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Effects of *in situ* CO_2 enrichment on structural characteristics, photosynthesis, and growth of the Mediterranean seagrass *Posidonia oceanica*

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Running head: *In situ* ocean acidification effects on *P. oceanica*

Abstract

Seagrass are expected to benefit from increased carbon availability under future ocean acidification. This hypothesis has been little tested by *in situ* manipulation. To test for ocean acidification effects on seagrass meadows under controlled CO_2/pH conditions, we used a Free Ocean Carbon Dioxide Enrichment (FOCE) system which allows for the precise manipulation of pH as an offset from the ambient. This system was deployed in a *Posidonia* oceanica meadow at 11 m depth in the Northwestern Mediterranean Sea. It consisted of two benthic enclosures, an experimental and a control unit both 1.7 m^3, and an additional reference plot in the ambient (2 m^2) to account for structural artifacts. The meadow was monitored from April to November 2014. The pH of the experimental enclosure was lowered by 0.26 pH units for the second half of the eight-month study. Changes in *P. oceanica* leaf biometrics, photosynthesis, and leaf growth accompanied seasonal changes recorded in the environment and values were similar between the two enclosures. Leaf thickness may change in response to lower pH but this requires further

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testing. Results suggest any benefit from ocean acidification, over the next century, on *Posidonia* physiology and growth may be minimal. The limited stimulation casts doubts on speculations that elevated CO_2 would confer resistance to thermal stress and increase buffering capacity of meadows.

Keywords: buffering capacity, leaf biometrics, meadows, ocean acidification, oxygen fluxes, PAM fluorescence, pH

1 Introduction

Ocean carbonate chemistry is being altered in ways that may affect future ocean ecology. The ocean absorbs carbon dioxide (CO_2) from the atmosphere which increases the concentrations of inorganic carbon and CO_2, and decreases pH in a process referred to as ocean acidification. Surface ocean pH has decreased by 0.1 units since the beginning of the industrial era and a further decline (0.06 to 0.32 units) is projected over the next century (Ciais et al., 2013). Through this process, the relative proportions of dissolved inorganic carbon species are concurrently being altered. By 2100, bicarbonate (HCO_3^-), already widely available, will increase along with CO_2, which will have the largest proportional increase from current day levels. An increase in carbon availability may benefit some marine producers (Koch et al., 2013). In contrast, the concentration of carbonate ions (CO_3^{2-}) needed by calcifying organisms will decrease. Thus, ocean acidification can alter competitive interactions which may cascade to alterations at the ecosystem level.

Seagrass meadows rank as one of the most productive ecosystems on Earth (Duarte et al., 2010; Duarte and Chiscano, 1999). They are highly valued for their ability to improve water quality, stabilize sediment, and provide habitat for a diversity of organisms. Human-driven changes to the seawater clarity and quality (e.g. eutrophication, ocean warming) are often related

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to meadow decline (Jordà et al., 2012; Waycott et al., 2009). However, these habitat-forming seagrasses are thought to benefit from ocean acidification because they are able to use both CO_2 and HCO_3^- for photosynthesis but, with a higher affinity for CO_2 and are often found to be carbon-limited (Invers et al., 2001; Koch et al., 2013).

Experiments under elevated CO_2 have shown an increase in seagrass photosynthesis (Apostolaki et al., 2010; Invers et al., 1997; Jiang et al., 2010; Ow et al., 2015; Zimmerman et al., 1997), below ground growth (Hall-Spencer et al., 2008; Zimmerman et al., 1997) and flowering frequency (Palacios and Zimmerman, 2007). Yet the majority of these studies were conducted in the laboratory over relatively short durations with single taxa or small groups of taxa isolated from their surroundings. Although studies along carbon dioxide vents allow for a whole ecosystem approach, the high spatial and temporal variability in CO_2 levels prevent the determination of a reliable dose-response relationship (Hall-Spencer et al., 2008; Kerrison et al., 2011). To the best of our knowledge, only Campbell and Fourqurean (2011, 2013a, 2014) have manipulated partial pressure of carbon dioxide (pCO_2) levels in a controlled manner *in situ* within a *Thalassia* meadow to test the response of seagrass to ocean acidification. After 6 months of exposure to lowered pH (-0.3 from mean ambient), the seagrass had increased non-structural carbohydrate content by 29% in below ground structures (Campbell and Fourqurean 2014). This finding generally supports the hypothesis that plant production will be stimulated from the increased carbon availability.

Posidonia oceanica is the foundation species for mono-specific meadows in the Mediterranean Sea where it covers up to 23% of shallow waters (0-50 m; Pasqualini et al., 1998) and provide services valued at 172 € m^{-2} $year^{-1}$ (Vassallo et al., 2013). These plants are largely dependent upon abiotic factors as evident by its seasonal growth and physiology (Alcoverro et al., 1995, 1998; Bay, 1984; Duarte, 1989). They have been studied under a range of pH in the

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laboratory as well as along pH gradients near CO_2 vents (Invers et al. 1997, 2001, 2002; Hall-Spencer et al. 2008; Cox et al. 2015). Around natural CO_2 vents in Ischia (Italy), *P. oceanica* biomass was greatest at the station nearest the CO_2 source with a mean pH_T of 7.6 and minimum of 6.98 (Hall-Spencer et al., 2008). Indeed, *P. oceanica* has a C3 photosynthetic pathway that is hypothesized to benefit from increased carbon availability and its photosynthesis is not saturated with respect to dissolved inorganic carbon at natural concentrations in seawater (Invers et al. 1997, 2001). This is evident by their enhanced productivity in the laboratory under a pH range from 9.0 to 7.9 and has been attributed to a less efficient use of widely available HCO_3^- and their reliance on CO_2 for about 50% of carbon for photosynthesis (Invers et al. 1997, 2001). External carbonic anhydrase acts to dehydrate HCO_3^- to CO_2 which enters the cell by a diffusive process (Invers et. al 2001). Thus CO_2 limitation depends upon the thickness of the boundary layer and can also occur at high pH with slow diffusion rates (Invers et al., 2001). However, the extent of the stimulation at pCO_2 levels projected for the coming decades appears limited (Cox et al., 2015; Invers et al., 2002). In addition, the environment and species dynamics in meadows are complex and interactions can alter outcomes. For example, the leaves and roots are colonized by small invertebrates and epiphytic algae (Borowitzka et al., 2006). These associated species, many sensitive to dissolution, compete with the plants for resources (Cebrián et al., 1999; Martin et al., 2008; Sand-Jensen et al., 1985). A laboratory investigation of this potential interaction under two elevated pCO_2 levels (on the total scale, pH_T 7.7 and 7.3) was performed (Cox et al., 2015). Despite loss of calcified photosynthetic epiphytes at pH_T 7.7, the effect on shoot productivity was limited and seagrass photosynthesis (without epiphytes) was only stimulated at pH_T of 7.3, a value unlikely to occur in the Mediterranean Sea in the next century (Cox et al., 2015). The long-lived plants, however, were maintained for a relatively short duration of six weeks and only under

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104 the irradiance, temperature, and nutrient conditions of February to March. From these studies it is
105 difficult to predict the impact of ocean acidification on *P. oceanica*.

106 Any alteration in *P. oceanica* productivity or abundance will likely have repercussions to
107 meadow function. Therefore the aim of the present study was to test the hypothesis that
108 Mediterranean seagrass, *P. oceanica,* will benefit from ocean acidification. We tested this
109 hypothesis *in situ* with a Free Ocean Carbon Dioxide Enrichment (FOCE) system (see Gattuso et
110 al., 2014) which consisted of two partially-open enclosures that were deployed in the Bay of
111 Villefranche (France) for eight months (April-November 2014). The pH was manipulated
112 continuously, in one enclosure, at a -0.26 pH unit offset from ambient between June and
113 November. Before and during pH manipulation, macrophyte abundance, *Posidonia* leaf
114 biometrics, photosynthesis, and growth were measured and environmental conditions were
115 monitored.

116 **2 Method**

117 **2.1 Experimental setup and system function**

118 This study used the European FOCE (eFOCE) system, an autonomous system which
119 allows for the *in situ* manipulation of pH in benthic enclosures as an offset from ambient pH
120 (Gattuso et al., 2014). The system was deployed in the Bay of Villefranche, approximately 300 m
121 from the Laboratoire d'Océanographie de Villefranche (NW Mediterranean Sea, France;
122 43°40.73′N, 07°19.39′E; Fig. 1). The eFOCE engineering design consisted of a surface buoy and
123 two underwater benthic enclosures (Fig. 1).

124 The underwater portion of eFOCE consisted of two clear, 1.7 m^3 (2 m long x 1 m width x
125 0.85 m tall) perspex enclosures that were open on the bottom to partially enclose a portion of a *P.*
126 *oceanica* meadow. They were located at 11 m depth, were placed end to end approximately 1.5 m
127 apart and faced south. The pH in one enclosure, referred to as the experimental enclosure, was

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lowered by ~0.25 units as an offset from ambient pH. The second enclosure served as a control. A third treatment consisted of an open fiberglass frame of the same dimensions as the enclosure footprint (2 m^2). It was placed nearby (3 m North of the experimental enclosure) and in the same meadow. It is referred to as a reference plot and accounts for any artifacts from the structure of the enclosures.

The surface component of eFOCE consisted of a buoy that housed solar panels, a wind turbine and 12 V batteries that provided energy to the system. It also housed three CO_2 tanks and a peristaltic pumping system which drew surface seawater into a 20 L container inside the buoy where pure CO_2 was added and mixed until a desired pH was reached (usually between 5.5 and 5.7 pH_T). A Seabird potentiometric 18-S pH sensor was used to monitor pH_T in this surface container.

