**Reply to comments:**

We thank Jason Hall-Spencer, Thomas Arnold, Joerg Ott, and Jon Havenhand for their insight and comments on the manuscript "Effects of *in situ* $CO_2$ enrichment on structural characteristics, photosynthesis, and growth of the Mediterranean seagrass *Posidonia oceanica*." by Cox, Gazeau, Alliouane, Hendriks, Mahacek, Le Fur, and Gattuso. We feel that the comments have been useful to improve the ideas and research put forth within the manuscript.

We have taken into account their comments and have revised the manuscript accordingly. We believe that, with these edits, our manuscript provides critical information, which improves the current knowledge on how seagrasses will respond to future ocean acidification. The strengths of our study have been discussed in each of the comments: it is a study on an intact community, it takes into account ambient conditions and natural environmental fluctuations, it compares growth and physiology within enclosures to their natural state (reference plot), and it is the longest manipulative study to date on *P. oceanica* under lowered pH.

We strongly feel that the focus of this paper on *P. oceanica* response will have a broad appeal to carbon research community, those interested in plant physiology and ecology, and the field of coastal conservation and human impacts.

We would like to address the general concerns of all four comments by Drs. Hall-Spencer, Arnold, Ott, and Havenhand, followed by line by line responses to some of the detailed reviews by Hall-Spencer and Ott.

The discussion has focused on three main issues (outlined below) and their implications to the main findings of the study.

1) Statistical issue of pseudoreplication with the study design
2) Short-term vs. long-term effects for *Posidonia oceanica,* which is a long-lived species with ability to store carbon reserves
3) The enclosures may have caused stress

We have made two major changes to the manuscript to address these concerns. First, we removed statistical analyses and referred to the lack of deviation in parameters between enclosures and reference plots with the lowered pH treatment. Second, we now mention the constraints of our study design in the abstract and within a new section (Summary, caveats, and perspectives) in the discussion.

More specifically, below are our rationale and comments:

1- ***Issue 1:Pseudoreplication***

We are aware that the study design results in pseudoreplication. Samples were collected or measured inside the plot or enclosure through time, often 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. This was no easy task to perform a 4-month *in situ* study with highly controlled pH at diving depth for a natural community. The logistics of ocean acidification experiments, as Arnold discusses, often requires a tradeoff between well replicated studies or well controlled pH. The scale of the system is an additional constraint as pH-control is increasingly difficult as the scale of the enclosure increases.

The challenge of true replication is further magnified for a clonal plant that relies heavily on vegetative propagation. These plants have little to no genetic diversity throughout the Mediterranean Sea (Procaccini et al. 1996). Although advancement in molecular methods have revealed more genetic structure than in earlier works, this species is still characterized by low genetic polymorphism (Procaccini et al. 2002; Micheli et al. 2005). From DNA fingerprinting it has been estimated that a single genet can occupy more than 20 m (Procaccini et al 1996), emphasizing the difficulties of true replication in any study on *P. oceanica* and the need addressed by this study for multiple lines of evidence to gauge the diversity of response.

We also agree with Havenhand that just because we have pseudoreplicated does not mean data we collected do not have value and that no conclusions can be drawn. We took extra steps within the study to try to account for the limitations of our design (see initial manuscript lines 649-653 of the discussion). In contrast to statements in the comments we never stated that there was "no effect" on *P. oceanica*. In the initial and revised version of the manuscript, we have tempered our implications and conclude within the confines of our study design and within the context of outcomes from other studies, stating that results support "minimal benefit" and "limited stimulation" at a pH predicted to occur by 2100.

We agree given the comments of Arnold and Havenhand that it is better to use appropriate analyses rather than applying statistics incorrectly to analyze our data. Thus we have removed the statistical analyses in the revised manuscript and refer to the figures to interpret the scale and magnitude of the effects observed. We have revised the initial manuscript to explain this approach, replacing section 2.9. We now raise attention to the caveats of our design and thus conclusions in a new section after the discussion. The following changes have been made.

- In the abstract we edited lines 30-34 (or 33 to 39 in revised manuscript) to read: "*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 $pH_T$ of ~7.7), 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 buffering capacity of meadows.* "

- Section 2.9 has been replaced with: "**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."*

- New section at the end of the Discussion: "**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).*"

**2- *Issue 2:* Short-term vs. long-term effects for *Posidonia oceanica,* which is a long-lived species with ability to store carbon reserves**

The authors are not naïve to the life-history of *P. oceanica* and are aware of the slow ability to colonize new space and the ability to store carbohydrates (see initial manuscript lines 102, 546-548, 611-633).

Most manipulative and published studies to date investigating or modeling the impacts of lowered pH on *P. oceanica* have relied on hourly incubations of leaf segments (see Invers et al. 1997, 2001, 2002). Prior to the present eFOCE experiment, the longest published manipulative study was a laboratory experiment conducted over six weeks on isolated shoots (Cox et al. 2015). In all experiments *P. oceanica* responded in the short-term and showed major increases in productivity at a $pH_T$ of 7.3, with no detectable effect at $pH_T$ 7.7. This suggests that the plants would be expected to respond in the short-term. It is also why we are suggesting that effects may be minimal at $pH_T$ above 7.7. It is true that we do not know the long-term response to ocean acidification for the next century. The closest approximations for this at this time would be the studies conducted along $CO_2$ vents and our study adds to the growing picture.

We have discussed the implications of our findings in terms of experiment duration and carbon storage extensively in the initial manuscript (lines 611-633). We have added this caveat to our new proposed section "Summary, caveats, and perspectives" (see above) and we have added some text to the abstract to highlight the confines of experimental length (see above). We also addressed some of the specific concerns of Ott in respect to lag time in the line by line response below.

***3- Issue 3: Enclosures may have caused stress***

We understand the concern about manipulative stress and drawing conclusions from stressed plants. However, plants were left *in situ* and not cut into leaf segments nor maintained outside of their natural setting to investigate the pH impact. These are greater steps that have been taken to limit stress than in any other publication that manipulated *P. oceanica* and pH. In our experimental design, we have compared the manipulative enclosure to a control enclosure and to a reference plot, thus we have taken greater steps than many studies to assess artifacts. In laboratory studies, the manipulative treatment is often compared to a control that is handled similarly and not compared to the response in un-manipulated, natural environmental conditions. Also, even along vents stations the stress of the habitat or organisms within control stations are often not measured. They are assumed to be at optimum at the time of study (discussion in Lauritano et. al, 2015).

To address this concern, we have added a new section entitled "Summary, caveats and perspectives" at the end of the manuscript (see above). We also clarified the issue in the abstract by adding text (see above). Briefly, we used information from studies conducted in the laboratory, *in situ* incubations with modeling, and along vents in comparison and concluded with caveats. We discussed combined evidence in the initial manuscript (lines 596 to 610). The combined evidence and lack of difference between enclosures supports the conclusion of limited stimulation for *P. oceanica*.

**References cited:**

Cox, T. E., Schenone, S., Delille, J., Díaz-Castañeda, V., Alliouane, S., Gattuso, J.-P. and Gazeau, F.: Effects of ocean acidification on *Posidonia oceanica* epiphytic community and shoot productivity, J. Ecol., 103(6)1594-1609, 2015.

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Invers, O., Tomas, F., Perez, M., Romero, J., Tomàs, F., Pérez, M. and Romero, J.: Potential effect of increased global $CO_2$ availability on the depth distribution of the seagrass *Posidonia oceanica* (L.) Delile: a tentative assessment using a carbon balance model, Bull. Mar. Sci., 71(3), 1191–1198, 2002.

Lauritano, C., Ruocco, M., Dattolo, E., Buia, M. C., Silva, J., Santos, R., Olivé, I., Costa, M. M. and Procaccini, G.: Response of key stress-related genes of the seagrass *Posidonia oceanica*; in the vicinity of submarine volcanic vents, Biogeosciences, 12(13), 4185–4194. 2015.

Micheli, C., Paganin, P., Peirano, A., Caye, G., Meinesz, A. and Bianchi, C. N.:Genetic variability of *Posidonia oceanica* (L.) Delile in relation to local factors and biogeographic patterns, Aquatic Bot., 82(3), 210–221, 2005.

Procaccini, G., Alberte, R.S., Mazzella, L.: Genetic structure of the seagrass *Posidonia oceanica* in the Western Mediterranean: ecological implications. Mar. Ecol. Prog. Ser. 140, 153–160, 1996.

Procaccini, G., Orsini, L., Ruggiero, M.V.: Genetic structure and distribution of microsatellite diversity in *Posidonia oceanica*. Biol. Mar. Medit. 7 (2), 115–118, 2000

**Line by Line response to reviews follows. Response is in bold text. A marked up text also follows where edited text referred to is in red font.**

**<u>In response to Jason Hall-Spencer:</u>**

The abstract mentions speculations about the potential for increased $CO_2$ levels to confer resistance to thermal stress, yet there is no reference any published work on this in the text. Either remove it, or explain the basis of this speculation backed up with references.

