



CO₂ 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 CO₂ 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 CO₂ benefits on *Z. noltii* leaf production were suppressed by epiphyte overgrowth, with no direct CO₂ effect on plant biochemistry or population-level traits. Multi-level meadow response to nutrients was faster and stronger than to CO₂. 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 CO₂ and nutrients separately resulted in reduced carbon storage in the sediment, probably due to enhanced microbial degradation of more labile organic matter. These changes, however, had no effect on herbivory or on community metabolism. Interestingly, individual effects of CO₂ or nutrient addition on epiphytes, shoot mortality, and carbon storage were attenuated when nutrients and CO₂ acted simultaneously. This suggests CO₂-induced benefits on eutrophic meadows. In the high-nutrient meadow, a striking shoot decline caused by amphipod overgrazing masked the response to CO₂ and nutrient additions. Our results reveal that under future scenarios of CO₂,

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 CO₂ and nutrient enrichment 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 have been published on the effects of ocean acidification, and reviewed by, among others, 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 CO₂ 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 CO₂ concentration has increased from pre-industrial levels of approximately 280 to 397 ppm in 2013

(NOAA, Mauna Loa Observatory, Hawaii), leading to a rise in the CO₂ 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 21st century, based on Intergovernmental Panel on Climate Change predictions (IPCC, 2013). 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 producer and alter inter-species competitiveness and producers' abundance (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 CO₂ effects has been mostly studied in terrestrial plants. Under elevated CO₂ 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, CO₂ enrichment may shift the biomass and composition of soil microbial communities, directly through different responses of microbial groups to high CO₂/low pH (Krause et al., 2012; Lidbury et al., 2012) or indirectly through reducing foliar and detritus quality (Drigo et al., 2007). Overall, high CO₂ 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 CO₂ and nutrient levels on marine coastal environments. Seagrass 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 producer (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 CO₂ 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 CO₂ and nutrient enrichments affect primary producers at the individual (plant biochemistry including CN contents 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 whether they react differently.

2 Methods

2.1 Study meadows

Samples of *Z. noltii* community were collected from two meadows located 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 (36°59'40 N 7°58'00 W; hereafter low-nutrient meadow) and the other under high nutrient levels (37°01'15 N 8°00'56.50 W; hereafter high-nutrient meadow). Low-intertidal samples exposed to a small immersion 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 (CCMAR) in the Ria Formosa lagoon. To assess the effects of CO₂ and nutrients on *Z. noltii* meadows, an enrichment experiment was conducted for 6 weeks during August–September 2011, after 4 days of acclimation to the experimental mesocosms. This time span is sufficient to detect any treatment-driven changes in physiological, morphological, and population traits of this fast-growing species (e.g. Peralta et al., 2002). Core samples of *Z. noltii* community, including sediment and algal, faunal, and microbial components, were randomly collected from each donor meadow and allocated to flowerpots of 20 cm diameter and height. Three flowerpots were placed in each of the 16 experimental mesocosms (tanks of 110 L), which were

Table 1. Environmental and *Zostera noltii* community traits (mean \pm SE) 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 Table S1 in the Supplement.

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

exposed to combinations of two CO₂ and two nutrient levels in a crossed design with two replicates.

Experimental levels of CO₂ encompassed present (pH 8.00 \pm 0.02, equivalent to ca. 400 ppm CO₂) and future conditions (pH 7.83 \pm 0.01, equivalent to ca. 800 ppm CO₂) in Ria Formosa lagoon. The mesocosms received sand-filtered seawater from two head tanks of 1000 L at a rate of 240 L h⁻¹. In one of the head tanks, the water CO₂ was manipulated to ensure fixed pH differences between treatment means within the range predicted for 2100 by the IPCC (pH decline = -0.18 units) following a commonly used method (e.g. Alsterberg et al., 2013). The CO₂ injection was controlled by an auto-analyser (Yokogawa, EXAxt 450, Tokyo, Japan), which continuously monitored the water pH and temperature. Total alkalinity, pH, temperature and salinity within the mesocosms, as well as the seawater dissolved inorganic carbon (DIC) and carbon speciation are provided in Table S2 in the Supplement.

