MOLECULAR PHYSIOLOGY REVEALS AMMONIUM UPTAKE AND RELATED GENE EXPRESSION IN THE SEAGRASS *ZOSTERA MUELLERI*

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ABSTRACT

Seagrasses are important marine foundation species, which are presently threatened by coastal development and global change worldwide. The molecular mechanisms that drive seagrass responses to anthropogenic stresses, including elevated levels of nutrients such as ammonium, remains poorly understood. Despite the evidence that seagrasses can assimilate ammonium by using glutamine synthetase (GS) / glutamate synthase (glutamine-oxoglutarate amidotransferase or GOGAT) cycle, the regulation of this fundamental metabolic pathway has never been studied at the gene expression level in seagrasses so far. Here, we combine (i) reverse transcription quantitative real-time PCR (RT-qPCR) to measure expression of key genes involved in the GS/GOGAT cycle, and (ii) stable isotope labelling and mass spectrometry to investigate ^{15}N-ammonium assimilation in the widespread Australian species *Zostera muelleri subsp. capricorni (Z. muelleri*). We demonstrate that exposure to a pulse of ammonium in seawater can induce changes in GS gene expression of *Z. muelleri*, and further correlate these changes in gene expression with ^{15}N-ammonium uptake rate in above- and below-ground tissue.

1. INTRODUCTION

Seagrasses are a group of vascular flowering plants whose evolutionary origins date back to the Cretaceous, approximately 100 million years ago (Larkum et al., 2006). They are composed of a true root-rhizome system (i.e. below-ground tissue) anchoring them to the seabed and a canopy of leaves (i.e. above-ground tissue) responsible for oxygen exchange, nutrient uptake and light capture (Larkum et al., 2006, Zimmerman et al., 1987) (Figure 1A). Worldwide, there are approximately 72 seagrass species in 12 genera (Short et al., 2011). Seagrasses play an important role in coastal ecosystems by (i) providing food and shelter for commercially important fish (Beck et al., 2001), (ii) enhancing sediment accretion (Koch et al., 2013), (iii) protecting the coastline from wave energy (Larkum et al., 2006) and (iv) sequestering carbon, being responsible for 10 to 18% of the carbon accumulation in the ocean (Macreadie et al., 2014a).

Despite their global importance, seagrasses are at risk worldwide. Significant losses of seagrass meadows have been reported (Calleja et al., 2007, Cambridge and McComb, 1984, Duarte, 2002) and Orth et al. (2006) declared a "global crisis for seagrass meadows". Previous studies have established a causal link between large die-off events and environmental stresses, having been triggered by climate change and anthropogenic impacts, e.g. ocean warming, increased frequency of heavy rainfall and severe weather as well as agricultural/urban runoff, eutrophication, boat mooring and dredging (Orth et al., 2006, Ralph et al., 2007, York et al., 2015). Eutrophication and enrichment of nutrients, especially nitrogen, in coastal waters have been shown to be one of the major factors of seagrass decline and have thus received increasing attention within the past decades (e.g. Short and McRoy, 1984, Touchette and

Burkholder, 2007, Alexandre et al., 2010, Apostolaki et al., 2012). A range of mechanisms have been proposed to explain reduced seagrass survival under anthropogenic nutrient enrichment including algal turbidity (Morris and Tomasko, 1993), i.e. stimulation of algal overgrowth, which shades the underlying seagrass, limiting light availability during daytime and reducing O_2 supply in darkness, both leading to diminished plant fitness (Kemp et al., 1983, Brodersen et al., 2015, Rasmussen et al., 2012). Direct nutrient toxicity effects on seagrass growth and survival have also been reported (Burkholder et al., 2007), but the underlying mechanisms have remained unclear.

Seagrasses are believed to have evolved in nitrogen-limited waters, and these plants may have maximized nitrogen uptake/assimilation processes during infrequent nutrients pulses (Burkholder et al., 1994). Previous research has shown that uptake of inorganic nitrogen from the water column or the sediment pore water can supply up to 73% of seagrass annual need (Pedersen and Borum, 1993) (Figure 1). This nitrogen supply is critical to balance the losses of nitrogen due to (i) denitrification in the rhizosphere and (iii) increase in meadow size and density (Pedersen and Borum, 1993). While the nutrients source (i.e. sediment pore-water or water column) and tissue (i.e. above or below-ground tissue) responsible for nutrients assimilation in seagrass are commonly considered to vary depending on availability and concentration, sediment pore water is often considered to be the primary source of nitrogen (see: Touchette and Burkholder, 2000 for review).

The physiology for ammonium assimilation to amino acids by seagrass tissues is complex involving the following two steps: (i) transport from seawater into the cell

and (ii) assimilation into amino acids. Ammonium transport into the cell is generally view as a passive movement through membrane channels but few studies have suggested the presence of a feedback inhibition mechanism indicative of a more active transport for certain seagrass species such as *Zostera marina* (Touchette and Burkholder, 2000, Short and McRoy, 1984). Among the molecular mechanisms driving nutrient assimilation into amino acids, the glutamine synthetase (GS) / glutamate synthase (glutamine-oxoglutarate amidotransferase or GOGAT) pathway of ammonium incorporation (Goodwin and Mercer, 1983) is an important and fundamental component of ammonium assimilation in seagrass (see: Touchette and Burkholder, 2000 for review). This metabolic pathway involves the incorporation of ammonium into glutamine by GS and the subsequent regeneration of glutamate from glutamine and α-ketoglutarate by GOGAT (Goodwin and Mercer, 1983) (Figure 1B). Indeed, several studies focusing on this metabolic pathway in seagrass have demonstrated the influence of the surrounding environment on the enzymatic activity of GS and its involvement in ammonium assimilation (Kraemer et al., 1997, Alexandre et al., 2010, see: Touchette and Burkholder, 2000 for review). However, the regulation of this fundamental metabolic pathway has never been studied at the gene expression level so far in seagrass.