**We have added the following reference and text to line 680 in the initial manuscript and now refer to the discussion by Jorda et al. (2012) (in revised manuscript lines 640-645).**

**Zimmerman, R. C., Hill, V. J. and Gallegos, C. L.: Predicting effects of ocean warming, acidification, and water quality on Chesapeake region eelgrass: Predicting eelgrass response to climate change, Limnol. Oceanogr., 60(5), 1781–1804, 2015.**

**Former line 680 now reads: "*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 draws 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.*"**

Line 67 states that variability in $CO_2$ prevents the determination of a reliable dose response relationship at seeps. This was true a few years ago but more recent work has been able to assess the $CO_2$ dose more accurately (Boatta et al. at Vulcano, Fabricius et al. in PNG, Kroeker et al. off Ischia. Change from prevents to hampers

**We have changed line 66 (in revised manuscript line 71) to "*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.*"**

Line 69: work has been carried out using the FOCE approach in Chesapeake Bay by Tom Arnold; I do not know if this has been published so this is worth checking.

**We cite Arnold et al. (2012) later in the discussion (line 579 in initial manuscript, line 531 in revised). Our FOCE system differs from Arnold et al. (2012) and differs from those cited by Campbell. In Arnold et al. (2012), $CO_2$ was bubbled directly in a free flow manner. The Campbell design delivers low pH seawater (instead of direct bubbling) but it does not control pH as a continuous offset from natural fluctuations. To be clear about our meaning we have revised the sentence at line 69 to read:**

***To the best of our knowledge, only Campbell and Fourqurean (2011, 2013a, 2014) have manipulated the partial pressure of carbon dioxide ($pCO_2$) in a controlled manner (ie. as opposed to free flow $CO_2$ bubbling) in situ within a Thalassia meadow to test the response of seagrass to ocean acidification.***

The authors have not mentioned an in situ study of the effects of increased CO2 levels on several seagrass species by Russell et al. (2013) Mar Poll Bull 73, 463-469 which I think would augment the introduction and discussion sections, especially as this investigates net primary production and respiration alongside biometrics.

**We have added Russell et al. (2013) to the citations in the introduction 63-64, and to the discussion. Line 638 in initial manuscript (now lines 598 to 593) now reads: *"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."***

**Russell, B. D., Connell, S. D., Uthicke, S., Muehllehner, N., Fabricius, K. E. and Hall-Spencer, J. M.: Future seagrass beds: Can increased productivity lead to increased carbon storage?, Mar. Pollut. Bull., 73(2), 463–469, 2013.**

I found the result provided on line 401 interesting and wonder if the authors could elaborate on what they think drove the seasonal change in seawater pH in the Discussion.

**We have added a sentence to the discussion. Line 661-662 of the initial manuscript now reads: "*In the current study, the decline in leaf length and 3°C difference in temperature likely contributed to the decline of ambient $pH_T$ from 8.10 to 8.01 from May to November."***

Line 441 confused me a little; were the plot quadrats not placed haphazardly? Please clarify.

**We had two methodological approaches (permanent vs haphazard) for two types of measurements (shoot density vs. surface cover). First type: 3 permanent quadrats, initially placed haphazardly and left in position to follow through time in order to determine shoot density. Second type: 3 to 5 quadrats placed haphazardly at each sampling interval to determine the % change in the surface cover of benthic macroflora or macrofauna. This is explained in the methods line 226 to 232 of the initial version of the manuscript and we have added text to lines 440 and 447-448 in the results section to remind the readers of the two approaches.**

***Lines 440 -441 (401-402 in revised) "There was no detectable change in shoot number (as determined in permanent quadrats re-sampled through time) related to the lowered pH in the experimental enclosure."***

**Lines 447-449(406 -407 in revised)** ***"The reference plot as well as the enclosures had very low diversity of benthic macrophytes as measured by estimates conducted within haphazardly placed quadrats at each sampling interval (Fig. 2)."***

Lines 444-451: when I read this section I began to understand perhaps why the findings of this study (little or no discernible effect of CO2 on seagrass in the test and control plots) differ from findings at various CO2 seeps. Posidonia oceanica in Italy, for example, tend to be heavily encrusted by Corallinaceae. At multiple Italian CO2 seeps this grass has much reduced calcareous epiphytic cover which presumably helps the Posidonia, as competitors for light and nutrients are removed. This may explain why seagrass is so abundant at CO2 seeps around the world. The results obtained in the high CO2 FOCE chamber in the current study may not be representative of what would be found in a more typical stand of Posidonia with its attendant coralline algal flora (see Martin et al. 2008 Biology Letters). Please consider this possibility in the Discussion section.

**This is an interesting point on interactions of species and how they may alter outcomes and this is a point that we have considered in an earlier publication (Cox et al., 2015) and hint at it in the discussion and introduction in this manuscript. We did assess aspects of the epiphyte-host interaction in Cox et al. (2015). In the laboratory, Cox et al. (2015) found that the loss of epiphyte competitors (at a similar percentage of leaf cover) did little to alter seagrass or shoot production. It is true that other locations could have greater epiphyte loads and thus more competition. This certainly indicates that more studies are needed throughout the Mediterranean to capture the diverse biology and interactions.**

**It is currently difficult to compare the degree of epiphyte competition between locations from published studies (see discussion in Borowitzka et al. (2006)). This difficulty arises from the different methods used to quantify amounts (biomass, percent surface cover, epiphyte index) and differences in sub-sampling (i.e. measures over entire shoot, random vs. oldest leaf, random portion of leaves, distal portions of older leaves, etc.), which may cause directional biases. Furthermore, studies were conducted at different times of the year and depths. Therefore, it is almost impossible to conclude whether differences and similarities between studies in epiphyte amounts are the result of method, season, or location.**

**We have edited the focus of the paragraph starting at 634 in the initial version of the manuscript to include biological and environmental variation that can alter outcomes. To specifically address this point we have added text to lines to 633, 638 to bring more emphasis to the potential variation in competition among meadows.**

- **633, start of paragraph (line 583 in revised manuscript): "*We caution that conclusions should not be applied to other seagrasses and that outcomes may vary with differences in community composition and environment.*"**

- **638 was changed to: "*Biological communities and environmental conditions are variable both within (e.g. depth) and among meadows (Hemming 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.*"**

- **Borowitzka, M. A., Lavery, P. S. and van Keulen, M.: Epiphytes of seagrasses, in Seagrasses: Biology, Ecology and Conservation, edited by A. W. D. Larkum, R. J. Orth, and C. M. Duarte, pp. 441–461, Springer, Dordrecht, The Netherlands., 2006.**

Line 558 has some discussion of the effects of increased CO2 on plant mechanical strength. Recent work by Newcombe et al. (2015) in Biology Letters showing that increased CO2 can weaken Acetabularia might be worthy of inclusion here.

**We have added the work and citation to line to 562 (line 512 in revised) of the initial manuscript "*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 at lower pH (Newcomb et al., 2015).*"**

**Newcomb, L. A., Milazzo, M., Hall-Spencer, J. M. and Carrington, E.: Ocean acidification bends the mermaid's wineglass, Biol. Lett., 11(9), 20141075, 2015.**

Line 569 unclear meaning 'discredits need for'?
**Changed to: "*However, photosynthesis measures were not elevated by the lowered pH and thus there would be no need for increased nutrients.*"**

Line 617 I don't think this paper should be drawing upon unpublished data, so the discussion of carbohydrates and carbon content can be left out for a future publication.

**We have removed and edited text to read: "*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 $pCO_2$ (Campbell and Fourqurean, 2013a).*"**

Line 631 'are mixed in support' meaning unclear

**We have removed the summary of conditions and "the mixed in support". We have focused the text at line 631 (line 580 in revised) to clarify meaning. It now reads "*Only two of six studies support a pulsed seasonal-pH interaction that could result in***

***long-term gains yet, these were found at $pH_T$ < 7.7 (see Hall-Spencer et al., 2008; Invers et al., 2002).*"**

Line 643 For the reasons set out above I do not think that this paper provides a "major advancement in our understanding of the response of Posidonia to ocean acidification" at all. It is a major advance in the use of the FOCE approach and can be presented as such, as in a methods paper.

**We respectfully disagree with the reviewer. This study does advance our understanding of the response of *Posidonia* to ocean acidification because we are addressing key needs of future perturbation experiments identified by the scientific community (see for example, Riebesell & Gattuso, 2015). The eFOCE experiment was manipulative, which is powerful to determine impacts, the duration was longer than any previous pH perturbation carried out on *P. oceanica,* it was conducted on the entire plant within its natural setting, it is the first to have pH fluctuate as it would in the natural environment. It showed that *in situ*, when pH is manipulated the response by *P. oceanica* is not overwhelming. When put into perspective with other studies, the results provide a clearer understanding of seagrass response. Each study has limitations but we do not claim that they do not advance our understanding. For example, the vent stations hamper our ability to define tipping points but they have value and can provide insight into the response of *Posidonia* or other organisms and communities to ocean acidification. The engineering and implementation are discussed in Gattuso et al. (2014) and it is not the focus of the present paper, which addresses the biological response of *Posidonia oceanica*.**

**Riebesell, U. and Gattuso, J.-P.: Lessons learned from ocean acidification research, Nat. Clim. Change, 5(1), 12–14.**

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

Line 680 – what speculation, where? Delete, or refer to published work on this.

