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Regulation of Nitrate Uptake by the Seagrass *Zostera marina* During Upwelling

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Abstract

Prolonged nitrogen (N) fertilization can impact seagrass survival and productivity; however, the effects of N enrichment pulses (e.g., upwelling or sediment resuspension) remain poorly understood. This study examined the effects of short-term (1 h) pulsing of nitrate (NO_3^-) enrichment, simulating an upwelling event, on dissolved inorganic carbon (DIC) and NO_3^- uptake capacities, critical in controlling eelgrass productivity. *Zostera marina* dominates submerged vegetation in coastal lagoons influenced by upwelling in the California Current system. Laboratory incubations were conducted in winter (non-upwelling) and spring (upwelling) with shoots collected from San Quintín Bay meadows, Baja California, Mexico, differentially exposed to upwelled NO_3^-. Results suggest that NO_3^- enrichment stimulated DIC and NO_3^- uptake in winter, reflecting the close relationship between carbon metabolism and NO_3^- assimilation. Eelgrass shoots showed reduced NO_3^- incorporation in spring; neither NO_3^- uptake nor photosynthesis increased when exposed to high NO_3^-. Saturation of spring shoots at lower ambient NO_3^- concentrations may be interpreted as a physiological strategy to restrict metabolically costly NO_3^- incorporation during upwelling; this regulation of NO_3^- uptake strongly contrasts to the apparently full exploitation of this nutrient by seaweeds also dominant within the bay, as indicated in previous works. Despite their reduced NO_3^- uptake, eelgrass meadows near the bay mouth acquire NO_3^- at rates up to 4.2 mmol N m^{-2} day^{-1}. This represents non-trivial water column NO_3^- removal compared to the estimated oceanic NO_3^- supply (~ 7.1 mmol m^{-2} day^{-1}) during upwelling, highlighting the importance of *Z. marina* beds in controlling the lagoonal N-budget.

Keywords *Zostera marina* · Upwelling · Pulsed nitrate fertilization · DIC uptake · N uptake

Introduction

Nitrogen availability and the capacity of seagrasses to acquire dissolved inorganic nitrogen (DIN) are key factors controlling seagrass productivity (Alexandre et al. 2011; Sandoval-Gil et al. 2015). Generally, seagrasses exhibit higher capacities to incorporate ammonium (NH_4^+) than nitrate (NO_3^-), as reflected by the uptake kinetics of both compounds (i.e., higher uptake $-\alpha$ affinities and greater maximum uptake rates, V_{max}, for NH_4^+; Touchette and Burkholder 2000; Alexandre et al. 2011). This has been related to differences in nutrient transport at the membrane level (including the number of uptake sites and their substrate affinity), the metabolic costs of NO_3^- assimilation, as well as the natural availability of DIN species (Touchette and Burkholder 2000; Rubio et al. 2007). However, DIN uptake can be highly variable within and among seagrass species. Furthermore, the availability of DIN sources in the water column and sediments is a primary source of such variability, which can also control DIN acquisition strategies at other physiological and vegetative levels (e.g., plant biomass allocation, internal N recycling; Hemminga et al. 1999; Lee and Dunton 1999). Other factors influencing DIN acquisition are the hydrodynamic regime and epiphyte cover (Cornelisen and Thomas 2004).

In coastal environments, aquaculture and sewage inputs can result in anthropogenic N enrichment in seagrass-dominated areas, causing, in most cases, negative effects on plant productivity and survival (Burkholder et al. 2007; Boudouresque et al. 2009). The effects of nitrogen (N) enrichment and euthrophication on seagrass beds have been well

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described for prolonged exposure (e.g., months, weeks), both in situ (e.g., aquaculture and/or urban discharges) and experimentally (Burkholder et al. 2007 and references therein). For instance, long-term exposure of seagrass meadows to N enrichment can lead to direct harmful effects, such as a decrease in leaf growth or shoot density, and to indirect adverse conditions, such as light limitation due to seaweed overgrowth and/or increase in herbivore pressure (Udy and Dennison 1997; Hauxwell et al. 2003; Ruiz et al. 2010). Alterations in photosynthesis, in the activity of nitrate assimilatory enzymes, enrichment in ^{15}N and N content have also been reported (Burkholder et al. 1992; Udy and Dennison 1997; Touchette and Burkholder 2007; Villazán et al. 2015; Marín-Guirao et al. 2017).

In addition to human activities, nitrogen enrichment of coastal systems can result from natural processes, such as sediment resuspension, upwelling, or increased runoff, although studies on their effects on seagrass physiology are much scarcer than those of anthropogenic inputs (Hemminga et al. 1993; Pedersen 1994; Hessing-Lewis and Hacker 2013). Nutrient-enriched waters transported nearshore during periods of upwelling in coastal systems affected by the California Current system have been found to influence seagrass and seaweed physiology and ecology (e.g., population dynamics, community interactions, and tissue N content; Fourqurean et al. 1997; Hessing-Lewis and Hacker 2013). For instance, Pedersen (1994) and Sandoval-Gil et al. (2015) demonstrated a transient surge of ammonium (NH_4^+) uptake in the seagrasses *Amphibolis antarctica* and *Zostera marina* based on higher values of V_{max} and the half-saturation constant, K_m, which allow these plants to efficiently exploit high concentrations (up to 50 μM) of this nutrient in the water column resulting from bottom resuspension (Hernández-Ayón et al. 2004). On the other hand, responses of *Z. marina* to anthropogenic N enrichment have been mostly studied during prolonged exposure (weeks to months), whereas there is limited understanding of the effects of short-term nutrient pulses typically associated with natural N fertilization (Burkholder et al. 1992, 1994). Indeed, the effects of pulsed (transient) high N availability on critical physiological processes of seagrasses, such as photosynthesis and N uptake, have been largely ignored.

