CO_2 and nutrient-driven changes across multiple levels of organization in *Zostera noltii* ecosystems

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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

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

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

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

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

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

134 Experimental levels of CO_2 encompassed present (pH 8.00±0.02, equivalent to ca. 400
135 ppm CO_2) and future conditions (pH 7.83±0.01, equivalent to ca. 800 ppm CO_2) in Ria
136 Formosa lagoon. The mesocosms received sand-filtered seawater from two head tanks
137 of 1000 L at a rate of 240 L h^{-1}. In one of the head tanks, the water CO_2 was
138 manipulated to ensure fixed pH differences between treatment means within the range
139 predicted for 2100 by the IPCC (pH decline = -0.18 units) following a commonly used
140 method (e.g. Alsterberg et al., 2013). The CO_2 injection was controlled by an auto-
141 analyzer (Yokogawa, EXAxt 450, Tokyo, Japan), which continuously monitored the
142 water pH and temperature. Total alkalinity, pH, temperature and salinity within the
143 mesocosms, as well as the seawater DIC and carbon speciation are provided on
144 Appendix B (Supplement).

145 Water nutrient levels encompassed the natural values found in the lagoon and the values
146 of highly eutrophic conditions (N: 45x and P: 11x natural, see Appendix B,
147 Supplement). The nutrient enrichment was obtained by adding a solubilised mixture of
148 the fertilizers ammonium nitrate and monoammonium phosphate directly into the water
149 column of each enriched mesocosm using a multi-channel dosing pump. Water samples
150 were collected weekly to analyze nutrient concentrations using a loop-flow analyzer
151 (μMac-1000; Systea, Anagni, Italy).

152 The water within mesocosms was homogenized using a submersible circulation pump
153 placed at leaf height. Pumps were stopped 2 h twice per day to simulate tidal currents.
154 Twice a week, the epiphytes growing in the mesocosm walls were removed and the
155 position of flowerpots within each mesocosm was reassigned to minimize potential
156 spatial differences. Natural settlement and growth of leaf epiphytes and small animals
157 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

190 ºC and determined with a spectrophotometer using chlorogenic acid as standard
191 following a modified Folin-Ciocalteu method (Bolser et al., 1998).

192 2.4 Community- and ecosystem-level traits

193 The response of the seagrass community to CO_2 and nutrient enrichments was
194 quantified weekly by: (1) the percentage of flowerpot surface covered by *Ulva* spp.; (2)
195 the feeding activity of mesograzers (percentage of leaves showing bite marks in the
196 same shoots used to measure the morphological traits); and (3) the whole-community
197 metabolism quantified from the oxygen evolution within benthic chambers of 17 cm
198 diameter (4.8±0.01 L incubated) fitted to the flowerpots for 30-45 minutes at midday
199 (12-14 h). A transparent acrylic chamber to estimate net production and a dark chamber
200 to estimate respiration were simultaneously deployed within each mesocosm. Dissolved
201 oxygen concentration was measured by spectrophotometry using the Winkler method
202 (Labasque et al., 2004) in three water samples collected before and after incubations
203 into 12 mL soda glass vials. Community metabolism was estimated from the net change
204 in oxygen concentration during incubations integrated by the chamber volume and
205 standardized by incubation time and bottom area. There were no effects of enclosure on
206 the water temperature within chambers (measured with onset HOBO loggers, Southern
207 MA, USA). All incubations were run under irradiances of photosynthetically active
208 radiation (PAR) averaging 283±8.6 μmol quanta m^{-2} s^{-1} (measured with a Li-192SA
209 underwater PAR quantum sensor, Li-Cor, USA), when *Z. noltii* photosynthesis is light
210 saturated and not photoinhibited (Peralta et al., 2002).

211 At the ecosystem level, detritus production (fresh weight of all floating material
212 collected during a 24h period) was quantified almost every week. Organic matter in the
213 sediment (loss of dry weight after combustion at 450 ºC, 4h) was measured at the end of
214 the experiment as indicator of the carbon sink capacity.

