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# Effect of Chronic Versus Pulse Perturbations on a Marine Ecosystem: Integration of Functional Responses Across Organization Levels

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## Abstract

Human impacts accelerate the intensity and frequency of perturbations on ecosystems; approaches that integrate responses across organization levels are, however, lacking, particularly in the ocean. We experimentally simulated the frequency of fertilization ('chronic' versus 'pulse' events) in orthogonal combinations of two intensities ('large' versus 'moderate' fertilization) to determine physiological and biological responses by the seagrass *Cymodocea nodosa* and associated flora (epiphytes and green seaweeds, specifically *Caulerpa prolifera*), as well as functional changes (community primary and secondary productivity) at the ecosystem level. We predicted that the absence of recovery time from chronic perturbation would more severely affect responses at population and ecosystem levels relative to discrete events (pulses). Nutrient enrichment increased the biomass of *C. prolifera* irrespective of its frequency, whereas seagrass biomass and shoot density particularly decreased under a chronic scenario. These demographic responses were connected with varying photophysiological performance of both *C. nodosa* and *C. prolifera*. Fertilization, regardless of its intensity and frequency, decreased the maximum photosynthetic rate of *C. nodosa*, concomitant with increased pigments, particularly under chronic fertilization, and decreased photoprotective (phenols) compounds. In contrast, fertilization boosted the maximum photochemical yield of *C. prolifera*, in addition to an increase in pigments and photoprotective compounds. Community primary and secondary productivity, however, did not vary under fertilization of varying intensity and frequency. In summary, fertilization precipitated population-level changes in physiological and biological attributes of vegetation. However, fertilization effects did not entirely cascade into ecosystem-level processes, that is, ecosystem productivity, which suggests a functional compensation (that is, increased algal per-

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formance to offset losses of seagrass production) during the initial stages of fertilization.

**Key words:** nutrients; ecosystem perturbation; frequency of perturbation; marine vegetation; temporal variability; marine angiosperms; green macroalgae; Atlantic Ocean.

## Introduction

Increasing human impacts globally accelerates the intensity and frequency of perturbations on ecosystems (Solomon and others 2007). Understanding the capacity of ecosystems to absorb and adapt to perturbations and retain their structure, function, and services is a vital goal of current ecology and conservation; this requires approaches incorporating responses from the organism to ecosystem scales. Holistic approaches that integrate responses across different organization levels are, however, majorly lacking, particularly in the ocean (Russell and others 2012). Human-mediated perturbations may occur chronically or through pulses and, as a result, the capacity of ecosystems to cope with perturbations does not only depend on mean values of whatever perturbation, but also on how perturbation events vary through time, for example, the temporal separation (frequency) of pulse events (Benedetti-Cecchi 2003). For example, gradients in the stress caused by water limitation are often described in terms of mean or maximum annual rainfall. Yet, terrestrial plants respond to the quantity of water available within a particular period of time (Cherwin and Knapp 2012) and to the time between succeeding rain events (Snyder and Tartowski 2006), rather than to total annual rainfall.

Human-mediated nutrient inputs (for example, from agricultural and urban sources) affect the structure and function of coastal food webs. As a global problem, continuous nutrient inflow into the coast negatively affects, for example, the health of salt marshes (Deegan and others 2012). Nutrient inflow into coastal habitats may be highly variable; the implications of varying frequency and duration of fertilization events (that is, chronic versus pulses) on coastal vegetation remain majorly unknown (Murphy and others 2012). Some studies have described the responses of slow-growing salt marshes to long-term fertilization (Kinney and Valiela 2013). However, responses by other types of submersed vegetation, seagrasses in particular, to varying types of fertilization remain untested, which have precluded generalizations. This is important, as even short-term increases in nutrients may have profound consequences on functioning of coastal ecosystems (Murphy and others 2012).

Landscapes dominated by marine angiosperms (seagrasses) worldwide deliver essential ecosystem goods and services (Duarte and others 2008). Conservation of seagrass meadows is, therefore, imperative, particularly in the context of the globally declining trends of seagrass meadows, predominantly in areas of large human-induced perturbations (Waycott and others 2009). Among the range of impacts derived from human activities, increases in the water column nutrient concentrations (eutrophication) have been pointed out as a key process negatively impacting seagrasses and their habitats (Hughes and others 2004; Burkholder and others 2007; Antón and others 2011; Tuya and others 2013b). Typically, nutrient enrichment directly promotes the growth of seagrass epiphytes, phytoplankton, and fast-growing macroalgae, which reduces the amount of light reaching seagrass photosynthetic tissue (Hughes and others 2004). Indirectly, eutrophication may increase herbivory on heavily epiphytized seagrass parts (Heck and others 2006; Leoni and others 2008; Bryars and others 2011; Tuya and others 2013b), as well as decrease benthic oxygen concentrations and increase concentrations of toxic sulfides in the sediment (Burkholder and others 2007). The combination of these mechanisms leads to seagrass demographic changes, ultimately promoting seagrass tissues necrosis and subsequent shoot mortality (Cabaço and others 2013).

A large body of literature has covered the morphological, demographic, and ecological implications of seawater fertilization over a range of seagrass species via experimental manipulations of nutrient delivery (Burkholder and others 2007; Cabaço and others 2013). Yet, there has been no experimental attempt to understand the effects of temporal variability (frequency) of fertilization events in regulating the direction and magnitude of seagrass morphological and functional responses, for example, seagrass photosynthetic performance, and further species interactions that drive the primary and secondary production of seagrass meadows. In general, experimental approaches in this field have followed the traditional strategy where mean (or maximum) values of nutrient concen-

trations are exclusively manipulated irrespective of their variation over the period of time in which estimates of ecological responses are studied. Fertilization events, however, greatly fluctuate through different time scales (Cabaço and others 2013), for example, from hours to days, and even from weeks to months. For example, nutrient inputs associated with coastal run-off change according to rain intensity and frequency. Similarly, sewage-associated nutrient delivery may temporally fluctuate according to the operational effectiveness of sewage treatment plants and local oceanographic patterns. Decreases in whatever seagrass ecological and demographic response may happen when populations are under chronic, rather than subjected to discrete perturbations through time, such as sewage release or organic enrichment, due to the absence of recovery time from continuous perturbation events (Cabaço and others 2013). Upward cascading effects of water column fertilization may concurrently alter the composition and abundance of associated fauna (Gil and others 2006). Nutrients may affect the nutritive quality and palatability of seagrasses and associated algae as food (Valentine and Heck 2001; Goecker and others 2005), as well as the quantity and quality of shelter provided by seagrasses for associated epifauna (Gil and others 2006; Antón and others 2011).

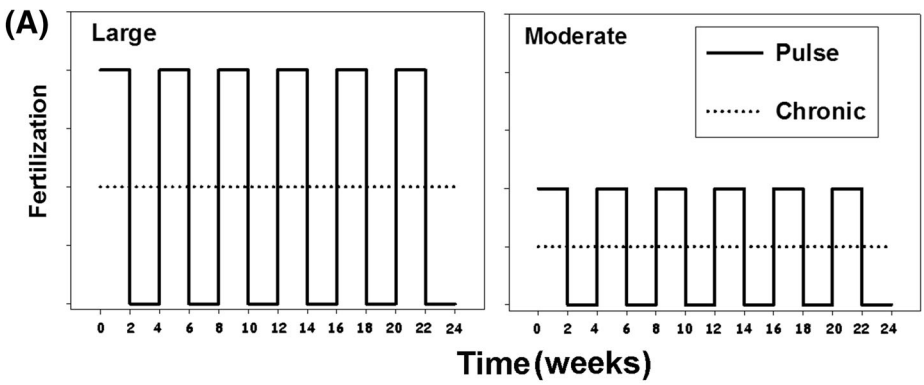
The seagrass *Cymodocea nodosa* (Ucria) Ascherson is distributed across the Mediterranean Sea and the adjacent eastern Atlantic coasts, including the Canary Islands (Barberá and others 2005), where meadows provide food and shelter for diverse invertebrate and fish assemblages (Espino and others 2011; Gartner and others 2013). Meadows constituted by *C. nodosa* are susceptible to a range of human-mediated stressors (Oliva and others 2012), including seawater fertilization, that actually have significantly reduced the presence of this seagrass at some coastal stretches of the Canary Islands (Tuya and others 2014a).