Our ability to predict the impacts of anthropogenic stresses such as nutrients enrichment on seagrass meadows is dependent on how well we understand the mechanisms that drive the cellular response to nutrients enrichment. Understanding these underlying mechanisms also has importance to policy and management responses, especially when it comes to understanding why some seagrass meadows appear to vary in their tolerance to nutrient loading. Seagrasses respond to changing

environments by regulating molecular pathways to prevent or abate physiological damage. These molecular events precede population-level changes and could be useful biomarkers if they can be linked to specific physiological or ecological events (Macreadie et al., 2014b). Thus, seagrass researchers have recently taken advantage of the power of genomics and transcriptomics to address questions about the molecular mechanisms of ecological responses to environmental perturbations (Franssen et al., 2011, Franssen et al., 2014, Dattolo et al., 2014, Serra et al., 2012, Lauritano et al., 2015, Salo et al., 2015, Brakel et al., 2014). Following the recent publication of *Zostera marina* genome (Olsen et al., 2016), the seagrass community is now eagerly awaiting the progress that will be promoted by integrating molecular biology and gene expression into the spectrum of physiological and metabolic changes involved in the response of seagrass to environmental stress.

In the present study, we conducted such a multi-parameter analysis by monitoring (i) the initiation of the GS/GOGAT cycle by differential expression of GS and GOGAT transcripts using RT-qPCR and (ii) the assimilation of ammonium by measuring the incorporation of ^{15}N using mass spectrometry in the above- and below-ground tissue of the seagrass *Zostera muelleri*. We hypothesised that seawater enriched in ammonium would affect levels of expression for these key genes in nitrogen assimilation in *Z. muelleri*. Moreover, for the first time, we correlated changes in gene expression to the overall uptake rate of nutrients within the above- and below-ground tissue.

2. MATERIAL AND METHODS

2.1. Collection and maintenance of seagrass

Zostera muelleri plants with intact sediment core of 5 cm in diameter and 10 cm in depth were collected at Pelican Banks, Port Curtis (Queensland, Australia, ambient salinity: 32 and water temperature: 24°C) and transported to the aquarium facility at University of Technology Sydney (UTS), where they were acclimatised for two months under an irradiance of 150 μmol photons m^{-2} s^{-1} over a 12:12 light:dark cycle. Ambient water temperature was maintained at 24°C by heater-chillers; practical salinity *sensu* (Lewis, 1980) was kept between 31.0 - 33.0 and adjusted when necessary using deionized water / seawater. During the acclimation period, nutrients concentration was monitored weekly in the aquaria and remained low (NO_2^-,<1 μM; NO_3^-,<5 μM ; NH_4^+, <1 μM). Further, seagrass leaves were cleaned using thumb and forefinger with gloves gently, and water was changed on a fortnightly basis to minimise epiphyte growth. After the initial acclimatisation period, the labelling experiment (as described below) was conducted to examine the effect of elevated nutrients on both ammonium assimilation and gene expression in *Z. muelleri*.

2.2. *Labelling experiment*

Plants were exposed to two experimental treatments (i.e. ammonium enriched and control treatments) across a total of six tanks (three 4 litres tank replicates per treatment with a total of 8 shoots per tank; closed water system yet continuously stirred using one powerhead pump for each tank). During the 36 h of the experiment, the conditions were as follows: practical salinity at 32.0, pH 8.1, irradiance of 150 μmol photons m-2 s-1 over a 12:12 light:dark cycle and 2mM of dissolved inorganic carbon. Three replicate samples of *Z. muelleri* (one plant for each of the three tank

166 replicates) were collected for each time point (0, 6, 12 and 36 h) and for each
167 treatment (ammonium enriched and control treatments) for further isotopic analysis
168 and gene expression analysis using RT-qPCR.
169 For the ammonium-enriched treatment, seawater used during acclimation was emptied
170 and replaced by dispensing artificial seawater enriched in ^{15}N-ammonium in the
171 aquarium tanks. The artificial seawater was prepared freshly using ultrapure water (no
172 detectable Dissolved Inorganic Nitrogen, recipe adapted from (Harrison et al., 1980)),
173 initially free of ammonium, which was then enriched in ^{15}N-labelled ammonium by
174 adding $^{15}NH_4Cl$ powder to a final concentration of 20 µM. This concentration is
175 above the natural concentration of ammonium in seawater and may represent elevated
176 environmental levels of ammonium caused by nutrients enrichments in coastal waters
177 (Koop et al. 2001). For example, a previous study reported that ammonium
178 concentration varied between <0.5 and 40 mM in northern Port Phillip Bay (VIC,
179 Australia) during periods of low runoff (January 1995) and high runoff (September
180 1993) respectively (Longmore et al., 2000). The $^{15}NH_4Cl$ powder had a ^{15}N isotopic
181 abundance of 98% (commercially available from Sigma, Castle Hill, NSW,
182 Australia).
183 For the control treatment, seawater used during acclimation was emptied and replaced
184 by dispensing artificial seawater, which was prepared freshly using ultrapure water as
185 described above (no detectable Dissolved Inorganic Nitrogen, recipe adapted from
186 Harrison et al., 1980), but this time free of ^{15}N- ammonium. It is important to note
187 that this experimental design could have induced nitrogen limitation in the control
188 treated plants. However, because our labelling experiment was focused on rapid
189 physiological response of seagrass (i.e. 36 hours experiment), this experimental
190 design was used to ensure that (i) the artificial seawater used for the control treatment

was initially nutrients-free and (ii) ^{15}N-labelled ammonium was the only source of nutrients in the ammonium-enriched treatment. For each plant sample collected, above- and below-ground tissues were separated using scissors and resulting samples were stored separately at -80°C for further isotopic and RT-qPCR analysis.