The two underwater enclosures (experimental and control) were mostly enclosed to maintain the desired pH offset, with the exception of two openings (12 cm) on the upper, side panels. The top of the enclosure could be removed to allow scuba divers to enter when needed. Each enclosure had 10 openings (8 cm diameter) along the bottom sides that allowed tubes to pass through. These 'injection' tubes passed through each enclosure into the ambient environment where they were connected to a set of three underwater brushless centrifugal pumps and a mixing tube (one for each enclosure). For the experimental enclosure, a hose ran from the surface to depth and connected the surface low pH container to the underwater mixing tube. A second peristaltic pump on the buoy controlled the flow rate (up to 0.12 L min^{-1}) of the low-pH water through this hose while the underwater centrifugal pumps (6.7 L min^{-1} each) continuously brought ambient seawater into the mixing tube. Each mixing tube also housed a potentiometric Seabird 18-S pH sensor that monitored pH. By sensing the pH of seawater before it enters the enclosure, the system, via a feedback loop, could adjust the CO_2-saturated seawater pumping rate to

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maintain seawater entering the experimental enclosure at the desired pH offset from ambient. Once seawater reached the subsurface mixing tubes, it then entered the enclosures via the injection tubes described above, where it was circulated by another set of centrifugal pumps (4 per chamber; 6.7 L min^{-1} each). Water could then exit enclosures through the two openings (12 cm diameter) on the upper, side panels. The complete renewal time of seawater in each enclosure was ca. 1.5 h.

2.2 Field sensors and system maintenance

The environment was characterized using sensors placed inside the enclosures and placed within 5 m from the reference plot. Sensors were connected by cables to the surface electronic hub. The surface electronic hub communicated 2 min averaged data by radio to the laboratory. Underwater sensors (with their sampling frequency) included 4 potentiometric Seabird 18-S pH sensors (8 measures in 1 s) located inside each enclosure and in each mixing tube, three Seabird 37 SMP-ODO CTD with SBE 63 oxygen (O_2) optodes one in each enclosure and one nearby in the ambient (one sample, each, for salinity and temperature every 2 min, two samples for O_2 every 2 min), and three LI-COR-192 PAR sensors (2000 irradiance measurements every 5 s) also located in each enclosure and in the nearby ambient environment.

The system required routine maintenance. Scuba divers lightly brushed the enclosure surfaces and sensor probes at least once per week to remove sediment and fouling. On four occasions throughout the experiment duration, CTDs were flushed by a syringe filled with clean seawater to remove any debris inside the sampling ports. Tubes and pumps on the buoy and subsea were also cleaned once a week of debris and replaced when heavily fouled.

The underwater 18-S pH sensors were calibrated one to three times per month by placing them together in the ambient environment for 45 min, followed by collection of three, 100 mL syringes of seawater drawn directly next to the probes. Seawater was immediately returned to the

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laboratory and pH determined spectrophotometrically as described in Dickson et al. (2007). Absorbances at peak wavelengths for purified meta-Cresol Purple (Liu et al., 2011) were measured using an Ocean Optics© spectrophotometer model USB2000+VIS+NIR. The pH of seawater samples was determined in triplicate (SD < 0.008) at 22 °C and recomputed at *in situ* temperature using the R package seacarb (function pHinsi, Gattuso et al., 2015, seacarb: seawater carbonate chemistry with R. R package version 3.0.2). The offset between the probe-sensed value at the time of water collection and laboratory determined measures was used for correction. In addition, pH sensors were refreshed every four to six weeks in a NBS buffer at pH 4 for 45 min.

2.3 Timeline

The experiment was conducted from April to November 2014. The experimental duration can be divided into three periods: (1) the pre-acidification period, before pH was manipulated, lasted from 5 April to 11 June, (2) the transition period from 12 to 21 June, where pH in the experimental enclosure was slowly lowered by no more than 0.05 units per day until an offset of approximately -0.25 units was reached and (3) the acidified period from 22 June to 3 November during which pH in the experimental enclosure was maintained at the targeted offset of -0.25 units. It should be noted that the pre-acidification period began on 5 April; however, data from all sensors were available from 15 May.

2.4 Environment characterization

All sensed data were initially screened for quality. Any obvious outliers or missing data that resulted from system or sensor malfunction were eliminated from the dataset. The mean (± SD) pH_T and median (± median absolute deviation, MAD) diel pH changes for the two enclosures and the ambient environment were calculated by time period and month.

Seawater samples for the determination of total alkalinity (A_T) levels in each enclosure were taken one to five times per month from May to October (n = 11 to 12). Samples (300 mL)

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were filtered on GF/F membranes (47 mm) and immediately poisoned with 100 µL of mercuric chloride ($HgCl_2$). A_T was determined on triplicate 50 mL subsamples by potentiometric titration on a Metrohm Titrando 888 titrator coupled to a glass electrode (Metrohm, ecotrode plus) and a thermometer (pt1000). The pH electrode was calibrated on the total scale using TRIS buffers of salinity 38, corresponding to salinity in the Bay of Villefranche. Measurements were carried out at 22 °C and A_T was calculated as described by Dickson et al. (2007). During the experiment, standards provided by A. Dickson (batch 132) were used to check precision (standard deviation) and accuracy (deviation from the certified value provided by Dickson); which was 0.889 and 1.04 µmol kg^{-1} (n = 6), respectively. As A_T variations during the experiment were very small, average A_T (mean ± SD, experimental enclosure, n = 12, A_T = 2545.5 ± 8.0 µmol kg^{-1}; control enclosure, n = 11, A_T = 2541.7 ± 12.2 µmol kg^{-1}) was used to calculate all carbonate chemistry parameters at a high frequency, together with sensed temperature, salinity and pH_T, using seacarb. To calculate carbonate chemistry of the ambient environment at high frequency, we used an A_T value of 2556 µmol kg^{-1} and the sensed ambient values of temperature, salinity, pH_T, using seacarb. This A_T value is the mean for 2014 determined from weekly measures of seawater collected at 1 m depth station, Point B, within the Bay (Point B data provided by Service d'Observation Rade de Villefranche and the Service d'Observation en Milieu Littoral). All these parameters, as well as the O_2 concentration (mean ± SD), median (± median absolute deviation, MAD) diel O_2 change and photosynthetically active radiation (PAR, mean ± SD, mol photons m^{-2} d^{-1}) were summarized by month and by time period for the two enclosures and the reference plot (ambient).

2.5 Shoot density and macrophyte abundance

After the enclosures had been deployed on the meadow for four weeks and before the acidification period, scuba divers counted the number of shoots within each treatment. Shoot density was determined twice by different divers and values were averaged, except for the

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224 experimental treatment where an observer error was made and one count was eliminated.
225 Permanent quadrats were then used to determine any change in shoot density through time. On 11
226 April, three 0.25 x 0. 25 m^2 permanent quadrats were haphazardly placed inside each enclosure
227 and in the reference plot. The number of shoots per quadrat was then determined every 2 to 4
228 weeks throughout the experiment.
229 Percentage cover of benthic macrophytes was estimated every two to four weeks in three
230 to five haphazardly placed, but not overlapping, 0.5 x 0.5 m^2 quadrats within each treatment. The
231 quadrats were also divided into four smaller squares 0.25 x 0.25 m^2 to assist with estimation.
232 Prior to estimation, researchers practiced estimates on the same quadrat location to inter-calibrate
233 and limit observer bias. On some occasions, the cover and shoot density could not be estimated in
234 all 9 to 15 quadrat locations in one day. In these instances, divers returned to the treatments
235 within 15 d (most within 8 d) to complete sampling.
236 To statistically test whether shoot density changed with time and by treatment
237 (experimental, control and reference plot), the number of shoots within three permanent quadrats
238 at each sampling interval was subtracted from the initial number. The data met parametric
239 requirements and were tested for differences between treatments (control, experimental, and
240 reference plot), months, and an interaction of treatment and month using a two-way ANOVA
241 with repeated-measures. A Tukey's honest significant difference (HSD) post-hoc test was used to
242 examine for pairwise differences when a significant main effect was found. To test whether the
243 macrophyte community differed by treatment (experimental, control and reference plot) and
244 month sampled, a similarity matrix was formed using the Bray-Curtis Index. A two-way
245 permutational-MANOVA (PERMANOVA) was run on the similarity matrix with 999
246 permutations of the residuals under a reduced model with a Monte Carlo simulation. The terms
247 tested in the model include treatment (experimental, control and reference plot), month and their

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248 interaction. A post-hoc multiple comparison test was used to examine pairwise differences when
249 significant main effects were found.

250 **2.6 Leaf biometrics**

251 The number of leaves per shoot, and leaf length, area, thickness and toughness were
252 monitored several times per month from April to November, before and during the acidification
253 period. On these occasions, scuba divers used a tape measure to measure the leaf length and
254 counted the number of leaves per shoot for five to fifteen shoots per enclosure and plot. In
255 addition, approximately every four weeks from 1 August, divers collected eight mature, six
256 intermediate and two to four young leaves from each enclosure and from the reference plot. To
257 limit destructive sampling yet get a baseline measurement, on 27 June (near the start of the
258 acidification period) leaves of about the same age were collected nearby. All leaves were
259 collected from different shoots and taken at their base above the meristem. They were brought
260 back to the laboratory and their length, width, and thickness measured with a tape measure and
261 caliper. The width and thickness was measured at the middle of the length of each leaf. On three
262 occasions (in July, September, and October), the toughness of each leaf was determined in the
263 middle of the leaf length with a penetrometer (see Cherrett, 1968).