**We have added the following reference and text to line 680 in the initial manuscript and now refer to the discussion by Jorda et al. (2012).**

**Zimmerman, R. C., Hill, V. J. and Gallegos, C. L.: Predicting effects of ocean warming, acidification, and water quality on Chesapeake region eelgrass: Predicting eelgrass response to climate change, Limnol. Oceanogr., 60(5), 1781–1804, 2015.**

**Former line 680 now reads: "*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 draws 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.*"**

Line 688 'amendable'? unclear and I think 'potentially powerful' is closer to the truth, given the difficulties of doing this sort of work and the limited sets of results to date.

**Because of the reviewer's concerns we have reworded the sentence- Line 710. However, we do think they are powerful tools and amendable (you can modify them to improve usability). There are difficulties in any field experiment but, each time they are implemented you learn to improve design and it becomes easier.**

***Line 710: "FOCE systems are tools that can be used to investigate these types of impacts."***

**In response to Ott:**

In parts the expectations of change induced by greater availability of CO2 appear a bit naïve. The life form of Posidonia resembles rather a "tree", than a "grass". With a life span of shoots of up to 50 years, as cited in line 611, little change in shoot density can be expected in an experiment lasting only 5 months. Furthermore, leaf growth is in part fueled by carbohydrate storage in the rhizomes, especially during the appearance of the new generation of leaves in fall and winter, rather than by photosynthesis alone (Pirc 1985 Marine Ecology, Pirc 1986 Aquatic Botany). The sequence of leaf appearance is probably an internal circannual rhythm (my paper in Mar. Biol. Letters 1, 1979). These properties may confound expected short-term changes and effects could possibly be found with a time lag after the end of the experiment (see for example the event cited in lines 683-684).

**See response to another comment above. We have edited the text and added "*prolonged to capture any lagging effect*" to line 626 which now reads in the revised manuscript: "*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*".**

Regarding the toughness experiments, where resistance to mechanical strain was tested in the middle of the leaf length: I have rarely observed leaves being torn at mid-leaf, when still green and healthy. Leaf erosion occurs at dead tips under heavy epiphyte cover leading to a progressive shortening of leavers in the later part of their life span. Leaves that are torn off by water movement generally break at the lunula, the preformed breaking line close to leaf base.

**It would have been a better choice to measure the toughness and thickness throughout the leaf. However, the thickness and toughness were always measured at a standard location. Thus the suggestion that they were thicker with lowered pH is still valid. The implications of this relationship are still unclear. We do not want to speculate or discuss any further and we put forth the finding as preliminary.**

Lines 415-416: What is meant by "amplification of a metabolic signal"?

**The metabolic signal is the change in $O_2$ that is driven by the metabolism of the plant. When plants are enclosed this fluctuation is amplified, that is the change in $O_2$ is larger. We have edited the revised version of the manuscript for clarity:**

**Line 415-416 changed to: "*The difference in diel change between the ambient and the enclosures was 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…*"**

Line 465: “leaf number” instead of “shoot number”
**Changed to: *“leaf number per shoot*** **“**

Line 625: I dearly miss a reference to my paper in Marine Ecology 1980 where most of the annual rhythms of leaf appearance, growth and decay, as well as production have been described for the first time.

**We apologize for the oversight. We have added the reference to line 625 of the initial manuscript.**

**Ott, J. A.: Growth and production in *Posidonia oceanica* (L.) Delile, Mar. Ecol., 1(1), 47–64, 1980.**

Lines 739-741: There is an error in the citation.
**Corrected.**

# 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 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 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 (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. 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

33 further testing. Results are congruent with other short-term and natural studies that have
34 investigated the response of *P. oceanica* over a wide range of pH. They suggest any benefit from
35 ocean acidification, over the next century (at a $pH_T$ of ~7.7), on *Posidonia* physiology and growth
36 may be minimal and difficult to detect without increased replication or longer experimental
37 duration. The limited stimulation, which did not surpass any enclosure or seasonal effect, casts
38 doubts on speculations that elevated $CO_2$ would confer resistance to thermal stress and increase
39 buffering capacity of meadows.
40
41 **Keywords:** buffering capacity, leaf biometrics, meadows, ocean acidification, oxygen fluxes,
42 PAM fluorescence, pH
43

## 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 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

68 studies were conducted in the laboratory over relatively short durations with single taxa or small
69 groups of taxa isolated from their surroundings. Although studies along carbon dioxide vents
70 allow for a whole ecosystem approach, the high spatial and temporal variability in $CO_2$ levels
71 hampers the determination of a reliable dose-response relationship (Hall-Spencer et al., 2008;
72 Kerrison et al., 2011). To the best of our knowledge, only Campbell and Fourqurean (2011,
73 2013a, 2014) have manipulated partial pressure of carbon dioxide ($pCO_2$) levels in a contained
74 (ie. as opposed to free flow $CO_2$ bubbling) manner *in situ* within a *Thalassia* meadow to test the
75 response of seagrass to ocean acidification. After 6 months of exposure to lowered pH (-0.3 from
76 mean ambient), the seagrass had increased non-structural carbohydrate content by 29% in below
77 ground structures (Campbell and Fourqurean 2014). This finding generally supports the
78 hypothesis that plant production will be stimulated from the increased carbon availability.
79 *Posidonia oceanica* is the foundation species for mono-specific meadows in the
80 Mediterranean Sea where it covers up to 23% of shallow waters (0-50 m; Pasqualini et al., 1998)
81 and provide services valued at 172 € $m^{-2}$ $year^{-1}$ (Vassallo et al., 2013). These plants are largely
82 dependent upon abiotic factors as evident by its seasonal growth and physiology (Alcoverro et al.,
83 1995, 1998; Bay, 1984; Duarte, 1989). They have been studied under a range of pH in the
84 laboratory as well as along pH gradients near $CO_2$ vents (Invers et al., 1997, 2001, 2002; Hall-
85 Spencer et al., 2008; Cox et al., 2015). Around natural $CO_2$ vents in Ischia (Italy), *P. oceanica*
86 biomass was greatest at the station nearest the $CO_2$ source with a mean $pH_T$ of 7.6 and minimum
87 of 6.98 (Hall-Spencer et al., 2008). Indeed, *P. oceanica* has a C3 photosynthetic pathway that is
88 hypothesized to benefit from increased carbon availability and its photosynthesis is not saturated
89 with respect to dissolved inorganic carbon at natural concentrations in seawater (Invers et al.,
90 1997, 2001). This is evident by their enhanced productivity in the laboratory under a pH range
91 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 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 eight 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 $m^3$ (2 m long x 1 m width x 0.85 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 $m^2$). 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 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 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.

187 **2.3 Timeline**

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

196 **2.4 Environment characterization**

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

201 Seawater samples for the determination of total alkalinity ($A_T$) levels in each enclosure
202 were taken one to five times per month from May to October (n = 11 to 12). Samples (300 mL)
203 were filtered on GF/F membranes (47 mm) and immediately poisoned with 100 µL of mercuric
204 chloride ($HgCl_2$). $A_T$ was determined on triplicate 50 mL subsamples by potentiometric titration
205 on a Metrohm Titrando 888 titrator coupled to a glass electrode (Metrohm, ecotrode plus) and a
206 thermometer (pt1000). The pH electrode was calibrated on the total scale using TRIS buffers of
207 salinity 38, corresponding to salinity in the Bay of Villefranche. Measurements were carried out
208 at 22 °C and $A_T$ was calculated as described by Dickson et al. (2007). During the experiment,
209 standards provided by A. Dickson (batch 132) were used to check precision (standard deviation)
210 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 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 x 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 x 0.5 $m^2$ quadrats within each treatment. The quadrats were also divided into four smaller squares 0.25 x 0.25 $m^2$ 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 one day. In these instances, divers returned to the treatments within 15 d (most within 8 d) 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 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 dataset 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, *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.

RLCs were produced following the procedures outlined in Cox and Smith (2015). The actinic irradiance levels ranged up to 895 $\mu$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 *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 ($\mu$mol electrons $m^{-2}$ $s^{-1}$ / $\mu$mol photons $m^{-2}$ $s^{-1}$), (2) the relative maximum electron transport rate, $r\mathrm{ETR}_{max}$ ($\mu$mol electrons $m^{-2}$ $s^{-}$

282 $^{1}$) and (3) $E_k$, optimal irradiance for maximal electron transport ($\mu$mol photons $m^{-2}$ $s^{-1}$) which is
283 determined by the equation $E_k = rETR_{max} / \alpha$.

