# Interactive comment on "$CO_2$ and nutrient-driven changes across multiple levels of organization in *Zostera noltii* ecosystems" by B. Martínez-Crego et al.

B. Martínez-Crego et al.

bmcrego@ualg.pt

REPLY TO REVIEWER #1 COMMENTS

In this reply version, our answer to technical corrections on pages 5256 (L2-4), 5257 (L10-11), and 5251 (L13-14) has been modified to jointly address interlinked Reviewer#1 and #2 comments (see below). Technical corrections accepted without further clarifications are also listed.

We greatly appreciate the comments and detailed suggestions by the anonymous referee. The referee provided two major comments, two specific comments, and several technical corrections for manuscript improvement or for detail clarifications. Regarding the general comments, as the referee correctly points out, our experimental design allows for determining the nature of the interaction between stressors (synergistic or antagonistic) but not the existence of thresholds or linear responses. This latter point was out of the scope of our study. The referee acknowledges that, in spite of the low replication of treatments, significant differences were detected that provided a general insight on the seagrass ecosystem responses at several levels of the biological organization. This general view is the major aim of the present work and it suggests ways forward for future research.

Referee's specific comment – "*The authors attribute the lack of grazer control of epiphyte growth to either an imbalance between epiphyte growth and grazing activity or a non-specialist grazer being the most abundant. Is it possible that the shift in epiphyte assemblages towards cyanobacteria which were less palatable for the grazers present?*"

This would be a particular case in which the most abundant mesograzer in our experiment, *Cymadusa filosa*, was not targeting the epiphytes that were mostly composed by cyanobacteria in the $CO_2$-enriched treatment. However, cyanobacteria are palatable and structurally accessible food for mesograzers (e.g. Vargas et al. 1998- J. Phycol. 34, 812–817; Nagarkar et al. 2004- Hydrobiologia 512: 89–95). At the same time, as stated in this paragraph, small invertebrate grazers (amphipods, isopods, gastropods) have been widely recognized as controllers of epiphyte overgrowth under nutrient enrichments (Hughes et al., 2004-Mar Ecol Prog Ser 282, 87-99), including cyanobacteria blooms (Neckles et al. 1993-Oecologia, 93, 285-295). We have revised the paragraph according the referee's suggestion, considering the particular control of the cyanobacteria overgrowth by the amphipod *Cymadusa filosa* rather than a general epiphyte control by grazers (L539-545 in the marked-up manuscript version, see below).

Referee's specific comment – "*Overall, the statistical analysis is sound, however I would question*

*why t-tests were used instead of post-hoc analysis (e.g. SNK, Tukeys or an appropriate test if variances were heterogeneous)? The ANOVA is the appropriate test to use and does not need to be confirmed by t tests. Nor do t tests tell you anything about interactions."*

Regarding the first part of this remark, post hoc comparisons were not possible in the factorial design because we only have two levels (unfertilized and enriched) within each factor ($CO_2$, Nutrients). When significant interactions were detected, we needed another test to detect significant differences between the 4 treatments separately considered (Unfertilized, $CO_2$-enrichmed, Nut-enriched, and $CO_2$-Nut-enriched). Given the unequal variances found for all variables under this design (1 factor: treatment, with 4 levels) and that the homogeneity of variances is an important assumption in the ANOVA (see Quinn and Keough 2002), we chose to use Welch's t test (robust against unequal variances) instead SNK or Tukey's comparisons after one-way ANOVA.

We agree with the referee that the ANOVA is the appropriate test and does not need to be confirmed by t tests. We have revised this paragraph to incorporate the remarks made by the referee (L272-280 in the marked-up manuscript version). We have clarified that within the design of the two-way ANOVA (two fixed crossed factors), a normal distribution with unequal variances was found for all variables, which is usual when the sample size is small. Following recommendation by Quinn and Keough (2002), we proceeded with the analyses but making significance level more restrictive to minimize the possibility of Type I error (mistakenly detection of differences). Fig. 2 and 5 (and Fig.2 legend), Appendix D, and text in the manuscript have been modified accordantly (e.g. changes in L364-386).

Regarding the technical corrections suggested by the referee, we have either accepted all edits and minor corrections or have added most suggested clarifications. Clarifications are addressed in detail as follows:

*"Page 5245, Line 18: It is not clear what three-five leaves means and why it is not standardised to a single number"* - Five shoots were used to quantify the number of leaves and the LAI at the beginning and at the end of the experiment, while three shoots were used in between sampling events. We have revised this paragraph to clarify this referee's remark (L202-207).

*"Page 5245, Line 28: How were epiphytes removed?"* - Epiphytes were removed with a glass slide to minimize damage on leaf surface. This has been clarified in the revised version (L216).

*"Page 5247, Line 8: I don't think export is the correct term. How do you know this carbon would not have settled back to the sediment within the bed?"* – The referee is right. In seagrass meadows, detritus production can be exported by currents and waves to neighboring systems (e.g. deep systems or beaches), enter in the trophic web through decomposers or detritivores, or be buried under the meadow becoming part of the carbon sink (Pergent et al. 1994-Mar Ecol Prog Ser 106, 139-146; Cebrián and Duarte 2001-Aquatic Botany, 70, 295-309; see also references already cited in the manuscript: Pidgeon 2009, Fourqurean et al. 2012). To avoid any confusion, we have removed the

allusion of detritus production as indicator of carbon export (L258).

*"Page 5247, Line 19: Greenhouse-Geisser adjustments actually correct the degrees of freedom, not the Significance levels (although significance levels will also change)"* - The referee is right. We have corrected this sentence (L270).

*"Page 5251, Lines 13-14: Should read "...with only purple bacteria appearing in them."* - This sentence has been altered to highlight the attenuation of nutrient-induced changes in the epiphyte assemblage by the simultaneous addition of $CO_2$ (L400-403).

*"Page 5255, Line 13: Surely there are more recent papers than (Fenchel 1977) to cite."* - Fenchel 1977 has been replaced by more recent references that highlight the relevance of detritus-based food webs in seagrass meadows (i.e. Pergent et al. 1994-Mar Ecol Prog Ser 106, 139-146; Moore and Fairweather 2006-Oikos 114, 329-337).

*"Page 5256, Lines 2-4: The sentence beginning with "To avoid toxicity, . . .." is confusing and the latter part needs revising for clarity."* - The paragraph containing this sentence (L604-609) has been rewritten in the revised version to address also Reviewer#2 remark about this paragraph (comment#10).

*"Page 5257, Lines 10-11: "The effect of nutrient enrichment was higher in the low-nutrient than in the high-nutrient meadow (Fig. 5a vs. b)." This belongs in results section."* - Reviewer#2 also made a similar comment (#13). We have revised the entire paragraph to incorporate a proper discussion and contextualization of the results as Reviewer#1 and #2 requested (L677-712).

*"Page 5258, Line 7: The sentence beginning with "Blooms of the, . . .." is confusing and the latter part needs revising for clarity."* - The sentence has been rewritten and the confusing part has been removed (L717-718).


List of technical corrections accepted without further clarifications:
Page 5243, Line 11: Should read "...assess if they react differently."
Page 5245, Line 28: Should read "...five shoots per pot."
Page 5247, Line 19: Greenhouse-Geisser adjustments actually correct the degrees of freedom, not the Significance levels (although significance levels will also change)
Page 5251, Line 28: Replace "low" with "minor" or "small".
Page 5255, Line 19: Replace "stronger" with "greater".
Page 5255, Line 27: Replace "a scarce" with "minimal".
Page 5258, Line 19: Should read "...CO2 or nutrient enrichment. . .. . ...." - This correction has also been incorporated through the manuscript (e.g. L387, 669, 713, 728)
Page 5258, Line 25: Replace "with no" with "without a".
Page 5262, Line 1: Hughes et al. 2004 – List of minor authors has first names rather than surnames

listed (e.g. Susan, L. W. should be Williams, S. L.)

List of technical corrections that refer to paragraphs that were modified in the revised version following Reviewer#2 suggestions:

Page 5253, Line 26: Replace "evidenced" with "observed".

Page 5254, Line 6: Remove ", however,".

Page 5256, Line 5: Replace "since" with "as".

Page 5257, Line 27: Should read "...meadow supports the hypothesis that nutrient. . .. . ...."

Page 5258, Line 20: Replace "of" with "in".

REPLY TO REVIEWER #2 COMMENTS

We thank the reviewer for the useful comments, which helped to improve the manuscript by flushing out our oversights and clarifying key aspects. The comments are presented below, followed by our responses.

Referee's specific comment #1 - *Lines 8-10, p5241: Please provide citations to support these statements.*

The reviewer refers to the sentence "This body of research has revealed that ocean acidification can be detrimental to most marine calcifying organisms, while increasing carbon dioxide ($CO_2$) concentration can benefit primary productivity of phytoplankton, cyanobacteria, fleshy algae, and seagrasses". This sentence is connected to the previous one, in which the reviews by Doney et al. (2009) and Kroeker et al. (2010) are cited for supporting the statements. No change done.

Referee's specific comment #2- *Lines 27-28, p5241: Please provide citations to support these statements.*

A reference has been added (Kroeker et al. 2013b-Nature Clim. Change 3, 156-159).

Referee's specific comment #3 - *Line 1, p5254: Any reason why all effects on epiphytes and sediments were attenuated under both $CO_2$ and nutrient enrichment.*

Regarding changes in the epiphyte community, we found that the interactive effect resulted in the attenuation of both, the nutrient-induced changes in the epiphyte assemblages and the $CO_2$-induced bloom of the cyanobacterium *Microcoleus* spp. The most likely explanation is that the interspecific competition between the species that dominated the epiphyte community under high $CO_2$ (the cyanobacterium *Microcoleus* spp.) and under high nutrient levels (diatoms of the genus *Navicula*) resulted in the observed attenuation under simultaneous addition of $CO_2$ and nutrients of the direct effects of individual stressors on certain taxa. Negative effects of interspecific competition on the involved species (i.e. symmetrical competition) have long been reported by ecologists (Connel, 1983)

and support this explanation.