264 For all leaf biometric parameters, data collected over several days were pooled into one
265 dataset for a comparison by month and among treatments (experimental, control and reference
266 plot). Additional samples collected in June in the nearby ambient (not collected within the
267 reference plot) were not included in statistical testing. The number of leaves per shoot was
268 transformed with an exponential function (e^x) to meet parametric requirements and a two-way
269 ANOVA was used to test for differences. When parameters could not be successfully transformed
270 to meet normality and homogeneity of variance requirements, a two-way PERMANOVA was
271 used instead. We applied the same model described to test for differences in macrophyte

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abundances to the leaf biometric data, except the similarity matrix was constructed with Euclidean distances. A post-hoc multiple comparison test was used to examine pairwise differences when significant main effects were found. Because of the correlative nature of leaf length (measured in the laboratory) and average shoot height (measured in the field), only the average shoot height was statistically tested. However, the lab and field determined leaf lengths were combined and averaged by month into a leaf length parameter that is included graphically. Similarly, because leaf area is not independent from leaf length, it was not statistically tested. Nevertheless, the leaf area is included because it is a frequent meadow descriptor (Pergent-Martini et al., 2005). The leaf length, thickness and toughness were investigated for relatedness with a linear regression.

2.7 Fluorescence, photosynthesis, and respiration

A diving pulse amplitude modulated fluorometer (diving-PAM, Walz, Germany) equipped with a red light emitting diode and an internal halogen lamp to provide actinic light, was used to measure the fluorescence in illuminated and dark-adapted leaves *in situ* throughout the experiment. These fluorescence values were used to produce rapid light curves (RLCs, *r*ETR, relative electron transport rate *vs* actinic light) and dark-adapted quantum yields (F_v/F_m).

All fluorescence and photosynthesis measures were performed on a randomly selected secondary leaf from enclosures and reference plot. Dark-adapted yields and RLCs were measured *in situ* between 10-12:00 hr (local time) over two to three consecutive days to produce a sample size of three to ten leaves per enclosure and reference for May (pre-acidification), July, September, and October (acidification period for experimental enclosure). For all fluorescence measures, the fiber optic cable was attached 8 cm above the leaf meristem and held at a standard distance of 3 mm and at a 90° angle from the blade.

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RLCs were produced following the procedures outlined in Cox and Smith (2015). The actinic irradiance levels ranged up to 895 μmol photons m^{-2} s^{-1} and were applied on the leaf surface for 10 s followed by a 0.8 s saturating pulse. Actinic range was also adjusted by month to account for the changing abilities of plants and corrected each time for battery decline. We determined the absorption factor (AF), used in *r*ETR calculations, following the methods and assumptions described in Beer and Björk (2000). Measurements were conducted one to three times each sampled month and monthly averages were used in calculations. Curves were fitted with the exponential model proposed by Platt et al. (1980). Parameters derived from the curves include (1) α, the initial slope before the onset of saturation (μmol electrons m^{-2} s^{-1} / μmol photons m^{-2} s^{-1}), (2) the relative maximum electron transport rate, $r\mathrm{ETR}_{max}$ (μmol electrons m^{-2} s^{-1}) and (3) E_k, optimal irradiance for maximal electron transport (μmol photons m^{-2} s^{-1}) which is determined by the equation $E_k = r\mathrm{ETR}_{max} / \alpha$.

For dark-adapted quantum yield, leaves were placed in the dark for five minutes using the dark-adapter then leaves were exposed to a 0.8 s white saturating light pulse (saturation intensity setting of 8). Then the maximum PSII quantum yield was calculated using the equation Genty et al. (1989) for dark adaption.

In addition, the photosynthesis versus irradiance (PE) curves of experimental and control leaf segments were produced in the laboratory using O_2 evolution within a series of incubations. These incubations were performed over two consecutive days in September and November to produce four PE curves per enclosure each month. Leaf segments (5 cm) collected from ~10 cm length leaf were collected from the enclosures in the morning and incubated in the afternoon (13:00 - 19:00 h, local time). Immediately after collection, leaves were stored underwater in plastic bags, and transported to the laboratory in a dark mesh bag. Leaves were held for up to 3 h in dim light within a temperature-controlled laboratory (20 ºC) in two open top cylindrical

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aquaria (1.5 L). Ambient water from the nearby bay was pumped into two header tanks that fed the aquaria and allowed excess water to overflow into a drainage basin. The pH in one header tank was maintained at a pH_T of ~7.8, corresponding to pH levels in the experimental enclosure by metered additions of pure CO_2 controlled by a pH-stat system (IKS, Aquastar Aquatic Products).

After carefully removing all epiphytes, segments were individually placed inside 60 mL biological oxygen demand (BOD) bottles submerged into a 50 L aquarium maintained 1 to 2º C to the mean monthly seawater temperature at the time of collection (21.2 ºC ± 0.2 SD). BOD bottles were filled between each incubation with fresh seawater from the respective header tank (ambient, or lowered pH) with a stirrer below. Light was provided at a 90º angle to the leaf surface by a 250 W metal-halide lamp and adjusted to nine increasing irradiance levels (5 to 200 μmol photons m^{-2} s^{-1} measured directly at the leaf surface). This range of irradiance is within and above irradiance observed at the depth of collection. Plants were maintained at each irradiance or in darkness (to measure respiration, R) for 15-30 min while the concentration of O_2 was continuously monitored with a PreSens OXY-4 O_2 meter with PSt3 fiber-optic mini-sensors. After the incubations, leaf segments were ground in a chilled room using a glass homogenizer with 90% acetone that had been previously chilled for 12 h. The extract was left for 24 h in darkness, centrifuged at 3000 rpm for 15 min, and the absorbance of the supernatant measured in quartz-glass cuvette with a UV/VIS spectrophotometer (Lambda 2, Perkin 366 Elmer). The concentrations of Chl *a* and *b* were determined by measuring the absorbance at 647 and 664 nm and the concentrations calculated from the equations in Jeffrey and Humphrey (1975).

Rates of changes in O_2 normalised to total chlorophyll (Chl a + b) were plotted against irradiance levels. Parameters of the PE curves were estimated using an hyperbolic tangent model (Jassby and Platt, 1976), assuming that R is similar in the light and dark:

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343 $P_{net} = P_{g\,max}$ x tanh $(-E/E_k) + R$

344 with:

345 P_{net}: rate of net photosynthesis (μmol O_2 (mg Chl)$^{-1}$ min^{-1})

346 $P_{g\,max}$, rate of maximal gross photosynthesis (μmol O_2 (mg Chl)$^{-1}$ min^{-1})

347 E, irradiance (μmol photons m^{-2} s^{-1})

348 E_k, irradiance at which α intersects $P_{g\,max}$ (μmol photons m^{-2} s^{-1})

349 R, respiration rate

350 The initial slope, α (μmol O_2 (mg Chl)$^{-1}$ min^{-1} / μmol photons m^{-2} s^{-1}) was calculated as P_g

351 $_{max}$ / E_k and E_c, the irradiance at which gross photosynthesis equals respiration and above which

352 plants exhibit a positive net photosynthesis, was determined from R/α.

353 Data were tested for normality and homogeneity of variance; a rank or $\log_{10}$

354 transformation was applied when necessary. Then a two-way ANOVA was used to test for

355 statistical differences between treatments (experimental, control and reference), months, and an

356 interaction of treatment and month for the RLC parameters, PE curve parameters, and total

357 chlorophyll. A Tukey's HSD post-hoc test was used to test for statistical differences when

358 significant main effects were found. Because data did not meet parametric requirements when

359 both months were included, the differences in chlorophyll *a* to *b* ratio among leaves from the

360 control and experimental enclosures were tested separately by month with a two-tailed student *t* -

361 test or a Mann-Whitney U-test (when non-parametric).

362 **2.8 Growth and biomass**

363 Leaf production and leaf plastochrone interval were determined using the Zieman method

364 modified by Short and Duarte (2001). Three to eight shoots in both enclosures and in the

365 reference plot were marked with a plastic tag with a unique number in July, August, and

366 September. A hypodermic needle was used to punch a hole in the meristem region. These tagged

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shoots were again located 33 to 46 d later. The distance from the puncture to the meristem was measured and any new leaves that lacked a puncture were enumerated. Using these methods, it was possible to calculate the number of days to produce a new leaf (plastochrone interval) and leaf production per day for each shoot. Leaf production incorporates the new length added to the shoot from both, the newly produced leaf (or leaves) and from the growth of older leaves.

Data were tested for parametric requirements and a two-way ANOVA was used to examine for statistical differences between treatments (control-, experimental enclosures, and reference plot), months, and an interaction of treatment and month for leaf production and leaf plastochrone interval. A Tukey's HSD post-hoc was used to examine for statistical pairwise differences when significant main effects were found.

Above-ground and below-ground biomass was determined for each enclosure and for the reference plot at the conclusion of the study. A fourth 2 m^2 area was also sampled for biomass in a nearby seagrass habitat located approximately 6 m from the enclosures. This area was added to further account for natural spatial variation. Three to five, 10 cm diameter cores of 12 cm height were hammered into haphazardly selected locations within the treatment area. They were brought back to the laboratory, stored in 5% formalin, and later sorted into above-ground and below-ground plant parts, blotted dry, and weighed. An one-way ANOVA was used to test for differences in above- and below- ground biomass when data met parametric requirements.

2.9 Accounting for pseudoreplication

Data collected in each month from the same treatment were considered as replicates in the statistical tests and values reported are mean (± standard error, SE), except when otherwise specified. This type of pseudoreplication inflates Type I error. However, for some tested parameters, monthly measures could be considered true replicates of the before and the acidification period. In these instances, the analyses were performed with and without

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391 pseudoreplication and the outcomes (not included) were the same. In this ecological system, it is
392 more cautious to assume that plant response could change by month. Thus the pseudoreplicated
393 analyses were used.

394 **3 Results**

395 **3.1 Environment characterization**

396 The pH in the experimental enclosure was maintained at a -0.26 unit offset from the
397 control enclosure during the acidification period (Table 1). Values summarized by month showed
398 that the difference between the two enclosures was maintained close to the targeted offset (range:
399 -0.22 to -0.29 pH units). Before the pH was manipulated the offset between enclosures was
400 smaller, -0.1 to 0.06 pH units.

