# Nitrogen uptake and internal recycling in *Zostera marina* exposed to oyster farming: eelgrass potential as a natural biofilter

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**Abstract**

Oyster farming in estuaries and coastal lagoons frequently overlaps with the distribution of seagrass meadows, yet there are few studies on how it/this aquaculture practice affects seagrass physiology. We compared *in situ* nitrogen uptake and the productivity of *Zostera marina* shoots growing near/alongside off-bottom longlines and at a site not affected by oyster farming in San Quintín Bay, a coastal lagoon in Baja California, Mexico. We used benthic chambers to measure leaf $NH_4^+$ uptake capacities by pulse-labeling with $^{15}NH_4^+$, and plant photosynthesis and respiration. The internal $^{15}N$ resorption/recycling was measured in shoots two weeks after incubations. The natural isotopic composition of eelgrass tissues and vegetative descriptors were also examined. Plants growing at the oyster farming site showed a higher leaf $NH_4^+$ uptake rate (33.1 mmol $NH_4^+$ $m^{-2}$ $day^{-1}$) relative to those not exposed to oyster cultures (25.6 mmol $NH_4^+$ $m^{-2}$ $day^{-1}$). We calculated that an eelgrass meadow of 15–16 ha (which represents only about 3–4% of the subtidal eelgrass meadow cover in the western arm of the lagoon) can potentially incorporate the total amount of $NH_4^+$ excreted by oysters (~$5.2 \times 10^6$ mmol $NH_4^+$ $day^{-1}$). This highlights the potential of eelgrass to act as a natural biofilter for the $NH_4^+$ produced by oyster farming. Shoots exposed to oysters were more efficient in re-utilizing the internal $^{15}N$ into the growth of new leaf tissues or to translocate it to belowground tissues. Photosynthetic rates were greater in shoots exposed to oysters, which is consistent with higher $NH_4^+$ uptake and more positive $\delta^{13}C$ values. Vegetative production (shoot size, leaf growth) was also higher in these shoots. Aboveground:belowground biomass ratio was lower in eelgrass beds not directly influenced by oyster farms, likely related to the higher investment in belowground biomass to incorporate sedimentary nutrients.

## Introduction

Aquaculture is a growing activity but its potential impacts on adjacent marine habitats, especially on seagrass-dominated ecosystems, are not well known (Pillay 2004; Ruiz et al. 2010). Seagrass meadows are amongst the most productive communities in coastal marine habitats, providing valuable ecological and socio-economic functions and services to coastal ecosystems (Green and Short 2003; Fourqurean et al. 2012). Aquaculture activities, such as oyster farming, often overlap with the distribution of seagrass meadows in estuaries and coastal lagoons (Everett et al. 1995; Simenstad and Fresh 1995). The effects of oyster farming on seagrasses have been investigated, particularly on the temperate species *Zostera marina* L. (Everett et al. 1995; Simenstad and Fresh 1995). Effects may include a decrease in growth, density and recruitment of plants, depending on the farming practice employed (e.g. on- *versus* off-bottom cultures), the effect of farming structures on the hydrology, sedimentation and shading of the meadows, the degree of physical disturbance during oyster placement and harvest, and the accumulation of oyster biodeposits (Everett et al. 1995; Wisehart et al. 2007; Tallis et al. 2009; Wagner et al. 2012; Skinner et al. 2014).

Nutrient inputs from bivalve excretion may have positive and negative effects on seagrasses (Reush et al. 1994; Vinther and Holmer 2008). Peterson and Heck (2001a, b) demonstrated through manipulative experiments that nutrient enrichment of the substrate caused by biodeposits of a suspension-feeding mussel can lead to an increase in biomass productivity of *Thalassia testudinum*. Reusch et al. (1994) also found a positive relation between ammonium concentration in sediment pore water originating from mussel biodeposition and the size of eelgrasses. On the other hand, biodeposit accumulation can lead to eelgrass metabolic toxicity due to an increase in sulfate

reduction rates and a consequent increase of sulfide in sediments (Vinther and Holmer 2008). Increased ammonium concentrations in the water column may result in large epiphyte loads on eelgrass leaves, affecting eelgrass performance probably due to a decrease in the incident irradiance on the leaves (Vinther and Holmer 2008; Vinther et al. 2008).

Seagrasses take up dissolved inorganic nitrogen (DIN: $NH_4^+$ and $NO_3^-$) from the water column and from sediment pore water (Touchette and Burkholder 2000; McGlathery 2008); they also take up organic nitrogen (urea, amino acids and peptides) as a complementary nitrogen (N) source (Vonk et al. 2008; Alexandre et al. 2015). N acquisition by seagrasses is mainly regulated by uptake kinetic properties of leaves and roots (i.e. maximum uptake rates, $V_{max}$, and affinity, $\alpha$, for DIN sources; Touchette and Burkholder 2000; Alexandre et al. 2011), which, in turn, can vary within and among seagrass species depending on a variety of natural factors that determine DIN availability (e.g. seasonal and local factors regulating external DIN pools, nutrient diffusion limits, type of substrate; Maier and Pregnall 1990; Stapel et al. 1996; Terrados and Williams 1997; Lee and Dunton 1999; Hasegawa et al. 2005).

Ammonium, which is directly excreted by bivalves or produced by the remineralization of their biodeposits (Newell et al. 2005; Hoellein and Zarnoch 2014), is the preferential DIN source for seagrasses (Alexandre et al. 2011 and references therein). Therefore, seagrasses may act as biofilters buffering the excess nutrient loading from oyster farming (and other aquaculture practices) by increasing their productivity and retaining N in belowground tissues (McGlathery et al. 2007). This biofiltering capacity has been widely demonstrated in seaweeds and halophytic plants, and has been applied in economic activities such as Integrated Multi-Trophic Aquaculture (Neori 2008; Buhmann and Papenbrock 2013). To the best of our knowledge, the potential of

seagrasses as biofilters of aquaculture-derived DIN has not been addressed. Seagrasses may be more efficient biofilters than macroalgae because they can acquire DIN not only from the water column but also from the substrate through their roots. In addition, seagrass tissues exhibit longer retention of nutrients over time and decompose slowly in sediments (McGlathery et al. 2007 and references therein).

The incorporation of DIN by seagrasses may depend on photosynthesis, since N assimilation can be limited by the availability of carbon skeletons of photosynthates required for such assimilation (Invers et al. 2004). The internal recycling of N within the shoot or within the plant clonal structure involves processes such as nutrient resorption from senescent tissues and nutrient translocation among ramets, which can limit the dependence on external DIN to sustain the N required for plant growth (Hemminga et al. 1999; Lepoint et al. 2002). Therefore, integrative studies involving physiological and vegetative responses of seagrasses exposed to bivalve aquaculture are needed for an in-depth understanding of the interaction between aquaculture practices and seagrass habitats. Furthermore, these studies could provide valuable insights for the assessment of the ecological status, management and restoration of seagrass habitats exposed to shellfish farming, and to elucidate their role as 'coastal filters' under potential conditions of N loading from such activity (McGlathery et al. 2007; Tallis et al. 2009; Buzzelli et al. 2015; Forde et al. 2015).

