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Nitrogen metabolism in the seagrass *Zostera noltii*

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"Agora que está começado vai ser preciso acabá-lo, é como uma fatalidade.

E as pessoas nem sonham que quem acaba uma coisa nunca é a aquela que a começou,

mesmo que ambas tenham nome igual, que isso só é que se mantém constante, nada mais."

José Saramago, *In* "O Ano da Morte de Ricardo Reis"

Prémio Nobel da Literatura 1998

RESUMO

O metabolismo do azoto é uma componente crucial na vida das plantas. Esta tese teve como principal objetivo investigar a ecofisiologia do metabolismo do azoto da erva marinha *Zostera noltii* na Ria Formosa. No contexto atual das alterações climáticas, foi também investigado o efeito do enriquecimento em CO_2 da água do mar na fotossíntese, crescimento e metabolismo do azoto na espécie. As taxas de incorporação de amónia e nitrato foram determinadas a três concentrações diferentes destes nutrientes, suplementados simultaneamente (NH_4NO_3) ou separadamente (NH_4Cl e KNO_3). A amónia foi identificada como a forma preferencial de azoto inorgânico para a *Z. noltii*. Contudo, a espécie apresentou taxas de incorporação de nitrato consideráveis quando suplementada com uma solução de nitrato sem amónia. Este resultado sugere o nitrato como uma fonte alternativa de azoto inorgânico. O fornecimento simultâneo de ambas as formas de azoto inorgânico à *Z. noltii* aumentou as taxas de incorporação de amónia e diminuiu as de nitrato comparativamente às taxas obtidas quando apenas uma das formas azotadas foi fornecida.

As taxas de incorporação de amónia e nitrato, as interações folhas-raízes no processo de incorporação de azoto e a translocação interna de azoto incorporado foram investigadas simultaneamente. As taxas de incorporação de azoto pelas folhas e raízes foram medidas usando câmaras de incubação de dois compartimentos que separaram fisicamente a parte aérea da parte subterrânea da planta, e foram quantificadas com base na incorporação de soluções de amónia e nitrato enriquecidas com o isótopo ^{15}N. As taxas de incorporação de amónia pelas folhas foram uma ordem de magnitude mais elevadas do que pelas raízes, designando as folhas como a principal via de entrada de amónia na planta.

Para além disso, as folhas apresentaram uma elevada afinidade para a incorporação de amónia (i.e. taxas elevadas de incorporação a concentrações muito baixas de azoto). Estas duas características demonstram que a *Z. noltii* possui capacidade não só para incorporar amónia de forma eficiente mas também para beneficiar de forma vantajosa de níveis elevados de azoto disponíveis de forma transitória na coluna de água. As folhas foram igualmente identificadas como sendo o local preferencial de redução da amónia e nitrato incorporados, tal como revelado pelas taxas mais elevadas de atividade das enzimas (nitrato redutase e glutamina sintetase) nas folhas do que as raízes. A incorporação de amónia ou nitrato por uma das partes da planta (ex. folhas) não afetou a taxa de incorporação pela outra parte (ex. raízes). Da mesma forma, não foi detetada translocação interna apreciável do azoto incorporado. A estimativa do orçamento total de azoto para a *Z. noltii* na estação de maior produtividade (primavera), calculada com base nas taxas de incorporação de amónia e nitrato das folhas e raízes, foi ligeiramente mais baixa do que o valor de requisito total de azoto para o crescimento, o que indica que o crescimento da *Z. noltii* na Ria Formosa não está limitado, ou está apenas ligeiramente limitado, por azoto.

As taxas de incorporação de azoto inorgânico à luz e no escuro foram determinadas ao longo do tempo utilizando o método da perturbação. Com base neste método, as taxas de incorporação de azoto de plantas expostas a concentrações iniciais elevadas de amónia ou nitrato (95 e 65 µM) foram medidas continuamente através da depleção dos nutrientes no meio ao longo do tempo de incubação. As taxas de incorporação de azoto foram semelhantes na luz e no escuro. Em ambas as condições de luz, o padrão temporal de incorporação de azoto caracterizou-se por taxas iniciais mais elevadas seguidas de taxas mais baixas mas relativamente constantes. Em condições de luz, as plantas suplementadas

quer com amónia quer com nitrato acumularam açúcares solúveis nas folhas, enquanto as plantas suplementadas apenas com nitrato apresentaram uma redução do conteúdo de amido, particularmente nos rizomas. Estes resultados sugerem que a energia e o carbono necessários para a assimilação do nitrato derivam não só diretamente da fotossíntese mas também dos açúcares solúveis provenientes da degradação das reservas de amido dos rizomas. A incorporação de azoto no escuro conduziu a uma utilização e mobilização adicional das reservas de carbohidratos, tanto de açúcares solúveis como de amido, particularmente ao nível dos rizomas. Ainda assim, o benefício decorrente da possibilidade de incorporar azoto inorgânico independentemente das condições de luz parece compensar o consumo adicional de carbohidratos de reserva associado à incorporação de azoto no escuro.

Os efeitos do enriquecimento em CO_2 da água do mar na fotossíntese, crescimento e metabolismo do azoto da *Z. noltii* foram investigados numa experiência em mesocosmos onde as plantas foram expostas durante cinco meses a concentrações atuais (360 ppm) e futuras (700 ppm) de CO_2. As taxas fotossintéticas das plantas expostas a condições de enriquecimento em CO_2 foram mais elevadas do que as das plantas expostas à concentração de CO_2 atual. Por outro lado, as taxas de crescimento e de incorporação de amónia não foram afetadas. A análise do conteúdo de azoto nas folhas das plantas de ambos os tratamentos experimentais de CO_2 revelou valores abaixo dos valores críticos indicativos de um deficiente fornecimento de azoto, o que sugere que o fornecimento de azoto inorgânico às plantas no mesocosmos poderá ter sido insuficiente para preencher os requisitos de azoto para o crescimento da espécie. Desta forma, as taxas de crescimento das plantas no mesocosmos podem ter sido controladas pela limitação da disponibilidade de azoto a que as

plantas foram sujeitas no mesocosmos. As taxas fotossintéticas mais elevadas registadas a concentrações de enriquecimento de CO_2 sugerem que a erva marinha *Z. noltii* poderá beneficiar de futuros incrementos da concentração de CO_2 na água do mar quando em condições de nutrientes não limitantes.

PALAVRAS-CHAVE: enriquecimento em CO_2, glutamina sintetase, nitrato redutase, azoto, Ria Formosa, erva marinha, incorporação, *Zostera noltii*.

(Este texto foi escrito ao abrigo do Novo Acordo Ortográfico da Língua Portuguesa)

ABSTRACT

Nitrogen metabolism is a vital component in plant's life. The main purpose of this thesis was to investigate the ecophysiology of nitrogen metabolism of the seagrass *Zostera noltii* in Ria Formosa lagoon. In the global change scenario, the effects of CO_2 enrichment on photosynthesis, growth and nitrogen metabolism of *Z. noltii* were also investigated. Ammonium was identified as the preferential N_i source for *Z. noltii*, although nitrate uptake rates were considerable in the absence of ammonium. The ammonium uptake rates through the leaves were one order of magnitude higher than through the roots. Leaves were also identified as the main site for the reduction of ammonium and nitrate, as revealed by the much higher activity of the enzymes nitrate reductase and glutamine synthetase in the leaves than in the roots. The simultaneous supply of both N_i forms to *Z. noltii* enhanced the ammonium uptake rates and decreased the rates of nitrate uptake comparatively to the rates obtained when N_i forms were supplied separately. The uptake of ammonium or nitrate by one plant part (e.g. leaves) did not affect the uptake rate of the other plant part (e.g. roots), and no internal translocation of incorporated nitrogen was detected. The estimated whole-plant nitrogen budget of *Z. noltii* in the peak production season (spring) was slightly lower than the total nitrogen requirement for growth, which indicates that the growth of *Z. noltii* in the lagoon is only slightly limited by nitrogen. *Z. noltii* took up ammonium and nitrate at similar rates in the light and in the dark. In both light conditions, the nitrogen uptake displayed a temporal pattern of enhanced initial rates followed by lower but relatively constant rates. The uptake of nitrogen in the dark represented an additional use and mobilization of carbohydrate reserves. *Z. noltii* plants exposed to CO_2-enriched conditions

enhanced the photosynthetic rates while growth and ammonium uptake rates were not affected, suggesting that *Z. noltii* may benefit from future increases in seawater CO_2 concentrations.

KEYWORDS: CO_2 enrichment, glutamine synthetase, nitrate reductase, nitrogen, Ria Formosa, seagrass, uptake, *Zostera noltii*.

CONTENTS

General Introduction

Seagrasses are marine flowering plants inhabiting shallow coastal areas, sheltered bays and coastal lagoons all over the world, except in polar regions. Most species colonize sandy or muddy sediments, although some can grow over rocks. Seagrasses are composed by modules that are repeated during clonal growth. Each module is composed by a piece of rhizome (horizontal or vertical), a group of leaves attached to the rhizome (shoot) and a root system. They can also reproduce sexually through hydrophilous pollination, with the production of flowers and fruits (Hemminga and Duarte, 2000).

Seagrasses are among the world's most productive and valuable ecosystems, along with coral reefs and mangroves (Duarte and Cebrián, 1996; Costanza et al., 1997; Duarte and Chiscano, 1999). The ecological value of seagrass ecosystems has become increasingly recognized (Duarte, 1999; Hemminga and Duarte, 2000). Services provided by seagrass ecosystems include food supply and shelter for a wide variety of species, from fishes to marine mammals, as well as breeding ground and nursery areas for important commercial species. The network of rhizomes and roots fix and stabilize the sediment over which seagrasses grow, reducing sediment resuspension by currents and waves and protecting against coastline erosion. Seagrasses are also highly efficient in removing nutrients, hence contributing to improve water transparency and quality (Duarte, 2000).

Nutrient recycling is one of the most relevant ecological functions of seagrass ecosystems being responsible for maintaining adequate nutrient balances in the coastal environments they dominate (Hemminga and Duarte, 2000). Nitrogen is a fundamental nutrient for seagrass growth, apart from light and inorganic carbon. Inorganic nitrogen is

available to seagrasses as ammonium and nitrate either in the water column or in the sediment porewater. Ammonium is the dominant form of N_i in the hypoxic or anoxic sediments which characterize seagrass habitats. Inorganic nitrogen concentrations in the water column are typically very low (< 5 µM) in habitats lacking appreciable anthropogenic influence as freshwater inflow (cf. Touchette and Burkholder, 2000). Seagrasses incorporate ammonium and nitrate both through the leaves and roots (Stapel et al., 1996; Lee and Dunton, 1999; Touchette and Burkholder, 2000). Although the uptake of ammonium through the roots is often considered the major source of N_i to seagrasses, research on nitrogen acquisition showed that 30 - 90% of the nitrogen requirements of several seagrass species can be supplied by the water column through leaf uptake (Hemminga et al., 1994, Terrados and Williams, 1997; Stapel et al., 1996). The assimilation of the incorporated nitrogen into aminoacids is a complex, energy-requiring process mediated by two key enzymes, glutamine synthetase and nitrate reductase. The conversion of ammonium into glutamine is catalyzed by the enzyme glutamine synthetase, with the use of ATP. The assimilation of nitrate is energetically more expensive because it must first be reduced to nitrite and then to ammonium. The first step of this process is mediated by the enzyme nitrate reductase, with the use of NADH (Taiz and Zeiger, 2002).

Nutrient uptake rates are usually determined by measuring the depletion of the nutrient from the medium at specific time intervals during the incubation. Alternatively, these rates can be determined by incubating plant tissues in nitrogen solutions enriched with the stable isotope ^{15}N. In this case, the rates are measured from the amount of incorporated ^{15}N in the tissues. The use of ^{15}N isotopes is a novel, powerful technique that allows following the flows and fates of nitrogen inside the plant without altering its natural

behavior (Dawson et al., 2002). This technique was used in two chapters of this thesis in the determination of the uptake rates of ammonium and nitrate, as well as its internal translocation in *Z. noltii*.

Zostera noltii is the dominant seagrass species in Ria Formosa lagoon (South Portugal). This fast-growing species develops dense meadows along the intertidal mudflats and plays a key role in the productivity of the lagoon (Santos et al., 2004). The photosynthetic ecology of *Z. noltii*, the environmental parameters determining the species growth and the main anthropogenic activities affecting the species in the lagoon were extensively investigated (Peralta, 2000; Alexandre, 2004; Silva, 2004; Cabaço, 2007). However, no information was yet available on the ecophysiology of the nitrogen metabolism of *Zostera noltii*. A review of the literature on the ecophysiology of nitrogen metabolism in seagrasses indicated substantial variation in the nutritional responses among seagrass species from different geographic regions, and highlighted the importance of examining species-specific aspects of a given species in its system (Touchette and Burkholder, 2000). In this perspective, the present thesis aimed to investigate the basic characteristics of the inorganic nitrogen metabolism of *Zostera noltii* in Ria Formosa lagoon, namely to identify the preferential N_i source (ammonium or nitrate) and the main via through which nitrogen is taken up and the primary site of nitrogen reduction and assimilation into aminoacids (leaves or roots). The presence of internal N_i translocation of incorporated nitrogen that could affect the uptake of each plant part was also investigated. Additionally, the thesis aimed to investigate the effect of light on nitrogen uptake (light versus dark uptake) and relate it with the carbohydrate metabolism. Finally, the emergent but yet less investigated problem of increased CO_2 availability in the ocean/acidification

was explored by evaluating the effects of CO_2 enrichment on the species photosynthesis, growth, and nitrogen uptake and assimilation.

The specific objectives were:

(i) to identify the preferential inorganic nitrogen source and the primary site of reduction and assimilation of incorporated nitrogen of *Z. noltii*. To achieve this, uptake rates of ammonium and nitrate were determined at different concentrations either supplied separately or simultaneously; in addition, the activity of nitrate reductase and glutamine synthetase was assessed in the leaves and roots and related to the inorganic nitrogen uptake.

(ii) to investigate simultaneously the three major processes involved in the uptake kinetics of inorganic nitrogen in *Z. noltii*, i.e. (1) the relative uptake rates of ammonium versus nitrate through the leaves and roots; (2) the leaf-root interactions in the nitrogen uptake and (3) the internal translocation of incorporated nitrogen. Specific uptake rates of leaves and roots and the internal translocation of incorporated nitrogen were assessed in two-compartment chambers using ^{15}N-labeled ammonium and nitrate.

(iii) to compare the light and dark uptake rates of ammonium and nitrate, and to assess the short-term effect of ammonium versus nitrate uptake on the carbohydrate levels. Uptake rates of ammonium and nitrate were determined in the light and in the dark following exposure of whole plants of *Z. noltii* to a high nutrient concentration.

(iv) to investigate the effects of CO_2 enrichment on photosynthesis, growth and nitrogen metabolism of *Z. noltii*. Rates of photosynthesis and growth were compared in plants exposed for five months to current (360 ppm) and future (700 ppm) seawater CO_2 concentrations in an outdoor mesocosm experiment. The effects of CO_2 enrichment on

nitrogen metabolism were assessed by comparing the rates of ammonium and nitrate uptake, as well as the activity of nitrate reductase and glutamine synthetase.

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Inorganic nitrogen uptake and related enzymatic activity in the seagrass *Zostera noltii*

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Inorganic nitrogen uptake and related enzymatic activity in the seagrass *Zostera noltii*

Abstract

The preferential inorganic nitrogen source for the seagrass *Zostera noltii* was investigated in plants from Ria Formosa, southern Portugal. Rates of ammonium and nitrate uptake were determined at different concentrations of these nutrients (5, 25 and 50 µM), supplied simultaneously (NH_4NO_3) or separately (KNO_3 and NH_4Cl). The activity of the enzymes nitrate reductase (NR) and glutamine synthetase (GS) were also assessed. The results showed that ammonium is the preferential inorganic nitrogen source for *Z. noltii*, but, in the absence of ammonium, the species also has a high nitrate uptake capacity. The simultaneous availability of both inorganic nitrogen forms enhanced the uptake rate of ammonium and decreased the uptake rate of nitrate compared to when only one of the nitrogen forms was supplied. The activity of both enzymes was much higher in the leaves than in the roots, highlighting the importance of the leaves as primary reducing sites in the nitrogen assimilation process.

Keywords: glutamine synthetase, nitrate reductase, nitrogen, seagrass, uptake.

Introduction

Seagrasses are marine angiosperms inhabiting shallow coastal areas, sheltered bays and coastal lagoons. Nutrient recycling is one their most relevant ecological functions (Hemminga and Duarte, 2000). Inorganic nitrogen is available both as nitrate and

ammonium, either in the water column or in sediment pore water. In seagrass habitats, the nitrate concentration in the water column typically ranges from 0 to 8 μM, whereas ammonium ranges from 0 to 3.2 μM. In sediment pore water, ammonium concentration is usually much higher, ranging from 1 to 180 μM, whereas nitrate concentration is almost negligible (cf. Touchette and Burkholder, 2000). Young, actively growing seagrass roots take up most of the pore water nitrogen as ammonium, whereas leaves take up both ammonium and nitrate from the water column (Pedersen and Borum, 1992; Hemminga et al., 1994; Stapel et al., 1996; Pedersen et al., 1997; Terrados and Williams, 1997). Typically, seagrasses have higher affinity and higher uptake rates for ammonium than for nitrate (Lee and Dunton, 1999; Touchette and Burkholder, 2000; Dudley et al., 2001) mostly because the ammonium assimilation process is energetically less expensive (Bloom et al., 1992). Leaves are more efficient than roots in absorbing low levels of ammonium (Lee and Dunton, 1999).

To avoid toxic effects inside the cells, ammonium must be rapidly converted into glutamate, a process driven by the enzyme glutamine synthetase (Touchette and Burkholder, 2000). Following its absorption into the tissues, nitrate is reduced to nitrite and then to ammonium. The first step of this process is the reduction of nitrate to nitrite, a reaction mediated by the enzyme nitrate reductase. Recently, the capacity for organic nitrogen uptake (urea and amino acids) was demonstrated in two seagrass species (Vonk et al., 2008). The organic nitrogen uptake rates were always lower than those of inorganic nitrogen, except the uptake of amino acids by the roots.

The objective of this study was to identify the preferential inorganic nitrogen source of the seagrass *Zostera noltii*. Rates of ammonium and nitrate uptake were determined at

different concentrations and when supplied separately or simultaneously. Leaf and root nitrate reductase and glutamine synthetase activity was assessed and related to the inorganic nitrogen uptake. To our best knowledge, this is the first study to investigate the inorganic nitrogen uptake of *Z. noltii*. The activities of nitrate reductase and glutamine synthetase in *Z. noltii* were also determined by Kraemer and Mazzella (1999) and Alexandre et al. (2004), but were not related to the nitrogen uptake.

Methods

Site description

Zostera noltii is the most abundant seagrass species in Ria Formosa coastal lagoon (South Portugal), spreading along its intertidal mudflats and playing an important role in the productivity of the lagoon (Santos et al., 2004). In this system, ammonium and nitrate concentrations in the water column are usually less than 5 μM. Ammonium concentrations in the sediment pore water are higher (12 - 38 μM), whereas nitrate concentrations are almost negligible (0.2 - 0.9 μM) (Loureiro et al., 2006; Cabaço et al., 2008).