San Quintín Bay (SQB) is a coastal lagoon located in the northwestern Baja California Peninsula, under the influence of the California Current. Northwesterly winds cause upwelling, and advection of upwelled waters into the lagoon during flood tides is frequent during spring–summer (Camacho-Ibar et al. 2003; Hernández-Ayón et al. 2004). *Zostera marina* L. is the dominant submerged vegetation in SQB and in other estuarine ecosystems influenced by upwelling events along the California Current system (Fourqurean et al. 1997; Cabello-Pasini et al. 2003; Ward et al. 2003). Pulsed, tidal injection of DIN supplied by coastal upwelling events, mostly as NO_3^-, plays a critical role in the lagoonal N budget. The oceanic NO_3^- supply during upwelling events in this region is ~ 7.1 mmol m^{-2} day^{-1}. This estimate is based on an unpublished salt budget calculation resulting in a water exchange flow of 9.5×10^6 m^3 day^{-1} and an average ocean NO_3^- concentration of 15 μM during intense upwelling, which result in a NO_3^- input of ~ 142,000 mol day^{-1}. It has been suggested that some abundant seaweeds within the bay (e.g., *Ulva* and *Gracilaria* sp.) can efficiently exploit these short-term (hours) NO_3^- fertilization pulses, as reflected by the increments in their biomass and N content (Zertuche-González et al. 2009; Sandoval-Gil et al. 2015; Ávila-López et al. 2016). *Zostera marina* was also found to efficiently exploit fluctuating/transient NH_4^+ pulses derived from oyster aquaculture or sediment resuspension within SQB (Sandoval-Gil et al. 2015, 2016). Shoots growing near oyster culture racks exhibited a higher photosynthetic capacity and photosynthesis:respiration (P:R) ratio than plants located further away, reflecting the fact that higher DIC fixation rates may be required to assimilate the NH_4^+ available from oyster excretion (Invers et al. 2004; Sandoval-Gil et al. 2015). In contrast, the uptake of transient/pulsed NO_3^- from upwelled waters by *Z. marina* or its effects on DIC uptake remains unknown.

Sandoval-Gil et al. (2015, 2016) demonstrated that shoots from different meadows within the bay exhibit different N acquisition strategies depending on their exposure to NH_4^+ excreted by cultivated oysters. Similarly, we hypothesize that different *Z. marina* populations could also differ in their NO_3^- uptake strategies depending on their proximity to the mouth of the bay and, thus, the availability of oceanic NO_3^-. *Zostera marina* populations growing near the bay mouth are exposed to high NO_3^- concentrations during upwelling (~ 9 μM in Camacho-Ibar et al. 2003; Hernández-Ayón et al. 2004; ~ 20 μM in this study, Fig. 1). This upwelled NO_3^- concentration rapidly decreases towards the inner portions of the bay mainly due to N uptake by primary producers (Camacho-Ibar et al. 2003). Thus, *Z. marina* populations growing a few kilometers from the bay mouth are exposed to NO_3^- concentrations typically below 5 μM. The present study examined the effects of short (1 h)-pulsed NO_3^- enrichment on *Z. marina* shoots from three populations in SQB: one located near the bay mouth (BM) and two (BSQ and BF) located ~ 5 km further into the lagoon (Fig. 1). Photosynthetic and nitrate uptake rates of *Z. marina* shoots were measured under such pulses by measuring DIC and ^{15}N-labeled nitrate incorporation, respectively. Nutrient content, shoot growth, and biomass were also measured. Since seagrass physiological responses can vary seasonally, experiments were carried out in spring, during upwelling events, and in winter, when upwelling is practically absent.

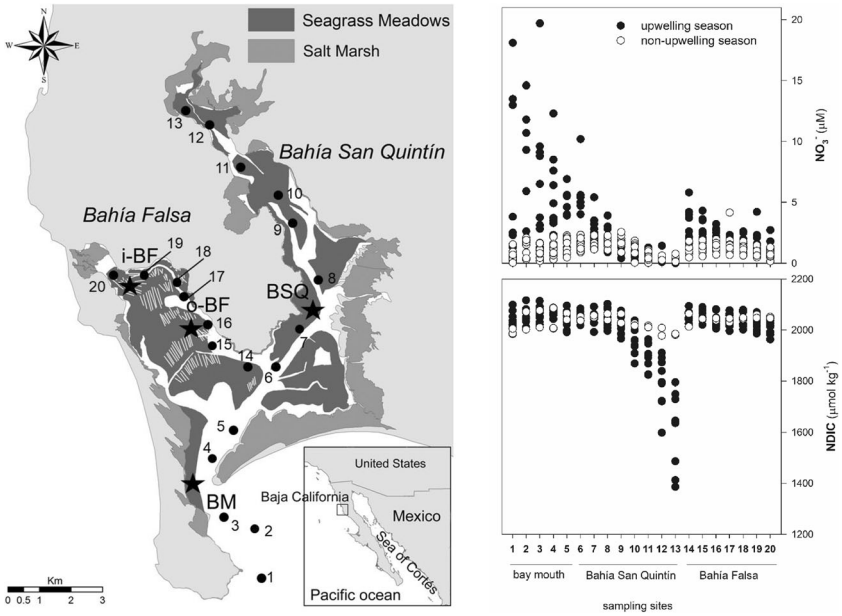

Fig. 1 Left panel, map of San Quintín Bay (SQB) indicating the location of the three *Z. marina* meadows in this study [stars indicate BM (bay mouth), BSQ (Bahía San Quintín), BF (Bahía Falsa)]; gray hatching within the western arm of Bahía Falsa shows the distribution of oyster culture racks. Right panel, seawater nitrate (NO_3^-) and normalized DIC (NDIC) concentrations measured at different sites in SQB (1–20 in the map) during seasons with or without upwelling

Methods

Study Area

Plants for experimental incubations were collected from SQB, which has extensive intertidal and shallow subtidal flats mainly occupied by monospecific *Z. marina* beds (~46% bottom areal cover) (Ward et al. 2003) (Fig. 1). The San Quintín Bay is a coastal lagoon located in the northwestern Pacific coast of the Peninsula of Baja California, Mexico (30° 30′ N, 116°W). This bay is Y-shaped (43 km^2, average 2 m depth) with a single mouth (~800 m wide) connecting with the ocean and with eastern and western arms known as Bahía San Quintín and Bahía Falsa, respectively (Fig. 1). The coastal system is under the influence of the California Current with a typical wind-driven upwelling system (from April to August); water exchange and circulation with SQB are mainly dominated by semidiurnal tidal flows. Water circulation within the bay largely occurs through narrow and deep (5–7 m) tidal channels extending along the length of both arms. Geomorphology and distance from the bay mouth lead to gradients of environmental parameters [e.g., temperature, salinity (Ribas-Ribas et al. 2011), and nutrients (Ávila-López et al. 2016; Camacho-Ibar et al. 2003]. Further details of biogeochemical characteristics of the bay are reported by Camacho-Ibar et al. (2003) and Hernández-Ayón et al. (2004).