In this study we investigate the potential of the seagrass *Z. marina* as a biofilter of the DIN (i.e. ammonium) released by oyster farming, by comparing the $NH_4^+$ uptake rates of shoots growing near oyster racks with those of shoots not directly affected by oyster farming. To this end, we quantified *in situ* leaf ammonium uptake rates by pulse-labeling with $^{15}N$ in benthic chambers. Among other vegetative descriptors, we measured eelgrass biomass to estimate the capacity of eelgrass meadows to incorporate

126 the $NH_4^+$ excreted by cultured oysters. The relationship between N uptake and other key
127 processes such as photosynthesis and internal recycling of N was also examined. Net
128 photosynthetic rates were measured *in situ* using benthic chambers, while the recycling
129 of $^{15}N$ was measured in plant tissues two weeks after pulse labeling.
130
131 **Materials and Methods**
132
133 **Study site**
134 The study was carried out in March 2015 at San Quintín Bay (30° 30' N, 116° 00' W), a
135 Y-shaped coastal lagoon (43 $km^2$, 2 m average depth) located on the northwestern
136 Pacific coast of the Baja California Peninsula, Mexico (Fig. 1a). The region has a
137 Mediterranean-type climate and arid conditions prevail throughout most of the year.
138 Land inputs of water and nutrients via runoff are non-existent except during the winters
139 of very wet years. Water exchange and circulation are mainly dominated by semidiurnal
140 tidal flows (average tidal amplitude of 1.6 m) between the lagoon and the coastal ocean,
141 which represents the main external source of nutrients. The bay connects with the ocean
142 through a single mouth and has two arms: an eastern, inner arm known as Bahía San
143 Quintín and a western arm known as Bahía Falsa. Water circulation largely occurs
144 through narrow and deep (5–7 m) channels that extend along the length of both arms. A
145 detailed description of the bay and its biogeochemical characteristics can be found in
146 Camacho-Ibar et al. (2003), Hernández-Ayón et al. (2004) and Ribas-Ribas et al.
147 (2011). Monospecific meadows of the seagrass *Z. marina* develop extensively along the
148 intertidal and shallow subtidal flats, occupying about 45 % of the total surface area of
149 the lagoon (about 2000 ha; Poumian-Tapia and Ibarra-Obando 1999; Ward et al. 2003).
150 In Bahía Falsa, intensive oyster aquaculture practices (mostly oyster racks and off-

151 bottom longlines) have co-occured with dense *Z. marina* meadows for more than 30
152 years (Ward et al. 2003; García-Esquivel et al. 2004). For this study, two eelgrass
153 meadows (~2 m depth during high tides) were selected in Bahía Falsa: one where shoots
154 grow alongside off-bottom longlines (Fig. 1b), hereafter referred to as the "oyster site"
155 (N 30° 25’ 53.4”, W115° 59’ 59.8”), and one where *Z. marina* shoots are not directly
156 affected by oyster aquaculture (~1 km from oyster farming structures), hereafter
157 referred to as the "reference site" (N 30° 25’ 22.5”, W116° 00’ 9.7”) (Fig. 1a). The
158 selected sites were in close proximity since Sandoval-Gil et al. (2015) recently
159 demonstrated that *Z. marina* exhibits high plasticity of its physiological properties,
160 including its DIN uptake kinetics, according to its location in the lagoon. Also,
161 environmental variables that may control DIN assimilation, including temperature and
162 salinity, show strong spatial gradients (Camacho-Ibar et al. 2003); thus, site proximity
163 would limit potential differences in the uptake kinetics due to spatial differences in
164 environmental parameters. Similar light availability, bottom depth and sediment
165 characteristics were also considered for the selection of the sites. Maintaining these
166 parameters as similar as possible allowed for a better evaluation of the effect of oyster
167 farming on DIN assimilation by seagrasses.

168 **Vegetative descriptors**

169 Leaf growth (g DW shoot$^{-1}$ day$^{-1}$; DW = dry weight) was determined using the
170 punching method described for seagrasses by Zieman et al. (1974). All leaves from ten
171 random shoots collected at each meadow site were punched with a hypodermic needle
172 immediately above the leaf sheath. After 14 days, the growth of all leaves of each shoot
173 was measured and summed and the average shoot growth was calculated ($n$ = 10). The
174 size (surface area) of each leaf (cm$^2$) was calculated from the length and three
175 measurements (taken with a digital vernier caliper) of the width at the basal,

intermediate and apical parts of the leaf. The percentages of new leaf tissue produced were calculated from the area of the new leaf tissue grown relative to the total area of each leaf. The leaf area ($cm^2$) corresponding to necrotic tissue and the coverage of epiphytes was also measured and expressed as the percentage of the entire surface of each leaf. Shoot density (number of shoots $m^{-2}$) was determined from shoot counts within 40 × 40 $cm^2$ quadrats ($n$ = 4), while above- and belowground biomass (g DW $m^{-2}$) were determined collecting all plant material within 3.5-cm-diameter cores placed in the sediment ($n$ = 4).

**Elemental composition and isotopic analysis**

The total carbon (C) and N contents and their natural stable isotopic composition ($\delta^{13}C$ and $\delta^{15}N$; Coplen 1994 and Sharp 2005) were determined in leaf and rhizome tissues of three shoots from each site. Elemental C and N composition was expressed as percentage per unit of dry weight and as the molar C/N ratio. Isotopic determinations were carried out at the University of California Davis Stable Isotope Facility using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer. The standard deviation was 0.2 ‰ for $\delta^{13}C$ and 0.3‰ for $\delta^{15}N$ . The analytical details can be found at http://stableisotopefacility.ucdavis.edu/13cand15n.html.

***In situ* plant incubations**

Plant incubations were carried out at each site to measure leaf $^{15}NH_4^+$ uptake using 4-L transparent Plexiglas benthic chambers (65 cm height, 10 cm diameter), each with a transparent lid to cover the top and held vertically by a tripod structure attached to the sediment (Fig. 2a). Each chamber containing one rooted *Z. marina* shoot was inserted 5 cm into the sediment avoiding damage to the rhizome. Prior to chamber insertion, epiphytes on leaves were carefully removed *in situ* with a razor blade (avoiding leaf damage) to limit their contribution to $^{15}NH_4^+$ uptake, since epiphytes on seagrasses can

incorporate large amounts of DIN (e.g. Dudley et al. 2001, Apostolaki et al. 2012). At each site, chambers ($n$ = 6) were deployed for 24 h without cover lid before the experimental incubations to allow stabilization of suspended sediments in the water within the chambers. Benthic chambers at each site were placed in close proximity, within an area of about 10 $m^2$, in order to avoid confounding factors in the comparison between sites, such as differences due to eelgrass physiology and/or changes in environmental conditions associated with heterogeneity at the meadow spatial scale. For incubations, chambers were covered at the top and 50 mL of tracer ($^{15}NH_4Cl$ at.% = 99, Cambridge Isotope Laboratories) were added with a polyethylene syringe to obtain an ammonium concentration of ~10 µM. The ammonium concentration in the incubations was higher than the natural concentration measured at the time of the experiment (~1 µM), but it is within the concentration range commonly experienced by *Z. marina* in San Quintín Bay (1.8–17 µM; Hernández-Ayón et al. 2004). During incubations, the seawater inside the chambers was continuously mixed with a submersible propeller pump to homogenize the tracer and reduce the leaf boundary layer (Cornelisen and Thomas 2004). At both sites, incubations were performed at similar irradiance, salinity, temperature and tidal conditions (906.8 ± 88 µmol photons $m^{-2}$ $s^{-1}$; S = 34 practical salinity scale; 18.1 ± 0.1 °C). Environmental parameters were monitored using an underwater spherical quantum sensor (LI-193, LI-COR, USA) and a submersible multi-parameter probe (YSI Pro Plus, USA). After 1 h of incubation ($T_i$) all chambers were removed but only three of the six incubated shoots at each site were collected. The leaves of the other three shoots were left rooted in the sediment for two weeks to follow the recycling of $^{15}N$ within plant tissues and to measure growth ($T_f$, see below). These shoots were tagged with plastic tape wrapped around the rhizome to facilitate recovery. Immediately after collection, leaves were cleaned of epiphytes and tissues were rinsed

with distilled water to remove any adsorbed tracer. The leaves were detached from belowground tissues and separated according to age. Leaves and rhizomes were dried at 60 ºC for 48 h and ground to a fine powder. Nutrient concentrations were determined from filtered seawater and sediment pore water samples ($n$ = 6) collected at the experimental sites and analyzed spectrophotometrically (Skalar SanPlus Analyzer). Nutrient samples were collected at high tide when ammonium concentrations are less variable (typically <10 µM), as during low tides, sediment resuspension may induce short-lived (< 1 h) ammonium pulses of up to 50 µM (Hernández-Ayón et al. 2004). Specific ammonium uptake rates by each leaf ($V_{leaf}$, expressed as µmol $^{15}N$ $g^{-1}$ DW $h^{-1}$) were calculated following Alexandre et al. (2011) and Sandoval-Gil et al. (2015):

(Eq. 1) $V_{leaf} = [(^{15}N_{exp}-^{15}N_{back}) \times N_c] / (M_N \times t)$

where the difference ($^{15}N_{exp}-^{15}N_{back}$, at. %) is the $^{15}N$ enrichment relative to natural $^{15}N$ levels of leaves of different age, $N_c$ is the N content (g N $g^{-1}$ DW), $M_N$ is the molar mass of N, and $t$ is the duration of the incubation (1 h). We assumed that during the 1-h incubation no significant water to sediment flux of $^{15}NH_4$ tracer occurred; thus, the amount of $^{15}N$ recovered in the rhizomes was included in the calculations to determine the rates of $^{15}N$ uptake by the leaves.