401 The pH_T in ambient ranged from a mean of 7.98 (± 0.06 SD) in September to 8.11 (± 0.04
402 SD) in June (Table 1). The ambient pH_T was similar to the pH_T in the control enclosure, which
403 ranged from 7.97 (± 0.07 SD) in September to 8.12 (± 0.06 SD) in June. The greatest difference
404 between control and ambient, in monthly mean pH_T values was 0.06 units. The differences in
405 pCO_2 reflected the magnitude of difference in pH_T, as A_T levels were rather constant during the
406 study (see method section).

407 The mean O_2 concentration was similar in enclosures and in the ambient (Table S1). For
408 example, the mean O_2 concentration (± SD) before acidification for ambient, control and
409 experimental respectively was 258 ± 18, 254 ± 34, 258 ± 32 μmol kg^{-1}. In the ambient and in the
410 enclosures, the O_2 concentration fluctuated over the course of the day (data not shown). After
411 sunset, O_2 concentration declined to a night-time minimum. In the morning, the O_2 began to
412 increase to a daily afternoon maximum; then it declined with decreasing irradiance. Over the
413 months of the experiment, this diel O_2 change ranged from 21 to 72 μmol kg^{-1} in the ambient, 34
414 to 95 μmol kg^{-1} in the control enclosure, and 34.5 to 100.5 μmol kg^{-1} in the experimental

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enclosure (Table 1). The difference in environment between the ambient and the enclosures was most likely due to the amplification of a metabolic signal inside a partially enclosed space as was evidenced by the more similar, and greater diel change in the two enclosures. The largest difference in median values between enclosures was 14 μmol kg^{-1} in May, prior to the perturbation.

The diel pH_T change in the meadow corresponded to the daily change in O_2 concentration. The natural diel pH_T for this meadow was evident from the measures in the ambient which median values show it fluctuated by 0.09 (± 0.02 MAD) and 0.08 (± 0.02 MAD) units per day in the pre- and acidification period, respectively. The diel change in pH_T for the control enclosure was slightly greater but consistent in the pre- and during acidification period (0.14 ± 0.06 MAD and 0.14 ± 0.06 MAD). In contrast, the diel pH_T change for the experimental enclosure increased from a median of 0.16 (± 0.06 MAD) before pH manipulation to 0.28 (± 0.14 MAD) during the acidification period.

Monthly differences were evident particularly for temperature, oxygen concentration, and PAR (Table S1) but were similar in the ambient, control and experimental enclosures. For example, the mean ± SD during the acidification period for temperature in ambient, control and experimental enclosures was 23.9 ºC ± 0.01 (for each) and for PAR, 4.6 ± 1.9, 4.6 ± 2.0, 4.1 ± 1.7 mol photons m^{-2} d^{-1}, respectively. Temperature increased approximately by 6 ºC from May through August and declined by 4 ºC until November. Oxygen concentrations and PAR fluctuated similarly with higher values in May to August (mean monthly range: 212 to 270 μmol kg^{-1}, 4.7 to 7.7 mol photons m^{-2} d^{-1}) and decreases in September to November (mean monthly range: 193 to 211 μmol kg^{-1}, 1.4 to 4.4 mol photons m^{-2} d^{-1}).

3.2 Shoot density and macrophyte abundance

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438 Initial shoot densities were similar in both enclosures and reference plot and ranged from
439 150 to 175 shoots m^{-2} (Fig. 2). There was no detectable change in shoot number related to the
440 lowered pH in the experimental enclosure. For both enclosures and the reference plot, the number
441 of shoots (initially 6 to 27 in permanent quadrats) tended to decline with time. The two-way
442 ANOVA with repeated measures did detect significant changes in shoot density by month (Table
443 S2) yet, the pairwise comparison test failed to find significant month-to-month differences.
444 The reference plot as well as the enclosures had very low diversity of benthic
445 macrophytes (Fig. 2). *Posidonia oceanica* was the dominant species, with a surface cover of 18 to
446 35%. *Peyssonnelia,* a red alga, covered between 1 and 11% of the substratum. Their abundances
447 were similar between months throughout the experiment. However, the experimental enclosure
448 had a different benthic structure than the control enclosure and reference plot (Table S2). The
449 difference was due to the slightly greater percentage of *P. oceanica* in the experimental enclosure
450 (experimental enclosure, 31.6 ± 0.6%; control enclosure, 27.9 ± 1.7%; reference plot, 28.9 ±
451 1.3%) throughout the experiment duration and was not related to pH manipulation.

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3.3 Leaf biometrics

There was no statistically significant difference in shoot height among the enclosures and reference plot but there were differences in shoot height between the sampled months (Fig. 3, Tables S3, S4). A similar monthly pattern in leaf length was observed between the three treatments, for the minimum, average and maximum leaf length. From April through August, average leaf length and average shoot height both increased and then declined between August and September. For example, the overall average shoot height increased from 40.6 cm in April to 73.4 cm in August then declined to 24.8 cm in November. Furthermore, the average shoot height in October and November was statistically different from the height measured in April through September (Table S4).

Shoots had between 2 and 8 leaves (Fig. 3). The number of leaves per shoot differed between treatments (enclosures and plot) and changed from month-to-month (Tables S3). However, the post-hoc test revealed that only the reference and control plants differed significantly in shoot number (reference, 5.4 ± 0.1 *vs* control, 5.1 ± 0.1), with no significant difference between control and experimental plants (5.2 ± 0.2) indicating an absence of pH effect (Table S4). Furthermore, the number of leaves per shoot in the experimental enclosure did not consistently increase or decrease after the pH was manipulated. Instead, leaf number per shoot in enclosures and plot increased during months when leaf height was lower (April, May and then October, November: 6 to 7) and tended to be lower in June and August (4 to 5) when leaf height was elevated.

Leaf thickness and leaf toughness increased with leaf length. Leaf thickness was moderately correlated to leaf length ($R^2 = 0.64$, $P < 0.001$) while leaf toughness was weakly correlated to leaf length ($R^2 = 0.17$, $P = 0.049$). However, leaf thickness was not correlated to leaf toughness. Both parameters significantly varied among the enclosures and reference plot, and

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between months (Fig. 3, Tables S3, S5). Leaves in the experimental enclosure were statistically thicker (2.5 ± 0.1 mm) than leaves in the control enclosure and the reference plot (2.2 ± 0.08 mm and 2.1 ± 0.1 mm, respectively). The pairwise statistics (Table S5) and mean values indicate that this pattern is driven by the decreased and more variable thickness for the leaves in control enclosure and the reference plot for the month of November. Pairwise comparisons (Table S5) also indicate that the leaves inside the enclosures were weaker than the leaves in the reference plot. Furthermore, leaves were significantly weaker in October compared to July and September. For example, the weakest leaf in July could withstand 34 g of force as compared to the weakest leaf in October which could only withstand 12 g of force. The ambient leaf samples collected in June also had a greater mean value of toughness than the October values from enclosures and the reference plot.

3.4 Fluorescence, photosynthesis, and respiration

The dark-adapted quantum yield statistically differed by month but not according to pH (Fig. 4, Table S6). The overall dark-adapted quantum yield ranged from 0.72 to 0.88 (n = 69). The mean values were similar in the enclosures and the reference plot. Mean yield was 0.8, 0.789, and 0.799 for leaves measured in the experimental, control, and reference treatments, respectively. Yield values increased over the duration of the experiment.

The AF factor for the calculation of *r*ETR changed with month. The determined values (as a mean ± SD) were as follows: May: 74.5; July: 65.0; September: 69.6 ± 1.5 (n = 3); October, 54.2 ± 0.0 (n = 2).

The photosynthetic RLCs in Fig. 4 (A-D), show that the shape of the curve changed with month. Leaves from the control and experimental enclosures have similar *r*ETR values that were slightly lower at high irradiance relative to the leaves in the reference plot. Nevertheless, there

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were no statistically significant differences among enclosures and plot for RLC parameters but there were clear statistical differences in parameters among months (Table S6).

The initial slope (α, μmol electrons m^{-2} s^{-1} / μmol photons m^{-2} s^{-1}) ranged between 0.23 and 0.58 (n = 57). It decreased substantially as a function of time (Table S6) from elevated values in May (0.43 ± 0.01) and July (0.48 ± 0.01) to lower values in September (0.31 ± 0.01) and October (0.27 ± 0.01). Overall (n = 57), $r\mathrm{ETR}_{max}$ values (in μmol electrons m^{-2} s^{-1}) ranged from 4.3 to 27.4 and E_k (μmol photons m^{-2} s^{-1}) ranged from 12.0 to 63.6. The leaves from the reference plot had $r\mathrm{ETR}_{max}$ (12.3 ± 0.6) and E_k (33.7 ± 2.0) that were more different than the leaves from the control ($r\mathrm{ETR}_{max}$ = 10.8 ± 0.7, E_k = 29.8 ± 2.0) and experimental ($r\mathrm{ETR}_{max}$ = 12.0 ± 0.7, E_k = 30.9 ± 0.7). However, these parameters differed by month and not among plants from the enclosures and the plot (Table S6). The post-hoc test (results seen on Fig. 4) revealed that $r\mathrm{ETR}_{max}$ values were substantially higher in May (22.1 ± 1.4) than in July (10.9 ± 0.8), September (7.2 ± 0.6), and October (7.5 ± 0.8). Overall, E_k was significantly greater in May (50.2 ± 2.2) than in July (23.1 ± 2.6), September (24.5 ± 2.1), and October (28.1 ± 2.5).

The parameters of the PE curves of leaves collected from the experimental and control enclosures also did not differ yet, month-to-month significant differences were found (Fig. 5, Table S6). α, $P_{g\,max}$, and R were greater for leaves measured in September than November.