Water nutrient levels encompassed the natural values found in the lagoon and the values of highly eutrophic conditions (N: 45 \times and P: 11 \times natural; see Table S2 in the Supplement). The nutrient enrichment was obtained by adding a solubilized mixture of the fertilizers ammonium nitrate and monoammonium phosphate directly into the water column of each enriched mesocosm using a multi-channel dosing pump. Water samples were collected weekly to analyse nutrient concentrations using a loop-flow analyser (μ Mac-1000; Systea, Anagni, Italy).

The water within mesocosms was homogenized using a submersible circulation pump placed at leaf height. Pumps were stopped for 2 h twice per day to simulate tidal currents. Twice a week, the epiphytes growing in the mesocosm walls were removed and the position of flowerpots within each mesocosm was reassigned to minimize potential spatial dif-

ferences. Natural settlement and growth of leaf epiphytes and small animals were allowed throughout the experiment.

2.3 Producers' traits at the individual or population level

Changes in producers at the individual or population level 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 was 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 between sampling events were randomly chosen within each flowerpot to quantify the number of leaves and the leaf area index (LAI) as indicators of above-ground production. 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 4 weeks of the 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 cm² of leaf area.

After 6 weeks, all shoots from each mesocosm were harvested and plant traits quantified in each flowerpot. Below-ground production 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 °C 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 °C until constant weight. Pooled material of five shoots was separated into leaves (without epiphytes) and rhizomes, freeze-dried, weighed, ground to fine powder, and used in subsequent analyses of plant biochemistry. Carbon and nitrogen concentrations were analysed using an elemental analyser (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 °C and determined with a spectrophotometer using chlorogenic acid as standard following a modified Folin-Ciocalteu method (Bolser et al., 1998).

2.4 Community- and ecosystem-level traits

The response of the seagrass community to CO₂ and nutrient enrichments was quantified weekly by: (1) the percentage of flowerpot surface covered by *Ulva* spp.; (2) the feeding activity of mesograzers (percentage of leaves showing bite marks in the same shoots used to measure the morphological traits); and (3) the whole-community metabolism quantified from the oxygen evolution within benthic chambers of 17 cm diameter (4.8 ± 0.01 L incubated) fitted to the flowerpots for 30–45 min at midday (12–14 h). A transparent acrylic chamber to estimate net production and a dark chamber to estimate respiration were simultaneously deployed within each mesocosm. Dissolved oxygen concentration was measured by spectrophotometry using the Winkler method (Labasque et al., 2004) in three water samples collected before and after incubations into 12 mL soda glass vials. Community metabolism was estimated from the net change in oxygen concentration during incubations integrated by the chamber volume and standardized by incubation time and bottom area. There were no effects of enclosure on the water temperature within chambers (measured with onset HOBO loggers, Bourne, MA, USA). All incubations were run under irradiances of photosynthetically active radiation (PAR) averaging 283 ± 8.6 μmol quanta m⁻² s⁻¹ (measured with a Li-192SA underwater PAR quantum sensor, Li-Cor, Lincoln, NE, USA), when *Z. noltii* photosynthesis was light saturated and not photoinhibited (Peralta et al., 2002).

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

2.5 Statistical analyses

The effects of CO₂ and nutrient treatments throughout the experiment were tested using three-way repeated-measures analyses of variance (RM ANOVAs). The subject repeatedly sampled was the mesocosm, CO₂ and nutrients were the among-subject factors (two fixed crossed factors), and time was the within-subject factor. To avoid the masking effect of the strong initial differences between meadows on the responses to experimental treatments, data for the low- and high-nutrient meadows were analysed separately. Data were checked for parametric assumptions and transformed where needed. When sphericity was not met, corrected degrees of freedom from Greenhouse-Geisser adjustment were used (Quinn and Keough, 2002).