Experimental Determination of DIC and NO_3^- Uptake

Zostera marina shoots with intact rhizomes and roots were randomly collected in winter (February) and spring (June) 2014 by scuba diving from three dense subtidal meadows (2.5 m max. depth at high tide). Shoots were collected at the mouth of the bay (BM), and at Bahía Falsa (BF) and Bahía San Quintín (BSQ) which are located ~5 km away from BM. Shoots at the BM site are directly exposed to upwelling events, while those at BF and BSQ show similar low exposure to NO_3^- fertilization during these upwelling events. The BF meadow is located within an active oyster aquaculture area and is exposed to higher NH_4^+ concentrations in the water column and sediment pore-water, due to oyster excretion and biodeposition (Sandoval-Gil et al. 2016). Entire shoots (including rhizome and roots) were transported to the laboratory in coolers filled with seawater. Within 3 h of collection, the shoots were cleaned of epiphytes and sediment, and were preincubated for 24 h in 20-L aquaria with seawater from their corresponding meadow site. Temperature and irradiance in the incubators were adjusted to mean values measured in the seagrass meadows during each season, to minimize stress associated with experimental conditions. Selected experimental irradiance values (see Table 1) were well above E_k (saturating irradiance) estimated for *Zostera marina* in SQB and other similar sites (50–150 μmol quanta m^{-2} s^{-1}; Cabello-Pasini et al. 2002, 2003), thus preventing light limitation during experimental incubations. Irradiance in the field was determined using underwater spherical quantum sensors (LI-193, LI-COR, USA), and water temperature was measured using a submersible, multi-parameter probe (YSI Pro Plus, USA). Light and temperature were continuously recorded for two to three consecutive weeks before the start of the experimental incubations. Sensors were positioned at mid-height of the leaf canopy to record the irradiance available to relatively young photosynthetic tissues.

Table 1 Experimental conditions during eelgrass incubations and field reference ranges of environmental variables; PPFD, photosynthetic photon flux density; DIC, concentration of dissolved inorganic carbon and its carbon species (HCO_3^- and $CO_{2(aq)}$). Data are shown as mean ± SE

	Winter	Spring (upwelling season)
Experimental incubations	Mean ± SE	
PPFD (μmol photon m^{-2} s^{-1})	353 ± 22	664 ± 48
Temperature (°C)	15 ± 0.2	18 ± 0.2
Salinity	33.2 ± 0.02	33.6 ± 0.01
DIC (μM)	2041 ± 8.5	2050 ± 9.8
HCO_3^- (μM)	1812.7 ± 9	1820.4 ± 8.2
$CO_{2(aq)}$ (μM)	16.3 ± 1.5	16.5 ± 1.4
Field conditions	Range	
Light dose (mol photon m^{-2} day^{-1})	18.5–23.4	32.3–38.7
Temperature (°C)	14.1–16.76	16.38–19.04
Salinity	33–33.9	33.6–34.5
DIC (μM)	1950–2067	2005–2210

Incubations to measure DIC and NO_3^- uptake rates were performed simultaneously using transparent split chambers placed in laboratory incubators (VWR 2015-2, USA). Complete shoots (leaves, rhizomes, and roots) of ~ 0.5–0.7 g dry weight (DW) were individually incubated for 1 h in the chambers; plant biomass:seawater ratio and incubation time were previously optimized to avoid carbon limitation. Chambers consisted of cylindrical acrylic tubes, divided into two compartments which separated the leaves (i.e., upper compartment, volume = 1.35 L) from the rhizomes and roots (i.e., lower compartment, volume = 0.66 L). To prevent seawater leakage, the connecting hole between compartments was sealed with a modeling clay and sterile vaseline. The upper compartment was filled with filtered (5 μm) and UV-treated seawater and closed by a tap. Nitrate and DIC uptake rates were determined for *Z. marina* leaves exposed to two NO_3^- concentrations (control vs high nitrate) in the upper compartment of the chamber. Within each season (winter and spring), NO_3^- level was the only experimental variable that differed among incubations, since all other physicochemical parameters were kept at similar levels. Seawater used for the incubations had low (< 1 μM) NO_3^- and NH_4^+ concentrations. The NO_3^- concentration in the control was adjusted by adding labeled $^{15}KNO_3$ (5 μM; at % = 99, Cambridge Isotope Laboratories, UK) to the seawater. The high $^{15}KNO_3$ treatment (20 μM concentration) of labeled NO_3^- was selected to simulate $^{15}KNO_3$ fertilization by intense upwelling, according to maximum values found within the bay (Fig. 1). Seawater in the leaf compartment was carefully agitated by hand without opening the chamber, to reduce the leaf boundary layer and to homogenize NO_3 concentrations during the incubation. To preserve the integrity of potential physiological interactions between leaves and roots during nutrient acquisition (Thursby and Harlin 1982; Alexandre et al. 2011), the nutrient concentration of the seawater in the lower compartment was adjusted to 200 μM of NH_4^+. This concentration was representative of the sediment pore-water around belowground tissues of *Z. marina* in SQB (Sandoval-Gil et al. 2016). Three incubations were performed for each nutrient level and meadow site.

Dissolved inorganic carbon uptake rates were compared among shoots exposed to the high NO_3^- treatment and those maintained in unenriched seawater. To obtain DIC uptake rates, discrete water samples were collected at the beginning and end of incubations (1 h) for carbonate analyses. Seawater samples were stored in 500 mL borosilicate bottles and preserved with 100 μL saturated mercuric chloride ($HgCl_2$). Dissolved inorganic carbon was measured following the colorimetric method described by Ávila-López et al. (2016). Prior to the analysis, accuracy and precision were assessed using the Certified Reference Material (CRM) for DIC provided by Andrew Dickson, Scripps Institution of Oceanography. Total alkalinity (TA) was measured with the potentiometric method described by Hernández-Ayón et al. (1999) and using standard reference materials. The concentration of DIC species (HCO_3^- and $CO_{2(aq)}$) was calculated with CO_2Sys.xls software, following Ávila-López et al. (2016). Uptake rates of DIC were expressed as μmol g^{-1} DW h^{-1}, while those for HCO_3^- and $CO_{2(aq)}$ were calculated as the percentage of total inorganic carbon (TIC) uptake. Shoots were also kept in the dark to assess plant respiration under both nutrient levels.

At the end of the incubations, whole shoots were removed from the chambers, and the leaves were immediately separated from belowground tissues. Plant tissues were rapidly rinsed with deionized water to remove nutrients from the tissue surface and were oven-dried at 60 °C to constant weight. Tissue samples were ground to a fine powder with mortar and pestle for isotopic analyses. Isotopic and nutrient content (%C, %N) 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 (EA-IRMS). Specific and absolute NO_3^- uptake rates were calculated according to Sandoval-Gil et al. (2015, 2016) and expressed as μmol N g^{-1} DW h^{-1} and mmol N m^{-2} day^{-1}, respectively. Total carbon and nitrogen content of shoot leaves from each sampling site were also analyzed and served as controls.