Ammonium and nitrate uptake

Z. noltii plants were collected in January 2009 from an intertidal mudflat. The leaves were cleaned of epiphytes and the roots were gently cleaned of any adherent sediment. The plants were kept for four days in seawater with negligible concentrations (< 1 μM) of ammonium and nitrate, in an acclimation chamber under light and temperature conditions similar to those used in the experiments. Plants were incubated in three different nitrogen media (NH_4NO_3, KNO_3 and NH_4Cl), under three different nutrient concentrations

(5, 25 and 50μM). The NH_4NO_3 medium contained equal concentrations of ammonium and nitrate (e.g. 50 μM of ammonium and 50 μM of nitrate).

Contrary to what occurred in our experiments, the below-ground conditions of *Z. noltii* meadows are anoxic, which may raise the question of the effects of this condition on the uptake of nitrogen. To discard this hypothesis, we ran a preliminary experiment in split-chambers where the below- and above-ground media were physically separated. We found no differences in the ammonium or nitrate uptake of leaves when roots were incubated either in anoxic or in oxygenated media (Fig. 1). Therefore, we incubated *Z. noltii* plants in whole containers filled with 300 ml of nutrient enriched artificial seawater, in an orbital shaker (125 rpm) at constant salinity (37.0), pH (8.0), temperature (16ºC) and light intensity (200 μmol quanta m^{-2} s^{-1}). The pH was adjusted using HCO_3^- in order to supply the media with a source of inorganic carbon. Nine containers were used for each nitrogen concentration, each containing three plants. Whole plant biomass in each container averaged 0.45 g FW, plant leaf length was about 15 cm and rhizome length was about 2 cm. The above:below-ground biomass ratio averaged 1.37. Incubations lasted one hour, the time interval where ammonium and nitrate uptake rates were highest. Previous experiments showed that after that period of time the uptake rates remained nearly constant throughout the next twenty-four hours. Short and McRoy (1984) showed the same pattern of nitrogen uptake in *Zostera marina*. After one hour of incubation, water samples were collected from each plant container, filtered through cellulose acetate filters and stored at -20ºC for ammonium and nitrate analysis. Nutrient analysis was performed in a loop-flow analyzer (μMAC-1000, Systea, Italy). Ammonium concentration was determined using the phenol-hypochlorite method and nitrate concentration was determined using the Cd-Cu column

reduction method. The detection limit of the analytical method for ammonium is 0.10 ± 0.03 µmol and for nitrate is 0.04 ± 0.01 µmol. The ammonium and nitrate uptake rates were calculated as the difference between the initial and final concentration of the nutrient in the medium per unit of plant dry weight and time (µmol g^{-1}DW h^{-1}). Dry weights were estimated from fresh weights using a previously obtained conversion factor of 0.18.

Interactions between above- and below-ground plant parts in the uptake of nitrogen as those reported in *Z. marina* (Thursby and Harlin, 1982) may confound our results as whole plants were used in the experiments. However, in recently ran experiments using split-chambers to separate the *Z. noltii* below- and above-ground incubation media, we did not find any interactions (Alexandre et al., 2011).

Enzymatic analysis

Plants from the nine replicates of each nutrient concentration were pooled to obtain 3 sub-samples, each with 0.2 g FW of tissue, for the analysis of nitrate reductase (NR) and glutamine synthetase (GS) activity. For NR activity measurements, plant material was used fresh, whereas for GS activity, plant tissues were frozen at -80ºC. Rhizomes were not considered in this experiment because measurements previously done *in vivo* in *Z. noltii* showed negligible NR activity in this tissue (< 0.1 µmol NO_2^- g^{-1}DW h^{-1}) (unpublished data). NR activity was measured *in vivo* using the method described by Corzo and Niell (1991), optimized for *Z. noltii* (Alexandre et al., 2004). This method is based on the colorimetric measure of nitrite, formed after the reduction of nitrate by NR. Plant tissues were incubated in 50 mM KNO_3, 0.1 M K_2HPO_4 (pH 8.0), 0.5 mM EDTA and 0.5% 1-propanol, in a final assay medium volume of 10 ml, flushed with N_2 for 2 min to remove

oxygen. Incubations lasted 30 min at 30ºC. The nitrite produced was measured spectrophotometrically (540 nm) after adding 1 ml of sulphanilamide and 1 ml of naphtyl-etylenediamine to the assay medium. The *in vivo* assay was preferred because in preliminary experiments it yielded consistently higher activity than the *in vitro* assay, which failed to provide reproducible results. Similar difficulties in using *in vitro* NR assays in seagrasses have been previously reported for other species (Touchette and Burkholder, 2007 and references therein). GS activity was measured *in vitro*, using the method described by Sagi et al. (2002). The normal biological activity of GS combines ammonium with glutamate to yield glutamine. This reaction is mimicked in the synthetase assay, in which hydroxylamine is substituted for ammonium to yield the product γ-glutamyl-hydroxamate, which can be quantified spectrophotometrically. Samples of 0.2 g FW of tissue were extracted in 1.6 ml of buffer containing 200 mM Tris buffer (pH 7.8), 2 mM EDTA, 3 mM dithiothreitol (DTT), 10 μM flavin adenine dinucleotide (FAD), 10 mM MgCl, 2% (w/v) casein, 10% (v/v) glycerol and 0.1 g polyvinylpyrrolidone (PVP). The homogenized plant material was centrifuged at 30 000 *g*, at 4ºC for 15 min. 100 μl of the enzyme extract were added to 250 μl of assay medium containing 18 mM ATP, 45 mM $MgCl_2 \cdot 6H_2O$, 25 mM hydroxylamine, 92 mM L-glutamate, and 50 mM imidazole HCl (pH 7.2), at 30ºC. After 20 min, the reaction was stopped by the addition of 0.5 mL of ferric chloride reagent (0.37 M ferric chloride, 0.67 M HCl and 0.2 M trichloroacetic acid). The reaction solution was then centrifuged, and the absorbance of the supernatant was read at 540 nm.

Data analysis

In order to meet the ANOVA criteria of equal variance, data were square root transformed. Two-way ANOVAs were used to test the effects of nitrogen form and nitrogen concentration on nitrogen uptake rates. Three tests were done: 1) the effects of the presence of both nitrogen forms in the medium on the ammonium uptake rate (ammonium plus nitrate vs ammonium alone), 2) the effects of the presence of both nitrogen forms in the medium on the nitrate uptake rate (ammonium plus nitrate vs nitrate alone) and 3) the effects of nitrogen form on the nitrogen uptake rate (ammonium alone vs nitrate alone). To compare the ammonium and nitrate uptake rates when both nitrogen forms are present in the medium, a t-test was used for each concentration. Differences among enzyme activities in leaves and roots under several nutrient concentrations were tested using two-way ANOVAs. Post hoc, multiple comparison analyses were done using Tukey tests. All comparisons were considered significant at p-values lower than 0.05 (Fowler and Cohen, 1990).

Results

The ammonium uptake rate was, on average, 10 times higher than the nitrate uptake rate, when both nitrogen forms were supplied (Fig. 2A, $p < 0.001$). The ammonium uptake rate increased sharply from 5 µM medium concentration to 25 and 50 µM, although rates were not significantly different at 25 and 50 µM. No significant differences in the nitrate uptake rate were observed among concentrations, even though there was an increasing tendency. In plants supplied only with nitrate (Fig. 2B), uptake rates were higher ($p <$

0.001) than when plants were supplied with both nitrogen forms (Fig. 2A) and increased significantly with nutrient concentration ($p < 0.001$).

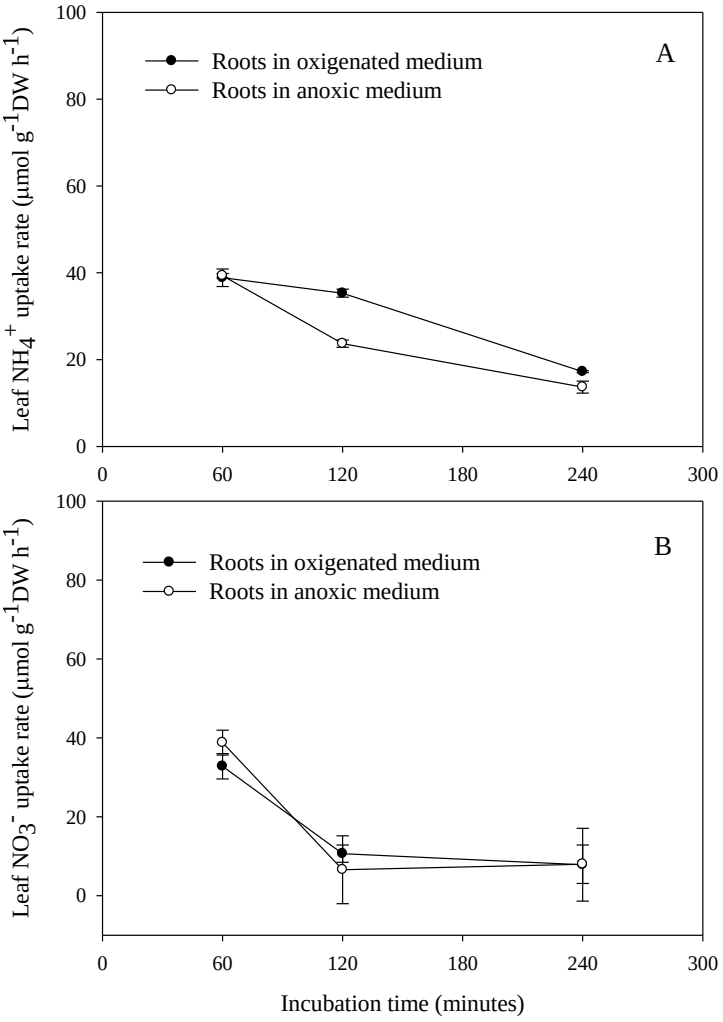

Figure 1. *Zostera noltii*. Effect of anoxic versus oxygenated conditions in the root medium on leaf uptake rates of ammonium (A) and nitrate (B). The effect was tested using split-chambers where roots + rhizomes were incubated separately from leaves, either in anoxic or in oxygenated media. Anoxic conditions were achieved by bubbling N_2 into the medium for five minutes. Ammonium and nitrate were supplied separately to the leaf chamber at an initial concentration of 90 µM. Values are mean ± SD (n = 3).

In plants supplied solely with ammonium (Fig. 2C), uptake rates also increased significantly with medium concentration ($p < 0.001$). Contrary to nitrate, ammonium uptake rates were lower than when both nitrogen forms were present (Fig 2A, $p < 0.001$). Nitrate and ammonium uptake rates were similar when only one form of nitrogen was supplied, except at low substrate concentrations where ammonium uptake was almost 10 times faster than the uptake of nitrate.

The activity of NR and GS enzymes was much higher in the leaves than in the roots (Fig. 3, $p < 0.001$). The enzyme activities were, respectively, 40-fold and 12-fold higher in the leaves than in roots. No significant effects of nitrogen concentration were detected in NR and GS activities, both in the leaves and roots, even though there was a tendency for leaf NR activity to peak at 25 μM and an increasing tendency of leaf GS activity with ammonium concentration.

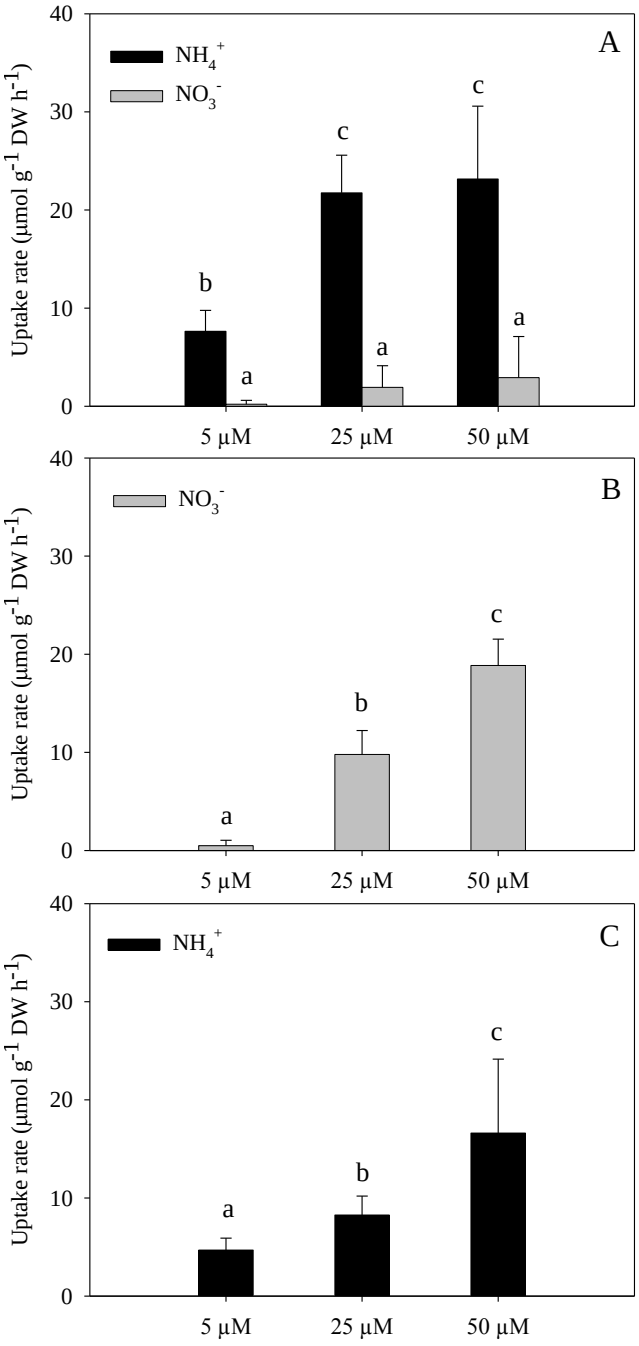

Figure 2. *Zostera noltii*. Effects of nitrogen source availability ((A) NH_4NO_3; (B) KNO_3; (C) NH_4Cl) on ammonium (black bars) and nitrate (grey bars) uptake rates at different nutrient concentrations. Values are mean ± SD (n = 9). Different letters indicate significant differences between treatments.

Discussion

The results of this study clearly show that ammonium is the preferential inorganic nitrogen source for the seagrass *Zostera noltii*, similarly to what is described for seagrasses elsewhere (e.g. Iizumi and Hattori, 1982; Short and McRoy, 1984; Hemminga et al., 1994; Lee and Dunton, 1999; Cornelisen and Thomas, 2004; Hasegawa et al., 2005). This finding is clearly supported by the results of the nutrient uptake experiments. When both inorganic nitrogen sources are available, *Z. noltii* relies preferentially on ammonium as the main nitrogen source, probably because this assimilation process is energetically less expensive than the nitrate uptake pathway (Turpin, 1991). However, we showed that *Z. noltii* has a remarkable nitrate uptake capacity when nitrate is the only nitrogen form available. Maximum uptake values will probably be higher than those found here as both the ammonium and nitrate uptake rates increased almost linearly with nutrient concentration (up to 50 μM), with the exception of the ammonium uptake rate of plants incubated in NH_4NO_3, which displayed saturation kinetics.

The nitrate uptake rate decreased when ammonium was present in the incubation medium, an effect that was also observed in other seagrass species (Iizumi and Hattori, 1982), aquatic plants (Thursby and Harlin, 1984; Dudley et al., 2001), seaweeds (Thomas and Harrison, 1987; Berges et al., 1995; Smit et al., 1997; Rees et al., 2007) and crop plants (Kronzucker et al., 1999). In this study, the presence of ammonium decreased the nitrate uptake rate by 40% at 5 μM NH_4NO_3 and by 85% at 50 μM NH_4NO_3 when compared to the nitrate uptake rates measured in the absence of ammonium. An inhibition mechanism mediated by a product of ammonium assimilation (e.g. glutamine) or a repression of the

active transport of nitrate across the membrane roots have been suggested to explain the reduced uptake of nitrate in the presence of ammonium among higher plants and seagrasses (Flynn, 1991; Iizumi and Hattori, 1982).

When nitrate was the sole nitrogen form available, its uptake rate by *Z. noltii* increased linearly with concentration and showed no evidence of saturation (product feedback inhibition), within the concentrations tested. This shows that nitrate is an effective alternative nitrogen source to ammonium, when it is available at high concentrations (25 µM and 50 µM). In Ria Formosa lagoon, such high concentrations are usually found near urban wastewater discharges (Cabaço et al., 2008). A linear relationship between nitrate uptake and concentration was also observed in *Z. marina* leaves incubated up to 25 µM of nitrate (Iizumi and Hattori, 1982), but not in *Thalassia testudinum* leaves exposed up to 160 µM (Lee and Dunton, 1999).

Interestingly, *Z. noltii* plants supplied with both ammonium and nitrate showed higher ammonium uptake rates than plants supplied with ammonium alone. This is probably because the presence of nitrate may alleviate ammonium toxicity, which has been determined to be above 25 µM for *Z. noltii*, with negative effects on the species survival and growth (Brun et al., 2002). The alleviation of ammonium toxicity by the addition of nitrate has been observed in crop plants (Feng and Barker, 1992; Adriaanse and Human, 1993; Kronzucker et al., 1999), and is related to the possible role of nitrate as a signal that optimizes several biochemical responses. In rice, plasma membrane fluxes of ammonium, cytosolic ammonium accumulation and ammonium metabolism are enhanced in the presence of nitrate. Additionally, the effect of rhizospheric alkalinization produced by the

nitrate uptake may help to limit the acidification associated with ammonium nutrition (Britto and Kronzucker, 2002).

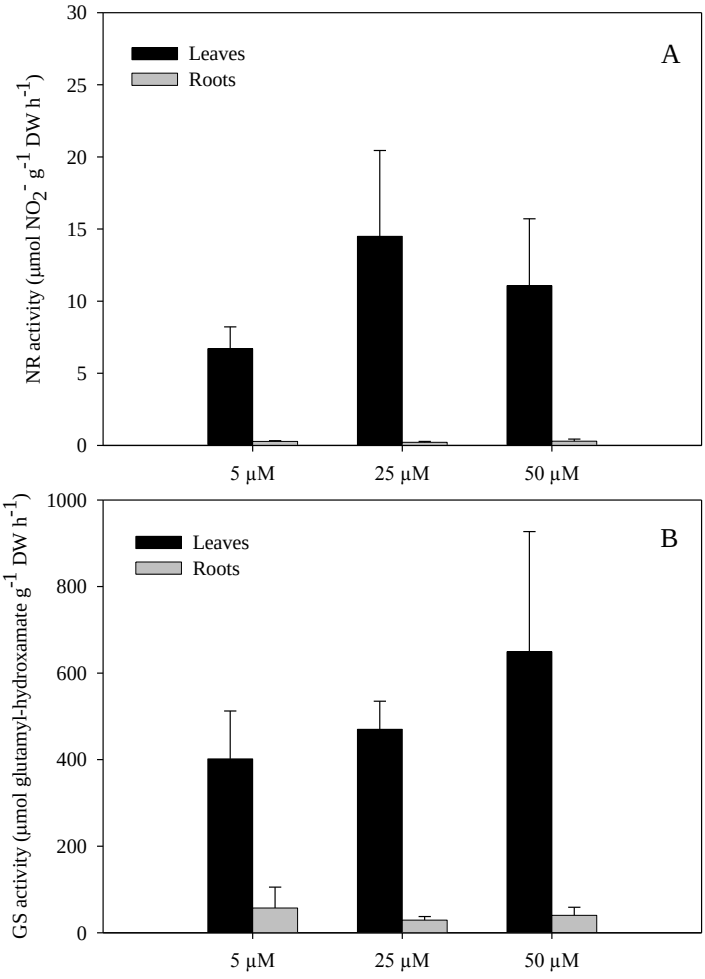

Figure 3. *Zostera noltii*. The activity of nitrate reductase (A) and glutamine synthetase (B) in leaves and roots of plants incubated in NH_4NO_3 at three different concentrations. Values are mean ± SD (n = 3).

