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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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Abstract. Seagrass is 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 manipulation of pH as continuous offset from ambient. It 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 environment (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 8-month study. The greatest magnitude of change 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 testing. Results are congruent with other short-term and natural studies that have investigated the response of *P. oceanica* over a wide range of pH. They suggest any benefit from ocean acidification, over the next century (at a pH of ∼7.7 on the total scale), on *Posidonia* physiology and growth may be minimal and difficult to detect without increased replication or longer experimental duration. The limited stimulation, which did not surpass any enclosure or seasonal effect, casts doubts on speculations that elevated CO_2 would confer resistance to thermal stress and increase the buffering capacity of meadows.

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 present-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.

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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 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; Russell et al., 2013) 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 hampers 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 contained (ie. as opposed to free flow CO_2 bubbling) 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 monospecific 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} yr^{-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 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 of 7.6 and minimum of 6.98 (on the total scale, pH_T, 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 (pH_T 7.7 and 7.3) was performed (Cox et al., 2015). Despite a 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 6 weeks and only under the irradiance, temperature, and nutrient conditions of February to March. From these studies it is difficult to predict the impact of ocean acidification on *P. oceanica*.

Any alteration in *P. oceanica* productivity or abundance will likely have repercussions to meadow function. Therefore the aim of the present study was to test the hypothesis that Mediterranean seagrass, *P. oceanica*, will benefit from ocean acidification. We tested this hypothesis in situ with a Free Ocean Carbon Dioxide Enrichment (FOCE) system (see Gattuso et al., 2014) which consisted of two partially open enclosures that were deployed in the Bay of Villefranche (France) for 8 months (April–November 2014). The pH was manipulated continuously, in one enclosure, at a -0.26 pH unit offset from ambient between June and November. Before and during pH manipulation, macrophyte abundance, *Posidonia* leaf biometrics, photosynthesis, and growth were measured and environmental conditions were monitored.

2 Method

2.1 Experimental setup and system function

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

The underwater portion of eFOCE consisted of two clear, $1.7\,\mathrm{m}^3$ ($2\,\mathrm{m}$ long $\times$ $1\,\mathrm{m}$ width $\times$ $0.85\,\mathrm{m}$ tall) perspex enclosures that were open on the bottom to partially enclose a portion of a *P. oceanica* meadow. They were located at 11 m depth, were placed end to end approximately 1.5 m apart and faced south. The pH in one enclosure, referred to as the experimental enclosure, was 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 ($2\,\mathrm{m}^2$). 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 $0.12\,\mathrm{L\,min^{-1}}$) of the low-pH water through this hose while the underwater centrifugal pumps ($6.7\,\mathrm{L\,min^{-1}}$ 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 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; $6.7\,\mathrm{L\,min^{-1}}$ 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 four potentiometric Seabird 18-S pH sensors (eight 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 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 4 to 6 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

