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Short-Term Response of Cytosolic NO_3^- to Inorganic Carbon Increase in *Posidonia oceanica* Leaf Cells

Lourdes Rubio[1]*, *Delia García-Pérez*[1], *Julia M. Davies*[2] and *José A. Fernández*[1]

[1] Departamento de Botánica y Fisiología Vegetal, Facultad de Ciencias, Universidad de Málaga, Málaga, Spain, [2] Department of Plant Sciences, University of Cambridge, Cambridge, United Kingdom

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***Correspondence:**
Lourdes Rubio
Lrubio@uma.es

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The concentration of CO_2 in the atmosphere has increased over the past 200 years and is expected to continue rising in the next 50 years at a rate of 3 ppm·year^{-1}. This increase has led to a decrease in seawater pH that has changed inorganic carbon chemical speciation, increasing the dissolved HCO_3^-. *Posidonia oceanica* is a marine angiosperm that uses HCO_3^- as an inorganic carbon source for photosynthesis. An important side effect of the direct uptake of HCO_3^- is the diminution of cytosolic Cl^- (Cl^-c) in mesophyll leaf cells due to the efflux through anion channels and, probably, to intracellular compartmentalization. Since anion channels are also permeable to NO_3^- we hypothesize that high HCO_3^-, or even CO_2, would also promote a decrease of cytosolic NO_3^- (NO_3^-c). In this work we have used NO_3^-- and Cl^--selective microelectrodes for the continuous monitoring of the cytosolic concentration of both anions in *P. oceanica* leaf cells. Under light conditions, mesophyll leaf cells showed a NO_3^-c of 5.7 ± 0.2 mM, which rose up to 7.2 ± 0.6 mM after 30 min in the dark. The enrichment of natural seawater (NSW) with 3 mM $NaHCO_3$ caused both a NO_3^-c decrease of 1 ± 0.04 mM and a Cl_c^- decrease of 3.5 ± 0.1 mM. The saturation of NSW with 1000 ppm CO_2 also produced a diminution of the NO_3^-c, but lower (0.4 ± 0.07 mM). These results indicate that the rise of dissolved inorganic carbon (HCO_3^- or CO_2) in NSW would have an effect on the cytosolic anion homeostasis mechanisms in *P. oceanica* leaf cells. In the presence of 0.1 mM ethoxyzolamide, the plasma membrane-permeable carbonic anhydrase inhibitor, the CO_2-induced cytosolic NO_3^- diminution was much lower (0.1 ± 0.08 mM), pointing to HCO_3^- as the inorganic carbon species that causes the cytosolic NO_3^- leak. The incubation of *P. oceanica* leaf pieces in 3 mM HCO_3^--enriched NSW triggered a short-term external NO_3^- net concentration increase consistent with the NO_3^-c leak. As a consequence, the cytosolic NO_3^- diminution induced in high inorganic carbon could result in both the decrease of metabolic N flux and the concomitant biomass N impoverishment in *P. oceanica* and, probably, in other aquatic plants.

Keywords: cytosolic NO_3^-, elevated inorganic carbon, NO_3^- efflux, anion channels, intracellular nitrate-selective microelectrodes, seagrasses

INTRODUCTION

Nitrate ion (NO_3^-) is the main source of inorganic nitrogen for plants in aerobic conditions. Compared with the concentrations in soils, the concentration of NO_3^- in seawater is persistently very low, particularly in the oligotrophic Mediterranean Sea (Lepoint et al., 2002; Bethoux et al., 2005). Seagrasses are the unique angiosperms that evolved from land plants to live submerged in the sea, forming the basis of the most productive and widespread coastal ecosystems on the planet (Larkum et al., 2006). For vascular plants, colonizing the sea implicates losses and gains to effect structural and physiological adaptations to complete the submerged life cycle, achieved by a reverse evolutionary trajectory in the seagrass lineage (Williams, 2016). Thus, key land angiosperm innovations were lost in seagrasses including the entire collection of genes involved in stomata differentiation, genes related to the synthesis and sensing of terpenoids and other volatile substances, genes for ultraviolet protection, and phytochromes for far-red sensing (Olsen et al., 2016). However, to survive in the conditions of low NO_3^- availability, seagrasses have evolved high affinity uptake systems to capture NO_3^- through their leaves. These systems have been characterized for *Zostera marina* in which the uptake of NO_3^- and inorganic phosphate (Pi) are driven by the inwardly directed electrochemical gradient for Na^+ (García-Sánchez et al., 2000; Rubio et al., 2005). In the Mediterranean, *Posidonia oceanica* is an endemic coastal species of huge ecological importance (Aires et al., 2011). Similar high-affinity and Na^+-dependent uptake mechanisms also operate in *P. oceanica* for both nutrients and some amino acids (Rubio et al., 2018). In both cases, the low semi-saturation constants observed (2.3 and 8.7 μM NO_3^- for *Z. marina* and *P. oceanica*, respectively: García-Sánchez et al., 2000; Rubio et al., 2018) indicate that those systems are very efficient for NO_3^- uptake at the very low concentrations of NO_3^- in seagrass meadows (Touchette and Burkholder, 2000; Romero et al., 2006). Nevertheless, those systems are energetically expensive because seagrass leaf cells have to keep low homeostatic Na^+ concentrations in the cytosol to maintain the Na^+ motive force (Rubio et al., 2011; Rubio et al., 2018). In this scenario, namely low availability of NO_3^- and the energetically expensive mechanism for its high-affinity uptake, the maintenance of NO_3^- inside the cells appears critical. Therefore, as with other vascular plants, seagrasses must maintain intracellular NO_3^- homeostasis to preserve the N metabolic flux.

In a previous work, besides cytosolic H^+ and Na^+, we also measured cytosolic Cl^- (using intracellular ion-selective microelectrodes) in the mesophyll cells of *P. oceanica* (Rubio et al., 2017). In that work, we demonstrated that this seagrass has a direct plasma membrane symporter to uptake HCO_3^- driven by the H^+ electrochemical potential gradient. A significant increase of photosynthesis in natural seawater supplemented by 3 mM HCO_3^- supported the role of HCO_3^- uptake in the photosynthetic activity of this species (Rubio et al., 2017). Furthermore, the enrichment of seawater with 3 mM HCO_3^- also evoked a delayed, but significant, diminution of the cytosolic Cl^- concentration (Rubio et al., 2017). Similar cytosolic Cl^- efflux has been observed in guard cells of the model land angiosperm *Arabidopsis thaliana* during stomatal closure in response to elevated CO_2 (Xue et al., 2011). This Cl^- efflux from guard cells is described as taking place through the plasma membrane S-type anion channels, whose activation responded to the cytosolic HCO_3^- concentration (Xue et al., 2011). Interestingly, in addition to Cl^- these anion channels have a high permeability to NO_3^- (Schmidt and Schroeder, 1994), which is also released from the guard cells during stomatal closure (Geiger et al., 2011; Demir et al., 2013; Maierhofer et al., 2014).