The activity of both nitrate reductase and glutamine synthetase was much higher in the leaves than in the roots, as have also been observed in other seagrasses (Roth and Pregnall, 1988; Kraemer et al., 1997; Kraemer and Mazzella, 1999; Invers et al., 2002; Touchette and Burkholder, 2007). These results highlight the importance of leaves as primary reducing sites of either nitrate or ammonium in the nitrogen assimilation process. *Z. noltii* leaf NR activity, measured *in vivo*, at 5 µM NH_4NO_3 (1.2 µmol $g^{-1}FW\ h^{-1}$, this study) was higher than that of *Z. marina* at less than 2 µM (< 0.5 µmol $g^{-1}FW\ h^{-1}$) (Touchette and Burkholder, 2007). Similarly, *Z. noltii* leaf GS activity, measured *in vitro*, at 5 µM NH_4NO_3 (72 µmol $g^{-1}FW\ h^{-1}$, this study) was higher than in both *Z. marina* (40 µmol $g^{-1}FW\ h^{-1}$) and *Cymodocea nodosa* (< 50 µmol $g^{-1}FW\ h^{-1}$, Kraemer and Mazzella, 1999; Touchette and Burkholder, 2007), suggesting a higher nitrogen assimilation capacity of *Z. noltii* leaves than in other seagrasses. *Z. noltii* leaf GS activity is within the range of that found for the same species in the Mediterranean (50-150 µmol $g^{-1}FW\ h^{-1}$) (Kraemer and Mazzella, 1999). On the other hand, *Z. noltii* root GS activity at 5 µM (10 µmol g^{-1} $FW\ h^{-1}$, this study) was lower than *Z. marina* (20 µmol $g^{-1}FW\ h^{-1}$), but higher than *Cymodocea nodosa* (< 0.4 µmol $g^{-1}FW\ h^{-1}$) (Kraemer and Mazzella, 1999; Touchette and Burkholder, 2007). The GS activity in roots of *Z. noltii* from Ria Formosa lagoon was higher than for the Mediterranean ecotype (i.e. < 4 µmol $g^{-1}FW\ h^{-1}$) (Kraemer and Mazzella, 1999).

In conclusion, *Z. noltii* plants clearly prefer ammonium over nitrate as the main inorganic nitrogen source. Despite this preference, the plants also possess an important capacity to take up nitrate in the absence of ammonium. The activity of NR and GS enzymes was much higher in the leaves than in the roots, highlighting the importance of the leaves as the primary reducing sites in the nitrogen assimilation process. Whether the

uptake of inorganic nitrogen is made preferentially by the leaves or it is translocated from the roots to the leaves, is a question to be solved.

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Inorganic nitrogen uptake kinetics and whole-plant nitrogen budget in the seagrass *Zostera noltii*

Ana Alexandre, João Silva, Tjeerd J. Bouma, Rui Santos (2011)

Journal of Experimental Marine Biology and Ecology 401: 7-12

Inorganic nitrogen uptake kinetics and whole-plant nitrogen budget in the seagrass *Zostera noltii*

Abstract

The uptake rates of ammonium and nitrate through the leaves and roots, the leaf-root interactions in the nitrogen uptake and the internal translocation of incorporated nitrogen were simultaneously investigated in the seagrass *Zostera noltii*. Leaf and root uptake rates, which were measured using two-compartment polyethylene chambers that physically separated the leaves from below-ground plant parts, were quantified based on tissue incorporation of ^{15}N-labeled ammonium and nitrate. The maximum leaf uptake rates (V_{max}) of ammonium were 100 times higher than those of nitrate. Both V_{max} and affinity for ammonium were one order of magnitude higher in the leaves (28.3 - 31.9 μmol g^{-1}DW h^{-1} and 0.93 - 0.99, respectively) than in the roots (2.3 - 3.0 μmol g^{-1}DW h^{-1} and 0.06 - 0.08, respectively). The uptake of ammonium and nitrate by one plant part did not affect the uptake of the other plant part, and no translocation of inorganic nitrogen was detected between plant parts. The ^{15}N enrichment detected in the rhizomes suggests either a direct uptake of inorganic nitrogen or its transference from the roots. The estimated total inorganic nitrogen uptake of *Z. noltii* (645 μmol m^{-2} h^{-1}) in the peak production season (spring) under typical nutrient concentrations, using the rates obtained during the surge uptake phase, exceeded by 3-fold the species estimated nitrogen requirement for growth (236 μmol N m^{-2} h^{-1}). However, using the stabilized values of the uptake rates obtained after several hours of incubation, the estimated whole-plant nitrogen budget (215 μmol m^{-2} h^{-1}) was slightly lower than the total nitrogen requirement for growth. We conclude that the

growth of *Z. noltii* in Ria Formosa lagoon is not limited, or is only slightly limited, by nitrogen.

Keywords: nitrogen, seagrass, translocation, uptake, *Zostera noltii*.

Introduction

Inorganic nitrogen sources such as ammonium and nitrate can be incorporated by seagrasses both through the leaves and through the roots (Stapel et al., 1996; Lee and Dunton, 1999; Touchette and Burkholder, 2000). The potential for rhizome uptake of nutrients has never been demonstrated as experimental procedures do not separate both components (i.e. rhizomes and roots). Ammonium uptake through the roots is often considered the major source of inorganic nitrogen uptake in seagrasses because the concentration of this nutrient in the sediment porewater is much higher than in the water column. However, leaf uptake rates can also contribute considerably to fulfill the total nitrogen requirement of seagrasses (Hemminga et al., 1994; Hasegawa et al., 2005). Physiological interactions between leaves and roots, i.e. whether the nutrient uptake rate by one plant part influences the nutrient uptake rate of the other plant part, were demonstrated for some seagrass species (Iizumi and Hattori, 1982; Thursby and Harlin, 1982; Thursby and Harlin, 1984). These studies showed that the uptake rate of either leaves or roots were affected by the availability of ammonium to the opposite plant part, and suggested the existence of internal translocation of ammonium between plant parts. However, the direct assessment of inorganic nitrogen translocation between leaves and roots following the distribution of the incorporated ^{15}N through leaves and roots to the opposite plant part was

done only twice (Iizumi and Hattori, 1982; Vonk et al., 2008). To our best knowledge the study developed here is the first one investigating simultaneously the three major processes involved in the uptake kinetics of inorganic nitrogen in seagrasses: i) the relative uptake rates of ammonium versus nitrate through the leaves and through the roots, ii) the leaf-root interactions in the nitrogen uptake and iii) the internal translocation of incorporated nitrogen. Specific uptake rates of leaves and roots of *Zostera noltii* of Ria Formosa lagoon (South Portugal) and the translocation of incorporated nitrogen between them were assessed in split-chambers that physically separated plant parts, through the incorporation of ^{15}N in the tissues using labeled ammonium and nitrate. The incorporation of ^{15}N in rhizomes was also measured to assess their potential to receive and accumulate inorganic nitrogen translocated internally from leaves or roots. Measured nitrogen uptake capacity was compared to the nitrogen requirement for growth during spring, when the species growth rate is highest (Peralta et al., 2005), to gain insight in the whole-plant nitrogen budget for *Z. noltii*.

Methods

Site description and plant material

Zostera noltii is the most abundant seagrass species in Ria Formosa coastal lagoon, South Portugal. This species develops along the intertidal flats and plays a major role in determining the lagoon's metabolism (Santos et al., 2004). Ammonium and nitrate concentration in the water column is usually less than 5 µM due to a high water exchange between the lagoon and the adjacent ocean in each tidal cycle. The inorganic nitrogen concentrations in the adjacent ocean waters are typically less than 1 µM. Ammonium

concentration in the sediment pore water is higher (12 - 38 µM), whereas nitrate concentration is almost negligible (0.2 - 0.9 µM). Above-ground biomass density at the site of plant collection in spring averaged 120 g DW m^{-2}, whereas below-ground biomass density averaged 150 g DW m^{-2} (Cabaço et al., 2008). *Z. noltii* plants were collected from an intertidal meadow during the spring of 2009. In the laboratory, the roots were carefully cleaned of adherent sediment avoiding damage of the roots hairs, and the leaves were cleaned of epiphytes. The plants were acclimated in lagoon water for two days at the same temperature and light conditions of the experiment.

Experimental procedure

Ammonium and nitrate uptake rates were measured separately in leaves and roots using two-compartment, polyethylene, cylindrical chambers that physically separated the above-ground from the below-ground plant parts. The volumes of the upper (leaves) and lower (roots plus rhizomes) compartments were 1.1 L and 0.55 L, respectively. Leakage between compartments was avoided using molding clay and sterile vaseline as sealants. The incubation medium in the leaf compartment was constantly mixed using a peristaltic pump creating a flow rate of ≈ 250 ml min^{-1}. On the other hand, root medium was mixed using a magnetic stirrer spinning slowly to avoid the damage of the delicate roots and root hairs, which are particularly important for nutrient absorption. The mixing was done to ensure a homogeneous concentration of the ^{15}N label throughout the incubation time.

In a first set of experiments, the ammonium or nitrate uptake rates of leaves were measured by incubating them during one hour in nitrogen free artificial seawater (35 PSU)

enriched with $^{15}NH_4Cl$ or $^{15}KNO_3$ (atom % = 99, Cambridge Isotope Laboratories), at four nutrient concentrations (5, 25, 50 and 100 µM). In this set of experiments the root compartment was left without nutrients. Incubations of the four nutrient levels were done simultaneously (one chamber for each nutrient concentration) and replicates (n = 3) were done sequentially. Incubations lasted one hour to maintain the nitrogen uptake rates at their highest. Experiments done subsequently to the present study where nutrient depletion in the medium was followed over time showed that after 1 hour the uptake rates decreased and stabilized at levels where rates were one third of the initial uptake rates. This decrease is related to an internal control over the uptake rates as the inorganic nitrogen is assimilated into organic molecules in the tissues (Harrison et al., 1989). Two plant modules (i.e. two shoots with respective rhizomes and roots) were incubated per chamber. The average leaf biomass per incubated shoot was 0.07 g DW, whereas the below-ground biomass was 0.05 g DW. The experiments were run in a walk-in culture chamber at constant temperature (14ºC) and light intensity (200 µmol quanta m^{-2} s^{-1}). The pH of the incubation media was adjusted to 8.1 using HCO_3^-. During the incubation period it was observed that the nutrient concentration in the media did not vary noticeably, i.e. the nutrient concentrations remained constant through the experiment. The ammonium and nitrate uptake rates of roots were determined using a similar procedure as for the leaves. Nitrogen enrichment was added to the root compartment, leaving the leaves in a nutrient-free medium. In a second set of experiments we studied leaf-root interactions by measuring the effects of ammonium (or nitrate) availability to a plant part on the ammonium (or nitrate) uptake rate of the other part. These experiments were performed similarly to the leaf and root uptake experiments, except that 50 µM of unlabeled ammonium (or nitrate) was added to the root compartment

when measuring leaf uptake or to the leaf compartment when measuring root uptake. Leaf-root interactions were analyzed by comparing these uptake rates to those obtained in the first set of experiments. To study the third process relevant to the nitrogen uptake, the internal translocation of labeled nitrogen (ammonium or nitrate) was determined in all experiments. The amount of ^{15}N that was recovered on the non-incubated (opposite) plant part was compared to the total amount of ^{15}N taken up (as atom %) by leaves and roots.

At the end of incubations, the plants were removed from the chambers, the leaves were immediately separated from the rhizomes and roots and the tissues were briefly rinsed with deionized water to remove adherent label. Tissues were dried at 60ºC for 48 h and reduced to a fine powder. Total nitrogen content and atom % ^{15}N of dried tissues were determined using a Thermo EA 1112 elemental analyzer coupled to a Thermo Delta V advantage isotope ratio mass spectrometer with a Conflo II interface (EA-IRMS). Leaf, root and rhizome ^{15}N background levels were measured in five replicate samples. Even though in natural conditions the rhizosphere of *Z. noltii* is mostly anoxic, in these experiments we incubated the whole plants in an oxygenated medium. Previous experiments reported elsewhere (Alexandre et al., 2010) showed no effects of rhizosphere oxygenation on the ammonium and nitrate uptake rates of leaves. Recent experiments have also showed that the ammonium uptake rates of *Z. noltii* roots incubated in anoxic seawater (purged with N_2) enriched with 30 µM of $^{15}NH_4Cl$ (1.25 ± 0.42 µmol $g^{-1}DW\ h^{-1}$) were not significantly different from the rates of roots incubated in oxygenated seawater (1.49 ± 0.50 µmol $g^{-1}DW\ h^{-1}$) (n = 3).

Data analysis

^{15}N enrichment (g) of tissues after incubations were calculated by subtracting the post-incubation ^{15}N levels from the initial background levels and multiplying by the total nitrogen content in the tissue (%) and by its weight (g DW). Nitrogen uptake rates were expressed in µmol N g^{-1} DW h^{-1}. Uptake rates were plotted against substrate concentration (µM) and the uptake kinetic parameters were derived using the Michaelis-Menten model

$$V = (V_{max} \times S) / (K_m + S)$$

where V is uptake rate (µmol g^{-1} DW h^{-1}), V_{max} is maximum uptake rate (µmol g^{-1} DW h^{-1}), S is substrate concentration (µM) and K_m is the half-saturation constant (µM).

To test for differences in the uptake rates by leaves and roots when the opposite plant part was exposed or not to the nutrient, the Michaelis-Menten model was Hanes-Woolf transformed (i.e. data were plotted as S/V against S) to obtain linearity, where S is substrate concentration and V is uptake rate. The differences between the slopes of the regression lines were evaluated using a t-test with a level of significance of 0.05 (n = 12) (Fowler and Cohen, 1990).

A whole-plant nitrogen budget of *Z. noltii* was developed based on the inorganic nitrogen uptake rates and the estimated nitrogen requirement for growth of each plant part. The uptake rates of ammonium and nitrate (µmol g^{-1} DW h^{-1}) through the leaves and the roots were calculated from the Michaelis-Menten equations obtained for each plant part at the range of natural nutrient concentrations in the lagoon. The value of 5 µM of ammonium and nitrate concentration in the water column was used to estimate the ammonium and

nitrate uptake rates by the leaves. The ranges of 12 - 38 µM of ammonium and 0.2 - 0.9 µM of nitrate concentrations in the sediment porewater were used to estimate the uptake rates thought the roots. These uptake rates were expressed per unit of surface area (µmol m^{-2} h^{-1}) using the spring biomass density (g DW m^{-2}) of the natural meadows where the plants were sampled (Cabaço et al., 2008) following the equation

$$V_{amb} = (V_{max} \times S_{amb}) / (K_m + S_{amb}) \times B$$

where V_{amb} is the ammonium or nitrate uptake rate at natural nutrient concentration (µmol g^{-1} DW h^{-1}), S_{amb} is the natural water column or sediment porewater nutrient concentration (µM) and B is the above- or below-ground biomass density (g DW m^{-2}). The inorganic nitrogen requirement for growth (µmol m^{-2} h^{-1}) of the above- and below-ground plant parts during spring, when the uptake experiments were performed, was calculated based on the species specific growth rates (mg DW h^{-1}) measured by Peralta et al. (2005) in Ria Formosa, combined with current measurements on the total nitrogen content (%) of the plant parts and the biomass density (g DW m^{-2}) of the respective plant parts, following the equation

$$N_{req} = GR \times TN \times B$$

where N_{req} is the nitrogen requirement for growth (µmol m^{-2} h^{-1}), GR is the specific growth rate (mg DW h^{-1}), TN is the total nitrogen content of the above- or below-ground tissues (%) and B is the biomass density of the respective plant part (g DW m^{-2}).

Results

Zostera noltii leaves showed much higher ammonium uptake rates than the roots (Fig. 1). On the other hand, the nitrate uptake rates by the leaves were only slightly higher than by the roots (Fig. 2). The ammonium and nitrate uptake followed Michaelis-Menten kinetics (Figs. 1 and 2A) except the nitrate uptake by the roots, which was best described by a linear regression model (Fig. 2B). The maximum ammonium uptake rate (V_{max}) of leaves (28.3 µmol g^{-1}DW h^{-1}) was one order of magnitude higher than of roots (3 µmol g^{-1} DW h^{-1}) (Table 1). As well, the ammonium uptake affinity (V_{max} / K_m) of leaves was one order of magnitude higher (0.99) than of roots (0.06). The maximum nitrate uptake rate and nitrate uptake affinity of leaves (0.26 µmol g^{-1}DW h^{-1} and 0.04, respectively) were considerably lower than those of ammonium. No significant differences were detected between the slopes of the linear regressions of the Hanes-Woolf plots (t = 0.72, 1.59, 1.38 and 0.50, df = 20 for leaf ammonium, root ammonium, leaf nitrate and root nitrate uptake, respectively) (Fig. 1 and 2, right insets), which indicates that ammonium and nitrate uptake rates of both leaves and roots were not affected by the exposure of the opposite plant part to the nutrient. No substantial inorganic nitrogen translocation occurred between leaves and roots. Less than 1% of the total inorganic nitrogen incorporated either by the leaves or the roots was translocated to the other plant part. Rhizome tissues showed some ^{15}N enrichment when both roots and rhizomes were incubated in labeled ammonium or nitrate. This enrichment corresponded respectively to 40% and 30% of the total ammonium and nitrate incorporated by the roots. The presence of ^{15}N in the rhizomes represents either direct uptake of inorganic nitrogen through the rhizomes or translocation from roots to rhizomes.

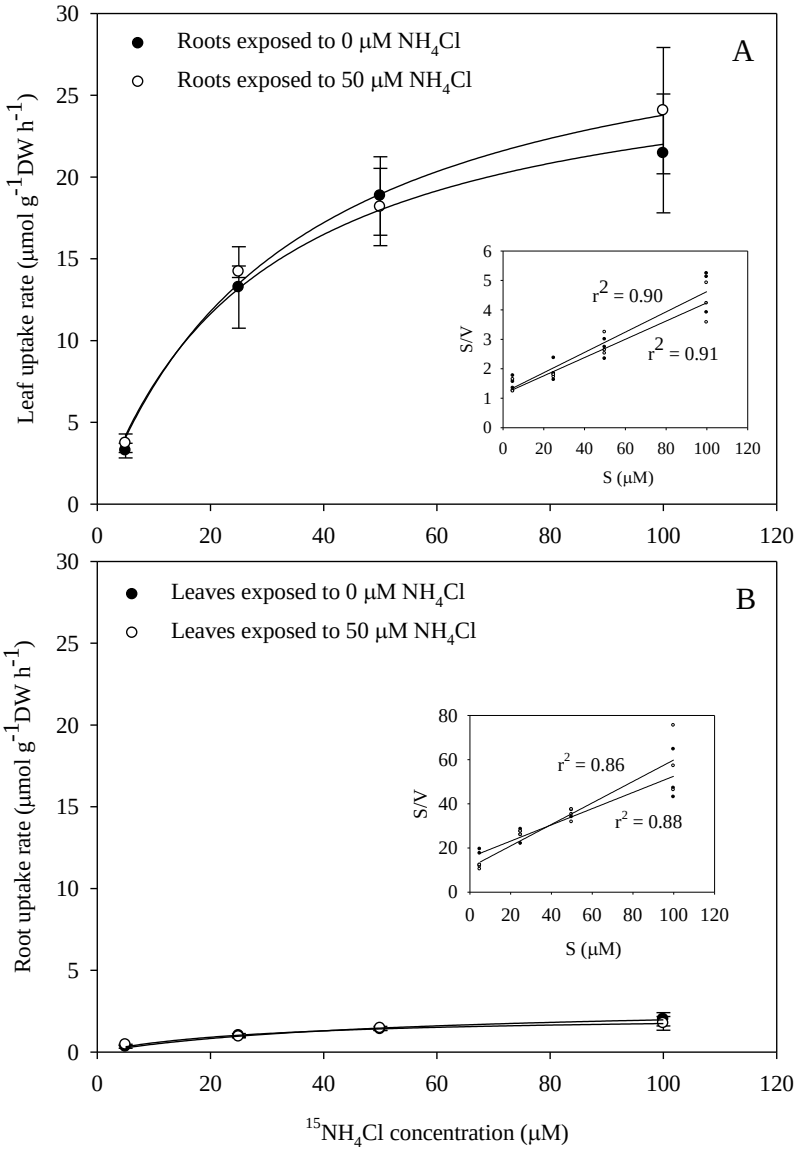

Figure 1. *Zostera noltii*. Ammonium uptake rates (μmol g^{-1} DW h^{-1}) of leaves (A) and roots (B) as a function of $^{15}NH_4Cl$ concentration (μM), with (○) and without (●) the opposite plant part exposed to unlabeled NH_4Cl. The curves represent the best fit of the Michelis-Menten model. Data were re-plotted as S/V against S to obtain linearity (right insets) and adjusted using linear regression (S = Concentration; V = Uptake rate).