Regarding changes in the sediments of the low-nutrient meadow, we observed similar organic matter content in the sediment under simultaneous $CO_2$ and nutrient addition and control conditions, which were lower than under individual enrichments. The simultaneous addition of $CO_2$ and nutrients might keep microbial decomposition rates in the sediment at control levels, as opposed to the probable acceleration observed under independent $CO_2$ or nutrient enrichment. Our results under individual enrichments contrast with previous studies conducted in situ with different seagrass species. Antón et al. (2011) found that meadow carbon sequestration was unaffected by nutrient addition, whereas Russell et al. (2013) observed increased carbon sequestration in response to $CO_2$ enrichment. These authors, however, did not assess the interactive effect of nutrients and $CO_2$ on carbon sequestration. Moreover, they used whole-community metabolism instead organic matter in the sediment as indicator of carbon sequestration.

We have better explained the responses to the simultaneous $CO_2$ and nutrient additions in the Discussion, highlighting the key role of species interactions in the attenuation of direct effects of individual stressors. The first paragraph of the discussion, to which the reviewer refers here, has been removed from this position, incorporated into the Abstract and discussed in each appropriate sub-section of the Discussion (see comments below and L657-675 and 728-788 in the marked-up manuscript version). In addition, we have highlighted the role of species interactions in mediating the individual and interactive effects of eutrophication and acidification in ecosystem functioning through the manuscript (Discussion, Results, and Abstract).

Referee's specific comments #4 and #8- *Line 11, p5254: However, this finding is in contradiction to much empirical research for seagrasses. Please address. (Jiang et al. 2010, Campbell & Fourqurean 2013). - Line 23, p.5254: Yet note that in a subsequent study Campbell 2013 Mar Biol document increases in carbohydrate content with elevated $CO_2$, along with Jiang et al 2010, Palacios 2007, and Zimmerman et al 1997. Clearly both nutrient regime and $CO_2$ levels can have an effect on carbohydrate levels and should be acknowledged. (Zimmerman et al. 1997, Jiang et al. 2010, Campbell & Fourqurean 2013)*

These two specific comments refer to the same paragraph and are interlinked, so we answered and addressed them together. We found that $CO_2$ enrichment had no direct effects on *Z. noltii* biochemistry, with no significant changes on the total carbohydrate reserves. This finding contrasts with observations in the seagrass *Thalassia hemprichii* (Jiang et al., 2010) and *T. testudinum* (Campbell and Fourqurean, 2013).

We have rewritten this entire paragraph to acknowledge that our results are different from others and to reinforce the idea that the plant responses to elevated $CO_2$ levels are highly context- and species-specific, and are not as readily consistent and predictable as resource availability hypotheses would suggest. References have been modified accordingly. The effects of nutrient regime on the

carbohydrate accumulation have been addressed in the sub-section ´High- vs. low- nutrient meadows´, where we discuss that the accretion of carbon based-compounds in *Z. noltii* might be driven by nutrient deficiency, thus, reinforcing the idea of the context-dependence of seagrass response to high $CO_2$ (L705-712).

Referee's specific comments #5 to #7 - *Line 12, p.5254: So the seagrasses from the low-nutrient meadows were not nutrient limited? What evidence do you have to suggest a trade-off between phenolic production and growth? I currently don't see data to support this conclusion. - Line 16, p.5254: Did $CO_2$ enrichment actually enhance LAI? Is this supported by your statistical analyses. - Line 20, p.5254: Were your plants really not under any degree of light limitation? Didn't the excessive epiphyte loading reduce light levels?*

Again, these three specific comments refer to the same paragraph than the previous remarks and are interlinked. We agree with the referee. As stated in the Results section, Figure 1b suggests that the *Z. noltii* leaf area index (LAI) tended to increase with $CO_2$ enrichment until the third week of the experiment. However, this trend was not statistically significant (no significant $CO_2$ effect or $CO_2$ x Time interaction in the RM ANOVA). Consequently, our results did not provide significant arguments to support a trade-off between phenolic production and growth. As the referee suggests, the lack of significant $CO_2$-induced changes in *Z. noltii* productivity can be explained by nutrient or light limitation to seagrass growth. Given the huge epiphyte overgrowth induced by $CO_2$ enrichment, we pointed out to light limitation mediated by epiphyte shading as the most likely reason.

We have revised and rewritten this entire paragraph to accommodate these and previous remarks (L475-524). We highlight that we found no significant increase of seagrass productivity under $CO_2$ enrichment to support the trade-off between phenolic accumulation and plant growth.

Referee's specific comment #9 - *Line 2, p.5255: Cite (Campbell & Fourqurean 2014)*
The reference has been added.

Referee's specific comment #10 - *Line 5, p. 5256: But don't you document declines in shoot recruitment and LAI?*
The referee is right. The paragraph has been modified and combined with the next paragraph to explain that the increased shoot mortality under nutrient enrichment can be linked to ammonium toxicity and/or to a reduction in light availability caused by the dense epiphytic layer of pennate diatoms and purple bacteria (L604-613). References have been modified accordingly. This change also addresses Reviewer#1 remark about this paragraph.

Referee's specific comment #11 - *Line 17 p. 5256: Any chance that this excess organic matter was simply exported out of the mesocosms due to the experimental set up?*
No chance. Any export of organic matter should equally affect all treatments, but it was not apparent in the control conditions as reflected by the relatively high organic matter content in the sediment of

the unfertilized mesocosms.

Referee's specific comment #12 - *Line 22 p.5256: Any explanation for this statement? The first sentence of this paragraph needs clarification / explanation.*

This paragraph has been modified also in reply to the referee´s comment #3. As explained above, the simultaneous addition of $CO_2$ and nutrients attenuated the nutrient-induced changes in the epiphyte assemblages and the $CO_2$-induced increase in the total epiphyte load through the alteration of competitive dynamics and species interactions within the epiphyte community.

Referee's specific comment #13 - *Line 4- 17 p.5257: This paragraph reads more like the results section. Please revise.*

Reviewer#1 also made a similar comment for lines 10-11 in p5257. We have revised the entire paragraph to incorporate a proper discussion and contextualization of the results as Reviewer#1 and #2 requested (L677-712).

Referee's specific comment #14 - *Line 19 p.5258: But if I understand correctly, combined $CO_2$ and nutrients had no effect on carbon sink capacity. Why might this be the case?*

See above answer to comment #3.

# CO$_2$ and nutrient-driven changes across multiple levels of organization in *Zostera noltii* ecosystems

**B. Martínez-Crego[1], I. Olivé[1], and R. Santos[1]**

[1]{Centre of Marine Sciences (CCMAR), Universidade do Algarve - Campus de Gambelas, 8005-139 Faro, Portugal}

Correspondence to: B. Martínez-Crego (bmcrego@ualg.pt)

**Abstract**

Increasing evidence emphasizes that the effects of human impacts on ecosystems must be investigated using designs that incorporate the responses across levels of biological organization as well as the effects of multiple stressors. Here we implemented a mesocosm experiment to investigate how the individual and interactive effects of CO$_2$ enrichment and eutrophication, scale-up from changes in primary producers at the individual- (biochemistry) or population-level (production, reproduction, and/or abundance) to higher levels of community (macroalgae abundance, herbivory, and global metabolism) and ecosystem organization (detritus release and carbon sink capacity). The responses of *Zostera noltii* seagrass meadows growing in low- and high-nutrient field conditions were compared. In both meadows, the expected CO$_2$ benefits on *Z. noltii* leaf production were suppressed by epiphyte overgrowth, with no direct CO$_2$ effect on plant biochemistry or population-level traits. Multi-level meadow response to nutrients was faster and stronger than to CO$_2$. Nutrient enrichment promoted the nutritional quality of *Z. noltii* (high N, low C:N and phenolics), the growth of epiphytic pennate diatoms and purple bacteria, and shoot mortality. In the low-nutrient meadow, individual effects of CO$_2$ and nutrients separately resulted in reduced carbon storage in the sediment, probably due enhanced microbial degradation of more labile organic matter. These changes, however, had no effect on herbivory nor on community metabolism. Interestingly, individual effects of CO$_2$ or nutrient addition on epiphytes, shoot mortality, and carbon storage were attenuated when both nutrients and CO$_2$ acted simultaneously. Thus reflecting CO$_2$-induced benefits on eutrophic meadows. In the high-nutrient meadow, a striking shoot decline caused by amphipod overgrazing

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masked the response to $CO_2$ and nutrient additions. Our results reveal that under future scenarios of $CO_2$, the responses of seagrass ecosystems will be complex and context dependent, being mediated by epiphyte overgrowth rather than by direct effects on plant biochemistry. Overall, we found that the responses of seagrass meadows to individual and interactive effects of $CO_2$ and nutrient enrichments varied depending on interactions among species and connections between organization levels.

## 1 Introduction

Understanding community and ecosystem responses to human impacts is a challenge that requires integrating not only the organism-level responses across populations and entire systems (Russell et al., 2012), but also synergistic or antagonistic effects of multiple stressors (Woodward et al., 2010). A large number of articles has been published on the effects of ocean acidification, and reviewed among others by Doney et al. (2009) and Kroeker et al. (2010). This body of research has revealed that ocean acidification can be detrimental to most marine calcifying organisms, while increasing carbon dioxide ($CO_2$) concentration can benefit primary productivity of phytoplankton, cyanobacteria, fleshy algae, and seagrasses. Our current understanding of these effects is largely based on the species-specific responses of individuals or populations. However, the broad variability in responses among organisms may influence species interactions and drive unforeseen impacts on marine communities and ecosystems (Hall-Spencer et al., 2008; Kroeker et al., 2013a).

The interactive effect of multiple stressors on ecological communities remains largely unknown (Crain et al., 2008). Atmospheric $CO_2$ concentration has increased from preindustrial levels of approximately 280 ppm to 397 ppm in 2013 (NOAA, Mauna Loa Observatory, Hawaii), leading to a rise in the $CO_2$ absorbed by the ocean with an associated pH decrease of 0.1 units. An additional pH decrease of 0.07-0.31 units is expected by the end of the $21^{th}$ century based on the `Intergovernmental Panel on Climate Change´ predictions (IPCC, 2013). In the marine environment, ocean acidification can locally interact with excess nutrients from coastal eutrophication to accelerate changes in ecosystem structure and functioning (Russell et al., 2009).