Nutrient Analyses in Seawater

Nitrate was measured from seawater samples ($n = 935$) collected in SQB during high tides (Fig. 1) and over consecutive days corresponding to periods characterized by the influence (or not) of upwelling events. Nutrients were determined photometrically (Skalar SanPlus Analyzer) from filtered (GF/F Whatman glass fiber filters) seawater samples as described by Camacho-Ibar et al. (2003). Dissolved inorganic carbon normalized for salinity (NDIC) was determined in the same water samples following the protocol described by Ávila-López et al. (2016).

Eelgrass Growth, Shoot Size, and Biomass

Leaf growth (g DW $shoot^{-1}$ day^{-1}) was determined for ten shoots ($n = 10$) using the punching technique described by Zieman (1974). The length and width of all leaves per shoot were also measured to estimate shoot size (cm^2 $shoot^{-1}$). Aboveground biomass (g DW m^{-2}) was collected using 36.5-cm-diameter cores ($n = 4$). Tissue samples were rinsed with deionized water and dried at 60 °C until constant weight.

Statistical Analyses

Statistical differences in the uptake rates of DIC and its species (HCO_3^- and $CO_{2(aq)}$) and N uptake rates were examined by two-way ANOVA after testing for normality and homoscedasticity of the data. This allowed testing of the effects of two fixed factors, "nitrate concentration" and "site," with two and three levels, respectively ("control" vs "high nitrate," and "BM vs BSQ vs BF"). Two-way analysis of variance (ANOVA) was also used to test the effect of two fixed factors on leaf growth, shoot size, leaf biomass, and leaf nutrient content (%C and %N); the two factors were "nitrate concentration" (with the two mentioned levels) and "season" (winter vs spring). *Post hoc* mean comparisons for the ANOVA (Student–Newman–Keuls test) were performed to identify specific treatment level(s) causing significant effects. Statistical analyses were performed using STATISTICA software (StatSoft Inc.) with a minimum significance level established at $p < 0.05$.

Results

In sites near BM, nitrate seawater concentration was 8-fold higher during the upwelling season than during the non-upwelling season (Fig. 1). During upwelling events, the NO_3^- concentration was higher at BM and decreased towards the head of the bay (BF and BSQ). Nitrate values at the BM averaged 8.9 μM (with maxima reaching ~20 μM), while those found at BF and BSQ averaged 1.9 ± 0.1 (SE) μM (with maxima attaining ~6 μM). During the non-upwelling season, NO_3^- concentrations were comparable among sites, with an average value for the three sites of 1.8 ± 0.1 μM, although maxima (~7 μM) were still obtained at BM. Values of NDIC were slightly higher at the mouth during upwelling events (average ~2050 μmol kg^{-1} and maximum of 2116 μmol kg^{-1}) compared to those measured during non-upwelling months (mean value and maximum of ~2035 and 2086 μmol kg^{-1}, respectively). Additionally, NDIC dropped to minimum values (~1400 μmol kg^{-1}) towards the head of the BSQ arm during upwelling events, but not during non-upwelling months. In contrast, there was no evidence of a decreasing gradient in NDIC from the mouth towards the head of the bay at BF.

In control shoots, mean (±SE) DIC uptake rates were higher in spring than in winter (143.1 ± 4.3 and 110.9 ± 10.4 μmol C g^{-1} DW h^{-1}, respectively). Uptake rates of DIC during the non-upwelling season (winter) were higher (by 50–90%) in shoots exposed to the high NO_3^- concentration compared to those exposed to the low NO_3^- nitrate level (Fig. 2a). The interaction of experimental factors was also significant ($p = 0.006$, $F = 7.945$), and the highest (90%) increase in DIC uptake was found in shoots from BF. During the upwelling season, shoots exposed to the high NO_3^- (90%) treatment did not show an increase in DIC acquisition rates (Fig. 2b). Respiration rates did not vary significantly among sites and nitrogen treatments within each season or between seasons. Shoot respiration rates ranged from 14.3 to 18.7 μmol C g^{-1} DW h^{-1}.

Specific and absolute ^{15}N uptake rates increased in shoots from BF and BSQ exposed to 20 μM NO_3^- compared to the control ($p < 0.01$, Fig. 3). Nitrate uptake rates also increased by ~42% in BM shoots exposed to high ambient NO_3^-, but the difference with the control was not significant ($p > 0.2$). Within each season, the lowest values of nitrate uptake were generally observed in shoots from BM, except for the absolute uptake rates in spring (Fig. 3d). Between seasons, nitrate uptake rates in the control and high NO_3^- concentrations decreased in spring, but the highest reductions (by 58–84%) were found when shoots were exposed to an ambient nitrate concentration of 20 μM.

Zostera marina leaf growth, shoot size, and biomass were significantly higher in spring than in winter ($p < 0.05$), and, within each season, higher values were generally determined

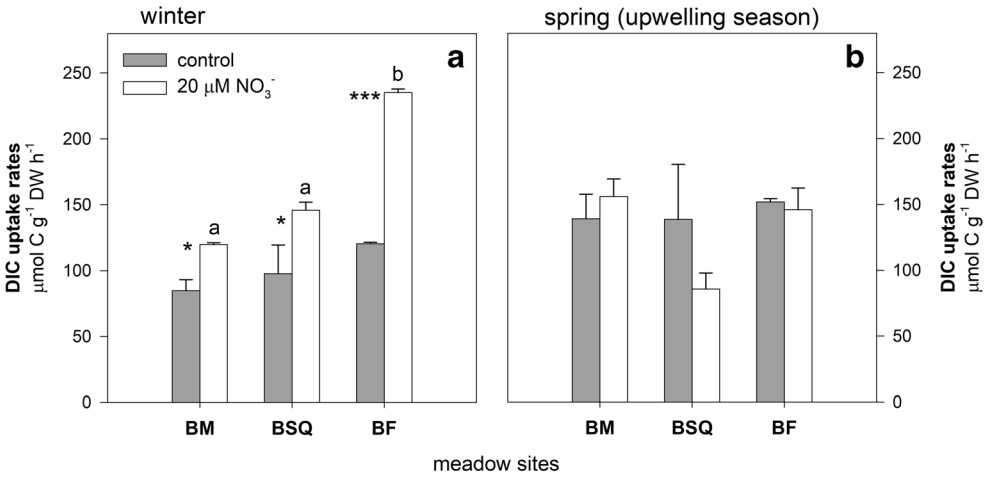

Fig. 2 Uptake rates of dissolved inorganic carbon (DIC) measured in eelgrass shoots from the various meadow study sites (see map in Fig. 1) under two different nitrate concentrations (i.e., control, 20 μM) in winter (**a**) and spring (upwelling season (**b**)) [site abbreviations (BM, BSQ, and BF) as in Fig. 1]. Within each season, statistical differences (2-way ANOVA and post hoc SNK analysis) among shoots from each meadow are indicated by different letters; within each season and site, asterisks represent statistical differences between the nitrogen (N) uptake rates measured in both N treatments (* $p < 0.05$; *** $p < 0.001$)

in shoots from BM ($p < 0.01$, Table 2). By contrast, leaf nutrient content (%C and %N) generally decreased in spring ($p < 0.01$), but no significant differences were found among shoots from different sites (Table 2).