The total concentration of chlorophyll in leaves did not differ among enclosures (Table S6). It was 0.36 ± 0.04, 0.38 ± 0.04 mg Chl cm^{-2} in the experimental and control enclosures, respectively. It was greater in November than in September (0.46 ± 0.03 *vs* 0.28 ± 0.04 mg Chl cm^{-2}; Table S6). For September and November, the Chl *a*:*b* ratio of leaves from the control and experimental enclosures did not statistically differ (Table S6), with an overall Chl *a*:*b* ratio of 0.64.

3.5 Growth and biomass

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523 Leaf production and plastochrone interval of shoots in the reference plot and in the
524 enclosures were statistically different (Fig. 6, Table S7). Pairwise significant differences indicate
525 the effect was caused by the structure of enclosures and not from the lowered pH. The shoots in
526 the reference plot were able to produce significantly more leaf material than in the experimental
527 and control enclosures. From July to September, reference shoots grew new leaf material at a
528 mean rate of 0.89 (± 0.06) cm d^{-1} compared to the reference plot and control enclosure, which
529 both produced 0.66 (± 0.05 to 0.06) cm d^{-1}. Furthermore, reference shoots produced a new leaf in
530 a significantly fewer number of days than shoots in the experimental and control enclosures ($P <$
531 0.05). From August to September, it took 11 days to produce a new leaf in the reference plot
532 while it took between 23 to 29 days for shoots that grew in the experimental and control
533 enclosures, respectively. Overall, leaf production (the growth of all leaves per shoot) was also
534 seasonal. It was significantly greater per day from September to October (1 cm d^{-1}) than during
535 the periods July-August (0.5 cm d^{-1}) and August-September (0.6 cm d^{-1}; $P < 0.05$).
536 At the end of the experiment, the above- and below-ground biomass was highly variable
537 and did not statistically vary between the reference plot and enclosures (Fig. 6, Table S7). The
538 above- and below-ground biomass ranged from 318 to 1484 and from 348 to 1584 g FW m^{-2},
539 respectively. Despite the lack of statistical significance, the control and experiment enclosures
540 tended to have less above-ground biomass (630 and 530 g FW m^{-2}) than the two external plots
541 (reference: 850 and extra ambient plot: 870 g FW m^{-2}).

542 **4 Discussion**

543 No overwhelming impact was detected on macrophyte abundance, *P. oceanica* leaf
544 biometrics and photosynthesis after four months of elevated pCO_2. Leaf thickness may change in
545 response to lowered pH but requires further testing. Many of the leaf biometrics and physiology
546 parameters varied seasonally with the varying temperature and irradiance. *Posidonia oceanica*

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547 abundance did not substantially change over eight months as expected for a seagrass with slow
548 rates of colonization (Marbà and Duarte 1998). However, under elevated pCO_2, no other benthic
549 macrophyte or epiphyte proliferated or decreased to alter the macro-community structure. The
550 similarity in leaf biometrics, photosynthesis, and growth between enclosures support the
551 conclusion of limited stimulation for *P. oceanica* under future ocean acidification.

552 Thickness and toughness are two structural factors related to mechanical strain (Harder et
553 al., 2006; Littler and Littler, 1980; Padilla, 1985) and both traits were altered. Flexibility and
554 strength are needed in environments with strong wave forces (de los Santos et al., 2013). In
555 *Cymodocea nodosa,* another Mediterranean seagrass, leaf cross-sectional area varies with
556 hydrodynamical forces (de los Santos et al., 2013). Therefore, differences in leaf toughness for
557 plants maintained in the enclosures support the notion that mechanical abrasion was less than in
558 ambient. This finding is an artifact of the structure that could not be avoided. In *P. oceanica*,
559 thickness changes along the leaf axis and leaves are thinner with depth (Colombo et al., 1983).
560 Given that the experiment was conducted at the same depth and leaves were measured at their
561 center, it is interesting to note that leaf thickness was greatest for the shoots collected from the
562 experimental enclosure and that this effect was driven by measures in November. There are
563 several possible interpretations of these results. First, leaves at the lower pH may have increased
564 their carbon content as observed for below-ground plant structure of the seagrass *Thalassia*
565 *testudinum* under elevated pCO_2 (Campbell and Fourqurean, 2013a). Secondly, lowered pH could
566 result in a delay of leaf shedding. Plants from the experimental enclosure had a tendency towards
567 relatively greater leaf length and maintenance of number of leaves in November. A prolonged
568 leaf life-span could allow plants to scavenge nutrients from senescing leaves to maintain C/N
569 ratio (Gobert et al., 2002). However, lack of stimulated photosynthesis discredits need for
570 increased nutrient demands and preliminary results (unpublished) of leaf carbon content do not

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support these hypotheses. Additionally, increased pCO_2 and high light increased leaf shedding for the seagrass *Amphibolis antarctica* (Burnell et al., 2014). The response was linked to proliferation of filamentous epiphytes, which did not occur in this study. Alternately, increased leaf thickness could be the result of chance. The plausible relationship warrants further investigation in field experiments with prolonged duration and increased replication.

If indeed leaf thickness increases with ocean acidification, it is unclear how this would impact herbivore feeding. The main herbivores, the fish, *Sarpa salpa*, and the sea urchin *Paracentrotus lividus*, feed preferentially on the adult and thicker leaves (Peirano et al., 2001). These herbivores were prevented from grazing in enclosures. Arnold et al. (2012) have reported increased rates of fish grazing on the plant at proximity of a CO_2 vent, presumably due to the significant decreases in the production of phenolics. To date, very few studies have focused on plant-herbivore interactions under elevated pCO_2 levels (Asnaghi et al., 2013; Campbell and Fourqurean, 2014; Poore et al., 2013) and as plant-herbivore interactions were not the focus of this study, it is not known how this would have impacted the results.

To our knowledge, this is the first *in situ* study to repeatedly and over several months (6) measure *P. oceanica* fluorescence to find that the second rank leaves showed a typical seasonal pattern of plant acclimation (Boardman, 1977). Leaves were more sun-adapted (relatively higher $rETR_{max}$ and E_k) in periods with elevated irradiance and more shade-adapted when irradiance and photoperiod were reduced. The relatively lowered $F\upsilon/F_m$ in May and July compared to October indicates a down-regulation of PSII activity (Campbell et al., 2003; Henley, 1993) that corresponds with elevated irradiance in warmer months. Findings are in agreement with Figuero (2002) where ETR and E_k were higher in September than in February. Although there have been some concerns on the ability of fluorescence techniques to indicate seagrass carbon stimulation

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(see Cox et al., 2015; Jiang et al., 2010), *P. oceanica* productivity as a function of increasing irradiance was in agreement with fluorescence results.

The results of the present study add to the growing evidence that the pH change predicted over the next century may result in limited production stimulation for *P. oceanica*. The relationship between pH and *P. oceanica* photosynthesis was established over wide range of pH_T from 9.0 to 7.9 (scale unknown, Invers et al., 1997), or with more extreme low levels (6.98 pH_T, Hall-Spencer et al., 2008; 7.5 scale unknown, Invers et al., 2002). Within the range 7.9 to 9.0, the slope of the pH-photosynthesis relationship was significant but, the two variables were moderately related (Invers et al., 1997). Along CO_2 vents, there was no indication of photosynthetic stimulation at stations with a pH range of 6.98 to 8.17 but, shoot density was 30% greater than nearby areas at the lowest mean pH station (Hall-Spencer et al., 2008). In a laboratory incubation of *P. oceanica* shoots with their attached epiphytes, at a similar pH_T as this study (~7.7-7.8), there was also limited stimulation of productivity (Cox et al., 2015). Similarly, modeled outcomes from laboratory studies of leaf segments by Invers et al. (1997, 2001) predicted that elevating pCO_2 by the amount used in this experiment would increase productivity by only 10%. This first *in situ* experiment confirms previous results obtained on isolated plants or leaf segments in the laboratory and is interpreted as in agreement with observations at CO_2 vents.

Posidonia oceanica has shoot lifespan estimated up to 50 years (Gobert et al., 2006). In carbon budgets there is thought to be asynchrony between fixation (photosynthesis) and use (respiration or growth), which is balanced by the storage of carbohydrate reserves (Alcoverro et al., 2001). Because of this asynchronicity, the photosynthetic benefit of CO_2 may translate into the following season or year as it did for the seagrass *Zostera marina* (Palacios and Zimmerman, 2007). In the present study, there was no indication of increased productivity and measures of root carbohydrates, leaf carbon content (both unpublished data), and chlorophyll do not support

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618 increased carbon storage as occurred for *T. testudinum* under elevated pCO_2 (Campbell and
619 Fourqurean, 2013a) Carbohydrates can be translocated to other ramets (Marbà et al., 2002) which
620 can lessen observed effects but, in this case, enclosure area captured the 20 cm maximum
621 translocation distance detected by Marbà and Duarte (1998) and edges severed (designed to
622 penetrate ~8 cm) several outside to inside shoot connections. The most productive period for
623 above-ground growth occurred from April to August; a pattern consistent with increased growth
624 induced from the greater availability of both light and nutrients in early spring and increased
625 storage in July to August (Alcoverro et al., 1995, 1998, 2001; Bay, 1984; Duarte, 1989).
626 Therefore it is possible that if the experiment were initiated earlier, in a period more conducive
627 for productivity, the long-term outcome may have been different. Results by Invers et al. (1997,
628 2001, 2002), Hall-Spencer et al. (2008), Cox et al. (2015), and this study for *P. oceanica*
629 conducted over a range of conditions (pH = 6.98 to 8.17, duration of study = hrs to four months,
630 months = February to November, depth = 3 to 14 m, hrs at saturation irradiance = 6.5 to 11.5,
631 epiphyte cover < 75%, shoot density =150 to 1000 m^2) are mixed in support. Two results support
632 a pulsed seasonal-pH interaction that could result in long-term gains yet, these were found at pH
633 < 7.7 (Hall-Spencer et al., 2008; Invers et al., 2002).

