TESIS DOCTORAL

Respuestas ecofisiológicas al enriquecimiento de amonio en angiospermas marinas

Ecophysiological responses to ammonium enrichment in seagrasses

Beatriz Villazán Peñalosa

Universidad de Cádiz

Facultad de Ciencias del Mar y Ambientales

Departamento de Biología-Área de Ecología

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Memoria presentada por Beatriz Villazán Peñalosa para optar al Grado de Doctor por la Universidad de Cádiz

Fdo. Beatriz Villazán Peñalosa

UCA Universidad de Cádiz
Facultad de Ciencias del Mar y Ambientales
Departamento de Biología (Área de Eología)
Polígono Río San Pedro s/n.11510 Puerto Real.
Tel. +34956016029. Fax: 956016019
http://www.dept.biologia@uca.es

Los directores: Dr. D. Juan José Vergara Oñate, catedrático de Ecología y Dr. D. Fernando G. Brun Murillo, Profesor Titular de Ecología de la Universidad de Cádiz.

CERTIFICAN:

Que la presente memoria titulada, "Respuestas ecofisiológicas al enriquecimiento de amonio en angiospermas marinas", presentada por Beatriz Villazán Peñalosa, ha sido realizada bajo su dirección y autorizan su presentación y defensa, para optar al Grado de Doctor por la Universidad de Cádiz.

Y para que así conste y surta los efectos oportunos, firman los presentes en Puerto real, a 19 de Mayo de dos mil catorce.

Prof. Dr. Juan. J. Vergara Oñate

Prof. Dr. Fernando G. Brun Murillo

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A la mujer a la que espero parecerme algún día

A mi madre

Asturias si yo pudiera

si yo supiera cantarte

Asturias verde de monte

y negra de minerales.

[…]

Victor Manuel

El mar

Necesito del mar porque me enseña:
no sé si aprendo música o conciencia:
no sé si es ola sola o ser profundo
o sólo ronca voz o deslumbrante
suposición de peces y navios.
El hecho es que hasta cuando estoy dormido
de algún modo magnético circulo
en la universidad del oleaje.
No son sólo las conchas trituradas
como si algún planeta tembloroso
participara paulatina muerte,
no, del fragmento reconstruyo el día,
de una racha de sal la estalactita
y de una cucharada el dios inmenso.
Lo que antes me enseñó lo guardo! Es aire,
incesante viento, agua y arena.