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

288 In addition, the photosynthesis versus irradiance (PE) curves of experimental and control
289 leaf segments were produced in the laboratory using $O_2$ evolution within a series of incubations.
290 These incubations were performed over two consecutive days in September and November to
291 produce four PE curves per enclosure each month. Leaf segments (5 cm) collected from ~10 cm
292 length leaf were collected from the enclosures in the morning and incubated in the afternoon
293 (13:00 - 19:00 h, local time). Immediately after collection, leaves were stored underwater in
294 plastic bags, and transported to the laboratory in a dark mesh bag. Leaves were held for up to 3 h
295 in dim light within a temperature-controlled laboratory (20 ºC) in two open top cylindrical
296 aquaria (1.5 L). Ambient water from the nearby bay was pumped into two header tanks that fed
297 the aquaria and allowed excess water to overflow into a drainage basin. The pH in one header
298 tank was maintained at a $pH_T$ of ~7.8, corresponding to pH levels in the experimental enclosure
299 by metered additions of pure $CO_2$ controlled by a pH-stat system (IKS, Aquastar Aquatic
300 Products).

301 After carefully removing all epiphytes, segments were individually placed inside 60 mL
302 biological oxygen demand (BOD) bottles submerged into a 50 L aquarium maintained 1 to 2º C
303 to the mean monthly seawater temperature at the time of collection (21.2 ºC ± 0.2 SD). BOD
304 bottles were filled between each incubation with fresh seawater from the respective header tank
305 (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 $\mu$mol 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:

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

with:

$P_{net}$: rate of net photosynthesis ($\mu$mol $O_2$ (mg Chl)$^{-1}$ min$^{-1}$)

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

E, irradiance ($\mu$mol photons $m^{-2}$ $s^{-1}$)

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

R, respiration rate

The initial slope, α ($\mu$mol $O_2$ (mg Chl)$^{-1}$ min$^{-1}$ / $\mu$mol photons $m^{-2}$ $s^{-1}$) was calculated as $P_{g\ max}$ / $E_k$ 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.

330 **2.8 Growth and biomass**

331 Leaf production and leaf plastochrone interval were determined using the Zieman method
332 modified by Short and Duarte (2001). Three to eight shoots in both enclosures and in the
333 reference plot were marked with a plastic tag with a unique number in July, August, and
334 September. A hypodermic needle was used to punch a hole in the meristem region. These tagged
335 shoots were again located 33 to 46 d later. The distance from the puncture to the meristem was
336 measured and any new leaves that lacked a puncture were enumerated. Using these methods, it
337 was possible to calculate the number of days to produce a new leaf (plastochrone interval) and
338 leaf production per day for each shoot. Leaf production incorporates the new length added to the
339 shoot from both, the newly produced leaf (or leaves) and from the growth of older leaves.

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

348 **2.9 Pseudoreplication**

349 Samples were collected or measured inside the plot or enclosure through time, often both
350 before and after the pH manipulation. Thus the replication is equal to one for each treatment.
351 True replication was sacrificed at the expense of controlling pH as an offset, at the spatial scale of
352 the plants. Traditional inferential statistics could, therefore, not be rigorously applied and we

353 compare results graphically, paying careful attention to any divergence in values between the
354 enclosures and the reference plot.

355 **3 Results**

356 **3.1 Environment characterization**

357 The pH in the experimental enclosure was maintained at a -0.26 unit offset from the
358 control enclosure during the acidification period (Table 1). Values summarized by month showed
359 that the difference between the two enclosures was maintained close to the targeted offset (range:
360 -0.22 to -0.29 pH units). Before the pH was manipulated the offset between enclosures was
361 smaller, -0.1 to 0.06 pH units.

362 The $pH_T$ in ambient ranged from a mean of 7.98 (± 0.06 SD) in September to 8.11 (± 0.04
363 SD) in June (Table 1). The ambient $pH_T$ was similar to the $pH_T$ in the control enclosure, which
364 ranged from 7.97 (± 0.07 SD) in September to 8.12 (± 0.06 SD) in June. The greatest difference
365 between control and ambient, in monthly mean $pH_T$ values was 0.06 units. The differences in
366 $pCO_2$ reflected the magnitude of difference in $pH_T$, as $A_T$ levels were rather constant during the
367 study (see method section).

368 The mean $O_2$ concentration was similar in enclosures and in the ambient (Table S1). For
369 example, the mean $O_2$ concentration (± SD) before acidification for ambient, control and
370 experimental respectively was 258 ± 18, 254 ± 34, 258 ± 32 $\mu$mol $kg^{-1}$. In the ambient and in the
371 enclosures, the $O_2$ concentration fluctuated over the course of the day (data not shown). After
372 sunset, $O_2$ concentration declined to a night-time minimum. In the morning, the $O_2$ began to
373 increase to a daily afternoon maximum; then it declined with decreasing irradiance. Over the
374 months of the experiment, this diel $O_2$ change ranged from 21 to 72 $\mu$mol $kg^{-1}$ in the ambient, 34
375 to 95 $\mu$mol $kg^{-1}$ in the control enclosure, and 34.5 to 100.5 $\mu$mol $kg^{-1}$ in the experimental
376 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 $\mu$mol $kg^{-1}$ 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 $\mu$mol $kg^{-1}$, 4.7 to 7.7 mol photons $m^{-2}$ $d^{-1}$) and decreases in September to November (mean monthly range: 193 to 211 $\mu$mol $kg^{-1}$, 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 very low diversity of benthic macrophytes as measured by estimates conducted within haphazardly placed quadrats at each sampling 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.

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 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.

446 The AF factor for the calculation of *r*ETR changed with month. The determined values (as
447 a mean ± SD) were as follows: May: 74.5; July: 65.0; September: 69.6 ± 1.5 (n = 3); October,
448 54.2 ± 0.0 (n = 2).
449 The photosynthetic RLCs in Fig. 4 (A-D), show that the shape of the curve changed with
450 month. Leaves from the control and experimental enclosures have similar *r*ETR values that were
451 slightly lower at elevated irradiance relative to the leaves in the reference plot.
452 The initial slope (α, $\mu$mol electrons $m^{-2}$ $s^{-1}$ / $\mu$mol photons $m^{-2}$ $s^{-1}$) ranged between 0.23
453 and 0.58 (n = 57). It decreased substantially as a function of time (Table S6) from elevated values
454 in May (0.43 ± 0.01) and July (0.48 ± 0.01) to lower values in September (0.31 ± 0.01) and
455 October (0.27 ± 0.01). Overall (n = 57), $r\text{ETR}_{max}$ values (in $\mu$mol electrons $m^{-2}$ $s^{-1}$) ranged from
456 4.3 to 27.4 and $E_k$ ($\mu$mol photons $m^{-2}$ $s^{-1}$) ranged from 12.0 to 63.6. The leaves from the reference
457 plot had $r\text{ETR}_{max}$ (12.3 ± 0.6) and $E_k$ (33.7 ± 2.0) that were more different than the leaves from
458 the control ($r\text{ETR}_{max}$ = 10.8 ± 0.7, $E_k$ = 29.8 ± 2.0) and experimental ($r\text{ETR}_{max}$ = 12.0 ± 0.7, $E_k$ =
459 30.9 ± 0.7). However, these parameters differed by a greater amount by month than among plants
460 from the enclosures and the plot. $r\text{ETR}_{max}$ values were substantially higher in May (22.1 ± 1.4)
461 than in July (10.9 ± 0.8), September (7.2 ± 0.6), and October (7.5 ± 0.8). Overall, $E_k$ was
462 obviously greater in May (50.2 ± 2.2) than in July (23.1 ± 2.6), September (24.5 ± 2.1), and
463 October (28.1 ± 2.5).
464 The parameters of the PE curves of leaves collected from the experimental and control
465 enclosures also did not greatly differ (Fig. 5). α, $P_{g\,max}$, and R were greater for leaves measured in
466 September than November.
467 The mean total concentration of chlorophyll in leaves did not greatly differ among
468 enclosures. It was 0.36 ± 0.04, 0.38 ± 0.04 mg Chl $cm^{-2}$ in the experimental and control
469 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 to 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 four months of elevated $pCO_2$. Leaf thickness may change in response to lowered pH but requires further testing. Many of the leaf biometrics and

494 physiology parameters varied seasonally with the varying temperature and irradiance. *Posidonia*
495 *oceanica* abundance did not substantially change over eight months as expected for a seagrass
496 with slow rates of colonization (Marbà and Duarte 1998). However, under elevated $pCO_2$, no
497 other benthic macrophyte or epiphyte proliferated or decreased to alter the macro-community
498 structure. The similarity in leaf biometrics, photosynthesis, biomass and growth between
499 enclosures support the conclusion of limited stimulation for *P. oceanica* under future ocean
500 acidification. However, due to tradeoffs related to experimental design, there were limitations to
501 our conclusions.