Human impacts that alter the availability of environmental resources are shifting the nutritional quality of primary producers through changes at the biochemical or

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90    individual levels of the biological organization (e.g. allocation of resources to growth,
91    storage, and chemical defences). In addition, changes in environmental resources can
92    favour different types of producers and alter the inter-species competitiveness and
93    producers´ abundances (Kroeker et al., 2013b). Overall, this may affect ecological
94    interactions and fluxes, leading to shifts at community and ecosystem levels. Under
95    elevated nutrient concentrations, aquatic and terrestrial ecosystems with higher producer
96    nutritional quality often support higher rates of herbivory, more rapid decomposition
97    rates and recycling of nutrients, and lower net accumulation of soil carbon (Wardle et
98    al., 2004; Cebrian et al., 2009). The effects of nutrient enrichment have been widely
99    described on terrestrial, freshwater, and marine ecosystems, whereas the scaling up of
100   elevated $CO_2$ effects has been mostly studied in terrestrial plants. Under elevated $CO_2$
101   levels, and especially if nutrient availability is limiting to growth, terrestrial plants
102   typically increase the accumulation of carbohydrates and/or carbon-based secondary
103   compounds (mostly phenolics). This increases C:N ratios ("nitrogen dilution" effect)
104   and sometimes leaf toughness through increasing indigestible polymers such as
105   cellulose and lignin (Zvereva and Kozlov 2006; Lindroth, 2010; Robinson et al., 2012).
106   Herbivores usually compensate for this lower food quality by eating more (Stiling and
107   Cornelissen, 2007). In addition, $CO_2$ enrichment may shift the biomass and composition
108   of soil microbial communities, directly through different responses of microbial groups
109   to high $CO_2$ / low pH (Krause et al., 2012; Lidbury et al., 2012) or indirectly through
110   reducing foliar and detritus quality (Drigo et al., 2007). Overall, high $CO_2$ levels may
111   have both positive and negative consequences on the decomposition of soil organic
112   matter and nutrient recycling (Lindroth, 2010).

113   In this study, we use seagrass meadows as model ecosystems to investigate the scaling
114   up of the effects of elevated $CO_2$ and nutrient levels on marine coastal environments.
115   Seagrasses beds are widely distributed habitats that host high biodiversity and provide
116   valuable ecosystem services (Orth et al., 2006). A rich epiphyte community usually
117   colonizes seagrass leaves, thus providing a useful system for studying how changes in
118   environmental resources can favour different types of producers (e.g. non-calcareous vs.
119   calcareous). They are highly productive systems that sequester larger amounts of carbon
120   per area than tropical forests, providing for a long-term removal of carbon dioxide from
121   the atmosphere (Pidgeon, 2009; Fourqurean et al., 2012). The maintenance of the key

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services provided by seagrass ecosystems under global change is thus of prime importance for human well-being.

A mesocosm experiment was conducted to assess: (1) how $CO_2$ and nutrient enrichments affect primary producers' at the individual- (plant biochemistry including CN and allocation of resources to carbohydrate reserves and carbon-based chemical defences) or population-level (plant allocation of resources to biomass and reproduction, and composition and abundance of seagrass epiphytes), and (2) whether these changes propagate to the community (macroalgae abundance, meso-herbivory, whole-community metabolism) and to the ecosystem (detritus production and organic carbon storage in sediment). The responses of meadows of the seagrass *Zostera noltii* Horneman developing in low- and high- nutrient conditions in the field were compared to assess if they react differently.

## 2 Methods

### 2.1 Study meadows

Samples of *Z. noltii* community were collected from two meadows separated 5.5 km from each other within the Ria Formosa lagoon (South Portugal). This shallow mesotidal lagoon is dominated by monospecific beds of the seagrass *Z. noltii* that occupy ca. 45% of the intertidal area. One meadow was developing under prior field conditions of low nutrient levels (36º59´40´´N 7º58´00´´W; hereafter low-nutrient meadow) and the other under high nutrient levels (37º01´15´´N 8º00´56.50´´W; hereafter high-nutrient meadow). Low-intertidal samples exposed to a small emersion period only during low spring tides were selected. Table 1 presents seawater nutrient concentrations and seagrass meadow traits that reveal the substantial initial differences between meadows.

### 2.2 Mesocosm experiment

The study was conducted in an outdoor mesocosm system at the Ramalhete field station of the Centre of Marine Sciences, which is located at the Ria Formosa lagoon. To assess the effects of $CO_2$ and nutrients on *Z. noltii* meadows an enrichment experiment was conducted for 6 weeks during August-September 2011, after 4 days of acclimation to

163 the experimental mesocosms. This time span is enough to detect any treatment-driven
164 changes in physiological, morphological and population traits of this fast-growing
165 species (e.g. Peralta et al., 2002). Core samples of *Z. noltii* community, including
166 sediment and algal, faunal and microbial components, were randomly collected from
167 each donor meadow and allocated to flowerpots of 20 cm of diameter and height. Three
168 flowerpots were placed in each of the 16 experimental mesocosms (tanks of 110 L),
169 which were exposed to combinations of two $CO_2$ and two nutrient levels in a crossed
170 design with two replicates.

171 Experimental levels of $CO_2$ encompassed present (pH $8.00\pm0.02$, equivalent to ca. 400
172 ppm $CO_2$) and future conditions (pH $7.83\pm0.01$, equivalent to ca. 800 ppm $CO_2$) in Ria
173 Formosa lagoon. The mesocosms received sand-filtered seawater from two head tanks
174 of 1000 L at a rate of 240 L $h^{-1}$. In one of the head tanks, the water $CO_2$ was
175 manipulated to ensure fixed pH differences between treatment means within the range
176 predicted for 2100 by the IPCC (pH decline = -0.18 units) following a commonly used
177 method (e.g. Alsterberg et al., 2013). The $CO_2$ injection was controlled by an auto-
178 analyzer (Yokogawa, EXAxt 450, Tokyo, Japan), which continuously monitored the
179 water pH and temperature. Total alkalinity, pH, temperature and salinity within the
180 mesocosms, as well as the seawater DIC and carbon speciation are provided on
181 Appendix B (Supplement).

182 Water nutrient levels encompassed the natural values found in the lagoon and the values
183 of highly eutrophic conditions (N: 45x and P: 11x natural, see Appendix B,
184 Supplement). The nutrient enrichment was obtained by adding a solubilised mixture of
185 the fertilizers ammonium nitrate and monoammonium phosphate directly into the water
186 column of each enriched mesocosm using a multi-channel dosing pump. Water samples
187 were collected weekly to analyze nutrient concentrations using a loop-flow analyzer
188 ($\mu$Mac-1000; Systea, Anagni, Italy).

189 The water within mesocosms was homogenized using a submersible circulation pump
190 placed at leaf height. Pumps were stopped 2 h twice per day to simulate tidal currents.
191 Twice a week, the epiphytes growing in the mesocosm walls were removed and the
192 position of flowerpots within each mesocosm was reassigned to minimize potential
193 spatial differences. Natural settlement and growth of leaf epiphytes and small animals
194 were allowed throughout the experiment.

## 2.3 Producers´ traits at the individual- or population-level

Changes on producers´ at the individual or population levels were assessed by measuring: (1) plant biochemistry and allocation of resources to biomass and reproduction; and (2) the composition and abundance of seagrass epiphytes.

Allocation of plant resources to biomass and reproduction at the population-level was monitored almost every week. Shoot recruitment or mortality were quantified within each flowerpot excluding shoots growing around the border to avoid edge effects. Allocation to reproduction was quantified as density of flowering shoots. Five shoots at the beginning and at the end of the experiment and three shoots in between sampling events were randomly chosen within each flowerpot to quantify the number of leaves and the leaf area index (LAI) as indicators of aboveground productivity. To estimate LAI, leaf area was measured on these shoots, averaged, multiplied by the number of shoots within the pot, and scaled per surface area.

After four weeks of experiment, leaf epiphyte composition was determined in the oldest leaves of three randomly chosen shoots. The surface covered by each taxon was quantified under a microscope and standardized per 10 cm$^2$ of leaf area.

After six weeks, all shoots from each mesocosm were harvested and plant traits quantified in each flowerpot. Belowground productivity was estimated from the vertical or horizontal rhizome length and from the total root length (number of roots multiplied by the average root length) of five shoots per pot. The above- and below-ground biomass allocation was quantified after drying at 60 ºC until constant weight. The pooled epiphyte load of three shoots was removed using a glass slide and quantified as relative to leaf area after drying at 60 ºC until constant weight. Pooled material of five shoots was separated into leaves (without epiphytes) and rhizomes, freeze-dried, weighted, ground to fine powder and used in subsequent analyses of plant biochemistry. Carbon and nitrogen concentrations were analyzed using an elemental analyzer (Carlo-Erba, Milan, Italy). Total non-structural carbohydrates were measured in rhizomes using the phenol-sulfuric acid colorimetric method (Dubois et al., 1956) with glucose as standard, after sugar extraction in hot ethanol and enzymatic conversion of starch to glucose equivalents (Smith and Zeeman, 2006). Total phenolics were quantified as indicators of plant allocation of resources to chemical defences. Phenolics were extracted from leaf material with methanol 50% for 24 h under constant agitation at 4

Bego Martínez 17/5/2014 13:57
**Deleted:** The number of leaves of three-five randomly chosen shoots within each flowerpot and the leaf area index (LAI) were quantified as indicators of aboveground productivity. To estimate LAI, leaf area was measured on these three-five shoots, averaged, multiplied by the pot number of shoots and scaled per surface area

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236 ºC and determined with a spectrophotometer using chlorogenic acid as standard

237 following a modified Folin-Ciocalteu method (Bolser et al., 1998).