In this study, we experimentally manipulated the frequency of fertilization ('chronic' versus 'pulse' events) of different intensity ('large' versus 'moderate' fertilization) to determine physiological and biological responses by the seagrass *C. nodosa* and associated flora, that is, epiphytes and accompanying green rhizophytic seaweeds, in particular *Caulerpa prolifera* (Forsskål) J.V. Lamouroux, an abundant submersed macrophyte in the study system. We then assessed functional changes at the ecosystem level by estimating changes in community primary and secondary production. We predicted that the absence of recovery time from chronic perturbation would more severely affect responses at both the population and the ecosystem levels relative to discrete events (pulses) through time.

## Materials and Methods

### Experimental Design

This study was carried out in the center of a *C. nodosa* seagrass meadow (900–1200 shoots $m^{-2}$, 70–80% of coverage), which also contained accompanying green rhizophytic seaweeds, in particular *C. prolifera*. The meadow is approximately 35 ha in size and located at 7–11 m depth off the east coast of Gran Canaria (N 27° 44.923′, W 15° 33.855′). We manipulated nutrient levels in square plots (~0.7 $m^2$) according to two intensities of fertilization ('large' vs. 'moderate') that were either consistent through time (chronic fertilization) or applied through 6 fertilization pulses of 2 weeks through 6 months (Figure 1A); these pulses had the same duration through the experimental time (Figure 1A). When integrated through time, both 'chronic' and 'pulse' treatments had similar released quantities of nutrients for each level of fertilization intensity (Figure 1A). This allowed us to disentangle the effect of fertilization intensity ('large' vs. 'moderate' enrichment) from their temporal frequency ('chronic' vs. 'pulse') (Benedetti-Cecchi 2003). These orthogonal combinations were identified as the following treatments: 'large' (chronic), 'large' (pulse), 'moderate' (chronic), and 'moderate' (pulse) (Figure 1A). Four plots of each treatment were randomly established; adjacent plots were separated by more than 4–5 m between edges to assure plot independence. At all plots, a 30-cm stake was hammered in the center, while 4 additional stakes were secured about 50 cm diagonally away from the central stake (Figure 1B). Nutrients (Osmocote™ slow-release fertilizer, 18% total N and 4.5% P) were added in five permeable (diffuser) bags per plot, one attached to each stake using cable-ties (Figure 1B). Bags were made from polyethylene mesh with a 1-mm mesh size; this semi-permeable membrane allows a gradual release of nutrients (Bryars and others 2011; Tuya and others 2013b). The amount of fertilizer differed between treatments: 'large' (chronic) nutrient enrichment plots had 500 g of fertilizer throughout the entire experiment; 'large' (pulse) enrichment plots had 1000 g of fertilizer during the 2-week pulses; 'moderate' (chronic) enrichment plots had 250 g of fertilizer throughout the entire experiment (6 months); 'moderate' (pulse) enrichment plots



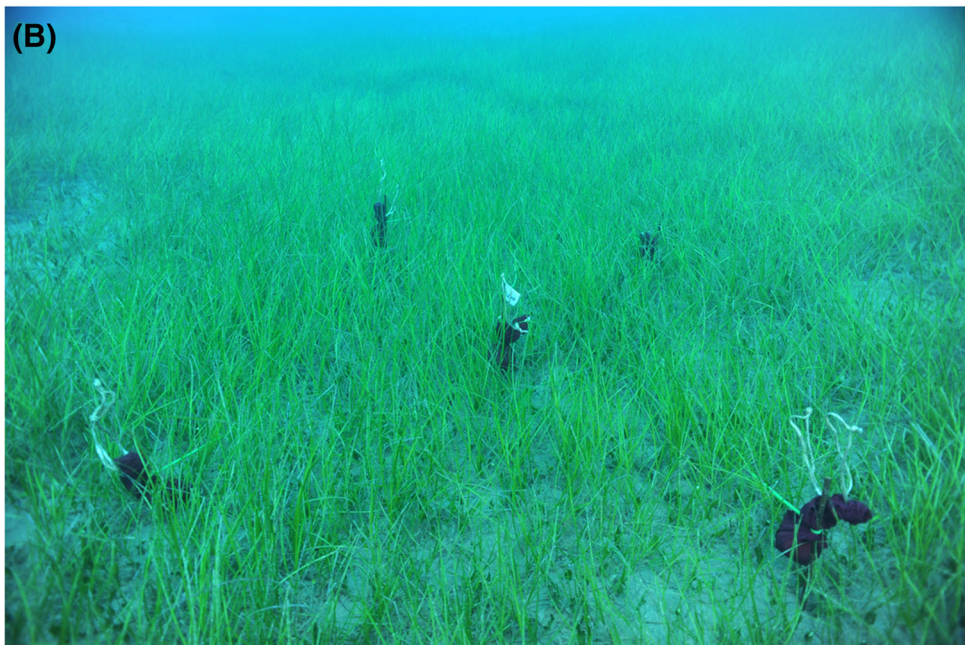




**Figure 1.** Distribution of (**A**) fertilization events over the course of the experiment for each level of fertilization intensity ('large' and 'moderate') and (**B**) nutrient bags within an experimental plot on a *Cymodocea nodosa seagrass* meadow. Six fertilization pulses of approximately 2 weeks were applied to those plots under the 'pulse' treatments. The amount of slow-release nutrients delivered was 6 and 3 kg for the overall experiment within 'large' and 'moderate' treatment plots, respectively.

had 500 g of fertilizer during the 2-week pulses. The volume of all diffusers was always the same by filling up the bags with inert gravel. Procedural controls (that is, placebo treatment containing gravel instead of fertilizer to account for manipulation artifacts) were not established, because it is experimentally impossible to distinguish 'chronic' from 'pulse' controls. Moreover, a previous experiment using similar in situ nutrient manipulations demonstrated that procedural artifacts had no influence over the performance of *C. nodosa* in the study region (Tuya and others 2013b). In any case, at the end of the experiment, we took samples/measurements from undisturbed controls 10 s of meters from the plots, for each of the responses indicated below.

The experiment started on the 17th June 2013 and lasted for almost 6 months (4th December 2013). This experimental duration is sufficient to detect morphological and ecological responses to experimental eutrophication, because epiphytes react rapidly to fertilization and because the mobility of epifauna in seagrass meadows is very high (Leoni and others 2006), particularly in the case of *C. nodosa* (Gartner and others 2013). The experiment was two months longer than a previous fertilization experiment performed within the same study region (Tuya and others 2013b). Bags containing fertilizer were replaced every 2 weeks to guarantee their effectiveness. During each visit, we additionally counted the number of seagrass shoots (shoot density) and *C. prolifera* fronds within each of $n = 3$, $25 \times 25$ cm, quadrats within each experimental plot, as well as in $n = 9$ control quadrats in the same meadow, but 10s of meters away from experimental manipulations.

Nutrient enrichment was measured by sampling water at approximately 5 cm from bags in situ before replacement ($n = 3$ samples per treatment at each of six times). In addition, water samples were

collected at about 30 cm, 90 cm, and more than 5 m from 'large' (pulse) bags to determine fertilization extent. Water samples were filtered on board and stored on ice and subsequently frozen (−20°C) until chemical analysis for phosphate and nitrate was conducted using an auto-analyzer. One-way ANOVA tested whether overall phosphate and nitrate concentrations differed between nutrient enrichment treatments (fixed factor), that is, integrated through the 6 sampling times. Data were $\ln(x + 1)$ transformed to achieve homogeneous variances. Among-treatment differences in temporal variation of nutrient concentrations were tested through the Levene's test. Linear regressions tested whether nutrient concentrations negatively decayed with distances away (5, 30, 90 cm and >5 m) from diffusers. Throughout the experiment, the amount of photosynthetically active radiation (PAR) reaching the top of the seagrass canopy (*ca.* 50 cm from the bottom) was measured by an Onset HOBO U12 4-channel external data logger away from the experimental plots; this device also registered seawater temperature. Both PAR light and seawater temperature may affect seagrass biology, and so these data were collected to put in context the environmental scenario of our study system to facilitate comparisons with other systems.