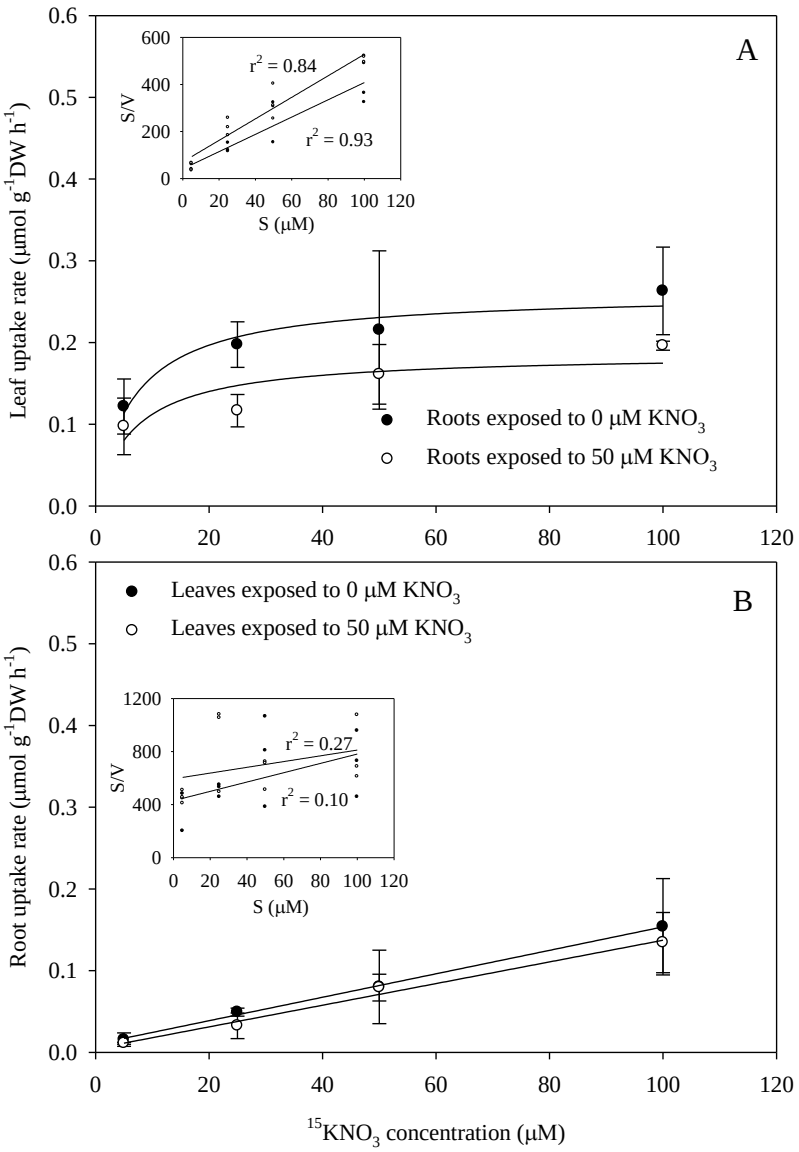

Figure 2. *Zostera noltii*. Nitrate uptake rates (µmol g^{-1} DW h^{-1}) of leaves (A) and roots (B) as a function of $^{15}KNO_3$ concentration (µM), with (○) and without (●) the opposite plant part exposed to unlabeled KNO_3. The curves represent the best fit of the Michelis-Menten model. Data were re-plotted as S/V against S to obtain linearity (right insets) and adjusted using linear regression (S = Concentration; V = Uptake rate).

The ammonium uptake rate through the leaves and the roots, as calculated from the Michaelis-Menten equations using the ranges of ambient ammonium concentrations in the water column and in the sediment porewater and the biomass density, was 480 µmol m^{-2} h^{-1} and 82.5 - 189 µmol g^{-1}DW h^{-1}, respectively (Table 2).

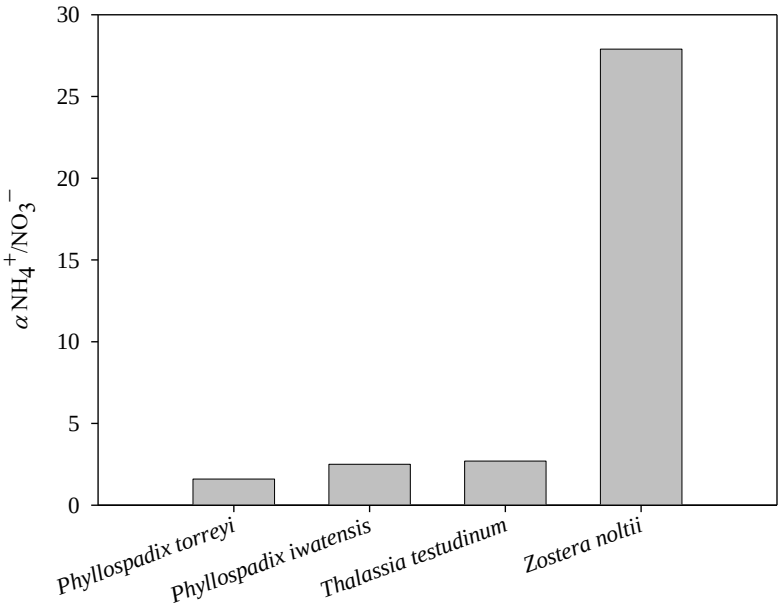

Figure 3. Relative uptake efficiencies ($\alpha = V_{max} / K_m$) of ammonium over nitrate for four seagrass species. The affinity coefficients α were calculated from the data presented in Table 2.

Table 1. *Zostera noltii*. Effects of nitrogen availability (0 or 50 µM) to a plant part on the uptake kinetics of the opposite part: maximum uptake rate V_{max} (µmol g^{-1} DW h^{-1}), half-saturation constant K_m (µM), and affinity constant α (V_{max} / K_m) of ammonium and nitrate in leaves and roots. The coefficient of determination (r^2) and the standard error of the estimates (in brackets) are given. Data not displaying saturation kinetics were fitted with linear regression model (V = uptake rate, S = substrate concentration).

	V_{max}	K_m	α	r^2	p
Ammonium					
Leaves (Roots 0 µM)	28.33 (2.35)	28.73 (6.60)	0.9	0.99	0.005
Leaves (Roots 50 µM)	31.91 (2.28)	34.17 (6.29)	0.93	0.99	0.003
Roots (Leaves 0 µM)	3.00 (0.31)	52.52 (11.69)	0.06	0.99	0.004
Roots (Leaves 50 µM)	2.27 (0.28)	29.99 (10.01)	0.08	0.98	0.013
Nitrate					
Leaves (Roots 0 µM)	0.26 (0.02)	6.48 (2.31)	0.04	0.93	0.036
Leaves (Roots 50 µM)	0.19 (0.03)	6.61 (5.04)	0.03	0.96	0.02
Roots (Leaves 0 µM)	V = 0.0014S	-	-	0.99	0.0008
Roots (Leaves 50 µM)	V = 0.0013S	-	-	0.99	0.0059

In a similar manner, it can be calculated that under field conditions, the nitrate uptake rate through the leaves would be 30 µmol m^{-2} h^{-1}, whereas through the roots it would be negligible (0.05 - 0.20 µmol m^{-2} h^{-1}). The ammonium uptake through the leaves accounted for 74 % of the total inorganic nitrogen acquisition, whereas the uptake through the roots accounted only for 21 %. The nitrate uptake through the leaves and the roots accounted for less than 5 % of the total nitrogen uptake. The total inorganic nitrogen uptake of *Z. noltii*, i.e. the sum of the ammonium and nitrate uptake through leaves and roots, was 645 µmol m^{-2} h^{-1}. The inorganic nitrogen requirement for growth calculated for *Z. noltii* leaves during its most productive season was 140 µmol N m^{-2} h^{-1}, whereas for the below-ground plant part was 96 µmol N m^{-2} h^{-1}.

Table 2. Whole-plant inorganic nitrogen budget for *Zostera noltii* growing in spring in Ria Formosa. Calculations of N-supply were derived from the inorganic nitrogen uptake rates of leaves and roots at the range of natural nutrient concentrations and plant biomass density. Calculations of N-demand were based on total nitrogen content, plant biomass density and the species growth rate. Values in brackets express the uptake fraction of the overall N uptake that accounts for the respective plant part.

Plant part	NH_4^+ uptake rate (µmol m^{-2} h^{-1})	NO_3^- uptake rate (µmol m^{-2} h^{-1})	N requirement for growth (µmol m^{-2} h^{-1})
Leaves	480 (74.3 %)	30 (4.6 %)	140
Roots	82.5 - 189 (21.1 %)	0.05 - 0.02 (0.02 %)	96

Table 3. Nitrogen uptake kinetic parameters of seagrass species, adapted and updated from Touchette and Burkholder (2000). The parameters were estimated based on the Michaelis-Menten model (V_{max} = maximum uptake rate in µmol g^{-1} DW h^{-1}; K_m = half-saturation constant in µM; α (V_{max} / K_m) = affinity coefficient).

Species	Nutrient	V_{max}	K_m	α	Source
Leaves					
A. antarctica	NH_4^+	5.9 - 43.1	9.5 - 74.3	0.6 - 0.8	Pedersen et al. (1997)
P. iwatensis	NH_4^+	2.2 - 35.5	12.7- 133.5	0.12 - 0.28	Hasegawa et al. (2005)
P. torreyi	NH_4^+	95.6 - 204.3	9.3 - 33.9	-	Terrados & Williams (1997)
R. maritima	NH_4^+	243 - 270	9.0 - 17.7	5.5	Thursby & Harlin (1984)
T. hemprichii	NH_4^+	32 - 37	21 - 60	0.52 - 0.85	Stapel et al. (1996)
T. testudinum	NH_4^+	8.3 - 16.4	7.6 - 15	0.57 - 2.82	Lee & Dunton (1999)
Z. marina	NH_4^+	20.5	9.2	2.2	Thursby & Harlin (1982)
Z. noltii	NH_4^+	28.3 - 31.9	28.7 - 34.2	0.93 - 0.99	This study
P. iwatensis	NO_3^-	1.0 - 2.1	13.9 - 21.1	0.05 - 0.11	Hasegawa et al. (2005)
P. torreyi	NO_3^-	24.9 - 75.4	4.4 - 17.0	-	Terrados & Williams (1997)
T. testudinum	NO_3^-	3.7 - 6.5	2.2 - 38.5	0.15 - 1.68	Lee and Dunton (1999)
Z. noltii	NO_3^-	0.19 - 0.26	6.48 - 6.61	0.03 - 0.04	This study

Roots					
A. antarctica	NH_4^+	1.1	4.7	0.2	Pedersen et al. (1997)
P.iwatensis	NH_4^+	0.5	61.3	0.01	Hasegawa et al. (2005)
R.maritima	NH_4^+	48 - 56	2.8 - 12.6	20.1	Thursby & Harlin (1984)
T. testudinum	NH_4^+	7.9 - 73.3	34.4 - 765.5	0.03 - 0.3	Lee & Dunton (1999)
Z. marina	NH_4^+	211	104	0.5	Thursby & Harlin (1982)
Z. noltii	NH_4^+	3.0	52.5	0.1	This study

Discussion

The results of this study demonstrated both the importance of ammonium as the major inorganic nitrogen source for *Zostera noltii* and of the leaves as the preferred site for the species ammonium uptake. A review of the existing literature on Michaelis-Menten kinetics for inorganic nitrogen uptake in seagrasses shows that most species present higher uptake rates for ammonium than for nitrate (cf. Touchette and Burkholder, 2000). The few available data also show that seagrass leaves have at least two times higher affinity for ammonium than for nitrate (Table 3). The affinity coefficient ($\alpha = V_{max} / K_m$) evaluates the efficiency of the nutrient uptake at low concentrations (Harrison et al., 1989). Of the four seagrass species for which the affinity coefficient was determined, *Z. noltii* stands out from the other three species by showing nearly thirty times more affinity for ammonium than for

nitrate (Fig. 3). Such affinity of *Z. noltii* leaves for ammonium makes this species particularly adapted to take up the pulses of ammonium observed from the sediment to the water column when the flood tide first covers the sediments that were exposed to the air during low tide (Falcão and Vale, 2003). The preference of ammonium over nitrate has also been demonstrated for *Z. noltii* elsewhere (Alexandre et al., 2010), but in this study the kinetic parameters of the nitrogen uptake were not determined. This preference is generally ascribed to the lower energetic cost associated with the uptake and assimilation of ammonium, comparatively to nitrate (Turpin, 1991; Bloom et al., 1992).

The few available studies on seagrass nitrogen uptake showed that ammonium uptake rates are usually higher in the leaves than in the roots (see Touchette and Burkholder, 2000 and references therein; Hasegawa et al., 2005), with only a few exceptions such as *Thalassia testudinum* (Lee and Dunton, 1999) and *Zostera marina* (Thursby and Harlin, 1982). Values of V_{max} for ammonium of *Z. noltii* leaves were much higher compared to roots, which reflect the species remarkable capacity to take up ammonium through the leaves. Additionally, the ammonium uptake affinity of *Z. noltii* leaves was much higher than that of roots. The high ammonium uptake rates in addition to the high ammonium affinity allows *Z. noltii* leaves not only to effectively take up pulsed ammonium at low concentrations but also to take advantage of transient high levels of ammonium in the water column.

The presence of physiological interactions between leaves and roots in seagrass nutrient acquisition was demonstrated for *Zostera marina* (Thursby and Harlin, 1982) and *Ruppia maritima* (Thursby and Harlin, 1984) with incubations that lasted for about 12 hours. In *Z. marina*, leaf ammonium uptake was not affected by the availability of

ammonium to the roots, but root uptake decreased significantly when leaves were exposed to ammonium (Thursby and Harlin, 1982). The authors suggested that basipetal translocation of ammonium dominated over acropetal translocation. On the other hand, in *R. maritima*, leaf ammonium and phosphate uptake was reduced when roots were exposed to these nutrients, but root uptake was not affected by the availability of these nutrients to the leaves, suggesting that acropetal translocation predominates in this species (Thursby and Harlin, 1984). On the contrary, with incubations that lasted between 1-5 hours there was no interactions between leaves and roots in nitrogen uptake in *Z. noltii* (this study), *Z. marina* (Short and McRoy, 1984), *Thalassia hemprichii* (Stapel et al., 1986) and *Phyllospadix torreyi* (Terrados and Williams, 1997). This raises the question that long incubations are needed to properly assess interactions between leaves and roots in seagrass nutrient acquisition.

As well, long incubations are probably needed to assess internal translocation. In the present study, no evident short-term (1h) basipetal or acropetal translocation of incorporated ammonium or nitrate was detected in *Z. noltii*, even when the incubation time was extended from 1 to 4 hours (data not shown). These results are similar to those found by Vonk et al. (2008) in *Thalassia hemprichii*, *Halodule uninervis* and *Cymodocea rotundata*, where less than 1% of the nitrogen incorporated by the leaves during 1h incubations was recovered in the below-ground plant parts. On the other hand, 8 to 20% of the nitrate incorporated by the roots was translocated to the leaves. Under longer incubation periods (24h), Iizumi and Hattori (1982) reported that most of the nitrogen incorporated by one plant part of *Z. marina* was recovered on the opposite plant part. The lack of interaction between leaves and roots in the uptake process and the absence of internal translocation of

incorporated nitrogen suggesting that *Z. noltii* leaves and roots act independently when acquiring inorganic nitrogen is thus hindered by the short term incubation times used (1- 4 h). However, *Z. noltii* may actually not need the internal translocation of inorganic nitrogen because both leaves and roots are able to take up inorganic nitrogen and to reduce it into organic nitrogen compounds (Alexandre et al., 2010).

A considerable ^{15}N enrichment of the rhizome tissues was observed when below-ground plant parts were incubated in labeled nitrogen. This enrichment represents either translocated nitrogen from the roots to the rhizomes and/or direct uptake through the rhizomes. The direct uptake of nitrogen by seagrass rhizomes has been frequently considered negligible (Stapel et al., 1996; Touchette and Burkholder, 2000) even though this hypothesis was never supported by experimental data. Our results suggest that the ^{15}N enrichment observed in *Z. noltii* rhizome tissues may originate from direct rhizome uptake, since the pattern of ^{15}N enrichment in the rhizome tissue varied with the nutrient concentration, both for ammonium and nitrate, similarly to the uptake rates of roots. This topic requires further studies to arrive at a conclusive answer.

The estimated total inorganic nitrogen uptake of *Z. noltii* (645 µmol m^{-2} h^{-1}) exceeded by 3-fold the species estimated nitrogen requirement for growth (236 µmol N m^{-2} h^{-1}). However, that uptake was estimated after one hour of incubation, which corresponds to the surge uptake phase, when uptake rates can exceed the requirement for growth several-fold (Harrison et al., 1989). A subsequent experiment to the present study, where both ammonium and nitrate uptake rates were followed over seven hours, showed that after the first hour of incubation, the uptake rates stabilized in values of approximately one third of the initial ones. The long term nitrogen uptake rate of *Z. noltii* will then be about 215 µmol

m^{-2} h^{-1}, a value slightly lower than the total nitrogen requirement for growth. We may conclude that the growth of *Z. noltii* in Ria Formosa lagoon is not limited, or it is only slightly, by nitrogen.

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Light versus dark nitrogen uptake of the seagrass *Zostera noltii*: integration with carbon metabolism

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To be submitted.