The effects of CO₂ and nutrient treatments on variables measured at the end of the experiment were tested using two-way ANOVAs (two fixed crossed factors) after testing parametric assumptions. A normal distribution with unequal variances was found for all variables, which is usual when the sample size is small. Following a 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 (mistaken detection of differences). Welch's *t* tests that are robust against unequal variances were used to interpret significant interactions. Again, data for the low- and high-nutrient meadows were analysed separately.

To assess the ordination of treatments based on differences in the composition of leaf epiphyte assemblages, a non-metric multi-dimensional scaling analysis (NMDS) with Bray-Curtis distances was carried out. Because NMDS axes are arbitrary, the final solution was rotated using a principal component analysis (PCA) to align the largest variance in the first axis. The significance of the effect of CO₂ and nutrient treatments on assemblage composition was tested with a two-way permutational analysis of variance (PERMANOVA; two fixed crossed factors). To perform the test, Bray-Curtis distances were calculated from untransformed data and 999 permutations were used under a reduced model.

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

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 2 weeks (Fig. 1, left). Shoot recruitment occurred mostly in unfertilized but also in CO₂-enriched conditions until the third week (Fig. 1a), after which shoot mortality progressively increased. Figure 1d suggests that the *Z. noltii* LAI tended to increase with CO₂ enrichment until the third week of the experiment. A positive, significant effect of the CO₂ enrichment was observed on detritus production throughout the experiment (Fig. 1c). Nutrient addition increased shoot mortality (Fig. 1a), whereas it decreased leaf number and LAI (Fig. 1b and d). Shoot mortality induced by the nutrient enrichment was attenuated by the simultaneous addition of CO₂, 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 Sect. S3 in the Supplement).

The responses of the high-nutrient meadow to CO₂ 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). CO₂ enrichment interacted with nutrients to alleviate the nutrient-induced reduction of the number of leaves (Fig. 1h). No effects of CO₂ or nutrient enrichment were observed through time on LAI, meso-herbivory, *Ulva* spp. cover, shoot flowering, and community production or respiration (Fig. 1j–l and Sect. S3 in the 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 (±SE) of 89 (±3.7)% of shoots died, 81 (±9.1)% of the seagrass leaves showed bite marks and leaf area was reduced from 5.0 (±0.2) to 1.0 (±0.4) cm² shoot⁻¹. Similarly, *Ulva* spp. cover progressively decreased, being close to 0% in all treatments after 6 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 CO₂-and-nutrient-enriched). However, net production and respiration in these pots were within the range observed in the other pots (Fig. S3d and e in the 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 CO₂ or nutrient enrichment on plant damage by meso-herbivory in the low-