Also, absolute uptake rates of eelgrass aboveground biomass ($V_{ab}$, expressed as mmol $^{15}N$ $m^{-2}$ $h^{-1}$) were calculated as:

(Eq. 2) $V_{ab} = \sum(V_{leaf} * ab_{leaf})$

where $ab_{leaf}$ is the aboveground biomass of the different leaves (g DW $m^{-2}$).

Internal recycling of $^{15}N$ was determined as the percentage of $^{15}N$ which remained in plant tissues at the end of the experiment by comparing the values of $^{15}N$ (µg DW) in the leaves and rhizomes of plants collected after the 1-h incubations ($T_i$) with those in plant tissues that remained rooted in the field for two weeks ($T_f$).

Net photosynthesis (net-P) and respiration (R) rates were also determined *in situ* by incubating whole plants (leaves plus rhizome and roots) in closed benthic chambers (Fig. 2b). Four plants from each site, collected by scuba diving, were carefully cleaned of epiphytes, avoiding leaf damage, and placed separately in two benthic chambers. During handling, shoots were kept in a Ziploc plastic bag filled with seawater to avoid emersion, and shaded with a dark plastic mesh to prevent exposure to excessive light. The above/belowground plant biomass ratios (1.9–2.2) were similar in all chambers. Incubations were performed simultaneously at the reference site to ensure that shoots were subjected to the same environmental conditions of irradiance, temperature and pH. For these experimental incubations, uprooting of shoots was unavoidable. To restrict the effects of uprooting on photosynthesis and respiration, the collection of plants and the incubations were done on the same day. In order to have a large number of net-P measurements, incubations were performed on four consecutive days at different tidal heights and, therefore, at different irradiance levels ranging from 750 to 2400 µmol photons $m^{-2}$ $s^{-1}$. On any given day, incubations were conducted with the same shoots collected that day. Rates of net-P by eelgrass leaves (expressed as µmol $O_2$ $g^{-1}$ leaves DW $h^{-1}$) were calculated from the increments in dissolved oxygen (DO) inside each chamber during short incubation periods (see below), measured with a multi-parameter probe with a DO polarographic sensor (Pro Series YSI) inserted in the upper lid cover. Following the recent study of Olivé et al. (2015), we conducted some trials to optimize the experimental incubation by using different amounts of tissue biomass within the chambers, and different incubation times. We finally selected a plant biomass per seawater volume of about 0.7–0.9 g DW $L^{-1}$ and short incubation times of 25 min to ensure accurate measurements of photosynthetic rates avoiding the underestimation of

photosynthesis due to oxygen over-saturation and/or C limitation. The seawater within chambers was completely renewed before each new incubation.

Respiration was measured as described above for photosynthesis, but in this case incubations were performed in the dark by covering the chambers with polyvinyl carbonate tubes. Incubations lasted for 40 minutes. Respiration rates (μmol $O_2$ g $DW^{-1}$ $h^{-1}$, $n$ = 3) were calculated from the decrease of DO inside the chambers at time intervals of ~10 min. The accuracy of the DO measurements obtained with the polarographic sensor method was tested against DO measurements using the Winkler titration method in preliminary trials. The DO values obtained with the different methods were similar, with a data variance of about 0.05%. P:R ratios were calculated as the integration of net-P and R during the daily photoperiod at the time of experiment (i.e. net-P × 11 hours of light; R × 13 hours of darkness). Although R may vary during the photoperiod (e.g. Rasmusson and Björk 2014), P:R ratios were used as a proxy of the plants daily C balance.

**Statistical analyses**

Statistical differences in the ammonium uptake rates, nutrient and isotopic composition of the different leaves of shoots from both sites were examined by two-way ANOVA with two fixed factors: meadow site (with two levels, reference site and oyster site) and leaf age (with five levels, leaf #1 to leaf #5). The application of this analysis has the implicit assumption of biological independence among leaves of different ages within a shoot, which is not the case for the natural situation in the field (see Alcoverro et al. 1998, 2001). However, since all the incubated shoots had the same number of leaves, and thus the same dependence effect is present in all replicates, the use of a general linear model is justified to test differences among leaves of shoots within sites. *Post-hoc* mean comparisons for the ANOVA (Student–Newman–Keuls) were performed to

identify specific treatment level(s) causing significant effects. Effects were considered statistically significant at $p < 0.05$. Significant differences in $V_{ab}$, the descriptors of eelgrass vegetative performance (i.e. shoot size, density, biomass, growth), and in photosynthesis and respiration between shoots from each meadow site (reference site and oyster site) were examined using Student t-tests. Prior to the analysis, data were checked for normality and homocedasticity, and transformed when necessary. Statistical analyses were performed using the SIGMAPLOT 11 statistical package (Systat Software Inc, USA).

## Results

Among the measured environmental parameters (Table 1), only $NH_4^+$ concentration was significantly different between sites, being higher at the oyster site, both in the water column and in pore water.

Values of $V_{leaf}$ varied significantly with site and leaf age (two-way ANOVA, $F_{4, 29} = 7.214$; $p < 0.001$) (Fig. 3). The uptake rates of older leaves (#3 to #5) were higher at the oyster site than at the reference site. In shoots from both sites, uptake rates by leaves #2 and #3 were ~2.5 fold higher than by leaves #4 and #5. On average, shoots from the oyster site took up ammonium at a rate 37% higher than those from the reference site (Fig. 3a). Values of $V_{ab}$ were also significantly higher ($t = -3.065$, $p = 0.022$) at the oyster site (Fig. 3b).

Values of net-P were, on average, 38% higher in plants from the oyster site than in those from the reference site ($p < 0.03$, Fig. 4a). The P:R ratio was higher at the oyster site (t-test; $t = -3.025$, $df = 34$; $p = 0.005$; Fig. 4b) as a result of similar leaf respiration rates at both sites (~15.5 µmol $O_2$ $g^{-1}$ DW $h^{-1}$) and a higher net-P rate at the oyster site.

All leaf and rhizome tissues of *Z. marina* from the oyster site showed significantly lower values of $\delta^{15}N$ (by 3.5‰: $p < 0.01$) (Fig. 5a) than those from the reference site. In contrast, $\delta^{13}C$ values were ~1.8‰ higher (except for leaf #4) (Fig. 5b) at the oyster site. Leaf %N was higher at the oyster site ($p < 0.01$), whereas no differences were detected in %C. Both %N and %C significantly decreased from the youngest leaf (#1) to the oldest leaf (#5). In general, %N and %C were lowest in rhizomes (Fig. 5c, d). The C/N molar ratio showed the opposite pattern, increasing by ~60% from the youngest to the oldest leaf at both sites, whereas maximum C/N values were found in rhizomes ($p < 0.01$; Fig. 5e). The C/N values were significantly higher in leaves and rhizomes from the reference site.