215 2.5 Statistical analyses

216 The effects of CO_2 and nutrient treatments throughout the experiment were tested using
217 three-way repeated-measures analyses of variance (RM ANOVA). The subject
218 repeatedly sampled was the mesocosm, CO_2 and nutrients were the among-subject
219 factors (two fixed crossed factors) and Time the within-subject factor. To avoid the
220 masking effect of the strong initial differences between meadows on the responses to

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

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

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

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

248

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

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

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

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

3.3 Responses of *Z. noltii* epiphytes

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

NMDS ordination of treatments based on the epiphyte composition showed clear CO_2 effects (Fig. 3b). CO_2 treatments were separated along axis I (51% of variance explained), whereas the other treatments were ordered along axis II (49% of variance explained) from unfertilized to CO_2-, CO_2-and-nutrient-, and nutrient-enriched. Separation of CO_2 enrichments along axis I was due to a higher epiphyte cover (mean $\pm$se: 22$\pm$2.3 cm^2 per 10 cm^2 of leaf) than the unfertilized, nutrient-enriched and CO_2-and-nutrient-enriched treatments (7.6$\pm$1.4, 11$\pm$1.3 and 8.4$\pm$3.2 cm^2 per 10 cm^2 of leaf, respectively). Treatments of both, low- and high-nutrient meadows were nearby in the

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.

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

4 Discussion

4.1 Effects of CO_2 enrichment in low-nutrient meadows

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

408 epiphyte overgrowth from the third week of experiment onwards. Photosynthesis
409 enhancements have been reported under CO_2 addition in *Z. noltii* (Alexandre et al.,
410 2012) and *Z. marina* (Zimmerman et al. 1997), but they do not always translate into
411 seagrass growth since other determinant factors such as light and nutrient availability
412 are also at play (Palacios and Zimmerman, 2007; Alexandre et al., 2012; Campbell and
413 Fourqurean, 2013). In accordance with the previously cited terrestrial studies, our
414 results suggest that the seagrass responses to elevated CO_2 levels are highly context-
415 and species-specific, and are not as readily consistent and predictable as resource
416 availability hypotheses would suggest.

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

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

441 reported under nutrient enrichment (see below). The acceleration of bacterial
442 degradation of organic matter polysaccharides at low pH reported by Piontek et al.
443 (2010) would also support this explanation. Our findings may have relevant
444 implications, since the organic carbon produced in seagrass meadows sustains important
445 detritus-based food webs (Pergent et al., 1994; Moore and Fairweather, 2006) and
446 provides a major global carbon sink (Pidgeon, 2009; Fourqurean et al., 2012).

447 4.2 Nutrient enrichment and interaction with CO_2 in low-nutrient
448 meadows

449 We found that nutrient enrichment had a faster and greater effect than CO_2 addition on
450 meadows developing in low-nutrient conditions. Nutrient enrichment enhanced leaf
451 nutritional quality (high nitrogen and low C:N ratio) and reduced the accumulation of
452 phenolic compounds (Fig. 5a). Both, the overall increase of plant nitrogen (e.g. Cabaço
453 et al., 2008; Invers et al., 2004) and the decrease of phenolics (e.g. van Katwijk et al.,
454 1997; Goecker et al., 2005) have been widely described in seagrasses as a result of
455 nutrient additions. The nitrogen increase was higher in leaves than in rhizomes, as
456 expected for this fast-growing species that acquires preferentially ammonium through
457 the leaves and shows minimal translocation of nitrogen to belowground tissues
458 (Alexandre et al., 2011).

