

Nutrient load and epiphytes are drivers of increased herbivory in seagrass communities

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ABSTRACT: Eutrophication is one of the major threats facing seagrasses, promoting effects in different compartments of the community (e.g. plants, epiphytes, fauna). In this study, we researched how *in situ* nutrient enrichment modified the consumption rates of *Cymodocea nodosa* plants during a period of 3 mo, by creating a set of mesocosm feeding choice experiments with the generalist herbivore *Paracentrotus lividus*. Nutrient enrichment intensified the consumption of *C. nodosa* leaves by increasing the palatability of their tissues at different levels. At a first level (i.e. the individual plant response), nutritional quality of the tissues increased (i.e. nitrogen content), while both biomechanical (i.e. absolute force-to-tear, F_{TA} , and specific force-to-tear, F_{TS}) and mechanical (i.e. fiber content) traits were reduced. At a second level, the presence of epiphytes coating the leaves increased their nutritional quality without actually increasing their biomechanical resistance, which rendered higher consumption rates. However, the presence of *Ulva* sp. (a highly palatable macrophyte) reduced the direct consumption of *C. nodosa* leaves, mainly when coated by epiphytes, a fact fully endorsed by the compensatory feeding theory. Therefore, the nutritional quality of the *C. nodosa* leaves is a key factor regulating their susceptibility to be consumed, although mechanical and biomechanical factors also gained importance when nutritional quality was low. In addition, the presence of other components of the community (epiphytes and macroalgae) may increase or reduce herbivore pressure, highlighting the complex nature of herbivore–plant interrelationships.

KEY WORDS: *Cymodocea nodosa* · Eutrophication · Grazing · Herbivore · Macroalgae · *Paracentrotus lividus* · Plant–herbivore interactions · Sea urchin

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INTRODUCTION

Herbivory is a key factor determining the structure and distribution patterns of seagrass communities, and largely influencing the transference of energy and matter through the whole ecosystem (Poore et al. 2012). Even though the diversity of organisms feeding in seagrass communities is large, from small mesograzers to mammals like dugongs (Thayer et al. 1984, Tomas et al. 2005, D'Souza et al. 2015), global estimates of herbivory rates on seagrasses are widely variable, from less than 10% up to 100% of the total production (Heck & Valentine 2006). Specific causes

of such variability are not well understood, since large inter- and intra-specific variability has been recorded (Cebrián & Duarte 1998, Valentine & Heck 1999, Bourque & Fourqurean 2013). The diversity and presence of herbivores and their feeding behaviour can, to some extent, explain this divergence (Prado & Heck 2011, Scott et al. 2018), but some characteristics of the seagrass tissues themselves can make them more or less appetizing (i.e. palatable) to the herbivores (Martínez-Crego et al. 2016, Jiménez-Ramos et al. 2018) and can also contribute to explaining such findings. Within the set of traits that can alter the palatability of seagrass tissues, leaf nutri-

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tional quality (e.g. nitrogen [N] and sugar content) is acknowledged as one of the most important factors, not only in seagrass ecosystems (McGlathery 1995, Cebrián & Lartigue 2004, Goecker et al. 2005), but also in terrestrial environments (Mattson 1980). However, morphological (e.g. width and thickness; Pagès et al. 2012, Martínez-Crego et al. 2016), structural (e.g. fiber and carbon [C] content; Mariani & Alcoverro 1999, Vergés et al. 2007a), biomechanical (de los Santos et al. 2012, Jiménez-Ramos et al. 2017a) and chemical (e.g. natural products; Vergés et al. 2007b, Hernán et al. 2017) traits can also influence tissue palatability and significantly modify the consumption rates of seagrasses. Since seagrass plants have the capacity to acclimate to local and global stressors by altering these traits, this can exert a significant impact on seagrass communities due to their influence on leaf palatability (Cebrián & Duarte 1998, Goecker et al. 2005, Prado & Heck 2011).

In this regard, eutrophication is a local stress that is acting globally, and it is currently considered one of the main causes of seagrass decline (Ralph et al. 2006, Short et al. 2006). Nutrient loads tend to increase the nutritional quality of the tissues (i.e. higher N content; van Katwijk et al. 1997, Villazán et al. 2013a,b), change leaf morphology (Short 1983, Lee & Dunton 2000, Brun et al. 2006, Romero et al. 2006), reduce the concentration of phenolic compounds (Ceccherelli et al. 2018) and make the leaves weaker from a biomechanical point of view (La Nafie et al. 2013, Jiménez-Ramos et al. 2017a, Soissons et al. 2018), which may eventually limit their resistance strategies and make seagrasses more vulnerable to herbivore pressure (Zieman et al. 1984, Neckles et al. 1993, Cebrián & Lartigue 2004, McGlathery 1995, Hughes et al. 2004, Goecker et al. 2005, Cebrián et al. 2009, Tomas et al. 2011, 2015). Seagrasses, however, do not live isolated in nature. They develop diverse communities (Duffy 2006), and the different components of the community (e.g. fauna, algae, bacteria) may have a differential capacity to respond to such nutrient enrichment (Cardoso et al. 2004, Jiménez-Ramos et al. 2017b). This may buffer or enhance the final response; for instance, nutrient load promotes the growth of macroalgae and epiphytes, which may compete with seagrasses for light and nutrients, and therefore increase the susceptibility of seagrasses to such harsh conditions (Dennison et al. 1993, Hauxwell et al. 2001, Brun et al. 2003, Rasmussen et al. 2013). Moreover, epiphytes may increase the feeding selectivity of herbivores (Heck & Valentine 2006, Marco-Méndez et al. 2015), because of the higher nutritional quality and lower structural and morphological defences they

bear (Alcoverro et al. 2000). In contrast, the presence of macroalgae may reduce the ammonium load in the water (Moreno-Marín et al. 2016) and even provide more diverse food sources for the herbivores, reducing the direct consumption of seagrass tissues (Hulme 1996, Jiménez-Ramos et al. 2018), which may benefit seagrasses. Therefore, the response to nutrient load takes place at different organization levels (individual, population and community), and involves both positive and negative consequences for seagrasses. This requires an understanding of the mechanistic responses underlying such interrelationships in order to explain the potential response of seagrass communities when they are subjected to a changing environment.

The combination of a nutrient enrichment field experiment followed by a set of feeding preference assays was used to research consumption rates and feeding preferences by a generalist herbivore on the temperate seagrass *Cymodocea nodosa* (Ucria) Ascherson. This species is largely distributed in shallow and soft substrates across the Mediterranean Sea and adjacent eastern Atlantic coasts (Ruiz et al. 2015), forming highly productive communities and providing food and shelter for diverse invertebrates and fish assemblages (Rueda et al. 2001, López de La Rosa et al. 2006). Moreover, the trophic importance of *C. nodosa* as a food resource for herbivores has been observed in several studies (Cebrián et al. 1996, Fernandez et al. 2012, Del Río et al. 2016, Jiménez-Ramos et al. 2017a), while the presence of fast-growing macroalgae such as *Ulva* sp., or large amount of epiphytes under nutrient load events were also recorded in these communities (Cebrián et al. 1999, Hauxwell et al. 2001, Rasmussen et al. 2013). Therefore, the individual response of this species to nutrient enrichment may be positive (Pérez et al. 1991, Pérez & Romero 1993) or negative (Pérez et al. 1994), while the final response at the community level can be more variable depending, for instance, on the balance of growth and consumption (Jiménez-Ramos et al. 2017b). In this framework, the response of this species to a 3 mo *in situ* nutrient enrichment was evaluated using a dual approach: firstly, by analysing how the response at the plant level (morphological, nutritional, structural and biomechanical) modifies its palatability, and thus the susceptibility to be consumed by the generalist herbivore *Paracentrotus lividus* (sea urchin). Secondly, by analysing the response at the community level, and how the presence of other photosynthetic organisms that develop under nutrient enrichment conditions may also alter the consumption rate of *C. nodosa*.