Epiphyte and necrotic tissue cover measured in the oldest leaves (#3 to #5) did not vary significantly between sites (Table 2). On the other hand, shoot morphology and meadow structure were significantly different between sites (Table 3). Shoot size and leaf growth were about two-fold higher in plants from the oyster site than from the reference site. On the contrary, belowground biomass and shoot density were significantly lower at the oyster site. Above/belowground biomass ratio was 1.7-fold higher in plants from the oyster site.

Values of $^{15}N$ content of plant tissues decreased in eelgrass shoots from both sities (Fig. 6) two weeks after the experimental incubations ($T_f$) until values close to those found at $T_0$ (i.e. natural isotope abundance). On the contrary, $^{15}N$ enrichment significantly increased in leaf #0 (by ~50%) and rhizome tissues (by 80%) of shoots from the oyster site, but not from the reference site. Within each site, we did not find significant differences between old and new tissues of each leaf.

## Discussion

In our study, *Z. marina* shoots growing around off-bottom oyster farming structures showed a higher capacity of leaf incorporation of $NH_4^+$ ($V_{leaf}$ and $V_{ab}$, Fig. 3) than those growing ~1 km from oyster farming structures, indicating physiological adjustments at the level of $NH_4^+$ uptake kinetics and/or its metabolic assimilation (Touchette and Burkholder 2000, 2007; Rubio et al. 2007). Although not assessed in this study, it is likely that shoots growing near oysters show specific uptake kinetics properties, such as higher $V_{max}$ (i.e. maximum uptake rates) and/or higher $\alpha$ (i.e. uptake affinity) for $NH_4^+$, as compared to those from the reference site. Differences in these descriptors of the uptake of DIN species among eelgrass plants from different sites in San Quintín Bay have recently been reported by Sandoval-Gil et al. (2015). Based on incubations under laboratory conditions of eelgrass shoots from four sites in the bay, using $^{15}N$ tracers ($^{15}NH_4^+$ and $^{15}NO_3^-$) Sandoval-Gil et al. (2015) obtained significantly higher $V_{max}$ and/or higher $\alpha$ values for the uptake of ammonium by *Z. marina* shoots collected at two oyster farming sites in Bahía Falsa compared to shoots collected at the mouth of the lagoon and at a site in the eastern arm, away from oyster farming activity. This physiological plasticity of *Z. marina* is consistent with the differences found between shoots from the reference and oyster sites in this work. Since there were no substantial differences in environmental parameters (e.g. salinity, temperature, irradiance) between sites, these differences can be related to the higher capacity of shoots at the oyster site to exploit a valuable source of $NH_4^+$ from oyster excretion.

Oysters release large amounts of $NH_4^+$ by direct excretion to the water column and also enhance $NH_4^+$ sediment–water fluxes through the remineralization of their biodeposits (Newell at al. 2005; Kellogg et al. 2014). This may represent an important DIN supply in the N budget of San Quintín Bay, particularly during weak or non-upwelling periods,

375 considering that the main external source of nutrients to this lagoon is the adjacent
376 ocean (Hernández-Ayón et al. 2004; Camacho-Ibar et al. 2003). Based on calculations
377 and assumptions reported by Hernández-Ayón et al. (2004), corrected with recent oyster
378 production figures for Bahía Falsa (~1240 t in 2013), we estimate that all oysters
379 cultivated in this bay excrete about $5.2 \times 10^6$ mmol $NH_4^+$ $day^{-1}$. According to our
380 measurements of eelgrass $NH_4^+$ uptake rates and aboveground biomass, eelgrass
381 meadows at Bahía Falsa located near oyster farms would have the potential to
382 incorporate about 33.1 mmol $NH_4^+$ $m^{-2}$ $day^{-1}$ and those located farther away, about 25.6
383 mmol $NH_4^+$ $m^{-2}$ $day^{-1}$. Consequently, it would take an area of 15–16 ha of eelgrass to
384 incorporate the whole amount of $NH_4^+$ excreted by oysters. This area represents only
385 about 3–4% of the 547 ha of subtidal eelgrass cover in Bahía Falsa (Ward et al. 2003),
386 clearly showing the biofiltration potential of *Z. marina* meadows in San Quintín Bay,
387 which may act as effective buffers of the $NH_4^+$ loading from oyster farms. It must be
388 noted, however, that seagrasses are not the sole sink of the ammonium derived from
389 oyster aquaculture. Opportunistic macroalgae (*Ulva* spp.) are also present in San
390 Quintín Bay and are particularly abundant in Bahía Falsa (Zertuche-González et al.
391 2009). As *Ulva* spp. is probably N-limited in San Quintín Bay (Zertuche-González et al.
392 2009), the uptake of $NH_4^+$ by eelgrass in Bahía Falsa may exert some control on the
393 growth of opportunistic macroalgae. These results highlight the critical role of
394 seagrasses in the N budget of this bay, and also their relevance for management
395 strategies developed to cope with potential sources of eutrophication (e.g. wastewater,
396 agriculture and groundwater, etc.) in other coastal ecosystems (McGlathery et al. 2007).
397 More research efforts must be conducted to assess the biofiltering role of *Z. marina* in
398 San Quintín Bay, because oyster production can, in turn, lead to alterations in vegetative
399 descriptors at shoot and meadow levels (Tallis et al. 2009), which have not been

400 evaluated for Bahía Falsa. Direct/indirect effects of oyster aquaculture like shading,
401 competition for space and increasing sedimentation caused by aquaculture structures, as
402 well as oyster harvesting methods, may have negative impacts on *Z. marina*. Negative
403 effects include a reduction in photosynthesis, the alteration of meadow structure (e.g.
404 shoot density, cover and aboveground biomass), and a decrease in recruitment (i.e. seed
405 production and seedling density) (Everett et al. 1995; Kelly and Volpe 2007; Wisehart
406 et al. 2007; Wagner et al. 2012; Skinner et al. 2014). Conversely, these descriptors can
407 be positively affected depending on oyster culture density, sediment characteristics or
408 cultivation practices employed (Booth and Heck 2009; Tallis et al. 2009; Wagner et la.
409 2012). In this study we observed that photosynthesis, leaf growth and plant size were
410 higher for shoots from the oyster site, where a higher concentration of $NH_4^+$ in the water
411 column and in the sediment pore water is available for plant tissues. In contrast, the
412 lower above/belowground biomass ratio of eelgrass at the reference site was probably
413 caused by a higher investment in belowground tissues to compensate for lower nutrient
414 availability (Short 1987; Lee and Dunton 1999). However, studies at different sites have
415 also reported that $NH_4^+$ excretion and biodeposition from bivalves (i.e. mussels) can
416 lead to adverse effects on eelgrass performance due to the decrease in light availability
417 by increasing leaf epiphyte cover and increased sulfide toxicity (Vinther and Holmer
418 2008; Vinther et al. 2008; Korhonen et al. 2012). As reported by Korhonen et al. (2012),
419 we observed that eelgrass beds were almost absent only directly under the oyster
420 structures; this may indicate that shoots at the oyster site (i.e. 1–4 m near oyster
421 longlines) were not directly exposed to the negative effects of oysters (e.g. sulfide and
422 $NH_4^+$ toxicity or structure shading). The average $NH_4^+$ concentration in sediment pore
423 water at the oyster site (422 µM) was much lower than the toxic levels observed to
424 cause detrimental effects on *Z. marina* productivity under experimental conditions