The release of Cl^- from *P. oceanica* in response to HCO_3^- is driven by the outwardly directed electrochemical potential gradient for Cl^- (Rubio et al., 2017). Considering Cl^--selective microelectrodes are also partially sensitive to NO_3^- (Miller and Zhen, 1991) we hypothesize that the increase of inorganic carbon in seawater could also lead to the decrease of cytosolic NO_3^- in *P. oceanica* leaf cells. Such diminution would affect assimilation (Bloom et al., 2010) and could partially be responsible for the plant biomass nitrogen impoverishment expected under elevated inorganic carbon in vascular plants with non-saturated photosynthesis (Taub and Wang, 2008).

Therefore, the aim of this work was to measure the cytosolic NO_3^- concentration in mesophyll leaf cells of *P. oceanica* to describe the responses to light and dark conditions and to the increase of dissolved inorganic carbon (CO_2 and HCO_3^-) in natural seawater. Ethoxyzolamide, the plasma membrane-permeable carbonic anhydrase inhibitor (Sültemeyer et al., 1993), has been used in combination with CO_2 to test for the effect of limiting any CO_2-dependent HCO_3^- generation. Since the first work in *Chara corallina* (Miller and Zhen, 1991), NO_3^--selective microelectrodes have been used in different plant species, such as barley (Zhen et al., 1991; Van Der Leij et al., 1998), *A. thaliana* (Cookson et al., 2005) and rice (Fan et al., 2007), plus the liverwort *Conocephalum conicum* (Trębacz et al., 1994). However, so far no direct measurement of cytosolic NO_3^- has been reported for marine vascular plants. Furthermore, external NO_3^- has been monitored, and the potential effects of elevated atmospheric CO_2 on the hypothesized diminution on the biomass nitrogen content are also discussed.

MATERIALS AND METHODS

Plant Material

Posidonia oceanica (L.) Delile plants were sampled in Punta de Calaburras (36°30′23.4′′N 4°38′37.6′′W) Málaga, southern Spain, at 2 m depth. Plants with 6 to 12 leaves attached to a piece of the rhizome were collected and transported to the laboratory in a thermos container in less than 30 min. Then, plants were placed in an aquarium filled with continuously aerated natural seawater (NSW). The air used for this purpose was obtained from the compressed air supply of the Faculty of Sciences building. Concentration of CO_2 in the air was regularly monitored (390 ± 10 ppm) using an IRGA, LICOR LI-820, Li, Nebraska (USA). Temperature was held at 15°C, and illumination was at a light intensity of 150 μmol photons·m^{-2}·s^{-1}

with a photoperiod 16L/8D. Renewing the seawater every three days, plants were used for experiments within two weeks after sampling.

Cytosolic NO_3^- and Cl^- Measurements

Cytosolic nitrate and chloride were measured by electrophysiological techniques using double-barreled intracellular microelectrodes. Glass capillary preparation details have been previously described (Fernández et al., 1999). In short, double-barreled capillaries with different diameter (1.5 and 0.75 mm o.d., respectively, Hilgenberg, Germany) were twisted before pulling using a Narishige PD-5 horizontal puller. Then, pulled double-barreled capillaries were heated for 30 min at 180°C and silanized by adding one drop of dichlorodimethylsilane dissolved in benzene (0.05% v/v) to the interior of the blunt end of the larger barrel, while the smaller barrel (that operates as voltage electrode) was not silanized. After that, the silanized double-barrelled capillaries were heated again for 60 min at 180°C. Once cool, the silanized barrel was backfilled with the appropriate chloride or nitrate sensor solution.

For Cl^--microelectrodes, the ionophore I (99408, Fluka), dissolved in a mixture of polyvinylchloride dissolved in tetrahydrofuran (PVC/THF, 4% w/v) was used and backfilled into the silanized barrel. Once THF evaporated, the remainder was filled with 0.5 M KCl (Planes et al., 2015). As described previously (Rubio et al., 2017), Cl^--microelectrodes were calibrated against NaCl solutions (1–100 mM) that contained 5 mM $NaNO_3$, the putative cytosolic NO_3^- concentration (Miller and Smith, 2008), to minimize the interference between Cl^- and NO_3^- in the cytosol (Felle, 1994). Calibration showed a linear relationship of 37 mV/pCl.

NO_3^--microelectrodes were backfilled using a NO_3^- sensor (Fluka 72549) based on the quaternary ammonium compound, methyltridodecylammonium nitrate ($MTDDA.NO_3^-$) containing the PVC/THF solution. These liquid ion-exchange based microelectrodes are highly selective for NO_3^-, maintaining a nitrate detection limit of 0.5 mM in the presence of 100 mM Cl^-, which means any interference will not be important in a physiological situation, as described in Miller and Zhen (1991). Once THF was evaporated from the nitrate-sensor cocktail and before use, the NO_3^--selective barrel was backfilled with 0.1 M $NaNO_3$ and 0.1 M KCl. Then, NO_3^--selective electrodes were calibrated against NO_3K solutions (0.1–20 mM) containing 10 mM KCl, to saturate the presumed interference using the Cl^- concentration reported previously in *P. oceanica* mesophyll leaf cells (Rubio et al., 2017), and KH_2PO_4 (from 15 to 50 mM), to give a constant background ionic strength in the calibration solutions (Miller and Zhen, 1991). NO_3^- calibration showed a linear relationship of 53 mV/pNO_3.

For measurements, the microelectrode voltage barrel was backfilled with 0.5 M KCl (Fernández et al., 1999; Rubio et al., 2005). Then, the NO_3^-- or Cl^--selective microelectrode and the reference electrode (containing agar 0.03% (w/v) in 0.5 M KCl) were fixed to Ag/AgCl electrode holders and connected to a high-impedance differential amplifier (FD223a, WPI, Sarasota, Florida, USA). Amplifier signals were continuously monitored on a double pen chart recorder (Linseis L250E).

Measurements were performed on leaf pieces (≈ 1 cm long), longitudinally peeled to remove part of the epidermis and fixed with paraffin wax on a Plexiglas transparent chamber (1.1 ml volume). A gravity-based flow-through system permitted controlled changes of the assay medium at a rate of 10 $ml{\cdot}min^{-1}$, which renewed chamber volume approximately 10 times every minute. This system kept the temperature, the ionic concentration, and gases constant during the experiments. Measurements were made under a microscope light of 150 $\mu mol\ photons{\cdot}m^{-2}{\cdot}s^{-1}$.