Thus, we hypothesised that nutrient enrichment can substantially increase the consumption rates of *C. nodosa* tissues because of the higher nutritional quality of the leaves (higher N content) and lower biomechanical traits displayed under enriched conditions. Moreover, the presence of epiphytes may also stimulate consumption of *C. nodosa* leaves independently of leaf properties, while the presence of *Ulva* sp. may divert feeding activity to that species, and therefore may relieve herbivore pressure on *C. nodosa*.

MATERIALS AND METHODS

Plant fertilization experiment setup

An *in situ* nutrient enrichment experiment was carried out in an inner section of the Bay of Cádiz (36° 1' N, 06° 15' W) over a period of 3 mo, from May to July, coinciding with the maximum growth period and biomass of *Cymodocea nodosa* in the area (Peralta et al. 2008). In the water column, nutrient peaks usually occur in spring–summer, with values up to 0.3 μM NO_2^- , 1 μM NO_3^- , 15 μM NH_4^+ and 2.3 μM PO_4^{3-} (Vergara et al. 2012). Two treatments (control and enriched plots) consisting of 3 replicates each (i.e. experimental plots) were distributed randomly in a large underwater *C. nodosa* meadow. Each experimental plot (50 × 50 cm) was separated from the others by at least 10 m and delimited by 8 sticks placed within the canopy of the seagrass bed. At the top of each stick (15 cm above the seafloor), a small mesh bag filled with slow-release fertilizer (Osmocote; N:P:K, 18:9:3) was employed to alter nutrient levels in the water column in the nutrient-enriched experimental plots. An empty mesh bag was attached to the sticks of the 3 control experimental plots representing ambient conditions. In each enriched experimental plot, 80 g of Osmocote were applied (0.5 kg m⁻²) and equally distributed in each mesh bag. Water samples were collected weekly in the central part of the square at 15 cm off the seafloor and within the canopy, using a silicone tube joined to sterilized plastic syringes and filtered through Whatman GF/F filters (0.45 μm) to measure nutrient concentrations. At this stage, mesh bags containing Osmocote® were checked and replaced, in case some of them were missing. Moreover, bags from control treatments were cleaned to avoid microbial and algal growth.

After 3 mo, above- and belowground biomasses were gathered manually using a 400 cm² quadrat

placed in the centre of each experimental plot. Shoots were collected very carefully with intact vertical rhizomes to minimize chemical changes occurring over time. *Ulva* sp. were also collected in the same area at the end of July. In the field and during transportation, all material was kept with water and aeration. Once in the laboratory, from each experimental plot, 10 experimental plant units (EPUs; a vertical shoot with its vertical rhizome segment and with no necrotic, bitten or broken leaves) were taken and carefully cleaned of epiphytes with a soft paper. Half of them were used to analyse morphological traits (length, width and thickness), while the other 5 EPUs were taken for biomechanical assays (cutting test) and subsequently used to analyse nutritional (i.e. N content) and structural (i.e. C and fiber contents) traits (Fig. 1). To determine how the presence of epiphytes can modify leaf palatability, another 5 EPUs from each experimental plot (from control and enriched treatments) were collected for biomechanical assays (cutting test) and subsequently used to analyse biochemical traits, but without cleaning off the attached epiphytes. Additionally, epiphyte biomass in control and enriched treatments was measured in 10 EPUs from each experimental plot. Epiphytes were carefully removed with a soft paper from each leaf of the shoot, freeze-dried and weighed afterwards. Epiphyte biomass was expressed as g dry weight [DW] shoot⁻¹. The remaining plant biomass from each experimental plot was kept alive in a 24 l tank with natural seawater, aeration and saturating light at room temperature (20°C), and used later for the feeding assays, conducted in the following 3 d (see Fig. 1).

Plant trait analysis

Morphological traits

Total leaf length, including sheath and blade length, was measured in all the leaves and expressed as an average for each shoot. Width and thickness were measured only in the 2 oldest leaves, 1 cm above the ligule. They were measured with a digital caliper (Mitutoyo 500 AOS) and a thickness gauge (Mitutoyo 7301).

Biochemical traits

Samples were freeze-dried and pulverized in a ball-grinder to determine N (nutritional features),