2.3. Nitrogen content and uptake rate

Prior to nitrogen content and ^{15}N/^{14}N analysis, samples of above- and below-ground seagrass tissue were freeze-dried and weighed. Nitrogen content (%) and ^{15}N/^{14}N (δ^{15}N, in ‰) were then analyzed in triplicate using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer at the Stable Isotope Facility, Department of Plant Sciences (UC Davis, California, US). The Increase in ^{15}N/^{14}N ratio for each sample was calculated in ‰ as follows:

$$\delta^{15}\mathrm{N} = (Nmes/Nnat) - 1) \times 10^3$$

where,

Nmes: ^{15}N/^{14}N measured in labelled samples;

Nnat: natural abundance of ^{15}N/^{14}N measured in unlabelled samples.

The nitrogen uptake rate per mg of tissue (ρ) was then expressed in ng N h^{-1} mg^{-1} and was calculated by normalizing the ^{15}N-incorporation measured (i) to the average nitrogen content (% of dry mass) of the tissue and (ii) to the time of incubation according to the equation previously described (Dugdale and Wilkerson, 1986):

$$\rho = ((Nmes - Nnat)/((Nenr - Nnat) \times Tinc)) \times Ncontent \times 10^3$$

where,

Nmes: ^{15}N/^{14}N measured in labelled samples;

Nnat: natural abundance of ^{15}N/^{14}N measured in unlabelled samples.

Nenr: ^{15}N-enrichment of the incubation medium

Tinc: incubation time

Ncontent: average nitrogen content (%) measured by using combustion (950°C) method.

2.4. Quantitative Real-Time PCR (qPCR)

The present study conforms to the Minimum Information for Publication of Quantitative Real-Time PCR guidelines (Bustin et al., 2009). In this section, we indicate the essential information, *sensu* (Bustin et al., 2009), required to allow reliable interpretation of the corresponding qPCR results.

2.5. Primer design

In-depth analysis of *Zostera muelleri* Transcriptomics Database (Hayward et al, in prep), revealed transcripts encoding proteins with high similarities to the domains of GS and GOGAT proteins already identified in the seagrass *Zostera marina* (Olsen et al., 2016). The sequences were blasted for isoforms (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and functional domains of two of these sequences, coding for Glutamine synthetase (GS; KMZ65307) and Glutamate synthase (GOGAT; KMZ61057) respectively, were used as a template to design sequence-specific primers for RT-qPCR using the software, Primer3 0.4.0 (Rozen and Skaletsky, 2000) (Source code available at http://fokker.wi.mit.edu/primer3/; Table 1).

2.6. RNA extraction and cDNA synthesis

Above-ground and below-ground samples (i.e. pieces from the second youngest leaf and youngest rhizome internode) from control and ammonium-enriched treatments were collected at 0, 6, 12, and 36 hours after incubation, snap-frozen in liquid nitrogen and stored in -80°C freezer until further analysis (n = 3). Samples were then ground into powder in liquid nitrogen using a mortar and pestle. RNA was then extracted using the RNA mini kit according to manufacturer's instructions (PureLink ™ RNA Mini Kit, Life Technologies). Column purification was carried out to remove gDNA using PureLink ™ DNase (Life Technologies). The RNA quantity was assessed using Qubit RNA HS assay kit (Invitrogen) and quality checks were performed using Nanochip technology (Agilent 2100 Bioanalyzer). High-quality RNA samples (integrity number >6) were used for further cDNA synthesis and RT-qPCR analysis. The cDNA was generated using a High Capacity Reverse Transcription kit (Applied Biosystems) in a 20 µl reaction using 200 ng of total RNA as template.

2.7. RT-qPCR

RT-qPCR gene expression quantification was performed in three technical replicates using SYBR Green PCR Master Mix (Applied Biosystems) on a Step One Plus Real-Time PCR System (Applied Biosystems). Amplification of 10 µL reactions with 1:50 dilutions of cDNA from control and treated seagrasses and 1 µM of each specific primers were placed in 96-well optical plates with the following PCR conditions: incubation at 95°C for 10 min, then 50 cycles of 95°C, 60°C, 68°C for 30 s each followed by 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The RT-qPCR efficiency for each gene and each treatment was determined from a cDNA dilution gradient of 243, 81, 27, 9 and 3 ng and a linear regression model (Pfaffl, 2001). The

corresponding RT-qPCR efficiencies were calculated according to the equation described by Radonic et al (Radonic et al., 2004):

$$PCR\ efficiency = (10^{[-\frac{1}{slope}]} - 1) \times 100$$

All of the RT-qPCR efficiencies obtained for the different primers were between 89-100% (Table 1; Supplementary Table S1), with a calibration coefficient >0.95 similarly to previous studies (Winters et al., 2011, Bergmann et al., 2010). A *no template control*, as well as a *no reverse transcription control* was generated for each gene and each treatment to ensure that the PCR reactions were free of DNA contamination. Replicate variability of the CT values between the 3 technical replicates, which were run on the same plate, was examined for each sample-gene combination. Repeatability of the assay between the technical replicates was consistent across the different genes with the replicate variability falling within the set limit of <0.5 cycles for all the sample-gene combinations tested.