NO_3^- Quantification in Assay Solutions

In order to monitor the net efflux and/or uptake of NO_3^- from assay medium, plants were previously adapted to N-sufficiency by incubation in NSW enriched with 100 µM $NaNO_3$ during 2 days. Then, excised leaves (2–3 g fresh weight) were placed separately in 250 ml flasks and incubated in 100 ml NSW (control) or NSW supplemented with 3 mM $NaHCO_3$. The assay was carried out at 25°C with gentle and constant agitation. For each treatment, samples of assay medium were taken at 0, 1, 2, 3, 5, 7, 10, 15, 20, and 30 min. The highly sensitive method for NO_3^- determination, based on vanadate NO_3^-reduction and the subsequent spectrophotometric determination of NO_2^- (García-Robledo et al., 2014) was used to quantify NO_3^- concentration in each sample. Net efflux and uptake rates were estimated as the slope of the linear phase of NO_3^--concentration time course, as a function of fresh weight (FW). Six replicates were conducted for each assay.

Assay Solutions

Natural seawater (NSW) supplemented with 3 mM HCO_3^- was prepared by adding the appropriate volume of a 0.5 M $NaHCO_3$ stock solution (pH 8.2). CO_2-enriched NSW was obtained by bubbling with artificial air (Air Liquide, Spain, 1000 ppm CO_2-air). A stock solution of the plasma membrane-permeable carbonic anhydrase inhibitor ethoxyzolamide (EZ, 10 mM) was prepared in 0.05 M NaOH. The addition of equivalent volumes of NaOH without the inhibitor had no effect on measurements. All chemicals were analytical grade and were purchased from Sigma-Aldrich.

Data Presentation and Statistical Analyses

Time-course measurements are shown as single traces, representative of a number of equivalent experiments carried out under the same conditions, as stated in the figure legends. Data are presented as means, and error bars are standard deviations. Number of repetitions (n) is indicated in every experiment. Data were analyzed using SPSS Statistics, version 21. The significance level was set at $P < 0.05$.

RESULTS

Effect of Light-Dark Transitions on *P. oceanica* Cytosolic NO_3^-

In NSW, mesophyll leaf cells of *P. oceanica* showed a stable plasma membrane potential of −174 ± 8 mV (as in Rubio et al., 2017) and

a cytosolic NO_3^- concentration of 5.7 ± 0.2 mM (n = 10). The transition from light to darkness evoked a fast depolarization of approximately 14 mV followed by a transient hyperpolarization of 22 mV and a subsequent, more prolonged, depolarization to level of at a lower membrane potential of −140 ± 5 mV (n = 12, P = 0.003, Student t test) after 25 min in the dark. With a delay of a few minutes, light-dark transition promoted the gradual increase of cytosolic NO_3^- concentration that stabilized at a higher value (7.2 ± 0.6 mM NO_3^-; n = 10, P = 0.02, Student t test) after 30 min in the dark (**Figure 1**).

Effect of HCO_3^--Enriched NSW on Cytosolic NO_3^-

We have previously reported that in *P. oceanica* mesophyll leaf cells, incubated in light conditions, the addition of NSW enriched with 3 mM HCO_3^- evoked an initial and transient plasma membrane depolarization that turned into a transient hyperpolarization to stabilize at a depolarized value (Rubio et al., 2017). The simultaneous measurement of cytosolic chloride showed a delayed but significant decrease of the cytosolic concentration of this anion concomitant with the extent of membrane depolarization (Rubio et al., 2017). The partial sensitivity of Cl^--selective microelectrodes to NO_3^- (Miller and Zhen, 1991; Felle, 1994) allows us to hypothesize that HCO_3^- enrichment not only produces the cytosolic Cl^- decrease but could also evoke a cytosolic NO_3^- shift. To test this hypothesis, cytosolic NO_3^- was measured in *P. oceanica* mesophyll leaf cells in the same conditions that we had reported for cytosolic chloride, the monitoring of which was used as a control in this work. **Figure 2** shows the membrane potential response to the enrichment of NSW with 3 mM HCO_3^- (black trace) and the simultaneous measurements of cytosolic Cl^- (green trace) or NO_3^- (blue trace), values and stats are presented in **Table 1**. As found previously (Rubio et al., 2017), the addition of 3 mM HCO_3^- caused an initial and transient membrane depolarization (black trace) of approximately 5 mV, which turned into a transient hyperpolarization (reaching a minimum membrane potential of −181 ± 2 mV, n = 5, P = 0.012, Student t test), followed by a prolonged depolarization that stabilized within approximately 40 min at a membrane potential of −168 ± 3 (n = 5). Also in agreement with the previous study, the addition of 3 mM HCO_3^- evoked a decrease of cytosolic Cl^- (after approximately 4 min) which continued progressively from 9.7 ± 0.2 mM to 6.3 ± 0.3 mM (green trace, n = 5; P = 0.004, Student t test) 20 min after HCO_3^- addition. Supporting the hypothesis of HCO_3^- enrichment's producing a cytosolic NO_3^- shift, *P. oceanica* mesophyll leaf cell cytosolic NO_3^- also decreased in response to the enrichment of NSW with 3 mM HCO_3^- (**Figure 2**, blue trace). In common with the response of cytosolic Cl^-, the diminution of cytosolic NO_3^- started approximately 4 min after HCO_3^- treatment. Cytosolic NO_3^- diminished gradually from 5.7 ± 0.2 mM to a steady lower value of 4.7 ± 0.1 mM after 35 min of HCO_3^- addition, resulting in a significant cytosolic NO_3^- shift of 0.9 ± 0.06 mM (n = 5; P = 0.03, Student t test). In both cases, time courses of cytosolic NO_3^- and cytosolic Cl^- decreases aligned with the recovery of the transient membrane hyperpolarization and profound membrane depolarization, supporting a cytosolic leak of negative charge induced by the HCO_3^- addition.