## 2.4 Community- and ecosystem-level traits

239 The response of the seagrass community to $CO_2$ and nutrient enrichments was

240 quantified weekly by: (1) the percentage of flowerpot surface covered by *Ulva* spp.; (2)

241 the feeding activity of mesograzers (percentage of leaves showing bite marks in the

242 same shoots used to measure the morphological traits); and (3) the whole-community

243 metabolism quantified from the oxygen evolution within benthic chambers of 17 cm

244 diameter (4.8±0.01 L incubated) fitted to the flowerpots for 30-45 minutes at midday

245 (12-14 h). A transparent acrylic chamber to estimate net production and a dark chamber

246 to estimate respiration were simultaneously deployed within each mesocosm. Dissolved

247 oxygen concentration was measured by spectrophotometry using the Winkler method

248 (Labasque et al., 2004) in three water samples collected before and after incubations

249 into 12 mL soda glass vials. Community metabolism was estimated from the net change

250 in oxygen concentration during incubations integrated by the chamber volume and

251 standardized by incubation time and bottom area. There were no effects of enclosure on

252 the water temperature within chambers (measured with onset HOBO loggers, Southern

253 MA, USA). All incubations were run under irradiances of photosynthetically active

254 radiation (PAR) averaging 283±8.6 μmol quanta $m^{-2}$ $s^{-1}$ (measured with a Li-192SA

255 underwater PAR quantum sensor, Li-Cor, USA), when *Z. noltii* photosynthesis is light

256 saturated and not photoinhibited (Peralta et al., 2002).

257 At the ecosystem level, detritus production (fresh weight of all floating material

258 collected during a 24h period) was quantified almost every week. Organic matter in the

259 sediment (loss of dry weight after combustion at 450 ºC, 4h) was measured at the end of

260 the experiment as indicator of the carbon sink capacity.

## 2.5 Statistical analyses

262 The effects of $CO_2$ and nutrient treatments throughout the experiment were tested using

263 three-way repeated-measures analyses of variance (RM ANOVA). The subject

264 repeatedly sampled was the mesocosm, $CO_2$ and nutrients were the among-subject

265 factors (two fixed crossed factors) and Time the within-subject factor. To avoid the

266 masking effect of the strong initial differences between meadows on the responses to

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268 experimental treatments, data for the low- and high-nutrient meadows were analyzed
269 separately. Data were checked for parametric assumptions and transformed where
270 needed. When sphericity was not met, corrected degrees of freedom from Greenhouse-
271 Geisser adjustment were used (Quinn and Keough, 2002).

272 The effects of $CO_2$ and nutrient treatments on variables measured at the end of the
273 experiment were tested using two-way ANOVAs (two fixed crossed factors) after
274 testing parametric assumptions. A normal distribution with unequal variances was found
275 for all variables, which is usual when the sample size is small. Following
276 recommendation by Quinn and Keough (2002), we proceeded with the analyses but
277 making significance level more restrictive to minimize the possibility of Type I error
278 (mistakenly detection of differences). Welch $t$ tests that are robust against unequal
279 variances were used to interpret significant interactions. Again, data for the low- and
280 high-nutrient meadows were analyzed separately.

281 To assess the ordination of treatments based on differences in the composition of leaf
282 epiphyte assemblages, a non-metric Multi-Dimensional Scaling analysis (NMDS) with
283 Bray-Curtis distances was carried out. Because NMDS axes are arbitrary, the final
284 solution was rotated using a Principal Component Analysis (PCA) to align the largest
285 variance in the first axis. The significance of the effect of $CO_2$ and nutrient treatments
286 on assemblage composition was tested with a two-way permutational analysis of
287 variance (PERMANOVA; two fixed crossed factors). To perform the test, Bray-Curtis
288 distances were calculated from untransformed data and 999 permutations were used
289 under a reduced model.

290 Finally, two PCAs, one for each meadow, were performed to assess links among the
291 several traits and the trajectory of treatment responses through time. Traits showing the
292 highest correlation with the components ($r \geq 0.7$) were selected for interpretations.
293 Since our variables were not dimensionally homogeneous, principal components were
294 computed from the correlation matrix.

295

## 3 Results

### 3.1 Responses of meadow traits measured through time

The response of the low-nutrient meadow showed a threshold at the third week of the experiment, when most variables responded differently from the first two weeks (Fig. 1, left). Shoot recruitment occurred mostly in unfertilized but also in $CO_2$-enriched conditions until the third week (Fig. 1a), after which shoot mortality progressively increased. Figure 1b suggests that the *Z. noltii* leaf area index (LAI) tended to increase with $CO_2$ enrichment until the third week of the experiment. A positive, significant effect of the $CO_2$ enrichment was observed on detritus production throughout the experiment (Fig. 1d). Nutrient addition increased shoot mortality (Fig. 1a), whereas it decreased LAI and leaf number (Fig. 1b and c). Shoot mortality induced by the nutrient enrichment was attenuated by the simultaneous addition of $CO_2$, especially from the third week onwards (Fig. 1a). A treatment and time interaction was detected on the community production and respiration (Fig. 1e and f). These variables showed high variability with similar ranges of variation in unfertilized and enriched conditions. No treatment effects were detected throughout the experiment on shoot flowering or meso-herbivory (see Appendix C, Supplement).

The responses of the high-nutrient meadow to $CO_2$ enrichment included an increased shoot mortality during the second and third weeks and an increased detritus production at the end of the experiment (Fig. 1g and i). Nutrient addition decreased the number of leaves per shoot and increased detritus production throughout the experiment (Fig. 1h and i). $CO_2$ enrichment interacted with nutrients to alleviate the nutrient-induced reduction of the number of leaves (Fig. 1h). No effects of $CO_2$ or nutrient enrichment were observed through time on LAI, meso-herbivory, *Ulva* spp. cover, shoot flowering, and community production or respiration (Figs. 1j-l and C, Supplement). Independently of the experimental treatments, overgrazing by the herbivorous amphipod *Cymadusa filosa* Savigny severely affected the plants from the high-nutrient meadow causing massive shoot mortality (Fig. 1k and g). At the end of the experiment a mean ($\pm$se) of 89 ($\pm$3.7)% of shoots died, 81 ($\pm$9.1)% of the seagrass leaves showed bite marks and leaf area was reduced from 5.0 ($\pm$0.2) to 1.0 ($\pm$0.4) $cm^2$ $shoot^{-1}$. Similarly, *Ulva* spp. cover progressively decreased, being close to 0% in all treatments after six weeks (Fig. 1l). At the end of the experiment, all *Z. noltii* shoots and *Ulva* spp. fronds disappeared

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from three flowerpots (one unfertilized and two $CO_2$-and-nutrient-enriched). However, net production and respiration in these pots were within the range observed in the other pots (Fig. Cd and e, Supplement), indicating that the metabolism of the sediment microbial community was similar to that of the *Z. noltii* community.

We did not detect any significant effect of $CO_2$ or nutrient enrichment on plant damage by meso-herbivory in the low- or high-nutrient meadows. However, at the end of the experiment plants from the high-nutrient meadow showed 81 ($\pm$9.1)% of leaves with bite marks, compared to only 6.9 ($\pm$3.2)% in the low-nutrient meadow (Figs. 1k and Cb, Supplement). These between meadow differences, as well as the link between shoot mortality and meso-herbivory in masking the enrichment effects in the high-nutrient meadow, were further confirmed by a PCA of responsive variables from the low- and high-nutrient meadows at the end of the experiment (see Appendix E, Supplement).

### 3.2   Responses of meadow traits measured at the end of the experiment

In plants from the low-nutrient meadow (Fig. 2, grey bars), nutrient enrichment enhanced the leaf nutritional quality (high leaf nitrogen and low leaf C:N ratio, Fig. 2a and c) and the accumulation of nitrogen in rhizomes (high rhizome nitrogen concentration and low rhizome C:N ratio, Fig. 2d and b), whereas it had a negative impact on the accumulation of leaf phenolics (Fig. 2f). A significant interaction of $CO_2$ and nutrient additions was detected for epiphyte load and sediment organic matter (Fig. 2g and h). The leaf epiphyte load increased significantly under $CO_2$ addition, whereas nutrient enrichment and especially the interactive $CO_2$ and nutrient additions had a lower and not significant effect (*t* test comparisons in Fig. 2g). Similarly, $CO_2$ and nutrient interaction resulted in maintenance of the organic matter content in the sediment, which tended to decrease with separated $CO_2$ and nutrient additions (*t* test comparisons in Fig. 2h).

In plants from the high-nutrient meadow (Fig. 2, black bars), $CO_2$ enrichment decreased rhizome C:N (Fig. 2b) and increased epiphyte loads (Fig. 2g). The $CO_2$-induced increase of the epiphyte load was maintained under the simultaneous addition of nutrients. Nutrient addition enhanced the leaf nutritional quality (high leaf nitrogen concentration, Fig. 2a). A reduction of leaf C:N ratio and phenolics was detected apparently in response to $CO_2$ and/or nutrient enrichments (Fig. 2c and f), but this was actually caused by an increase of these traits in the unfertilized plants at the end of the

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385 experiment in relation to the initial field conditions (Table 1). A synergistic interaction
386 between $CO_2$ and nutrient additions caused an increase of the rhizome length (Fig. 2e).

387 Variables for which no significant effects of $CO_2$ or nutrient addition were detected are
388 shown in Appendix D (Supplement).