2.8. Data acquisition

Data from RT-qPCR was analysed using the Step One Plus Software (Ver. 2.3; Applied Biosystems). Expression levels were determined as the number of cycles needed for the amplification to reach a fixed threshold in the exponential phase of the RT-qPCR reaction (Walker, 2002). The cycle threshold (CT) was set at 0.03 for all genes. To validate changes in target genes expression, CT were imported into the qbase+ software package (Biogazelle). Expression data for target genes was first normalized against the best combination of 3 reference genes as previously described (Adenosylhomocysteinase, AHCY; Glyceraldehyde 3-phosphate dehydrogenase, GADPH; and Translation initiation factor 1 subunit beta, Elof1; Supplementary Table

S1, Supplementary Figure S1) (Schliep et al., 2015) using GeNorm algorithm within qbase+ software package (Biogazelle) and then transformed into quantities using maximum efficiency of 1.00 (or 100%) to obtain Calibrated Normalized Relative Quantities (CNRQ) (Hellemans et al., 2007).

2.9. Statistical analysis

Statistical analyses were done using Repeated Measures Analyses of Variance with PERMANOVA+ software in PRIMER v6 (Anderson et al., 2008). The analyses tested the null hypothesis that there no difference in these data among ammonium enriched and control treatments and/or among sampling time (0, 6, 12 and 36 hours). Univariate analyses of variance of ^{15}N-enrichment and gene expression data were undertaken based on Euclidean dissimilarity matrices (Anderson et al., 2008) using the factors described in Supplementary Table S2. Where significant factors' effects were detected, post hoc permutational pair-wise comparison using PERMANOVA+ were undertaken to identify the levels of factors in which differences occurred. P-values were based on 4999 permutations and results were considered significant at $P < 0.05$. Differential gene expression data are presented on a log scale relative to control. Throughout the paper, values given are mean±SE (n=3 plants). Because of biomass limitation, we used a random design for the factor aquaria and only one plant was collected for each of the aquaria replicate (1, 2 and 3) for each time point (0, 6, 12 and 36 h) and for each treatment (ammonium enriched and control treatments). Therefore, we could not add aquaria as a factor in our statistical analysis (i.e. n=1 plant per aquaria per time point per treatment but n=3 plants per time point per treatment in total). While adding aquaria as an additional factor and nesting it under treatment would have potentially increased the statistical power as well as the testing

313 of specific temporal variation, our experimental design allowed to testing for
314 treatment-specific temporal variation in ^{15}N enrichment and gene expression.

3. RESULTS AND DISCUSSION

3.1. Nitrogen uptake in above- and below-ground tissue of Zostera muelleri

The dynamics of ^{15}N-ammonium incorporation was followed and quantified in the above- and below-ground tissue fractions of *Z. muelleri* plants by measuring the $^{15}N/^{14}N$ ratio after tissue separation. The $^{15}N/^{14}N$ mass spectrometry data indicated rapid uptake of ^{15}N-ammonium by *Z. muelleri* plants (Figure 2). The above-ground tissue displayed a substantial ^{15}N-enrichment after incubation in enriched seawater with a 11093 ± 1412 ‰ ^{15}N-enrichment within the first 6 h of labelling experiment (pair-wise comparison between control and treated seagrass plants at t=6 h, p=0.003, Supplementary Table S2B), followed by further increase in the next 6 h of the experiment (14457 ± 438 ‰ ^{15}N-enrichment at t=12 h, pair-wise comparison between control and treated seagrass plants, $p<0.001$, Supplementary Table S2B) and accompanied by progressive increase from 12 h onward (21960 ± 5868 ‰ ^{15}N enrichment at t=36 h, pair-wise comparison between control and treated seagrass plants, p= 0.016, Figure 2A, Supplementary Table S2B). In contrast, the ^{15}N-labeling of the below-ground tissue remained substantially weaker during the entire experiment (Figure 2B), with an average ^{15}N-enrichment 3 to 4 times weaker than the one observed in the above-ground tissue. The below-ground tissue showed a significant increase in $^{15}N/^{14}N$ ratio in response to enriched seawater with a 2582 ±636 ‰ ^{15}N-enrichment within the first 6 h of labelling experiment (pair-wise comparison between control and treated seagrass plants at t=6 h, p= 0.017, Supplementary Table S2D), followed by further increase (4935 ± 1700 ‰ ^{15}N-enrichment at t=12 h ; pair-wise comparison between control and treated seagrass plants, p= 0.021, Supplementary Table S2D, Figure 2B). Because ^{15}N-ammonium was added in the water column, the slight delay observed in ^{15}N-enrichment in below-

ground tissue compared to the above-ground tissue is likely to reflect the time needed for (i) basipetal translocation of ^{15}N-compounds (Zimmerman and Alberte, 1996) and/or (ii) ^{15}N-ammonium diffusion from the water column into the sediment pore water directly in contact with seagrass below-ground tissue.

By normalizing the ^{15}N-incorporation to the average nitrogen content of the above- and below-ground fractions and to the time of incubation following the equation of (Dugdale and Wilkerson, 1986), it was possible to estimate the ^{15}N taken up per hour by the above- and below-ground tissue, respectively. In the first 12 hours of the experiment, the above-ground tissue took up nitrogen with a specific rate of 267 ± 27 ngN h^{-1} mg^{-1}. This uptake rate is in the range of values found previously in the leaves of *Z. marina* at the same initial ammonium concentration (Thursby and Harlin, 1982), confirming the capacity of *Z. muelleri* above-ground tissue to uptake nitrogen rapidly in response to a pulse of nutrients in the water column. Comparison between above- and below-ground tissue uptake rates further allows establishing the relative contribution of above- and below-ground tissue to the ammonium uptake capabilities. In this respect, our data indicates that the above-ground tissue incorporated 8 times more nitrogen than below-ground tissue during the first 12 h of the labelling experiment (specific uptake rates of: 267 ± 27 ngN h^{-1} mg^{-1}, n=6 for above-ground tissue; 33 ± 11 ngN h^{-1} mg^{-1}, n=6 for below-ground tissue; Figure 2C). This particular result indicates a significant heterogeneity in the access and therefore ability of above- and below-ground tissue to uptake nitrogen from a pulse of nutrients in the water column; the above-ground fraction being the most active, but not the only site of assimilation. It is important to note that these uptake rate calculations assume that the ^{15}N isotope fraction of the ammonium was the same in the water above and below the sediment. Therefore, the uptake reported for the below-ground tissue is likely to be