502 Thickness and toughness are two structural factors related to mechanical strain (Harder et
503 al., 2006; Littler and Littler, 1980; Padilla, 1985) and both traits were altered. Flexibility and
504 strength are needed in environments with strong wave forces (de los Santos et al., 2013). In
505 *Cymodocea nodosa,* another Mediterranean seagrass, leaf cross-sectional area varies with
506 hydrodynamical forces (de los Santos et al., 2013). Therefore, observed differences in leaf
507 toughness for plants maintained in the enclosures support the notion that mechanical abrasion was
508 less than in ambient. This finding is an artifact of the structure that could not be avoided. In *P.*
509 *oceanica*, thickness changes along the leaf axis and leaves are thinner with depth (Colombo et al.,
510 1983). Given that the experiment was conducted at the same depth and leaves were measured at
511 their center, it is interesting to note that leaf thickness was greatest for the shoots collected from
512 the experimental enclosure and that this effect was driven by measures in November. An increase
513 in seagrass leaf thickness would be an opposing effect to those observed for the upright calcified
514 alga, *Acetabularia acetabulum*, which lost skeletal support under ocean acidification conditions
515 (Newcomb et al., 2015). There are several possible interpretations of these results. First, leaves at
516 the lower pH may have increased their carbon content as observed for below-ground plant
517 structure of the seagrass *Thalassia testudinum* under elevated $pCO_2$ (Campbell and Fourqurean,

518 2013a). Secondly, lowered pH could result in a delay of leaf shedding. Plants from the
519 experimental enclosure had a tendency towards relatively greater leaf length and maintenance of
520 number of leaves in November. A prolonged leaf life-span could allow plants to scavenge
521 nutrients from senescing leaves to maintain C/N ratio (Gobert et al., 2002). However,
522 photosynthesis measures were not elevated by the lowered pH and thus there would be no need
523 for increased nutrients. Additionally, increased $pCO_2$ and high light increased leaf shedding for
524 the seagrass *Amphibolis antarctica* (Burnell et al., 2014). The response was linked to proliferation
525 of filamentous epiphytes, which did not occur in this study. Alternately, increased leaf thickness
526 could be the result of chance. The plausible relationship warrants further investigation in field
527 experiments with prolonged duration and increased replication.
528 If indeed leaf thickness increases with ocean acidification, it is unclear how this would
529 impact herbivore feeding. The main herbivores, the fish, *Sarpa salpa*, and the sea urchin
530 *Paracentrotus lividus*, feed preferentially on the adult and thicker leaves (Peirano et al., 2001).
531 These herbivores were prevented from grazing in enclosures. Arnold et al. (2012) have reported
532 increased rates of fish grazing on the plant at proximity of a $CO_2$ vent, presumably due to the
533 significant decreases in the production of phenolics. To date, very few studies have focused on
534 plant-herbivore interactions under elevated $pCO_2$ levels (Asnaghi et al., 2013; Campbell and
535 Fourqurean, 2014; Poore et al., 2013) and as plant-herbivore interactions were not the focus of
536 this study, it is not known how this would have impacted the results.
537 To our knowledge, this is the first *in situ* study to repeatedly and over several months (6)
538 measure *P. oceanica* fluorescence to find that the second rank leaves showed a typical seasonal
539 pattern of plant acclimation (Boardman, 1977). Leaves were more sun-adapted (relatively higher
540 $rETR_{max}$ and $E_k$) in periods with elevated irradiance and more shade-adapted when irradiance and
541 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 (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 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 $pCO_2$ (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 $pCO_2$ 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

590 both species had increased productivity along the lowered pH gradient, it was only *C. serrulata*
591 with dense below ground biomass that had increased abundance (Russell et al., 2013);
592 demonstrating that outcomes may be species specific, related to the plant physiology and
593 structure, and vary with competition. Biological communities and environmental conditions are
594 variable both within (e.g. depth) and among meadows (Hemming and Duarte, 2000). For
595 example, epiphyte coverage and thus level of competition were reported to be greater along
596 control stations at Ischia, Italy (Martin et al., 2008) than in our study site, however, differences in
597 methodology prevent direct coverage comparisons. Nutrient concentration can also alter the
598 response of seagrass to $CO_2$ additions (Burnell et al., 2014; Martínez-Crego et al., 2014). Clearly
599 our understanding of meadow dynamics under ocean acidification conditions could benefit from
600 repeated *in situ* studies that address issues such as species differences, more prolonged durations,
601 herbivore-plant interactions, and temporal and spatial effects.
602 Performing this experiment *in situ,* over several months, is an advancement for
603 understanding the response of *P. oceanica* to ocean acidification. The eFOCE design has
604 advantages to other mesocosm systems such as its large size which allows for measuring
605 processes at the scale of a meadow, its ability to monitor the environment in real-time, and its
606 ability to maintain pH as an offset. Though replicated enclosures would have been preferred and
607 are recommended for future use, their implementation was not feasible at this stage. However,
608 several steps were taken to eliminate possible erroneous conclusions including: (1) the
609 environment was continuously monitored to ensure comparisons were valid, (2) repeated
610 measurements were made at the same location through time both before and after acidification (3)
611 comparisons from the pH manipulated enclosure were made to at least two different spatial
612 locations and (4) results obtained in laboratory and natural experiments were compared and are in
613 general agreement. The duration of this study was longer than any previous pH perturbation

614 carried out on *P. oceanica* and it was performed in the most natural conditions possible. This
615 study addresses a need for manipulative experiments done *in situ* for longer durations to make
616 best predictions of future marine ecology (see Gattuso et al., 2014).
617 Our findings have implications for the function of future meadows. Seagrasses through
618 their metabolic activity alter the chemical properties of the meadow. In daylight, seagrasses draw
619 down the available dissolved inorganic carbon and at night their respiration has the opposite
620 effect (Hendriks et al., 2014a). The daily change in pH has been shown to be up to 0.24 pH units
621 and to be related to the density and length of leaves (Hendriks et al., 2014a). In the current study,
622 the decline in leaf length and 3°C difference in temperature likely contributed to the decline of
623 ambient $pH_T$ from 8.10 to 8.01 from May to November. Hendriks et al. (2014b) has suggested
624 that (1) organisms within the meadow may not be as vulnerable to ocean acidification because
625 they are adapted to large diel pH changes (2) the productivity of *Posidonia* during the day may
626 buffer the impacts of ocean acidification, particularly for calcifiers by providing a daily window
627 of maximum calcium carbonate saturation where calcification can be more efficient and (3) ocean
628 acidification could stimulate seagrass productivity and thus increase buffering capacity; which
629 was not supported by the results of this present study. Considering the two previous proposed
630 hypotheses, the median diel pH variation for the meadow in this study was ~0.1 and also
631 appeared to be driven by plant metabolism. However, the median diel pH range in the
632 experimental enclosure was two to three times larger than the control (0.09 to 0.29 pH units) and
633 exhibited greater variability; a finding that would be missed in typical experiments which lower
634 pH and maintain it at a constant future level(s). The variation in diel pH cannot solely be
635 explained by $O_2$ fluxes. The increased diel pH fluctuation could largely be the result of the
636 reduced buffering capacity of seawater at lowered pH (Shaw et al., 2013). The lowered and larger

637 diel pH variation and lack of productivity stimulation casts doubt on the adaptability of organisms
638 to future pH change and the ability of a *P. oceanica* meadow to serve as a future refuge.
639 Ocean acidification is not occurring in isolation, warming has been predicted to result in a
640 complete extinction of *P. oceanica* meadows by the year 2049 (Jordà et al., 2012). The
641 speculation that increased $CO_2$ availability would enhance seagrass production and help to
642 alleviate thermal stress (Zimmerman et al., 2015) was not supported. Jordà et al. (2012) also
643 draws attention to the continuing decline of *P. oceanica* meadows from 1990 despite the increase
644 in $CO_2$ as a demonstration of the limited capacity of ocean acidification to buffer seagrass
645 vulnerability to disturbances. It confirms observations after an explosive episode at a $CO_2$ vent
646 which resulted in an extreme lowering of pH (4.7 to 5.4) and elevated temperatures (28-30 °C, 3
647 to 5 °C above ambient). Along this vent, *P. oceanica* experienced a decrease in growth that
648 persisted for three years (Vizzini et al., 2010). The extreme nature of the vent activity,
649 confounding biological differences found at vent sites (e.g. Vizzini et al., 2013), and the possible
650 change in physiology under combined stressors make it difficult to predict future meadow
651 ecology. It underscores the need to investigate stressors concurrently and *in situ*. The FOCE
652 systems are tools that can be used to investigate these types of impacts.
653 4.1 Summary, caveats, and perspectives
654 Any benefit from ocean acidification, over the next century, on *Posidonia* physiology and
655 growth appears minimal. This conclusion is supported by the similarity of measures between
656 enclosures and in context of results from other studies. We have cautioned that the eFOCE study,
657 like all studies, has limitations. There may be small gains in plant productivity which are masked
658 by an enclosure effect or difficult to identify without replication or more prolonged duration. We
659 recommend that future *in situ* manipulative efforts use FOCE systems to control pH as an offset,
660 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).

*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 S. Alliouane, T.E. Cox, J.-P. Gattuso, and F. Gazeau. 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.