425 (~1200 µM; Vinther and Holmer 2008). Also, epiphyte coverage on leaves was similar
426 between sites. A positive interaction between mussel culture and the productivity of *Z.*
427 *marina* and *Thalasssia testudinum* has also been reported, mainly due to the fertilization
428 of sediments by mussel biodeposition and a reduction of epiphytic loads on the leaves
429 (Reusch at al. 1994; Peterson and Heck 2001a, b).
430 Photosynthesis and the P:R ratio of *Z. marina* were higher near oyster cultures, probably
431 fuelled by the higher $NH_4^+$ uptake since N and C metabolism are closely related
432 (Burkholder et al. 1992; Invers et al. 2004; Touchette and Burkholder 2007). Higher
433 carbon fixation rates and higher mobilization of storage carbohydrates may be required
434 by shoots near oyster farming structures in order to assimilate the $NH_4^+$ available from
435 oyster excretion and to incorporate it into organic compounds like free amino acids
436 (Invers et al. 2004). Photosynthesis decreased in shoots from both sites at high
437 irradiances when tidal height decreased and pH and temperature increased, probably due
438 to the combined effects of photoinhibiton and C limitation (Invers et al. 1997; Olivé et
439 al. 2015).
440 Nitrogen content decreased with leaf age in *Z. marina*. This pattern has been previously
441 described for seagrasses, and results from the dilution of the N pool by a higher relative
442 abundance of structural components as suggested by the higher C/N ratio, and/or the N
443 resorption from senescent leaves (Pedersen and Borum 1993; Lepoint et al. 2002).
444 Observing consistently higher %N in leaves and rhizomes of shoots from the oyster site
445 was an expected response likely related to the storage of excess N for plant growth
446 (Burkholder et al. 2007). Although C content declined in older leaves, the C/N molar
447 ratio gradually increased from younger to older leaves, reflecting that structural C
448 compounds are less mobile than N-containing components (Stapel and Hemminga
449 1997).

*Zostera marina* shoots from the oyster site showed natural $\delta^{15}N$ values of around 12.5‰, which were lower than the $\delta^{15}N$ values for the reference site (~16‰). This can be a consequence of the lighter isotope being preferentially uptaken over the heavier isotope at the oyster site due to a greater availability of DIN. It may also be related to differences in the isotopic signal of different sources of dissolved organic/inorganic N (Alkhatib et al. 2012). On the other hand, we did not find significant differences in the natural N isotopic signal among the leaves of different ages and rhizomes of eelgrass shoots within each site. This suggests that even with different uptake rates, leaves of different ages do not exhibit differences in discrimination against the heavier isotope, or such differences are masked by other processes such as intra-plant fractionation due to N translocation or exudation (Evans 2001; Yamamuro et al. 2004).

Values of $\delta^{13}C$ were higher in shoots at the oyster site, reflecting a higher C demand at this site where photosynthetic production was higher. Under high demand, C isotopic discrimination during photosynthesis decreases, leading to higher isotopic ratios in leaf tissues (Hemminga and Mateo 1996). The decrease in $\delta^{13}C$ values of leaves with age suggests the effects of a progressive decrease in growth and thus in C demand. Similar trends have been documented elsewhere, reflecting the reduction in the photosynthetic rates and the change in the carbohydrate composition with leaf ageing (Lepoint et al. 2003).

Different leaves of *Z. marina* shoots from both sites exhibited different $NH_4^+$ uptake rates, a probable consequence of different levels of growth activity. We found higher uptake rates for intermediate, actively-growing leaves (#2 and #3), while lower rates corresponded to the older, less active leaves (#4 and #5). These leaves showed larger necrotic areas and higher epiphyte cover, which also limit $NH_4^+$ uptake (Cornelisen and Thomas 2004). The youngest leaf (#1) showed lower $NH_4^+$ uptake capacity than leaves

#2 and #3, in contrast to previous studies reporting higher uptake in the youngest leaves of *Z. marina* and other seagrasses after experimental incubation using labeled $NH_4^+$ (Iizumi and Hattori 1982; Pedersen et al. 1997; Lepoint et al. 2002). This discrepancy may be explained by the larger incubation periods used in the cited studies (from 24 hours to 7 days), which potentially allowed for translocation and/or internal N recycling that could have increased the $^{15}N$ abundance in the youngest tissues that act as N sinks (Hemminga et al. 1999; Lepoint et al. 2002; Marbá et al. 2002). In addition, it must be noted that most of the surface of the youngest leaf was within the sheath, probably restricting the incorporation of $NH_4^+$ directly from the water column.

The abundance of $^{15}N$ in eelgrass leaf tissues generally decreased two weeks after pulse labeling ($T_f$). This resulted from the dilution of the internal $^{15}N$ pool by active uptake of unlabeled external N sources. This is consistent with the notion that fast growing species like *Z. marina* depend to a lesser extent on internal N recycling compared to slow-growing species (e.g. *Posidonia oceanica*) that have a longer leaf life span (Hemminga et al. 1999). Also, within each leaf, there were no significant differences in the $^{15}N$ content between old and new tissues, except for leaf #0 (i.e. entirely new leaf) of shoots from the oyster site which showed the highest enrichment compared to the rest of plant tissues; rhizome tissues were also enriched in $^{15}N$ after two weeks incubation ($T_f$) but only in shoots exposed to oysters. This may indicate that the greater availability of $NH_4^+$ from oyster excretion allows shoots at the oyster site to use the N incorporated to satisfy the N requirements of younger actively growing tissues, as well as to store excess N in belowground tissues through basipetal translocation (Pedersen et al. 1997; Stapel et al. 2001).

In summary, *Z. marina* plants directly influenced by oyster aquaculture in the western arm of San Quintín Bay showed higher rates of $NH_4^+$ uptake than shoots located ~1 km

500 from oyster longlines. This indicates that eelgrass growing near oysters are
501 physiologically adapted to efficiently acquire $NH_4^+$ from bivalve excretion. We
502 calculate that about 3% of the area covered by eelgrass meadows in Bahía Falsa could
503 incorporate the total of $NH_4^+$ excreted by oysters, which highlights the biofiltering
504 potential of eelgrass meadows in this bay. Vegetative productivity and photosynthesis
505 of eelgrass were also higher at the oyster site, reflecting the higher metabolic utilization
506 of N compounds for plant growth. However, the capacity of eelgrass as a biofilter of
507 oyster aquaculture-derived nutrients in Bahía Falsa may also depend on other spatio-
508 temporal factors that must be addressed in future studies. For instance, the biofiltering
509 capacities of eelgrass meadows can be influenced by changes in vegetative productivity,
510 which changes seasonally (Cabello-Pasini et al. 2003), or by environmental factors that
511 show spatial gradients within the bay (e.g. salinity, temperature; Ribas-Ribas et al.
512 2011). Aditionally, the interaction between oyster aquaculture and submerged
513 vegetation must be examined, given that the abundance of opportunistic macroalgae
514 (e.g. *Ulva* spp.) has increased recently in San Quintín Bay, probably as a result of oyster
515 cultivation (Zertuche et al. 2009). The complete understanding of the complex
516 relationship between co-occuring oyster farming and eelgrass, which provides important
517 ecosystem and economic services, will provide valuable scientific criteria urgently
518 needed for the management, conservation and restoration of coastal lagoons and
519 estuaries worldwide (Buzzelli et al. 2015; Dumbauld and McCoy 2015; Forde et al.
520 2015; Sharma et al. 2016).
521
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523