underestimated, but the uptake rate for above-ground tissue remains valid. It is also possible that nitrogen-levels and fauna present in the sediment might have had an impact on our data but we did not measure these parameters as our labelling experiment was focused on the physiological responses of seagrass to a short pulse of nutrients (i.e. 36 hours experiment after 2 months acclimation under laboratory conditions). Further studies focussing on longer term response should therefore investigate the effects of different nitrogen-levels and fauna present in the sediment on ammonium assimilation in *Z. muelleri*. Because the pulse of ^{15}N-ammonium was experimentally introduced into the water column, the difference in the intensity and the kinetics on which the ^{15}N-label reached the above- and the below-ground tissue suggest that the ^{15}N incorporated by below-ground tissue derives either (i) from rapid assimilation by the leaves and then a delayed basipetal translocation of nitrogenous compounds (Kaldy et al., 2013, Thursby and Harlin, 1982) or (ii) from the delayed diffusion of ^{15}N-ammonium into sediment pore water surrounding the below-ground tissue.

3.2. Expression of key assimilation genes in above- and below-ground tissue of Zostera muelleri

In parallel to ^{15}N-ammonium assimilation, we were able to detect changes in the expression of transcripts coding for GOGAT and GS in the different tissue fraction of *Z. muelleri* over the course of the experiment using RT-qPCR (Figure 3). Among the transcripts targeted in this study, GS transcripts was significantly upregulated in above- and below-ground tissue of *Z. muelleri* in response to enriched seawater with a significant effect of the incubation time (above-ground tissue: PERMANOVA analysis, $p<0.001$, Figure 3A, Supplementary Table S2E; below-ground tissue: PERMANOVA analysis, $p=0.042$, Figure 3C, Supplementary Table S2H).

Ammonium enriched seawater induced different temporal dynamics of GS transcripts regulation in the above- and below-ground tissue. Indeed, the earliest significant gene regulation occurred within the above-ground tissue, with GS being significantly upregulated from 6 h onward (treatment: 3.3 up-regulation; pair-wise comparison, p=0.001, Figure 3A, Supplementary Table S2F) while in the below-ground tissue GS was significantly upregulated only after 12 hours (treatment: 9.2 up-regulation; pair-wise comparison, p= 0.012, Figure 3C, Supplementary Table S2I). Furthermore, in the above-ground tissue, this increase in expression level of GS transcript remained stable and significant from 6 hours onwards (Figure 3A) while in the below-ground tissue, the upregulation of GS transcript was only significant at 12 hours and decreased back to control level after that (Figure 3C). In the light of nitrogen uptake data, these gene expression data suggest that the delay observed in ^{15}N incorporation in below-ground tissue could be linked (i) to the time needed for ^{15}N-ammonium to diffuse into the sediment pore water directly in contact with seagrass and/or (ii) basipetal translocation. Because the level of expression GS transcript declined concomitantly with the ^{15}N-enrichment in the below-ground tissue, our results further suggest that during our experiment, very little of the ^{15}N-ammonium diffused into the porewater, indicating that the second hypothesis is more likely. Although sediment pore water is generally considered to be the primary source of ammonium for seagrasses (Touchette and Burkholder, 2000), our results are supported by previous studies indicating that ammonium uptake by below-ground tissues can be limited by diffusion into sediment pore water (Short and McRoy, 1984, Stapel et al., 1996). It is important to note that our experiment was focused on investigating rapid physiological and molecular response of seagrass and represents a very unrealistic case, since most sediments contain very high concentrations of ammonium (up to mM

levels). Further, the relative contribution of seagrass below-ground tissue to ammonium assimilation is highly variable and might depend on (i) the presence of microbial population associated with sediment or seagrass tissue and (ii) on the level of ammonium in the water column and on the type of substrata the plants are growing on. For instance, (Thursby and Harlin, 1982, Terrados and Williams, 1997) indicated that maximum rates of root ammonium uptake were substantially diminished when leaves were exposed to increased ammonium concentration, suggesting that under these conditions, (i) most of nutrient absorption was occurring from the water column into the leaves and (ii) basipetal translocation of nitrogenous products was higher relative to acropetal translocation. Future experimental studies combining stable isotope incubation, characterisation of associated microbial population and gene expression over a range of environmental conditions should also include simultaneous measurement of (i) N-levels in the water column and in the sediment pore-water as well as (ii) bioturbation in order to determine more precisely the environmental factors affecting nutrient assimilation and exchanges in *Z. muelleri* and the molecular regulations involved in the different tissues.

As for land plants, GS and GOGAT are widely recognized as key enzymes in seagrass nitrogen metabolism. GS drives the assimilation of ammonium into glutamine first, which is then followed by addition of carbon skeletons by GOGAT to produce two glutamate molecules. However, the diversity of isoforms present in seagrasses for these key genes in nitrogen assimilation remains unknown and future studies characterizing the diversity of GS isoforms and their localisation within the different tissues of seagrass are needed to reveal specific functional roles and possible adaptations.