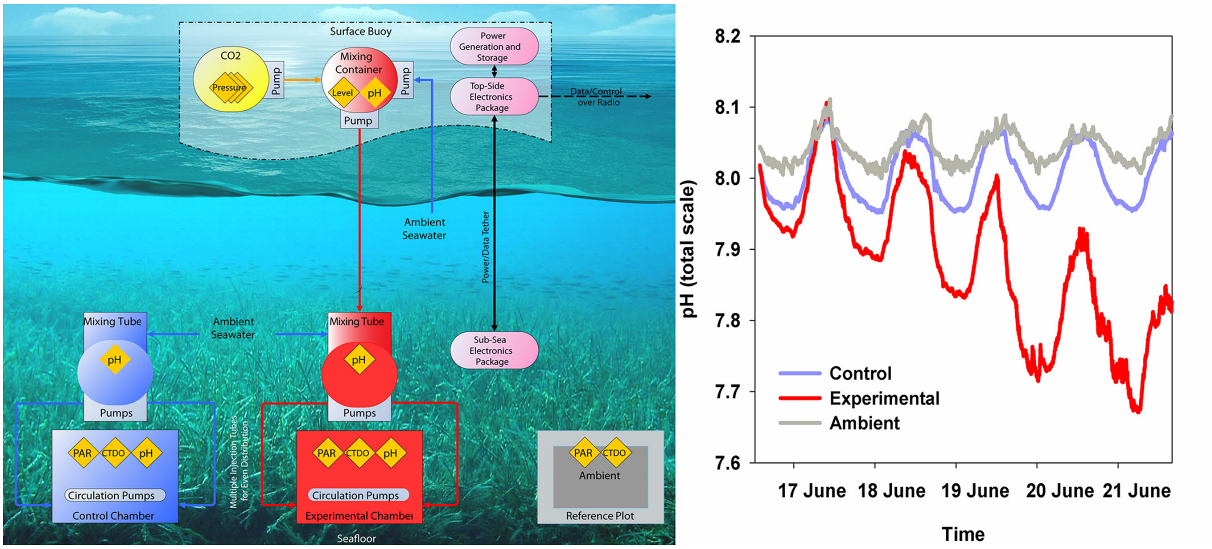

Figure 1. Schematic of the system and study design (left), see text for details; (right) the pH (total scale) inside the enclosures and in ambient during the week-long transition to the targeted offset (−0.25 units).

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 data set. 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) 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 $\mu mol\,kg^{-1}$ ($n = 6$), respectively. As A_T variations during the experiment were very small, average A_T (mean ± SD, experimental enclosure, $n = 12$, $A_T = 2545.5 \pm 8.0\,\mu mol\,kg^{-1}$; control enclosure, $n = 11$, $A_T = 2541.7 \pm 12.2\,\mu mol\,kg^{-1}$) 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 $\mu mol\,kg^{-1}$ 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 4 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 experimental treatment where an observer error was made and one count was eliminated. Permanent quadrats were then used to determine any change in shoot density through time. On 11 April, three $0.25 \times 0.25\,m^2$ permanent quadrats were haphazardly placed inside each enclosure and in the reference plot. The number of shoots per quadrat was then determined every 2 to 4 weeks throughout the experiment.

Percentage cover of benthic macrophytes was estimated every two to four weeks in three to five haphazardly placed, but not overlapping, $0.5 \times 0.5\,m^2$ quadrats within each treatment. The quadrats were also divided into four smaller squares $0.25 \times 0.25\,\mathrm{m}^2$ to assist with estimation. Prior to estimation, researchers practiced estimates on the same quadrat location to inter-calibrate and limit observer bias. On some occasions, the cover and shoot density could not be estimated in all 9 to 15 quadrat locations in 1 day. In these instances, divers returned to the treatments within 15 days (most within 8 days) to complete sampling.

2.6 Leaf biometrics

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

For all leaf biometric parameters, data collected over several days were pooled into one data set for a comparison by month and among treatments (experimental, control and reference plot). Lab and field determined leaf lengths were combined and averaged by month into a leaf length parameter that is included graphically. 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 scatter plot.

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, rETR, relative electron transport rate vs. actinic light) and dark-adapted quantum yields (F_{υ}/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 10:00 and 12:00 LT (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.

RLCs were produced following the procedures outlined in Cox and Smith (2015). The actinic irradiance levels ranged up to $895\,\mu\mathrm{mol\,photons\,m^{-2}\,s^{-1}}$ 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 rETR 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 ($\mu\mathrm{mol\,electrons\,m^{-2}\,s^{-1}}/\mu\mathrm{mol\,photons\,m^{-2}\,s^{-1}}$), (2) the relative maximum electron transport rate, $r\mathrm{ETR_{max}}$ ($\mu\mathrm{mol\,electrons\,m^{-2}\,s^{-1}}$) and (3) E_k, optimal irradiance for maximal electron transport ($\mu\mathrm{mol\,photons\,m^{-2}\,s^{-1}}$) 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 5 min using the dark-adapter and 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 in Genty et al. (1989) for dark adaption.

In addition, the photosynthesis vs. 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 LT). 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 °C) in two open top cylindrical 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 $\mathrm{pH_T}$ 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 2 °C to the mean monthly seawater temperature at the time of collection (21.2 °C ± 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 90° 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 normalized to total chlorophyll (Chl *a* and *b*) were plotted against irradiance levels. Parameters of the PE curves were estimated using a hyperbolic tangent model (Jassby and Platt, 1976), assuming that R is similar in the light and dark:

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

with P_{net}: rate of net photosynthesis (µmol O_2 (mg Chl)$^{-1}$ min^{-1}), $P_{\text{g max}}$, rate of maximal gross photosynthesis (µmol O_2 (mg Chl)$^{-1}$ min^{-1}), E, irradiance (µmol photons $m^{-2}\,s^{-1}$), E_k, irradiance at which α intersects $P_{\text{g max}}$ (µmol photons $m^{-2}\,s^{-1}$), R, respiration rate.

