates of hatching, metamorphosis, larval and juvenile growth,
83 and early survival from predation (Gazeau et al., 2010; Gazeau et al., 2013; Kurihara, 2008;
84 Talmage et al., 2009). Furthermore, the sensitivity of echinoderms and molluscs in their earlier
85 stages may be greater than other taxa (Kroeker et al., 2013). However, effects on early life-
86 history stages in mixed populations are poorly understood because of the difficulty of culturing
87 and rearing larvae.

88 Seagrass meadows are a model benthic system to examine effects on multispecies
89 assemblages and ecological processes. They are composed of diverse taxa and are highly valued
90 for the ecosystem services they provide (Hemminga and Duarte, 2000). For example, seagrass
91 meadows play a fundamental role in maintaining populations of exploited fisheries (Jackson et
92 al., 2015) and are global contributors to carbon sinks, with a net primary production (NPP) of
93 490 Tg C yr⁻¹ (Mateo et al., 2006).

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

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

110 Observations and conclusions drawn from naturally acidified ecosystems (volcanic CO₂
111 vents, spring inputs, and upwelling areas) predict changes in benthic community composition
112 and abundances under ocean acidification (e.g. Hall-Spencer et al., 2008) although such shifts
113 have been attributed to a variety of ecological processes. Most observations examining
114 community assembly or abundances on artificial substrata support a reorganization of
115 communities as a direct result of taxa sensitivity, tolerance, or benefit (Allen et al., 2016;
116 Cigliano et. al., 2010; Crook et al., 2016; Donnarumma et al., 2014). A few studies indicate shifts
117 in benthic abundances related to indirect effects of lowered pH, such as loss of habitat
118 complexity or changes in competitors and prey (Garrard et al., 2014; Kroeker et al., 2012). In
119 one study, species interactions in later successional stages amplified direct effects (Kroeker et al.,
120 2012) to result in an altered community assembly at lowered pH from ambient. While these
121 observations at naturally acidified ecosystems capture diverse species interactions and
122 acclimation, they are not perfect predictors of future ocean ecology. Conclusions are often
123 hindered by large temporal pH variability (Kerrison et al., 2011) and outcomes can also be
124 confounded by other environmental conditions that vary across the pH gradient. Therefore,
125 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 m³ (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 (\pm SD) pH_T of 7.75 \pm
178 0.13 and an increase in *p*CO₂ to 971 \pm 323 μ atm. 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 m²) 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 CO₂ 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 \pm SD, pH-manipulated enclosure, $n = 12$, $2545.5 \pm 8.0 \mu\text{mol kg}^{-1}$; control enclosure, $n = 11$,
204 $2541.7 \pm 12.2 \mu\text{mol kg}^{-1}$; open plot (or ambient), $2556 \mu\text{mol kg}^{-1}$, 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-

217 and during acidification period (median \pm median absolute deviation 0.14 ± 0.06 and $0.14 \pm$
218 0.06).

219 During the acidification period, the pH in the pH-manipulated enclosure was maintained
220 at a mean -0.26 unit offset (monthly mean values ranged from -0.22 to -0.29 pH units) from the
221 control enclosure (Table S1). In the pH-manipulated enclosure, the monthly mean values of
222 saturation state with respect to aragonite (Ω_a) ranged from as low as $2.0 (\pm 0.05 \text{ SD})$ in October
223 to a high of $2.5 (\pm 0.06 \text{ SD})$ in August and saturation state with respect to calcite (Ω_c) ranged
224 from $3.0 (\pm 0.07 \text{ to } 0.008 \text{ SD})$ in September and October to $3.8 (\pm 0.09 \text{ SD})$ in August. The
225 median diel pH range in the pH-manipulated enclosure was two to three times larger than the
226 control (monthly median ranged from 0.09 to 0.29 pH units).

227 Monthly differences were evident, particularly for temperature (mean monthly range:
228 17.7 to 24.2 °C) and PAR (mean monthly range: 1.3 to 7.3 mol photons $\text{m}^{-2} \text{d}^{-1}$, Table S2) but
229 these variables were similar in the ambient, control and pH-manipulated enclosures.

230 *2.3. eFOCE timeline*

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

237 *2.4. Artificial collectors*

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

240 adult or early life history stages of benthic organisms (e.g. Cigliano et al., 2010; Kroeker et al.,
241 2012).