Uptake rates of HCO_3^- were significantly higher than those for $CO_{2(aq)}$ ($p < 0.001$). Although HCO_3^- acquisition represented about ~ 96.5% of the TIC uptake by *Z. marina* leaves, the percentage of $CO_{2(aq)}$ acquired was only about 4.2%

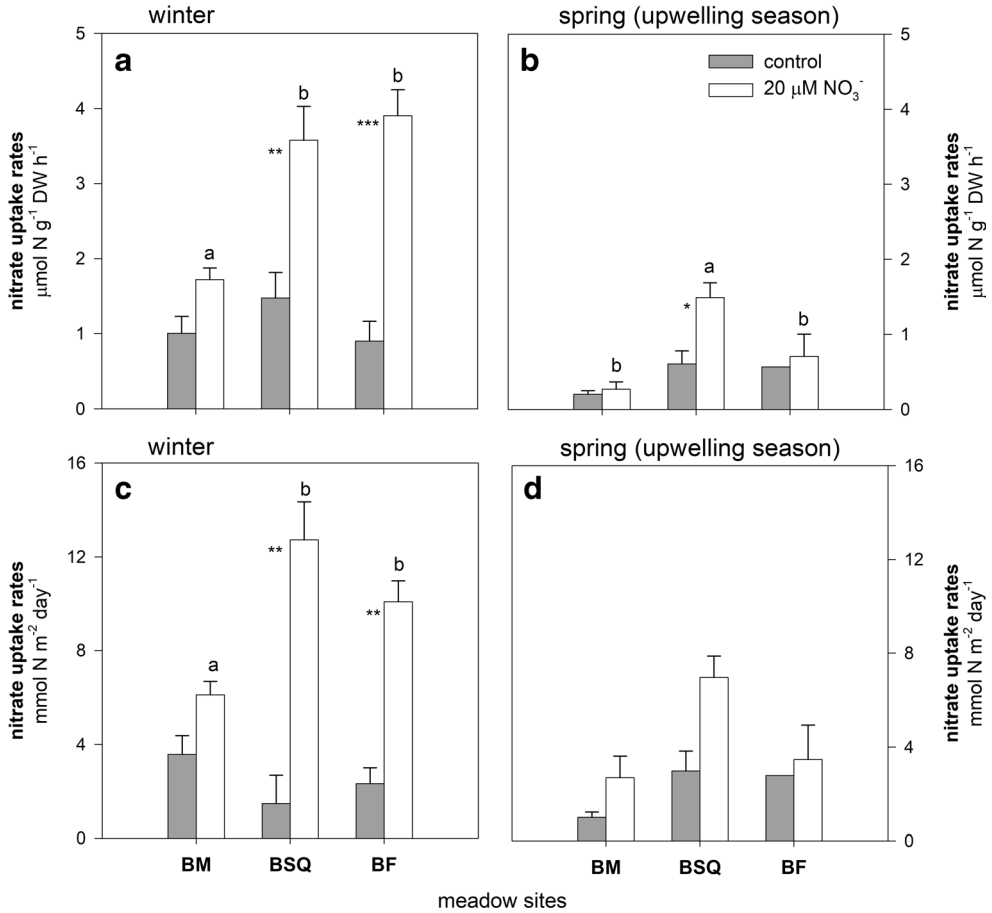

Fig. 3 Specific (**a** and **b**) and absolute (**c** and **d**) nitrate (NO_3^-) uptake rates measured in eelgrass shoots from the three *Z. marina* meadow sites (see map in Fig. 1) under two different NO_3^- concentrations (i.e., control, 20 μM), in winter (left panels) and spring (right panels). Within each season and NO_3^- treatment, statistical differences (2-way ANOVA and post hoc analysis SNK) among shoots from each meadow are indicated by different letters; within each season and site, asterisks represent statistical differences between the nitrogen uptake rates measured in both treatments (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

Table 2 Leaf growth, aboveground biomass, shoot size, and leaf carbon and nitrogen (C and N) percent content measured in eelgrass shoots from each meadow site (BM, BSQ, and BF abbreviated as in Fig. 1) and season (winter and spring); statistical differences (2-way ANOVA and post hoc analysis SNK) among shoots from the three meadows during each season are indicated by different letters. Data are shown as mean ± SE

	BM	BSQ	BF
Winter			
Leaf growth (mg DW shoot^{-1} day^{-1})	0.014 ± 0.001 b	0.0087 ± 0.0008 c	0.0044 ± 0.0005 a
Leaf biomass (g DW m^{-2})	148.4 ± 32.5	148.2 ± 13.4	107.6 ± 8.6
Shoot size (cm^2 shoot^{-1})	235.7 ± 13.5 b	123.1 ± 8.3 c	46.1 ± 5.9 a
Leaf N content (%)	2.51 ± 0.03	2.41 ± 0.09	2.71 ± 0.04
Leaf C content (%)	40.1 ± 0.9	39.9 ± 0.1	39.9 ± 0.2
Spring (upwelling season)			
Leaf growth (g DW shoot^{-1} day^{-1})	0.025 ± 0.001 b	0.02 ± 0.002 a	0.018 ± 0.001 a
Leaf biomass (g DW m^{-2})	412 ± 9.1 b	194.4 ± 27.3 b	205 ± 5.1 a
Shoot size (cm^2 shoot^{-1})	347.7 ± 18.5 b	296.6 ± 14.6 a	271.4 ± 20.9 a
Leaf N content (%)	2.07 ± 0.11	1.79 ± 0.09	1.92 ± 0.09
Leaf C content (%)	36.6 ± 1.3	38.8 ± 0.3	38.05 ± 0.9

(Table 3). The highest NO_3^- concentration did not generally change the relative percentage of acquisition of DIC species ($CO_{2(aq)}$ and HCO_3^-). In both control and fertilized shoots, HCO_3^- uptake ranged from 90 to 235 and 123 to 206 μmol C g^{-1} DW h^{-1} in winter and spring, respectively; CO_2(aq) uptake varied from 4.2 to 11.8 μmol C g^{-1} DW h^{-1} in winter, while in spring, these values ranged from 3.4 to 6.2 μmol C g^{-1} DW h^{-1}.