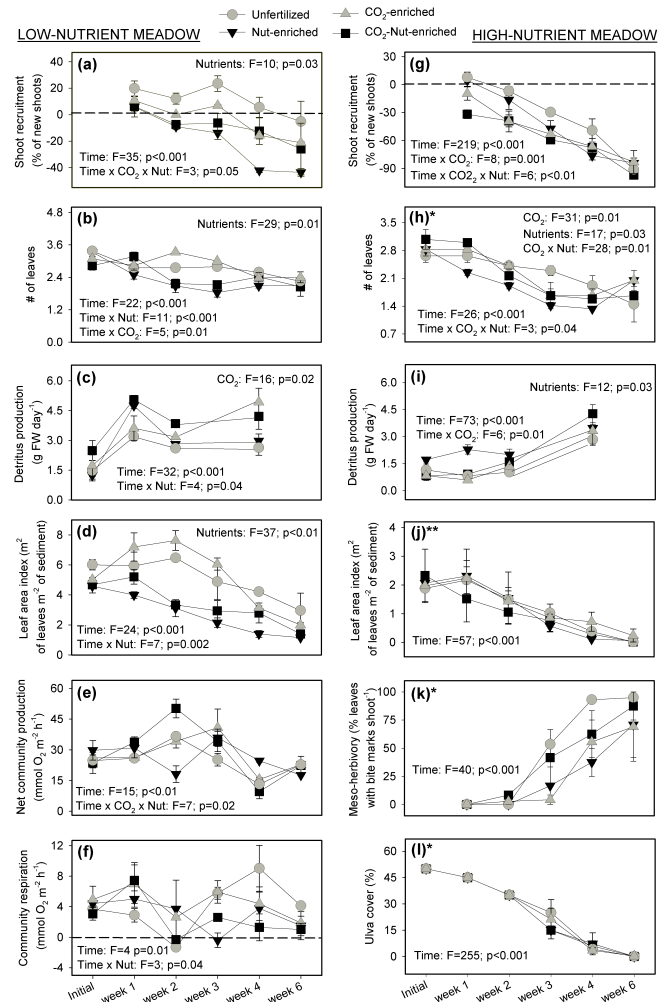


Figure 1. Effects of CO₂ and nutrient additions on *Zostera noltii* plant-, community-, and ecosystem-level traits from the low-nutrient (left graphs) and high-nutrient meadow (right graphs) through time. Symbols are means (±SE, n = 2). F statistics and p levels from RM ANOVA tests are shown for among-subject factors (CO₂; Nut: nutrients) and their interaction (CO₂ × Nut), and for within-subject factor (Time) and interactions (Time × CO₂, Time × Nut, Time × CO₂ × Nut). Only the significant effects (p < 0.05) and useful traits for results interpretation are shown here; the non-significant effects are shown in Sect. S3 (in the 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.

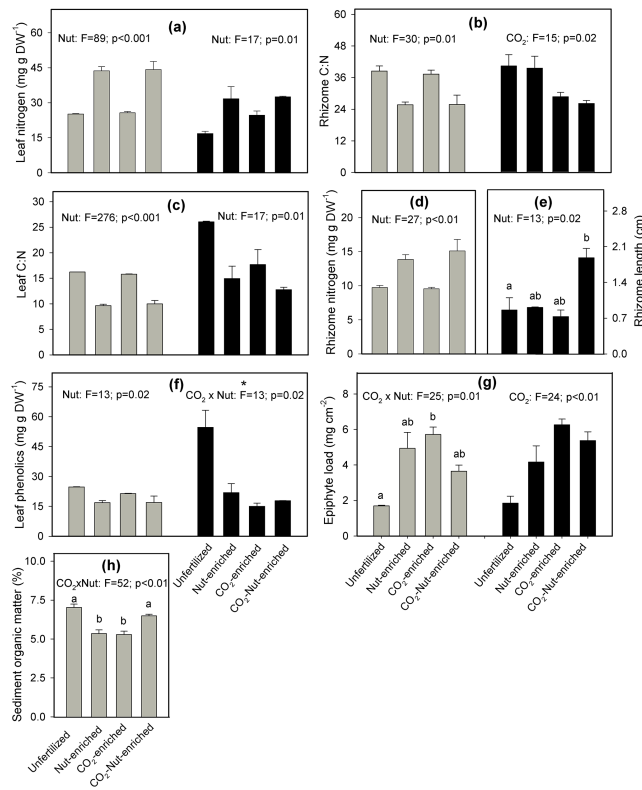


Figure 2. Effects of CO_2 and nutrient additions on *Zostera noltii* plant-, community-, and ecosystem-level traits measured at the end of the experiment from the low-nutrient (grey bars) and high-nutrient meadow (black bars). Bars are means (\pm SE, $n = 2$). The F statistics and p levels from two-way ANOVA tests are shown for each fixed crossed factor (CO_2 ; Nut: nutrients) or interaction ($\text{CO}_2 \times \text{Nut}$). Lowercase letters above bars show significant differences between treatments for significant interactions (Welch's 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 in Sect. S4 (in the Supplement).

or high-nutrient meadows. However, at the end of the experiment plants from the high-nutrient meadow showed 81 (± 9.1)% of leaves with bite marks, compared to only 6.9 (± 3.2)% in the low-nutrient meadow (Figs. 1k and S3b in the 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 Sect. S5 in the 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