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

250 2.5. Scourer pre-settlement and calcein staining for growth measurements

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

256 Thirteen other scourers were placed in a similar manner within a 5 m² area on 6 February
257 2014 and collected after ~4 months (19 June 2014). These settled scourers were transported to
258 the Laboratoire d'Océanographie de Villefranche, held in circulated seawater from the bay for 72
259 h under ca. 150 $\mu\text{mol photons m}^2 \text{s}^{-1}$ (13:11 h light:dark cycle) and maintained at seawater
260 temperature conditions (22-23 °C). After 24 h, the free-flowing addition of Bay water to the
261 holding container was stopped, calcein (50 mg L⁻¹) was added, and organisms were fed *ad*
262 *libitum* with the microalga *Isochrysis galbana* Parke. The staining lasted for 48 h then scourers

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

265 *2.6. Collector placement, collection intervals, and study design*

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

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

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

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

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

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

293 *2.7. Organism identification and quantification*

294 The 15 settlement tiles were kept in a temperature controlled (20 – 22 °C) dark room for
295 less than 24 h until scanning could be completed. A ZooScan (Hydroptic, France; Gorsky et al.,
296 2010) was used to produce high resolution colour images (2400 dpi) of settled tiles. There were
297 no canopy-forming macroalgae or large sessile macrofauna on tiles, thus “layering” did not occur
298 and images could be directly analyzed.

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

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

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

317 2.8. *Growth rates*

318 The bivalve *Lyonsia* sp. (Family Lionsiidae) within pre-settled, stained scourers was the
319 only organism where a calcein mark was visible. Individuals were carefully placed in a
320 horizontal position (hinge horizontal), in visible proximity to a scale bar, and photographed
321 directly from above, through the binocular microscope. This resulted in 15, 5, and 8 imaged
322 individual *Lysonia* sp. from 5, 2, and 3 scourers collected from the pH-manipulated enclosure,
323 the control enclosure and the open plot, respectively. Images were analyzed using ImageJ. The
324 complete bivalve shell and new growth were outlined by tracing the perimeter and their surface
325 areas were estimated with the measure tool. Growth (area in mm²) was provided as a proportion
326 of new growth to total size (as area) and expressed as a rate (per day).

327 2.9. *Statistical analyses*

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

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

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

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

350 A multivariate approach following the recommendations outlined in Anderson and Willis
351 (2003) was taken to analyze scourer assemblages. A $\log_{10}(X+1)$ transformation was applied and
352 the Bray-Curtis Index was used to create a resemblance matrix. A two-way permutational-
353 MANOVA (PERMANOVA) was run on each matrix with 999 permutations and type III sum of

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

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

366 **3. Results**

367 *3.1. Settlement on tiles*

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

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

382 There was a significantly greater coverage of “Other taxa” on tiles from the open plot and
383 control enclosure than on tiles from the pH-manipulated enclosure (Table 1, Fig. 3B).
384 Differences were largely driven by the lower coverage observed at T2 on pH-manipulated tiles
385 (0.23 vs 0.54 to 1.10 cm^2). For all treatments, the coverage of “Other taxa” increased from
386 September (T2) to October (T3) and declined again in November (T4).

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

392 *3.2. Overall description of scourer assemblages*

393 Out of a combined 106 scourers, 7,220 individuals from eight phyla were found and
394 identified to 106 different OTUs. At least 77 families were identified with 33 identifications
395 (31%) performed at the level of genus or species. The most abundant group of organisms were
396 annelids (2,891 individuals) closely followed by arthropods (2,646 individuals), then by molluscs
397 (1,569 individuals), echinoderms (28 individuals), cnidarians (6 individuals), ascidians (6
398 individuals), nematodes (5 individuals), nemertean (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 \pm 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

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

437 Many taxa were common in all treatments (Tables S3, S4). The SIMPER routine was
438 performed on the dataset from monthly collected scourers. It indicated dissimilarities between
439 enclosures and the open plot assemblages (80-82 % dissimilar) were based largely (85-86 % of
440 cumulative contribution) upon the density of the shared common species and less upon unique
441 species to enclosures or open plots. Nevertheless, out of 83 taxa found in monthly collected
442 scourers, 18 were exclusively found in enclosures (total individuals per OTU < 7, combined) and
443 11 were exclusively found in the open plot (total individuals per OTU < 2, except 19 individuals
444 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 enclosure 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

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

470 3.3.4. Enclosure comparison - settlement within four months

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

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

496 3.4. Pre-settled scourers

497 The pre-settled scourers had 26 to 44 different taxa and 73 to 461 total individuals per
498 scourer. The total number of individuals tended to be greater within scourers settled and
499 deployed for longer duration. For example, the pre-settled scourers collected at T4 had between
500 366 to 461 (mean \pm SD, 400 \pm 92) individuals per scourer while the pre-settled scourers
501 collected at T2 had between 73 to 414 (mean \pm SD, 190 \pm 85) individuals per scourer. In addition
502 to polychaetes and arthropods the calcifiers *Spirobanchus* sp. and molluscs (*Lyonsia* sp.,
503 Gastropoda juveniles, class Bivalvia, family Trochidae, and *Musculus costulatus*) tended to be
504 abundant (Table S5, S6).