The initial slope, α (µmol O_2 (mg Chl)$^{-1}$ min^{-1}/ µmol photons $m^{-2}\,s^{-1}$) was calculated as $P_{\text{g max}}/E_k$ and E_c, the irradiance at which gross photosynthesis equals respiration and above which plants exhibit a positive net photosynthesis, was determined from R/α.

2.8 Growth and biomass

Leaf production and leaf plastochrone interval were determined using the Zieman method modified by Short and Duarte (2001). Three to eight shoots in both enclosures and in the reference plot were marked with a plastic tag with a unique number in July, August, and September. A hypodermic needle was used to punch a hole in the meristem region. These tagged shoots were again located 33 to 46 days 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.

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. A one-way ANOVA was used to test for differences in above- and below-ground biomass when data met parametric requirements.

2.9 Pseudoreplication

Samples were collected or measured inside the plot or enclosure through time, often both before and after the pH manipulation. Thus the replication is equal to one for each treatment. True replication was sacrificed at the expense of controlling pH as an offset, at the spatial scale of the plants. Traditional inferential statistics could, therefore, not be rigorously applied and we compare results graphically, paying careful attention to any divergence in values between the enclosures and the reference plot.

3 Results

3.1 Environment characterization

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

The pH_T in ambient ranged from a mean of 7.98 (±0.06 SD) in September to 8.11 (±0.04 SD) in June (Table 1). The ambient pH_T was similar to the pH_T in the control enclosure, which ranged from 7.97 (±0.07 SD) in September to 8.12 (±0.06 SD) in June. The greatest difference between control and ambient, in monthly mean pH_T values was 0.06 units. The differences in $p\text{CO}_2$ reflected the magnitude of difference in pH_T, as A_T levels were rather constant during the study (see method section).

The mean O_2 concentration was similar in enclosures and in the ambient (Supplement Table S1). For example, the mean O_2 concentration (±SD) before acidification for ambient, control and experimental was 258 ± 18, 254 ± 34, and 258 ± 2 µmol kg^{-1}, respectively. In the ambient and in the enclosures, the O_2 concentration fluctuated over the course of the day (data not shown). After sunset, O_2 con-

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

		pH_T								pCO_2 (µatm)						Δ Diel pH_T							Δ Diel O_2 (µmol kg^{-1})					
	N	Ambient		Control		Experimental		Diff		Ambient		Control		Experimental		*N*	Ambient		Control		Experimental		Ambient		Control		Experimental	
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	MAD
Before																												
May	11 840	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	14.0
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	9.0
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	11.0
July	21 007	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	18.0
August	22 682	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	12.0
September	21 854	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	11.5
October	22 420	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	5.0
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	29.5
Before	24 334	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	13.0
Acidification	95 711	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	23.0

centration declined to a night-time minimum. In the morning, the O_2 began to increase to a daily afternoon maximum; then it declined with decreasing irradiance. Over the months of the experiment, this diel O_2 change ranged from 21 to 72 µmol kg^{-1} in the ambient, 34 to 95 µmol kg^{-1} in the control enclosure, and 34.5 to 100.5 µmol kg^{-1} in the experimental enclosure (Table 1). The difference in diel change between the ambient and the enclosures was most likely due to the amplification of a metabolic signal inside a partially enclosed space (similar to the example of a larger O_2 fluctuation when a similar sized plant is contained in a relatively smaller volume of water) 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 °C ± 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 °C from May through August and declined by 4 °C 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

Initial shoot densities were similar in both enclosures and reference plot and ranged from 150 to 175 shoots m^{-2} (Fig. 2). There was no obvious change in shoot number (as determined in permanent quadrates re-sampled through time) related to the lowered pH in the experimental enclosure. For both enclosures and the reference plot, the number of shoots (initially 6 to 27 in permanent quadrats) tended to decline with time.