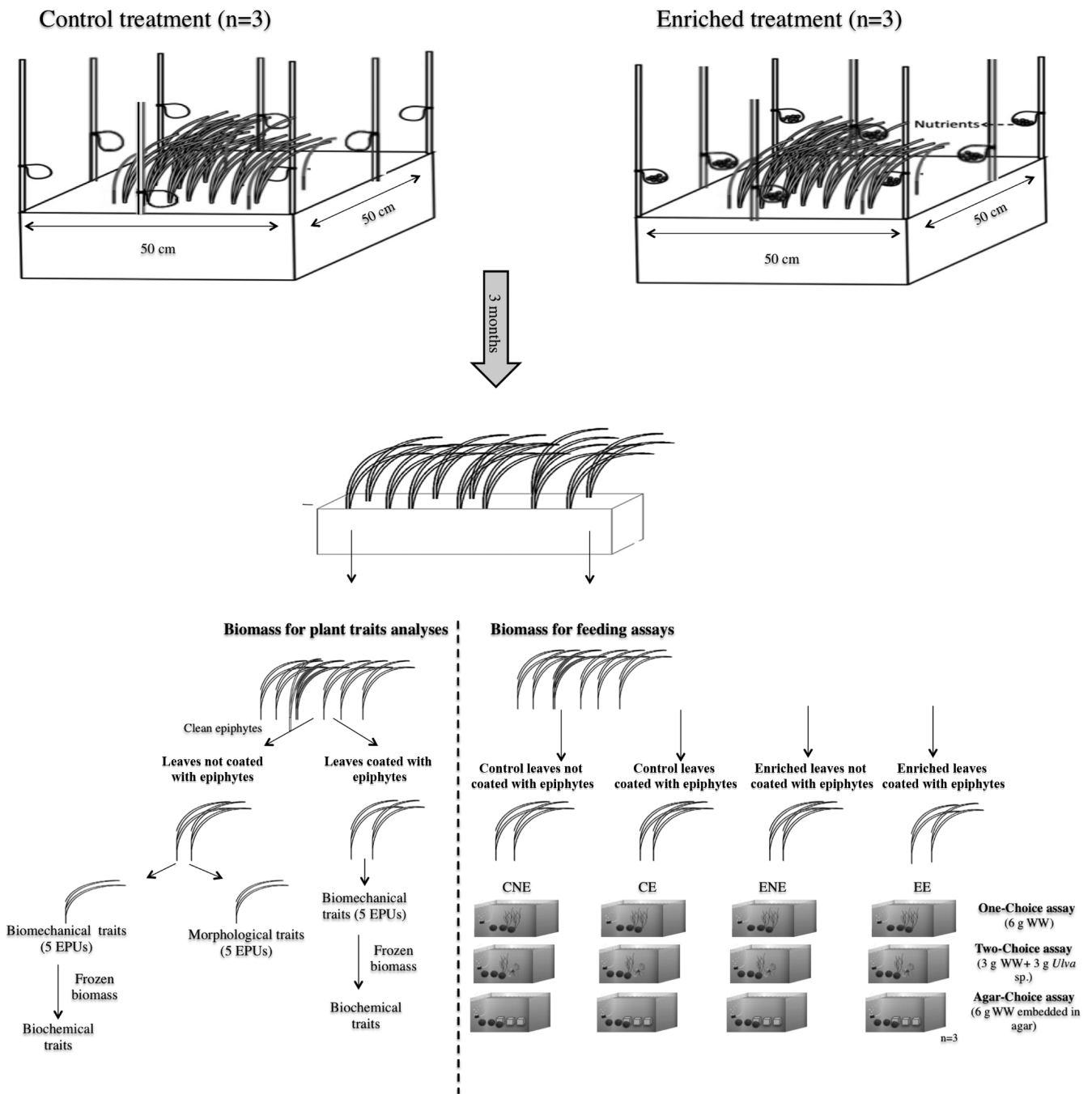


Fig. 1. Experimental set-up from *in situ* enrichment experiment to feeding assays in mesocosms

C, and fiber (structural traits) contents in leaves (not-coated and coated with epiphytes in each treatment). Elemental analysis was performed in a Perkin-Elmer 2400 elemental analyser, while fiber content was determined using the method of Van Soest et al. (1991) modified by de los Santos et al. (2012).

Biomechanical traits

Biomechanical properties were measured with an Instron universal testing machine (model 5542) and BlueHill software (v.2.18). The leaves were measured within 2 d of sampling and the specimens were tested in the same sequence in which they were collected, so

that the time of storage was homogeneous among samples and treatments. The first outermost, fully-developed leaf of the shoot was selected (normally the second leaf), and a portion of the leaf blade (4 to 5 cm above the ligule, not including the leaf sheath) was used for testing. Leaf-fracture properties were evaluated by using a cutting test, since this test measures the force required for foliar breakage (Wright & Vicent 1996, Aranwela et al. 1999). Since the whole leaf was cut transversally during the test, the force applied to cut the lamina included the leaf veins (de los Santos et al. 2012). During the tests, the tissue fragments were cut at a constant velocity of 10 mm min⁻¹, while the displacement (δ , in mm) and the force (F , in N) were recorded every 0.1 s until total fracture, when the maximum force (absolute force-to-tear, F_{TA} , in N) and displacement (δ , in mm) were recorded. From the force–displacement curve and the morphological traits of the specimens, the following mechanical properties were obtained: (1) total force needed to cut a single leaf blade, which depends on the leaf size and its mechanical properties at the material level (F_{TA} , in N); (2) material mechanical traits, normally called ‘material properties’, which is an inherent property of the material (F_{TS} [specific force-to-tear]; in N mm⁻²) since it takes into account the size of the assayed specimen. That is, material properties define the attributes of the matter the plants are made of, without regard to their dimensions. Regarding the ecological significance of these traits, whole-leaf mechanical traits indicate the force needed in absolute terms to cut or tear a single leaf blade by a herbivore, whereas material properties show the invested work or force required to ingest an amount of material, giving an idea of the cost-efficiency of the feeding process (de los Santos et al. 2012).

Herbivory preference assays

Sea urchin collection

Sea urchins *Paracentrotus lividus* were collected from a nearby rocky shore, La Caleta, in Cádiz (SW Spain, 36° 31' 39" N, 6° 18' 46" W). A stable population of *P. lividus* inhabits this location and therefore this area was chosen for the extraction of the individuals. Once permission was granted by local environmental authorities, individuals were collected at a depth of 2 m. Harvesting was carefully carried out by snorkelling, avoiding damage to the organisms. Sizes varied between 3 and 5 cm in diameter (adult size). Collected individuals were kept in coolers with seawater

and brought to the laboratory within 1 h. Once in the laboratory, sea urchins were distributed amongst 5 tanks (30 l; 18 ind. tank⁻¹) with aeration, and were fed with *Ulva* sp. for 3 d before starting the assays, to acclimate sea urchins to laboratory conditions.

Feeding preference assays

Feeding assays were run in a temperature-controlled climate room adjusted to 20°C, in a set of aquaria (volume = 20 l). Lighting was established with cool fluorescent tube lamps (T5 High Output Blau Aquaristic aquarium colour extreme fluorescents) in an 8 h light:16 h dark cycle because *P. lividus* usually exhibits nocturnal activity (Boudouresque & Verlaque 2001). Aeration pits were placed in all aquaria to ensure adequate mixing of water and air. Experimental sea urchins were selected from the large pool of collected sea urchins and were starved for 72 h prior to starting each assay. Three sea urchins were used in each experimental aquarium, and new ones from the acclimated pool were selected for each new feeding trial. Three different types of feeding assays were performed: (1) 1-choice feeding assay, (2) 2-choice feeding assay and (3) agar feeding assay, in which a total of 5 different types of foods were offered to *P. lividus*: *Ulva* sp. (*U*); control *Cymodocea nodosa* leaves, no epiphytes (CNE); control *C. nodosa* leaves plus epiphytes (CE); enriched *C. nodosa* leaves, no epiphytes (ENE); and enriched *C. nodosa* leaves plus epiphytes (EE). Leaves were of similar age in each feeding assay (i.e. oldest leaves). *Ulva* sp. fulfils 2 different roles in this experimental design. Firstly, as mentioned in the ‘Introduction’, it is a commonly found species during coastal eutrophication events that is readily consumed by sea urchins due to its high palatability. Secondly, the *Ulva* sp. treatment also served as a control of the feeding capacity of the sea urchins among the different treatments, in order to monitor their condition of health.

One-choice feeding assay. One-choice assays were done to elucidate whether feeding choice (2-choice assays, see next section) was in accordance with the feeding preference or whether compensatory feeding took place (i.e. if the consumption rate for *Ulva* sp. or enriched plant material was lower than that for control leaves or material with low nutritional value). In this type of assay, only one type of food (6 g fresh weight [FW] aquarium⁻¹) was supplied to the sea urchins in each aquarium (i.e. *U*, CNE, CE, ENE and EE). Simultaneously, autogenic controls (i.e. the same experimental

setup without adding sea urchins to the aquaria) were performed to account for potential changes in weight not due to grazing by sea urchins. Results showed no significant autogenic changes in any of the food types and were thus not considered further in the analysis (Student's *t*-test: $t_U = 1.18$, $df = 23$, $p > 0.05$; $t_{CNE} = -0.35$, $df = 23$, $p > 0.05$; $t_{CE} = -1.55$, $df = 23$, $p > 0.05$; $t_{EE} = -0.45$, $df = 23$, $p > 0.05$; $t_{ENE} = -1.12$, $df = 23$, $p > 0.05$).