## Photo-Physiological Responses

On the 4th December 2013 (end of the experiment), we randomly collected between 9–12 *C. nodosa* leaves and 9–12 *C. prolifera* fronds from each plot. In vivo chlorophyll a fluorescence of photosystem II (PSII) was immediately assessed through a portable pulse amplitude modulation fluorometer (diving PAM-2000; Waltz GmbH, Germany); PAM fluorometry is widely used to evaluate the physiological status of macrophytes, including these two macrophytes (García-Sánchez and others 2012). After 15 min of dark adaptation, the minimum (basal) fluorescence was measured (Fo) and the maximum fluorescence (Fm) obtained immediately after applying a saturated pulse of actinic light (2350 µmol photons $m^{-2}$ $s^{-1}$, 0.8 s); the optimum quantum yield was therefore calculated as Fv/Fm = Fm − Fo/Fm ($n$ = 9), which is an indicator of physiological stress (Maxwell and Johnson 2000). A rapid light curve (RLC, $n$ = 9) was initiated involving 20 s of exposure to nine successive irradiances, from 20 to 1418 µmol photons $m^{-2}$ $s^{-1}$ (García-Sánchez and others 2012). RLCs were then obtained by calculating the electron transport rate (ETR) through the PSII for each level of actinic light through the formula: ETR (µmol electrons $m^{-2}$ $s^{-1}$) = ($\Delta F/F'm$) * $E$*$A$*FII, where '$E$' is the irradiance, '$A$' is the absorptance of each macrophyte (0.88 ± 0.02 for *C. nodosa* and 0.93 ± 0.03 for *C. prolifera*), and 'FII' is the fraction of chlorophyll associated with the PSII (0.5 for green seaweeds, according to Grzymski and others 1997). The absorptance, $A = 1 - (E_t/E_o)$, was calculated from the light transmitted through the thallus of each species ($E_t$) placed on a cosine-corrected PAR sensor (Licor 192 SB, Lincoln, NE, USA) connected to a data logger (Licor-1000); $E_o$ is the incident irradiance in the absence of the macrophyte. RLCs were fitted through the model provided by Jassby and Platt (1976) to obtain the initial slope of the curve ($\alpha_{ETR}$, that is, the photosynthetic efficiency), the saturation irradiance ($E_k$), and maximal ETR ($ETR_{max}$). All RLCs were performed at midday within a 1 h temporal window.

## Internal Contents in P, Phenols, and Pigments

Subsamples of dried seagrass rhizomes ($n$ = 2), as well as *C. prolifera* fronds ($n$ = 3), from each plot collected at the end of the experiment (4th December 2013) were grounded to a fine powder and subsequently analyzed for P content, which was determined spectrophotometrically after 3 h ignition of material at 500°C and 30 min of boiling of the ash in 0.2 N HCl (Fourqueran and others 1992). P provides a straightforward response in seagrass rhizomes when subjected to experimental fertilization in the study region, whereas N shows a less clear response (Tuya and others 2013b).

To measure phenolic compounds, leaves of *C. nodosa* and thalli of *C. prolifera* (*ca.* 0.25 g FW, $n$ = 3) from each plot were grounded with a mortar and a pestle in sand at 4°C, and extracted overnight in centrifuge tubes with 2.5 ml of 80% (v/v) methanol. The mixture was centrifuged at 4000 rpm for 30 min and the supernatants were collected (Sigma 2-16PK, Göttingen, Germany). Total phenolic compounds, expressed in mg $g^{-1}$ DW, were determined using gallic acid as a standard (Folin and Ciocalteu 1927). The reaction was complete after 120 min in darkness at 4°C, and the absorbance was measured at 760 nm in a spectrophotometer (Thermo Scientific Evolution 201, UV–Visible, China).

The content of chlorophyll a and b (chl-a and chl-b) was determined spectrophotometrically. The analyses were carried out by extracting pigments from plants (*ca.* 20 mg FW) using 1 mL of saturation solution of acetone 90% + $C_4Mg_4O_{12}$ and maintained in darkness at 4°C for 12 h. After cen-

trifugation (4,000 rpm for 20 min), each supernatant was used to measure pigments in a spectrophotometer (absorption spectra between 480 and 750 nm). The chl-a and chl-b concentration, expressed as mg $g^{-1}$ DW, were calculated using equations provided by Ritchie (2008).

## Community Metabolism and Harvesting

Net community production and respiration was assessed through the oxygen evolution method (Antón and others 2011) at the end of the experimental period (4th December 2013). At each experimental plot, incubations were performed underwater, by randomly fixing 1 clear and 1 dark Plexiglas cylindrical chambers (*ca*. 1 l of volume, 30 cm long, 6.6 cm of diameter) on the soft bottom around the central stake of each experimental plot, encompassing vegetation. Chambers were slowly inserted in the sediment without cutting belowground material. The oxygen concentration of the water was measured at the start and the end of incubations using a portable optical oxygen meter (Hanna HI9829, Germany). The duration of incubations (2 h) was established after a previous study (Tuya and others 2014b) and performed at midday; surface PAR was between 1000 and 1500 µmol of photons $m^{-2}$ $s^{-1}$. Once incubations were finished, all vegetation inside chambers was harvested by hand. Collections included below- and aboveground material to assess morphological and demographic responses by both *C. nodosa* and *C. prolifera* (see below); all material from each plot was transferred to a labeled bag. Differences between initial and final $O_2$ concentration in the clear chambers were compared to differences between initial and final $O_2$ concentration in the dark chambers, to calculate net/gross community primary production and respiration using the following formulas:

$$\text{Benthic Net Primary Production} = (F_{cb} - I_{cb}) \times V \times C \times Q_p^{-1} \times t^{-1}$$

$$\text{Benthic Respiration} = (F_{db} - I_{db}) \times V \times C \times Q_r \times t^{-1}$$

$I_{cb}$ and $F_{cb}$ are the initial and final water $O_2$ concentrations (clear chambers); $I_{db}$ and $F_{db}$ are the initial and final water $O_2$ concentrations (dark chambers); $V$ is the chamber volume (1 l); $C$ is the oxygen to carbon conversion factor of 0.375 mg C per mg $O_2$; $Q_p^{-1}$ and $Q_r$ are the photosynthetic and respiratory quotients (1.2 and 1, respectively); and t is the incubation time (2 h, Tuya and others 2014b). All concentrations are in mg $O_2$ $l^{-1}$. Final values were expressed on per area ($m^2$) and aboveground vegetation biomass (g DW). For each sample, we oven-dried (24 h at 70°C) all vegetation to obtain dry weight biomasses for each macrophyte. For each experimental plot, we additionally sampled epifauna by lowering an unbleached woven cotton bag with a quadrat (0.04 $m^2$) over the vegetation, cutting the macrophytes immediately above the sediment surface, and then closing the bag to retain the mobile fauna within (Gartner and others 2013). Epifaunal samples were labeled and preserved in a 10% seawater formaldehyde solution.

## Epiphytic and Grazing Responses

We counted the number of marks left by herbivore bites on every leaf per sample, as an indirect way to estimate herbivory at the end of the experiment (4th December 2013). No major herbivorous invertebrates (for example, sea-urchins) are found in the study area. Most bite marks were clearly crescent-shaped, a doubtless indication of consumption by herbivorous fishes (White and others 2011). When the apical part of either seagrass leaves or *C. prolifera* fronds was damaged, we omitted to record these as bite marks, due to the difficulty of ascertaining if these marks resulted from herbivory or other type of damage (for example, currents and/or swells).