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

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

475 The simultaneous addition of CO_2 and nutrients did not modify the individual effects of
476 nutrient enrichment on plant biochemistry, but attenuated the proliferation of certain
477 epiphyte taxa (also occurring under high CO_2) and subsequent nutrient-induced *Z. noltii*
478 mortality. The interactive attenuation of epiphyte overgrowth may result from an
479 increase in the interspecific competition between the species that dominated the
480 epiphyte community under elevated CO_2 (i.e. the cyanobacterium *Microcoleus* spp.) and
481 under elevated nutrient levels (i.e. diatoms of the genus *Navicula*). Our findings are in
482 agreement with the negative effects of interspecific competition on the involved species
483 (i.e. symmetrical competition) that have long been reported in ecological studies
484 (Connel, 1983). Together, attenuation by simultaneous CO_2 and nutrient additions of the
485 overgrowth of certain epiphytes and of nutrient-induced *Z. noltii* mortality, reduced the
486 amount of more labile organic matter reaching the sediments compared to the individual
487 CO_2 or nutrient enrichment. This probably resulted in the maintenance at control levels
488 of the bacterial decomposition rates and of the sediment capacity to store organic
489 matter. To our knowledge, this is the first report of the interactive effect of CO_2 and
490 nutrient enrichments on the meadow carbon sink capacity. Overall, we found that under
491 simultaneous addition of CO_2 and nutrients, species interactions attenuated the direct
492 effects of individual stressors on *Z. noltii* and on sensitive epiphyte species or
493 taxonomic groups.

494 ### 4.3 High- vs. low- nutrient meadows

495 Our results revealed that the expected benefits of high CO_2 predicted for the end of the
496 century on seagrass productivity might be restrained by epiphyte overgrowth and by the
497 interaction with local eutrophication. In both low- and high-nutrient meadows, CO_2
498 effects were more important in epiphyte populations than in the seagrass *Z. noltii*. These
499 findings strengthen the increasingly recognized importance of species interactions in
500 modulating the direct effects of eutrophication or acidification in single species,
501 populations, and ultimately in ecosystem functioning (Orth et al., 2006; Kroeker et al.,
502 2013b). The effect of nutrient enrichment was greater in the low- than in the high-
503 nutrient meadow (Fig. 5a vs. b), with nutrient-induced mortality of *Z. noltii* only
504 appearing in the former. When CO_2 and nutrient enrichments interacted, an increase of

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

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

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

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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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 (μM)[b]	< 0.01	1.1±0.2
Seawater ammonium (μM)	0.7±0.2	3.0±0.4
Seawater phosphate (μM)	0.5±0.1	1.2±0.1

782 **Figure captions**

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

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

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

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

814 rectangle to highlight the range of initial natural variability. Variable loadings on the
815 two principal components are depicted in right graphs. LAI refers to leaf area index,
816 NCP to net community production and CR to community respiration.

817 Fig. 5. Summary of the effects of CO_2 (blue line) and nutrient (green line) additions, and
818 when significant of their interaction (red line), on *Zostera noltii* plant- community and
819 ecosystem- level traits of low-nutrient (a) and high-nutrient (b) meadows. Solid lines
820 indicate significant effects on variables measured at the end of the experiment and
821 dashed lines on variables measured through time. Dotted lines indicate no significant
822 effects. Letters below the x-axis denote unfertilized (U) and enriched (E) treatment,
823 which is the pooled mean response to the respective enrichment over the other when
824 there was no significant interaction (no red line). For shoot mortality and net community
825 production (NCP) in the low-nutrient meadow a significant Time x CO_2 x Nutrients
826 interaction was detected and values were represented as for CO_2 x Nutrients significant
827 interactions. * Apparent effect due to a drastic increase in unfertilized plants in relation
828 to the initial field conditions (see results).
829