Two-choice feeding assay. The 2-choice feeding assays were performed using *Ulva* sp. (*U*) and different types of *C. nodosa* leaves (i.e. CNE, CE, EE, ENE), to test how the presence of other photosynthetic organisms (such as the fast-growing macroalgae *Ulva* sp. that develops under nutrient-enriched conditions) may also alter the consumption rate of *C. nodosa*. A total of 3 g of fresh *Ulva* sp. and 3 g of each of the fresh seagrass leaves from the different treatments (i.e. CNE, CE, ENE, EE) were placed in the aquaria together (i.e. *U*+CNE; *U*+CE; *U*+EE; *U*+ENE), rendering 4 different assays. Likewise, autogenic controls were performed to account for potential changes not related to sea urchin grazing activity. Results showed no significant autogenic changes in *Ulva* sp. and seagrass during control tests, and therefore were not considered further in the analysis.

Experiments were conducted on consecutive days for each feeding assay (1-choice and 2-choice feeding assays) due to time and space restrictions. Three replicates were done for each feeding assay and were conducted over a 24 h period. Both *Ulva* sp. and seagrass biomass were distributed randomly and secured to the bottom (sandy bottom). Seawater temperature was monitored during the experimental period and averaged $22.5 \pm 0.7^\circ\text{C}$. Once the testing period was complete (24 h), the remaining material was removed from each aquarium, blot-dried and weighed, and consumption rates were calculated as the difference between initial and final wet weight biomass per sea urchin and day (i.e. g WW ind.⁻¹ d⁻¹).

Agar preference assay

Agar diets were used to test the capacity of consumers to detect differences in plant nutritional features, since the effect of tissue structural characteristics are eliminated in this type of diet (Ojeda & Caceres 1993, Prado & Heck 2011). A total of 6 g of each type of food (i.e. *U*, CNE, CE, ENE and EE) was placed independently in a heated mixture of 100 ml distilled water (as indicated by Goecker et al. 2005)

and 2 g of agar (Carolina Biological Supply). The mixture was poured into small moulds (2 cm diameter) and allowed to cool for 1 h in a refrigerator. Samples were then removed from the moulds and weighed to the nearest 0.001 g. The experimental design was conducted as 1-choice feeding assays with 3 replicates each (i.e. 5 types of assays: *U*, CNE, CE, EE, ENE). Autogenic controls were conducted simultaneously as described above, and revealed a significant decline in the weight of the agar blocks (Student's *t*-test: $t_U = 29.87$, $df = 23$, $p < 0.05$; $t_{CNE} = 40.98$, $df = 23$, $p < 0.05$; $t_{CE} = 40.67$, $df = 23$, $p < 0.05$; $t_{EE} = 39.56$, $df = 23$, $p < 0.05$; $t_{ENE} = 42.12$, $df = 23$, $p < 0.05$) over the experimental period (ca. 4.5% of the initial weight). In addition to autogenic controls, an agar diet control was also run ($n = 3$), where under the same experimental conditions, pure agar blocks were offered to the sea urchins. This control was run in order to discriminate whether the addition of ground macrophyte biomass or the agar itself was responsible for the change in the consumption rates of the sea urchins. Consumption rates were estimated as the difference between the initial and final wet weight of the agar blocks during 24 h per individual (g WW ind.⁻¹ d⁻¹), taking into account the biomass loss in the agar blocks measured in the autogenic controls.

Statistical analyses

Prior to any statistical analysis, data were checked for normality (Shapiro-Wilk normality test) and homoscedasticity (Bartlett test of homogeneity of variances test). Nutrient concentration in seawater, epiphyte biomass on leaves and morphological traits of *C. nodosa* from each treatment (i.e. control vs. enriched) were analysed using a 1-way ANOVA. Biochemical and biomechanical traits of *C. nodosa* were analysed using a 2-way ANOVA (nutrients: 2 levels and epiphyte presence: 2 levels, both considered fixed factors). When significant differences were found, a post hoc Tukey test was applied.

C. nodosa consumption rates in the 1-choice and agar feeding assays were analysed by means of a 2-way ANOVA (nutrients: 2 levels and epiphyte presence: 2 levels, both considered fixed factors). Differences in the 2-choice assays between *Ulva* sp. and *C. nodosa* leaves were analysed following Prince et al. (2004), using Hotelling's multivariate (T^2) test. Then, to find differences in *C. nodosa* consumption rates in the different treatments of 2-choice assays, a 2-way ANOVA was performed (nutrients: 2 levels

and epiphyte presence: 2 levels, both considered fixed factors). Meanwhile, to analyse the differences in consumption of each type of leaf (i.e. CNE, CE, ENE and EE) between 1- and 2-choice assays, analyses using a 1-way ANOVA were done. When significant differences were found, a post hoc Tukey test was applied. Data were both log and square-root transformed (i.e. consumption rates in 1- and 2-choice assays respectively) when necessary to meet normal distribution assumptions.

In addition, to assess the existence of a power issue because of the limited sample size ($n = 3$), a statistical meta-analysis of the size effect was used to avoid the possibly misleading influence of sample size. While null hypothesis significance testing only informs about the probability of an observation, the presentation of the effect size along with its standard error (SE) provides the 2 most important pieces of statistical information for biologists: the magnitude estimate of an effect of interest and the precision of that estimate (Nakagawa & Cuthill 2007). Thus, if there are non-significant differences but large effects, it may suggest further research with greater power is required (Fritz et al. 2012). To estimate the effect size of the parameters under study, the Hedges' d metric was chosen (Hedges & Olkin 1985), as it is an unbiased estimator that provides a better assessment for small sample sizes. The effect size was presented as Hedges' $d \pm$ asymptotic SE for the effect size according to Nakagawa & Cuthill (2007). Hedges' d metric values above 0 indicate a positive effect, below 0 indicate a negative effect and a value equal to 0 indicates no effect on the parameter under investigation. The bigger the number, either in the positive or neg-

ative direction, the higher the magnitude of the effect. Cohen (1988) proposed 'conventional' values as benchmarks for what are considered to be small, medium and large magnitude effects ($d = 0.2, 0.5$ and 0.8 respectively) (Nakagawa & Cuthill 2007).

Data are presented as means \pm SE. The significance level (α) in all tests was 0.05. Statistical analyses were computed with R v.3.0.2 (R Development Core Team 2013).

RESULTS

Nutrient concentration in seawater

Nutrient concentration *in situ* differed significantly between enriched and control treatments over time, revealing large differences in nutrient availability for the plant community depending on the experimental treatment (Table 1), as indicated by the 1-way ANOVA analyses. Nutrient enrichment significantly increased phosphate (0.37 ± 0.04 vs. 0.83 ± 0.31 μM ; $p = 0.038$) and ammonium concentrations (5.69 ± 3.43 vs. 23.32 ± 6.68 μM ; $p = 0.041$) in comparison to control treatments.

Epiphyte biomass on leaves

Epiphyte biomass increased on leaves in the nutrient-enriched treatments, although the differences were not significant when compared to controls (10.4 ± 1.6 vs. 8.5 ± 0.5 g DW shoot⁻¹; 1-way ANOVA $F_{1,8} = 7.642$, $p = 0.072$).