The reference plot as well as the enclosures had a very low diversity of benthic macrophytes as measured by estimates conducted within haphazardly placed quadrats at each sam-

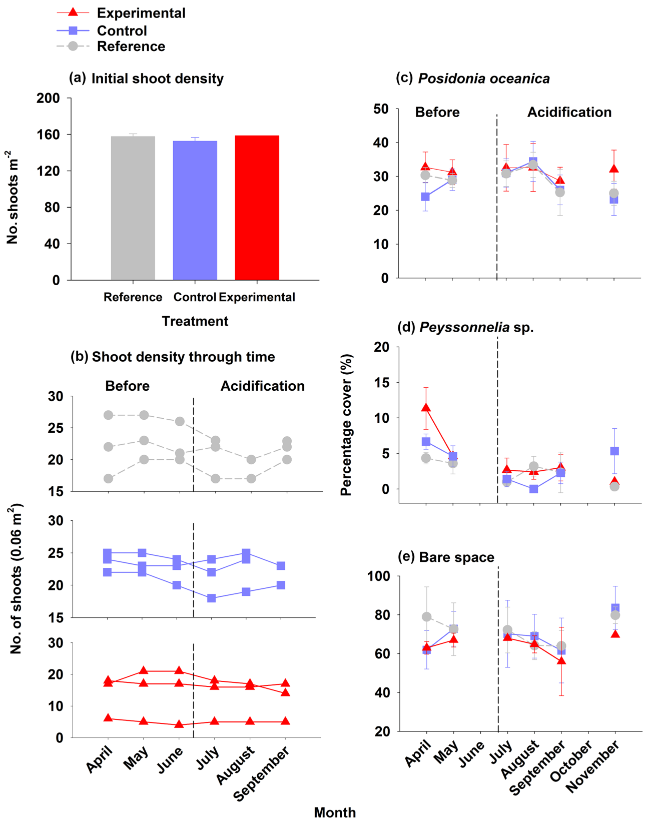

Figure 2. Macrophyte abundance throughout the experiment; **(a)** enclosures and reference plot had initially similar *P. oceanica* shoot density m^{-2} (mean ± SE). **(b)** mean shoot number with time within three permanently located quadrats (0.06 m^2) per reference plot (top), control (middle) and experimental (bottom) enclosures. **(c, d, e)** coverage (%) of benthic macrophytes and unoccupied sediment or rocks (bare space) before and during the acidification period (x axis after the dashed vertical line).

pling interval (Fig. 2). *Posidonia oceanica* was the dominant species, with a surface cover of 18 to 35 %. *Peyssonnelia*, a red alga, covered between 1 and 11 % of the substratum. Their abundances were similar between months throughout the experiment. There was a slightly greater percentage of *P. oceanica* in the experimental enclosure (experimental enclosure, 31.6 ± 0.6 %; control enclosure, 27.9 ± 1.7 %; reference plot, 28.9 ± 1.3 %) throughout the experiment duration that did not appear to be related to the timing of the pH manipulation.

3.3 Leaf biometrics

There was no large difference in shoot height among the enclosures and reference plot but there were large differences in shoot height between the sampled months (Fig. 3). 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.

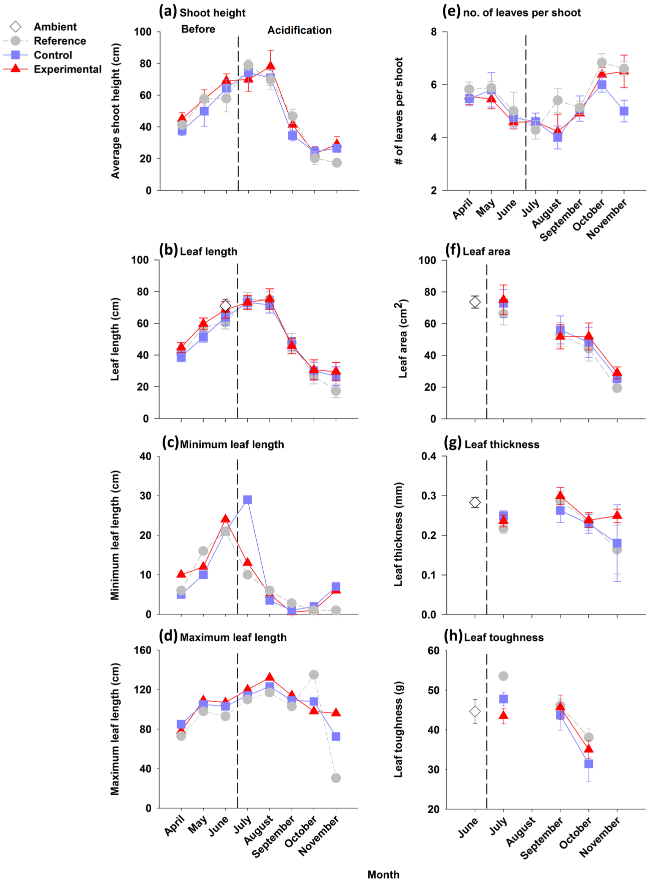

Figure 3. Leaf biometrics (mean ± SE) before and during the acidification period for the reference and enclosure plants. Measures through time: average shoot height **(a)**, leaf length **(b)**, minimum **(c)** and maximum leaf length **(d)**, number of leaves per shoot **(e)**, leaf area **(f)**, leaf thickness **(g)** and leaf toughness **(h)** are shown. The dashed vertical line indicates when the pH was lowered in the experimental enclosure. Additional leaves were collected in June from the meadow and are referred to as ambient leaves.