505 There was no indication of an enclosure or pH effect on pre-settled scourers collected
506 after two months of exposure (Fig. 7). Pre-settled assemblages did not statistically differ among
507 treatments for all considered parameters (Table 2). The most numerous OTUs in the pre-settled
508 scourers at T2 were the bivalve *Lyonsia* sp., the polychaetes *Psamathe fusca*, *Sphaerosyllis* sp.,
509 and Copepoda (an arthropod; Table S5). Calcareous members of the assemblage (calcareous
510 tube-forming polychaetes, gastropods, and bivalves) that were predicted to decline with lowered
511 pH, actually tended to be more numerous in the pH-manipulated enclosure than in the control
512 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 mm²). 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

536 al., 2014; Jokiel et al., 2008; Kuffner et al., 2008) at a $\text{pH}_T < 7.8$. Although the spores and larvae
537 lack calcified structures, settled CCA and tubes of *Spirorbis* are susceptible to dissolution
538 because they are composed of calcite and/or aragonite (Ippolitov & Rzhavsky, 2015) or
539 magnesian calcite; 11.3 to 11.7 mol% magnesium in carbonate (MgCO_3) for CCA (Cox et al., in
540 press) and calcite with ~15% MgCO_3 reported for spirorbid tubes (Bornhold and Milliman,
541 1973). Several studies have shown dissolution, reduced calcification, or a loss of coverage for
542 CCA under lowered pH (reviewed by Hofmann and Bischof, 2014; Koch et al., 2013; McCoy
543 and Kamenos, 2015; Nelson, 2009).

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

554 The *in situ* control of pH (present study) could explain any contrasting conclusions drawn
555 from CO_2 vent observations which are relied upon extensively to predict future ocean ecology.
556 For example, meadow ecology has been studied along CO_2 vents in *Posidonia* meadows in
557 Ischia, Italy where the pH_T varies in a gradient from 8.1 to 6.6 nearest the CO_2 source (e.g. Hall-
558 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 $\text{pH}_T \sim 6.7$); 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 CO₂ 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

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

608 Other studies focused on invertebrate adult stages (Kroeker et al., 2011) or seasonal
609 influence (over similar months as the present study, Garrard et al., 2014) at the Ischia CO₂ vents
610 mirror the observations of Cigliano et al. (2010). However, these authors attribute increases in
611 particular arthropods or polychaetes to indirect effects, such as release of invertebrate predators
612 or interactions with macrophytes. Predation has long been thought to be a structuring factor of
613 invertebrate assemblages in seagrass meadows (Edgar, 1990; Heck and Thoman, 1981; Heck and
614 Orth, 2006). In *P. oceanica* meadows, Gambi et al. (1998) attributed seasonal differences in
615 polychaetes to differences in fish predation and Stuardo et al. (2016) found total density and
616 biomass of amphipods to be greater in ambient than within fish inclusion plots. In the present
617 study, indirect effects from fishes cannot be addressed because they were excluded from
618 enclosures. Furthermore, ocean acidification effects on fish predatory behavior have largely been
619 ignored. However, predator foraging ability, as well as invertebrate density, are often related to
620 seagrass density (Heck and Thoman, 1981; Orth et al., 1984). Elevated seagrass density and
621 canopy structure has been suggested to facilitate habitat heterogeneity and lower predation
622 ability (Garrard et al., 2014; Orth et al., 1984). In the present study, seagrass density was similar
623 between enclosures and *Posidonia* leaf biometrics and photosynthesis unaffected by lowered pH
624 (Cox et al., 2016).

625 Results from the present study indicate that gastropods and bivalves can settle under
626 moderate ocean acidification scenarios. These groups also occurred at similar mean pH at the
627 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

651 elevated $p\text{CO}_2$ which are dependent upon whether the producer density or complexity is
652 increased, replaced, or reduced. However, at low pH near volcanic CO_2 vents in the
653 Mediterranean Sea, elevated seagrass density was not correlated with increases in local diversity
654 (Sunday et al., 2016). The results from the present study do not support changes in local diversity
655 via indirect effects with changes in the habitat complexity. Seagrass physiology and abundance
656 as well as algal abundance were not affected by the pH manipulation in eFOCE (Cox et al.,
657 2016). The reduction of calcifiers on tiles, however, would be congruent with a loss of local
658 diversity through direct effects. Yet, the only decline in taxonomic richness associated with the
659 lowered pH treatment was in the epibont assemblage found within the unreplicated, pre-settled
660 scourers left in lowered pH for the duration of study.

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

671 *4.3. Caveats*

672 Large-scale unreplicated experiments, such as eFOCE, because of their unique qualities
673 (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