Table 1. Mean \pm SE phosphate and ammonium concentrations in seawater along experimental time in control and enriched treatments

Period	Phosphate (μM)		Ammonium (μM)	
	Control	Enriched	Control	Enriched
2–8 May	0.376 ± 0.015	0.793 ± 0.353	5.99 ± 1.785	25.92 ± 8.113
9–15 May	0.386 ± 0.056	0.886 ± 0.225	3.51 ± 2.31	21.573 ± 6.5
16–17 May	0.346 ± 0.032	0.573 ± 0.360	3.88 ± 1.483	16.78 ± 9.268
23–29 May	0.373 ± 0.030	0.946 ± 0.241	5.35 ± 4.63	20.643 ± 9.663
30 May–5 Jun	0.384 ± 0.028	0.847 ± 0.207	3.5 ± 2.290	31.326 ± 2.367
6–12 Jun	0.322 ± 0.061	0.95 ± 0.199	4.85 ± 2.157	18.34 ± 5.33
Jun 13–19	0.336 ± 0.041	0.943 ± 0.198	5.97 ± 3.142	24.226 ± 4.084
Jun 20–26	0.343 ± 0.025	0.786 ± 0.242	3.67 ± 4.097	21.396 ± 8.987
Jun 27–Jul 3	0.333 ± 0.025	0.944 ± 0.259	4.81 ± 3.617	22.33 ± 6.339
Jul 4–5	0.36 ± 0.081	0.73 ± 0.334	6.52 ± 1.476	18.39 ± 4.344
Jul 11–12	0.362 ± 0.05	0.953 ± 0.144	7.016 ± 4.062	24.332 ± 5.645
Jul 18–24	0.36 ± 0.026	0.663 ± 0.342	8.29 ± 3.67	21.326 ± 3.402
Jul 25–31	$0.38 \pm 6.80 \times 10^{-7}$	0.732 ± 0.297	7.99 ± 1.90	22.39 ± 4.361

Plant traits

Morphological traits varied significantly between control and enriched treatments in terms of width and thickness (Fig. 2). Enriched plants were significantly broader and thicker than control plants (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m599p049_supp.pdf), with a positive effect size value of 1.64 for width and 3.21 for thickness (see Fig. 4).

With respect to biochemical traits, the nutritional quality of the plants (i.e. N content) was significantly increased in enriched plants, showing a 46.1% higher N content in leaves from enriched treatments than in those in the controls (2-way ANOVA $F_{1,8} = 155.57$, $p < 0.01$; Table S1). Moreover, the presence of epiphytes on *Cymodocea nodosa* leaves significantly increased their nutritional quality, increasing the N content from 2.20 ± 0.09 to $4.41 \pm 0.18\%$ (control and enriched plants, respectively), with an effect size of 1.98 and 4.55 for CE (control leaves plus epiphytes) and EE (enriched leaves plus epiphytes) (Figs. 3 & 4). Regarding structural traits, fiber content was significantly higher in control plants, while no significant differences between treatments were recorded for C content in leaves (Fig. 3, Table S1).

The response of biomechanical traits showed that control plants had the highest values of F_{TA} and F_{TS} (the whole-leaf and material mechanical traits), while nutrient enrichment decreased leaf resistance significantly (i.e. both F_{TA} and F_{TS} ; Fig. 3, Table S1). Epiphyte presence on the leaves also significantly decreased the force needed to cut or bite the leaves compared to leaves with epiphytes removed (Figs. 3 & 4).

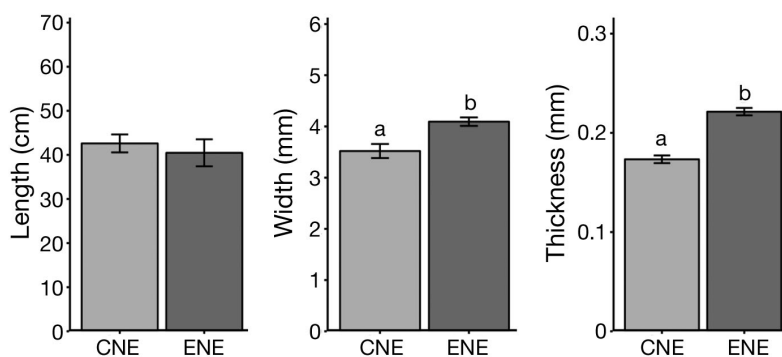


Fig. 2. Morphological traits of *Cymodocea nodosa* leaves from control and enriched treatments. CNE: control plants, no epiphytes; ENE: enriched plants, no epiphytes. Data are expressed as means \pm SE; letters indicate significant differences between treatments ($p < 0.05$)

Herbivore feeding assays

One-choice feeding assay

In the 1-choice assay, *Paracentrotus lividus* showed different consumption rates for *Ulva* sp. and all the *C. nodosa* leaves from each treatment (i.e. CNE, CE, ENE and EE), the macroalgae being the most consumed (Fig. 5, Table 2). Therefore, sea urchins did not exhibit compensatory feeding behaviour, as they tended to eat the more nutritional tissues like *Ulva* sp. and nutrient-enriched plants (Fig. 5A). Regarding seagrass leaves, *P. lividus* had the highest preference for both enriched and enriched-plus-epiphytes leaves (effect size of 22.96; Fig. 6).

Two-choice feeding assay

The Hotelling's test showed a significantly higher consumption of *Ulva* sp. with respect to each type of *C. nodosa* leaf (i.e. CNE, CE, ENE and EE), maintaining the same pattern as in the 1-choice feeding assay (Table 2). Regarding *C. nodosa* leaves, epiphyte presence increased leaf consumption compared to the same treatments without epiphytes, although this effect was lower in enriched leaves (16.79% increase between ENE vs. EE; Fig. 5B, Table S2 in the Supplement) than in 1-choice assays. Additionally, despite the fact that the total amount ingested (i.e. the sum of *Ulva* sp. plus leaves) by the sea urchins throughout the experimental period (24 h) was higher than in the 1-choice assays, the consumption rate of each type of *C. nodosa* leaves was lower, although only significantly lower in those leaves coated by epiphytes (i.e. CE and EE; Table 3).

Agar feeding assays

The consumption of blocks of pure agar (i.e. without *Ulva* sp. or *C. nodosa*) was marginal (0.094 ± 0.027 g WW ind.⁻¹ d⁻¹), and significantly lower than the consumption of those agar blocks containing *C. nodosa* (e.g. for the less consumed leaf, in 1-choice assay CNE; 1-way ANOVA $F_{1,4} = 35.46$, $p = 0.004$).