Shoots had between 2 and 8 leaves (Fig. 3). The reference and control plants differed slightly in leaf number per shoot (reference, 5.4 ± 0.1 vs. control, 5.1 ± 0.1), but control and experimental plants (5.2 ± 0.2) were highly similar indicating an absence of pH effect. 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. However, leaf thickness did not appear to be correlated to leaf toughness. Both parameters varied among the enclosures and reference plot, and between months (Fig. 3). Leaves in the experimental enclosure were slightly 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). Leaves in control enclosure and the reference plot for the month of November had a decreased and more variable thickness. Leaves inside the enclosures appeared to be weaker than the leaves in the reference plot. Furthermore, leaves appeared 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 obviously differed by month but not according to pH (Fig. 4). 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 rETR 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. 4a–d, show that the shape of the curve changed with month. Leaves from the control and experimental enclosures have similar rETR values that were slightly lower at elevated irradiance relative to the leaves in the reference plot.

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 ($r\mathrm{ETR}_{\max} = 10.8 \pm 0.7$, $E_k = 29.8 \pm 2.0$) and experimental ($r\mathrm{ETR}_{\max} = 12.0 \pm 0.7$, $E_k = 30.9 \pm 0.7$). However, these parameters differed by a greater amount by month than among plants from the enclosures and the plot. $r\mathrm{ETR}_{\max}$ 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 obviously 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 greatly differ (Fig. 5). α, $P_{\mathrm{g\,max}}$, and R were greater for leaves measured in September than November.

The mean total concentration of chlorophyll in leaves did not greatly differ among enclosures. 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}). The Chl $a:b$ ratio of leaves from the control and experimental enclosures did not obviously differ, with an overall Chl $a:b$ ratio of 0.64.

3.5 Growth and biomass

Leaf production and plastochrone interval of shoots in the reference plot and in the enclosures appeared to differ (Fig. 6). Differences are most congruent with an effect caused by the structure of enclosures and not from the lowered pH. The shoots in the reference plot were able to produce more leaf material than in the experimental and control enclosures. From July to September, reference shoots grew new leaf material at a mean rate of 0.89 (±0.06) cm d^{-1} compared to the reference plot and control enclosure, which both produced 0.66 (± 0.05 to 0.06) cm d^{-1}. Furthermore, reference shoots produced a new leaf in a fewer number of days than shoots in the experimental and control enclosures. From August to September, it took 11 days to produce a new leaf in the reference plot while it took between 23 and 29 days for shoots that grew in the experimental and control enclosures, respectively. Overall, leaf production (the growth of all leaves per shoot) was also seasonal. It was greater per day from September to October (1 cm d^{-1}) than during the periods July–August (0.5 cm d^{-1}) and August–September (0.6 cm d^{-1}).

At the end of the experiment, the above- and below-ground biomass was highly variable (Fig. 6). The above- and below-ground biomass ranged from 318 to 1484 and from 348 to 1584 g FW m^{-2}, respectively. The control and experiment enclosures tended to have less above-ground biomass (630 and 530 g FW m^{-2}) than the two external plots (reference: 850 and extra ambient plot: 870 g FW m^{-2}).

4 Discussion

No overwhelming impact was observed on macrophyte abundance, *P. oceanica* leaf biometrics, biomass, and photosynthesis after 4 months of elevated $p\mathrm{CO}_2$. Leaf thickness may change in response to lowered pH but requires further testing. Many of the leaf biometrics and physiology parameters varied seasonally with the varying temperature and irradiance. *Posidonia oceanica* abundance did not substantially change over 8 months as expected for a seagrass with slow rates of colonization (Marbà and Duarte, 1998). However, under ele-