Seagrass epiphytes were removed from six random leaves from each sample (that is, plot) collected at the end of the experiment using a razor blade and the composition (presence of epiphytic taxa) registered. All epiphytic material and their corresponding seagrass leaves were then dry-weighted (24 h at 70°C), and the amount (load) of epiphytes (dry weight) was expressed per g (dry weight) of leaf biomass.

## Epifaunal Identification and Estimation of Secondary Production

Samples from each plot collected at the end of the experiment were decanted through a 0.5-mm mesh sieve. The fraction retained was separated into different taxonomical groups and preserved in 70% ethanol. Macrofaunal specimens were determined to species level, whenever possible, by means of a binocular microscope. Epifaunal (secondary) production was calculated using the mean ash-free dry weight of animals retained by sieves of differing mesh size, the abundances of animals in different sieve size classes, and the general equations provided by Edgar (1990), in particular:

$$\text{Secondary Production}\left(\mu g\ d^{-1}\right) = 0.0049 \times B^{0.80} \times T^{0.89}$$

$B$ is the ash-free dry weight of animals (μg) and $T$ is the local seawater temperature (°C).

## Statistical Analyses

Because the number of living seagrass shoots and *C. prolifera* fronds within plots was followed through time, repeated measures ANOVAs (RM-ANOVAs) tested whether seagrass shoot density and *C. prolifera* frond density differed between fertilization intensity ('large' vs. 'moderate') that was either consistent or variable ('chronic' vs. 'pulse' fertilization) through the experimental duration time. The model, therefore, included orthogonal combinations of intensity ('large' vs. 'moderate') and frequency of fertilization ('chronic' vs. 'pulse' fertilization); both factors were considered fixed. Controls were ignored in this particular case, as there is no sense to include 'chronic' and 'pulse' controls. To test for differences in biological and physiological responses at the end of the experiment between orthogonal combinations of fertilization intensity ('large' vs. 'moderate') and its frequency ('chronic' vs. 'pulse' events), two-way ANOVAs were applied. Again, both factors were treated as fixed. Pairwise comparisons between each treatment and controls were carried out through a 1-way ANOVA with five levels corresponding to the four fertilization treatments plus the control plots; Student-Newman-Keuls (SNK) tests then resolved differences between each pair of treatments. For all ANOVAs, data were transformed to avoid heterogeneous variances whenever necessary (see results for specific transformations); the Cochran's $C$ test was used in this sense.

# Results

## Experimental Scenario: Nutrient Enrichment

Phosphate concentrations in the water column were higher adjacent to 'large' (including both pulse and chronic fertilization) enrichment relative

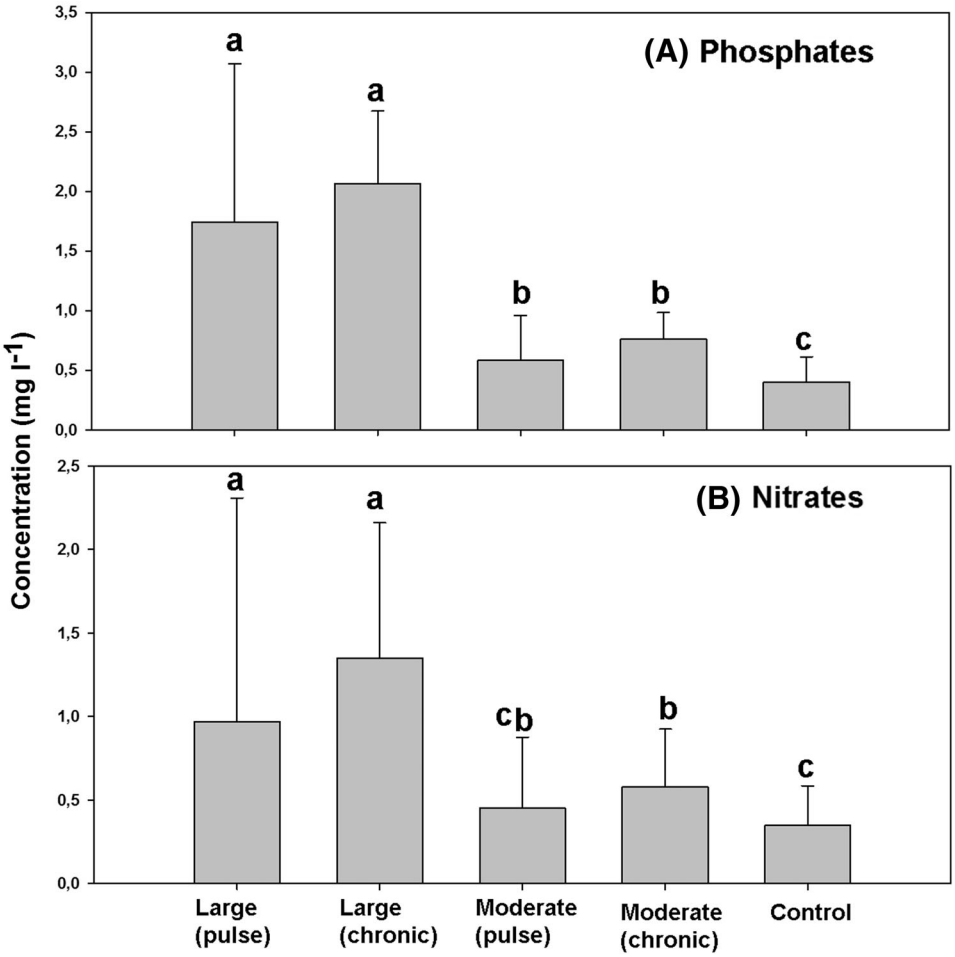



**Figure 2.** Concentrations (+SE) of (**A**) phosphates and (**B**) nitrates at approximately 5 cm from permeable bags within fertilized and control plots. Data are means of 6 times; at each time, $n = 3$ samples were taken before bag replacement. Different *letters* above *bars* denote significant differences.

to 'moderate' and 'ambient' plots (Figure 2A; $F_{4,25} = 5.73$, $P = 0.0020$). Differences in nitrate concentrations between fertilization treatments were more subtle (Figure 2B; $F_{4,25} = 3.51$, $P = 0.021$). Temporal variation of nutrient concentrations in pulse fertilization treatments exceeded that of the chronic fertilization for phosphates and nitrates, respectively (Figure 2A, B; Levene's test: $F_{4,25} = 11.13$, $P = 0.00002$, $F_{4,25} = 2.88$, $P = 0.0431$, respectively). Generally, but not always, there was an abrupt and significant decay of nutrient concentrations with distances away from diffusers (Online Appendix 1). The amount of PAR reaching the bottom typically decreased through time, as the experiment was initiated in summer, but harvested in early winter (Online Appendix 2). Seawater temperature varied between 20 and 23°C.