Effect of CO_2 Increase on Cytosolic NO_3^-

As we have previously reported, in *P. oceanica* mesophyll leaf cells the direct uptake of HCO_3^- and the subsequent use of CO_2 for photosynthesis has consequences for anion homeostasis (Rubio et al., 2017). In this work, we show that HCO_3^- use in this plant has an effect not only on cytosolic Cl^- but also on

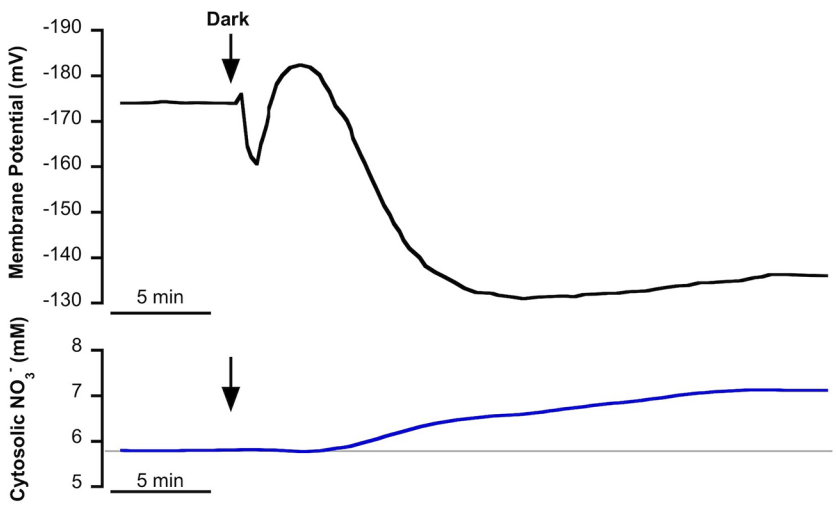

FIGURE 1 | Effect of light-dark transition on plasma membrane potential (*Em*, mV) and cytosolic NO_3^- (mM) in *Posidonia oceanica* mesophyll leaf cells, incubated in natural seawater. Traces are representative examples of intracellular nitrate-selective microelectrode recordings from 10 independent experiments. Arrows indicate the onset of dark treatment. Auxiliary grey line shows the standard cytosolic NO_3^- concentration under light conditions (150 µmol photons m^{-2} s^{-1}). Mean values and statistics are indicated in the text.

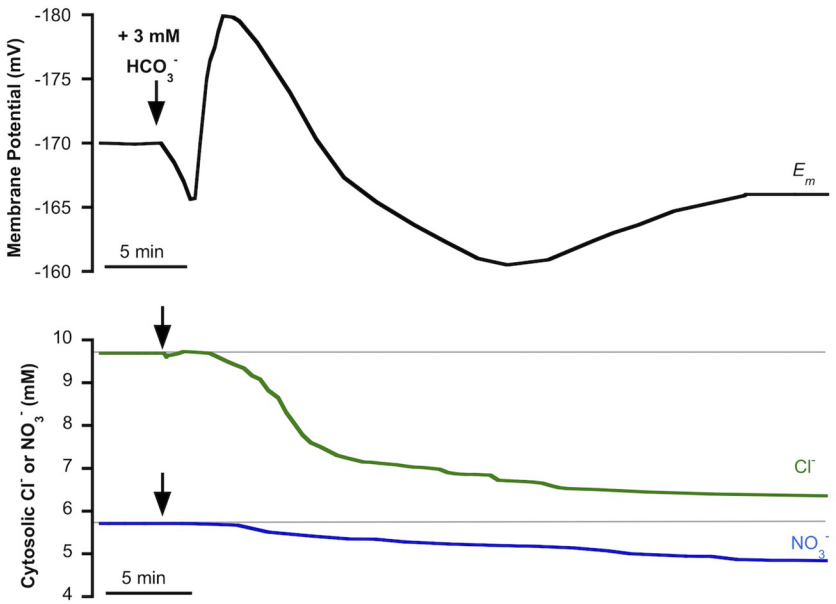

FIGURE 2 | Effect of the addition of 3 mM HCO_3^- on the plasma membrane potential (*Em*, mV), cytosolic chloride (Cl^-, mM) or cytosolic NO_3^- (NO_3^-, mM) measured in mesophyll leaf cells of *Posidonia oceanica*. Assay medium consisted of natural seawater and arrows indicate the addition of 3 mM HCO_3^-. Traces are representative examples from five independent recordings using intracellular Cl^--selective (green trace) or NO_3^--selective (blue trace) microelectrodes, respectively. Auxiliary grey lines indicate the normal cytosolic Cl^- or NO_3^- concentrations before HCO_3^- addition. Mean values and statistics are indicated in the text and in **Table 1**.

TABLE 1 | Membrane potential (mV), cytosolic NO_3^- (mM), and cytosolic Cl^- (mM) measured in mesophyll leaf cells of *P. oceanica* incubated in natural seawater (NSW) supplemented with different inorganic carbon concentrations.

	Membrane Potential (mV)	Cytosolic NO_3^- (mM)	Cytosolic Cl^- (mM)
NSW	−170 ± 8 initial value	5.7 ± 0.2 initial concentration	9.7 ± 0.2 initial concentration
+3 mM HCO_3^-	−181 ± 2* initial hyperpolarization −168 ± 3 final value	4.7 ± 0.1* final concentration	6.3 ± 0.3* final concentration
+1000 ppmCO_2	−174 ± 3 initial hyperpolarization −165 ± 3 final value	5.3 ± 0.6 final concentration	
+1000 ppmCO_2 (0.1 mM EZ)	−178 ± 2 final value	5.6 ± 0.1 final concentration	

EZ is the carbonic anhydrase inhibitor, ethoxyzolamide. Values are mean ± SD of a number of experiments indicated in the text. Asterisks (*) denote significant differences with respect to control conditions (NSW), the statistical values are indicated in the text.

cytosolic NO_3^- (**Figure 2**). The match of membrane depolarization and the onset of cytosolic Cl^- and NO_3^- diminution suggests the involvement of plasma membrane S-type anion channels, which are permeable to both anions and whose activation takes place when cells depolarize (Schmidt et al., 1995; Roelfsema et al., 2004; Roberts, 2006). In terrestrial angiosperm guard cells, these channels are activated when the concentration of HCO_3^- (not CO_2) increases and the cytosolic pH (pHc) is alkaline (Xue et al., 2011). These are similar conditions to those observed in *P. oceanica* in the presence of high HCO_3^- (Rubio et al., 2017). In order to rule out the role of CO_2, cytosolic NO_3^- was measured in mesophyll leaf cells of *P. oceanica* incubated in NSW supplemented with 1000 ppm CO_2, in the absence or in the presence of the plasma membrane-permeable carbon anhydrase inhibitor ethoxyzolamide (0.1 mM EZ; Sültemeyer et al., 1993). As shown in **Figure 3** (data are summarized in **Table 1**), the increase of CO_2 in NSW evoked a gradual plasma membrane hyperpolarization, reaching a value of −174 ± 3 mV (n = 5) after 7 min of 1000 ppm CO_2 treatment. This maximum hyperpolarization was lower and almost 2 min delayed compared to that induced by the treatment with 3 mM HCO_3^-, included in **Figure 3** as control (black trace). Cytosolic NO_3^- also decreased after the addition 1000 ppm CO_2; however, in these conditions cytosolic NO_3^- diminution was lower than that induced by 3 mM HCO_3^- treatment. After 15 min in the presence of 1000 ppm CO_2, cytosolic NO_3^- stabilized at a concentration of 5.3 ± 0.6 mM, a statistically non significant shift (n = 5; P = 0.37, Student t test). Furthermore, in the presence of 0.1 mM EZ, the enrichment of NSW with 1000 ppm CO_2 evoked a marked plasma membrane hyperpolarization that reached a steady maximum value of −178 ± 2 mV after 12 min treatment. In the presence of EZ, cytosolic NO_3^- showed a minimum, statistically non-significant, shift from 5.7 ± 0.2 to 5.6 ± 0.1 mM NO_3^- (n = 4; P = 0.53, Student t test). That neither