### 3.3 Responses of *Z. noltii* epiphytes

390 Both, $CO_2$ and nutrient additions altered the relative abundance of epiphyte populations,
391 whereas elevated nutrient levels also modified the epiphyte composition (Fig. 3a). In the
392 unfertilized plants, the epiphyte cover was low and the most abundant leaf epiphytes
393 were the fanlike diatoms *Licmophora* spp. The second-most-abundant epiphyte in plants
394 from the low-nutrient meadow was the encrusting coralline algae *Melobesia*
395 *membranacea*, whereas in plants from the high-nutrient meadow it was the
396 cyanobacterium *Microcoleus* spp. The response to the $CO_2$ enrichment in both, low- and
397 high-nutrient meadows was a great increase of epiphyte cover, mostly due to a bloom of
398 *Microcoleus* spp. (73% of the total cover) that outcompeted the diatoms *Licmophora*
399 spp. and the encrusting corallines. Under nutrient-enrichment pennate diatom
400 populations dominated by *Navicula* spp. outcompeted the other taxa. In the nutrient- and
401 $CO_2$-and-nutrient- treatments the composition of epiphyte assemblages was similar, but
402 with a reduced replacement of *Licmophora* spp. by pennate diatoms in the $CO_2$-and-
403 nutrient- treatment. Chlorophytes (mainly *Ulva prolifera*) and filamentous rhodophytes
404 (mainly *Bangia* spp. and *Stylonema alsidii*) were also present in all treatments.
405 Temporal changes in epiphyte abundances within the enriched mesocosms involved a
406 shift from relatively low epiphyte loads until the second week to increasing epiphyte
407 loads from the third week onwards, with the occurrence of purple bacteria in nutrient-
408 and $CO_2$-and-nutrient-enriched treatments during the fourth week.

409 NMDS ordination of treatments based on the epiphyte composition showed clear $CO_2$
410 effects (Fig. 3b). $CO_2$ treatments were separated along axis I (51% of variance
411 explained), whereas the other treatments were ordered along axis II (49% of variance
412 explained) from unfertilized to $CO_2$-, $CO_2$-and-nutrient-, and nutrient-enriched.
413 Separation of $CO_2$ enrichments along axis I was due to a higher epiphyte cover (mean
414 $\pm$se: $22\pm2.3$ cm$^2$ per 10 cm$^2$ of leaf) than the unfertilized, nutrient-enriched and $CO_2$-
415 and-nutrient-enriched treatments ($7.6\pm1.4$, $11\pm1.3$ and $8.4\pm3.2$ cm$^2$ per 10 cm$^2$ of leaf,
416 respectively). Treatments of both, low- and high-nutrient meadows were nearby in the

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ordination diagram, reflecting minor differences among meadows in the response of the epiphyte assemblage. NMDS pattern was further confirmed by the PERMANOVA results, which showed significant effects of $CO_2$, nutrients and their interaction (Fig. 3b).

The above-mentioned PCA of traits from the low- and high-nutrient meadows further confirmed the increased epiphyte load and the change in epiphyte composition as main drivers of the meadow responses to $CO_2$ and nutrient enrichments (see Appendix E, Supplement).

### 3.4 Response trajectories through time

The first two PCA components of the low-nutrient meadow traits measured through time, explained 41% (component I) and 20% (component II) of the variance. The *Z. noltii* traits that highly correlated with component I were the LAI and the number of leaves, which were negatively correlated with shoot mortality and herbivory (Fig. 4a, right graph). Flowering, community production and community respiration highly correlated with component II (variable loadings are presented in Table E2, Supplement). The variability of all treatment scores on the component I during the first week and of unfertilized and $CO_2$-enriched treatments during the second and third weeks were within the initial range of natural variability (week 0, grey rectangle in Fig. 4a, left graph). At this time, *Z. noltii* plants showed higher LAI and higher number of leaves. The time series ordination of the rest of treatments along component I revealed that the effects of nutrient addition started during the second week, when the scores of nutrient- and $CO_2$- and-nutrient-treatments suddenly shifted to higher values. These nutrient effects were dominated by high mortality of *Z. noltii* shoots and to a less extent by high meso-herbivory. The highest scores on component I were attained by the nutrient-treatment during the fourth to sixth weeks. The system response to the $CO_2$ treatment was slower (starting at week 4) and of lower magnitude than the response to nutrient- and $CO_2$-and-nutrient-treatments. This analysis supported the previous indication of a temporal threshold for the meadow responses, which was the second week for elevated nutrients and the fourth week for elevated $CO_2$. No clear ordination of treatments was detected along component II, indicating that traits highly correlated with this component were substantially influenced by natural variability.

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456  The first two PCA components of the high-nutrient meadow traits measured through
457  time explained 64% (component I) and 12% (component II) of the variance. The *Z.*
458  *noltii* traits that highly correlated with component I were the LAI, the number of leaves,
459  the community production and the abundance of *Ulva*, which were negatively correlated
460  with mortality, herbivory and detritus production (Fig. 4b, right graph). Community
461  respiration highly correlated with component II (see variable loadings in Table E2,
462  Supplement). The range of initial natural variability of all treatment scores was
463  narrower than for the low-nutrient meadow (Fig. 4b, left graph). The system was
464  initially dominated by high seagrass LAI and number of leaves, cover of *Ulva* spp., and
465  community production. This progressively shifted to a later stage (week 6) dominated
466  by high *Z. noltii* mortality, herbivory and detritus production. Contrary to the response
467  of the low-nutrient meadow, there were no relevant differences in the time course and in
468  the final stage attained by PCA scores of both unfertilized and enriched treatments. The
469  shoot mortality of *Z. noltii* was positively correlated with meso-herbivore activity and
470  detritus production, and negatively correlated with LAI, number of leaves and *Ulva*
471  cover. No clear ordination of treatments was detected along component II.

472

## 4  Discussion

### 4.1  Effects of $CO_2$ enrichment in low-nutrient meadows

475  The $CO_2$ enrichment had no direct effects on *Z. noltii* biochemistry (Fig. 5a). We found
476  no evidence of increased nonstructural carbohydrates and subsequent nitrogen dilution
477  effect (increased C:N ratio) as has been previously observed in the seagrass *Thalassia*
478  *hemprichii* (Jiang et al., 2010) and *T. testudinum* (Campbell and Fourqurean, 2013). As
479  well, there was no increase of phenolic contents as predicted by the carbon-nutrient
480  balance hypothesis and no propagation to susceptibility to herbivory. Several studies in
481  terrestrial plants reveal that this lack of response is not uncommon (reviewed by
482  Peñuelas and Estiarte, 1998 and Bidart-Bouzat and Imeh-Nathaniel, 2008). The lack of
483  accumulation of carbohydrates and phenolics that we observed could be explained by
484  the trade-off between secondary metabolism and plant growth predicted by the growth-
485  differentiation balance hypothesis under no light and nutrient limitation (review by
486  Stamp, 2003). However, we found no significant increase of seagrass productivity under
487  $CO_2$ enrichment to support this trade-off, probably due to light limitation induced by

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epiphyte overgrowth from the third week of experiment onwards. Photosynthesis enhancements have been reported under $CO_2$ addition in *Z. noltii* (Alexandre et al., 2012) and *Z. marina* (Zimmerman et al. 1997), but they do not always translate into seagrass growth since other determinant factors such as light and nutrient availability are also at play (Palacios and Zimmerman, 2007; Alexandre et al., 2012; Campbell and Fourqurean, 2013). In accordance with the previously cited terrestrial studies, our results suggest that the seagrass responses to elevated $CO_2$ levels are highly context- and species-specific, and are not as readily consistent and predictable as resource availability hypotheses would suggest.

The most striking response of *Z. noltii* meadows to the $CO_2$ enrichment was the increase of the epiphyte load, with changes in the relative abundance but not in the identity of the main epiphyte taxa. Interestingly, epiphyte-induced shading did not cause seagrass mortality as occurred under nutrient enrichment (see below). This suggests attenuation of the negative effect of reduced light availability by increased $CO_2$ availability, which may reduce the energy cost of carbon uptake (Koch et al., 2013). The epiphyte bloom was mostly caused by the proliferation of the colonial and filament-forming cyanobacterium *Microcoleus* spp. at the expense of a reduction of coralline algae crusts of *Melobesia membranacea* and fanlike diatoms *Licmophora* spp. This is in accordance with previous studies that showed elevated $CO_2$ / low pH to stimulate cyanobacteria growth and photosynthesis (Liu et al., 2010) and to decrease abundance of coralline algae (Hall-Spencer et al., 2008; Martin et al., 2008; Kuffner et al., 2008, Campbell and Fourqurean, 2014). As well, Hervé et al. (2012) reported negative effects of low pH on diatom valve formation and porosity, which were alleviated by a simultaneous nutrient addition. We found that the activity of mesograzers was insufficient to regulate the epiphyte proliferation in response to increased $CO_2$ levels, despite their recognized capacity of controlling epiphyte biomass (Hughes et al., 2004) and particularly cyanobacteria blooms (Neckles et al., 1993). Likely explanations are that the feeding capacity of the most abundant mesograzer in the experiment, the amphipod *C. filosa*, was exceeded by the cyanobacterium overgrowth or that the amphipod was not targeting these particular epiphytes.

Epiphyte overgrowth resulted in increased detritus production and decreased organic matter accumulated in the sediment. This suggests that bacterial decomposition in the sediment was accelerated due to the highly labile organic matter of epiphytes, as

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reported under nutrient enrichment (see below). The acceleration of bacterial degradation of organic matter polysaccharides at low pH reported by Piontek et al. (2010) would also support this explanation. Our findings may have relevant implications, since the organic carbon produced in seagrass meadows sustains important detritus-based food webs (Pergent et al., 1994; Moore and Fairweather, 2006) and provides a major global carbon sink (Pidgeon, 2009; Fourqurean et al., 2012).

## 4.2 Nutrient enrichment and interaction with $CO_2$ in low-nutrient meadows

We found that nutrient enrichment had a faster and greater effect than $CO_2$ addition on meadows developing in low-nutrient conditions. Nutrient enrichment enhanced leaf nutritional quality (high nitrogen and low C:N ratio) and reduced the accumulation of phenolic compounds (Fig. 5a). Both, the overall increase of plant nitrogen (e.g. Cabaço et al., 2008; Invers et al., 2004) and the decrease of phenolics (e.g. van Katwijk et al., 1997; Goecker et al., 2005) have been widely described in seagrasses as a result of nutrient additions. The nitrogen increase was higher in leaves than in rhizomes, as expected for this fast-growing species that acquires preferentially ammonium through the leaves and shows minimal translocation of nitrogen to belowground tissues (Alexandre et al., 2011).