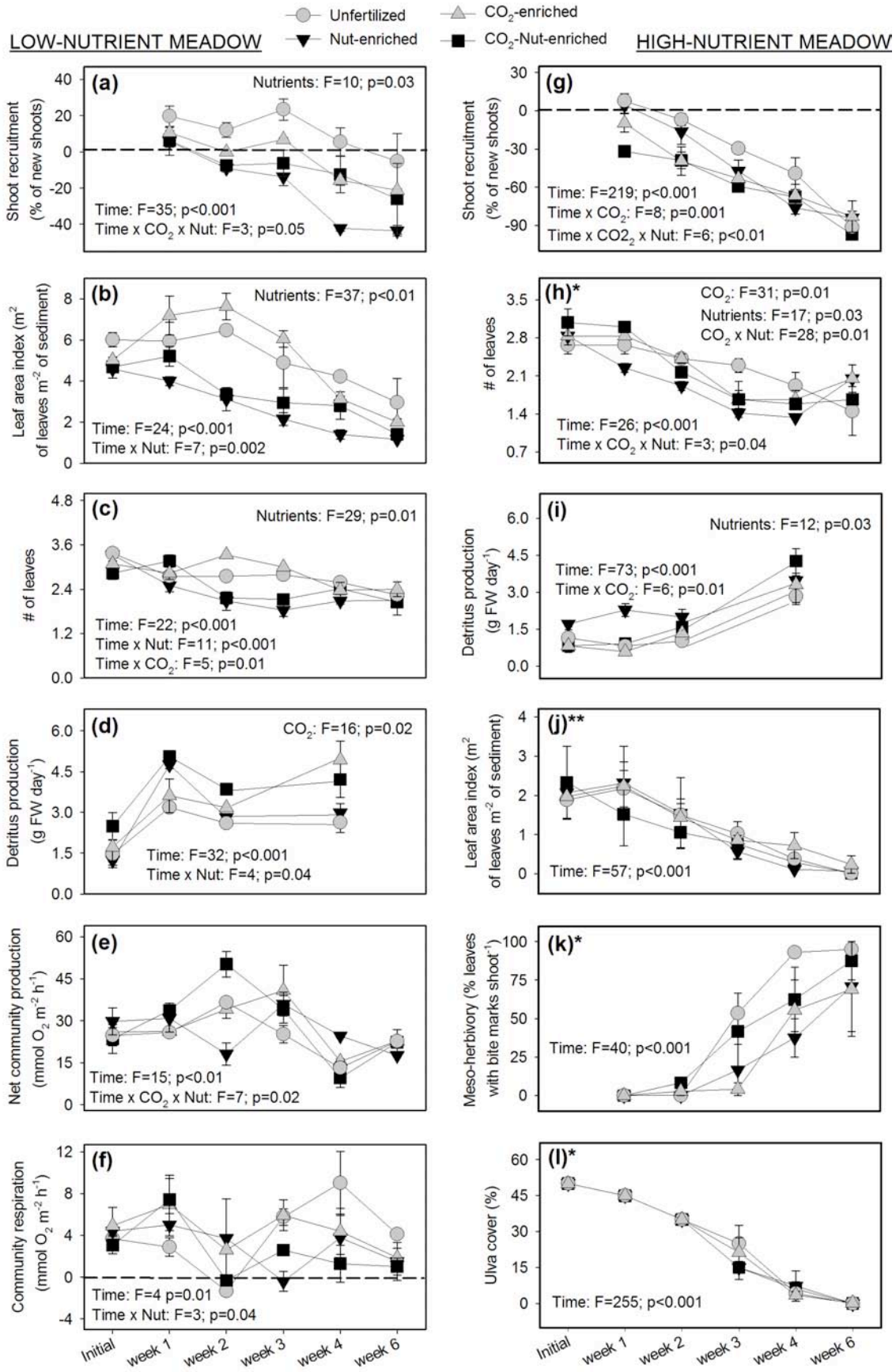