Light versus dark nitrogen uptake of the seagrass *Zostera noltii*: integration with carbon metabolism

Abstract

We showed here that *Zostera noltii* may take up ammonium and nitrate from the water column at high rates, both under light and dark conditions. This is an important advantage over some seaweed species where these rates are severely reduced at night. Furthermore, our results suggest that the nitrate uptake, at least during the day, requires the mobilization of starch whereas the uptake of ammonium does not. Both the ammonium and nitrate uptake rates of *Z. noltii* were similar in the light and in the dark. In the light, the ammonium uptake rates were initially higher (15 and 20 µmol g^{-1} h^{-1}) and stabilized at a rate of 5 µmol g^{-1} h^{-1} after 1h, whereas in the dark the rates remained at a constant rate of 10 µmol g^{-1} h^{-1} along the first 180 minutes of incubation. The nitrate uptake rates in the light were high within the first 120 minutes of incubation (7.2 - 11.1 µmol g^{-1} h^{-1}) and decreased afterwards to lower values (0.8 - 3.9 µmol g^{-1} h^{-1}), whereas in the dark, rates fluctuated around 0.0 - 11.1 µmol g^{-1} h^{-1} throughout the whole incubation time (7 hours). The soluble sugar content of *Z. noltii* leaves increased significantly during the light incubations of both ammonium and nitrate but not in the dark, indicating the metabolic outcome of photosynthesis. Contrary to expected, both the soluble sugar and the starch content of plants incubated in the dark with either ammonium or nitrate showed no significant reduction. However, the total starch content of plants decreased whereas the total soluble sugars increased, suggesting a process of starch catabolism to generate energy with the consequent production of smaller monosaccharide products. On the other hand, the

starch content of rhizomes decreased significantly during the light incubations with nitrate but not with ammonium. These results suggest that carbohydrate mobilization is necessary for *Z. noltii* to account for extra energetic costs needed for the uptake and assimilation of nitrate.

Keywords: carbohydrates, dark, light, nitrogen, seagrass, uptake.

Introduction

The incorporation of nitrogen by plants during the day is considered energetically less expensive than at night because the energy and carbon skeletons necessary for the assimilation process are provided directly by photosynthesis, whereas in the dark it involves the use of accumulated carbohydrates (Turpin, 1991; Huppe and Turpin, 1994). This must be particularly crucial for nitrate as its assimilation process is energetically more costly than the assimilation of ammonium because nitrate must first be reduced to nitrite and then to ammonium in reactions catalyzed by the enzymes nitrate reductase and nitrite reductase, respectively (Bloom et al., 1992; Lobban and Harrison, 1994). Consequently, the rates of nitrogen uptake and assimilation in the dark are expected to be lower than the rates in the light.

Diurnal changes in nitrogen uptake rates were described for terrestrial angiosperms and seaweed species. For example, the uptake rates of ammonium and nitrate in the dark were reduced by one-third to one-half of the rates measured during the day in the red and brown algae *Hypnea musciformis* and *Laminaria groenlandica* (Haines and Wheeler, 1978; Harrison et al., 1986). Similarly, the nitrogen uptake rates were reportedly higher during the

light period compared to darkness in angiosperm grasses (Macduff et al., 1997) and in tomato (Cárdenas-Navarro et al., 1998). In another study with tomato plants, the carbohydrate level increased in the leaves during the light periods and decreased at night (Magaña et al., 2009). The authors related the dark growth of these plants with the use of carbohydrates accumulated during the day, which were the main source of carbon for growth at night. In this species, growth was not restricted during darkness because there was sufficient carbohydrate reserves accumulated during the light period. In contrast, the ammonium uptake rates of three seaweed species (*Ulva lactuca*, *Soliera robusta* and *Dictyota dichotoma*) showed no diurnal variation, i.e. the rates of ammonium uptake in the light were similar to the rates in the dark (Raikar and Wafar, 2006). Surprisingly, the light versus dark uptake of inorganic nitrogen by seagrasses was assessed only twice, for *Zostera marina* and *Thalassia testudinum* (Iizumi and Hattori, 1982; Lee and Dunton, 1999). In these species, no diurnal variation was reported for the ammonium and nitrate uptake by the leaves or for the ammonium uptake by the roots. The role of stored carbohydrates in the nitrogen assimilation process has also been investigated. In *Zostera marina*, the activity of nitrate reductase in the leaves was sustained during darkness following the addition of nitrate, and the intensity and duration of the enzyme activity was directly related to the carbohydrate availability in the leaves (Touchette and Burkholder, 2001; Touchette and Burkholder, 2007). Barley seedlings containing higher carbohydrate levels also showed higher nitrate assimilation during darkness (Aslam and Huffaker, 1984).

Here we investigate the ammonium and nitrate uptake rates of the seagrass *Zostera noltii* in the light and in the dark at non-limiting nitrogen concentrations. The uptake rates were continuously recorded over time in plants exposed to initial high nutrient

concentrations. We hypothesize that the nitrogen uptake by *Z. noltii* during darkness may be low or even not any because the non-structural carbohydrate levels of both leaves and belowground tissues of *Z. noltii* are amongst the lowest compared to other seagrass species (Touchette and Burkholder, 2000). We also investigate how the ammonium and nitrate assimilation is related to the short-term carbon metabolism, i.e. the carbohydrate levels, in both light conditions. We hypothesize that the production of soluble sugars by *Z. noltii* under ammonium rich conditions will be higher and that the mobilization of starch will be lower when compared to nitrate rich conditions.

Methods

Zostera noltii plants were collected from an intertidal meadow of Ria Formosa lagoon, southern Portugal, in January 2010. The plants were cleaned of epiphytes and any adherent muddy sediment and were acclimated to experimental conditions for 2 - 3 days in filtered seawater (N_i levels < 5 µM) in a walk-in chamber under constant photoperiod (12h:12h) and temperature (17º C). The ammonium and nitrate uptake rates were determined over time using the perturbation method (Pedersen, 1994), in which the plants are incubated in a relatively high initial nitrogen concentration and the depletion of nitrogen from the medium is continuously recorded over short time intervals. The uptake rates are estimated for each time interval and plotted against time. In these experiments, whole plants were incubated in an oxygenated medium. Even though the rhizosphere of *Z. noltii* is mostly anoxic in its natural environment, previous experiments showed no effects of rhizosphere oxygenation in the ammonium and nitrate uptake by the leaves (Alexandre et al., 2010). The uptake rates resulting from incubating whole plants not separating the

rhizosphere from the leaves will reflect mostly the leaf uptake rates as those of the rhizosphere are very low (Alexandre et al., 2011).

Plants were incubated in transparent cylindrical containers (n = 3, 20 plants per container), each filled with 1.5 L of filtered seawater enriched with NH_4Cl or KNO_3 added separately to achieve initial concentrations of 95 µM or 65 µM, respectively. The nitrogen pulse was added to the medium approximately one hour after the onset of the light cycle. Light incubations were performed at constant saturating light intensity (300 µmol quanta m^{-2} s^{-1}) (Silva et al., 2005). The incubation media were continuously homogenized in an orbital shaker (125 rpm). The biomass to volume ratio in each container ranged 1.5 - 3 g fresh weight L^{-1}, whereas the above:below-ground biomass ratio of incubated plants averaged 1.5. The depletion of ammonium or nitrate from the medium was followed throughout time by removing triplicate 10 ml - aliquots from each container at specific sampling times (15, 30, 60, 120, 240, 300 and 420 minutes after nitrogen addition). Control media without plants showed non-significant variation (< 1%) in the initial nitrogen concentration during the incubation period. The experimental procedure described above was repeated for the dark incubations. These were initiated with the addition of the nitrogen pulses shortly after the start of the dark cycle.

The nitrogen uptake rates (V, µmol g^{-1} h^{-1}) were derived from the depletion of the nutrients in the medium at each time interval, using the following equation:

$$V = (S_o - S_f) * vol / t * B$$

where S_o is the nutrient concentration at the beginning of a sampling interval (µM), S_f is the nutrient concentration at the end of a sampling interval (µM), vol is the water volume at the end of a sampling interval (L), t is the time elapsed between two successive samplings, and B is the plant biomass (g DW).

Leaf, rhizome and root tissue samples of *Z. noltii* plants were taken prior to and after incubations and immediately frozen at -80ºC for carbohydrate analysis. The samples were lyophilized at -80ºC and ground into a fine powder. Soluble sugars were extracted from 5 - 10 mg of ground sample in 3 ml of ethanol 80 % (v/v) at 80ºC for 10 min and centrifuged for 5 min at 2000 rpm (Longstaff et al., 1999; Burke et al., 1992). The supernatant was collected and the pellet was ressuspended in ethanol for additional extraction. This procedure was repeated a third time to allow full extraction of soluble sugars. The supernatant was mixed with more ethanol to reach a final volume of 10 ml. The sugar content of the extract was determined by the phenol-sulphuric acid method, using glucose as standard (Dubois et al., 1956), by incubating 1 ml of extract with 1 ml of phenol (5%) and 1 ml of sulphuric acid for 30 minutes. The final coloration was read spectrophotometrically at 540 nm. For starch quantification, the pellet of the previous soluble sugar extraction was homogenized in 1 ml deionised water and centrifuged at 12000 rpm for 2 min. The supernatant was discarded and the pellet was ressuspended in 1 ml deionised water. This procedure was repeated two more times to fully wash the pellet before autoclaving for 15 min. Starch was hydrolyzed to glucose overnight at 37ºC in an enzymatic suspension (α-amylase and amyloglucosidase) and determined as glucose equivalents following the phenol-sulphuric assay described above.

Statistical analysis

Significant differences between uptake rates determined in the light and in the dark were assessed for each nitrogen source using a two-way analysis of variance with repeated measures (ANOVAR). Differences in the rate of change (final amount - initial amount / initial amount) of soluble sugars and starch of plant tissues incubated with different nitrogen sources were detected using t-tests. Significance levels were tested at $p < 0.05$ (Sokal and Rohlf, 1995).

Results

The ammonium uptake rates of *Zostera noltii* plants incubated in the light were not significantly different from those of plants incubated in darkness ($F = 0.051$; $p = 0.83$) (Fig. 1). Given the high deviation of the initial uptake rates, no significant differences in the ammonium uptake rates were detected throughout the incubation time ($F = 1.51$; $p = 0.22$). However, the mean values of the ammonium uptake rates in the light were higher within the first 30 minutes of incubation (15 and 20 µmol g^{-1} h^{-1}) and stabilized at a rate of 5 µmol g^{-1} h^{-1} in the subsequent time intervals, whereas in the dark the means remained high within the first 180 min of incubation at a constant rate of 10 µmol g^{-1} h^{-1}. No significant differences were also detected between the nitrate uptake rates measured in the light and in the dark ($F = 0.13$; $p = 0.74$) or throughout the incubation time ($F = 0.79$; $p = 0.59$) (Fig. 2). Yet, the mean values of these rates were higher within the first 120 min of incubation (7.2 - 11.1 µmol g^{-1} h^{-1}) and decreased afterwards to lower values (0.8 - 3.9 µmol g^{-1} h^{-1}) (Fig. 2A). In the dark, the nitrate uptake rates fluctuated throughout the incubation time, with maximum values ranging from 9 to 11.1 µmol g^{-1} h^{-1} (Fig. 2B).

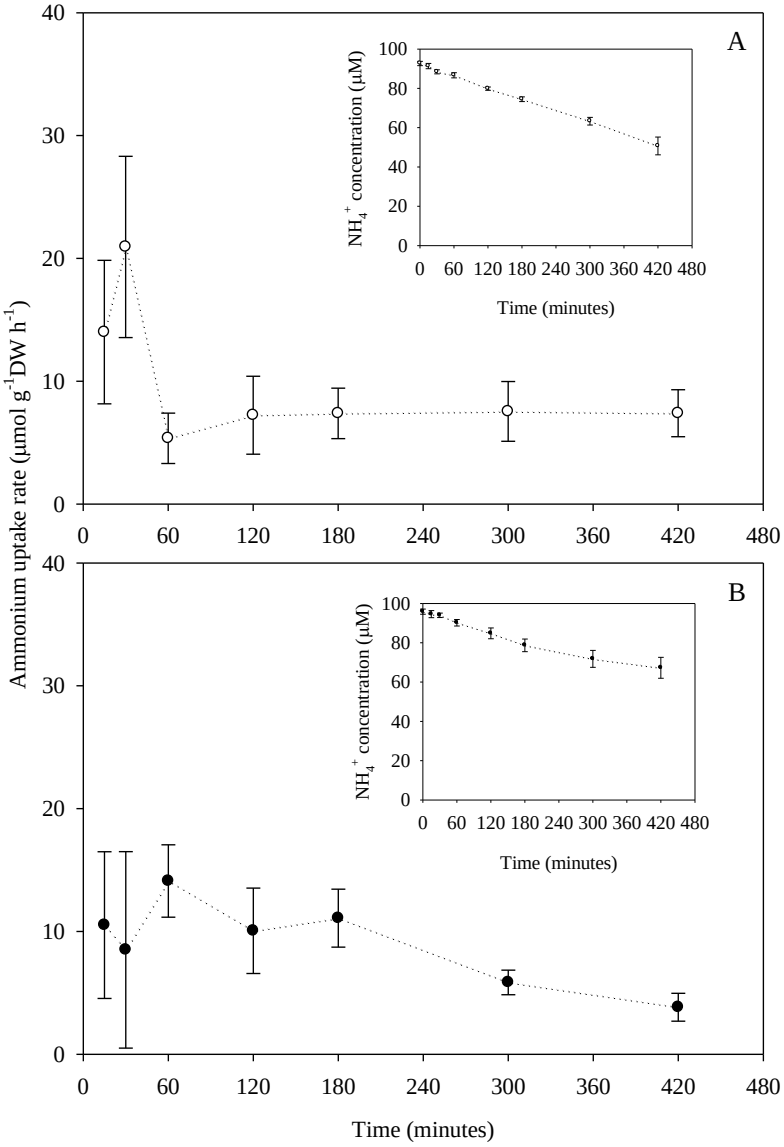

Figure 1. *Zostera noltii*. Time course of the ammonium uptake rate in the light (A) and in the dark (B). Inset graphs represent the ammonium depletion pattern over time. Values are mean ± SE (n = 3).

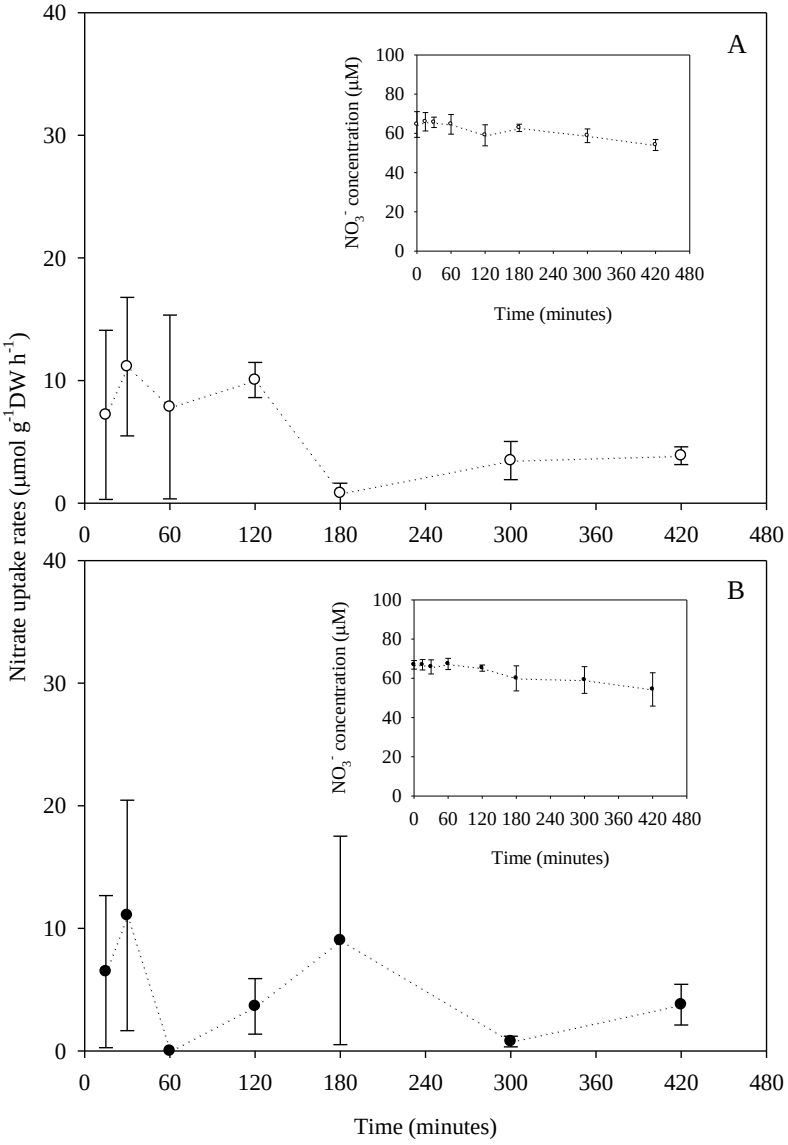

Figure 2. *Zostera noltii*. Time course of the nitrate uptake rate in the light (A) and in the dark (B). Inset graphs represent the nitrate depletion pattern over time. Values are mean ± SE (n = 3).

The leaf soluble sugar content of *Z. noltii* plants incubated in either ammonium or nitrate increased significantly at the end of the light incubations ($p = 0.02$), whereas the increase in sugar content of rhizomes and roots was not statistically significant (Fig. 3). The starch content decreased significantly only in the rhizomes of plants incubated with nitrate ($p = 0.008$) (Fig. 4). Plants incubated either with ammonium or nitrate in the dark showed no significant reduction in the carbohydrate content, both as soluble sugar and starch.

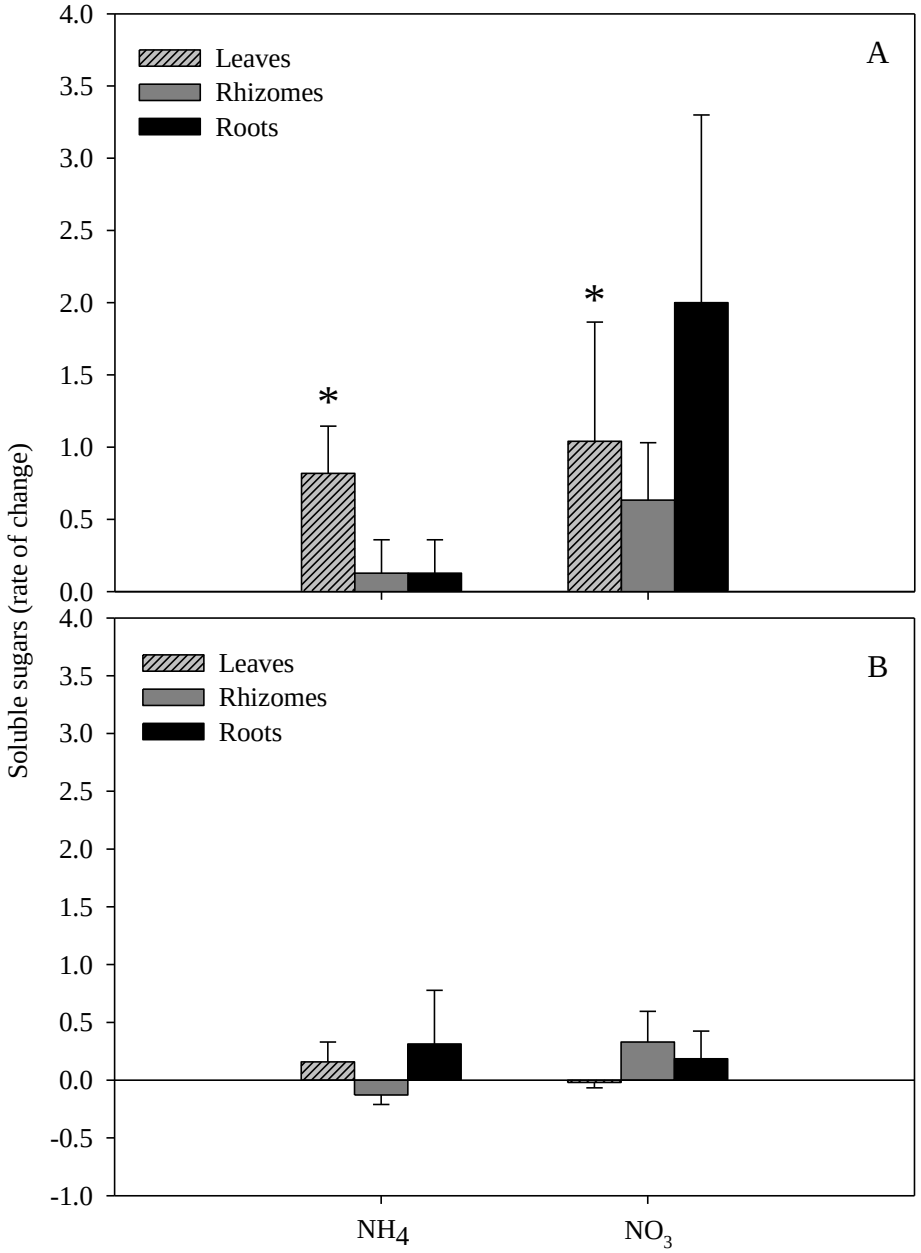

Figure 3. *Zostera noltii*. Variation of the soluble sugar content of plant tissues incubated with ammonium and nitrate in the light (A) and in the dark (B). Values are means ± SD (n = 3). The rate of change indicates the percentage of decrease (< 0) or increase (> 0) in the soluble sugar content. Significant rates of change are indicated by asterisks.