634 We caution that conclusions should not be applied to other seagrasses. Presumably due to
635 differences in their evolutionary past, some species are comparatively more responsive to lowered
636 pH (Campbell and Fourqurean, 2013b; Invers et al., 2001; Koch et al., 2013). *Posidonia oceanica*
637 is less sensitive to pCO_2 and can rely heavily on bicarbonate compared to two other Pacific
638 seagrass species (Invers et al., 2001). Nutrient concentration can also alter the response of
639 seagrass to CO_2 additions (Burnell et al., 2014; Martínez-Crego et al., 2014). Clearly our
640 understanding of meadow dynamics under ocean acidification conditions could benefit from

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repeated *in situ* studies that address issues such as species differences, more prolonged durations, herbivore-plant interactions, and temporal and spatial effects.

Performing this experiment *in situ,* over several months, is a major advancement for understanding the response of *P. oceanica* to ocean acidification. The eFOCE design has advantages to other mesocosm systems such as its large size which allows for measuring processes at the scale of a meadow, its ability to monitor the environment in real-time, and its ability to maintain pH as an offset. Though replicated enclosures would have been preferred and are recommended for future use, their implementation was not feasible at this stage. However, several steps were taken to eliminate possible erroneous conclusions including: (1) the environment was continuously monitored to ensure comparisons were valid, (2) repeated measurements were made at the same location through time, (3) comparisons from the pH manipulated enclosure were made to at least two different spatial locations and (4) results obtained in laboratory and natural experiments were compared and are in general agreement. The duration of this study was longer than any previous pH perturbation carried out on *P. oceanica* and it was performed in the most natural conditions possible. This study addresses a need for manipulative experiments done *in situ* for longer durations to make best predictions of future marine ecology (see Gattuso et al., 2014).

Our findings have implications for the function of future meadows. Seagrasses through their metabolic activity alter the chemical properties of the meadow. In daylight, seagrasses draw down the available dissolved inorganic carbon and at night their respiration has the opposite effect (Hendriks et al., 2014a). The daily change in pH has been shown to be up to 0.24 pH units and to be related to the density and length of leaves (Hendriks et al., 2014a). Hendriks et al. (2014b) has suggested that (1) organisms within the meadow may not be as vulnerable to ocean acidification because they are adapted to large diel pH changes (2) the productivity of *Posidonia*

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during the day may buffer the impacts of ocean acidification, particularly for calcifiers by providing a daily window of maximum calcium carbonate saturation where calcification can be more efficient and (3) ocean acidification could stimulate seagrass productivity and thus increase buffering capacity; which was not supported by the results of this present study. Considering the two previous proposed hypotheses, the median diel pH variation for the meadow in this study was ~0.1 and also appeared to be driven by production. However, the median diel pH range in the experimental enclosure was two to three times larger than the control (0.09 to 0.29 pH units) and exhibited greater variability; a finding that would be missed in typical experiments which lower pH and maintain it at a constant future level(s). The variation in diel pH cannot solely be explained by O_2 fluxes. The increased diel pH fluctuation could largely be the result of the reduced buffering capacity of seawater at lowered pH (Shaw et al., 2013). The lowered and larger diel pH variation and lack of productivity stimulation casts doubt on the adaptability of organisms to future pH change and the ability of a *P. oceanica* meadow to serve as a future refuge.

Lastly, ocean acidification is not occurring in isolation, warming has been predicted to result in a complete extinction of *P. oceanica* meadows by the year 2049 (Jordà et al., 2012). The speculation that increased CO_2 availability would enhance *P. oceanica* production and help to alleviate thermal stress was not supported. It confirms observations after an explosive episode at a CO_2 vent which resulted in an extreme lowering of pH (4.7 to 5.4) and elevated temperatures (28-30 °C, 3 to 5 °C above ambient). Along this vent, *P. oceanica* experienced a decrease in growth that persisted for three years (Vizzini et al., 2010). The extreme nature of the vent activity, confounding biological differences found at vent sites (e.g. Vizzini et al. 2013), and the possible change in physiology under combined stressors make it difficult to predict future meadow ecology. It underscores the need to investigate stressors concurrently and *in situ*. The FOCE systems are amendable, powerful tools that can be used to investigate these types of impacts.

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Author contribution

All authors contributed to the research in this manuscript. J.-P. Gattuso and F. Gazeau were co-principle investigators that had the idea, oversaw the project, and were involved in data collection. P. Mahacek was responsible for eFOCE system design. A. Le Fur ensured the system functioned with assistance from F. Gazeau, T.E. Cox, S. Alliouane, and J.-P. Gattuso. T.E. Cox was responsible for the seagrass protocol and data collection with assistance from S. Alliouane and advice given by I.E. Hendriks who contributed to fluorescence measures. T.E. Cox wrote the manuscript with J.-P. Gattuso and F. Gazeau and all other authors contributed editorial comments.

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927 **Figure captions**

928 **Figure 1**. Schematic of the system and study design (A) see text for details (B): the pH (total
929 scale) inside the enclosures and in ambient during the week-long transition to the targeted offset
930 (-0.25 units).

931

932 **Figure 2**. Macrophyte abundance throughout the experiment; A: enclosures and reference plot
933 had initially similar *P. oceanica* shoot density m^{-2} (mean ± SE). B: mean shoot number with time
934 within three permanently located quadrats (0.25 m^2) per reference plot (top), control (middle) and
935 experimental (bottom) enclosures. C, D, E: coverage (%) of benthic macrophytes and unoccupied
936 sediment or rocks (bare space) before and during the acidification period (x-axis after the dashed
937 vertical line).

938

939 **Figure 3**. Leaf biometrics (mean ± SE) before and during the acidification period for the
940 reference and enclosure plants. Measures through time: average shoot height (A), leaf length (B),
941 minimum (C) and maximum leaf length (D), number of leaves per shoot (E), leaf area (F), leaf
942 thickness (G) and leaf toughness (H) are shown. The dashed vertical line indicates when the pH
943 was lowered in the experimental enclosure. Additional leaves were collected in June from the
944 meadow and are referred to as ambient leaves.

945

946 **Figure 4**. Photosynthetic rapid light curves (RLCs, A-D), dark-adapted quantum yield (E), and
947 the derived RLC parameters (F-H) measured on 2nd rank leaves in enclosures and reference plot
948 before (May) and during (July, September, and October) the acidification period. Symbols
949 represent the mean (±SE) relative electron transport rate (*r*ETR) at each mean photosynthetic
950 active radiation (PAR) value. Curved lines represent the Jassby and Platt (1976) regression based

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951 on mean values. The dashed outline encloses the acidification period. Letter groups above bars in
952 E-H are the months that have statistically similar values as determined by Tukey's HSD post-hoc
953 tests.
954
955 **Figure 5.** Photosynthesis versus irradiance (PE) curves produced from laboratory incubations of
956 *P. oceanica* leaf segments collected from the enclosures after two (September, A) and four
957 (November, B) months of acidification. The derived parameters from the curves are shown in
958 panels C-G. Letters above or below bars represent statistically similar groups as determined from
959 Tukey's HSD post-hoc test when a two-way ANOVA found significant main effects ($P<0.05$).
960
961 **Figure 6.** Growth as *P. oceanica* leaf production (A) and leaf plastochrone interval (B) during the
962 acidification period. After 4 months of acidification, biomass (above-ground, C; below-ground,
963 D) was determined from replicate cores collected from enclosures and the reference plot. A fourth
964 nearby ambient area was additionally sampled to better account for spatial variation. Letter
965 groups above bars represent the results of Tukey's HSD post-hoc test when a two-way ANOVA
966 found significant main effects ($P<0.05$).
967

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968 **Table 1.** A comparison of the carbonate chemistry and diel changes within the ambient and enclosures: the mean (± standard deviation, SD)
969 pH (on the total scale), the maintained pH offset between experimental and control enclosures as a difference (Diff), the partial pressure of
970 carbon dioxide (pCO_2), and the median (Med ± median absolute deviation, MAD) diel pH and oxygen (O_2) change for each month and the
971 period before and during the acidification.

		pH_T									**pCO_2 (μatm)**							**Δ Diel pH_T**								**Δ Diel O_2**			
		N	Ambient		Control		Experimental		Diff		Ambient		Control		Experimental		N	Ambient		Control		Experimental		Ambient		Control		Experiment	
Months in *Period*		Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Days	Med	MAD	Med	MAD	Med	MAD	Med	MAD	Med	MAD	Med	MA
Before																													
	May	11840	8.10	0.03	8.12	0.06	8.01	0.05	-0.10	0.03	374	30	358	55	477	74	17	0.08	0.02	0.10	0.06	0.12	0.05	52.0	11.0	68.0	7.0	82.0	14
	June	8119	8.11	0.04	8.04	0.05	8.10	0.06	0.06	0.05	369	38	443	63	378	65	11	0.10	0.03	0.15	0.04	0.16	0.02	72.0	9.0	91.0	8.0	101.0	9
Acidification																													
	June	6226	8.05	0.03	8.02	0.04	7.79	0.13	-0.23	0.13	430	42	470	57	868	318	9	0.12	0.03	0.12	0.04	0.27	0.08	72.0	7.0	85.0	10.0	92.0	11
	July	21007	8.03	0.03	8.03	0.06	7.79	0.12	-0.24	0.11	454	46	453	81	870	254	30	0.09	0.02	0.17	0.05	0.27	0.06	60.5	14.0	95.5	18.0	100.5	18
	August	22682	8.00	0.03	8.04	0.07	7.81	0.12	-0.23	0.09	489	42	445	85	834	253	31	0.09	0.02	0.18	0.05	0.29	0.06	55.0	8.0	77.0	12.0	86.0	12
	September	21854	7.98	0.07	7.97	0.06	7.70	0.11	-0.27	0.10	521	96	536	87	1098	288	30	0.07	0.01	0.11	0.06	0.28	0.10	37.5	5.5	62.5	15.5	54.0	11
	October	22420	8.01	0.04	8.00	0.04	7.70	0.13	-0.29	0.14	480	52	497	64	1086	390	31	0.06	0.02	0.09	0.04	0.29	0.08	27.0	3.0	34.0	5.0	44.0	5
	November	5377	8.02	0.03	8.02	0.02	7.80	0.15	-0.22	0.15	469	48	467	22	836	305	10	0.04	0.01	0.06	0.03	0.09	0.05	21.0	5.5	34.0	22.5	45.5	29
Before		24334	8.10	0.04	8.05	0.07	8.06	0.07	0.01	0.09	380	39	434	85	426	87	34	0.09	0.02	0.14	0.06	0.16	0.07	63.5	13.0	80.5	13.5	88.0	13
Acidification		95711	8.01	0.05	8.01	0.06	7.75	0.13	-0.26	0.11	483	67	482	86	971	323	132	0.08	0.02	0.14	0.06	0.28	0.14	44.0	14.5	68.5	23.5	74.0	23