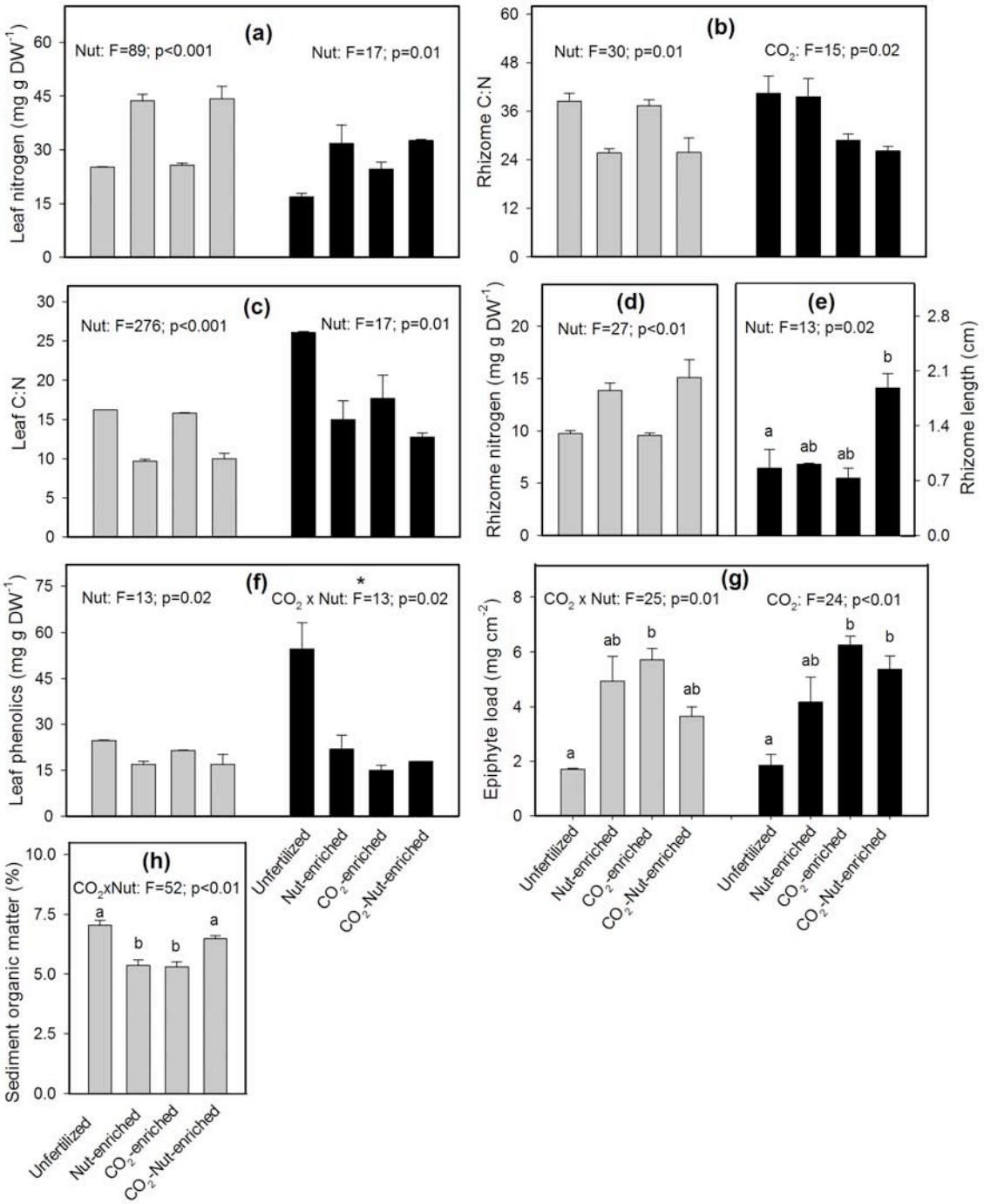