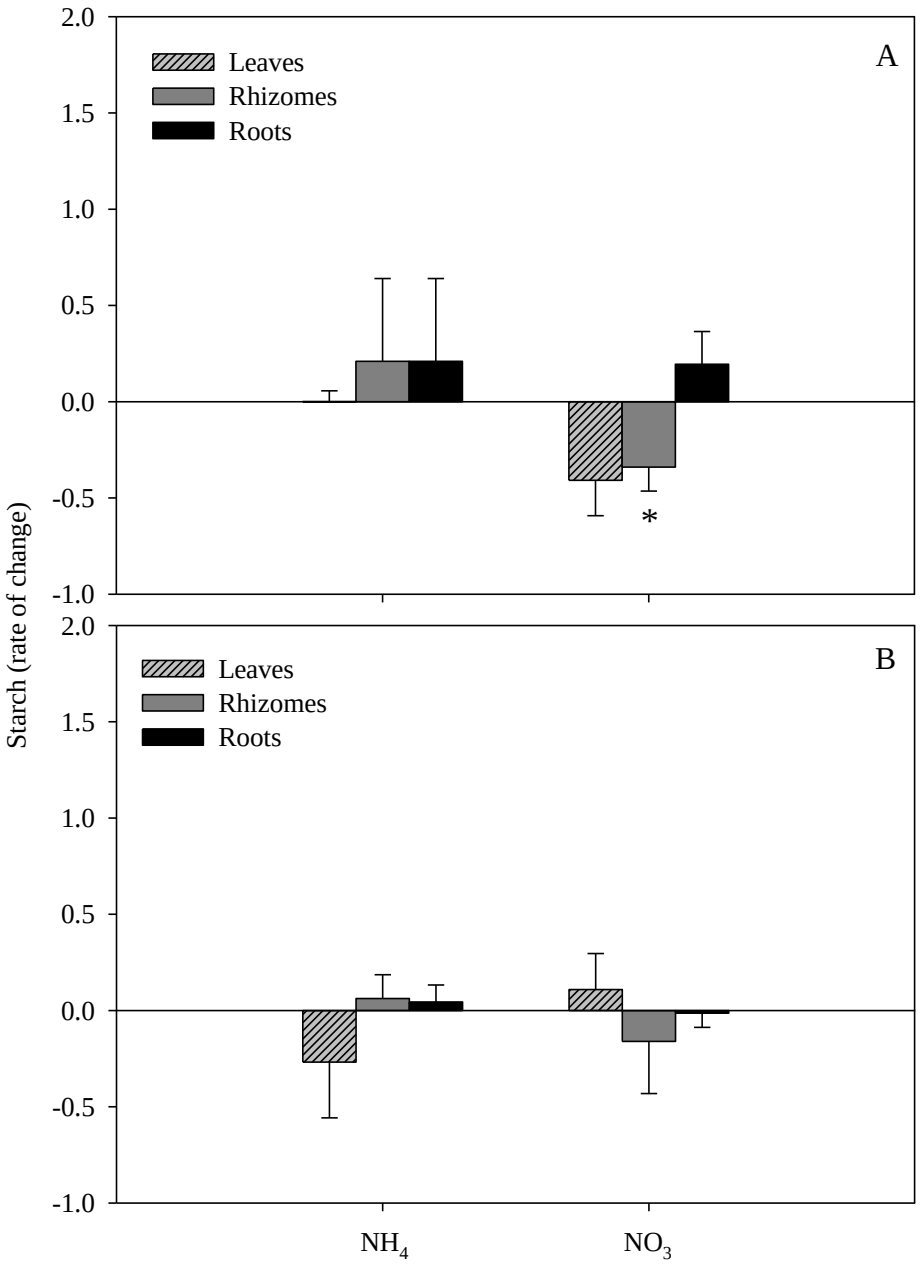

Figure 4. *Zostera noltii*. Variation of the starch content of plant tissues incubated with ammonium and nitrate in the light (A) and in the dark (B). The rate of change indicates the percentage of decrease (< 0) or increase (> 0) in the starch content. Values are mean ± SD (n = 3). Significance is indicated by asterisks. Note that the scale of yy axis is different from Figure 3.

Discussion

Our first hypothesis that the nitrogen uptake by *Zostera noltii* during darkness may be low because its carbohydrate levels are amongst the lowest of seagrass species (Touchette and Burkholder, 2000) was not supported. The results of this study revealed that *Z. noltii* is able to take up inorganic nitrogen at similar rates in the light and in darkness, showing the same behavior of the other two seagrass species where this comparison was done, *Zostera marina* and *Thalassia testudinum* (Iizumi and Hattori, 1982; Lee and Dunton, 1999). This capacity may represent an advantage over opportunistic seaweed species that may out-compete seagrasses. An example of an opportunistic species that displays clear diurnal cycles for the uptake of nitrogen is *Ulva pertursa* (Gevaert et al., 2007). Other seaweeds, such as *Laminaria groenlandica* (Phaeophyceae), also showed lower ammonium and nitrate uptake rates in the dark than at light (Harrison et al., 1986).

In both light and dark conditions, the nitrogen uptake of *Z. noltii* displayed a temporal pattern of enhanced initial rates followed by lower but relatively constant rates in response to the ammonium or nitrate supply at high concentration. High initial uptake rates are expected when the availability of inorganic nitrogen suddenly increases. This was shown both for phytoplankton and seaweed species (Conway et al., 1976; Thomas and Harrison, 1987; Pedersen, 1994; Dy and Yap, 2001; Raikar and Wafar, 2006). Initial rapid accumulation of ammonium by the leaves of the seagrass *Zostera marina* was also shown (Short and McRoy, 1984). During the surge uptake phase, the uptake rates are maximal and exceed the internal nitrogen utilization rates by several-fold (Conway et al., 1976; Fujita, 1985).

As an intertidal species, *Z. noltii* undergoes periods of reduced nitrogen availability during low tide, when sediment porewater becomes the only source of inorganic nitrogen. When the incoming tide floods air-exposed sediments important ammonium pulses occur (Falcão and Vale, 2003), which may be crucial to meet the nitrogen requirements necessary to sustain the species high growth rates (Peralta et al., 2005). We showed here that *Z. noltii* is able to maximize the use of nitrogen when available at high concentration either during the day or during the night. The nitrogen acquisition during the surge uptake phase by *Z. noltii* plants exposed to high initial concentrations (65 - 95 µM) was 2 to 4-fold higher than the nitrogen acquisition at lower initial concentrations (5 µM), which are the typical nitrogen concentrations in the water column environment of Ria Formosa (Alexandre et al., 2010).

Following the surge uptake phase, the nitrogen uptake of *Z. noltii* dropped quickly and stabilized at rates that were half the initial uptake rates. A similar pattern was observed in the red algae *Kappaphycus alvarezii* exposed to initial concentrations of 30 µM NH_4Cl (Dy and Yap, 2001). The ammonium uptake rates of the green algae *Ulva lactuca* exposed to an initial concentration of 50 µM NH_4Cl decreased by 10-fold after 1h relative to the initial uptake rates (Pedersen, 1994). This second temporal phase, characterized by lower but constant uptake rates, is called internally controlled phase. These rates are equivalent to the rates of nitrogen conversion into amino acids (Harrison et al., 1989) and depend on the enzymatic activity (NR and GS), the availability of carbon skeletons, ATP and reducing energy (Huppe and Turpin, 1994).

The metabolic energy (as ATP, NADH and carbon) for nitrogen assimilation in the light is provided directly by photosynthesis, whereas the assimilation of nitrogen in the

dark is energetically more costly because it involves the mobilization of stored carbohydrates through respiration of previously fixed carbon compounds such as starch (Turpin, 1991; Huppe and Turpin, 1994). In the light, *Z. noltii* plants incubated with either ammonium or nitrate showed significant accumulation of soluble sugars in the leaves, a consequence of the metabolic outcome of photosynthesis. On the other hand, only the plants incubated with nitrate showed starch content reduction, which was particularly significant in the rhizomes. These results indicate that starch reserves may be involved in the nitrate assimilation process during the light period. The necessary energy and carbon to assimilate nitrate in the light may derive not only directly from photosynthesis, as seems to be the case for the assimilation of ammonium, but also from the degradation of starch reserves. This may also explain the higher accumulation of soluble sugars in the leaves of plants incubated at light with nitrate, despite the lower uptake rates of nitrate relatively to ammonium.

The assimilation of nitrate is considered energetically more costly than ammonia because nitrate must first be reduced to nitrite and then to ammonia (Taiz and Zieger, 2002). This energy may be obtained from photosynthesis or from the mobilization of starch. Although starch degradation does not generally occur during photosynthesis, it may occur during the light period if the carbon demand of nitrogen assimilation exceeds the rate of photosynthetic CO_2 fixation (Turpin, 1991). The mobilization and translocation of starch from the rhizome to the leaf tissue, which is the primary site of nitrate reduction in *Z. noltii* (Alexandre et al., 2010), may have generated extra energy and carbon supply for the nitrate assimilation. The use of storage reserves, alone or combined with recently fixed

photosynthates, was shown to drive nitrate reduction in other plants (e.g. barley seedlings), both in light and dark conditions (Aslam et al., 1979).

Several plants store excess photoassimilates as starch in the chloroplast, which is later mobilized together with sucrose to support respiration and other metabolic needs during darkness (Taiz and Zieger, 2002). It is known that the seagrass *Z. marina* can sustain dark nitrate reductase activity if adequate carbohydrate supply is available (Touchette and Burkholder, 2001). If carbohydrate levels remain high during dark periods, *Z. marina* can assimilate and reduce nitrate at rates comparable to those measured in the light. In *Z. noltii*, both the soluble sugar and starch content were not significantly reduced in plants supplied either with ammonium or nitrate during darkness. However, in dark incubations both with nitrate and ammonium, the total starch content of plants decreased by 1.0-1.1 fold whereas the total soluble sugars increased by 1.1-1.2 fold. This suggests a process of starch catabolism occurring at night to generate energy with the consequent production of soluble monosaccharide products.

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Effects of CO_2 enrichment on photosynthesis, growth and nitrogen metabolism of the seagrass *Zostera noltii*

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Effects of CO_2 enrichment on photosynthesis, growth and nitrogen metabolism of the seagrass *Zostera noltii*

Abstract

Seagrass ecosystems are expected to benefit from the global increase of CO_2 in the ocean because the photosynthetic rate of these plants may be C_i-limited at the current CO_2 level. On the other hand, no reports are available on the effects of CO_2 increase on the nitrogen metabolism of seagrasses. Here we investigate the long-term effects of CO_2 enrichment on both carbon and nitrogen metabolism of the seagrass *Zostera noltii* in a mesocosm experiment where plants were exposed to two experimental CO_2 concentrations (current levels of 360 ppm and future levels of 700 ppm) for five months. The specific response variables of the experiment were photosynthesis and growth, ammonium and nitrate uptake rates and the activities of nitrate reductase and glutamine synthetase. Both the maximum photosynthetic rate (P_m) and photosynthetic efficiency (α) were higher (1.3- and 4.1-fold, respectively) in plants exposed to CO_2-enriched conditions. Surprisingly, no significant effects of CO_2 enrichment on leaf growth rates were observed. This was probably due to nitrogen limitation experienced by the plants in the mesocosm, as their low leaf nitrogen content revealed. This suggests that the global effects of CO_2 on seagrass growth may not be spatially homogeneous and will depend on the specific nitrogen availability of each system. The leaf ammonium uptake rate and the glutamine synthetase activity were not significantly affected by increased CO_2 concentrations. On the other hand, the leaf nitrate uptake rate of plants exposed to CO_2-enriched conditions was 4-fold lower than the uptake of plants exposed to current CO_2 levels. In contrast, the activity of nitrate

reductase was 3-fold higher in plant leaves grown at high CO_2concentrations. The decrease of the nitrate uptake rates observed at high CO_2 (low pH, higher proton concentration) suggests that in the seagrass *Z. noltii* nitrate is not co-transported with H^+ as in terrestrial plants. The CO_2-driven stimulation of the nitrate reductase activity of *Z. noltii* plants that were exposed to enriched-CO_2 concentrations and low nitrogen availability was probably related with high internal carbohydrate levels, which contribute with energy and carbon skeletons for nitrate reduction.

Keywords: CO_2 enrichment, glutamine synthetase, growth, nitrate reductase, nitrogen uptake, photosynthesis.

Introduction

Projections that the current atmospheric CO_2 concentration will double by the end of this century and that oceanic CO_2 level will rise (Houghton et al., 2001; Caldeira and Wickett, 2003) have caused increasing interest in the research of the direct impacts of elevated CO_2 on the marine environment (Gattuso et al., 1998; Feely et al., 2004; Guinotte and Fabry, 2008; Pörtner, 2008; Hall-Spencer et al., 2008; Porzio et al., 2011). It is expected the seawater pH to decrease 0.3-0.4 units relative to present values before the year 2100 (Caldeira and Wickett, 2003; Feely et al., 2004). The acidification of the seawater will render changes in the carbonate chemistry, i.e. in the relative proportions of the inorganic carbon species, dioxide (CO_2) bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), shifting the total dissolved inorganic carbon away from CO_3^{2-} towards more bicarbonate HCO_3^- and CO_2 (Riebesell et al., 2007).

Seagrasses are highly productive ecosystems with an important role in the carbon cycle of coastal areas (Duarte and Chiscano, 1999; Hemminga and Duarte, 2000). The scientific knowledge on how seagrasses will respond to elevated CO_2 concentrations must be considered for an effective management of coastal regions in the future. Seagrasses are reported as one of the few ecosystems that may benefit from rising CO_2 levels because their photosynthetic rates have been considered C_i-limited at the current oceanic CO_2 concentration (Thom, 1996; Beer and Koch, 1996; Zimmerman et al., 1997; Invers et al., 2001). Consequently, increases in seagrass production and growth are expected in a future high CO_2 scenario.

CO_2 enrichment may also affect the nitrogen metabolism, both at the uptake and the assimilation level, since growth enhancement at high CO_2 concentrations is expected to increase the nitrogen demand of plants. In addition, the relative uptake rates of ammonium and nitrate may be altered by the acidification of the seawater resulting from CO_2 enrichment, due to the involvement of protons (H^+) in the nitrogen transport across plasma membrane. In terrestrial plants, nitrate is co-transported with H^+ through the membrane and consequently lower external pH facilitates nitrate uptake because of the higher H^+ gradient outside the cell (e.g. Vessey et al., 1990). On the other hand, the lower external pH affects the ammonium uptake because the higher content of H^+ reduces the activity of H^+-ATPase, which is involved in the cation transport into the cells (Marschner, 1995). From the ionic balance perspective, lower pH levels in the seawater may reduce the ammonium uptake rates of seagrasses, while nitrate uptake rates may be unaffected or even increased.

The effects of CO_2 enrichment on seagrasses are poorly studied and have focused mainly on how elevated CO_2 concentrations will affect seagrass productivity and light

requirements (Beer and Koch, 1996; Thom, 1996; Zimmerman et al., 1997; Palacios and Zimmerman, 2007; Jiang et al., 2010). However, these effects were investigated with short-term (days) laboratory experiments, except the study of Palacios and Zimmerman (2007), in which experiments were run in outdoor aquaria for one year. Long-term studies are thus needed to account for the acclimation potential of seagrass species to increasing CO_2. Here we investigate the long-term effects of CO_2 enrichment on the carbon and nitrogen metabolism of the seagrass *Zostera noltii* in a mesocosm experiment where plants were exposed for five months to current (360 ppm) and future (700 ppm) seawater CO_2 concentrations. We specifically aimed to assess the long-term effects of CO_2 enrichment on photosynthesis and growth, on the ammonium and nitrate uptake rates (at two concentrations) and on the activity of nitrate reductase and glutamine synthetase. These are the two key enzymes of nitrogen assimilation. Nitrate reductase catalyses the first reduction of nitrate to nitrite within the cells and glutamine synthetase the incorporation of ammonium into the amino acid glutamine. To the best of our knowledge this is the first report on the effects of the global CO_2 increase on the nitrogen metabolism of seagrasses.

Methods

Plant collection and experimental design

Corers of *Zostera noltii* community were collected from an intertidal meadow in Ria Formosa coastal lagoon, South Portugal (37º00′N, 7º58′W), in March 2010. The corers were carefully collected to avoid damage to the belowground structures of plants, placed inside plastic boxes (55 cm x 35 cm x 14 cm) with the sediment and associated community and transported to an outdoor mesocosm facility located at the Ramalhete field station of

CCMAR, near the donor meadow. The outdoor mesocosm consisted of two flow-through open systems running in parallel, one with seawater at the current CO_2 concentration (360 ppm) and the other with 2 x the current CO_2 concentration (700 ppm). Each system consisted of one head tank (1500 L) connected to two independent tanks (660 L each). Each tank included four plastic boxes of *Z. noltii* and its associated community. Consequently, the experiment consisted of 2 CO_2 levels x 2 replicates (660 L tanks), within each 4 plant units were available. The seawater used in the mesocosm was pumped from the lagoon into the head tanks after passing through a sand filter. The flow rate of the seawater to each replicate unit was about 210 L h^{-1}. CO_2 was bubbled into the head tanks from a CO_2 tank to achieve the experimental CO_2 concentrations (360 ppm and 700 ppm). The rate of CO_2 injection into the system was controlled by the pH level of the seawater using pH probes connected to CO_2 controllers (Yokogawa, EXAxt 450). The plants were exposed to the experimental CO_2 levels for five months.

Seawater chemistry

The daily fluctuations of dissolved inorganic carbon (CO_2, HCO_3^- CO_3^{2-}), pH and total alkalinity of the seawater in both CO_2 treatments were monitored throughout the experiment and during a 24h cycle in July. Triplicate water samples were collected inside the seagrass canopy in each mesocosm replicate unit every two hours. Total alkalinity was determined by measuring pH directly (Multimeter 340, WTW) in 4 ml water samples before and after acidification with 1 ml of HCl 0.01 M, according to Parsons et al. (1984) and modified by Semesi et al. (2009). The concentration of dissolved inorganic carbon (CO_2, HCO_3^- and CO_3^{2-}) was calculated from total alkalinity, temperature and salinity of

the seawater using the Excel implemented program CO_2SYS.XLS 1.0 (Pelletier et al., 1997). Water samples for nutrient analysis were also collected in triplicate, filtered through cellulose acetate filters and stored at -20ºC. The concentrations of ammonium, nitrate and phosphate in the seawater were determined in a loop-flow analyzer (μMac-1000; Systea, Anagni, Italy). Ammonium concentration was determined using the hypochlorite method and nitrate concentration was determined by the Cd-Cu column reduction method. Phosphate was determined by the molybdate and ascorbic acid colorimetric method.