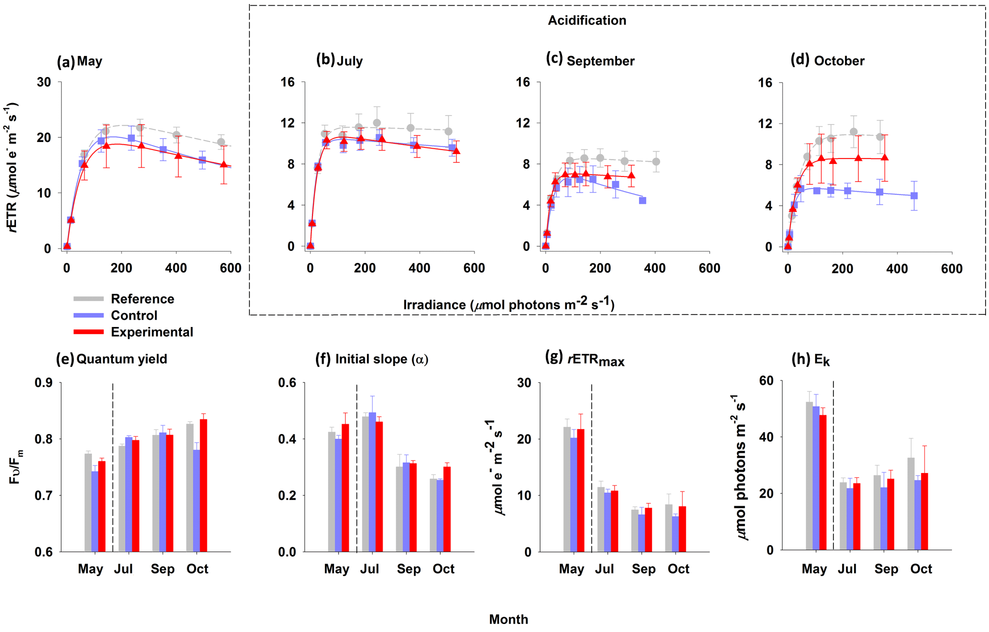

Figure 4. Photosynthetic rapid light curves (RLCs, **a–d**), dark-adapted quantum yield **(e)**, and the derived RLC parameters **(f–h)** measured on 2nd rank leaves in enclosures and reference plot before (May) and during (July, September, and October) the acidification period. Symbols represent the mean (±SE) relative electron transport rate (*r*ETR) at each mean photosynthetic active radiation (PAR) value. Curved lines represent the Jassby and Platt (1976) regression based on mean values. The dashed outline encloses the acidification period.

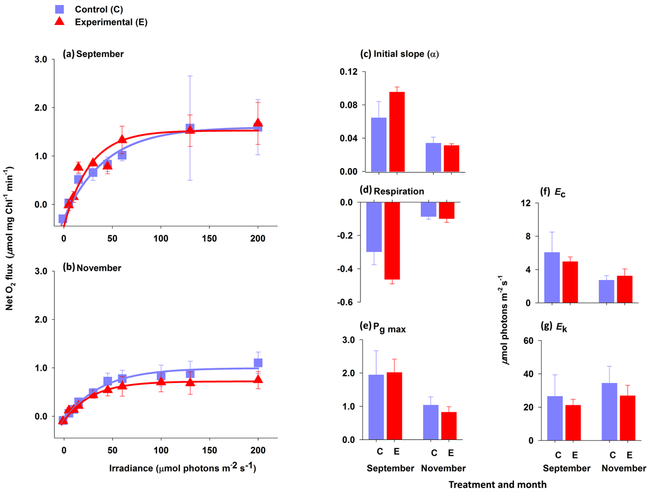

Figure 5. Photosynthesis vs. irradiance (PE) curves produced from laboratory incubations of *P. oceanica* leaf segments collected from the enclosures after two (September, **a**) and four (November, **b**) months of acidification. The derived parameters from the curves are shown in **(c)**–**(g)**.

vated $p\mathrm{CO_2}$, no other benthic macrophyte or epiphyte proliferated or decreased to alter the macro-community structure. The similarity in leaf biometrics, photosynthesis, biomass and growth between enclosures support the conclusion of limited stimulation for *P. oceanica* under future ocean acidification. However, due to tradeoffs related to experimental design, there were limitations to our conclusions.