*Acknowledgements*

We would like to acknowledge the following people who assisted in the laboratory, in the field, or with system engineering or maintenance: E. Beck Acain, J. Acain, J. Delille, L. van der Heijden, M. Maillot, F. Moullec, S. Schenone, L. Urbini, K. Walzyńska. We also thank J.-J. Pangrazi, R. Patrix, and E. Tanguy for aide in construction of the enclosures. Éric Béraud, G. de Liege, D. Luquet, L. Mangialajo, S. Reynaud, and D. Robin kindly assisted in diving activities. We are grateful to C. Ferrier-Pagès and her research team for use of their PAM fluorometer. We also thank B. Kirkwood at Monterey Bay Aquarium Research Institute who advised in system design. We thank the Service d'Observation Rade de Villefranche and the Service d'Observation en Milieu Littoral for their kind permission to use Point B data. We also thank the Service National d'Analyse des Paramètres Océaniques du $CO_2$ for performing the determination of $A_T$ at Point B. This work was funded by the 'European Free Ocean Carbon Enrichment' (eFOCE; BNP

685 Paribas Foundation), the European Commission through the project 'Mediterranean Sea
686 Acidification in a changing climate' (MedSeA; grant agreement 265103) and the MISTRALS-
687 MERMEX (INSU, CNRS) program.

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915 **Figure captions**

916 **Figure 1**. Schematic of the system and study design (A) see text for details (B): the pH (total
917 scale) inside the enclosures and in ambient during the week-long transition to the targeted offset
918 (-0.25 units).

919

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

926

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

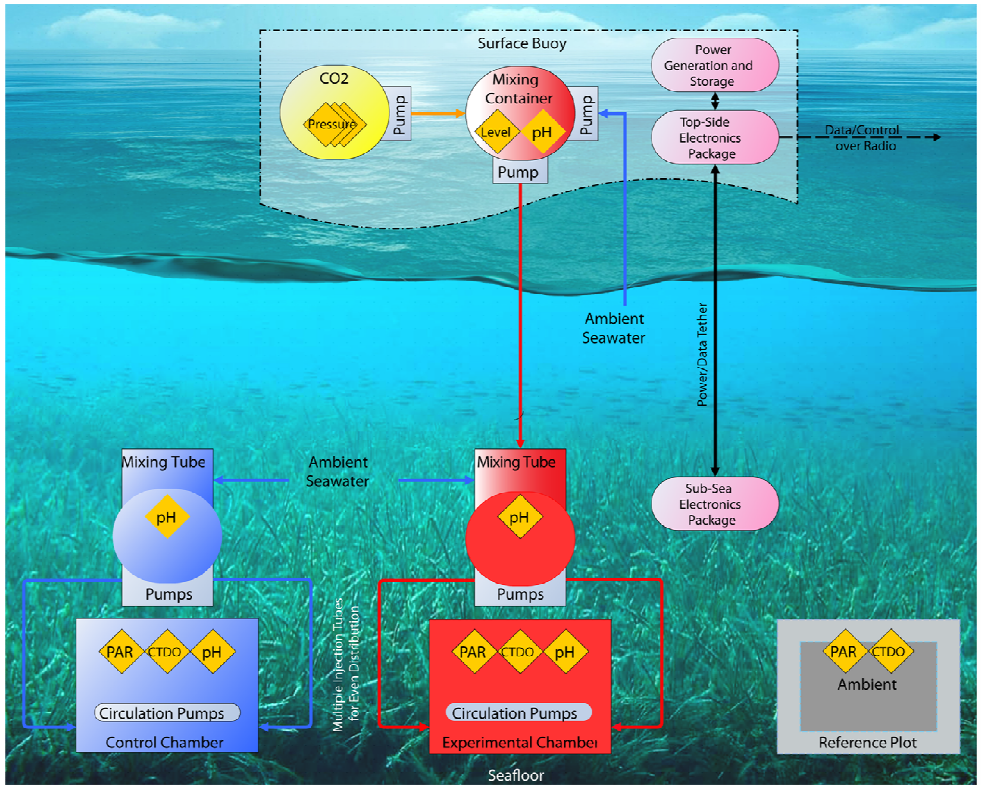
933

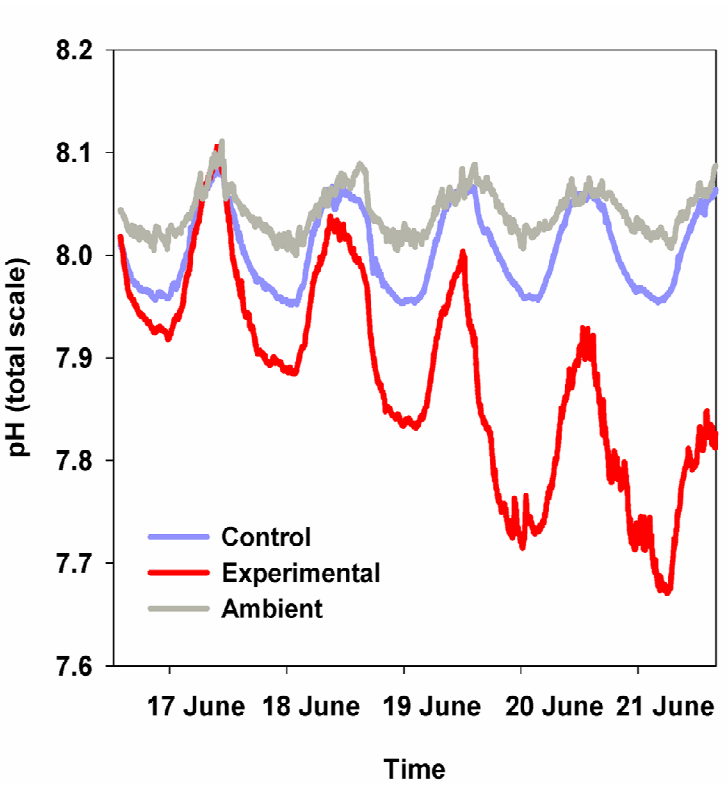
934 **Figure 4**. Photosynthetic rapid light curves (RLCs, A-D), dark-adapted quantum yield (E), and
935 the derived RLC parameters (F-H) measured on 2$^{nd}$ rank leaves in enclosures and reference plot
936 before (May) and during (July, September, and October) the acidification period. Symbols
937 represent the mean (±SE) relative electron transport rate (*r*ETR) at each mean photosynthetic

938 active radiation (PAR) value. Curved lines represent the Jassby and Platt (1976) regression based
939 on mean values. The dashed outline encloses the acidification period.
940
941 **Figure 5.** Photosynthesis versus irradiance (PE) curves produced from laboratory incubations of
942 *P. oceanica* leaf segments collected from the enclosures after two (September, A) and four
943 (November, B) months of acidification. The derived parameters from the curves are shown in
944 panels C-G.
945
946 **Figure 6.** Growth as *P. oceanica* leaf production (A) and leaf plastochrone interval (B) during the
947 acidification period. After 4 months of acidification, biomass (above-ground, C; below-ground,
948 D) was determined from replicate cores collected from enclosures and the reference plot. A fourth
949 nearby ambient area was additionally sampled to better account for spatial variation.

**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.

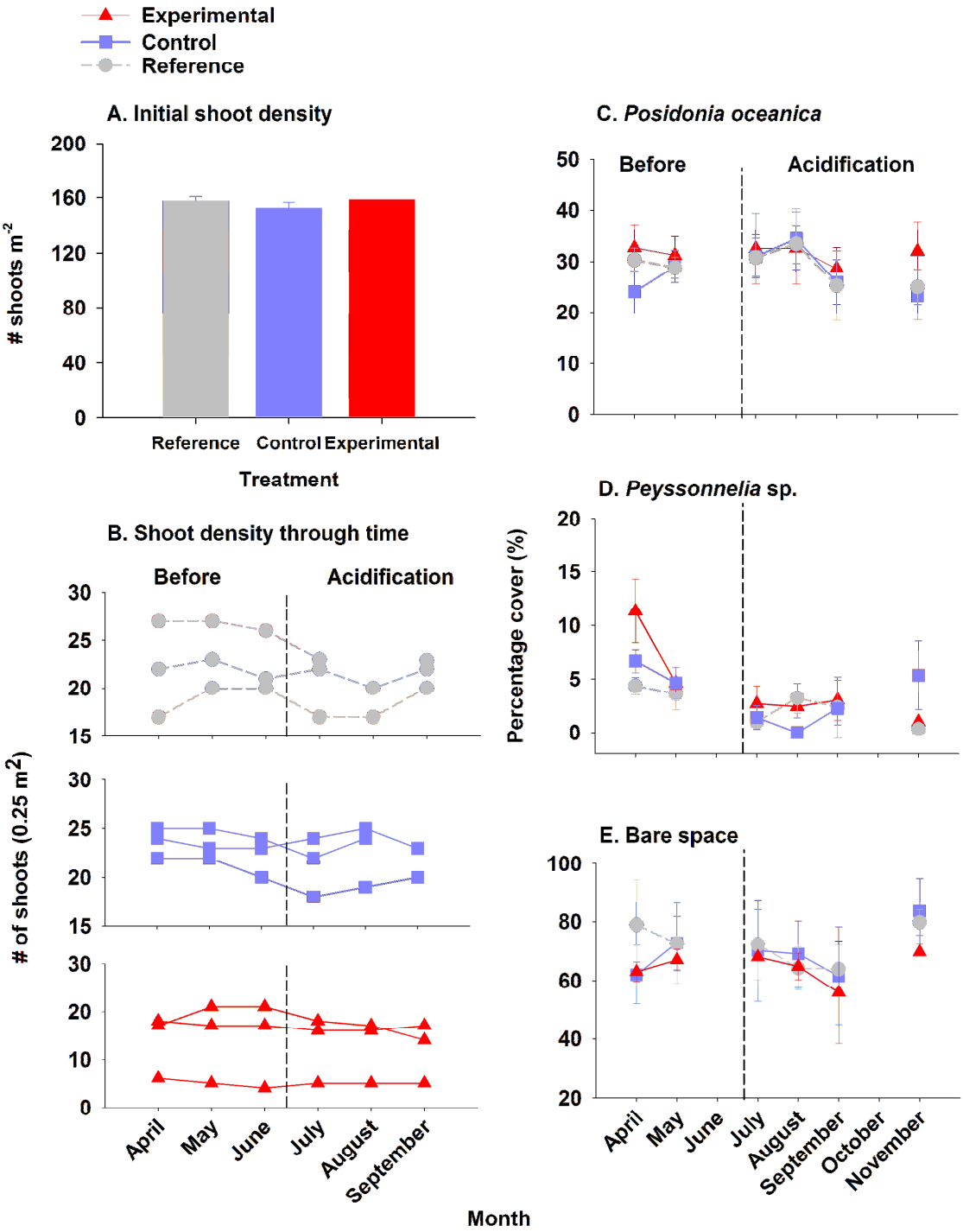
| | | **$pH_T$** | | | | | | | | **$pCO_2$** ($\mu$atm) | | | | | | **Δ Diel $pH_T$** | | | | | | | **Δ Diel $O_2$** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Ambient | | Control | | Experimental | | Diff | | 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 | 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 | 14.0 |
| 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 | 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 | 18.0 |
| 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 | 12.0 |
| 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 | 11.5 |
| 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 | 5.0 |
| 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 | 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 | 13.0 |
| 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 | 23.0 |