Photosynthetic measurements

Net photosynthetic rates were measured following oxygen evolution as a function of irradiance in square section incubation chambers (15 ml) coupled to a Clark-type oxygen electrode (DW3/CB1, Hansatech, Norfolk, UK). Actinic light was provided by a slide projector (Pradovit 150, Leica, Germany) equipped with a halogen lamp (Osram Xenophot 150W). Different light intensities were achieved using a series of neutral density filters. The oxygen evolution of *Z. noltii* plants exposed to the two CO_2 concentrations (360 ppm and 700 ppm) was measured simultaneously in GF/F filtered seawater from the respective CO_2 treatment. For each measurement (n = 4 for the current CO_2 and n = 3 for the enriched CO_2 concentration), two independent segments (≈ 2 cm long) of *Z. noltii* leaves were held vertically inside the chamber. During the measurements, the water in the incubation chamber was continuously stirred and the temperature was kept constant at 20°C. Irradiance varied between 0 and 875 μmol photons m^{-2} s^{-1}. Each light step took approximately seven minutes to reach steady-state photosynthesis, after which the water in

the reaction chambers was replaced by new water. After each measurement, the area of the leaf segments was measured (30 - 40 mm^2 each) and leaf tissues were dried at 60°C for 24h. The adapted hyperbolic tangent model equation of Jassby & Platt (1976) was fitted to the net photosynthesis versus irradiance data plots:

$$P = P_m \times \tanh (\alpha \times I / P_m)$$

where P_m is the maximum photosynthetic rate (µmol O_2 g^{-1}DW h^{-1}), I is irradiance (µmol photons m^{-2} s^{-1}) and α is the ascending slope at limiting irradiances (µmol O_2 g^{-1}DW h^{-1}/µmol photons m^{-2} s^{-1}).

Growth measurements

Leaf growth rates were determined in *Z. noltii* plants exposed to the two CO_2 levels (360 and 700 ppm) using the classical punching method described for seagrasses by Zieman (1974) and modified by Peralta et al. (2000). For each CO_2 level, the leaves of five random shoots in each mesocosm replicate unit were marked with fine plastic fibers immediately above the leaf sheath. The total length of non-marked leaves (small or new leaves) was also recorded. After three days, the length from the leaf base to the punching mark and the total leaf length of non-marked leaves were recorded. Leaf growth rate (LGR) (cm d^{-1} $shoot^{-1}$) was calculated following the equation:

$$LGR = (\Sigma G_{nm} + \Sigma G_m) / t,$$

where G_{nm} is the growth rate of non-marked leaves, G_m is the growth rate of marked leaves and t is the time elapsed (days) between the punching and the final measurements (t_f - t_0). G_{nm} (cm d^{-1} $shoot^{-1}$) = TLL_f - TLL_i, where TLL_i and TLL_f is the total leaf length at t_0 and t_f, respectively. G_m (cm d^{-1} $shoot^{-1}$) = MLL_f - MLL_i, where MLL_i and MLL_f is the length from the leaf base to the punching mark at t_0 and t_f, respectively.

Nitrogen uptake rates and enzymatic analysis

Leaf nitrogen uptake rates were estimated using two-compartment cylindrical chambers that physically separated the leaves from the below-ground plant parts. Leakage between compartments was avoided using molding clay and sterile vaseline as sealants. The leaves of plants grown at 360 and 700 ppm CO_2 were simultaneously incubated for 2h in seawater enriched with $^{15}NH_4Cl$ or $^{15}KNO_3$ solutions (atom % = 99, Cambridge Isotope Laboratories) in a walk-in culture chamber at constant temperature (21ºC) and light intensity (200 µmol quanta m^{-2} s^{-1}) . The seawater used in the incubations was collected from the respective CO_2 treatment. The uptake rates were determined at two nitrogen concentrations (5 and 30 µM). Incubations at different CO_2 levels were done simultaneously for each nitrogen concentration, and replicate incubations (n = 3) were done sequentially. One single shoot with the respective rhizome and roots was placed inside each split-chamber. In the leaf compartment, an average leaf biomass of 0.04 g dry weight was incubated within 1.5 L of seawater, which was constantly mixed with a flow rate of ≈ 250 ml min^{-1}using a peristaltic pump. The nitrogen concentration (ammonium or nitrate) in the media did not vary noticeably throughout the incubation period. Root compartments were left without nutrients. Even though in natural conditions the rhizosphere of *Z. noltii* is

mostly anoxic, in these experiments we incubated the whole plants in an oxygenated medium. Previous experiments reported elsewhere (Alexandre et al., 2010) showed no effects of rhizosphere oxygenation on the ammonium and nitrate uptake rates of leaves.

At the end of incubations, the plants were removed from the chambers, the leaves were immediately separated from the rhizomes and roots and were briefly rinsed with deionized water to remove adherent label. Leaf tissues were dried at 60ºC for 48h and reduced to a fine powder. Total nitrogen content and atom % ^{15}N of dried tissues were determined using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (UC Davis, CA). Leaf ^{15}N background levels were measured in three replicate samples.

The incubations described above were repeated for the determination of nitrate reductase (NR) and glutamine synthetase (GS) activity. In these incubations, the leaf media were enriched with 30 µM of non-labeled NH_4Cl and KNO_3. The leaf incubations at different CO_2 level were performed simultaneously for each nitrogen concentration, while replicate incubations (n = 3) were done sequentially. NR activity was measured *in vivo* using the method described by Corzo and Niell (1991), optimized for *Z. noltii* (Alexandre et al., 2004). This method is based on the colorimetric measure of nitrite, formed after the reduction of nitrate by NR. Leaf tissue (0.12 g fresh weight) was incubated in 50 mM KNO_3, 0.1M K_2HPO_4 (pH 8.0), 0.5 mM EDTA and 0.5% 1- propanol, in a final assay medium volume of 10 ml, flushed with N_2 for 2 min to remove oxygen. Incubations lasted 30 min at 30ºC. The nitrite produced was measured spectrophotometrically (540 nm) after adding 1 ml of sulphanilamide and 1 ml of naphtyl-etylenediamine to the assay medium. The *in vivo* assay was preferred because it yielded consistently higher activity than the *in*

vitro assay, which failed to provide reproducible results (see also Touchette & Burkholder 2007 and references therein). GS activity was measured *in vitro*, using the method described by Sagi et al. (2002) optimized for *Z. noltii*. The normal biological activity of GS combines ammonium with glutamate to yield glutamine. This reaction is mimicked in the synthetase assay, in which hydroxylamine is substituted for ammonium to yield the product γ-glutamyl-hydroxamate, which can be quantified spectrophotometrically. Samples of 0.12 g fresh weight of leaf tissue were extracted in 1.6 ml of buffer containing 200 mM Tris buffer (pH 7.8), 2 mM EDTA, 3 mM dithiothreitol (DTT), 10 μM flavin adenine dinucleotide (FAD), 10 mM MgCl, 2% (w/v) casein, 10% (v/v) glycerol and 0.1 g polyvinylpyrrolidone (PVP). The homogenized plant material was centrifuged at 30 000 *g*, at 4ºC for 15 min. 100 μl of the enzyme extract were added to 250 μl of assay medium containing 18 mM ATP, 45 mM $MgCl_2 \cdot 6H_2O$, 25 mM hydroxylamine, 92 mM L-glutamate, and 50 mM imidazole HCl (pH 7.2), at 30ºC. After 20 min, the reaction was stopped by the addition of 0.5 ml of ferric chloride reagent (0.37 M ferric chloride, 0.67 M HCl and 0.2 M trichloroacetic acid). The reaction solution was then centrifuged, and the absorbance of the supernatant was read at 540 nm.

Data analyses

The effects of CO_2 enrichment on the photosynthetic parameters, rates of leaf growth and enzymatic activity were tested using t-tests. Differences in the rates of ammonium and nitrate uptake between the two CO_2 levels were also tested using a t-test for each nutrient concentration. When necessary, data were square-root transformed to fit the

assumptions of normal distribution and homogeneity of variance. The statistical test was performed at a level of significance of 0.05 (Fowler and Cohen, 1990).

Results

The pH and CO_2 concentration of the seawater in the two CO_2 treatments varied throughout the daily cycle as a consequence of the photosynthetic and respiration processes of *Zostera noltii* plants and its associated community (Fig. 1A and B). The concentration of CO_2 and pH in the current CO_2 treatment (control) averaged 360 ± 128 ppm and 8.13 ± 0.12, whereas in the enriched-CO_2 treatment it averaged 695 ± 167 ppm and 7.91 ± 0.08, respectively.

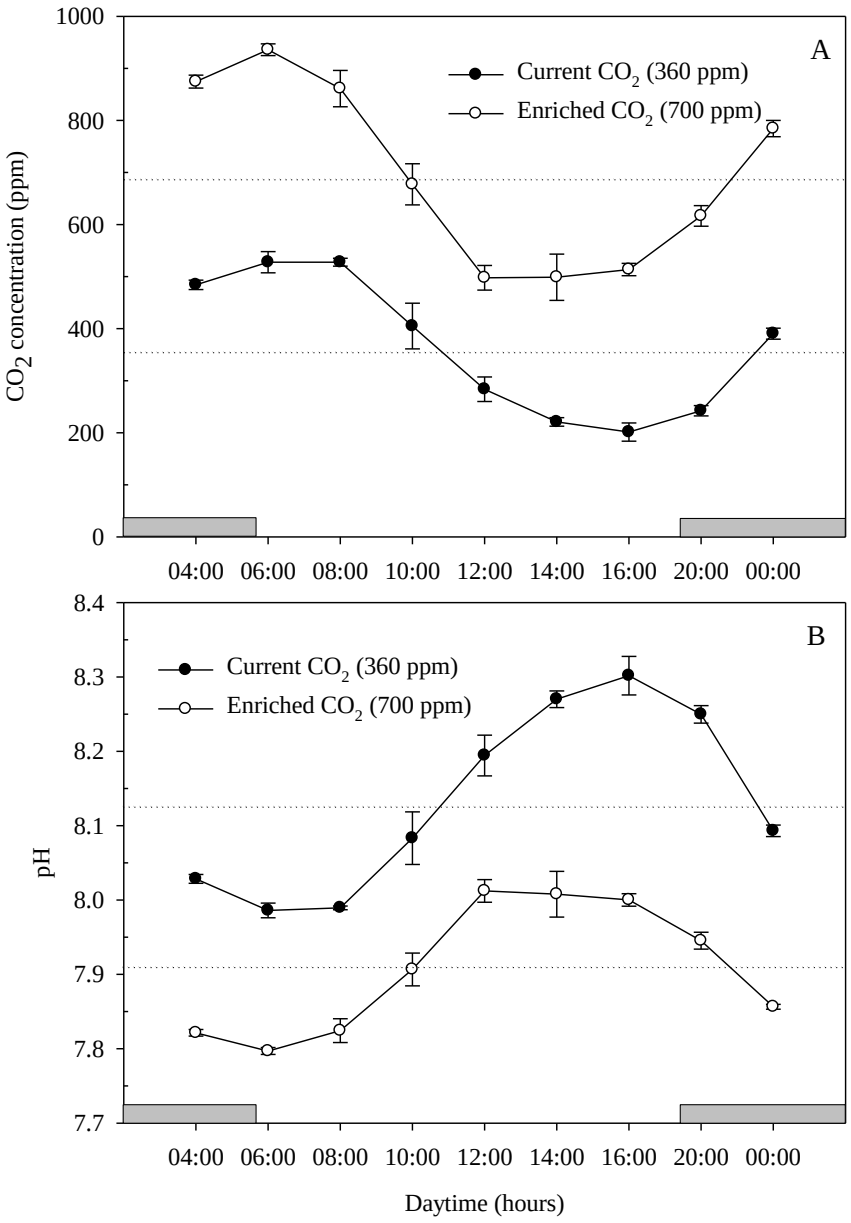

Figure 1. Daily fluctuation of (A) CO_2 concentration (ppm) and (B) pH of the seawater in the control (open circle) and the CO_2-enriched (closed circle) treatments. Dark areas represent night-time. Values are mean ± SD (n = 6).

As a consequence of the CO_2 addition to the system, the concentration of CO_2 and HCO_3^- increased in the enriched-CO_2 treatment compared to the control, while the concentration of CO_3^{2-} was reduced (Table 1). Total alkalinity was not significantly different between treatments and did not vary much along the day. The concentration of ammonium and nitrate in both treatments was nearly undetectable throughout the daily cycle (< 0.01 µM) suggesting that all the available inorganic nitrogen was being taken up by the plants. Phosphate concentration in the control and CO_2-enriched treatment averaged 0.18 ± 0.03 µM and 0.24 ± 0.04 µM, respectively.

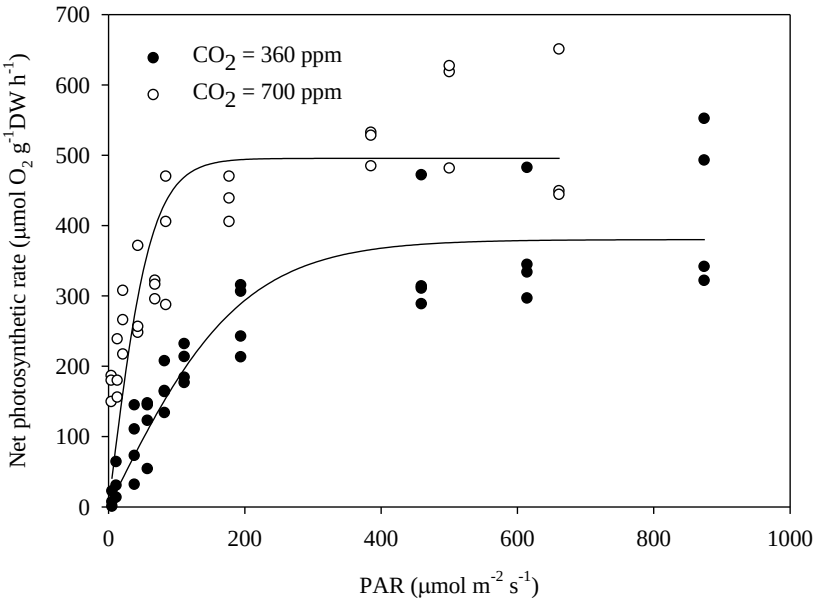

Figure 2. *Zostera noltii*. Net photosynthetic rate (µmol O_2 g^{-1}DW h^{-1}) versus photosynthetic active radiation (PAR; µmol m^{-2} s^{-1}) measured following oxygen evolution determined at 20ºC in leaf segments of plants exposed at 360 ppm (closed circles) and 700 ppm (open circles). Values are mean ± SD (n = 3 - 4).

The irradiance-saturated photosynthetic rate (P_m) of plants exposed to CO_2-enriched conditions (495.6 ± 26.4 µmol O_2 g^{-1}DW h^{-1}) was 1.3-fold higher than the rate of plants exposed to current CO_2 concentration (380.1 ± 17.7 µmol O_2 g^{-1}DW h^{-1}) (Fig. 2).

Table 1. Daily fluctuation of the seawater carbonate speciation in the two experimental CO_2 levels (360 ppm and 700 ppm). Values of total carbon (TC), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) were calculated using total alkalinity (TA), pH, salinity and temperature of the seawater. Values are mean ± SD (n = 6) and represent pooled data from the two replicate mesocosm units. Units are µmol/Kg.

	360 ppm				700 ppm			
Daytime (h)	TA	TC	HCO_3^-	CO_3^{2-}	TA	TC	HCO_3^-	CO_3^{2-}
04:00	2722 ± 17	2340 ± 16	2040 ± 15	285 ± 3	2760 ± 18	2510 ± 17	2288 ± 16	198 ± 2
06:00	2629 ± 34	2278 ± 40	2015 ± 36	255 ± 3	2765 ± 14	2529 ± 13	2314 ± 12	190 ± 2
08:00	2656 ± 21	2295 ±13	2032 ± 19	259 ± 2	2738 ± 24	2488 ± 20	2266 ± 20	198 ± 7
10:00	2661 ± 19	2260 ± 39	1926 ± 54	305 ± 17	2697 ± 10	2398 ± 9	2153 ± 17	227 ± 10
12:00	2653 ± 9	2121 ± 24	1730 ± 36	376 ± 16	2697 ± 15	2300 ± 22	2001 ± 26	286 ± 7
14:00	2622 ± 14	2035 ± 12	1613 ± 20	411 ± 6	2660 ± 16	2277 ± 24	1986 ± 35	277 ± 15
16:00	2627 ± 15	2028 ± 29	1582 ± 45	428 ± 14	2680 ± 14	2307 ± 13	2020 ± 13	273 ± 5
19:30	2702 ± 25	2145 ± 20	1695 ± 57	400 ± 18	2743 ± 29	2409 ± 26	2141 ± 24	251 ± 6
00:00	2743 ± 15	2238 ± 4	1878 ± 17	360 ± 5	2774 ± 42	2456 ± 39	2192 ± 35	244 ± 4

Similarly, the photosynthetic rates at limiting irradiances (α), expressed as photosynthetic efficiency, were much higher in CO_2-enriched plants (8.0 ± 1.2) than in plants exposed to current CO_2 concentration (1.95 ± 0.2). On the other hand, no significant effect of CO_2 enrichment was detected on the leaf growth rate of *Z. noltii* (Fig. 3). The leaf growth rate of plants exposed to elevated CO_2 concentration was 1.12 ± 0.27 cm d^{-1} $shoot^{-1}$, whereas the rate of plants grown at current CO_2 conditions was 1.18 ± 0.21 cm d^{-1} $shoot^{-1}$.

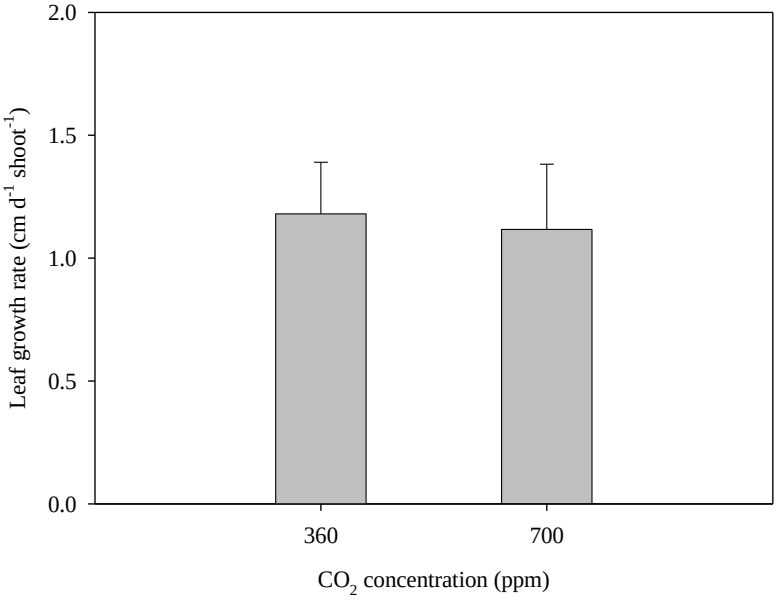

Figure 3. *Zostera noltii*. Leaf growth rate (cm d^{-1} $shoot^{-1}$) of plants exposed to CO_2 concentrations of 360 and 700 ppm. Values are mean ± SE (n = 10).