Thickness and toughness are two structural factors related to mechanical strain (Harder et al., 2006; Littler and Littler, 1980; Padilla, 1985) and both traits were altered. Flexibility and strength are needed in environments with strong wave forces (de los Santos et al., 2013). In *Cymodocea nodosa*, another Mediterranean seagrass, leaf cross-sectional area varies with hydrodynamical forces (de los Santos et al., 2013). Therefore, observed differences in leaf toughness for plants maintained in the enclosures support the notion that mechanical abrasion was less than in ambient. This finding is an artifact of the structure that could not be avoided. In *P. oceanica*, thickness changes along the leaf axis and leaves are thinner with depth (Colombo et al., 1983). Given that the experiment was conducted at the same depth and leaves were measured at their center, it is interesting to note that leaf thickness was greatest for the shoots collected from the experimental enclosure and that this effect was driven by measures in November. An increase in seagrass leaf thickness would be an opposing effect to those observed for the upright calcified alga, *Acetabularia acetabulum*, which lost skeletal support under ocean acidification conditions (Newcomb et al., 2015). There are several possible interpretations of these results. First, leaves at the lower pH may have increased their carbon content as observed for below-ground plant structure of the seagrass *Thalassia testudinum* under elevated $p\mathrm{CO_2}$ (Campbell and Fourqurean, 2013a). Secondly, lowered pH could result in a delay of leaf shedding. Plants from the experimental enclo-

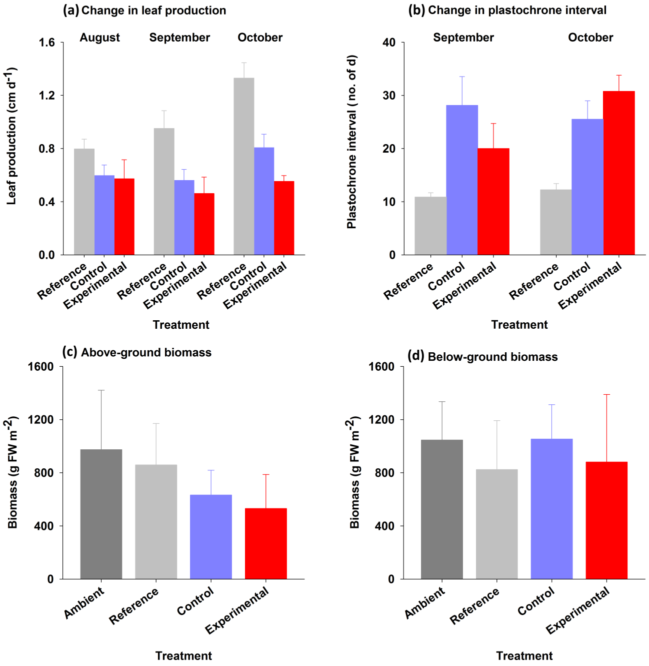

Figure 6. Growth as *P. oceanica* leaf production **(a)** and leaf plastochrone interval **(b)** during the acidification period. After 4 months of acidification, biomass (above-ground, **c**; below-ground, **d**) was determined from replicate cores collected from enclosures and the reference plot. A fourth nearby ambient area was additionally sampled to better account for spatial variation.

sure had a tendency towards relatively greater leaf length and maintenance of number of leaves in November. A prolonged leaf life-span could allow plants to scavenge nutrients from senescing leaves to maintain C / N ratio (Gobert et al., 2002). However, photosynthesis measures were not elevated by the lowered pH and thus there would be no need for increased nutrients. 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 a 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$_{\text{max}}$ and E_k) in periods with elevated irradiance and more shade-adapted when irradiance and photoperiod were reduced. The relatively lowered F_υ/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 Figueroa (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 (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 a wide range of pH$_{\text{T}}$ from 9.0 to 7.9 (scale unknown, Invers et al., 1997), or with more extreme low levels (6.98 pH$_{\text{T}}$, Hall-Spencer et al., 2008; 7.5 scale unknown, Invers et al., 2002). Within the range of 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$_{\text{T}}$ as this study ($\sim$7.7–7.8), there was also a limited stimulation of productivity (Cox et al., 2015). Similarly, modeled outcomes from laboratory studies of leaf segments by Invers et al. (1997, 2001) predicted that elevating pCO$_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 a shoot lifespan estimated up to 50 years (Gobert et al., 2006). In carbon budgets it is thought that there is 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 as gauged by RLCs, PE curves, and measures of leaf chlorophyll. Therefore, there is no available evidence that carbon availability translated into increased carbon storage as occurred for *T. testudinum* under elevated $p\mathrm{CO}_2$ (Campbell and Fourqurean, 2013a). Carbohydrates can be translocated to other ramets (Marbà et al., 2002) which can lessen observed effects but, in this case, enclosure area captured the 20 cm maximum translocation distance detected by Marbà and Duarte (1998) and edges severed (designed to penetrate ∼ 8 cm) several outside to inside shoot connections. The most productive period for above-ground growth occurred from April to August; a pattern consistent with increased growth induced from the greater availability of both light and nutrients in early spring and increased storage in July to August (Alcoverro et al., 1995, 1998, 2001; Bay, 1984; Duarte, 1989; Ott, 1980). Therefore it is possible that if the experiment were initiated earlier, in a period more conducive for biomass production, or prolonged to capture any lagging effects the outcome may have been different. Only two of six studies support a pulsed seasonal-pH interaction that could result in long-term gains yet, these were found at pH < 7.7 (see Hall-Spencer et al., (2008); Invers et al., 2002).