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

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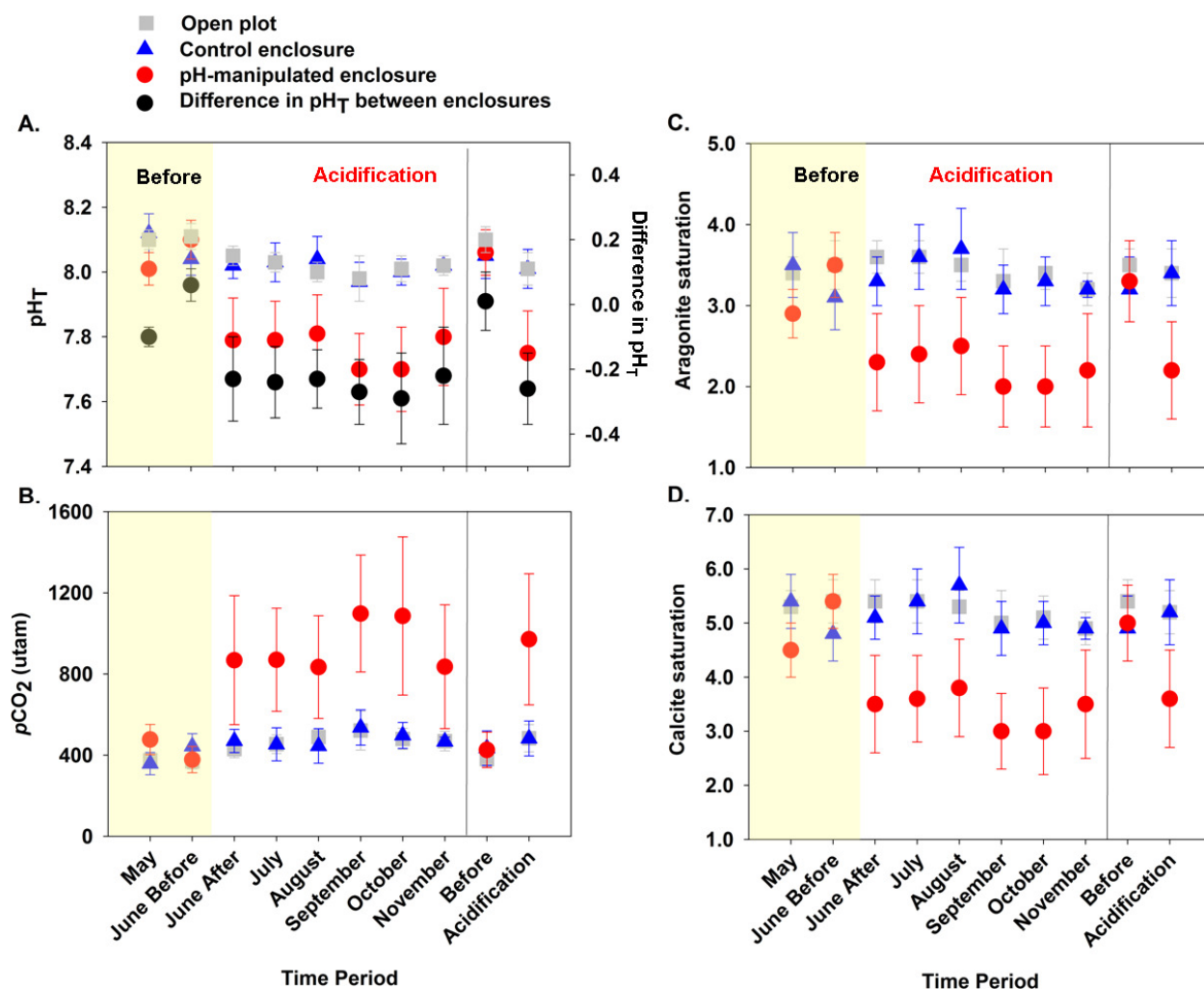
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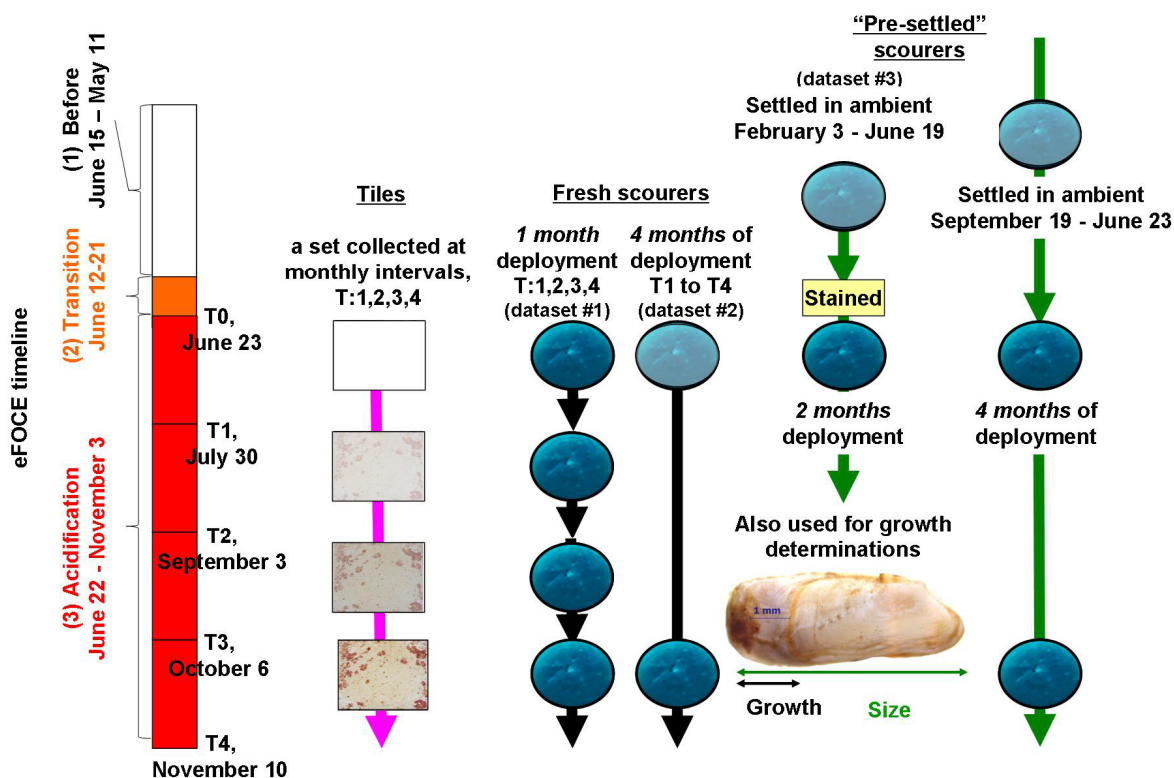
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929 **Figures**