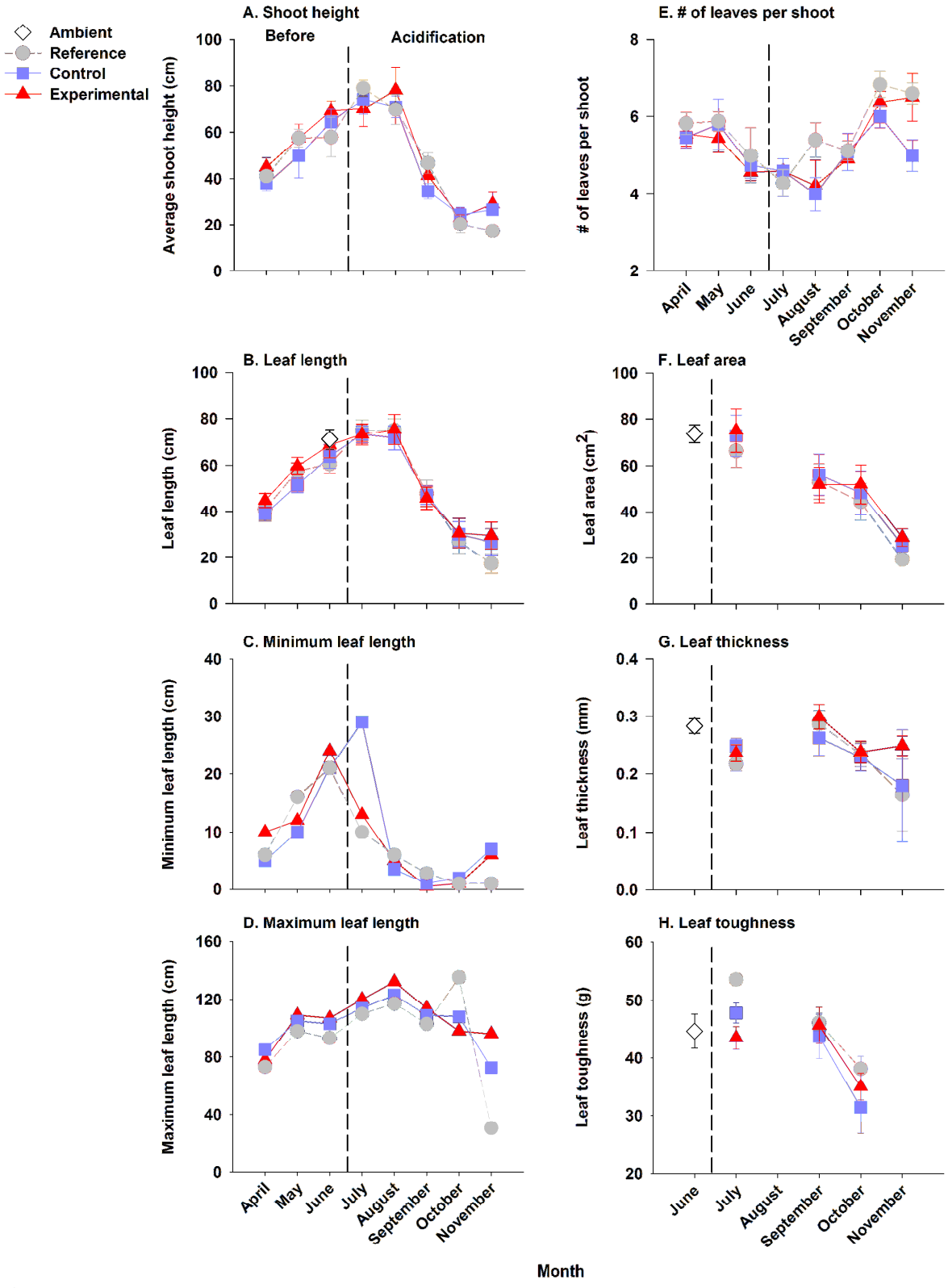




954
955 **Figure 1**.