A reduction of the number of *Z. noltii* leaves and of leaf area index, and an increase of shoot mortality were observed after the second week in response to nutrient addition. These effects can be linked to ammonium toxicity, which has previously been reported in *Z. noltii* (Brun et al., 2002) and other seagrass species (Santamaría et al., 1994, van Katwijk et al., 1997). In addition, nutrient-induced changes in the epiphyte assemblage may also contribute to shoot mortality by reducing light availability to seagrass leaves. This seems supported by the abrupt increase of shoot mortality after the third week, coinciding with the shift of the epiphyte assemblage from coralline algae and fanlike diatoms to a dense layer of pennate diatoms (mostly of the genus *Navicula*), with a purple bacteria biofilm developing as well during the fourth week. Towards the end of the experiment, excess organic matter was released within the system due to increased shoot mortality and epiphyte shifts. Coincidently, the accumulation of organic carbon in the sediments decreased, suggesting that an accelerated microbial decomposition was promoted by the higher nutritional quality of producers as reported elsewhere for

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655 terrestrial systems (Wardle et al., 2004) and seagrass beds (López et al., 1998; Holmer
656 et al., 2004; Spivak et al., 2007).

657 The simultaneous addition of $CO_2$ and nutrients did not modify the individual effects of
658 nutrient enrichment on plant biochemistry, but attenuated the proliferation of certain
659 epiphyte taxa (also occurring under high $CO_2$) and subsequent nutrient-induced *Z. noltii*
660 mortality. The interactive attenuation of epiphyte overgrowth may result from an
661 increase in the interspecific competition between the species that dominated the
662 epiphyte community under elevated $CO_2$ (i.e. the cyanobacterium *Microcoleus* spp.) and
663 under elevated nutrient levels (i.e. diatoms of the genus *Navicula*). Our findings are in
664 agreement with the negative effects of interspecific competition on the involved species
665 (i.e. symmetrical competition) that have long been reported in ecological studies
666 (Connel, 1983). Together, attenuation by simultaneous $CO_2$ and nutrient additions of the
667 overgrowth of certain epiphytes and of nutrient-induced *Z. noltii* mortality, reduced the
668 amount of more labile organic matter reaching the sediments compared to the individual
669 $CO_2$ or nutrient enrichment. This probably resulted in the maintenance at control levels
670 of the bacterial decomposition rates and of the sediment capacity to store organic
671 matter. To our knowledge, this is the first report of the interactive effect of $CO_2$ and
672 nutrient enrichments on the meadow carbon sink capacity. Overall, we found that under
673 simultaneous addition of $CO_2$ and nutrients, species interactions attenuated the direct
674 effects of individual stressors on *Z. noltii* and on sensitive epiphyte species or
675 taxonomic groups.

## 4.3 High- vs. low- nutrient meadows

677 Our results revealed that the expected benefits of high $CO_2$ predicted for the end of the
678 century on seagrass productivity might be restrained by epiphyte overgrowth and by the
679 interaction with local eutrophication. In both low- and high-nutrient meadows, $CO_2$
680 effects were more important in epiphyte populations than in the seagrass *Z. noltii*. These
681 findings strengthen the increasingly recognized importance of species interactions in
682 modulating the direct effects of eutrophication or acidification in single species,
683 populations, and ultimately in ecosystem functioning (Orth et al., 2006; Kroeker et al.,
684 2013b). The effect of nutrient enrichment was greater in the low- than in the high-
685 nutrient meadow (Fig. 5a vs. b), with nutrient-induced mortality of *Z. noltii* only
686 appearing in the former. When $CO_2$ and nutrient enrichments interacted, an increase of

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**Deleted:** reduced the nutrient-induced changes in the epiphyte assemblages and the $CO_2$-induced increase in epiphyte loads. This led to maintenance at control levels of the capacity of the sediments to store organic matter. Most studies of the interactive effect of $CO_2$ and nutrients in terrestrial systems are focused on nutrient levels that alleviate nutrient limitation and increase plant growth at the expense of carbon reserves as predicted by the Growth-Differentiation Balance hypothesis (Johnson and Lincoln, 1991; Coley et al., 2002). However, the potential alleviation by $CO_2$ of the plant mortality that may occur due to imbalances induced by excess nutrients has not, to our knowledge, been previously reported.

epiphyte load was observed in the high-nutrient meadow as opposed to the low-nutrient meadow. These results highlight the context-dependence of the effects of multiple stressors in agreement with the meta-analysis of Crain et al. (2008). We observed that the accumulation of phenolics and carbohydrates was higher under lower nutrient regimes in the initial field conditions, and also in the experimental conditions for phenolics. This suggests that nutrient deficiency rather than a direct effect of high $CO_2$ drives the accretion of carbon-based compounds in *Z. noltii*. These observations are in agreement with previous studies in both, terrestrial plants (Lambers, 1993; Peñuelas and Estiarte, 1998) and seagrasses (Campbell et al., 2012), and reinforce the idea of the context-dependence of seagrass responses to $CO_2$ enrichment.

Interestingly, we detected little evidence that $CO_2$ or nutrient addition affected seagrass herbivory by mesograzers. However, clear differences in herbivory between meadows were observed. Plants from the high-nutrient meadow experienced a 12-fold higher amphipod grazing than plants from the low-nutrient meadow, which resulted in a massive loss of shoots. Blooms of the amphipod *C. filosa* may occur in warmer months (Appadoo and Myers, 2004). This tube-building amphipod is widely distributed and uses macroalgae for feeding (Ceh et al., 2005) and shelter (Appadoo and Myers, 2003). To our knowledge, these observations constitute the first report of *C. filosa* using the seagrass *Z. noltii* for both feeding and shelter-construction. We found that the consequences of plant-specific vulnerability to grazing on seagrass meadows can be stronger than the effects of $CO_2$ and eutrophication. This result concurs with findings by Alsterberg et al. (2013), which showed that the presence of grazers masked the response of benthic microalgae to ocean acidification and warming. Further studies aiming to identify the factors underlying the plant-specific seagrass vulnerability to grazers are thus of vital importance.

Our results showed that separated $CO_2$ or nutrient enrichment individually result in a loss of the carbon sink capacity of the low-nutrient meadow, as opposed to the high-nutrient meadow. This loss contrasts with results of previous studies conducted in situ with other seagrass species, which found that the meadow carbon sequestration capacity was unaffected by nutrient addition (Antón et al., 2011) or increased due to $CO_2$ enrichment (Russell et al., 2013). In both studies, the whole-community metabolism was used as indicator of the carbon storage capacity of seagrass meadows. In our study, however, the loss of carbon storage occurred without a significant response of the

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whole-community metabolism, indicating a metabolic compensation between the *Z. noltii* populations and the leaf epiphyte and sediment microbial communities. A similar dynamic global balance has also been reported for marine pelagic systems under $CO_2$ addition (Silyakova et al., 2013).

Overall, we found that shifts in the community dynamics of leaf epiphytes or sediment bacteria mediated the multi-level responses of *Z. noltii* meadows to independent $CO_2$ or nutrient addition. They also modulated the attenuation of individual effects under simultaneous $CO_2$ and nutrient enrichments. Overgrazing masked the response to $CO_2$ enrichment and eutrophication but only in the high-nutrient meadow. Our findings highlight the importance of integrative multi-level and ecosystem-based approaches considering not only species interactions and connections between organization levels, but also the effect of interactive stressors, to anticipate the evolution of seagrass meadows in the near future and to endorse conservation efforts.

## Appendices

Appendix A. Full comparative description of the low- and high-nutrient meadows of *Zostera noltii* in June-August 2011, prior to the start of the experiment.

Appendix B. Seawater chemistry within the experimental mesocosms.

Appendix C. Full results of the response to $CO_2$ and nutrient additions of *Zostera noltii* plant-, community-, and ecosystem-level traits measured through time.

Appendix D. Full results of the response to $CO_2$ and nutrient additions of *Zostera noltii* plant-, community-, and ecosystem-level traits measured at the end of the experiment.

Appendix E. Results of principal component analyses.

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**Deleted:** Our study revealed that $CO_2$ or nutrient enrichments may lead to a loss of the carbon sink capacity of low-nutrient meadows. These results are contradictory with studies by Antón et al. (2011) and Russell et al. (2013), who found no effect and an increase in the meadow carbon sequestration capacity of other seagrass species due to in situ nutrient and $CO_2$ enrichments, respectively. They used the whole-community metabolism as indicator of the carbon storage capacity of seagrass meadows. However, in our study, the loss of carbon storage occurred with no response of the whole-community metabolism, which showed high variability. Our results suggest a dynamic balance of the community metabolism in response to high $CO_2$ and nutrients, in which the changes in the *Z. noltii* metabolism are compensated by the metabolism of fast-growing leaf epiphyte and sediment microbial communities. A similar dynamic global balance has also been reported for marine pelagic systems under $CO_2$ addition (Silyakova et al., 2013).

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**Deleted:** Overall, the role of herbivory in driving differences between low- and high-nutrient meadows, as well as the role of epiphyte and microbial communities in mediating meadow responses to $CO_2$ and nutrients, illustrated the importance of interactions among species and connections between organization levels in ecosystem-based studies. The alleviation of nutrient effects by high $CO_2$ highlighted the relevance of incorporating responses to multiple stressors. This integrative ecosystem-based approach is an important challenge to endorse future conservation efforts.