930

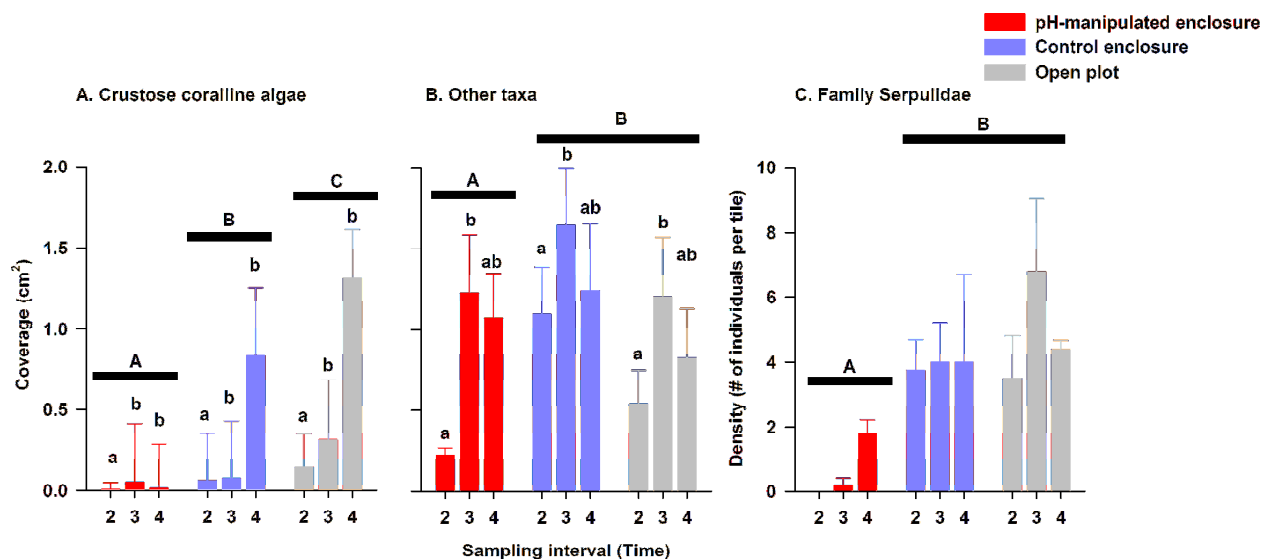
931 **Fig. 1.** Carbonate chemistry within ambient (open plot) and enclosures: averaged (\pm standard
 932 deviation, SD): Panel A, pH (on the total scale; pH_T), Panel B, partial pressure of carbon dioxide
 933 (pCO₂) and Panel C and D, saturation states with respect to aragonite (Ω_a) and calcite (Ω_c) for
 934 each month and for the period before and during acidification. The difference in pH_T between the
 935 pH-manipulated and the control enclosure is also shown in Panel A.