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767 **Figure captions**

768 **Fig. 1** (a) Map of San Quintín Bay indicating the location of the study sites (reference
769 and oyster sites) and the approximate distribution of oyster farms. (b) Photo of the
770 oyster site at low tide showing *Zostera marina* growing alongside off-bottom longlines
771 **Fig. 2** Benthic chambers used to measure *Zostera marina in situ* (a) leaf uptake of
772 $^{15}NH_4^+$ and (b) leaf photosynthesis/respiration
773 **Fig. 3** (a) Specific ($V_{leaf}$) and (b) absolute ($V_{ab}$) uptake rates of $^{15}NH_4^+$ by *Zostera*
774 *marina* at the reference and oyster sites. Leaves are ranked from the youngest (leaf #1)
775 to the oldest (leaf #5). Significant differences are indicated by different letters. Values
776 are means and standard errors
777 **Fig. 4** Variation of *Z. marina* net photosynthesis (net-P) with irradiance at the reference
778 and oyster sites. The upper panel (a) shows the variation in tide height, pH, and
779 temperature throughout the day. The daily P:R ratio of *Z. marina* leaves at each site is
780 presented in (b). Values are means and standard errors
781 **Fig. 5** Natural isotopic nitrogen and carbon composition ($\delta^{15}N$, a; $\delta^{13}C$, b) and nutrient
782 content (% N, c; % C, d) of *Z. marina* leaves and rhizomes at the reference and oyster
783 sites. Leaves are ranked from the youngest (leaf #1) to the oldest (leaf #5). Significant
784 differences are indicated by different letters. Values are means and standard errors
785 **Fig. 6** $^{15}N$ content of *Z. marina* leaves and rhizomes after *in situ* incubations ($T_i$, panel
786 a) and two weeks after incubations ($T_f$, panel b). Lines within the bars in panel a)
787 indicate the natural $^{15}N$ content in eelgrass tissues at $T_0$. Leaves are ranked from the
788 youngest (#1) to the oldest (#5). In the upper panels, schematic representations of the
789 different leaves within a shoot are presented; new tissue that developed during the two
790 weeks is differentiated (dashed columns) from old tissue. Leaf #0 corresponds to an
791 entirely new leaf produced after the incubation. Leaf #5 was lost two weeks after

incubation. Significant differences are indicated by different letters. Values are means and standard errors

## Tables

**Table 1** Environmental parameters measured at the reference and oyster sites. Significant differences (Student t-test) between sites are indicated by asterisks (*$p$<0.05). Values are means and standard errors

| | **Ref. site** | **Oyster site** |
|---|---|---|
| **Irradiance** (mol photons $m^{-2}$ $day^{-1}$), $n$=10 | 19.5 ± 2.1 | 18.2 ± 1.3 |
| **Temperature** ($^{o}$C), $n$=62 | 18.1 ± 0.2 | 18.0 ± 0.1 |
| **Salinity** (practical salinity scale), $n$=62 | 33.7 ± 0.3 | 33.8 ± 0.5 |
| **Water-column [$NH_4^+$]** (µM), $n$=6 | 0.6 ± 0.2 | 1.6 ± 0.3* |
| **Water-column [$NO_3^-$]** (µM), $n$=6 | 0.47 ± 0.1 | 0.49 ± 0.1 |
| **Pore-water [$NH_4^+$]** (µM), $n$=6 | 192 ± 16 | 422 ± 31* |

**Table 2** Vegetative descriptors and epiphyte coverage measured in leaves of *Z. marina* at the reference and oyster sites, and collected two weeks after *in situ* incubations (see Fig. 4). Leaves are ranked from the youngest (#0) to the oldest (#4). Values are means and standard errors

| | **Ref. site** | | | | |
|---|---|---|---|---|---|
| | **Leaf #0** | **Leaf #1** | **Leaf #2** | **Leaf #3** | **Leaf #4** |
| **Leaf size** ($cm^2$) | 10.5 ± 4.4 | 28.1± 3.7 | 36.4 ± 1.4 | 36.2 ± 2.5 | 25.5± 4.2 |
| **New leaf tissue** (%) | 100 | 57.9 ±10.3 | 38.6 ± 7.4 | 10.2 ± 2.6 | 0 |
| **Necrotic tissue** (%) | 0 | 0 | 5.3 ± 1.01 | 12.7 ± 3.34 | 32.2 ± 5.7 |
| **Epiphyte coverage** (%) | 0 | 0 | 0 | 18.1 ± 4.1 | 39.2 ± 3.3 |
| | **Oyster site** | | | | |
| | **Leaf #0** | **Leaf #1** | **Leaf #2** | **Leaf #3** | **Leaf #4** |
| **Leaf size** ($cm^2$) | 7.4 ± 2.2 | 33.9 ± 2.6 | 43.9 ± 0.8 | 49.6 ± 3.6 | 38.3± 1.1 |
| **New leaf tissue** (%) | 100 | 71.6 ± 8.3 | 49.3 ± 7.4 | 5.3 ± 0.8 | 0 |
| **Necrotic tissue** (%) | 0 | 0 | 6.8 ± 2.01 | 8.5 ± 4.3 | 35.2 ± 8.7 |
| **Epiphyte coverage** (%) | 0 | 0 | 2.3 ± 0.2 | 13.2 ± 6.2 | 36.7 ± 9.1 |

**Table 3** Vegetative parameters of *Z. marina* shoots and meadow structure measured at the reference and oyster sites. Significant differences (Student *t*-test) between sites are indicated by asterisks (*$p$<0.05; **$p$<0.01; ***$p$<0.001). Values are means and standard errors

| | **Ref. site** | **Oyster site** | ***t*** | ***p*** |
|---|---|---|---|---|
| **Shoot size** (leaf-cm$^2$ shoot$^{-1}$), $n$=10 | 165.8 ± 7.6 | 210.6 ± 8.1 | -4.052 | *** |
| **Shoot growth** (leaf-g DW shoot$^{-1}$ day$^{-1}$), $n$=10 | 0.005 ± 0.0002 | 0.009 ± 0.0004 | 6.295 | *** |
| **Meadow density** (shoots m$^{-2}$), $n$=4 | 525 ± 27.3 | 340.6 ± 32.3 | 4.39 | ** |
| **Aboveground biomass** (g DW m$^{-2}$), $n$=4 | 178.7 ± 0.2 | 144.7 ± 7.3 | 2.068 | ns |
| **Belowground biomass** (g DW m$^{-2}$), $n$=4 | 93.1 ± 6.1 | 45.7 ± 4.7 | 6.181 | *** |
| **Biomass ratio** | 1.92 ± 0.1 | 3.3 ± 0.5 | -2.799 | * |