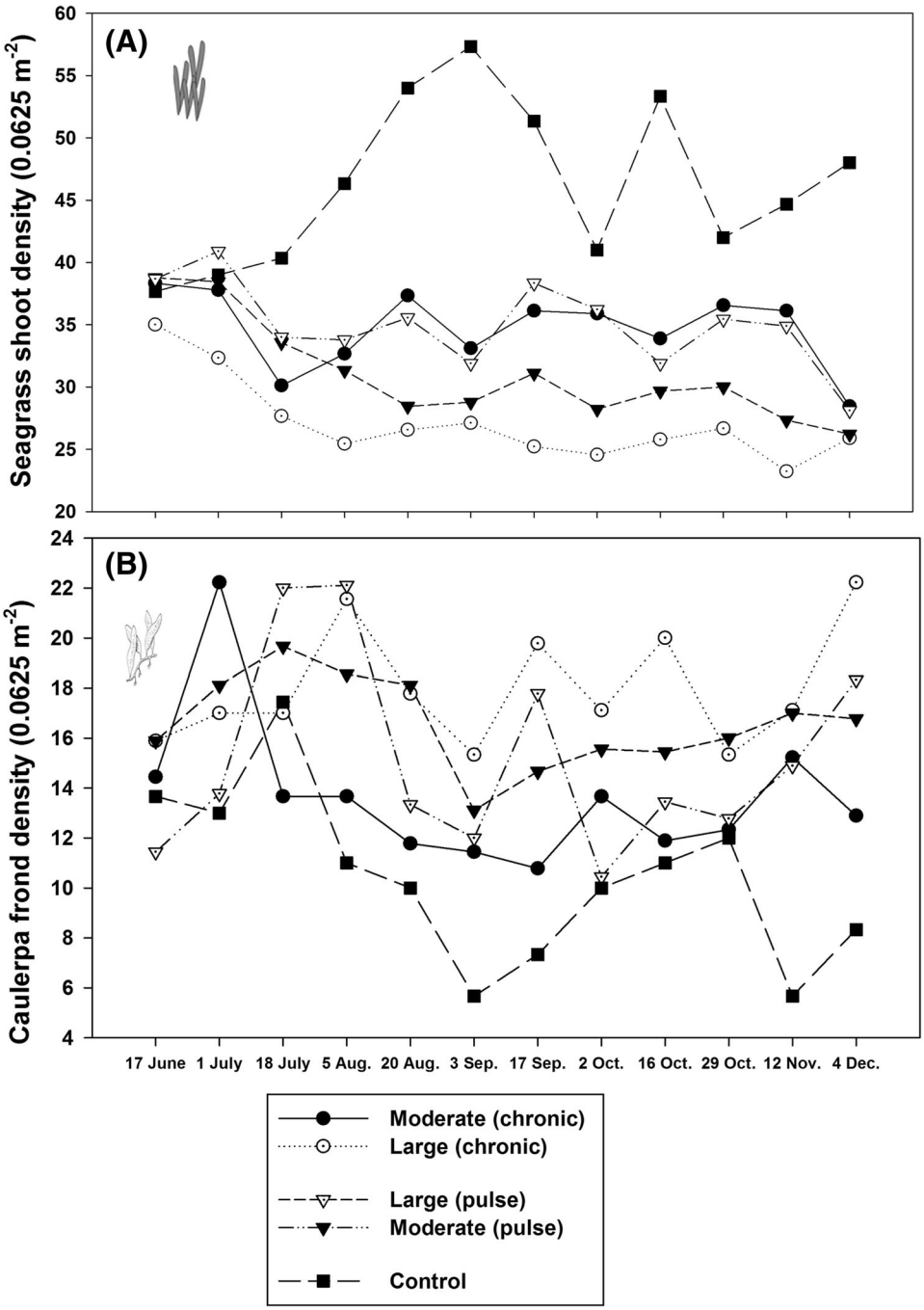



**Figure 3.** Changes in (**A**) seagrass shoot density and (**B**) the density of *Caulerpa prolifera* fronds throughout the experiment under varying fertilization treatments.

**Table 1.** Repeated Measures ANOVAs Testing the Effects of Fertilization Intensity (Int) and Frequency (*F*) on Seagrass Shoot Density and the Density of *Caulerpa prolifera* Fronds

| Seagrass shoot density | df | MS | *F* | *P* |
|---|---|---|---|---|
| *Between fertilization treatments* | | | | |
| Intensity | 1 | 387.2 | 1.293 | 0.264 |
| Frequency | 1 | 450.2 | 1.503 | 0.2291 |
| Int × *F* | 1 | 4137.9 | 13.813 | **0.0007** |
| Res | 32 | 299.6 | | |
| *Between times within fertilization treatments* | | | | |
| Time | 11 | 269.2 | 3.538 | **0.0001** |
| T × Int | 11 | 10.9 | 0.144 | 0.9994 |
| T × *F* | 11 | 32.3 | 0.425 | 0.9445 |
| T × Int × *F* | 11 | 96.4 | 1.267 | 0.2417 |
| Res | 352 | 76.1 | | |
| *C. prolifera* fronds | | | | |
| *Between fertilization treatments* | | | | |
| Intensity | 1 | 223.9 | 2.455 | 0.1270 |
| Frequency | 1 | 0.5 | 0.006 | 0.9402 |
| Int × *F* | 1 | 933.4 | 10.234 | **0.0031** |
| Res | 32 | 91.2 | | |
| *Between times within fertilization treatments* | | | | |
| Time | 11 | 131.9 | 5.187 | **0.0000** |
| T × Int | 11 | 89.2 | 3.507 | **0.0001** |
| T × *F* | 11 | 57.1 | 2.247 | **0.0119** |
| T × Int × *F* | 11 | 21.3 | 0.836 | 0.6039 |
| Res | 352 | 25.4 | | |

*Significant values are highlighted in bold.*

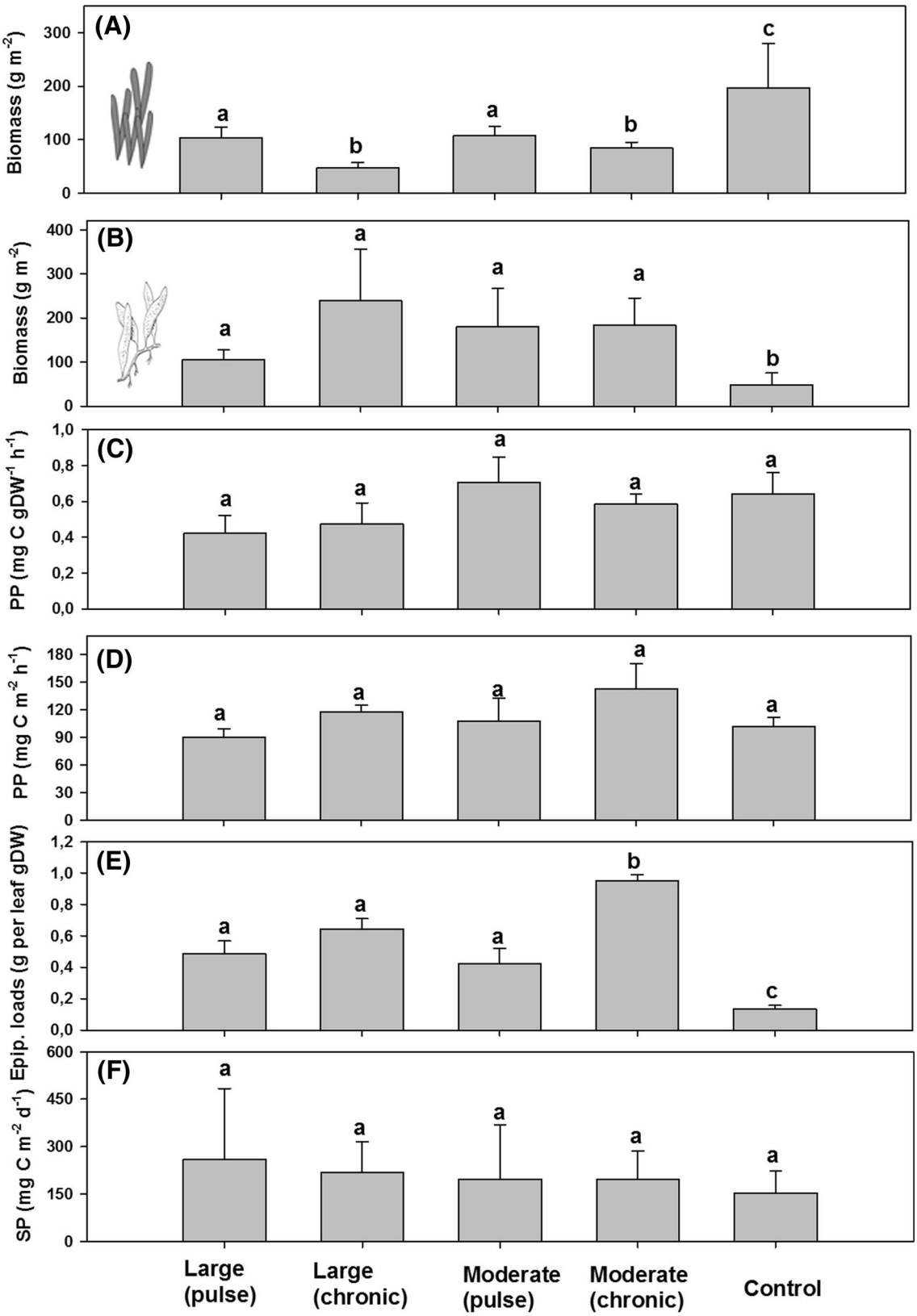
## Biological Responses and Community Primary Production

The number of seagrass shoots decreased through time for all fertilization treatments (Figure 3A; Table 1, 'Int × *F*', $P = 0.0007$). In general, there was a progressive increase in the number of *C. prolifera* fronds through time under a 'Large (chronic)' scenario relative to the other treatments (Figure 3B; Table 1, 'Int × *F*', $P = 0.0031$).