In higher plants, GOGAT catalyses the production of glutamate from glutamine and α-ketoglutarate (Lam et al., 1996). While an increasing trend was observed in the expression level of this transcript in both tissues in response to seawater enriched in ammonium, no statistical difference was observed (above-ground tissue: PERMANOVA analysis, p=0.204, Figure 3B, Supplementary Table S2G; below-ground tissue: PERMANOVA analysis, p=0.249, Figure 3D, Supplementary Table S2G), suggesting either (i) that the variability in our RT-qPCR assay was too high to allow detecting statistical difference in the expression level of this transcript or (ii) that the role of GOGAT regulation in ammonium assimilation in *Z. muelleri* may depends on other modifications (e.g. post-translational) that may not be followed by differential gene expression.

3.3. Expression of key assimilation genes as potential bioindicators of nutrients levels

Ammonium assimilation in seagrasses is complex and variable, the first step of this process, transport of ammonium into the cell, being active for certain species and population of seagrass, while passive for others (Touchette and Burkholder, 2000). As a result, sensitivity to nutrients pulse can vary among different species and populations of seagrass (Touchette and Burkholder, 2000) and ammonium concentration in seawater might not be a good predictor for seagrass ammonium assimilation concentration. Given that GS/GOGAT cycle is widely recognized as the universal route for the second step of this process, i.e. incorporation of ammonium into amino acids through glutamate, the regulation of key genes involved in this pathway could conceivably be used as an indicator for exposure of different species and populations of seagrass to a sudden pulse of ammonium. In this respect, by

combining nitrogen uptake with gene expression data from our labelling experiment (t=6, 12 and 36 hours), we built a series of regressions in an attempt to predict the relationship between the expression level of GS and GOGAT transcripts and the uptake rate measured in the above- and below-ground tissue of *Z. muelleri*. Although regression might not be the best way to describe these relationships because of our experimental sampling design, this supplementary data can be useful to suggest correlation between ^{15}N-enrichment and differential expression of certain genes (Supplementary Table S3 and Supplementary Figure S2). Among the different transcripts investigated in this study, the level of expression of GS transcript was the most strongly correlated to ^{15}N-enrichment in the different tissue fractions of *Z. muelleri* (Supplementary Figure S2A and C). The ^{15}N-ammonium uptake rate explained more than 70% of the variation in GS transcripts in the above-ground tissue (Supplementary Figure S2A) and more than 50% in the below-ground tissue (Supplementary Figure S2C). Such molecular markers could be used (i) to characterize the nutritional history of different populations and species of seagrass in order to make informed management decisions about the likely impact of anthropogenic nutrient enrichment and (ii) to monitor changes in nutritional conditions of seagrass meadows rapidly (i.e. within 6 hours of the onset of nutrients enrichment), before any epiphyte growth and/or morphological changes occur (2 to 6 weeks) (Murray et al., 1999). However, it is important to note that such markers will be more complex to implement and interpret than a simple environmental assay for ammonium concentration. Further, given the dynamic kinetic that occurred during our study, further studies need to investigate these gene regulation on an hourly basis, especially in the context of diurnal cycle.

With the future advances of digital qPCR, the use of these markers might not even require the step of housekeeping genes selection that is normally needed to normalize RT-qPCR data (Schliep et al., 2015, Zmienko et al., 2015). Consequently, as long as a threshold database can be established and maintained for different species, our results may have implications for the management of seagrass meadows. Indeed, the regulations in the expression level of transcripts such as GS has potential to be developed as integrative indicators of nutrient conditions and anthropogenic nutrient enrichment. However, more work is clearly needed regarding the applicability of this technology as a management tool to monitor seagrass condition adjacent to coastal development and associated nutrient inputs, more particularly including (i) experiments with various environmental conditions to determine if the relationship between ammonium levels and the expression level of GS and GOGAT transcripts in seagrass can be influenced by others factors, especially light, temperature and salinity (Touchette and Burkholder, 2000), (ii) field collections and experiments with a broader range of nutrients levels in order to establish more precisely this dose-response relationship and (iii) experiments over longer period to assess if these target genes can provide early warnings of epiphyte growth and if their level of expression can integrate nutrient stress over a longer timeframe (i.e. remain altered after exposure to the pulsed event). In this respect, our results suggest that this molecular toolkit may be used as early warning of nutrient-stress only and may require a different set of genes for longer term nutrient impact assessment as they are more likely to affect epiphytes, thereby creating also an issue of competition for light. Further, because seagrass species have shown considerable variation in nutritional response, their acclimation or adaptation on differing nutrient levels could have potential effects on gene expression of GS and GOGAT, the response of these molecular signatures

remains therefore to be examined in other seagrass species and compared across geographic regions. Nevertheless, further research combining isotopic labelling experiments with gene expression analysis should enable us to significantly advance our knowledge of the metabolism and nutritional physiology that lie at the very heart of seagrass physiology.

In conclusion, this is the first study to investigate in parallel the expression of key genes involved in GS/GOGAT cycle and the assimilation of ammonium in the seagrass *Z. muelleri* in response to a pulse of nutrients. The present data constitute the first evidence that exposure of a seagrass to a pulse of ammonium in seawater can induce fast change in GS gene expression and suggest potential physiological importance of this gene regulation for nitrogen metabolism in the different tissues of this seagrass. These results not only provide new insights into the nutritional physiology of *Z. muelleri* but also have implications for the development of molecular markers for reactive monitoring of *Z. muelleri* meadows in response to anthropogenic nutrient enrichment.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MP and PR. Performed the experiments: SS, AC and MP. Analysed the data: GS, SS, AC and MP. Contributed reagents/materials/analysis tools: PR, PM and MR. Wrote the paper: MP with comments and suggestions from all authors.