Discussion

Eelgrass DIC/NO_3^- Acquisition Under Short-Term Nitrate Exposure

Zostera marina collected in winter increased its photosynthetic (μmol C g^{-1} DW h^{-1}) and nitrate uptake capacities (μmol N g^{-1} DW h^{-1} and mmol N m^{-2} day^{-1}) in response to an experimental pulse (1 h) of nitrate with concentration comparable to that of upwelled waters (20 μM). Longstaff et al. (2002) also found that in situ short-term (hours) fertilization can stimulate photosynthesis (e.g., P_{max}, ETR, α) in the seaweed *Ulva lactuca*. The direct and/or indirect effects of exposure to N enrichment for longer periods (days to months) on carbon metabolism of seagrasses have been previously reported (see subsequent discussion). As far as we know, however, this is the first experimental evidence of such high plasticity and rapid acclimation of seagrasses photosynthesis (DIC uptake) under a transient increase of ambient NO_3^- concentration.

Table 3 Calculated eelgrass uptake rates of HCO_3^- and $CO_{2(aq)}$ (% of total DIC uptake) in the two treatments: nitrate concentrations (i.e., control, 20 μM) in winter (February) and spring (June, upwelling season). Data are shown as mean ± SE. Site abbreviations as in Fig. 1

	BM	BSQ	BF
Winter			
% HCO_3^- (control)	94.9 ± 0.3	95.9 ± 0.6	96.1 ± 0.3
% HCO_3^- (20 μM)	95.5 ± 0.2	95.2 ± 0.2	96.5 ± 0.4
% $CO_{2(aq)}$ (control)	5.1 ± 0.3	4.03 ± 0.6	3.93 ± 0.2
% $CO_{2(aq)}$ (20 μM)	4.5 ± 0.2	4.8 ± 0.2	3.5 ± 0.4
Spring (upwelling season)			
% HCO_3^- (control)	97.4 ± 0.4	95.9 ± 1.1	97.4 ± 0.05
% HCO_3^- (20 μM)	95.6 ± 0.4	96.6 ± 0.2	97.5 ± 0.1
% $CO_{2(aq)}$ (control)	2.5 ± 0.4	4.03 ± 1.1	2.5 ± 0.05
% $CO_{2(aq)}$ (20 μM)	4.4 ± 0.4	3.3 ± 0.2	2.5 ± 0.1

A reduction of tissue carbohydrates and an increase in photochemical capacities (e.g., electron transport rate, ETR) have been observed in the Mediterranean seagrass *Posidonia oceanica* when exposed to nitrogen fertilization (Invers et al. 2004; Marín-Guirao et al. 2017). These responses have also been observed in *Z. marina* by means of field and laboratory experiments (Burkholder et al. 1992: van Katwijk et al. 1997; Villazán et al. 2013). Furthermore, week-long exposure to high ambient NH_4^+ concentration (10–25 μM) can also cause a drop in non-structural carbohydrates in leaves and rhizomes, as well as an increase in tissue N compounds and photosynthetic rates in this species (Villazán et al. 2013). These responses can be explained by the close relationship between C and N metabolism (Burkholder et al. 2007). Indeed, studies indicate that N enrichment can increase the demand for energy and carbon skeletons from photosynthates to support the assimilation of DIN (Invers et al. 2004; Villazán et al. 2013). The increased energy requirements associated with elevated assimilation of DIN, or even its direct toxicity, can adversely affect seagrass productivity (e.g., growth, density), morphology and survival, and can also increase deleterious effects of other potential stressful conditions, such as hyposalinity and low light (Burkholder et al. 1992: van Katwijk et al. 1997; Villazán et al. 2013, 2015). Positive and neutral effects of N

enrichment on seagrass' growth and physiology have also been reported, mostly induced by the presence of environmental limiting factors, including N availability (Udy and Dennison 1997; Touchette and Burkholder 2000; Villazán et al. 2013).

In this study, photosynthesis was mainly stimulated by high nitrate concentrations in shoots from BF, relative to those growing in BM and BSQ, suggesting that shoots from BF exhibited a higher capacity to assimilate external inorganic nitrogen. This is supported by results from in situ incubations which demonstrated that shoots growing adjacent to oyster cultures (BF) exhibit a higher physiological capacity to exploit NH_4^+ from oyster excretions and, also, higher photosynthetic capacities, compared to shoots growing away from oyster aquaculture (Sandoval-Gil et al. 2016).

Generally, *Z. marina* spring shoots showed lower capacities to acquire NO_3^- (i.e., lower specific and absolute nitrate uptake rates; Fig. 3) than winter shoots in control treatments. Additionally, and in contrast to results with winter shoots, exposure to high NO_3^- concentration (20 μM) did not stimulate NO_3^- uptake in spring. This indicates that NO_3^- uptake kinetics of shoots collected in spring saturated at lower ambient NO_3^- concentration; thus, incorporation rates remained similar to the control even at higher ambient NO_3^- concentration. This finding agrees with results of a prior study which demonstrated that *Z. marina* exhibited a reduced capacity for NO_3^- acquisition (i.e., lower maximum uptake rates, V_{max}, and half-saturation constant, K_m) during the upwelling season (Sandoval-Gil et al. 2015). The availability of external DIN can drive changes in the uptake kinetics of seagrasses, at least partially determining the DIN acquisition strategies and whole-plant N budget in seagrasses (Hemminga et al. 1999; Lee and Dunton 1999; Marbá et al. 2002). Therefore, since oceanic NO_3^- availability increases during spring upwelling events (Fig. 1), the reduced capacity to acquire NO_3^- in this season can be interpreted as an effective shutdown mechanism by which these plants can avoid the potential harmful effects of unregulated incorporation of NO_3^- (e.g., direct toxicity, metabolic costs associated with its assimilation; Burkholder et al. 1992; Sandoval-Gil et al. 2015, 2016). Similarly, roots exhibited reduced capacities to incorporate NH_4^+ (lower V_{max}, K_m, and α) compared to leaves, due to the very high availability of this nutrient in sediments (Sandoval-Gil et al. 2015). It is noteworthy that in the present study shoots from BM showed an even lower capacity to incorporate NO_3^- in both seasons but higher growth rates and N content than shoots from BSQ and BF. This can be attributed to the relatively small contribution of NO_3^- uptake by leaves to the total N budget (5–25%) and N demand for growth, when compared with the contribution of NH_4^+ (75–95%), which is highly available in the water column and sediment pore-water in SQB (up to ~30 and ~700 μM, respectively) (Sandoval-Gil et al. 2015).