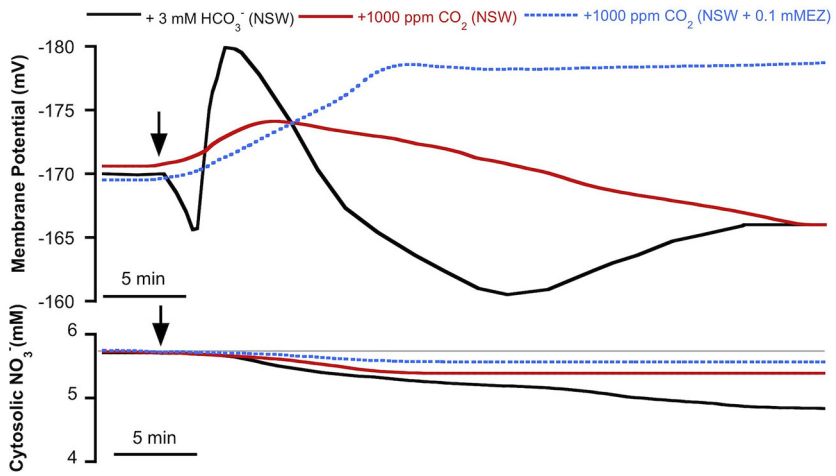

FIGURE 3 | Effect of inorganic carbon increase on the plasma membrane potential (*Em*, mV) and cytosolic NO_3^- (mM) measured in *Posidonia oceanica* mesophyll leaf cells. Arrows indicate the onset of 3 mM HCO_3^- addition to natural seawater (NSW, black traces, control conditions) and the addition of 1,000 ppm CO_2 to NSW (red traces) or to NSW containing 0.1 mM ethoxyzolamide (EZ, dashed blue traces). Traces are representative records of a minimum of four independent experiments using intracellular NO_3^--selective microelectrodes. Mean values and statistics are indicated in the text and **Table 1**. Auxiliary grey line represents the normal cytosolic NO_3^- concentration before the inorganic carbon additions.

the long-term depolarization nor the drop in cytosolic NO_3^- content evoked by HCO_3^- were evident with CO_2 in the presence of EZ (thus restricting HCO_3^- production from the CO_2 source) points to the need for substantial cytosolic HCO_3^- accumulation to effect ionic fluxes. Accordingly, these results strongly indicate that the cytosolic NO_3^- decrease is caused by the HCO_3^- enrichment and point to the S-type anion channel activation by the cytosolic HCO_3^- (not CO_2) increase.

Effect of HCO_3^- Increase on External NO_3^-

The activation of S-type anion channels may allow the efflux of NO_3^- from mesophyll leaf cells; such a phenomenon of NO_3^- efflux should be higher in the case of NO_3^--replete cells. Thus, in order to investigate if the HCO_3^- enrichment of NSW promoted NO_3^- efflux from *P. oceanica* leaves, the time course of external NO_3^- concentration change was monitored in assay medium containing leaves from plants pre-incubated for 2 days in NSW containing 100 μM NO_3^-. NO_3^--supplied leaves were then incubated in NSW containing the standard NO_3^- concentration (10 μM). After the addition of 3 mM HCO_3^-, external NO_3^- concentration increased significantly (10.8 ± 0.04 μM NO_3^-; n = 6; P <0.001, Student t test) within the first minute of incubation. Maximum net efflux rate was 18 ± 2 nmol $NO_3^-{\cdot}g_{FW}^{-1}{\cdot}min^{-1}$. Then, external NO_3^- decreased at a net rate of 8 ± 1 nmol $NO_3^-{\cdot}g_{FW}^{-1}{\cdot}min^{-1}$ until 5 min of incubation to stabilize at a concentration of 9.2 ± 0.1 μM NO_3^- (**Figure 4**). Under control conditions (no HCO_3^- addition), no external NO_3^- increase was detected, but NO_3^- concentration progressively decreased at a net rate of 11 ± 2 nmol $NO_3^-{\cdot}g_{FW}^{-1}{\cdot}min^{-1}$ during the first 10 min of incubation to reach a steady lower value of 7.6 ± 0.2 μM NO_3^- (n = 6;

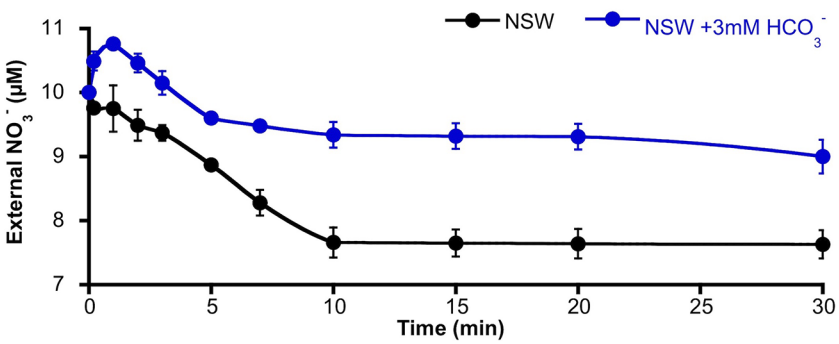

FIGURE 4 | Time course of the external nitrate concentration around *Posidonia oceanica* leaf pieces incubated in NSW. Plants were previously incubated in NSW containing 100 μM NO_3^-, then excised leaves were incubated in NSW (black trace, control condition) or NSW supplemented with 3 mM HCO_3^- (blue trace). At different times within the first 30 min of incubation, samples were taken and used to determinate external NO_3^- concentration. Data are mean ± SD of six independent assays. Mean values, net efflux, and influx NO_3^- rates and statistics are indicated in the text.

P = 0.002, Student t test). External NO_3^- concentrations showed higher values in all samples from leaves incubated in NSW enriched with 3 mM HCO_3^- suggesting a net NO_3^- efflux from *P. oceanica* leaves under these conditions.