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Table 1. Environmental and *Zostera noltii* community traits that revealed significant differences between the low- and high-nutrient donor meadows during June-August 2011, prior to the start of the experiment, tested using unpaired t-tests. [a]Sqrt-transformed data to meet normality. [b]Mann-Whitney rank tests were conducted for variables that did not meet normality even after transformation. All measured traits and methods are shown in Appendix A (Supplement).

| | Low-nutrient meadow | High-nutrient meadow |
|---|---|---|
| Leaf phenolics (mg $(gDW)^{-1}$) | 48±1.2 | 29±3.2 |
| Leaf nitrogen (mg $gDW^{-1}$) | 21±0.8 | 25±0.6 |
| Leaf C : N | 19±0.7 | 16±0.4 |
| Rhizome starch (mgGlu $(gDW)^{-1}$) | 473±14 | 355±27 |
| Rhizome TNC (mg Glu g $(DW)^{-1}$) | 668±18 | 532±28 |
| Shoot area ($cm^2$ $shoot^{-1}$)[a] | 7.8±0.6 | 4.5±0.7 |
| *Z. noltii* density (shoots $m^{-2}$) | 5517±755 | 2664±411 |
| *Z. noltii* cover (% of sediment surface)[b] | 96±2.2 | 18±9.8 |
| *Ulva* spp. cover (% of sediment surface)[b] | absent | 38±12 |
| Seawater nitrate ($\mu M$)[b] | < 0.01 | 1.1±0.2 |
| Seawater ammonium ($\mu M$) | 0.7±0.2 | 3.0±0.4 |
| Seawater phosphate ($\mu M$) | 0.5±0.1 | 1.2±0.1 |

**Figure captions**

Fig. 1. Effects of $CO_2$ and nutrient additions on *Zostera noltii* plant-, community-, and ecosystem-level traits from the low-nutrient (left graphs) and high-nutrient meadow (right graphs) through time. Symbols are means ($\pm$se, $n = 2$). $F$ statistics and $p$ levels from RM ANOVA tests are shown for among-subject factors ($CO_2$; Nut: nutrients) and their interaction ($CO_2$ x Nut), and for within-subject factor (time) and interactions (Time x $CO_2$, Time x Nut, Time x $CO_2$ x Nut). Only the significant effects ($p < 0.05$) and useful traits for results interpretation are shown here; the non-significant effects are shown in Appendix C (Supplement). *Variables that did not meet normality after transformation, for which the significance level was more restrictive ($p < 0.03$) to minimize the possibility of Type I error. **Variable sqrt-transformed to meet normality.

Fig. 2. Effects of $CO_2$ and nutrient additions on *Zostera noltii* plant-, community-, and ecosystem-level traits measured at the end of the experiment from the low-nutrient (grey bars) and high-nutrient meadow (black bars). Bars are means ($\pm$se, $n = 2$). The $F$ statistics and $p$ levels from two-way ANOVA tests are shown for each fixed crossed factor ($CO_2$; Nut: nutrients) or interaction ($CO_2$ x Nut). Lowercase letters above bars show significant differences between treatments for significant interactions ($t$ tests, see Methods). Only restrictive significant effects ($p < 0.03$) selected to minimize the possibility of Type I error due to unequal variances are shown here; the non-significant effects are shown on Appendix D (Supplement).

Fig. 3. Responses of *Zostera noltii* epiphyte populations to experimental addition of $CO_2$ and nutrients: (a) Relative abundance of the main epiphyte taxa of plants exposed to experimental treatments; and (b) NMDS ordination of experimental treatments (see symbol legend below treatment names in Fig. 3a) based on leaf epiphytes of plants from the low-nutrient (L; grey symbols) and high-nutrient meadow (H; black symbols). Pseudo-$F$ statistics and $p$ levels from two-way PERMANOVA test are shown in the NMDS diagram for each fixed crossed factor ($CO_2$; Nut: nutrients) and their interaction ($CO_2$ x Nut).

Fig. 4. Principal components analysis of *Zostera noltii* plant-, community, and ecosystem- level responses to treatments through time: (a) low-nutrient and (b) high-nutrient meadow. Numbers inside the symbols indicate sampling weeks from 0 to 6. The initial variability (week 0) along component I is incorporated within a grey