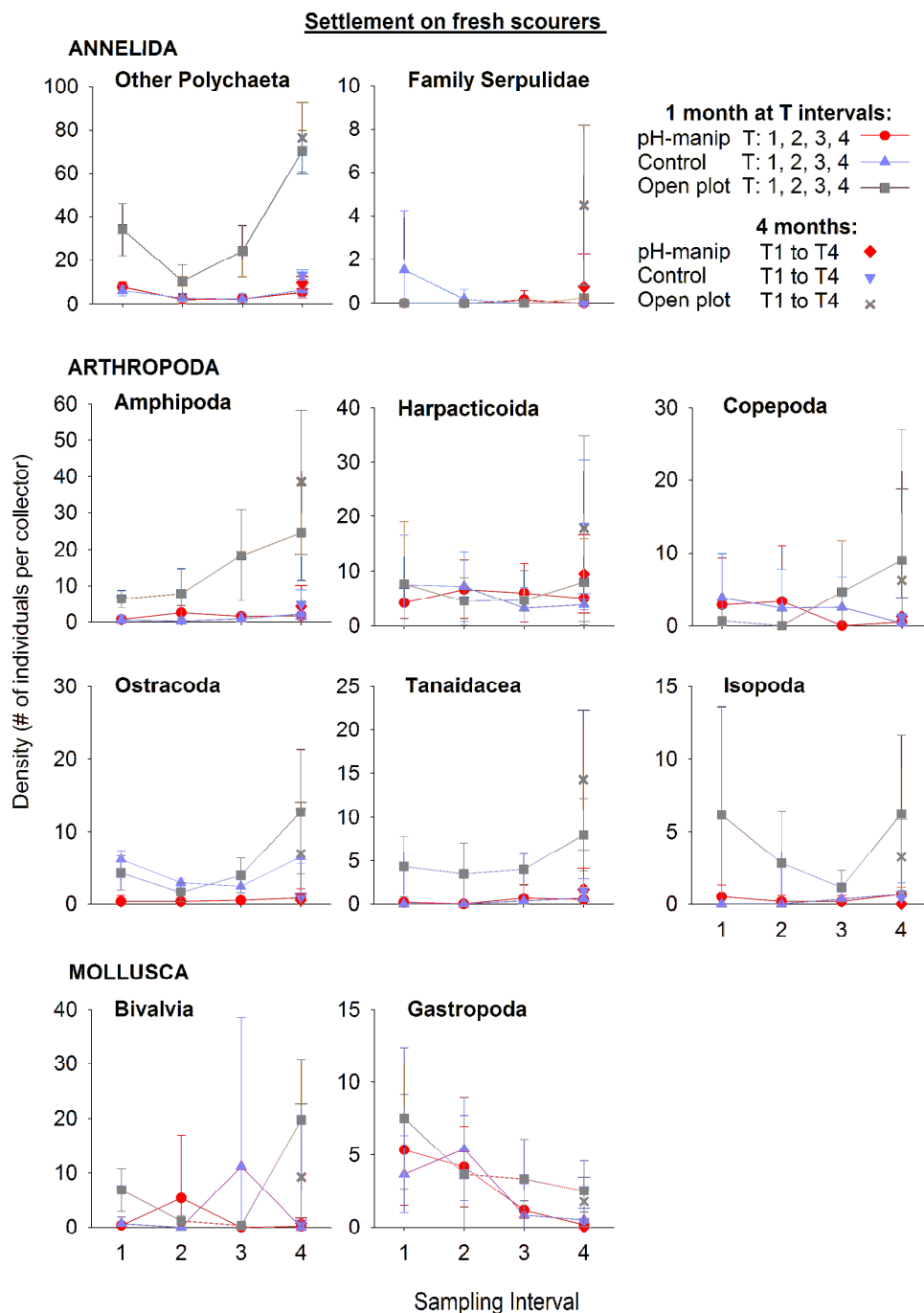
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937 **Fig. 2.** Artificial collectors used and collection intervals. Tiles were placed in the three eFOCE
 938 treatments (control and pH-manipulated enclosures and open plot) at the start of the acidification
 939 period (T0) and a set of three to 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.