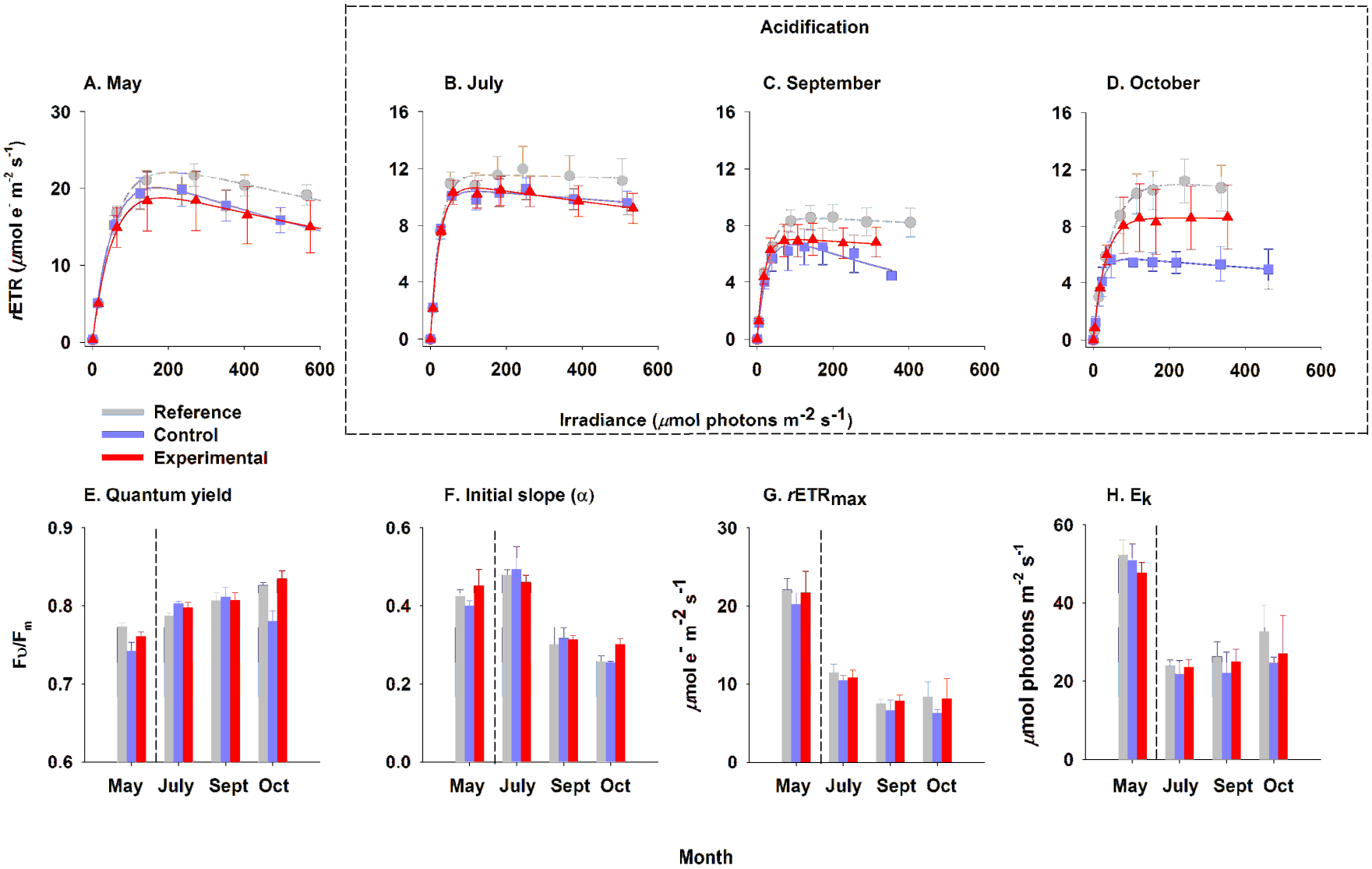
956



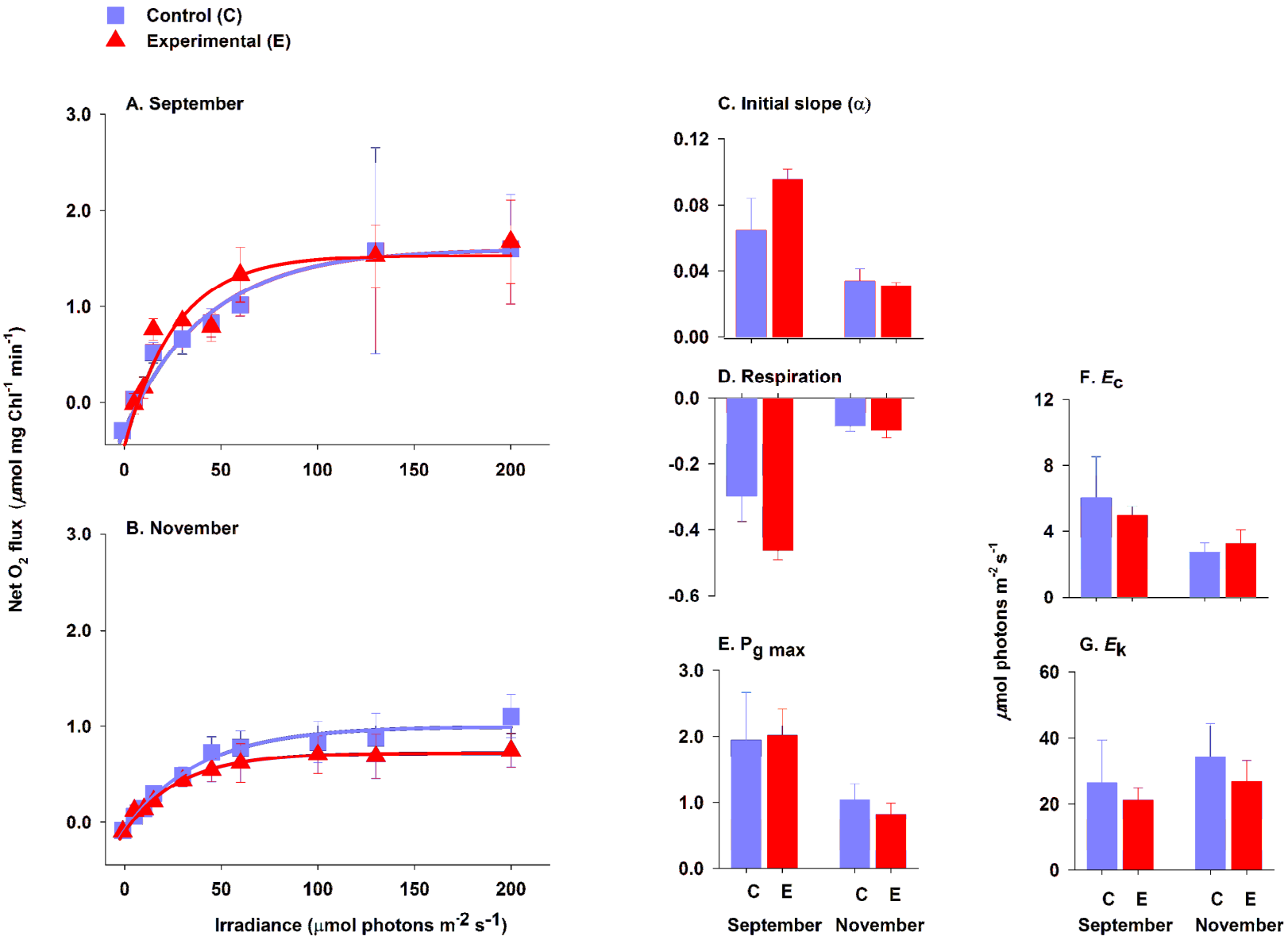

957
958 **Figure 2.**




959
960 **Figure 3.**




961
962 **Figure 4.**




963
964 **Figure 5.**

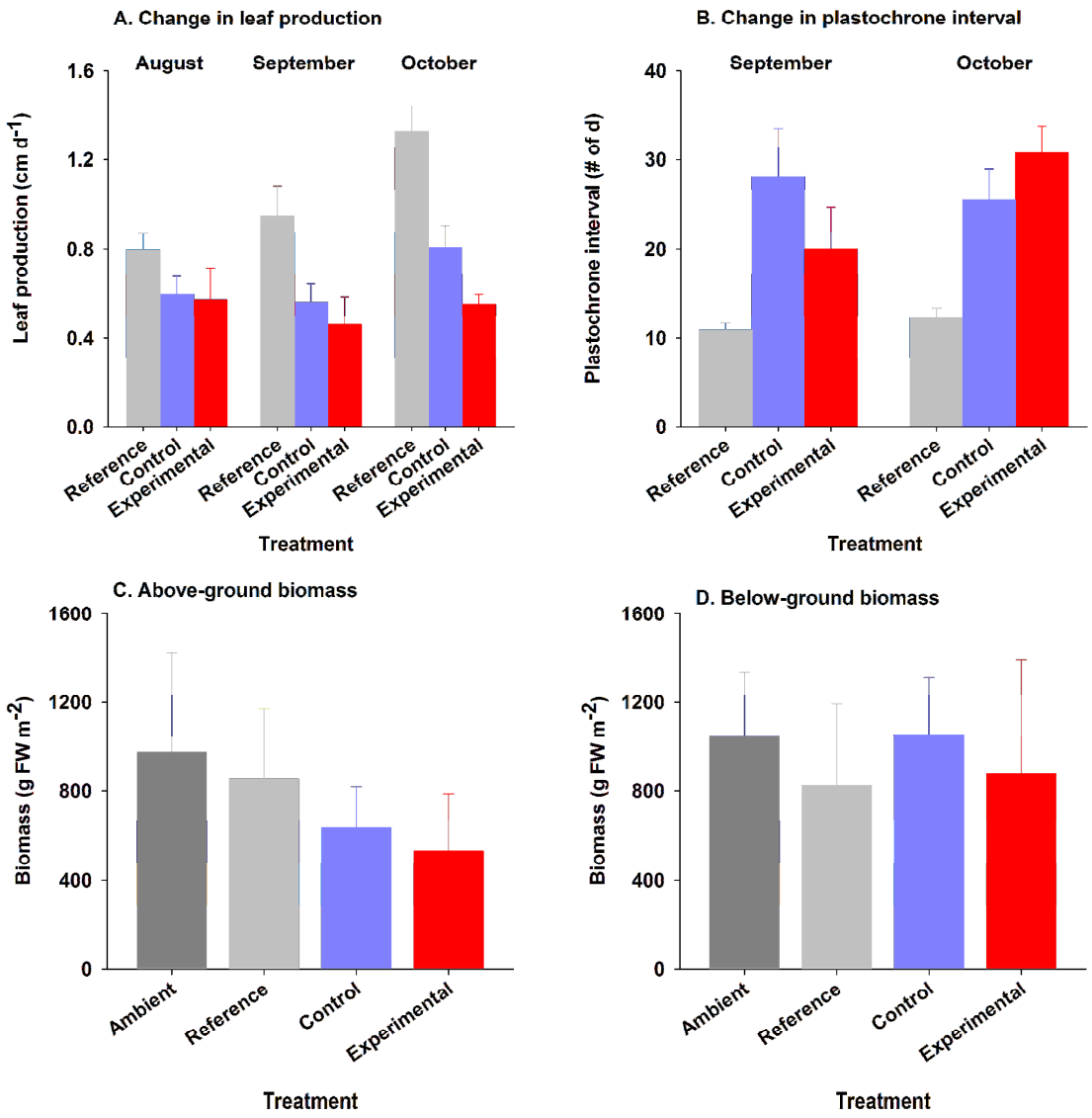

A. Change in leaf production
August
September
October
Leaf production (cm d-1)
1.6
1.2
0.8
0.4
0.0
Reference
Control
Experimental
Treatment
B. Change in plastochrone interval
September
October
Plastochrone interval (# of d)
40
30
20
10
0
Reference
Control
Experimental
Treatment
C. Above-ground biomass
Biomass (g FW m-2)
1600
1200
800
400
0
Ambient
Reference
Control
Experimental
Treatment
D. Below-ground biomass
Biomass (g FW m-2)
1600
1200
800
400
0
Ambient
Reference
Control
Experimental
Treatment

**Figure 6.**