DISCUSSION

Cytosolic NO_3^- Changes During Light–Dark Transitions in *P. oceanica* Leaf Cells

The concentration of NO_3^- in the cytosol (NO_3^-c) depends on a series of highly regulated processes at the cellular level that includes sensing and transport at the plasma membrane (uptake and efflux), subcellular compartmentalization and metabolism. Depending on the technique and the plant system used, a wide range of cytosolic NO_3^- values has been reported with also a large degree of variability. Therefore some authors suggest that NO_3^-c would not be subject to homeostasis, as would be the case for cytosolic pH or Ca^{2+} for example (for discussion see Siddiqi and Glass, 2002 or Miller and Smith, 2008). After the development of intracellular NO_3^--selective microelectrodes by Miller and Zhen (1991) it is possible to perform continuous measurements of the cytosolic free ion activity of NO_3^- with the possibility to obtain instantaneous responses to changes in the experimental conditions. This approach has been used here to report the first cytosolic NO_3^- values of a marine vascular plant.

Under light conditions the cytosolic NO_3^- measured using intracellular NO_3^--selective microelectrodes in *P. oceanica* mesophyll leaf cells was 5.7 ± 0.2 mM (n = 10), almost double the value reported for both epidermal and mesophyll leaf cells of *A. thaliana* (2.2 and 2.8 mM NO_3^-c, respectively; Cookson et al., 2005). This cytosolic NO_3^- in mesophyll leaf cells of *P. oceanica* is also much higher than that reported for the intermodal cells of the freshwater alga *C. corallina* (1.6 mM NO_3^-; Miller and Zhen, 1991) or in the liverwort *C. conicum* (0.63 mM NO_3^-; Trębacz et al., 1994), using the same technique. However, the NO_3^-c value found in *P. oceanica* is similar to those observed in root cells of barley (5.4 mM NO_3^-; Zhen et al., 1991) or maize (3.1 mM NO_3^-; Miller and Smith, 1996).

The NO_3^-c value in mesophyll leaf cells of *P. oceanica* is lower than the cytosolic Cl^- concentration (Cl^-c) of 9.7 mM Cl^-, yielding a NO_3^-c/Cl^-c of 0.6. This ratio is higher than that calculated for *C. conicum,* NO_3^-c/Cl^-c ≈ 0.1, taking 7.4 mM as the Cl^-c (Trębacz et al., 1994), but it is in the range of the estimates for root cells of barley (NO_3^-c/Cl^-c ≈ 0.9) or *A. thaliana* mesophyll leaf cells (NO_3^-c/Cl^-c ≈ 0.3). Those values have been calculated considering Cl^-c as 6 mM for barley root cells (Britto et al., 2004) and assuming that the reported cytosolic Cl^- in *A. thaliana* root cells (8.7 mM Cl^-c; Planes et al., 2015) would be the same in mesophyll leaf cells.

Changes in environmental conditions have been related to NO_3^-c alterations, supporting the idea that the cytosol operates a strong ion homeostasis not only for pH, Pi or Ca^{2+}, but also for NO_3^- (reviewed by Miller and Smith, 2008). Light-dark transition triggers a slight increase of NO_3^-c in the liverwort *C. conicum* (Trębacz et al., 1994) and a transient increase, from 2 to 3.5 mM, which peaked after 7 min of darkness in the case of *A. thaliana* leaf cells (Cookson et al., 2005). A similar response has been found here in *P. oceanica* mesophyll leaf cells, in which cytosolic NO_3^- reached a maximum value of 7.2 mM after 25 min of dark treatment. In the case of *A. thaliana*, since the effect was not observed when measurements were performed in the nitrate reductase (NR) mutant (*nia1nia2*) leaf cells, the NO_3^-c increase was explained because the shift to the dark inactivates NR, leading to a transient build-up of NO_3^-c due to a slower reduction rate (Cookson et al., 2005). A higher NO_3^-c in *A. thaliana nia1nia2* mesophyll leaf cells and a similar time course of cytosolic increase to the rate of NR activity change in response to illumination transitions (half-life of 2 to 15 min in spinach; Huber et al., 1992; Kaiser et al., 1992; Riens and Heldt, 1992) support the evidence for the role of NR in regulating NO_3^-c (Cookson et al., 2005). In *P. oceanica*, the NO_3^-c increase observed in the dark could also explain the one-half diminution of the maximum high affinity NO_3^- uptake observed previously in mesophyll leaf cells of this plant (Rubio et al., 2018), due to the apparent substrate inhibition of the transporter.

Inorganic Carbon Increase Triggers Cytosolic NO_3^- Decrease

In a previous work we demonstrated that a plasma membrane nH^+/HCO_3^- symporter mediates the uptake of HCO_3^- in *P. oceanica* mesophyll leaf cells. Further, the direct uptake of HCO_3^- followed by its internal dehydration renders CO_2 (used for photosynthesis) and hydroxyl anions, promoting membrane potential and cytosolic pH and Cl^- variations (Rubio et al., 2017). In the present work, we have also verified that uptake of HCO_3^- promotes the decrease of NO_3^-c in *P. oceanica* mesophyll leaf cells. The increases of cytosolic HCO_3^- and cytosolic pH have been related to promoting the opening of plasma membrane S-type anion channels in guard cells (Xue et al., 2011). A similar pathway seems to explain the NO_3^-c diminution observed in response to HCO_3^- addition in *P. oceanica* mesophyll leaf cells, as was previously proposed in the case of Cl^-c (Rubio et al., 2017), used as a control in this work. In both cases, the onset of NO_3^-c and Cl^-c decreases matched that of the plasma membrane depolarization, and indeed depolarization is a required initial phase to activate S-type anion channels operating at the plasma membrane of guard cells (Schmidt et al., 1995; Roelfsema et al., 2004; Roberts, 2006).

S-type anion channels are encoded by the small *SLAC/SLAH* gene family, that share homology to transport systems found in different kingdoms (Dreyer et al., 2012). In *A. thaliana*, apart from *SLAC1* that is exclusively expressed in guard cells, four additional homologs (*SLAH1-4*) are present (Negi et al., 2008). SLAC1 and SLAH3 channels exhibit a permeability preference for NO_3^- and Cl^- but not for malate (Schmidt and Schroeder, 1994; Geiger et al., 2009; Chen et al., 2010). The SLAH3 channel has the highest permeability for NO_3^- (NO_3^-/Cl^- permeability ratio of 20) and in contrast to SLAC1, SLAH3 also requires extracellular NO_3^- to induce its activity (Geiger et al., 2011).

Furthermore, a role in NO_3^--dependent alleviation of ammonium toxicity in *A. thaliana* roots has been proposed for SLAH3 (Zheng et al., 2015).