Pablo Neruda

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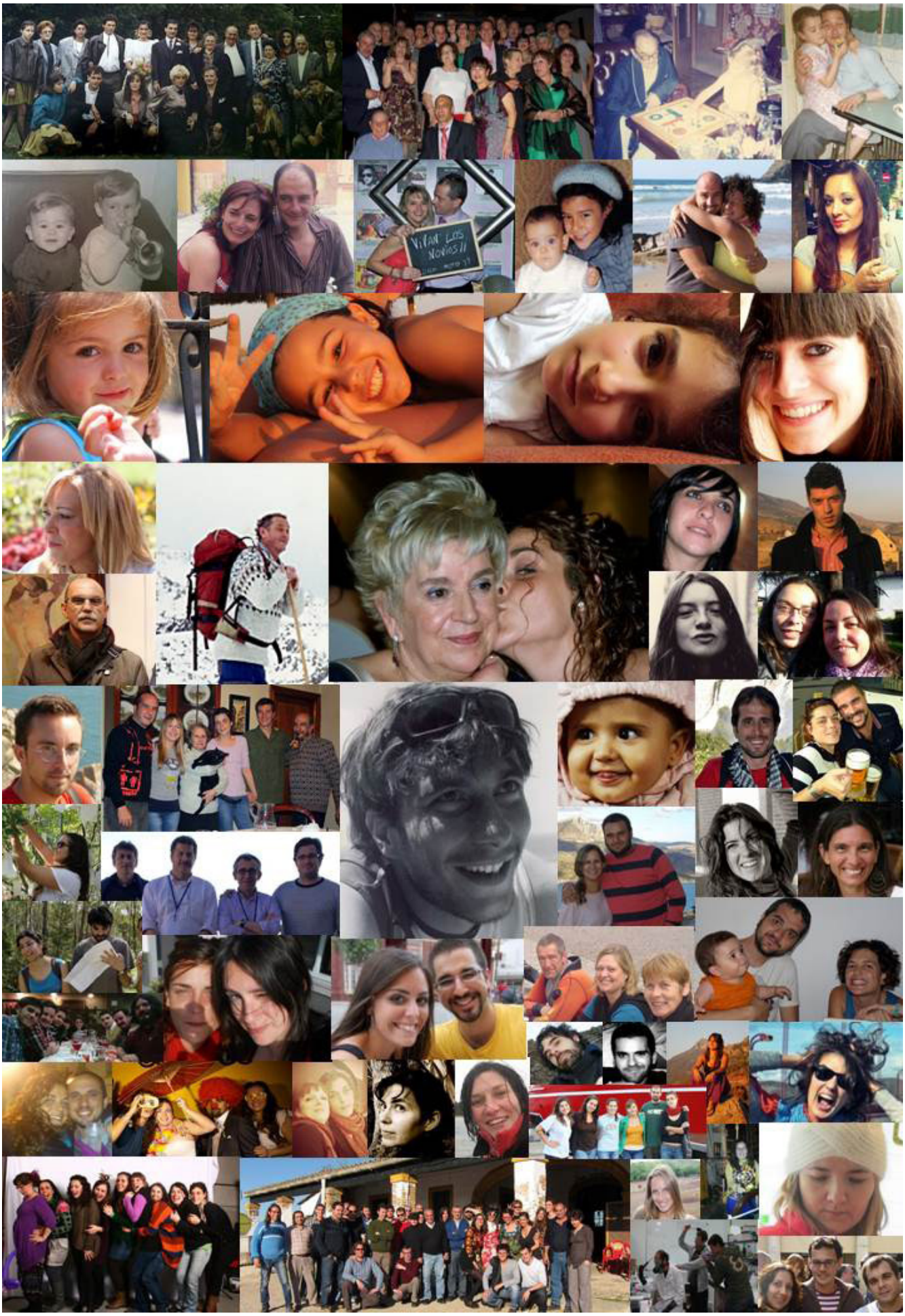
Los científicos dicen que estamos hechos de átomos, pero a mí un pajarito me contó que estamos hechos de historias

Eduardo Galeano


Viva los novios!!

Y después de todos estos años me encuentro ante el folio en blanco, ese folio que tanto ansiaba para poder daros las gracias por todo el cariño y comprensión que he recibido a lo largo de estos años. Volviendo la vista atrás millones de momentos, imágenes, sentimientos… se agolpan en mi cabeza. ¿Cómo describir brevemente lo que cada uno de vosotros me habéis brindado en este viaje? ¿Cómo resumir todo lo que he aprendido, disfrutado, reído, vivido a vuestro lado? Simplemente deciros que sin vosotros ni yo sería quien soy, ni esta tesis sería lo que es.

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Abstract

Resumen/Resumo

If you can´t explain simply,

you don´t understand it well enough

Albert Einstein

Anthropogenic nutrient loading in coastal areas is one of the major causes of seagrass decline worldwide. One of the main negative effects is indirectly caused by the proliferation of fast-growing species promoting light attenuation, sediment anoxia and sulphide intrusion risks. However, the parallel direct toxic effect caused by high ammonium (NH_4^+) concentrations on seagrasses has received little attention, in spite of some pioneer works. Adverse effects of NH_4^+ enrichment have been traditionally explained by internal accumulation of NH_4^+. To prevent toxic effects, plants must assimilate this nutrient into amino acids and other non-toxic organic compounds, generating strong internal demand for ATP and organic carbon skeletons, which must be provided by photosynthesis or from C-reserves. Thus, any factor affecting NH_4^+ uptake and/or assimilation could intensify or ameliorate NH_4^+ toxicity in seagrasses. This PhD Thesis studies the interactive effect of NH_4^+ enrichment and different environmental factors in the genus *Zostera*. In the first two chapters, the interaction between NH_4^+ and phosphate uptake rates and the interactive effect of light, hydrodynamics and NH_4^+ enrichment was studied in *Z. noltei*. In these assays ammonium was taken up following a diffusive trend, and while a shortcoming of phosphate uptake was found in leaves of *Z. noltei* when NH_4^+ was present, NH_4^+ uptake was unaffected by phosphate presence (chapter 1). As well, a non-linear response to NH_4^+ enrichment with flow velocity was recorded, with the strongest negative effect at intermediate flow (chapter 2). The effects of light and NH_4^+ on morphology, physiology, nitrogen metabolism and carbon reserves were studied in *Z. marina* (chapter 3). Light reduction had negative and synergistic effects with NH_4^+ enrichment, which were related with a drastic drop in carbon reserves and a remarkable increase in amino acid concentrations, indicating a tight coupling between carbon an nitrogen metabolisms. The response to the interaction between NH_4^+ loading and hyposaline stress was analyzed on different physiological and morphological properties of *Z. marina* (chapter 4). The negative and interactive effect between high NH_4^+ concentrations and low salinity was correlated with an increase in intracellular NH_4^+ content and a decrease in photosynthetic rates and non-structural carbohydrates, causing a drop in growth and survival of plants. Thus, hyposaline stress enforced the toxic effect of NH_4^+ by increasing the competition between ammonium assimilation and osmotic regulation for energy and C-skeletons. In summary, this Thesis highlights that toxic effects promoted by NH_4^+ on seagrasses can be intensified or alleviated depending in the presence of other natural stressors. In nature, organisms are rarely stressed by only one factor, and the interaction among multiple stressors may drive synergistic, antagonist or even non- linear responses. The understanding of nature of such interactions is important for developing better predictions and managing policies for seagrass ecosystems, since NH_4^+ load is expected to increase in the near future in coastal areas, jointly with the modification of a broad range of environmental factors as a consequence of the global change.