## Figures

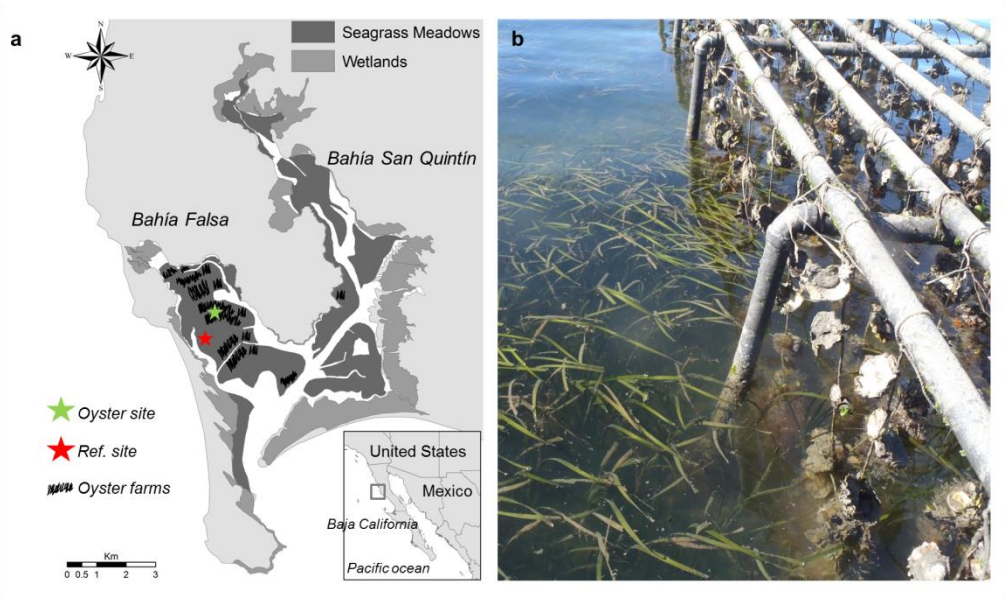


Fig. 1

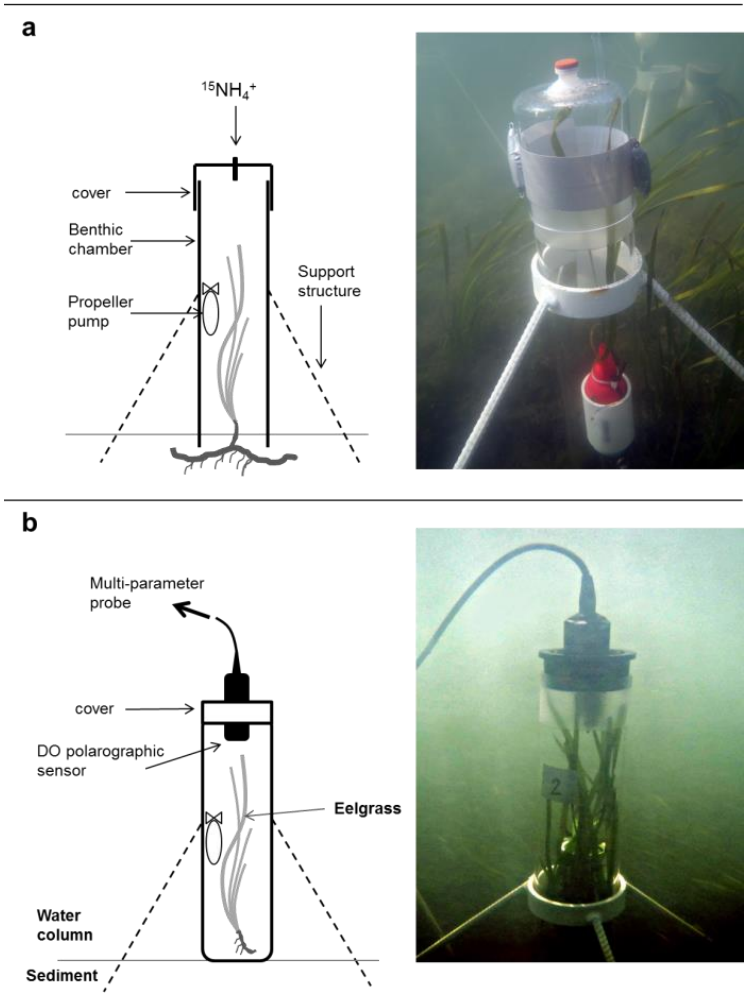
a
$^{15}NH_4^+$
cover
Benthic chamber
Propeller pump
Support structure
b
Multi-parameter probe
cover
DO polarographic sensor
Eelgrass
Water column
Sediment

Fig. 2

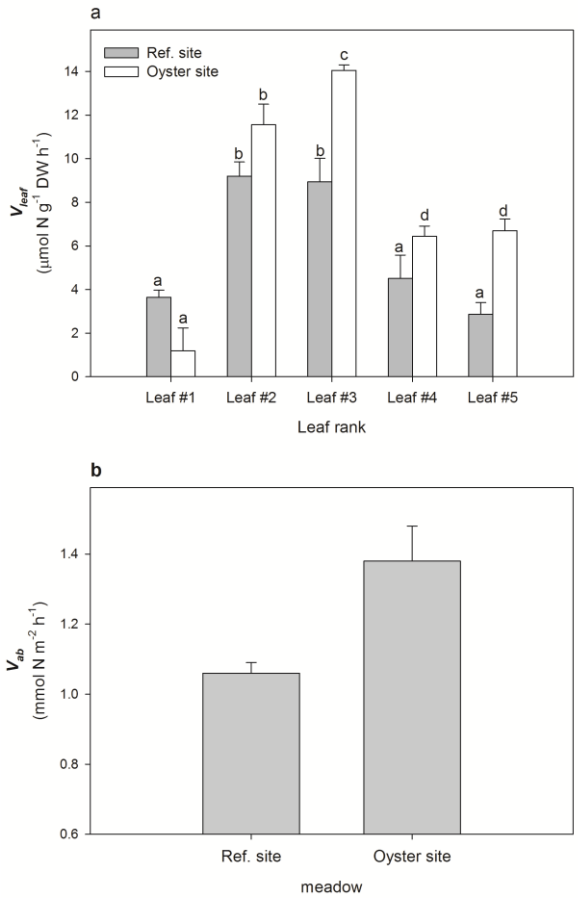
a
Ref. site
Oyster site
$V_{leaf}$ (µmol N g$^{-1}$ DW h$^{-1}$)
0
2
4
6
8
10
12
14
a
a
b
b
b
c
a
d
a
d
Leaf #1
Leaf #2
Leaf #3
Leaf #4
Leaf #5
Leaf rank
b
$V_{ab}$ (mmol N m$^{-2}$ h$^{-1}$)
0.6
0.8
1.0
1.2
1.4
Ref. site
Oyster site
meadow

Fig. 3.

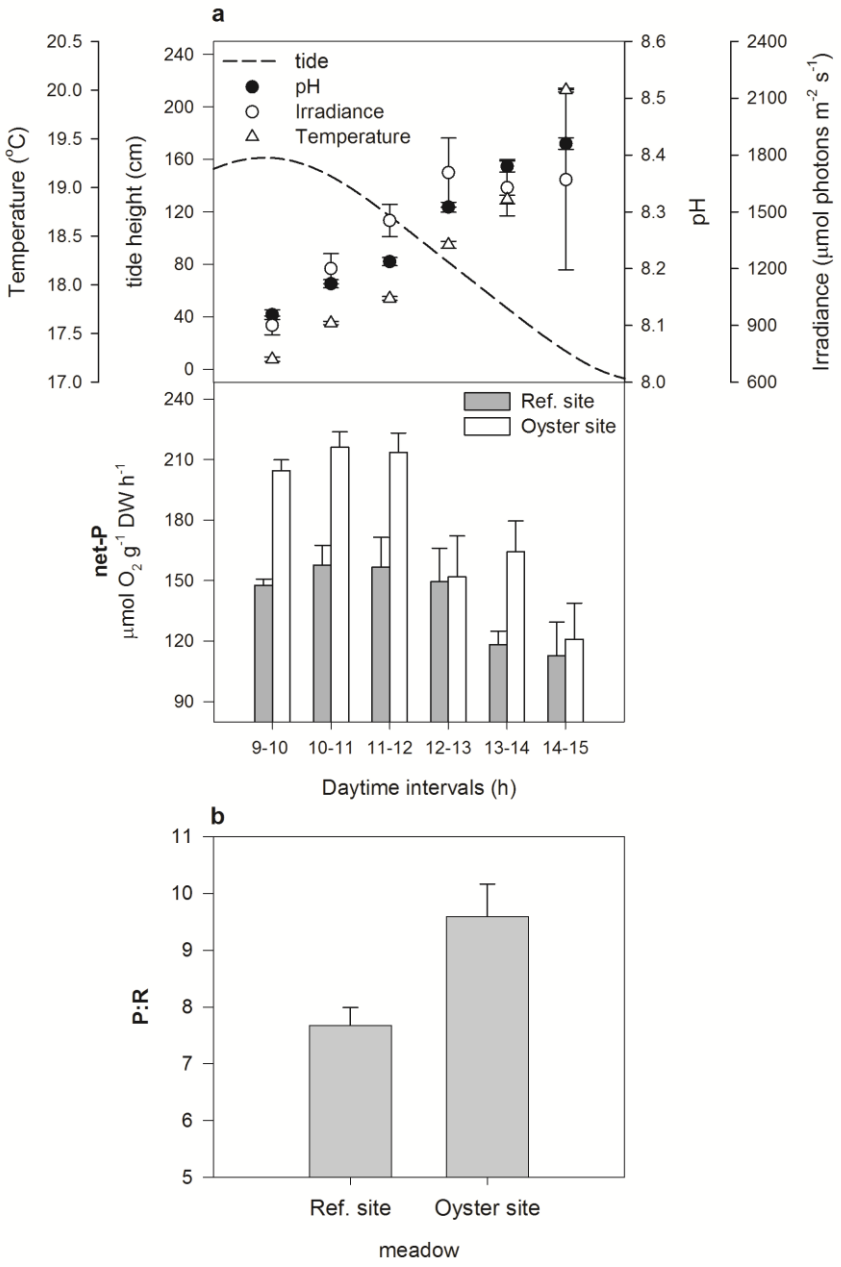
a
tide
pH
Irradiance
Temperature
Temperature (°C)
tide height (cm)
pH
Irradiance (µmol photons m-2 s-1)
Ref. site
Oyster site
net-P
µmol O2 g-1 DW h-1
9-10
10-11
11-12
12-13
13-14
14-15
Daytime intervals (h)
b
P:R
Ref. site
Oyster site
meadow