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TABLES AND FIGURES LEGEND

Table 1

Target genes investigated in *Zostera muelleri* by using RT-qPCR. Accession numbers, primers sequences, amplicon length, melting temperature, and RT-qPCR efficiency are indicated. GS: Glutamine synthetase; GOGAT: Glutamate synthase.

Name	Accession number	Primer forward sequence	Primer reverse sequence	Length (bp)	Tm	Efficiency (%)
GS	KMZ65307	TGACCCTAAGCCAATTCCAG	CTTCATACCCACCAGGTGCT	92	60.0	97
GOGAT	KMZ61057	TGTCAGCTGGACCAAAGATG	AGGGCCAAGAAATCCCATAC	112	59.8	89

Figure 1

Ammonium as a nitrogen source for seagrass.
A Relative contributions of inorganic nitrogen (N_i: nitrate, NO_3^-; ammonium, NH_4^+; and urea) dissolved in water column (49%) and in porewater (51%), to the annual external nitrogen requirements of seagrass (Pedersen and Borum, 1993).
B Reactions involved in ammonium uptake and assimilation in seagrass cells. Ammonium enters the GS / GOGAT cycle, where (i) glutamate is aminated by the enzyme glutamine synthetase (GS) to synthesize glutamine, which is then (ii) transaminated by glutamate synthase (GOGAT) with the addition of carbon skeletons (α-ketoglutarate) to finally produce two glutamate molecules. One of these glutamate molecules can be recycled through the GS / GOGAT pathway, whereas the other can be used to form more complex amino acids (Touchette and Burkholder, 2000). Black boxes indicate key enzymes targeted in this study with coding genes identified in *Z. muelleri* transcriptome (Hayward et al, in prep) and in *Z. marina* genome (Olsen et al., 2016) and corresponding expression levels measured by RT-qPCR assay.

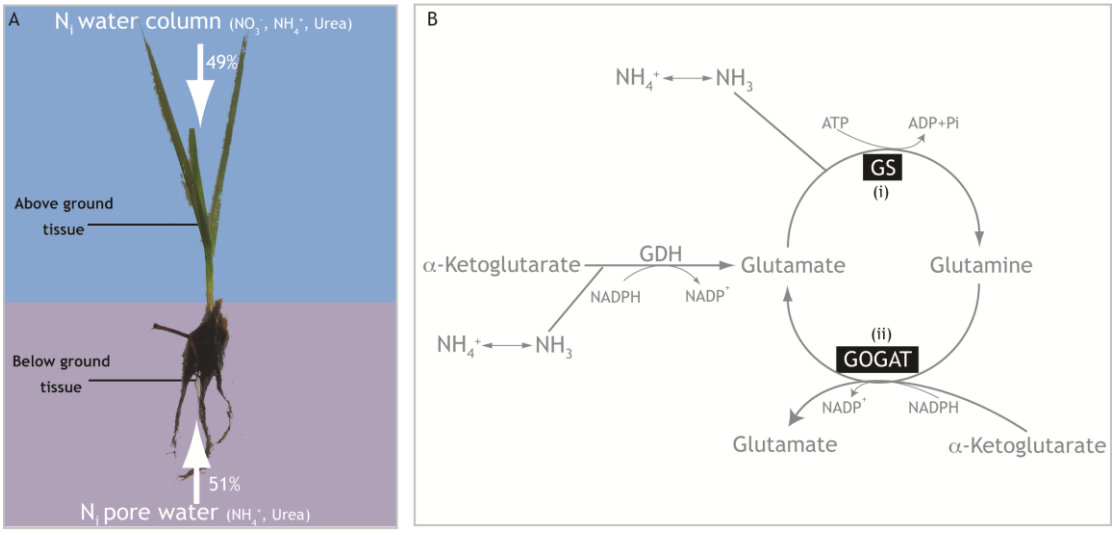


Figure 2

Quantification of nitrogen uptake in above- and below-ground tissue of *Zostera muelleri* in response to seawater enriched in ^{15}N-ammonium.

Time series of ^{15}N- enrichment in above- (A) and below-ground (B) tissue of *Zostera muelleri* for the duration of the experiment (36 hours). *Significant difference (pair-wise comparison, P<0.05) between control treatment (black circle; no ^{15}N-enrichment in seagrass tissue above the natural fluctuations of the ^{15}N/^{14}N ratio, 0.00385 ± 0.00015) and seawater enriched in ^{15}N-ammonium (red triangle; average ^{15}N-enrichement in ^{15}N/^{14}N ratio in seagrass tissue, 0.04009 ± 0.00649). Error bars represent ± SEM (3 plants). (C) Tissue specific nitrogen assimilatory capacity in *Zostera muelleri.* The total nitrogen uptake rate was calculated in above- (green) and below (white) ground tissue by normalizing the ^{15}N-incorporation to the average nitrogen content (% of dry mass) of *Z. muelleri* tissue and to the time of incubation according to the equation of Dugdale and Wilkerson (Dugdale and Wilkerson, 1986). The boxes indicate the mean value ± SEM (6 plants).