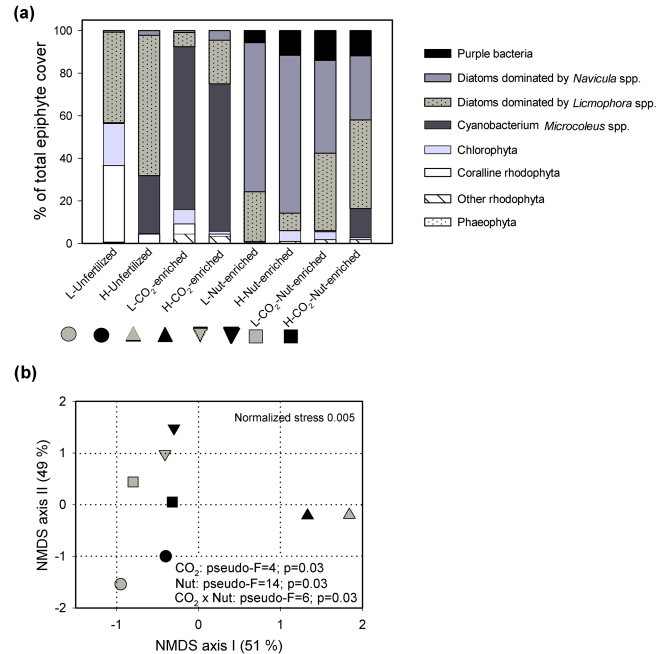


Figure 3. Responses of *Zostera noltii* epiphyte populations to the addition of CO_2 and nutrients: (a) Relative abundance of the main epiphyte taxa on plants exposed to experimental treatments; and (b) NMDS ordination of experimental treatments (see symbol legend in (a)) based on leaf epiphytes of plants from the low-nutrient (L; grey symbols) and high-nutrient meadow (H; black symbols). Pseudo- F statistics and p levels from two-way PERMANOVA testing are shown in the NMDS diagram for each fixed crossed factor (CO_2 ; Nut: nutrients) and their interaction ($\text{CO}_2 \times \text{Nut}$).

(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 (Welch's 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 (Welch's 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 (Fig. 2g and Table S4 in the Supplement). 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 CO₂ 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 CO₂ and nutrient additions caused an increase of the rhizome length (Fig. 2e).

Variables for which no significant effects of CO₂ or nutrient addition were detected are shown in Sect. S4 (in the Supplement).

3.3 Responses of *Z. noltii* epiphytes

Both, CO₂ 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 fan-like 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 CO₂ 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 CO₂-and-nutrient treatments the composition of epiphyte assemblages was similar, but with a reduced replacement of *Licmophora* spp. by pennate diatoms in the CO₂-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 CO₂-and-nutrient treatments during the fourth week.

NMDS ordination of treatments based on the epiphyte composition showed clear CO₂ effects (Fig. 3b). CO₂ 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 CO₂-, CO₂-and-nutrient, and nutrient enriched. Separation of CO₂ enrichments along axis I was due to a higher epiphyte cover (mean ±SE: 22 ± 2.3 cm² per 10 cm² of leaf) than the unfertilized, nutrient-enriched and CO₂-and-nutrient-enriched treatments (7.6 ± 1.4, 11 ± 1.3 and 8.4 ± 3.2 cm² per 10 cm² 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