When morphological, structural and biomechanical traits were removed by using the agar preference assays, *P. li-*

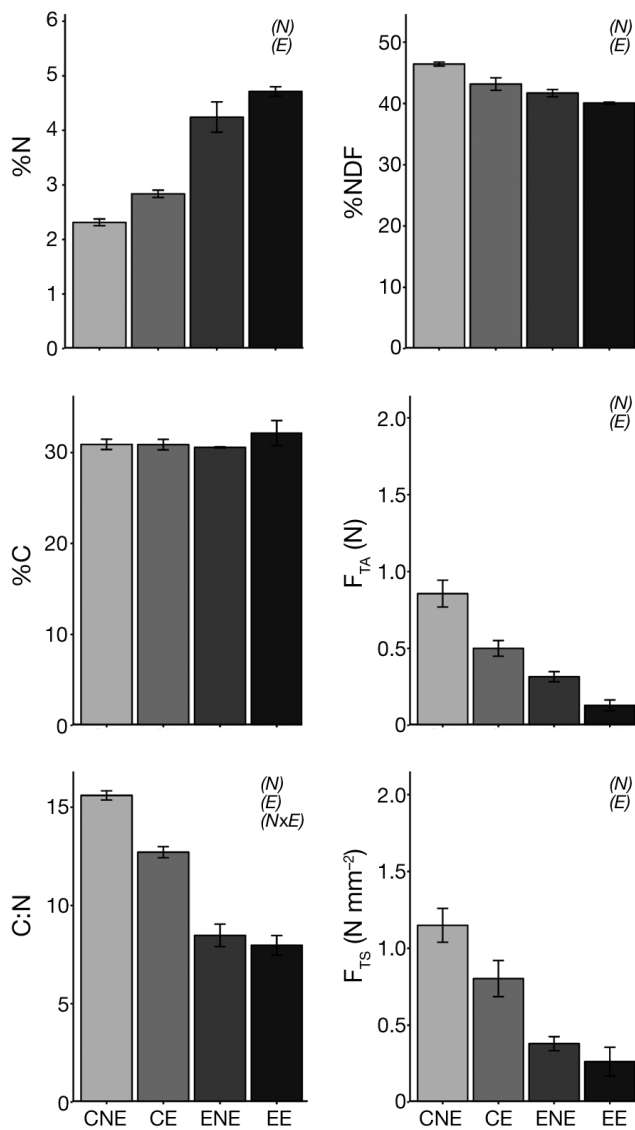


Fig. 3. Biochemical and biomechanical traits of *Cymodocea nodosa* leaves coated and not coated with epiphytes from control and enriched treatments. CNE: control plants, no epiphytes; CE: control plants plus epiphytes; ENE: enriched plants, no epiphytes; EE: enriched plants plus epiphytes. For biochemical traits: %N: nitrogen content; %C: carbon content; C:N: carbon to nitrogen ratio, %NDF: fiber content. For biomechanical traits: F_{TA} (N): absolute force-to-cut; F_{TS} (N mm⁻²): specific force-to-cut. Data are expressed as means \pm SE; factors resulting in significant differences in the 2-way ANOVA ($\alpha = 0.05$; fixed factors, N: nutrient enrichment; E: epiphyte presence) are shown in brackets. Absence of brackets indicates no significant differences

vidus maintained the same order of preferences as in previous assays using intact vegetal material (i.e. *Ulva* sp. and *C. nodosa* leaves). As in previous assays, *Ulva* sp. was the most consumed, followed by EE plants (i.e. enriched leaves plus epiphytes) while

CNE treatment (control leaves, no epiphytes) was consumed the least (Fig. 5C, Table 2). However, it is important to note the significant increase in consumption rate recorded in control plants (CNE) compared to the 1-choice feeding assays (0.33 ± 0.035 vs. 0.06 ± 0.004 g WW ind.⁻¹ d⁻¹). This resulted in a significantly higher consumption rate in agar feeding assays compared to CNE in 1-choice feeding assays (1-way ANOVA $F_{1,8} = 32.84$, $p = 0.007$; Fig. 5A,C).

DISCUSSION

This study clearly demonstrated not only that nutrient load increased the chance of *Cymodocea nodosa* tissues being consumed by herbivores, but also that this enhanced consumption was ruled both by the increase in palatability of the leaves (i.e. higher nutritional quality, lower biomechanical and structural traits) and by the presence of epiphytes on the leaves. Moreover, *Ulva* sp., a highly palatable macrophyte occurring during eutrophication events, was always preferred by the generalist herbivore *Paracentrotus lividus*, which significantly reduced the consumption of *C. nodosa* leaves as hypothesised — but only those leaves coated by epiphytes.

Coastal eutrophication is one of the main factors leading to the decline of seagrass populations because of the direct (e.g. toxicity effects by nutrients) and indirect effects (e.g. organic matter load, shading by epiphytes or macroalgae) it promotes (Orth et al. 1984, van Katwijk et al. 1997, Brun et al. 2002, 2008, Coll et al. 2011, Unsworth et al. 2014, Moreno-Marín et al. 2016). In addition to negative effects, higher nutrient availability under eutrophication events raise the nitrogen content of plant tissues (Invers et al. 2004, Burkholder et al. 2007, Villazán et al. 2015), yielding a greater nutritional quality. As a consequence, some studies have recorded a higher palatability of N-enriched seagrass leaves, and therefore higher consumption rates by herbivores (McGlathery 1995, Cebrián & Lartigue 2004, Goecker et al. 2005, Heck & Valentine 2006). Hence, nutritional quality is considered the main factor regulating feeding decisions by consumers in terrestrial and aquatic ecosystems (Elser et al. 2000, Cebrián et al. 2009). Our results are fully aligned with these previous studies, since N-enriched *C. nodosa* plants were consumed more than control ones by *P. lividus*. However, our data indicated that not only were *C. nodosa* leaves higher in nutritional quality under nutrient enrichment, but also the structural (e.g. lowering fiber content and C/N ratios) and biomechanical