Overall, these results indicate that *Z. marina* is able to regulate/restrict the acquisition of NO_3^- in this coastal lagoon; this finding strongly contrasts with the paradigm of full exploitation of this nutrient attributed to seaweeds within the bay and other ocean upwelling-influenced estuaries along the NE Pacific (Zertuche-González et al. 2009; Hessing-Lewis and Hacker 2013). Nevertheless, this interpretation must be treated with caution and more experimental studies are required to explore the influence of other factors on the NO_3^- incorporation strategies of *Z. marina*, such as the availability of internal N resources or the influence of seasonally-dependent acclimation patterns (Stapel et al. 1996; Terrados and Williams 1997; Lee and Dunton 1999; Lepoint et al. 2002; Cornelisen and Thomas 2004; Apostolaki et al. 2012).

Contrary to winter observations, photosynthesis (DIC uptake) did not increase when BF and BM shoots were exposed to high ambient NO_3^- in spring (Fig. 2); this was consistent with the absence of the NO_3^- uptake enhancement observed in these shoots (see subsequent discussion; Fig. 3). However, shoots collected from BSQ exhibited higher capacities to incorporate NO_3^- when exposed to high concentrations of this nutrient, without an increase in photosynthesis. This response was unexpected and may be explained by differences in physiological status of winter and spring plants. Specifically, Cabello-Pasini et al. (2003) found that *Z. marina* in SQB can occasionally be light-limited in winter due to high water turbidity. Light limitation, likely combined with other factors such as lower temperatures, results in a reduction in plant vegetative productivity and C internal reserves, reflected as a decrease in leaf and rhizome non-structural carbohydrates (Cabello-Pasini et al. 2004). In the present study, winter plants may have been light-limited in the field, and, thus, soluble sugar concentration may have been lower in their tissues. Under this physiological condition, winter plants exposed to high ambient NO_3^- should have increased their photosynthetic activity to provide photosynthates and, thus, C-skeletons, essential for assimilating DIN into organic N compounds (free amino acids). Alternatively, field light conditions and plant-endogenous reserves may have been optimized in spring plants, thus allowing N assimilation without the activation of photosynthesis.

Even though temperature differed between the two study seasons, respiration rates were similar, indicating no detectable temperature effect on this metabolic process. Respiration responses to seasonal and experimental warming are inconsistent (Koch et al. 2013; Ruiz and Romero 2001) and depend on several factors, such as the experimental temperature condition applied, the species or population (ecotype/genotype), and the synergistic effects associated to other biotic and abiotic factors.

Similar to nitrate uptake, *Z. marina* leaf N content was reduced in spring, which could be indicative of N limitation. However, recent estimations of N demand for shoot growth vs

N uptake rates under ambient conditions of DIN availability (N_{demand} and V_{amb}, respectively, in Sandoval-Gil et al. 2015) indicated that shoot N limitation is unlikely to occur in SQB. In addition, the %N measured in leaf tissues (Table 2) was generally above or close to the threshold value of 1.82% suggested by Duarte (1990) as indicative of N-limiting conditions. Consequently, N reduction could be related to other seasonally dependent factors, such as the leaf N pool dilution by the increase in structural components during maximum seasonal growth (Pedersen and Borum 1992, 1993; Kraemer and Mazzella 1999; Lepoint et al. 2002).

Uptake of DIC Species

The uptake of HCO_3^- and $CO_{2(aq)}$, expressed as the percentage of total DIC acquired, showed that HCO_3^- is the major source of DIC ~ 95% for *Z. marina*, while $CO_{2(aq)}$ contributed only marginally. This DIC utilization behavior is typical of marine macrophytes, which are able to develop C concentration mechanisms based on intra- or extracellular conversion of HCO_3^- (the main species of DIC present in seawater at pH ~ 8) to $CO_{2(aq)}$, via the activity of carbonic anhydrase (Invers et al. 1997; Beer et al. 2002). On the other hand, we did not find changes in the percent utilization of DIC species due to the experimental NO_3^- pulse. Anion exchange mechanisms to acquire DIC species that depend on N metabolism by-products have been previously described in macroalgae (e.g., *Ulva* sp., Beer 1994; Drechsler et al. 1994), although their operation in seagrasses has not been demonstrated. Upwelled waters advected into SQB are characterized by relatively high $CO_{2(aq)}$ (Fig. 1; $pCO_2 > 700$ μatm in Ribas-Ribas et al. 2011). During upwelling events, it is, thus, expected that differences in the incorporation of HCO_3^- and $CO_{2(aq)}$ must be driven by the proportional availability of these DIC species rather than their interaction with NO_3^-. In other coastal systems, changes in the isotopic signal of leaf carbon ($\delta^{13}C$) indicated this differential acquisition of HCO_3^- and $CO_{2(aq)}$ by *Z. marina* when exposed to different C sources, including upwelled waters (Fourqurean et al. 1997; Papadimitriou et al. 2005; Ruesink et al. 2015).

Removal of Upwelled Nitrate by Eelgrass Meadows

Despite their reduced capacities to incorporate NO_3^- in spring (upwelling season), *Z. marina* meadows growing near the bay mouth acquired this nutrient at rates up to 4.2 mmol N m^{-2} day^{-1} (Fig. 3d). Since *Z. marina* populations cover ~ 910 ha of the bottom near the bay mouth and the