At the end of the experiment, nutrient enrichment decreased seagrass biomass, particularly under a chronic scenario (Figure 4A; Online Appendix 3). Overall, the biomass of *C. prolifera* increased under any type of nutrient enrichment relative to controls, despite a lack of statistically significant differences among nutrient enrichment treatments (Figure 4B, $F_{4,25} = 2.1662$, $P = 0.1032$). Community primary productivity did not vary between fertilization treatments (Figure 4C, D; Online Appendix 3) and was similar to that of the controls (Figure 4C, D, $F_{4,10} = 1.1428$ and $F_{4,10} = 1.2291$, $P = 0.3982$ and $P = 0.361$, for primary productivity on an area and biomass basis, respectively). Epiphytic loads (see Online Appendix 4 for a taxonomic description) were larger under chronic fertilization (Figure 4E; Online Appendix 3); in all cases, epiphytic loads under fertilization exceeded epiphytic loads in controls (Figure 4E; $F_{4,25} = 21.31$, $P = 0.00001$, pairwise tests). The number of grazing marks did not differ between fertilization treatments and controls ($F_{4,10} = 0.57$, $P = 0.689$).

## Physiological Responses

At the end of the experiment, the maximum quantum yield ($F_v/F_m$) of both *C. nodosa* and *C. prolifera* neither differed between fertilization treatments (Figure 5A, Online Appendix 5) nor relative to controls (Figure 5A; $F_{4,40} = 1.39$ and $F_{4,40} = 1.10$, $P = 0.2551$ and $P = 0.3678$, for *C. nodosa* and *C. prolifera*, respectively). Fertilization decreased the maximum photosynthetic rate ($ETR_{max}$) of *C. nodosa* relative to controls (Figure 5B; Online Appendix 5, $F_{4,40} = 2.73$, $P = 0.0421$), which was concomitant with increased quantum efficiency (Figure 5C, $F_{4,40} = 2.83$, $P = 0.0372$), as well as



◀**Figure 4.** Population and community-level responses to varying fertilization treatments at the end of the experiment, including: (**A**) seagrass biomass, (**B**) *Caulerpa prolifera* biomass, (**C**) community primary productivity (PP, per area), (**D**) community primary productivity (PP, per vegetation biomass), (**E**) epiphytic loads on *C. nodosa* leaves, and (***F***) secondary productivity (SP). Different *letters* above *bars* denote significant differences.

increased pigment contents under chronic fertilization (Figure 6B, C; Online Appendix 6; $F_{4,40}$ = 7.82, $F_{4,40}$ = 13.76, $P$ = 0.0001, $P$ = 0.00001, for chl-a and chl-b, respectively) and decreased phenol concentration under large fertilization (Figure 6D; Online Appendix 4; $F_{4,40}$ = 4.77, $P$ = 0.003). Fertilization did not increase the internal $P$ contents of below-ground seagrass compartments relative to

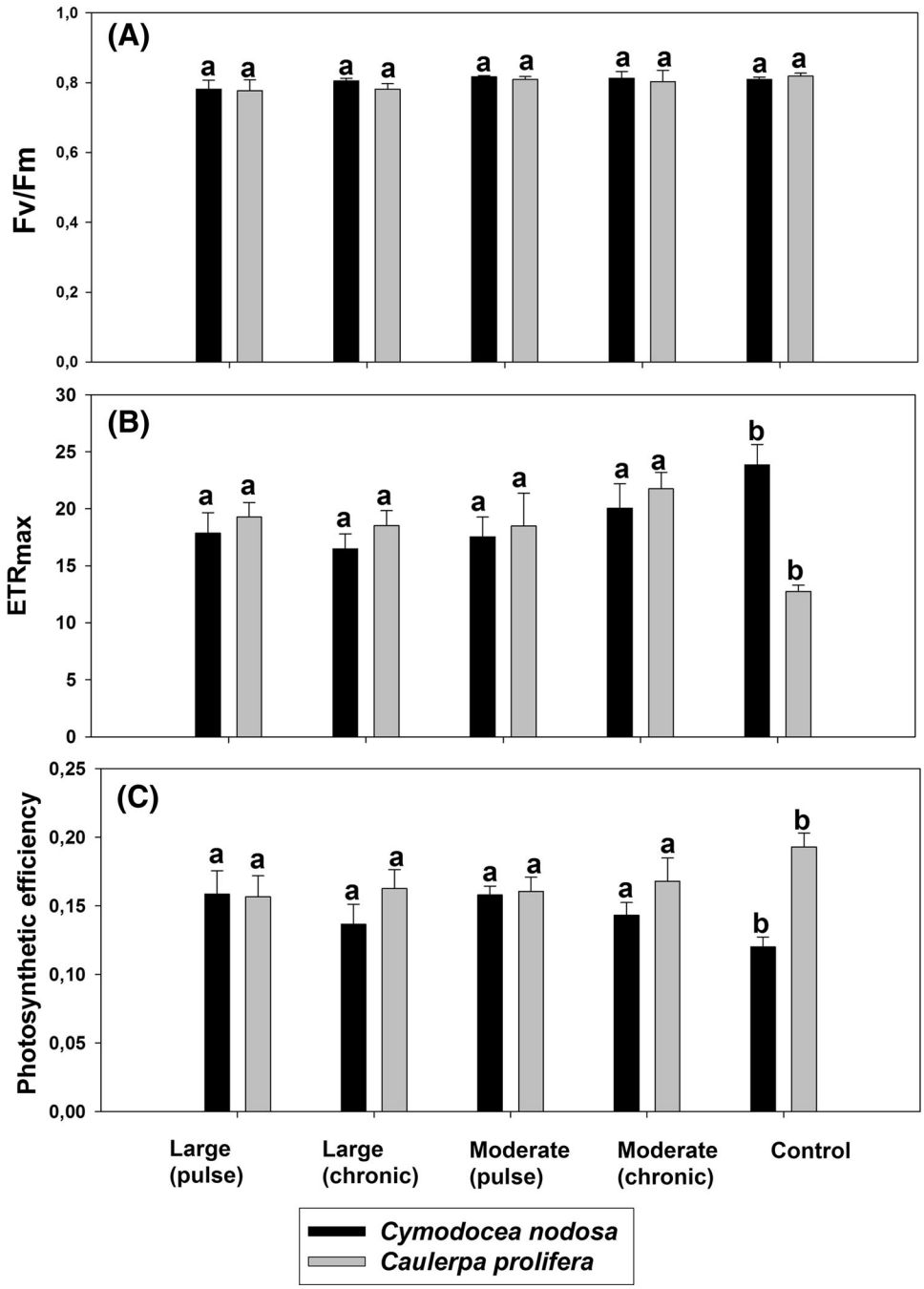



**Figure 5.** Photophysiological performance of *Cymodocea nodosa* and *Caulerpa prolifera* under varying fertilization treatments at the end of the experiment: (**A**) maximum quantum yield (Fv/Fm). (**B**) $ETR_{max}$ (maximum Electron Transport Rate) and (**C**) photosynthetic efficiency. Different *letters* above *bars* denote significant differences separately for each macrophyte.