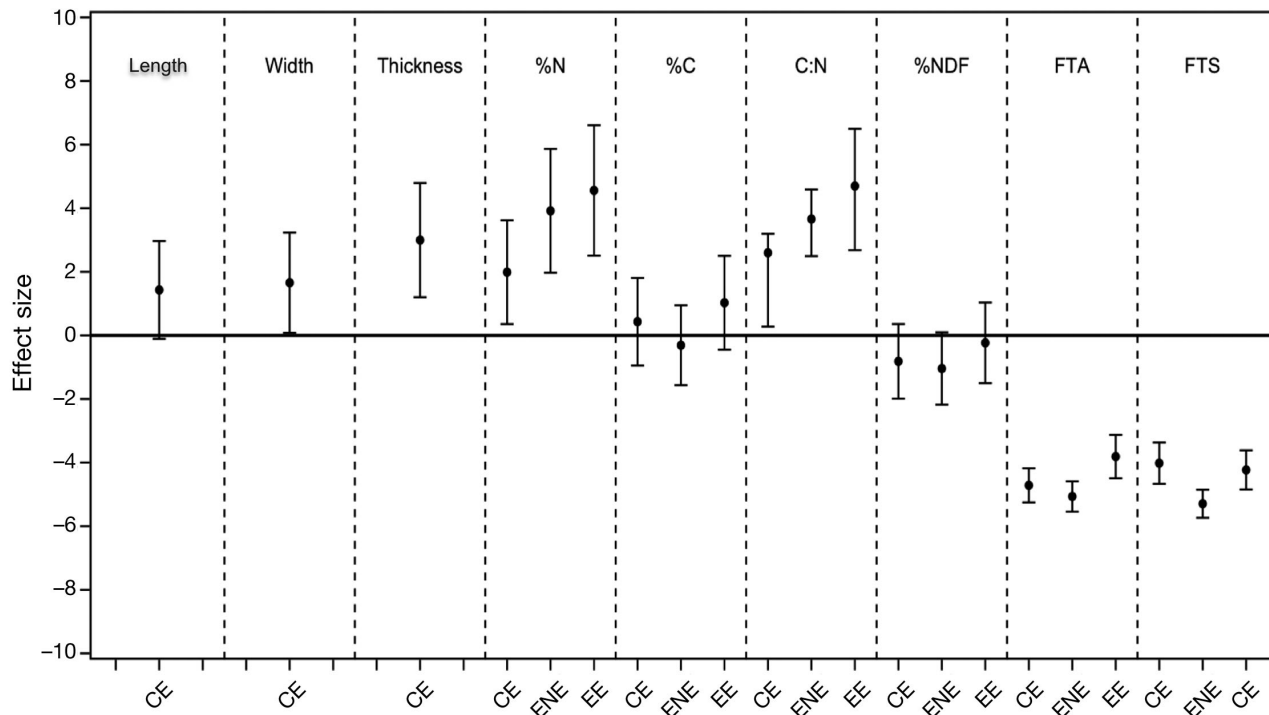


Fig. 4. Effect size ($n = 3$) of *Cymodocea nodosa* leaf traits of leaves coated and not coated with epiphytes (in biochemical and biomechanical analyses) from control and enrichment treatments. Error bars: 95 % confidence intervals (CIs). Effects are significantly different from zero if CIs do not overlap with zero. Morphological traits: length (cm); width (mm); thickness (mm). Biochemical traits: %N: nitrogen content; %C: carbon content; C:N: carbon to nitrogen ratio, %NDF: fiber content. Biomechanical traits: F_{TA} (N): absolute force-to-cut; F_{TS} ($N\text{ mm}^{-2}$): specific force-to-cut

traits (both F_{TA} and F_{TS}) were both reduced; all these traits are known to directly affect the palatability of the tissues (Mariani & Alcoverro 1999, Prado & Heck 2011, Tomas et al. 2015, Martínez-Crego et al. 2016, Jiménez-Ramos et al. 2017a). Previous studies have already demonstrated that nutrient enrichment may affect the biomechanical performance of seagrass leaves (de los Santos et al. 2013, La Nafie et al. 2013, Soissons et al. 2018), which would suggest a potential increase in the susceptibility of plants to mechanical damage. Unexpectedly, the presence of leaf epiphytes also significantly reduced the force-to-cut the leaf (i.e. F_{TA} and F_{TS}). This suggests that the coverage of epiphytes over the seagrass leaves could have an effect on the material properties. At this stage, however, we do not have enough knowledge to predict

Table 2. Results of the 1-way ANOVA examining the differences between *Ulva* sp. and *Cymodocea nodosa* consumption by *Paracentrotus lividus* in the 1-choice and agar feeding assays. Hotelling's multivariate test was used to examine differences in consumption rates between *Ulva* sp. and *C. nodosa* leaves in the 2-choice feeding assays. Values in **bold** indicate significant differences between treatments ($p < 0.05$). *U*: *Ulva* sp.; CNE: control plants, no epiphytes; CE: control plants plus epiphytes; ENE: enriched plants, no epiphytes; EE: enriched plants plus epiphytes

	1-way ANOVA	df	MS	<i>F</i>	<i>p</i>
1-choice feeding assay	Treatment	4,10	0.2789	31.93	<0.001
	Hotelling's test	df	T^2	<i>F</i>	<i>p</i>
2-choice feeding assay	<i>U</i> × CNE	4	13.27	2.965	<0.01
	<i>U</i> × CE	4	8.493	3.124	0.001
	<i>U</i> × ENE	4	9.419	3.177	0.037
	<i>U</i> × EE	4	7.394	4.254	0.029
	1-way ANOVA	df	MS	<i>F</i>	<i>p</i>
Agar feeding assay	Treatment	4,10	0.073	11.12	0.002

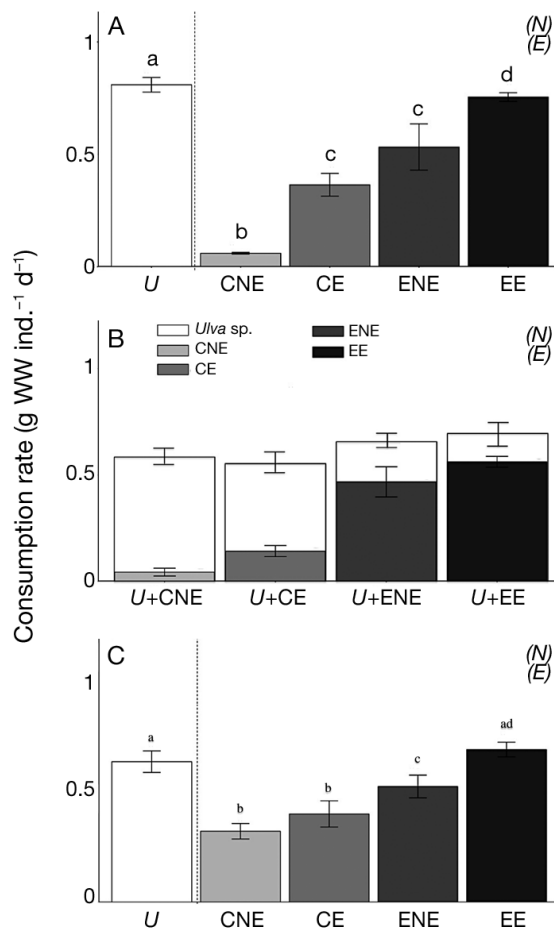


Fig. 5. Consumption rates of *Ulva* sp. and *Cymodocea nodosa* leaves (U: *Ulva* sp.; CNE: control plants, no epiphytes; CE: control plants plus epiphytes; ENE: enriched plants, no epiphytes; EE: enriched plants plus epiphytes) by the sea urchin *Paracentrotus lividus* (g wet weight [WW] ind.⁻¹ d.⁻¹; mean \pm SE). (A) One-choice feeding assay (n = 3); (B) 2-choice feeding assay (n = 3); (C) agar feeding assay (n = 3). Different letters above bars indicate significant differences between consumption rates of *Ulva* sp. and *C. nodosa* (1-way ANOVA, $\alpha = 0.05$); 2-way ANOVA factors resulting in significant differences in consumption rates among *C. nodosa* leaves are shown in brackets ($\alpha = 0.05$; fixed factors: N: nutrient enrichment; E: epiphyte presence)

what effect the epiphyte influence on seagrass biomechanical features will have at the community level. In this regard, our work clearly indicates that biomechanical and structural traits have a great importance with respect to tissue palatability, since when both traits were removed using agar treatments, the consumption rates in control plants increased prominently. However, the nutritional quality of the tissues seemed to play a major role in affecting the behaviour of the herbivores, as indicated by the high consumption rates recorded over