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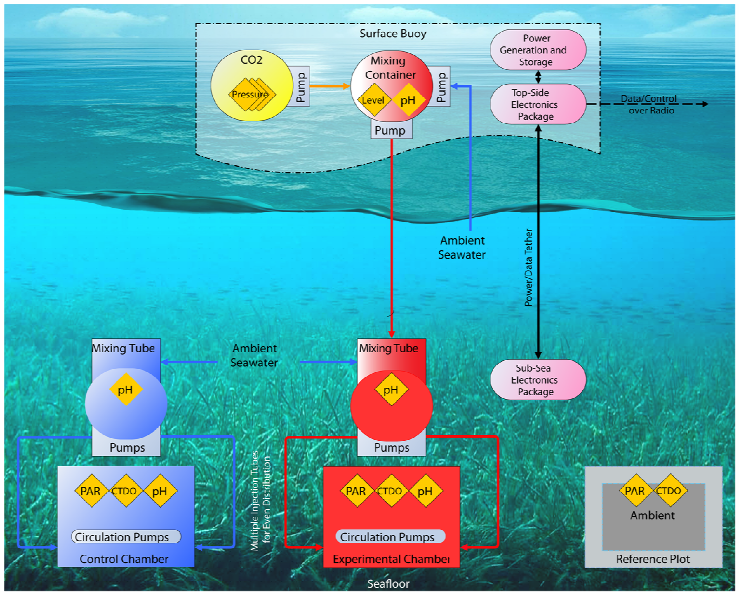

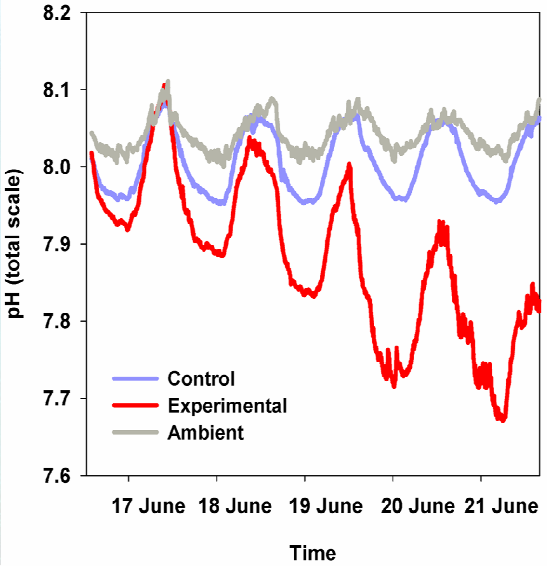

Figure 1.

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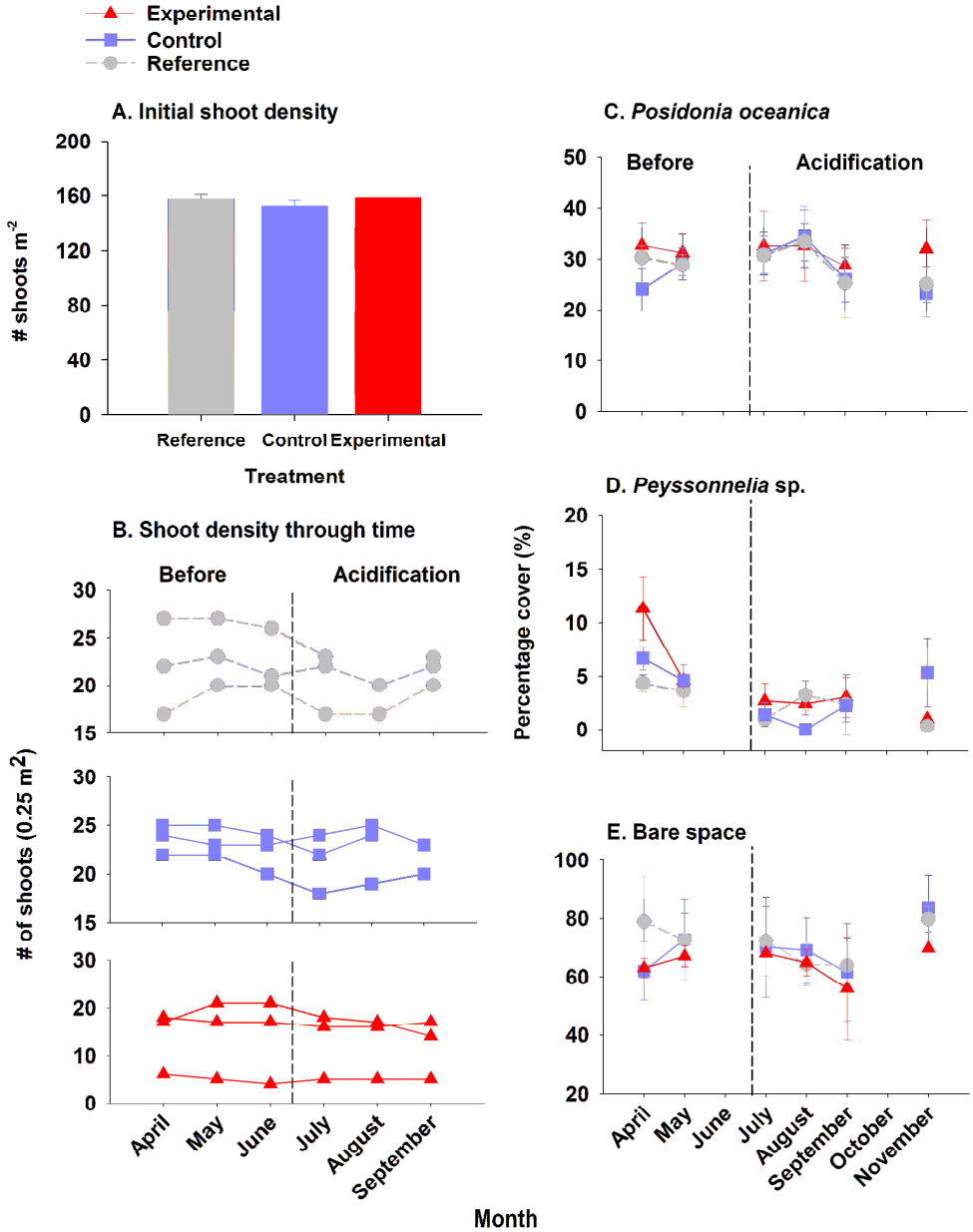

975
976 **Figure 2.**

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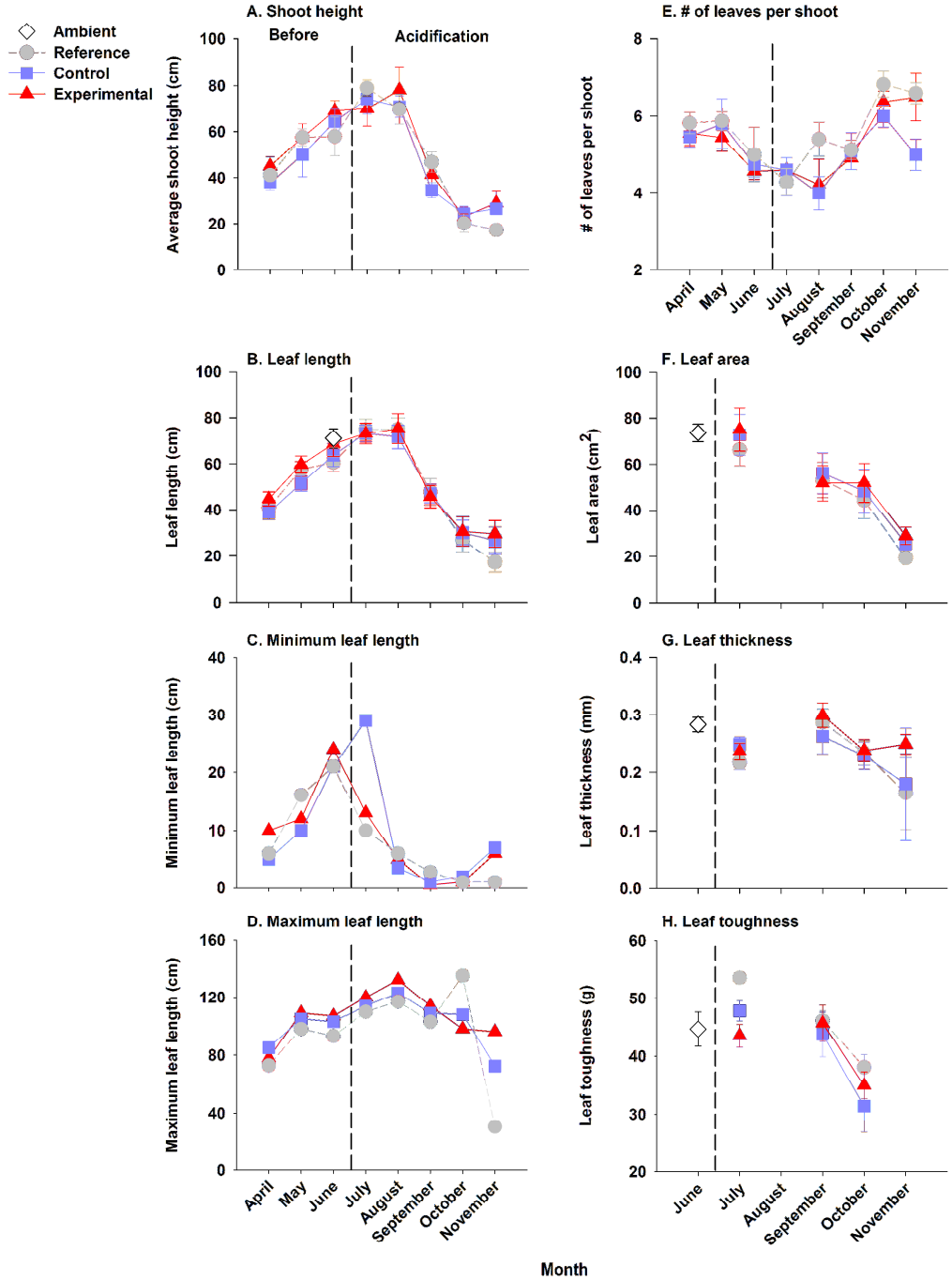