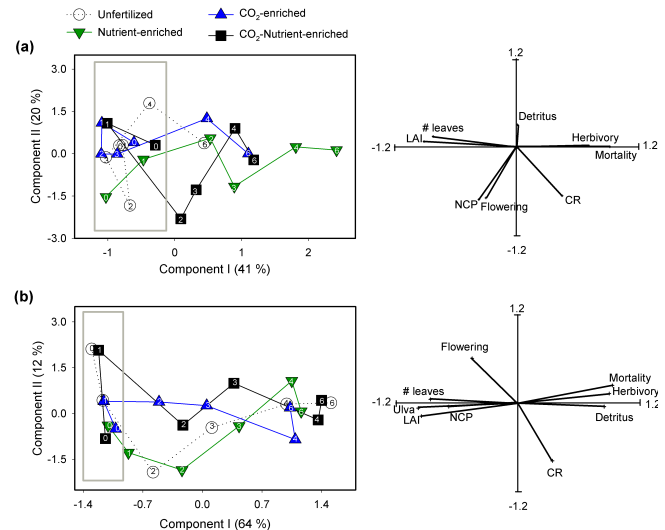


Figure 4. PCA of *Zostera noltii* plant-, community-, and ecosystem-level responses to treatments through time: (a) low-nutrient and (b) high-nutrient meadow. Numbers inside the symbols indicate sampling weeks from 0 to 6. The initial variability (week 0) along component I is incorporated within a grey rectangle to highlight the range of initial natural variability. Variable loadings on the two principal components are depicted in the right-hand graphs. NCP, net community production; CR, community respiration.

significant effects of CO₂, nutrients, and their interaction (Fig. 3b).

The abovementioned 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 CO₂ and nutrient enrichments (see Sect. S5, in the 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 correlated highly with component I were the LAI and the number of leaves, which were negatively correlated with shoot mortality and herbivory (see right-hand graph in Fig. 4a, and variable loadings presented in Table S5 in the Supplement). The variability of all treatment scores on component I during the first week and of unfertilized and CO₂-enriched treatments during the second and third weeks were within the initial range of natural variability (week 0, grey rectangle in Fig. 4a, left-hand graph). Initially, *Z. noltii* plants showed higher LAI and higher number of leaves. The time series ordination of the treatments along component I revealed that the effects of nutrient addition started during the second week, when the scores of nutrient and CO₂-and-nutrient treatments suddenly shifted to higher values. These nutrient effects were dominated by high mortality of

Z. noltii shoots and to a lesser 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 CO₂ treatment was slower (starting at week 4) and of lower magnitude than the response to nutrient and CO₂-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 CO₂. Flowering and net community production correlated highly with component II. These traits were substantially influenced by natural variability since no clear ordination of treatments was detected along component II.

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 correlated highly with component I were the LAI, the number of leaves, and the abundance of *Ulva*, which were negatively correlated with mortality, herbivory, and detritus production (Fig. 4b, right-hand graph). The system was initially dominated by high seagrass LAI and number of leaves, and cover of *Ulva* spp. This progressively shifted to a later stage (week 6) dominated by high *Z. noltii* mortality, herbivory, and detritus production (Fig. 4b, left-hand graph). Only community respiration highly correlated with component II, along which no clear ordination of treatments was detected. The range of initial natural variability of all treatment scores was narrower than for the low-nutrient meadow. 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 either unfertilized or enriched treatments.

4 Discussion

4.1 Effects of CO₂ enrichment in low-nutrient meadows

The CO₂ enrichment had no direct effects on *Z. noltii* biochemistry (Fig. 5a). We found no evidence of increased non-structural carbohydrates and subsequent nitrogen dilution effect (increased C : N ratio) as have 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 total phenolics 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 nonexistence 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 (re-

view by Stamp, 2003). However, we found no significant increase of seagrass productivity under CO₂ enrichment to support this trade-off, probably due to light limitation induced by epiphyte overgrowth from the third week of experiment onwards. Photosynthesis enhancements have been reported under CO₂ 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 (Palcios 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 CO₂ levels are highly context- and species-specific, and are not as consistent and predictable as resource availability hypotheses would suggest.

The most striking response of *Z. noltii* meadows to the CO₂ 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 the next sub-section). This suggests attenuation of the negative effect of reduced light availability by increased CO₂ 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 fan-like diatoms *Licmophora* spp. This is in accordance with previous studies that showed elevated CO₂/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. Despite the recognized capacity of mesograzers in controlling epiphyte biomass (Hughes et al., 2004), and particularly cyanobacteria blooms (Neckles et al., 1993), we found that their activity was insufficient to regulate the CO₂-induced proliferation of leaf epiphytes. Likely explanations are that the feeding capacity of the most abundant mesograzers 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 reported under nutrient enrichment (see the sub-section 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 CO₂ in low-nutrient meadows

We found that nutrient enrichment had a faster and greater effect than CO₂ 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 ammonium preferentially through the leaves and shows minimal translocation of nitrogen to below-ground tissues (Alexandre et al., 2011).