La eutrofización constituye una seria amenaza para las zonas costeras y se postula como una de las principales causas del declive de las praderas de angiospermas marinas a nivel global. De un modo indirecto, una elevada carga de nutrientes favorece la proliferación de especies de rápido crecimiento causando una reducción en los niveles de luz o un incremento de la materia orgánica, aumentando el riesgo de anoxia en el sedimento y la intrusión de sulfuro en las plantas. Asimismo, las propias concentraciones elevadas de amonio (NH_4^+) pueden resultar tóxicas. A pesar de la existencia de algunos estudios pioneros, la toxicidad del NH_4^+ en angiospermas marinas es aún un fenómeno poco estudiado. El efecto tóxico del NH_4^+ está ligado al incremento de dicho nutriente en el interior celular. Para evitar su acumulación el amonio ha de ser asimilado rápidamente en aminoácidos u otros compuestos orgánicos, generando una fuerte demanda de energía en forma de ATP e hidratos de carbono no estructurales que son proporcionados a través de fotosíntesis o bien suministrados a partir de las reservas internas. Por lo tanto, cualquier factor que afecte tanto a los procesos de incorporación como a la asimilación de NH_4^+, podría potenciar o aliviar su toxicidad. El objetivo principal de esta tesis doctoral es el estudio de la interacción entre diferentes variables ambientales y elevadas dosis de NH_4^+ en dos especies de fanerógamas del genero *Zostera (Z. noltei* y *Z. marina)*. El **capítulo 1** se centró en el estudio de la interacción entre las tasas incorporación de fosfato y amonio en hojas de *Z. noltei***.** Bajo elevadas concentraciones de NH_4^+ se produjo una disminución en las tasas de incorporación de fosfato; a su vez, la incorporación de NH_4^+ mostró un claro comportamiento difusivo y no fue afectada por la presencia de fosfato en el medio. El **capítulo 2** abordó el estudio de los efectos de una alta disponibilidad de NH_4^+ bajo diferentes condiciones hidrodinámicas y lumínicas en *Z. noltei*. La interacción entre estos factores dio lugar a una respuesta no lineal, registrándose una mayor toxicidad en velocidades intermedias. En el **capítulo 3**, se estudiaron los efectos producidos por diferentes niveles de luz y concentraciones de NH_4^+ en diversas propiedades morfológicas y fisiológicas, y las implicaciones sobre el metabolismo del nitrógeno y del carbono en *Z. marina*. Se observó un efecto sinérgico negativo entre las altas concentraciones de NH_4^+ y las condiciones de luz limitantes, que se correspondieron con una drástica disminución en el contenido de hidratos de carbono no estructurales y con un notable incremento en las concentraciones de aminoácidos libres, lo que puso de manifiesto la estrecha relación existente entre el metabolismo del carbono y del nitrógeno. En el **capítulo 4** se evaluó la interacción entre las condiciones de enriquecimiento de NH_4^+ y el estrés hiposalino, sobre diferentes variables fisiológicas y morfológicas en *Z. marina*. El hallazgo más relevante fue el efecto negativo y aditivo entre las altas concentraciones de NH_4^+ y las condiciones de baja salinidad. Esto se relacionó con un incremento en el contenido intracelular de NH_4^+ y una disminución en las tasas fotosintéticas y en las concentraciones de hidratos de carbono no estructurales, provocando una reducción en el crecimiento y la supervivencia de *Z. marina*. Por lo tanto, las condiciones hiposalinas potenciaron el efecto tóxico del NH_4^+, debido a que los procesos de osmorregulación compiten por los mismos recursos que los requeridos para la asimilación del NH_4^+ (ATP e hidratos de carbono no estructurales). En conclusión, los resultados de esta tesis mostraron que los efectos tóxicos producidos por el NH_4^+ sobre las angiospermas marinas podrían intensificarse o aminorarse en función de las condiciones ambientales que coocurren de un modo natural en las zonas costeras. En las próximas décadas, se prevé tanto un incremento en la carga de NH_4^+, como cambios en las condiciones ambientales a consecuencia del cambio global. La interacción entre múltiples factores pueden conducir a respuestas sinérgicas, antagónicas o incluso no lineales en sistemas dominados por angiospermas marinas; la comprensión de cómo múltiples factores ambientales interactúan entre sí resulta por tanto trascendental para el desarrollo de políticas que conduzcan a una correcta gestión de los ecosistemas dominados por estas especies tan carismáticas.