977
978 **Figure 3.**

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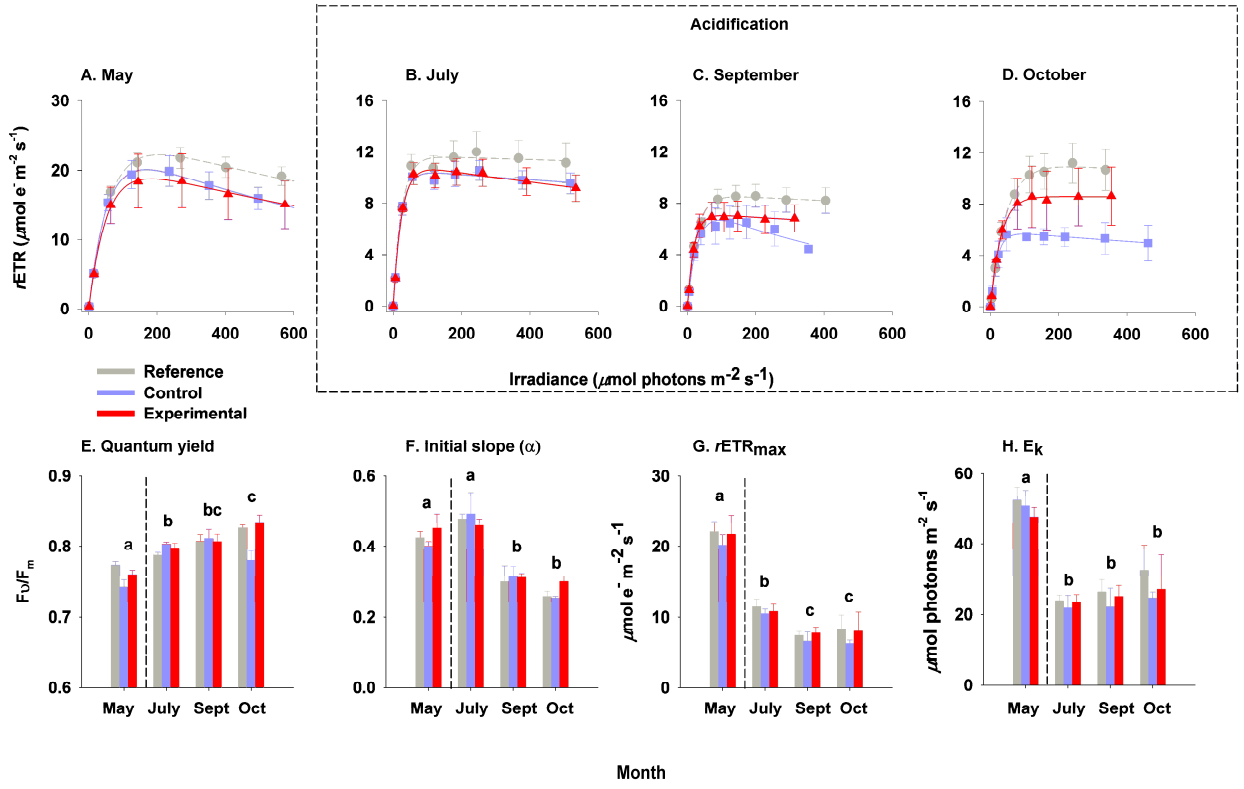

979
980 **Figure 4.**

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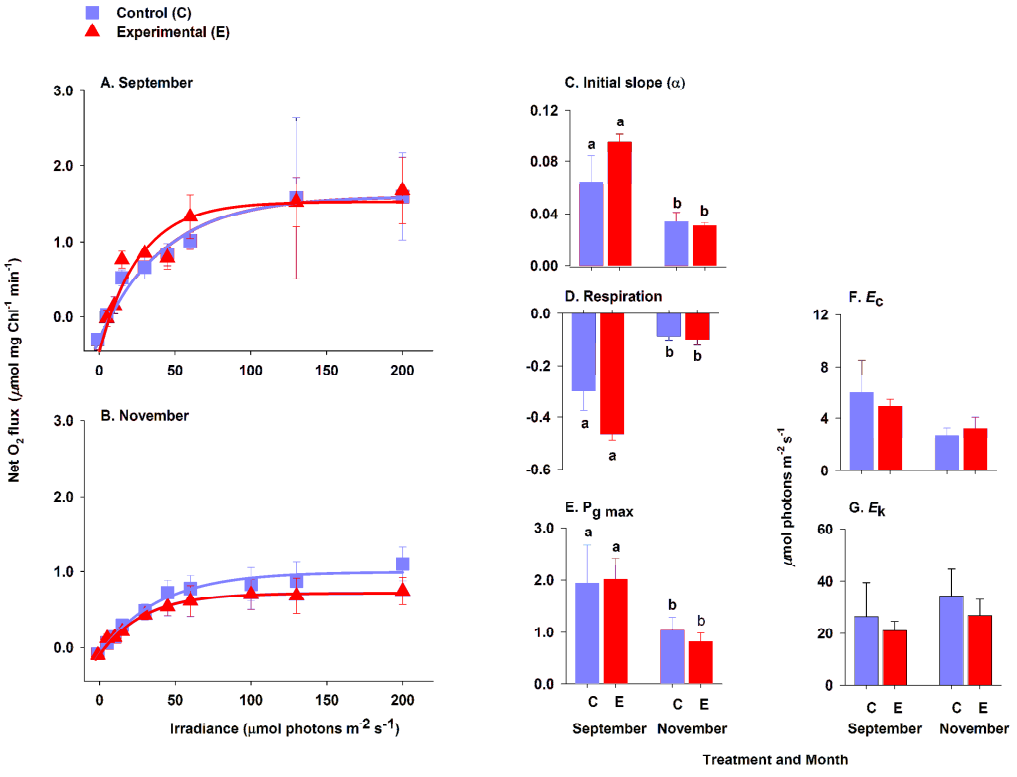

981
982 **Figure 5.**

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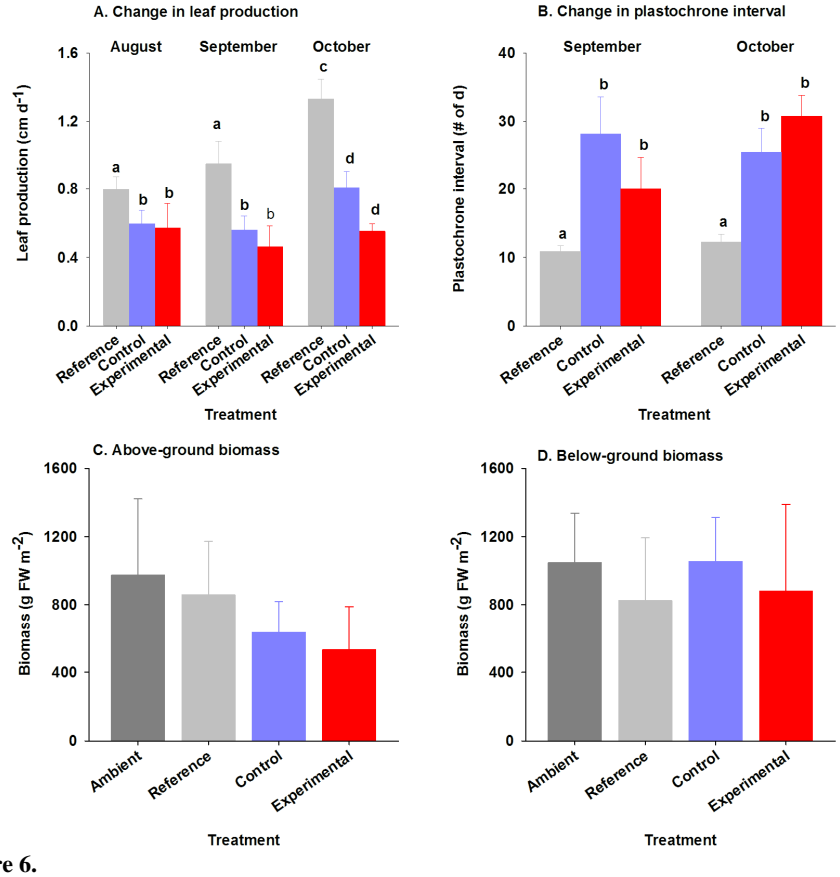

983
984 **Figure 6.**