Although seagrasses have lost stomata differentiation genes (Olsen et al., 2016) the presence of S-type anion channels cannot be ruled out in those plants since, presumably, these anion channels have evolved as emergency valves, rapidly releasing excess osmolytes under stress conditions (Roelfsema et al., 2012). The *Zostera marina* genome, the first one available from a seagrass (Olsen et al., 2016), contains two homologs for the *A. thaliana SLAC/SLAH* gene family (*Phytozome v12.1*, data base), one *SLAH1* homolog (*Zosma91g00860.1*) and one *SLAC1* homolog (*Zosma76g00610.1*). The presumed homologs of these channels in *P. oceanica* should be good candidates for the proposed channels for the leak of NO_3^- and Cl^- ions from the cytosol, but further molecular analyses are needed to characterize the specific role of these anion channels to mediate anion efflux in response to high HCO_3^-.

In guard cells, stomatal closure in response to high CO_2 is mediated by the activation of S-type (SLAC1/SLAH3) anion channels (reviewed by Hedrich and Geiger, 2017). Interestingly, guard cells do not sense CO_2 themselves, but instead HCO_3^- synthesized from CO_2 within the cytosol by carbonic anhydrases (Xue et al., 2011). The same case could be proposed in *P. oceanica* mesophyll leaf cells, since no significant NO_3^-c decrease was found in response to 1000 ppm CO_2 treatment in either the absence or the presence of the plasma membrane-permeable carbon anhydrase inhibitor ethoxyzolamide, suggesting that HCO_3^-, not CO_2, is the inorganic carbon species that triggers NO_3^-c efflux from *P. oceanica* mesophyll leaf cells (**Figure 5**). Furthermore, probably due to the activation of the H^+-pump in response to the cytosolic accumulation of CO_2, a weak acid, the 1000 ppm CO_2 treatment renders the hyperpolarization of the plasma membrane and the cytosolic acidification (see Rubio et al., 2017). Both responses are prolonged in the presence of ethoxyzolamide, that impairs HCO_3^- production from the CO_2 source, leading to conditions that not support the activation of S-type anion channels described above and could explain the non-significant NO_3^-c diminution observed in response to CO_2 increase.

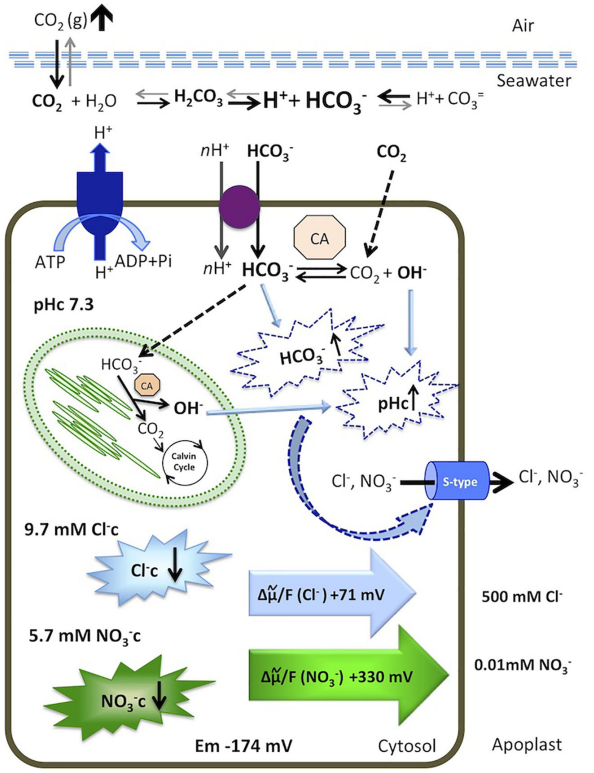

FIGURE 5 | Model for cytosolic NO_3^- and Cl^- responses to an increase of dissolved inorganic carbon in the marine angiosperm *Posidonia oceanica* leaf cells. Elevated atmospheric CO_2 level increases total dissolved inorganic carbon, shifting the equilibrium in favor of HCO_3^- in the seawater. The HCO_3^- is taken up by an H^+-symport and dehydrated in the cytosol or transported to the chloroplast serving as inorganic carbon source for photosynthesis. Internal dehydration, catalyzed by the carbonic anhydrases (CA) renders CO_2, consumed by the Calvin Cycle, and OH^- ions that increase cytosolic pH (pHc). Excess of cytosolic HCO_3^- and elevated pHc activates S-type anion channels, through which Cl^- and NO_3^- leave the cells by an outwardly directed motive force of +71 mV and +330 mV, respectively. Consequently, the cytosolic concentrations of both anions decrease. Membrane potential, ion concentrations, calculations, and discussion are included in the text.

Cytosolic NO_3^- Efflux Under High Inorganic Carbon Could Contribute to Biomass N-Impoverishment in Seagrasses

As we showed previously, direct HCO_3^- uptake has consequences for cytosolic ion homeostasis in *P. oceanica* mesophyll leaf cells (Rubio et al., 2017). The short-term net efflux of NO_3^- and the low NO_3^- net uptake rate observed in the present work also indicate that natural seawater HCO_3^- enrichment may be important not only for cytosolic ion homeostasis but for the metabolic flux of NO_3^- in this seagrass. In natural seawater, containing 500 mM Cl^- and 10 μM NO_3^-, the efflux of Cl^- and NO_3^- from *P. oceanica* mesophyll leaf cells is driven by the outwardly directed anion electrochemical potential gradients for both anions (**Figure 5**). This electrochemical potential gradient (in mV) is almost five-fold higher for NO_3^- (+330 mV) than for Cl^- (+71 mV). However, in response to high HCO_3^- the Cl^-c decrease observed in *P. oceanica* mesophyll leaf cells (ΔCl^-c = 3.4 mM) was much higher than for NO_3^-c (ΔNO_3^-c = 1 mM). This lower NO_3^-c leak than the expected from electrochemical potential gradient comparison with Cl^-c could be explained by a different membrane permeability for both anions and/or different capability of compartmentalization. Nevertheless, even the observed decrease of NO_3^-c, the short-term NO_3^- efflux, and the low NO_3^- uptake rate could impair N-assimilation in *P. oceanica* leaf cells in natural seawater containing high inorganic carbon.