A reduction of the number of *Z. noltii* leaves and of LAI 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 contribute to shoot mortality by reducing light availability to seagrass leaves (Neckles et al., 1993). 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 fan-like 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. Coincidentally, 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 terrestrial systems (Wardle et al., 2004) and seagrass beds (López et al., 1998; Holmer et al., 2004; Spivak et al., 2007).

The simultaneous addition of CO₂ and nutrients did not modify the individual effects of nutrient enrichment on plant biochemistry, but attenuated the proliferation of certain epiphyte taxa (also occurring under high CO₂) and subsequent nutrient-induced *Z. noltii* mortality. The interactive attenuation of epiphyte overgrowth may result from an increase in the interspecific competition between the species that dominated the epiphyte community under elevated CO₂ (i.e. the cyanobacterium *Microcoleus* spp.) and under elevated nutrient levels (i.e. diatoms of the genus *Navicula*). Our findings are in agreement with the negative effects of interspe-

(a) Low-nutrient meadow

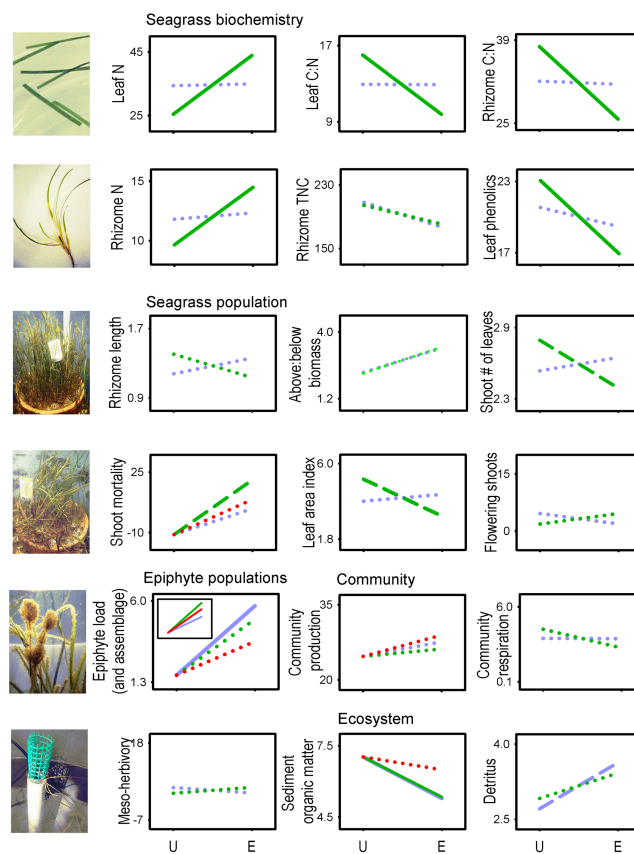


Figure 5. Summary of the effects of CO₂ (blue line) and nutrient (green line) additions and, when significant, of their interaction (red line) on *Zostera noltii* plant-, community-, and ecosystem-level traits of low-nutrient (a) and high-nutrient (b) meadows. Solid lines indicate significant effects on variables measured at the end of the experiment and dashed lines on variables measured through time. Dotted lines indicate no significant effects. Letters below the x axis denote unfertilized (U) and enriched (E) treatment, which is the pooled mean response to the respective enrichment over the other when there was no significant interaction (no red line). For shoot mortality and NCP in the low-nutrient meadow a significant time × CO₂ × nutrients interaction was detected and values were represented as for CO₂ × nutrients significant interactions. * Apparent effect due to a drastic increase in unfertilized plants in relation to the initial field conditions (see results).