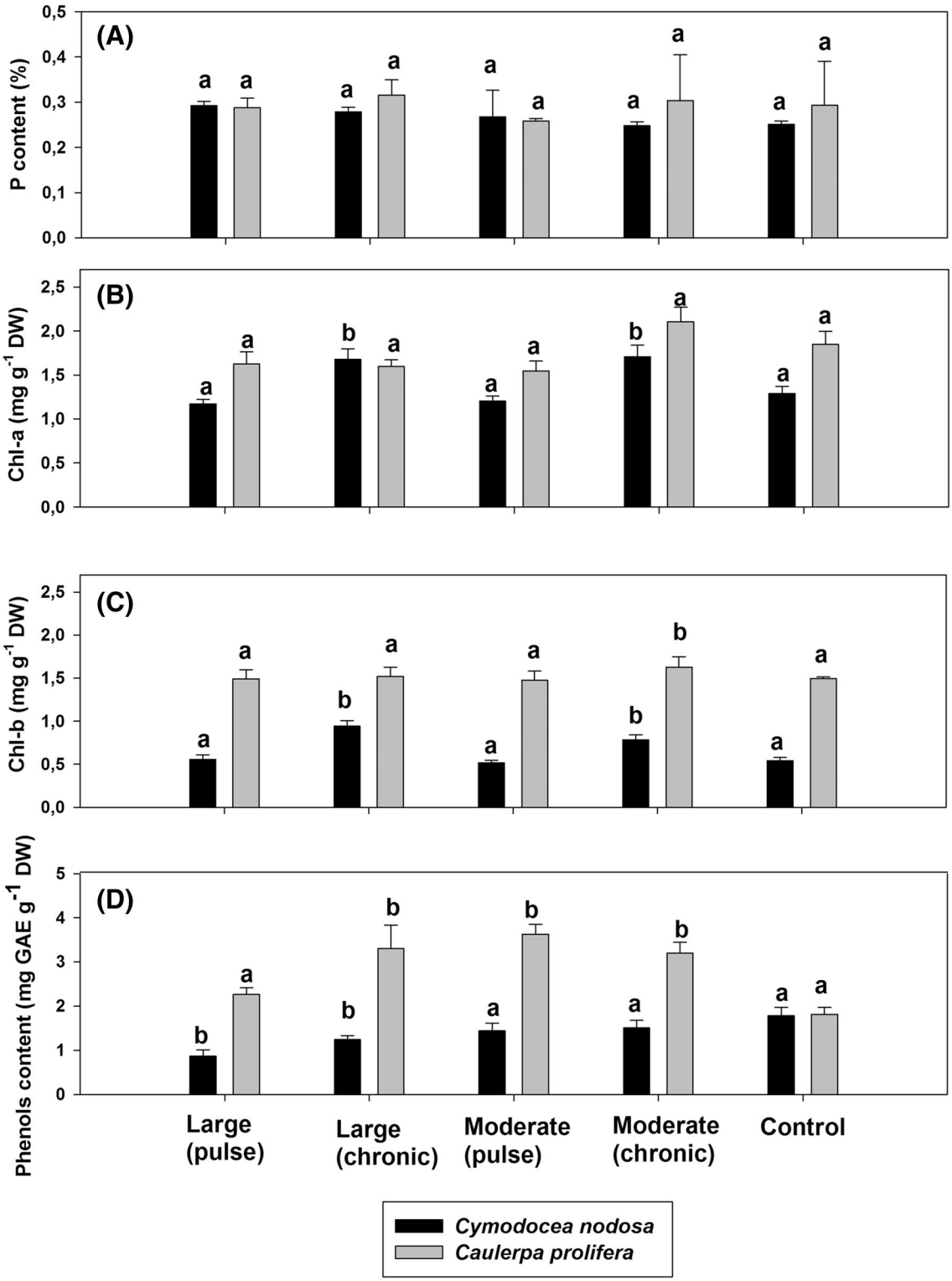



**Figure 6.** Physiological responses under varying fertilization treatments at the end of the experiment by *Cymodocea nodosa* and *Caulerpa prolifera*, including (**A**) internal P contents, (**B**) chlorophyll a contents, (**C**) chlorophyll b contents, and (**D**) total phenolic contents, expressed as gallic acid equivalents (mg GAE $g^{-1}$ DW). Different *letters* above *bars* denote significant differences separately for each macrophyte.

controls (Figure 6A, Online Appendix 6; $F_{4,25} = 1.84$, $P = 0.1533$).

In contrast to the seagrass, fertilization boosted the maximal ETR of *C. prolifera* relative to controls ($ETR_{max}$, Fig 5b; $F_{4,40} = 5.56$, $P = 0.0021$), concomitant with decreased photosynthetic efficiency ($\alpha_{ETR}$, Figure 5C, $F_{4,40} = 2.66$, $P = 0.0468$). Fertilization did not increase the internal P contents of *C. prolifera* (Figure 6A; Online Appendix 6). The concentration of chl-a of *C. prolifera* did not vary with fertilization (Figure 6B; Online Appendix 6, $F_{4,40} = 0.34$, $P = 0.8519$), but the concentration of chl-b slightly increased under fertilization relative to controls under a moderate (chronic) scenario (Figure 6C, $F_{4,40} = 3.12$, $P = 0.0253$). In general, the concentration of phenols in tissues of *C. prolifera* increased relative to controls (Figure 6D; Appendix 6 in Electronic Supplementary Material; $F_{4,40} = 8.74$, $P = 0.0001$).

Overall, these responses suggest that fertilization promoted *C. nodosa* to behave as a 'shade'-adapted plant relative to controls, regardless of the intensity and frequency of fertilization, whereas *C. prolifera* altered its photochemical performance under fertilization, regardless of the intensity and frequency of fertilization, towards a 'sun'-adapted behavior relative to ambient conditions.

### Epifaunal Responses: Secondary Production

Secondary productivity (see Online Appendix 7 for a taxonomic description of epifaunal assemblages) neither differed between experimental treatments (Figure 4F; Online Appendix 3, all terms, $P > 0.05$) nor relative to controls ($F_{4,10} = 0.05$, $P = 0.99$).

## Discussion

### Physiological and Biological Responses of the Seagrass *C. nodosa*

The negative impacts of eutrophication events over the health and function of seagrass meadows have been largely demonstrated across the globe (Burkholder and others 2007; Leoni and others 2008; Cabaço and others 2013). Our results have shown that under chronic fertilization, elevated water column nutrients caused a reduction in the biomass of seagrass above-ground tissues, which was less accentuated under pulse fertilization events. In contrast, under these circumstances, epiphytic loads and, importantly, the biomass and frond density of the green seaweed *C. prolifera* increased. A large body of literature has covered internal imbalances in the C metabolism of seagrasses when epiphytic blooms occur, promoting suffocation and energetic collapse of seagrasses (reviewed by Burkholder and others 2007), including our seagrass case-study, *C. nodosa*, in the study region (Tuya and others 2013b). At the end of the experimental period, *C. nodosa* was apparently not stressed under fertilization relative to controls (that is, the maximal quantum yield of photosynthesis remained unaltered). The seagrass, however, altered its photosynthetic performance relative to ambient conditions, so the plant overall behaved as a 'shade'-adapted plant under increased nutrients, regardless of the intensity and frequency of fertilization. This photosynthetic plasticity is typical of seagrasses under light deficiency (Durako and others 2003; Collier and others 2012), for example, as a result of large epiphytic loads on seagrass above-ground tissues. In the case of *C. nodosa*, acclimation to reduced light was accomplished by altering the structure of its photosynthetic apparatus, particularly by increasing the contents in pigments (chl-a and chl-b) to raise light capture, in addition to increased photosynthetic efficiency (Beer and others 2001). As a result, the plant does not need to protect against an excess of light, what would explain the decrease of photoprotective compounds (phenols), particularly since the intensity of herbivory remained unaltered during experimentation relative to controls. In this sense, and contrary to a previous experiment in the study region (a *C. nodosa* seagrass meadow at *ca.* 30 km away), the decrease of seagrass above-ground parts under chronic fertilization was not related to overgrazing of leaves, which in the area may be induced by large abundances of juvenile parrotfish *Sparisoma cretense* (Tuya and others 2013b). This was despite fertilization putatively enhanced the palatability of seagrass leaves through larger epiphytic biomass under fertilization.