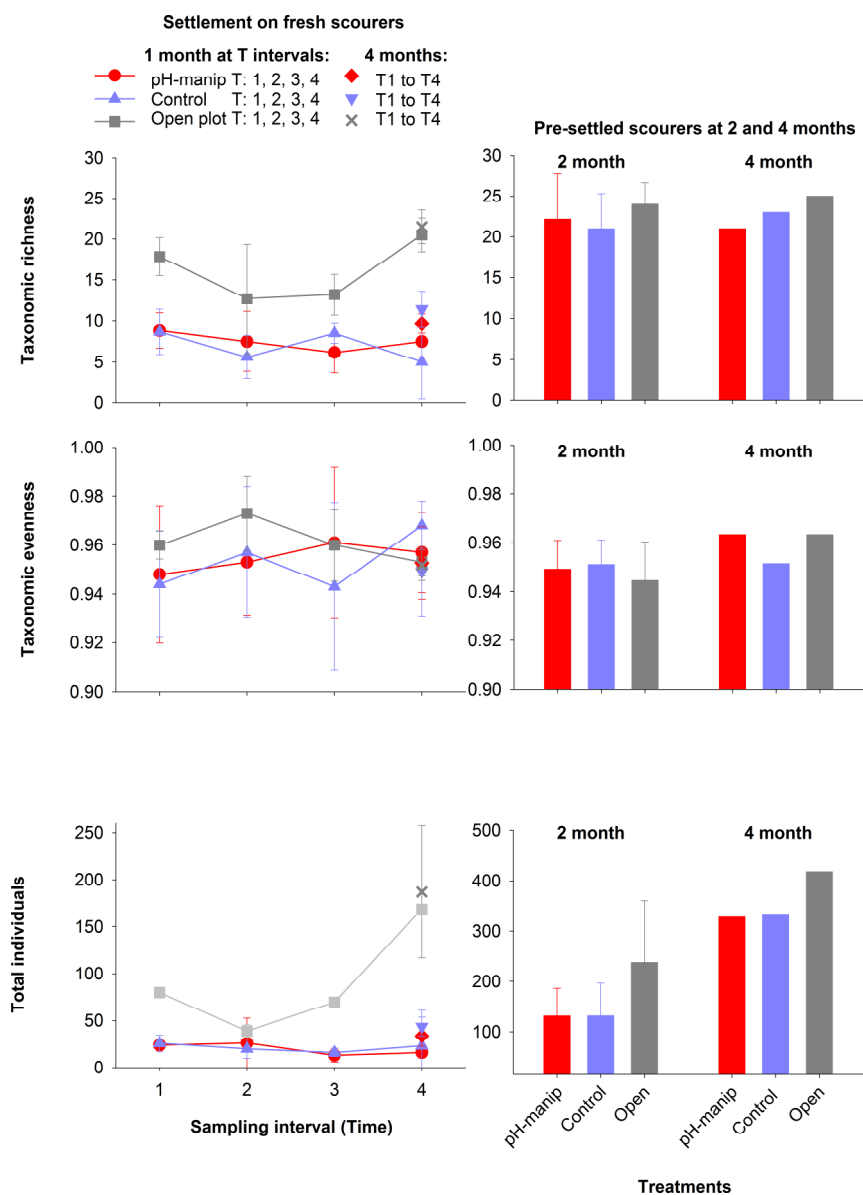
951
 952 **Fig. 3.** Mean abundance (+ SD) of operational taxonomic units (OTU) found on tiles placed
 953 initially during the pH manipulation and collected ($n = 5$) every four weeks (sampling intervals
 954 T2 to T4) from the pH-manipulated and control enclosure and the open plot. Letter groups and
 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.



958

959 **Fig. 4.** Mean density (individuals per scourer, \pm SD) of taxonomic groups on scourers collected
 960 and replaced at sampling intervals T1, T2, T3, T4 (symbols connected by line) and collected
 961 after four months (placed at T0 left until T4, symbols only) from the pH-manipulated, control
 962 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 (\pm SD)

966 found within fresh scourers (left) and pre-settled scourers (right) deployed within the pH-

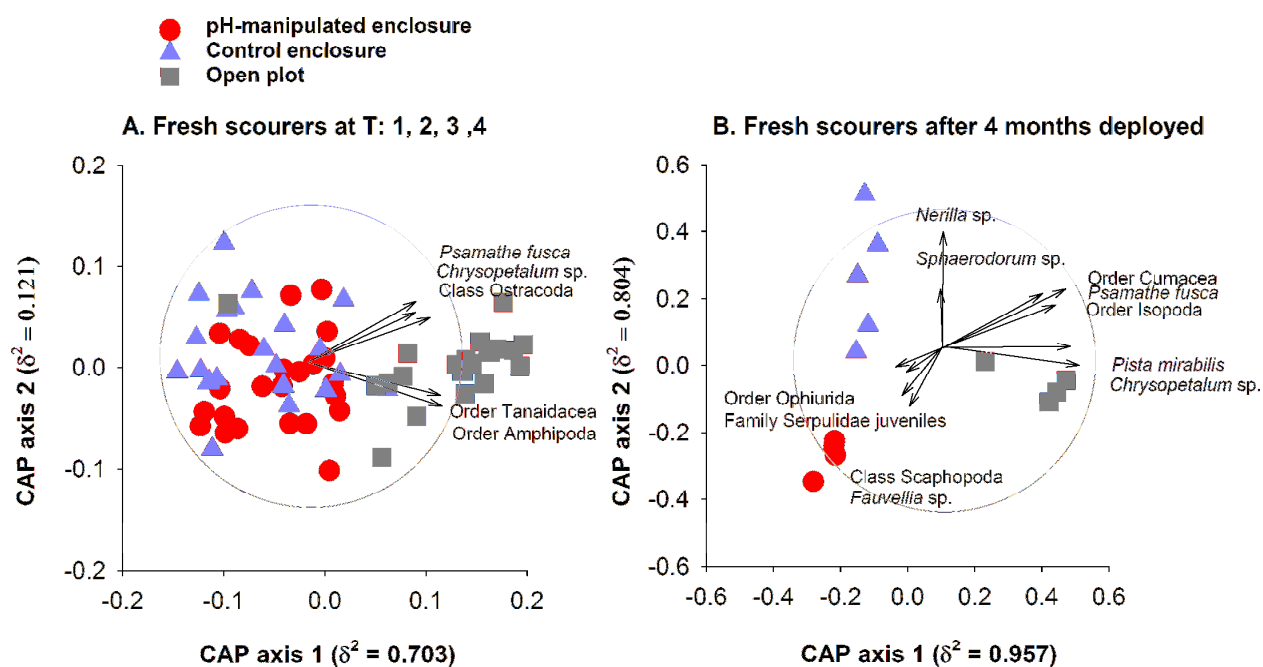
967 manipulated, control enclosures and open plot for one to four months. Fresh scourers were