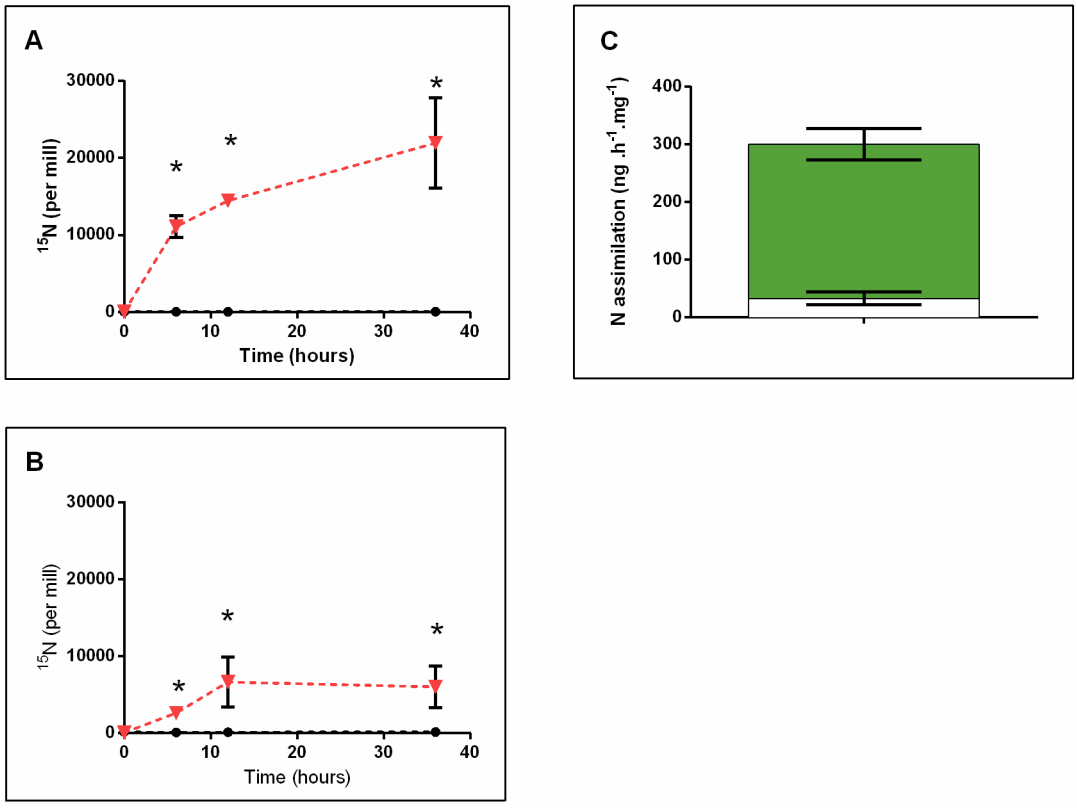
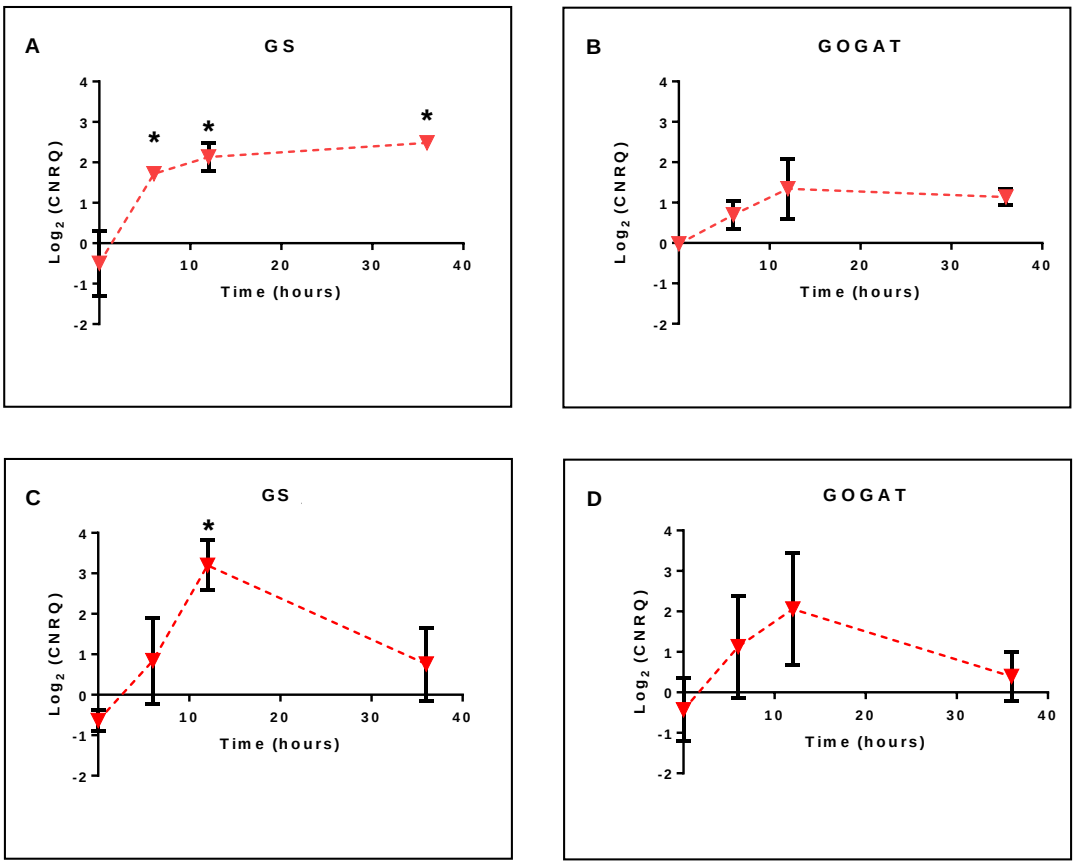


Figure 3

Ammonium-mediated expression of key assimilation genes in above- and below-ground tissue of *Zostera muelleri*

Differential gene expression as Log_2 (Calibrated Normalized Relative Quantities) in ammonium-enriched treatment relative to control treatment in above-ground tissue (A: GS; B: GOGAT) and below-ground tissue (C: GS; D: GOGAT) of *Zostera muelleri* for the duration of the experiment (36 hours). *Significant difference (pair-wise comparison, P<0.05). Error bars represent ± SEM (3 plants).



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