Unfertilized
Nut-enriched
CO2-enriched
CO2-Nut-enriched
LOW-NUTRIENT MEADOW
HIGH-NUTRIENT MEADOW
(a)
Nutrients: F=10; p=0.03
Shoot recruitment (% of new shoots)
Time: F=35; p<0.001
Time x CO2 x Nut: F=3; p=0.05
(g)
Time: F=219; p<0.001
Time x CO2: F=8; p=0.001
Time x CO22 x Nut: F=6; p<0.01
(b)
Nutrients: F=37; p<0.01
Leaf area index (m2 of leaves m-2 of sediment)
Time: F=24; p<0.001
Time x Nut: F=7; p=0.002
(h)*
CO2: F=31; p=0.01
Nutrients: F=17; p=0.03
CO2 x Nut: F=28; p=0.01
# of leaves
Time: F=26; p<0.001
Time x CO2 x Nut: F=3; p=0.04
(c)
Nutrients: F=29; p=0.01
# of leaves
Time: F=22; p<0.001
Time x Nut: F=11; p<0.001
Time x CO2: F=5; p=0.01
(i)
Nutrients: F=12; p=0.03
Detritus production (g FW day-1)
Time: F=73; p<0.001
Time x CO2: F=6; p=0.01
(d)
CO2: F=16; p=0.02
Detritus production (g FW day-1)
Time: F=32; p<0.001
Time x Nut: F=4; p=0.04
(j)**
Leaf area index (m2 of leaves m-2 of sediment)
Time: F=57; p<0.001
(e)
Net community production (mmol O2 m-2 h-1)
Time: F=15; p<0.01
Time x CO2 x Nut: F=7; p=0.02
(k)*
Meso-herbivory (% leaves with bite marks shoot-1)
Time: F=40; p<0.001
(f)
Community respiration (mmol O2 m-2 h-1)
Time: F=4 p=0.01
Time x Nut: F=3; p=0.04
(l)*
Ulva cover (%)
Time: F=255; p<0.001
Initial
week 1
week 2
week 3
week 4
week 6