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

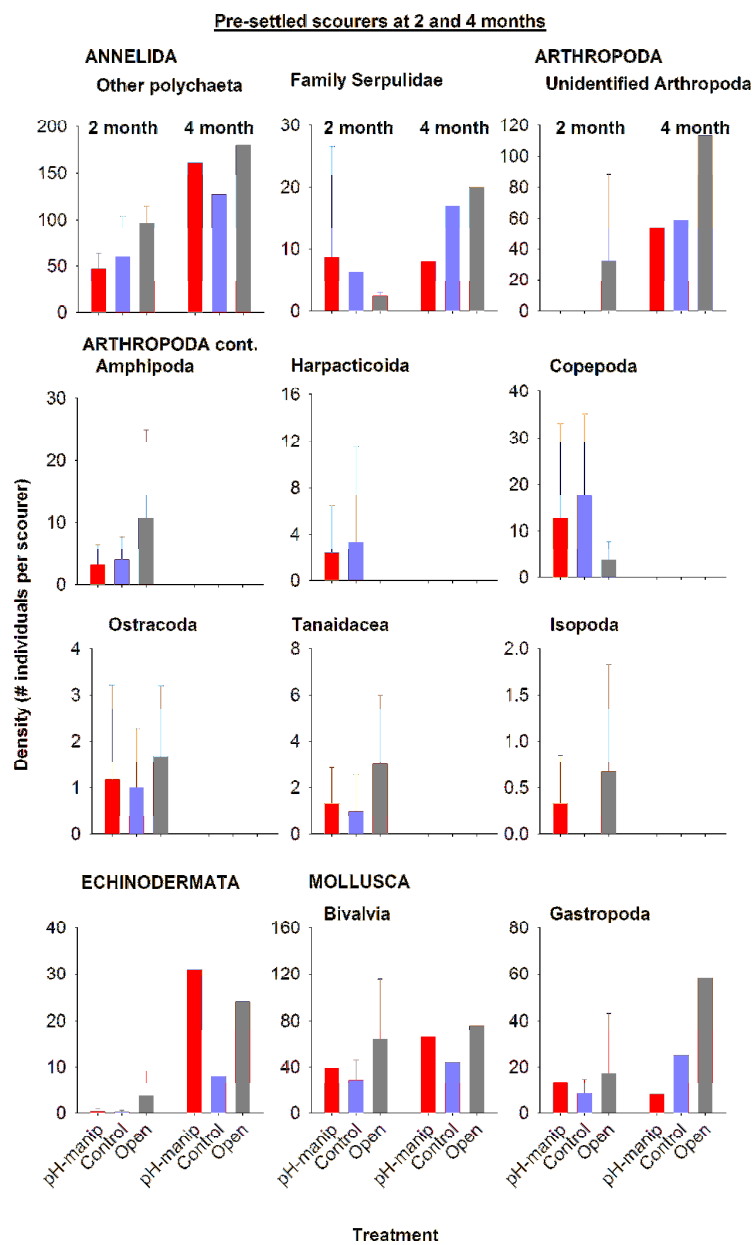
970 line). Pre-settled scourers were collected after two and four months of exposure.

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 \neq 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.



984

985

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

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

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.

Source	Assemblage structure				Taxonomic richness			Taxonomic evenness			Total # of individuals		
	<i>Df</i>	MS	<i>F</i>	<i>P</i>	MS	F or H	<i>P</i>	MS	F or H	<i>P</i>	MS	F or H	<i>P</i>
<u>Settlement within one month at T1-T4 (dataset #1)</u>													
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		
<u>Settlement within four months (dataset #2)</u>													
Treatment	2	3845.7	2.9	0.002		8.54	0.005		2.79	0.26		8.63	0.005
Residuals	10	1336.3											
<u>Pre-settled scourers collected at T2 (dataset #3)</u>													
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													
	<i>T</i>	<i>P</i>			<i>Q</i>	<i>P</i>		<i>q</i>	<i>P</i>		<i>q</i>	<i>P</i>	
<u>Settlement within one month at T1-T4 (dataset #1)</u>													
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	
<u>Settlement within four months (dataset #2)</u>													
pH manipulated,	0.9	0.6			<i>Q</i>	<i>P</i>					<i>Q</i>	<i>P</i>	
					-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 $\log_{10}(X + 1)$ 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, $m = 7$				Treatment classification, $m = 5$				
Classified	T1	T2	T3	T4	Total	% Correct	% Correct	
Original								
T1	13	2	1	2	18	72.2	Original	
T2	3	2	1	2	17	23.5	pH-manipulated	
T3	2	4	6	6	18	33.3	Control	
T4	4	1	3	8	16	50.0	Open	
Total correct %	44.9						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				