The ammonium uptake rates of leaves exposed to higher CO_2 concentrations, either incubated with 5 µM (2.42 ± 0.18 µmol g^{-1}DW h^{-1}) or 30 µM $^{15}NH_4Cl$ (10.27 ± 0.83 µmol g^{-1}DW h^{-1}), were not significantly different from the rates of plants exposed to the current CO_2 conditions (2.69 ± 0.44 µmol g^{-1}DW h^{-1} and 9.07 ± 0.64 µmol g^{-1}DW h^{-1}, respectively)

(Fig. 4). On the other hand, the leaf nitrate uptake rates of CO_2-enriched plants, either incubated at 5 µM (0.02 ± 0.01 µmol $g^{-1}DW\ h^{-1}$) or at 30 µM $^{15}KNO_3$ (0.05 ± 0.03 µmol $g^{-1}DW\ h^{-1}$) were significantly lower than the control (0.08 ± 0.02 µmol $g^{-1}DW\ h^{-1}$ and 0.16 ± 0.04 µmol $g^{-1}DW\ h^{-1}$, respectively).

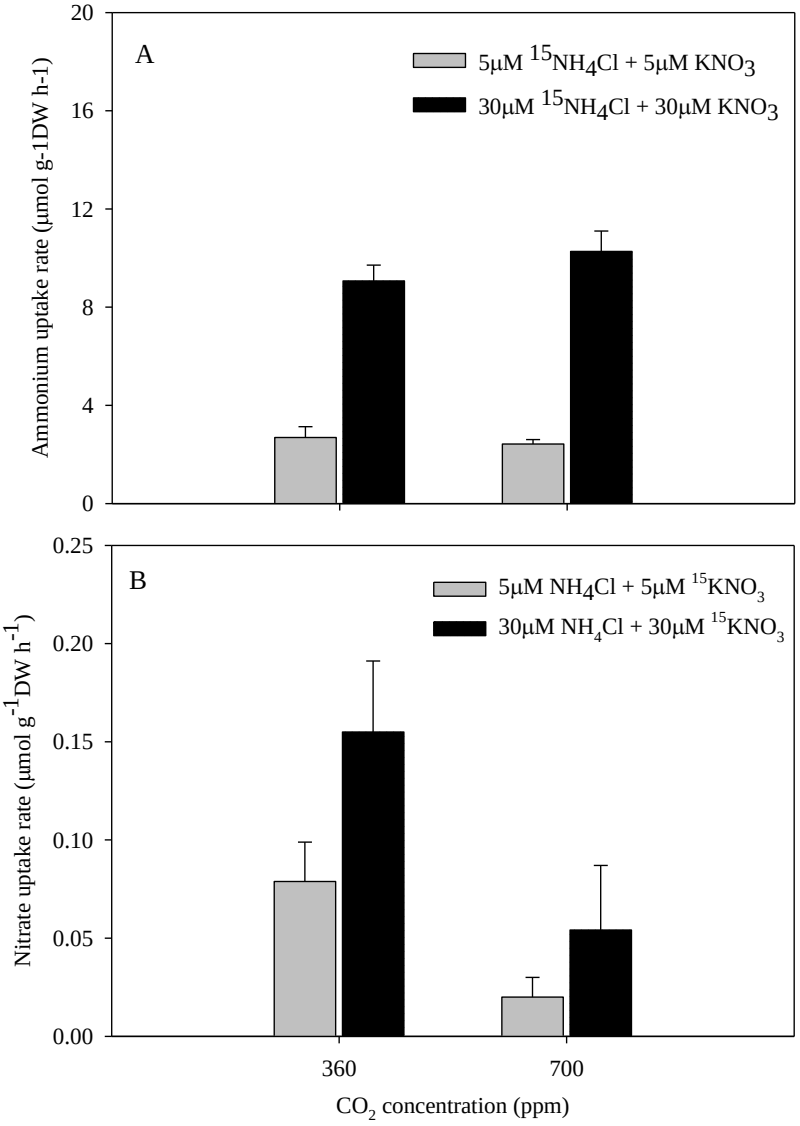

Figure 4. *Zostera noltii*. Ammonium (A) and nitrate (B) uptake rates (µmol $g^{-1}DW\ h^{-1}$) of plants leaves exposed to CO_2 concentrations of 360 and 700 ppm when incubated at 5 and 30 µM of NH_4Cl + KNO_3. Values are mean ± SE (n = 6).

The activity of the enzyme glutamine synthetase of plant leaves grown under CO_2 enrichment (923 ± 58 µmol glutamil-hydroxamate $g^{-1}DW\ h^{-1}$) was not significantly different from that of plants from the control treatment (907 ± 48 µmol glutamil-hydroxamate $g^{-1}DW\ h^{-1}$) (Fig. 5).

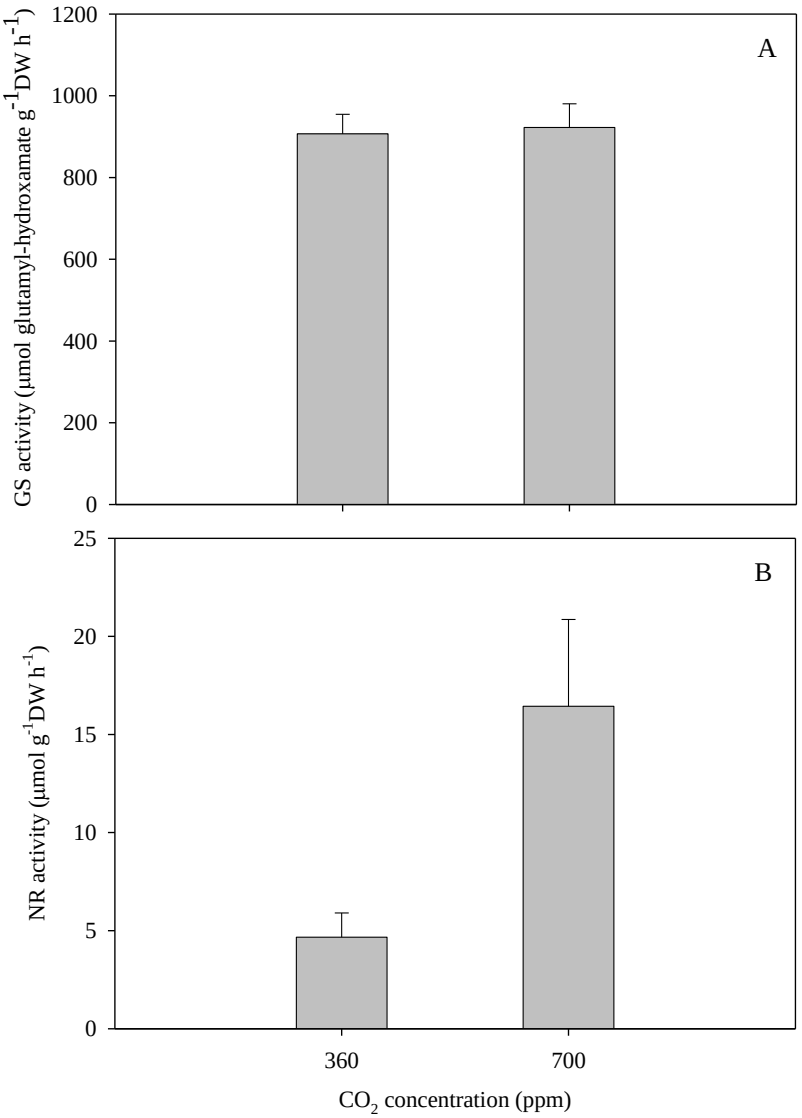

Figure 5. *Zostera noltii*. Effect of CO_2 enrichment on the activity of the enzymes glutamine synthetase (A) and nitrate reductase (B). Values are mean ± SE (n= 3).

On the other hand, the activity of nitrate reductase was 3-fold higher (16.4 ± 4.4 µmol NO_2^- g^{-1}DW h^{-1}) in the leaves of plants exposed to higher CO_2 concentration than in control plants (4.7 ±1.2 µmol NO_2^- g^{-1}DW h^{-1}).

Discussion

The present study showed that the net photosynthetic rates of *Zostera noltii* were positively affected by the CO_2 enrichment of the seawater. Plants exposed to CO_2-enriched conditions showed higher photosynthetic rates at saturating irradiances and were photosynthetically more efficient at limiting light intensities compared to plants exposed to the current CO_2 concentration. These results indicate that photosynthesis of *Z. noltii* is C_i-limited at the current inorganic carbon concentration of seawater, confirming the conclusions previously obtained for the same species (Silva et al., 2005), and for other seagrass species (Beer and Koch, 1996; Zimmerman et al., 1997; Invers et al., 2001). Collectively, these results indicate that *Z. noltii* plants may benefit from future CO_2 enrichment by enhancing the photosynthetic rates at higher CO_2 concentrations. The CO_2-stimulated increase of photosynthesis found here for *Z. noltii* is consistent with the findings reported for the temperate and tropical seagrass species *Z. marina* and *Thalassia testudinum*, where positive photosynthetic responses to CO_2 enrichment were also found (Beer and Koch, 1996; Thom, 1996; Zimmerman et al., 1997; Jiang et al., 2010).

The CO_2 enrichment did not stimulate the growth rate of *Z. noltii* leaves, despite the higher photosynthetic rates of plants exposed to elevated CO_2 concentrations. This finding is inconsistent with other seagrass studies where CO_2 enrichment reportedly enhanced leaf growth rates (Thom, 1996; Jiang et al., 2010). Growth rates were also enhanced in several

seaweed species cultured at CO_2 levels two to three times higher than the current seawater CO_2 concentration (Gao et al., 1991; Gao et al., 1993; Gordillo et al., 2001; Zou, 2005, Xu et al., 2010). However, there is evidence that the stimulation of growth by elevated CO_2 concentrations can be strongly curtailed in plants grown under nitrogen-limited conditions (Stitt and Krapp, 1999 and references therein; Liu et al., 2010). For example, nitrogen-sufficient cultures of *Ulva sp.* grown at CO_2-enriched conditions more than doubled the growth rate, whereas growth rates of nitrogen-limited cultures were only slightly increased (Gordillo et al., 2001). The nitrogen status of the plants, which is a consequence of the nitrogen growth conditions, has also been used to determine the expression of effects of CO_2 enrichment on growth rates (Andría et al., 1999; Gordillo et al., 2001). The leaf nitrogen content of *Z. noltii* plants from both experimental CO_2 treatments (1.4%) was below the critical level of 1.8% reported as indicative of low nitrogen supply (Duarte, 1990), suggesting that the nitrogen available for the plants in the mesocosm was insufficient to fully meet the species nitrogen requirements for growth. Therefore, we hypothesize that the growth rates of *Z. noltii* plants were primarily controlled by the low nitrogen availability rather than by the elevated CO_2 concentration in the mesocosm. An important corollary of this is that the global effects of CO_2 on seagrass growth may not be spatially homogeneous and will depend on the specific nitrogen availability of each system.

The effect of CO_2 enrichment on the nitrogen uptake rates of *Z. noltii* was more evident for nitrate than for ammonium. Surprisingly, the nitrate uptake rates of CO_2-enriched plants were significantly lower, whereas the ammonium uptake rates were slightly enhanced. These findings are the opposite of our initial hypothesis that the lower pH of CO_2-enriched seawater would decrease the ammonium uptake rates because the higher

content of H^+ reduces the activity of H^+-ATPase, which is involved in the cation transport into the cells (Marschner, 1995). On the other hand, nitrate uptake rates were expected to increase under lower pH, because in higher plants nitrate is co-transported with H^+ through the membrane (Vessey et al., 1990). Research on the nitrate transport system of *Z. marina* leaves (García-Sánchez et al., 2000) suggested that the uptake of nitrate in this species is probably not coupled with H^+, as in other angiosperms (Ullrich, 1992). A similar situation may occur in *Z. noltii*.

Factors other than pH might be involved in the decrease of the nitrate uptake rates of *Z. noltii* observed at high CO_2. A nitrogen-limited seaweed *Ulva lactuca* also showed much lower nitrate uptake rates when exposed to elevated CO_2 conditions (Magnusson et al., 1996). The authors also concluded that the decreasing effect of CO_2 enrichment on the nitrate uptake rates was not related to the pH level of the seawater and suggested that uncontrolled CO_2 entering the cellular compartments may affect regulatory mechanisms and enzyme functioning with consequences for the nutrient uptake rates.

The nitrate assimilatory capacity of *Z. noltii* was positively affected by the CO_2 enrichment, as revealed by the higher nitrate reductase activity of plant leaves grown under CO_2-enriched conditions. The CO_2-driven stimulation of the activity of this enzyme was also reported for terrestrial plants and seaweeds (Fonseca et al., 1997, Mercado et al., 1999; Gordillo et al., 2001; Zou, 2005). In terrestrial plants, it has been suggested that elevated CO_2 controls nitrate assimilation indirectly through the amount of accumulated carbohydrates (Fonseca et al., 1997). Increased accumulation of carbohydrates, as soluble sugars and starch, has been observed in both seagrasses and seaweed species grown at elevated CO_2 concentrations as a consequence of limiting nitrogen regimes (Zimmerman et

al., 1995, 1997; Andría et al., 1999; Jiang et al., 2010). Under nitrogen limitation, the increased photosynthetic activity demonstrated by *Z. noltii* may have caused an imbalance between the carbon supply and its utilization for growth, leading to an accumulation of carbohydrates. We hypothesize that *Z. noltii* plants exposed to elevated-CO_2 concentrations may have accumulated higher levels of carbohydrates, which contributed to increase the nitrate reductase activity by supplying energy and carbon skeletons for the nitrate reduction process. On the other hand, it has also been suggested that the CO_2-driven increase in the maximum nitrate reductase activity is regulated not by the carbohydrate level or internal carbon content but rather through a direct action on the enzyme synthesis, which is triggered by nitrate signaling (Gordillo et al., 2001). This is an interesting topic that deserves further investigation.

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Final synthesis

1. Ammonium is the preferential inorganic nitrogen source for the seagrass *Zostera noltii*. When both inorganic nitrogen forms are available, *Z. noltii* relied preferentially on ammonium as the main nitrogen source. However, the species displayed a high nitrate uptake capacity in the absence of ammonium. This was demonstrated in two chapters of this thesis, where nitrate uptake rates were nearly as high as those of ammonium when nitrate was supplied alone. This result suggests that nitrate is a possible alternative to ammonium as a nitrogen source.

2. Leaves are the preferred site for ammonium uptake, with roots functioning minimally in the N_i uptake. Both V_{max} and α for ammonium were one order of magnitude higher in the leaves (28.3 µmol g^{-1}DW h^{-1}) compared to roots (3 µmol g^{-1} DW h^{-1}). These two characteristics allow *Z. noltii* not only to effectively take up pulsed ammonium at low concentrations but also to take advantage of transient high levels of ammonium in the water column.

3. Leaves are the primary reducing site in the nitrogen assimilation process. The activity of two important enzymes in this process, glutamine synthetase and nitrate reductase, was 12 to 40-fold higher in the leaves than in the roots.

4. The simultaneous availability of both inorganic nitrogen forms slightly enhanced the uptake rate of ammonium and decreased the uptake rate of nitrate by 50-80% compared to when only one of the nitrogen forms was supplied. The enhancement of the ammonium uptake rates was attributed to an effect of alleviation of ammonium toxicity by nitrate whereas the decrease of the nitrate uptake rates was probably related with inhibitory feedback mechanisms associated with the production of glutamine or repression of the active transport of nitrate across the plasma membrane.

5. The uptake of ammonium or nitrate by one plant part was not affected by the uptake of the other plant part, at least in incubations that lasted four hour. Additionally, no appreciable translocation of inorganic nitrogen occurred between plant parts. The ^{15}N enrichment detected in the rhizomes suggests either a direct uptake of inorganic nitrogen or its transference from the roots. We hypothesize that *Z. noltii* may lack internal translocation of inorganic nitrogen because both leaves and roots are able to take up inorganic nitrogen and to reduce it into organic nitrogen compounds.

6. The estimated whole-plant nitrogen budget of *Z. noltii* (215 μmol m^{-2} h^{-1}) in the peak production season (spring) was slightly lower than the total nitrogen requirement for growth (236 μmol N m^{-2} h^{-1}), indicating that the growth of *Z. noltii* in Ria Formosa lagoon is not limited, or is only slightly limited, by nitrogen.

7. *Z. noltii* takes up ammonium and nitrate at similar rates in the light and in the dark. In both light and dark conditions, the nitrogen uptake displayed a temporal pattern of

enhanced initial rates followed by lower but relatively constant rates in response to ammonium or nitrate supplies of high concentration (95 and 65 µM, respectively).

8. In the light, *Z. noltii* plants incubated with either ammonium or nitrate accumulated soluble sugars in the leaves but plants incubated only with nitrate reduced their starch content, particularly in the rhizomes. These results indicate that the energy and carbon necessary to assimilate nitrate in the light derive not only directly from photosynthesis but also from the degradation of starch reserves.

9. In the dark, *Z. noltii* plants incubated with either ammonium or nitrate showed no significant reduction in the soluble sugars, except in the rhizomes of plants incubated with ammonium. *Z. noltii* plants incubated with nitrate showed reduction of the starch content of rhizomes. These results suggest that the assimilation of ammonium during darkness relies on energy and carbon derived from the degradation and mobilization of stored soluble sugars, whereas the assimilation of nitrate requires the degradation of starch reserves stored in the rhizomes.

10. The CO_2 enrichment of the seawater affected positively the photosynthetic rates of *Z. noltii*. Plants exposed for five months to elevated CO_2 concentrations (700 ppm) enhanced their maximum photosynthetic rate (P_m) and photosynthetic efficiency (α) when compared to plants exposed to current CO_2 concentrations (360 ppm). These results indicate that photosynthesis of *Z. noltii* is C_i-limited at the current inorganic carbon

concentration of seawater and suggest that *Z. noltii* may benefit from future CO_2 enrichment by enhancing the photosynthetic rates at higher CO_2 concentrations.

11. The absence of a significant effect of CO_2 enrichment on leaf growth rates of *Z. noltii* is inconsistent with the reports of other CO_2 enrichment studies on seagrasses. This absence was attributed to a certain level of nitrogen limitation experienced by *Z. noltii* plants in the mesocosm, which was revealed by the leaf nitrogen content.

12. The rate of ammonium uptake through the leaves and the activity of glutamine synthetase were not affected by elevated-CO_2 concentrations but nitrate uptake rates decreased by 4-fold. The hypothesis that the pH level of the seawater could be involved in this decrease was discarded. The CO_2-driven stimulation of the nitrate reductase activity was attributed to the probable higher carbohydrate level of the plants exposed to enriched-CO_2 concentrations and low nitrogen availability.

13. Collectively, the results of this thesis show that *Z. noltii* is physiologically adapted to take up inorganic nitrogen mainly as ammonium available in the water column at very low concentrations. The lack of physiological interactions in the nitrogen uptake between plant parts and the absence of internal translocation of the incorporated nitrogen suggest that leaves and roots act independently in the uptake and assimilation process. This hypothesis is also corroborated by the individual capacity for inorganic nitrogen reduction displayed by the leaves and roots. The energy and carbon necessary to assimilate ammonium in the light derive directly from photosynthesis whereas nitrate assimilation

also involves the degradation of starch reserves. On the other hand, a process of starch catabolism appears to generate the energy for the uptake and assimilation of ammonium and nitrate during darkness. Finally, the enhancement of photosynthetic rates and the lack of negative effects on the ammonium uptake rates of *Z. noltii* plants exposed to enriched-CO_2 conditions suggest that the species may benefit from future increases in seawater CO_2 concentration.