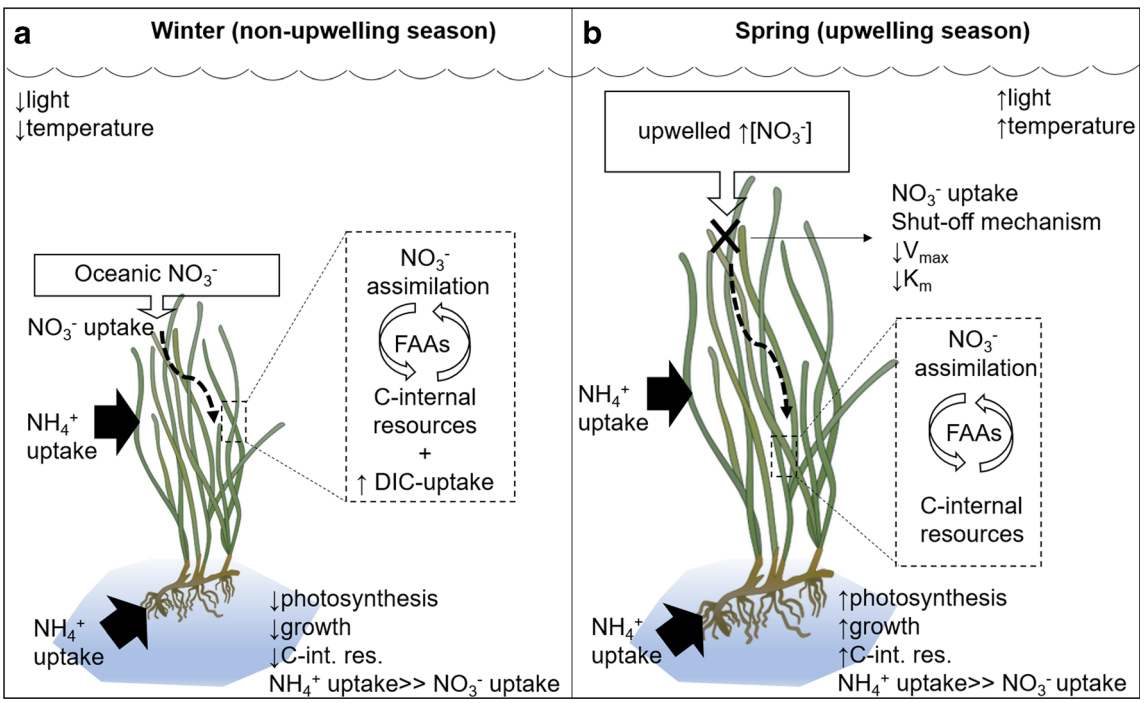

Fig. 4 Conceptual representation of *Z. marina* responses in winter (**a**) and spring (upwelling season; **b**) in SQB. The figures contain the eelgrass' responses found in this study, as well as other related responses obtained in previous studies (p.e. Sandoval-Gil et al. 2015; Cabello-Pasini et al. 2003, 2004; see the "Discussion" section). In summary, this study provides experimental evidence that high external nitrate concentration simulating upwelling stimulated nitrate uptake and photosynthesis (DIC uptake) in winter plants; such rapid photosynthetic plasticity could allow these plants to assimilate inorganic N under limited C reserves. By contrary, spring plants generally did not increase nitrate uptake and, thus, photosynthesis when exposed to external high nitrate. This reduced capacities to acquire nitrate, based on reduced V_{max} and K_m, which was interpreted as a shut-off mechanism to restrict excessive incorporation of nitrate and its potential harmful effects on plant metabolism. Eelgrass image was obtained from http://ian.umces.edu/

outermost parts of both arms (Ward et al. 2003), these meadows can potentially remove ~ 38.2×10^3 mol N day^{-1}. This represents a non-trivial removal rate of NO_3^- from the water column when compared to an estimated oceanic nitrate supply of ~ 142×10^3 mol N day^{-1} during intense upwelling events. The other dominant submerged macrophyte within the bay, *Ulva* sp., can exhibit a higher potential for NO_3^- acquisition (~ 137×10^3 mol N day^{-1}), based on its biomass and cover in spring (431 ha, 3.3 tons DW ha^{-1}; Zertuche-González et al. 2009), and assuming incorporation rates as high as ~ 31.7 mmol N m^{-2} day^{-1} (unpub. data; pers. obs.). Therefore, among other biotic components (e.g., macroalgae) and biogeochemical processes, such as denitrification (Camacho-Ibar et al. 2003), NO_3^- incorporation by *Z. marina* meadows can partly contribute to the marked reduction of this nutrient within the initial portion of the bay (20 km^2 area closest to the bay mouth; Fig. 1). The biofiltration potential of eelgrass meadows to buffer the NH_4^+ loading from oysters farms is also noteworthy (Sandoval-Gil et al. 2016), highlighting that *Z. marina* beds are an essential biological component controlling the N budget of SQB.

Conclusions

This is among the first research studies to empirically demonstrate the capacity of seagrass to adapt to high baseline N levels, actively regulate their NO_3^- uptake capacity, and make an important contribution to the N budget of upwelling-influenced estuaries. This study provides experimental evidence that *Z. marina* photosynthesis (DIC uptake) can be stimulated by short-term (1 h) pulses of nitrate (20 μM) simulating upwelling. This response was directly related to the higher acquisition of NO_3^- by eelgrass leaves (see conceptual representation in Fig. 4). In winter, when plant internal C resources may be exhausted by environmental limiting factors (e.g., light, temperature), the utilization of C skeletons from photosynthates can be critical to assimilate NO_3^- into organic compounds and sustain plant growth. In spring (upwelling season), eelgrass responses contrast greatly with those found in winter, since NO_3^- uptake (and, thus, photosynthetic capacity) did not increase under high ambient NO_3^-; this indicated that NO_3^- uptake kinetics saturates at lower ambient NO_3^- concentration (Sandoval-Gil et al. 2015). Results of the present study suggest that these responses represent a shutdown acclimation mechanism that allows *Z. marina* to restrict the incorporation of NO_3^- during intense upwelling events, thus avoiding metabolic costs and direct toxicity of its excessive accumulation. The fact that the lowest capacity to incorporate NO_3^- corresponded to shoots growing near the bay mouth reinforced this suggestion.

Overall, these findings highlight the marked physiological plasticity of *Z. marina* in response to upwelled NO_3^- and, as indicated earlier, strongly contrast to the full exploitation of this nutrient by seaweeds also dominant in SQB and other systems (Hurd et al. 2014). Despite the conservative strategy exhibited by *Z. marina* to acquire NO_3^-, it is estimated that meadows growing within ~ 20 km^2 of the bay mouth are able to remove ~ 30% of the available oceanic NO_3^- during intense upwelling events, which partially explains the marked decrease in the concentration of this nutrient within the outermost sector of the bay. This capacity, together with other recent evidences which stated that *Z. marina* meadows can act as effective biofilters of oyster excreta (Sandoval-Gil et al. 2016), demonstrates that these eelgrass beds are an essential biological component controlling the N budget of SQB. These results contribute to enhanced understanding of the role of seagrasses in the N cycle of coastal lagoons influenced by upwelling. They also serve to emphasize the relevance of seagrass communities for the implementation of management strategies to cope with potential anthropogenic sources of eutrophication (e.g., wastewater, agriculture, and groundwater) in other coastal ecosystems (McGlathery et al. 2007).

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