the enriched and epiphyte-coated leaves, even in agar assays. The presence of epiphytes on the leaves did not increase their mechanical resistance, but in contrast, substantially improved the nutritional quality of the tissues. This may clearly explain why treatments with epiphytes were preferred by sea urchins. In this respect, the external location of epiphytes, the minor structural and morphological defences they bear, and their higher nutritional quality have been pointed out as factors that increase feeding selectivity by herbivores when epiphytes are present (Greenway 1995, Alcoverro et al. 2000, Heck & Valentine 2006, Marco-Méndez et al. 2012, 2015). Specifically, Tomas et al. (2004), using stable isotope analyses, indicated that approximately 90% of the nitrogen assimilated by *P. lividus* in *Posidonia oceanica* meadows came from epiphytes. Therefore, our results show that overgrazing may occur in *C. nodosa* communities when epiphyte growth takes place, which may be detrimental for this species. However, we should also consider that epiphyte use is preferentially allocated to the oldest external leaves of the shoots, and that overgrazing of these leaves may reduce the competition between seagrasses and epiphytes for light and nutrients, as suggested by Alcoverro et al. (1997). Although moderate grazing can stimulate seagrass growth, shoot production (Valentine et al. 1997) and nutrient recycling (Drifmeyer 1981, Koike et al. 1987, Vonk et al. 2008), intensive grazing can substantially decrease seagrass production and distribution to the level of complete denudation of meadows, as has been demonstrated in previous works (e.g. Camp et al. 1973, Peterson et al. 2002, Eklöf et al. 2008, Ling et al. 2015). These contrasting effects of epiphytes in the seagrass community may also depend on the diversity and identity of herbivores, since, for instance, during nutrient enrichment the presence of mesograzers may reduce the density of epiphytes and may benefit seagrasses (Hughes et al. 2004), while the presence of macrograzers may increase seagrass consumption and negatively affect these species (Campbell et al. 2018). Therefore, our results suggest that the selectivity of *P. lividus* for enriched and epiphyte-containing leaves of *C. nodosa* under nutrient enrichment may have a negative effect on this species if density and/or activity of the sea urchins is high, but perhaps low to moderate grazing pressure on these leaves may benefit seagrasses by improving light conditions.

One of the first consequences of nutrient enrichment is the proliferation of epiphytes and ephemeral macroalgae such as the genus *Ulva* (Sand-Jensen & Borum 1991, Borum & Sand-Jensen 1996, Hernández

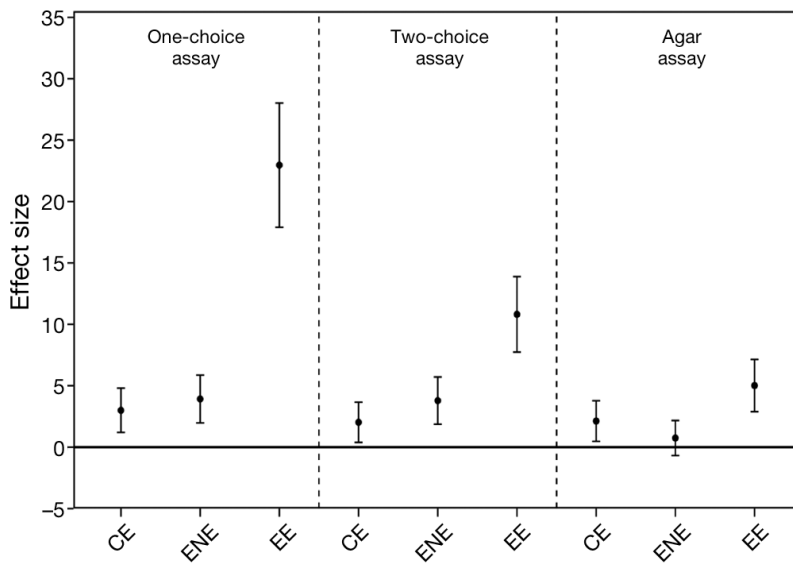


Fig. 6. Effect size ($n = 3$) of consumption rates by the sea urchin *Paracentrotus lividus* of *Cymodocea nodosa* leaves coated and non-coated by epiphytes from control and enrichment treatments. (A) One-choice feeding assay ($n = 3$); (B) 2-choice feeding assay ($n = 3$); (C) agar feeding assay ($n = 3$). Error bars: 95% confidence intervals (CIs). Effects are significantly different from zero if CIs do not overlap with zero. Negative values indicate preference for the control leaves (control plants, no epiphytes [CNE]), while positive values denote a significant preference for the enriched leaves and/or leaves coated by epiphytes (control plants plus epiphytes [CE], enriched plants, no epiphytes [ENE], enriched plants plus epiphytes [EE])

Table 3. Results of the 1-way ANOVA test examining the differences in consumption rates of *Cymodocea nodosa* by *Paracentrotus lividus* among treatments (CNE: control plants, no epiphytes; CE: control plants plus epiphytes; ENE: enriched plants, no epiphytes; EE: enriched plants plus epiphytes) from 1- and 2-choice feeding assays. Values in **bold** indicate significant differences ($p < 0.05$)

Variables, factors	df	MS	F	p
CNE (1- vs. 2-choice assays)	1,4	0.00041	0.769	0.423
CE (1- vs. 2-choice assays)	1,4	0.073	15.24	0.017
ENE (1- vs. 2-choice assays)	1,4	0.0061	0.264	0.634
EE (1- vs. 2-choice assays)	1,4	0.055	36.07	0.003

et al. 1997, Rasmussen et al. 2013). It can create mixed meadows (i.e. those containing seagrasses and green seaweeds) where herbivores have several food choices, which may generate different patterns of vegetation consumption as demonstrated in this work. *Ulva* sp. was always preferred in all experi-

mental assays (1-choice, 2-choice and agar assays), which can be clearly explained by the high nutritional quality and low structural and biomechanical traits it bears (see Table S3 in the Supplement). However, it is remarkable that the presence of *Ulva* sp. resulted in reduced consumption of *C. nodosa* leaves, mostly in those leaves coated by epiphytes. This fact is consistent with the compensatory theory (sensu Cruz-Rivera & Hay 2000a), where the consumption rate is higher in foods of low nutritional quality compared to those with higher quality. In other words, although the generalist herbivore *P. lividus* is able to feed on different species (Boudouresque & Verlaque 2001), higher quality foods are generally preferred when available in sufficient supply (because they tend to enhance fitness; Berner et al. 2005), and thus when consuming leaves coated by epiphytes, the higher nutritional quality of this type of food source made it possible to reduce the total consumption of seagrass leaves. This strategy (i.e. compensatory feeding) has already been recorded in

P. lividus in previous experiments (Mazzella et al. 1992, Tomas et al. 2011, Boada et al. 2017). Therefore, the presence of *Ulva* sp. at moderate loads, when the main herbivore has a similar behaviour as *P. lividus*, may divert the consumption to this macroalga and reduce grazing pressure on seagrasses.

CONCLUSIONS

The present study showed that nutrient enrichment had consequences at the physiological level of the plant (morphological, biochemical and biomechanical traits), increasing its palatability and, therefore, its consumption. In addition, nutrient loads generated changes at the community level, enhancing epiphyte growth and thus improving the nutritional quality of the whole leaf (i.e. leaf plus epiphytes), increasing the vulnerability of the seagrass to being consumed. However, the feeding patterns of *Paracentrotus lividus* seemed to react to the proliferation of the highly palatable macroalga *Ulva* sp., which could result in reduced seagrass consumption due to compensatory feeding. Hence, the among-species

patterns of food use also suggest that the variable ability of herbivores to use alternative macrophyte resources will determine how primary producer populations will be affected when, for instance, preferred foods become limiting (see Cruz-Rivera & Hay 2000b) or under overgrowth of herbivore populations caused by overfishing (see Lafferty 2004). Finally, as the frequency and intensity of anthropogenic impacts such as eutrophication continues to increase, our findings have significant implications for conservation, and highlight the importance of maintaining biodiversity of primary producers to support the provision of key ecological processes such as herbivory.

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