A mechanism that ameliorates deleterious effects of perturbations on the structure and demographic decay of seagrasses, for example, *C. nodosa*, is the clonal integration of the plant. Translocation of resources between adjacent seagrass ramets is a survival strategy of *C. nodosa* (Terrados and others 1997), which in turn has been demonstrated to buffer the impact of fertilization over *C. nodosa*; that is, severed ramets experienced a much quicker

degradation as a result of fertilization relative to unsevered ramets (Tuya and others 2013b). In our study, we did not experimentally manipulate the clonal integration of the seagrass, so we may have underestimated the pervasive effects of fertilization if the clonal integration had been eliminated.

In general, these results suggest that although seagrass physiological responses fluctuated inconsistently between fertilization treatments according to varying intensity and temporality of fertilization, these intra-treatment differences were subtle compared to unfertilized controls. Importantly, from a demographic perspective, there was a significant decrease in *C. nodosa* biomass under chronic fertilization.

## Physiological and Biological Responses of *C. prolifera*

The green seaweed *C. prolifera*, native in the study region, typically behaves as a 'shade'-adapted macrophyte (Malta and others 2005). In our study, this alga altered its photochemical performance under fertilization, regardless of the intensity and frequency of fertilization. This macrophyte adopted a less 'shade'-adapted behavior relative to unmanipulated controls. This seaweed is a nitrophilic alga with clonal modular morphology (Collado-Vides 2002) that often takes advantage of fertilization (Malta and others 2005; Garcia-Sanchez and others 2012), although our results have not shown a statistically significant increase in the internal content of P under fertilization. Species in the genus *Caulerpa* show rapid growth in high nutrient conditions (Lapointe and others 2005). Under experimental fertilization, *C. prolifera* increased its photosynthetic capacity ($ETR_{max}$), as reported for other green macroalgae (Gómez-Pinchetti and others 1998; Figueroa and others 2009). This is directly connected with promoting physiological mechanisms to protect the photosynthetic machinery from excess light, for example, via increasing the concentration of phenolic compounds, as we here have demonstrated. The increment in the pigment contents under fertilization (N enrichment) has also been reported for green macroalgae, for example, *Ulva rigida* and *Ulva lactuca* (Gómez-Pinchetti and others 1998; Figueroa and others 2009), suggesting that pigment contents were controlled by nutrient availability rather than by light (Gómez-Pinchetti and others 1998).

## Ecosystem-Level Responses: Integration of Results

Seagrass ecologists have typically overlooked whether the separation and/or succession of perturbation events, that is, what we here consider chronic versus pulse perturbation episodes, may affect the functioning of seagrass meadows, particularly via experimental approaches. Without a doubt, experiments are context-dependent and limited to short durations, and may result in conclusions that cannot be entirely extrapolated to a long-term ecosystem scale (Cabaço and others 2013). Our results suggest a certain degree of similarity between fertilization treatments and controls for ecosystem-level attributes, that is, for primary and secondary productivity. This result contrasts with long-term experimental enhancement of nutrients in salt marshes, where both plants and herbivores initially responded positively to fertilization (Murphy and others 2012). Our result, however, should be taken with caution, as our experiment was limited to 6 months. It is thus plausible that longer experimentation would have resulted in larger effect sizes for ecosystem-scale attributes under fertilization relative to ambient conditions. At a global scale, for example, continuous nutrient enrichment impacts the health of salt marshes (Deegan and others 2012). In this sense, community primary productivity is eroded when luxurious *C. nodosa* seagrass meadows are replaced by green seaweeds, for example, *C. prolifera* (Tuya and others 2014b). In fact, this reinforces the notion that alterations at physiological and population levels in response to perturbations, for example, those experimentally simulated here, occur before cascading up towards ecosystem (for example, meadow) level responses (Collier and others 2012). In fact, when the capacity to absorb impact is surpassed, severe events can have sudden and long lasting effects, often pushing ecosystems towards other regimes or ecosystem states, where recovery may be prolonged, or even impossible (Holling 1973).

The structure and physiognomy of seagrasses underpins their role as 'foundation' species, mediating key ecological processes, such as their 'fish nursery' function. Although long-term perturbations, for example, nutrient enrichment, can have profound ecological implications (reviewed by Burkholder and others 2007), many ecological roles of macrophytes may depend on the species'

specific peculiarities; for example, some species may be more relevant as food source and others as biogenic habitat providers (Heck and others 2008). For example, *C. prolifera* may locally increase epifaunal abundances relative to *C. nodosa* on a per area basis (Tuya and others 2014b), for example, through inducing organic enrichment of the seabed (Holmer and others 2009). In addition, epifaunal richness and abundance may locally increase under moderate fertilization, as a result of increased food quantity (epiphytes) and palability (Tuya and others 2013b). In this study, the lack of significant differences in secondary production between experimental treatments may be caused by the local dominance of epifaunal assemblages by small-sized amphipods (1–3 mm of total length; this study, Tuya and others 2014b), which may not respond quickly to increases in food availability (epiphytes). Moreover, the low amount of drifting debris found in *C. nodosa* meadows, relative to other seagrass species (*Posidonia*, *Amphipholis*), does not support abundant epifaunal assemblages. The lack of responses for secondary production may also have been the result of predation limiting the abundance of epifaunal organisms, particularly as a result of the presence of abundant fish populations in the study area (Tuya and others 2014b). Finally, it should be noted that size class data were used as a proxy to estimate secondary production and may not provide an accurate estimate of secondary production.

Local changes in environmental conditions, for example, turbidity and water column nutrient concentrations, have facilitated the replacement of *C. nodosa* by *C. prolifera* at some locations from the Atlantic and Mediterranean Sea (Morris and others 2009; García-Sánchez and others 2012). In the study region, severe declines in the presence and abundance of the seagrass *C. nodosa* between the 1990s and 2011 have been concurrently accompanied by increases in the abundance of *C. prolifera* (Tuya and others 2013a). This observation was merely correlative, so a mechanistic understanding is missing. Our study has demonstrated that fertilization facilitated the biomass and productivity of *C. prolifera*. What still remains untested is whether interactions between both coexisting macrophytes may have been severely altered through fertilization on a long-term basis, as previously annotated for the Mediterranean Sea (Pérez-Ruzafa and others 2012). It is plausible that *C. prolifera* may interfere with nutrient acquisition, particularly as a result of its high demand for N, and light availability by *C. nodosa* under nutrient enrichment, and/or affecting seagrass photosynthesis through allelopathic products (Raniello and others 2009).

## Conclusions

In this study, we predicted that the absence of recovery time from chronic perturbation events would more severely affect responses at physiological, population, and ecosystem levels relative to events (pulses) separated through time. From a seagrass demographic perspective, our data confirm this, despite ecosystem-level responses remained elusive. Importantly, physiological changes precluded population and ecosystem-level responses, with varying responses under different scenarios of fertilization intensity and frequency. In summary, fertilization firstly precipitated changes in physiological responses that varied under different intensities and frequency of fertilization. Seagrass population-level responses (for example, shoot density) only varied under chronic fertilization, that is, inter-treatment variation under varying intensity and frequency of fertilization were small relative unfertilized controls. Finally, fertilization effects did not entirely cascade into ecosystem-level processes, that is, community productivity, which somehow suggests a functional compensation (that is, increased algal performance to offset losses of seagrass production) at initial stages of fertilization. As a result, both chronic and pulse fertilization may similarly impact seagrass-dominated ecosystems.

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