A carga de nutrientes em áreas costeiras é uma das mais importantes causas do declive mundial de ervas marinhas. Um dos principais efeitos negativos é indiretamente causado pela proliferação de espécies de crescimento rápido, que ocasionam atenuação da luz, anóxia do sedimento e riscos de intrusão de compostos reduzidos de enxofre. De qualquer modo, o efeito tóxico paralelo causado pelas altas concentrações de amónio (NH_4^+) nas ervas marinhas não tem sido estudado em detalhe, embora alguns estudos pioneiros sugeriram a importância de este problema. Os efeitos adversos do enriquecimento em NH_4^+ hão sido tradicionalmente explicados pela acumulação interna de NH_4^+. Para prever os efeitos tóxicos, as plantas devem assimilar este nutriente em forma de aminoácidos e outros compostos orgânicos não tóxicos, o que gera uma grande demanda interna de ATP e esqueletos de carbono orgânico que têm que ser provisionados pela fotossíntese ou por médio de reservas de carbono. Pelo tanto, qualquer factor que afecte a assimilação de NH_4^+ pode intensificar ou facilitar a toxicidade do NH_4^+ em ervas marinhas. Esta tese de doutoramento estuda o efeito interativo do enriquecimento em NH_4^+ com diferentes factores ambientais no género *Zostera*. Nos dos primeiros capítulos presenta-se um estudo da interação entre as taxas de assimilação de fosfato, a luz, a hidrodinâmica e o enriquecimento em NH_4^+ em *Zostera noltei.* Observou-se uma limitação de assimilação de fosfato nas folhas de *Z. Noltei* por causa do NH_4^+, em tanto que a assimilação de NH_4^+ presentou uma tendência difusiva e não se viu afectada pelo fosfato. Também, observamos uma resposta não-linear ao enriquecimento de NH_4^+ com a velocidade do fluxo, encontrando o efeito negativo mais destacado no caso do fluxo intermédio. Os efeitos da luz e o NH_4^+ na morfologia, fisiologia, metabolismo do nitrogénio e reservas de carbono foram estudadas em *Z. marina* no capítulo 3. A redução da luz teve um efeito negativo e efeitos sinérgicos com o enriquecimento em NH_4^+, que foram relacionados com o declive drástico em reservas de carbono e um notável incremento nas concentrações de aminoácidos, o que indica um acoplamento entre os metabolismos do carbono e do nitrogénio. No capítulo 4, a resposta à interação entre enriquecimento de NH_4^+ e stresse hiposalino foi analisada em distintas propriedades fisiológicas e morfológicas de *Z. marina*. O efeito negativo e interativo entre as altas concentrações de NH_4^+ e a baixa salinidade correlacionaram com o aumento em contido intracelular de NH_4^+ e a diminuição nas taxas de fotossíntese e contido em carboidratos estruturais, ocasionando um declive no crescimento e a supervivência das plantas. Assim, o stresse hiposalino forçou o efeito tóxico do NH_4^+ a través da competição entre a assimilação de NH_4^+ e a regulação osmótica para a energia e os esqueletos de carbono. Resumidamente, esta tese destaca que o efeitos tóxicos do NH_4^+ em ervas marinhas pode verse intensificado ou aliviado em combinação com outros factores. No médio ambiente, estes organismos são raramente stressados por um único factor, e a interação entre múltiplos factores stressantes pode ocasionar respostas sinérgicas, antagónicas ou não-lineares. A compreensão de estas interações é importante para desarrolhar predições e políticas de gestão dos ecossistemas de ervas marinhas mais eficientes, tendo em conta que a carga de NH_4^+ em áreas costeiras pode incrementar num futuro próximo, e um grande variedade de factores ambientais pode ver-se modificada em consequência do cambio global.