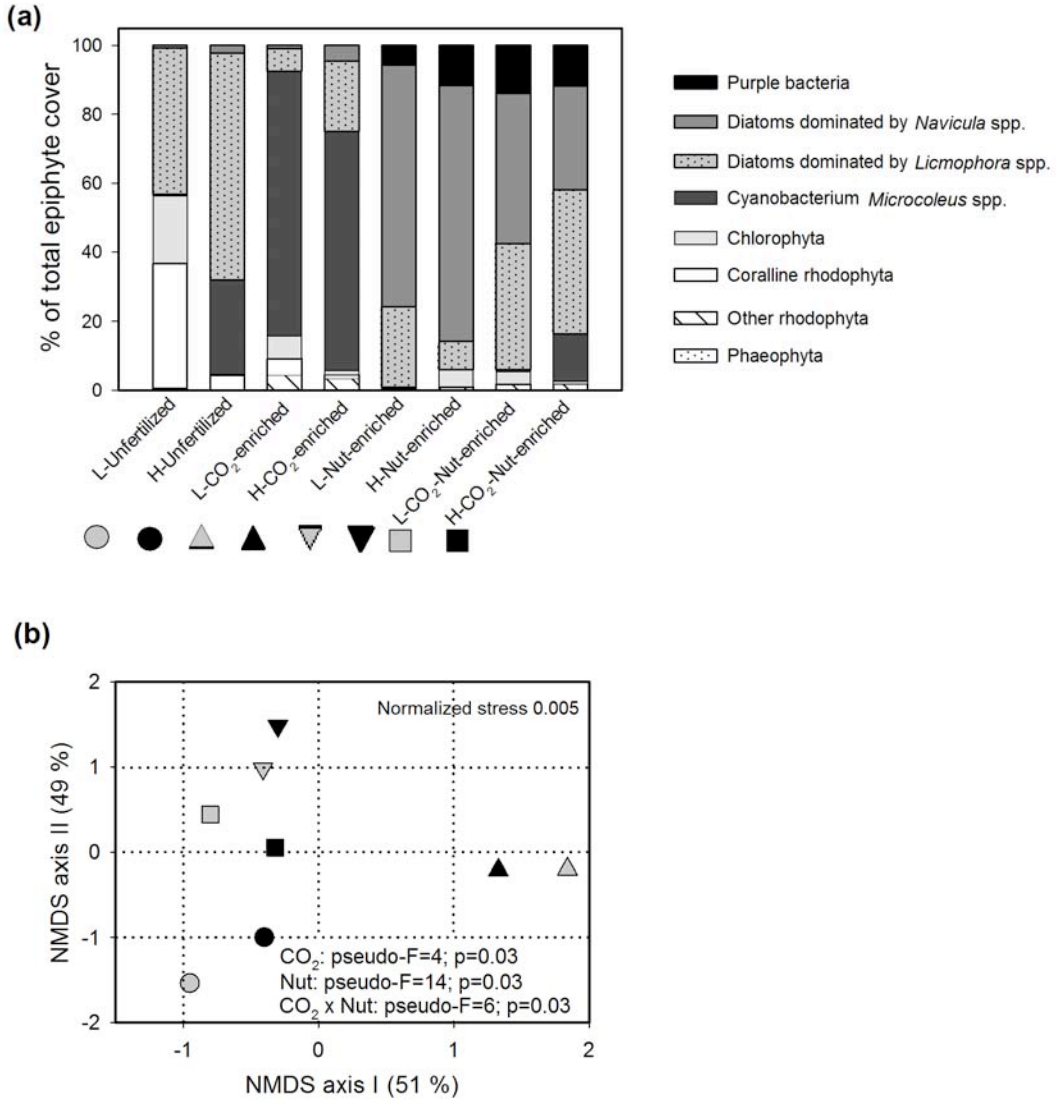
Figure 1

833



834

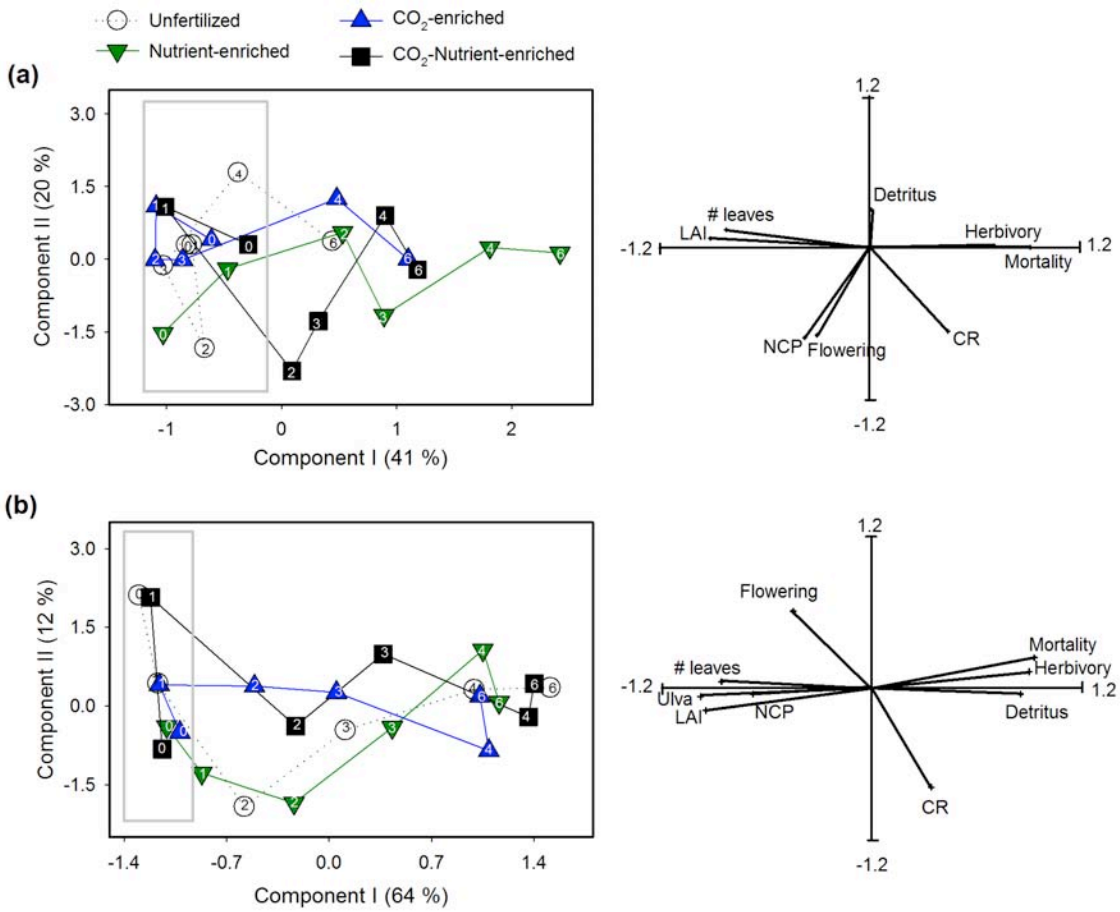
835 Figure 2



836

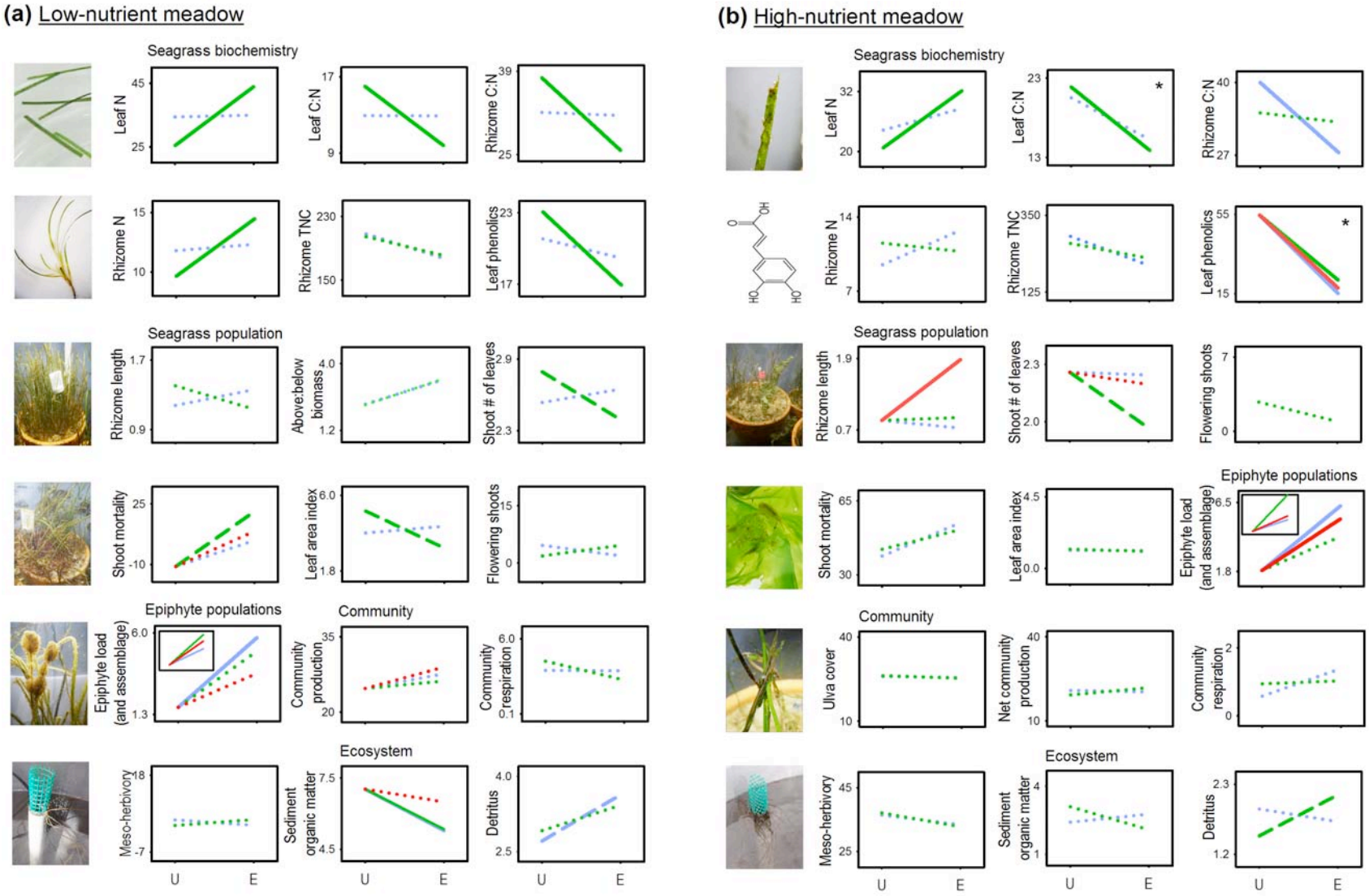
837 Figure 3

838



839

840 Figure 4.


(a) Low-nutrient meadow
(b) High-nutrient meadow
Seagrass biochemistry
Leaf N
Leaf C:N
Rhizome C:N
Rhizome N
Rhizome TNC
Leaf phenolics
Seagrass population
Rhizome length
Above:below biomass
Shoot # of leaves
Flowering shoots
Shoot mortality
Leaf area index
Epiphyte populations
Epiphyte load (and assemblage)
Community
Community production
Community respiration
Ulva cover
Net community production
Ecosystem
Meso-herbivory
Sediment organic matter
Detritus
U
E

841 Figure 5