We caution that conclusions should not be applied to other seagrasses and that outcomes may vary with differences in community composition and environment. Presumably due to differences in their evolutionary past, some species are comparatively more responsive to lowered pH (Campbell and Fourqurean, 2013b; Invers et al., 2001; Koch et al., 2013). *Posidonia oceanica* is less sensitive to $p\mathrm{CO}_2$ and can rely heavily on bicarbonate compared to two other Pacific seagrass species (Invers et al., 2001). In addition, at CO_2 seeps in Papua New Guinea, two seagrass species (*Cymodocea serrulata* and *Halophila ovalis*) occur in mixed stands and while both species had increased productivity along the lowered pH gradient, it was only *C. serrulata* with dense below-ground biomass that had increased abundance (Russell et al., 2013); demonstrating that outcomes may be species specific, related to the plant physiology and structure, and vary with competition. Biological communities and environmental conditions are variable both within (e.g. depth) and among meadows (Hemminga and Duarte, 2000). For example, epiphyte coverage and thus level of competition were reported to be greater along control stations at Ischia, Italy (Martin et al., 2008) than in our study site, however, differences in methodology prevent direct coverage comparisons. Nutrient concentration can also alter the response of seagrass to CO_2 additions (Burnell et al., 2014; Martínez-Crego et al., 2014). Clearly our understanding of meadow dynamics under ocean acidification conditions could benefit from 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 an 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 the following: (1) the environment was continuously monitored to ensure comparisons were valid; (2) repeated measurements were made at the same location through time both before and after acidification; (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). In the current study, the decline in leaf length and 3 °C difference in temperature likely contributed to the decline of ambient $\mathrm{pH_T}$ from 8.10 to 8.01 from May to November. 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* 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 plant metabolism. 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.

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 seagrass production and help to alleviate thermal stress (Zimmerman et al., 2015) was not supported. Jordà et al. (2012) also draw attention to the continuing decline of *P. oceanica* meadows from 1990 despite the increase in CO_2 as a demonstration of the limited capacity of ocean acidification to buffer seagrass vulnerability to disturbances. 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 3 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 tools that can be used to investigate these types of impacts.

4.1 Summary, caveats, and perspectives

Any benefit from ocean acidification, over the next century, on *Posidonia* physiology and growth appears minimal. This conclusion is supported by the similarity of measures between enclosures and in context of results from other studies. We have cautioned that the eFOCE study, like all studies, has limitations. There may be small gains in plant productivity which are masked by an enclosure effect or difficult to identify without replication or more prolonged duration. We recommend that future in situ manipulative efforts use FOCE systems to control pH as an offset, as we did, and increase replication. The field of ocean acidification and future seagrass ecology could benefit from further in situ experiments that focus on combined stressors, extended experiment duration, and differences which occur over varying spatial and temporal scales (eg. within a season promoting above-ground biomass).

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Author contributions. All authors contributed to the research in this manuscript. Jean-Pierre Gattuso and Frédéric Gazeau were co-principle investigators that had the idea, oversaw the project, and were involved in data collection. Paul Mahacek was responsible for eFOCE system design. Arnaud Le Fur ensured the system functioned with assistance from Samir Alliouane, T. Erin Cox, Jean-Pierre Gattuso, and Frédéric Gazeau. T. Erin Cox was responsible for the seagrass protocol and data collection with assistance from Samir Alliouane and advice given by Iris E. Hendriks who contributed to fluorescence measures. T. Erin Cox wrote the manuscript with Jean-Pierre Gattuso and Frédéric Gazeau and all other authors contributed editorial comments.

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