28

1144 rectangle to highlight the range of initial natural variability. Variable loadings on the
1145 two principal components are depicted in right graphs. LAI refers to leaf area index,
1146 NCP to net community production and CR to community respiration.
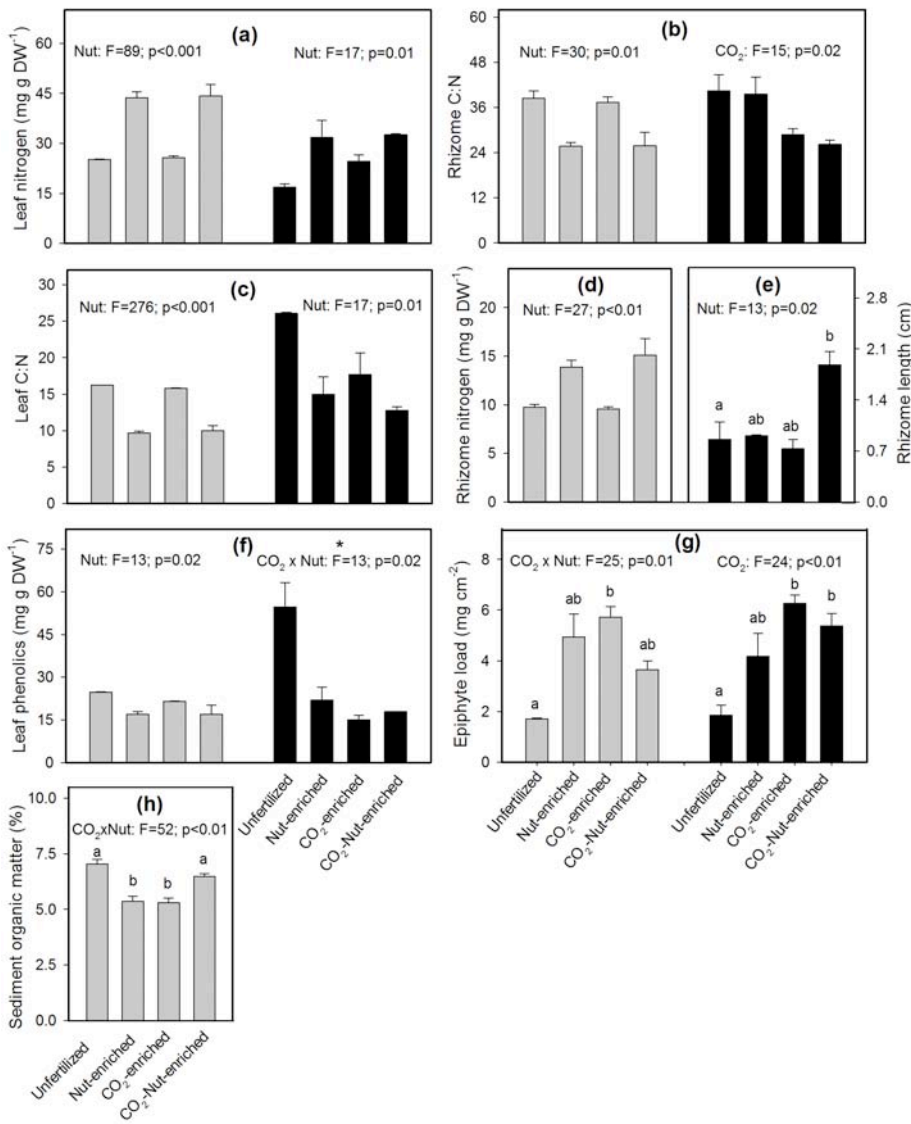
1147 Fig. 5. Summary of the effects of $CO_2$ (blue line) and nutrient (green line) additions, and
1148 when significant of their interaction (red line), on *Zostera noltii* plant- community and
1149 ecosystem- level traits of low-nutrient (a) and high-nutrient (b) meadows. Solid lines
1150 indicate significant effects on variables measured at the end of the experiment and
1151 dashed lines on variables measured through time. Dotted lines indicate no significant
1152 effects. Letters below the x-axis denote unfertilized (U) and enriched (E) treatment,
1153 which is the pooled mean response to the respective enrichment over the other when
1154 there was no significant interaction (no red line). For shoot mortality and net community
1155 production (NCP) in the low-nutrient meadow a significant Time x $CO_2$ x Nutrients
1156 interaction was detected and values were represented as for $CO_2$ x Nutrients significant
1157 interactions. * Apparent effect due to a drastic increase in unfertilized plants in relation
1158 to the initial field conditions (see results).
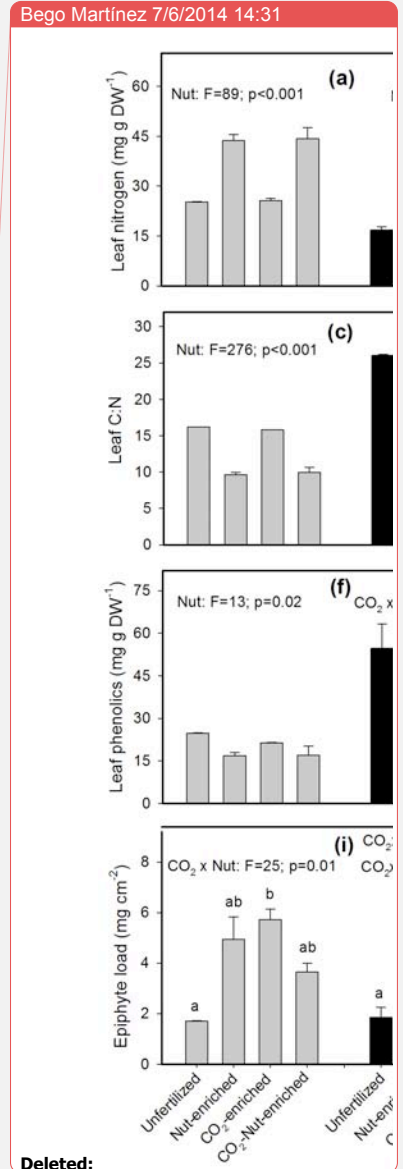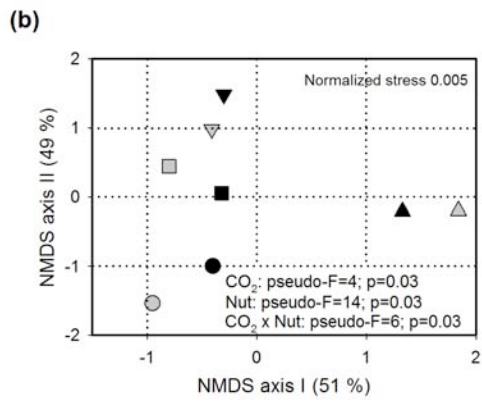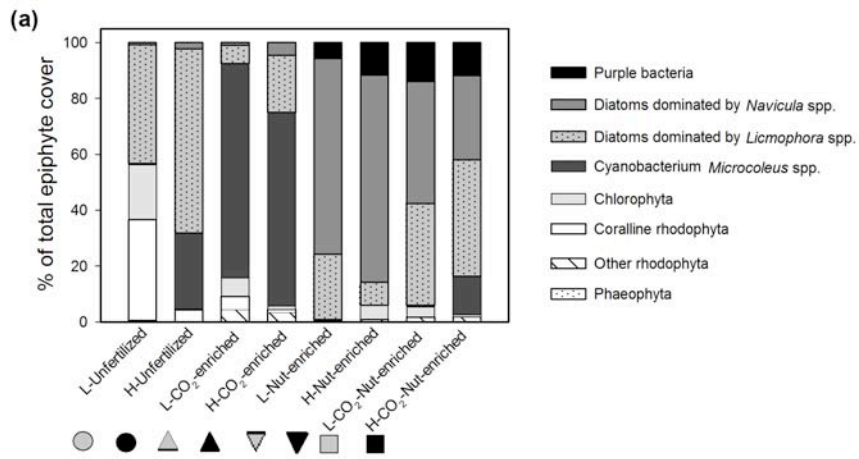
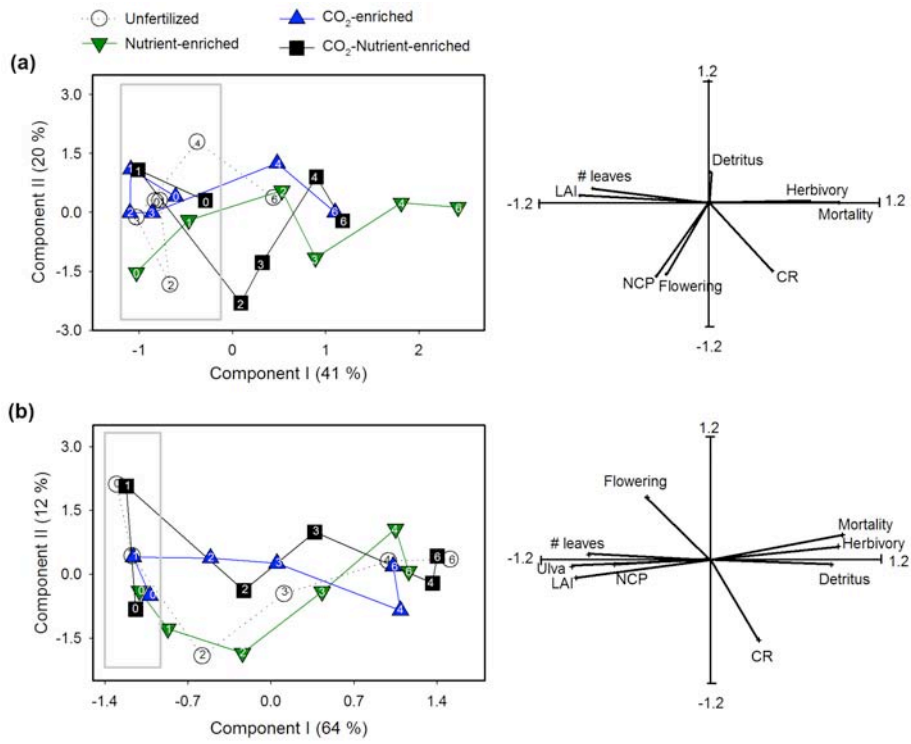1159
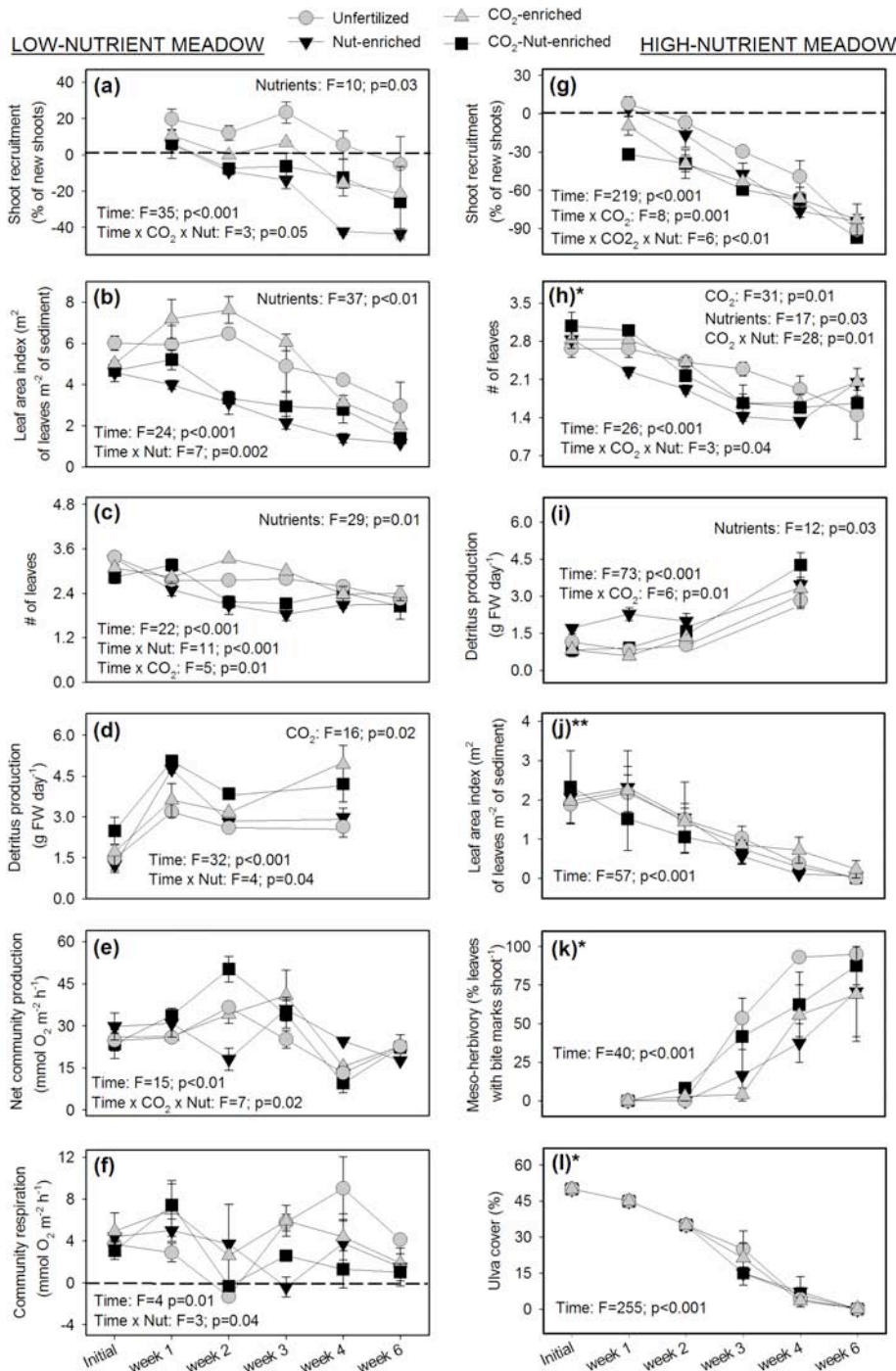
1160

1161    Figure 1

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1163



1164

1165    Figure 2

(a)

Legend:
- Purple bacteria
- Diatoms dominated by *Navicula* spp.
- Diatoms dominated by *Licmophora* spp.
- Cyanobacterium *Microcoleus* spp.
- Chlorophyta
- Coralline rhodophyta
- Other rhodophyta
- Phaeophyta

(b)

Normalized stress 0.005

$CO_2$: pseudo-F=4; p=0.03
Nut: pseudo-F=14; p=0.03
$CO_2$ x Nut: pseudo-F=6; p=0.03
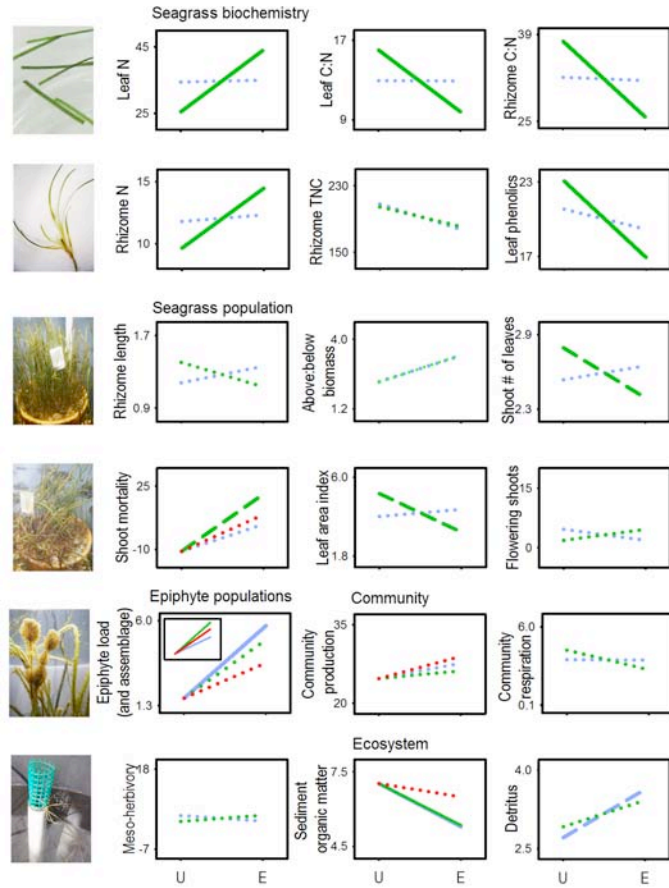
1167

1168    Figure 3

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32

1170

1171    Figure 4.

**(a)** <u>Low-nutrient meadow</u>

**(b)** <u>High-nutrient meadow</u>

1172 | Figure 5