An impaired metabolic flux of N should be relevant in the context of atmospheric CO_2 rise. Oceans have been the sink for 30% of CO_2 released during the industrial era, at a higher rate (3.8 GTons·year^{-1}) than the 1.8 GTons·year^{-1} fixed by photosynthesis or the 2 GTons·year^{-1} removed by abiotic absorption (Behrenfeld et al., 2002). Atmospheric CO_2 is

exchanged into aquatic environments rendering the dissolved inorganic carbon (DIC) equilibrium. Controlled by pH, this equilibrium generates the distribution of DIC species: dissolved CO_2, bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions. Consequently, elevated atmospheric CO_2 concentration increases the total DIC and lowers the pH, shifting the relative proportion of each DIC species. Under current ocean pH (~8.04) and atmospheric CO_2 (~ 410 ppm), the smallest pool of DIC is dissolved CO_2, but this will have the greatest increase (> 250%) among the DIC constituents as the pH drops (~ 0.3–0.4 pH units) under the predicted rise in atmospheric CO_2 (1000 ppm) for 2100. In contrast, the HCO_3^- pool will only increase by 15% at that date (Koch et al., 2013). However, in terms of absolute concentration ($mol{\cdot}kg^{-1}$) HCO_3^- levels will rise more than dissolved CO_2 (Raven et al., 2005). Using the predicted data for 2100, calculations render 2.05 $mmol{\cdot}kg^{-1}$ HCO_3^- and only 0.03 $mmol{\cdot}kg^{-1}$ CO_2 (Koch et al., 2013). Considering such a HCO_3^- rise (1.2 fold higher than actual concentration) and that the addition of 3 mM HCO_3^- corresponds to an increase by 2.3 fold of the HCO_3^- concentration in natural seawater, a NO_3^-c leak of 0.5 mM would be expected in *P. oceanica* mesophyll leaf cells by 2100. However, long-term effect of elevated atmospheric CO_2 concentration on seagrasses N content needs further investigation, since N-deficiency induces high-affinity NO_3^- uptake, which contributes to NO_3^- homeostasis (reviewed by Rubio and Fernández, 2019).

Furthermore, the concomitant natural seawater pH decrease under elevated atmospheric CO_2 could alter N availability in seawater and even the energy cost for nutrient uptake. A pH change from 8.1 to 7.8 evokes a decrease in the amount of NH_3 in the NH_4^+/NH_3 ratio (Raven, 1986), whereas the amount of NO_3^- would not be affected (Zeebe and Wolf-Gladrow, 2001). Instead, the 0.3 external pH unit decrease renders a rise of the proton motive force at the plasma membrane of *P. oceanica* leaf cells of −18 mV, considering −174 mV as membrane potential, 7.3 as the cytosolic pH and constant temperature (Rubio et al., 2017). This amount represents, approximately, a 15% increase of the proton motive force (inwardly directed), which could prompt the activity of H^+-dependent transport systems in this seagrass, including the plasma membrane H^+/HCO_3^- symporter (Rubio et al., 2017). As discussed by Rubio and Fernández (2019), ocean acidification could increase both the H^+ and even the Na^+ motive force due to changes in the activity of the plasma membrane Na^+/H^+ antiporter found in seagrass species (Rubio et al., 2011). The activity of this antiporter generates a lower cytosolic Na^+ concentration at acid external pH and, consequently, a rise of the inwardly directed Na^+ motive force could be also expected, favoring high-affinity NO_3^- uptake based on Na^+-dependent transport systems in seagrasses (reviewed by Rubio and Fernández, 2019). In fact, a recent study in *P. oceanica* beds from the Gulf of Naples (Italy) shows that the long-term exposure (9 months) to acidified seawater (pH 7.82) under constant DIC conditions promotes the diminution of the C/N molar ratio, due to the increase of N content by 21% and 70% in leaves and rhizome, respectively, whereas C content of those organs is not affected by external pH acidification (Scartazza et al., 2017). Those authors suggest that seawater acidification promoted a feed-forward long-term effect on N accumulation in *P. oceanica*, especially at the rhizome, although they recognize the need to specify the effect of acidification on the nutrient availability in their study (Scartazza et al., 2017). Interestingly, long-term nutrient enrichment seems to modulate the effects of ocean acidification on *P. oceanica*. Molecular analysis indicates that after 18 months at low pH (7.78) conditions the expression of nitrate transporter genes in *P. oceanica* leaves is altered; while *NRT1_6.3* and *NRT1_2.13* (involved in NO_3^- sensing and low-affinity transport, respectively) are overexpressed the high-affinity NO_3^- transporter gene *NRT2* shows a down-regulated expression (Ravaglioli et al., 2017). Thus, long-term overexpression of nitrogen transporter genes following nutrient additions at low pH suggests enhanced nutrient uptake and proposes that the effects of ocean acidification on *P. oceanica* depend upon local nutrient concentration (Ravaglioli et al., 2017).

Contrary to the acidification effect in *P. oceanica* meadows, several lines of evidence show that the most common effect of elevated CO_2 is a decrease in the dry mass concentration of N in plant tissue (Cotrufo et al., 1998; Curtis and Wang, 1998; Norby et al., 1999; Jablonski et al., 2002; Ainsworth and Long, 2005; Taub et al., 2008). This suggests that physiological changes leading to decreased biomass N under elevated CO_2 predominate in their effects over factors that would tend to increase N content (reviewed by Taub and Wang, 2008). Consequently, plants with non-saturated photosynthesis at actual atmospheric CO_2, show an ionomic imbalance, with N the main nutrient that decreased at high C assimilation (Loladze, 2014). The physiological mechanisms responsible for this phenomenon have not been well established yet, although different hypotheses are proposed to account for it. In terrestrial vascular plants, the best-supported are the decrease of specific N uptake and assimilation due to a diminution of the transpiration-driven mass flow of N by a decreased stomatal conductance at elevated CO_2 and the biomass dilution of N by increased photosynthetic assimilation of C (reviewed by Taub and Wang, 2008).

Seagrass meadows rank among the most productive ecosystems on Earth (Duarte and Chiscano, 1999), which largely contribute to C uptake in coastal waters and with most species capable of utilizing HCO_3^- as a CO_2 source for photosynthesis (Raven et al., 2014). With the exception of *Cymodocea nodosa* (considered C4), seagrasses show C3 photosynthetic pathways and are not saturated at the current ocean DIC concentration (reviewed by Koch et al., 2013). As occurs in C3 terrestrial plants, a higher C assimilation occurs in seagrasses due to the rise of DIC discussed above (Borum et al., 2016). The unpredicted effect of HCO_3^- enrichment on anion homeostasis in *P. oceanica* mesophyll leaf cells, leading to the short-term efflux of NO_3^- from the cytosol, possibly through the activation of S-type anion channels, supports a new mechanism as a key consideration in understanding the expected biomass N-alteration in seagrasses under elevated DIC, and probably, for terrestrial plants growing in waterlogged alkaline environments.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

LR, DG-P, and JF performed the experiments. DG-P and LR accomplished data analysis. LR, JD, and JF discussed the results, wrote and edited the manuscript. LR and JF conceived the project. All authors contributed to the article and approved the submitted version.

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