List of abbrevations

Un científico en su laboratorio no es sólo un técnico:

es también un niño colocado ante fenómenos naturales

que le impresionan como un cuento de hadas

Marie Curie

AG	Aboveground biomass
AG/BG	Ratio aboveground/belowground biomass
ASW	Artificial seawater
BG	Belowground biomass
C	Carbon
Chl	Chlorophyll
Cl^-	Chloride
EPUs	Experimental plant units
FAA	Free amino acids
Fv/Fm	Maximum quantum yield
GS	Glutamine synthetase
HAST	High affinity transport system
HL	High light
HV	High velocity
IA	Internode abundance
IAR	Internode appearance rate
IL	Internode length
I_{sec}	Internode cross section
K^+	Potassium
K_m	Half-saturation constant
LA	Leaf abundance
LAST	Low affinity transport system
LER	Leaf elongation rate
LL	Leaf length
LL	Low light
L_{sec}	Leaf cross section
LV	Low velocity
LW	Leaf width
MV	Medium velocity
Na^+	Sodium

NH_3	Ammonia
NH_4^+	Ammonium
N_i	Inorganic nitrogen
NiR	Nitrite reductase
NO_2^-	Nitrite
NO_3^-	Nitrate
NP	Net production
NR	Nitrate reductase
NSC	Non-structural carbohydrates
P	Phosphorus
PI	Plastochrome interval
P_{max}	Maximun net photosynthetic rate
PO_4^{3-}	Phosphate
Resp	Dark respiration
RL	Root length
RR	Root/Rhizome
SAR	Shoot appearance rate
SR	Survival
TSP	Total soluble protein
V	Uptake rate
V_{max}	Maximum uptake rate

General introduction

Everyone is a genius.

But if you judge a fish by its ability to climb a tree,

it will live its whole life believing that it is stupid

Albert Einstein

Seagrass ecosystems and functions

Seagrasses are marine flowering angiosperms developing their whole life cycle in shallow coastal areas, sheltered bays and coastal lagoon all over the world (Fig. 1) (den Hartong & Kuo 2006). Unlike other marine macrophytes, they possess both its vascular system (Kuo & den Hartong 2006) and its ability to produce flowers, fruits and seeds (Ackerman 2006). All seagrass species share analogous architectural patterns. They are modular plants composed by units (ramets), which are constituted by a piece of rhizome (horizontal or vertical) and a group of leaves attached to the internode, which bear a set of roots (Brun et al. 2006). Although reproductive strategies of seagrasses vary widely among species (Heminga & Duarte 2000), it is commonly assumed that maintenance and expansion of seagrass meadows proceed mainly through vegetative reproduction (Tomasko & Dawes 1989, Williams 1990, Hemminga & Duarte 2000). Despite this, sexual reproduction through hydrophilous pollination is essential for the formation of new clones and necessary for the development and maintenance of seagrass beds (Hemminga & Duarte 2000, Olesen at al. 2004, Short et al. 2011).

Seagrasses evolved from terrestrial monocotyledonous flowering plants 70-100 million of years ago (Duarte & Gattuso 2010). The taxa regarded as seagrasses belong to a limited number of families, all classified within the superorder Alismatiflorae (Monocotyledonae). There are four families of seagrasses; three of them are constituted exclusively by seagrasses, the Zosteraceae, the Cymodoceaceae and the Posidoniaceae (den Hartong & Kuo 2006). The fourth family, the Hydrocharitaceae, contains three genera considered as seagrasses, but other 14 genera are confined to fresh-water habitats (Cook 1990, 1998).