(b) High-nutrient meadow

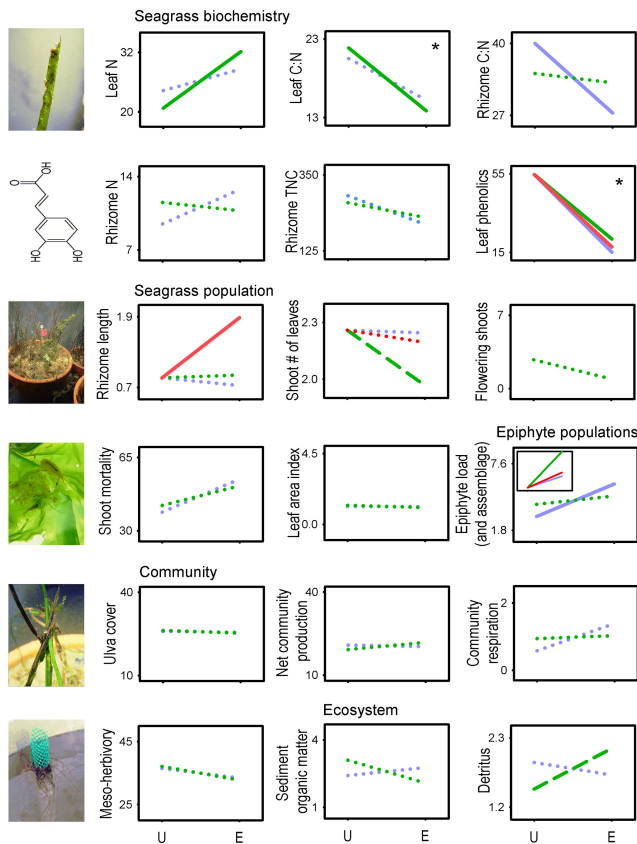


Figure 5. Continued.

cific competition on the involved species (i.e. symmetrical competition) that have long been reported in ecological studies (Connel, 1983). Together, attenuation by simultaneous CO_2 and nutrient additions of the overgrowth of certain epiphytes and of nutrient-induced *Z. noltii* mortality reduced the amount of more labile organic matter reaching the sediments compared to the individual CO_2 or nutrient enrichment. This probably resulted in the maintenance at control levels of the bacterial decomposition rates and of the sediment's capacity to store organic matter. To our knowledge, this is the first report of the interactive effect of CO_2 and nutrient enrichments on the meadow carbon sink capacity. Overall, we found that under simultaneous addition of CO_2 and nutrients, species interactions attenuated the direct effects of individual stressors on *Z. noltii* and on sensitive epiphyte species or taxonomic groups.

4.3 High- vs. low- nutrient meadows

Our results revealed that the expected benefits of high CO_2 predicted for the end of the century on seagrass productivity might be restrained by epiphyte overgrowth and by the interaction with local eutrophication. In both low- and high-nutrient meadows, CO_2 effects were more important in epiphyte populations than in the seagrass *Z. noltii*. These findings strengthen the increasingly recognized importance of species interactions in modulating the direct effects of eutrophication or acidification in single species, populations, and ultimately in ecosystem functioning (Orth et al., 2006; Kroeker et al., 2013b). The effect of nutrient enrichment was greater in the low- than in the high-nutrient meadow (Fig. 5a vs. b), with nutrient-induced mortality of *Z. noltii* only appearing in the former. When CO_2 and nutrient enrichments interacted, the CO_2 -induced increase of epiphyte load was maintained 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 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 results 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 CO₂ enrichment (Russell et al., 2013). In both studies, the whole-community metabolism was used as an 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 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 been reported for marine pelagic systems under CO₂ addition (Silyakova et al., 2013).

Overall, we found that shifts in the dynamics of leaf epiphyte or sediment communities mediated the multi-level responses of *Z. noltii* meadows to independent CO₂ or nutrient addition. They also modulated the attenuation of individual effects under simultaneous CO₂ and nutrient enrichment. Overgrazing masked the response to CO₂ 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.

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