Fig. 4

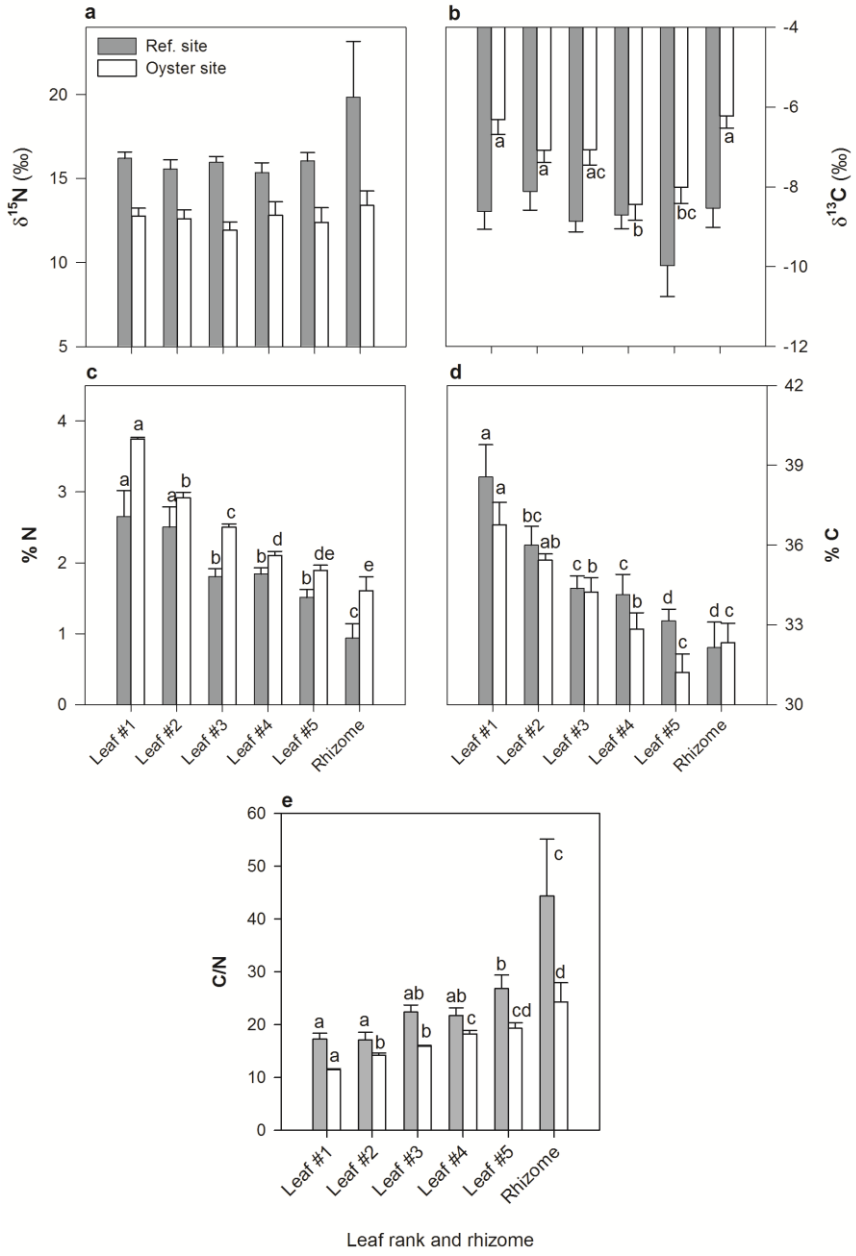
a
Ref. site
Oyster site
δ15N (‰)
b
δ13C (‰)
c
% N
d
% C
e
C/N
Leaf #1
Leaf #2
Leaf #3
Leaf #4
Leaf #5
Rhizome
Leaf rank and rhizome

Fig. 5

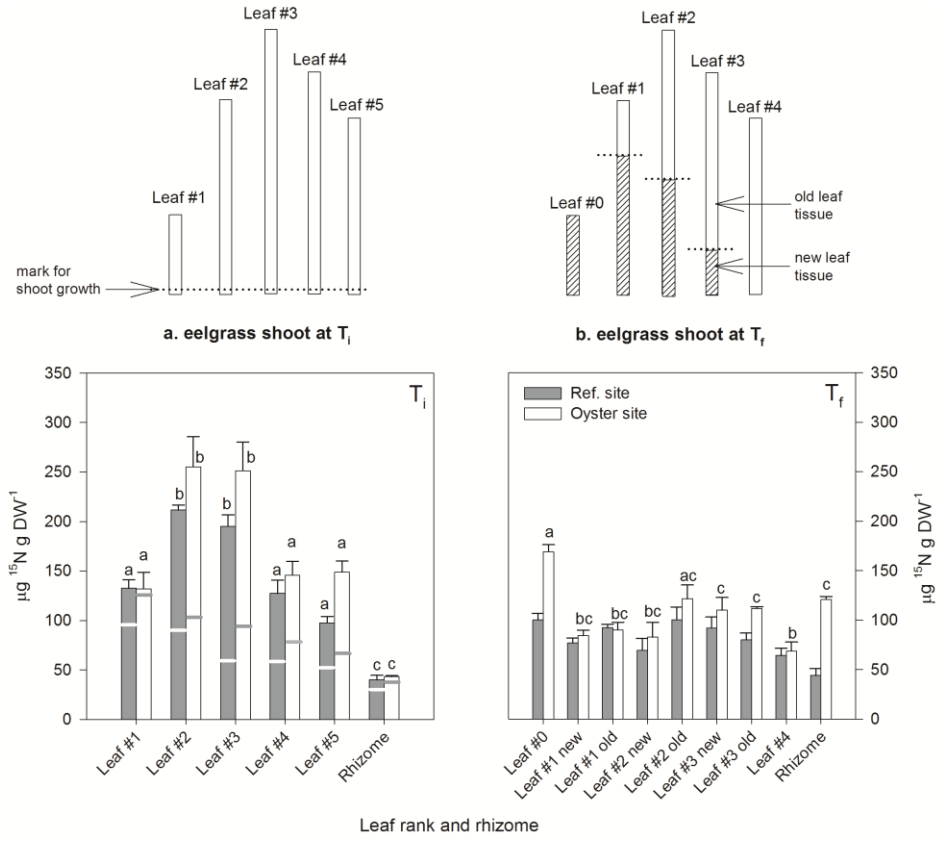
Leaf #1
Leaf #2
Leaf #3
Leaf #4
Leaf #5
mark for shoot growth
a. eelgrass shoot at $T_i$
Leaf #0
Leaf #1
Leaf #2
Leaf #3
Leaf #4
old leaf tissue
new leaf tissue
b. eelgrass shoot at $T_f$
$T_i$
$T_f$
Ref. site
Oyster site
µg $^{15}$N g DW$^{-1}$
Leaf #1
Leaf #2
Leaf #3
Leaf #4
Leaf #5
Rhizome
Leaf #0
Leaf #1 new
Leaf #1 old
Leaf #2 new
Leaf #2 old
Leaf #3 new
Leaf #3 old
Leaf rank and rhizome

Fig. 6.