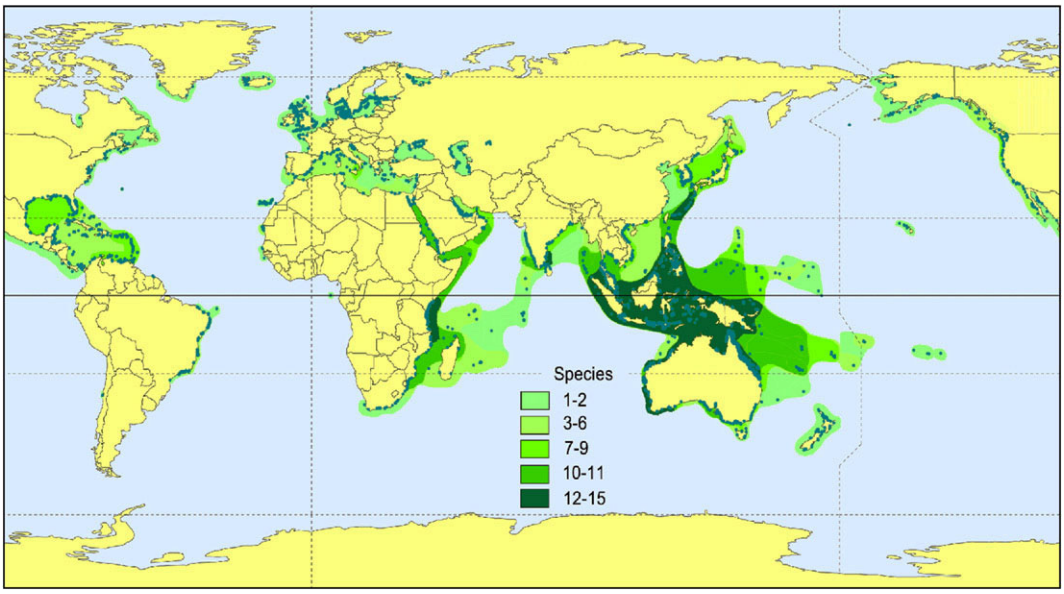


Fig. 1. Global map of seagrass species richness and distribution. Areas shaded of green indicate numbers of species reported for an area; blue points and polygons indicate documented reports of seagrass occurrence (taken from Short et al. 2007).

Despite seagrasses comprise less than 0.02% of the angiosperm species (Short et al. 2007) and their meadows cover less than a 0.15% of the seabed (Charpy-Roubaud & Sournia 1990), they are among the most productive ecosystems of the earth (on area basis), reaching a global estimate of primary production of 0.49 PgC $year^{-1}$ (c.a. 1.1% of the total marine primary production; Duarte & Chiscano 1999). Furthermore, seagrass meadows have important ecological roles in coastal ecosystems (Costanza et al. 1997, Waycott et al. 2009, Barbier et al. 2011). For instance, they provide habitats for a broad number of invertebrate and fish species, many of which are of commercial importance (Fig. 2) (Duarte 2000, Heck & Valentine 2006). They also serve as substrata for a diverse array of epiphytic organisms that contribute to the productivity of the seagrass beds in different ways (Borowitzka et al. 2006). Moreover, they contribute to nutrient recycling since they act like biofilters by stripping nutrients and other contaminants from the water through foliar uptake. Seagrass meadows improve water transparency since they enhance the settling of suspended particles, both by the passive action of the canopy or by the active filter-feeding activity of those organisms living within the meadows (Hemminga & Duarte 2000, Pérez-Lloréns et al. 2014, González-Ortiz et al. in press). They contribute to shoreline protection since belowground network of roots and rhizomes stabilizes sediment, and canopy reduces the erosive forces of waves and tides (Orth 2006, Peralta et al. 2008). Seagrasses also provide oxygen to water and sediment (Duarte 2002) and represent a large carbon sink. Seagrass communities have a net metabolism, removing inorganic CO_2 from the atmosphere (Kennedy et al. 2010, Fourqurean et al. 2012, Egea et al. in preparation), and a significant fraction of this carbon is buried and stored in the sediment, being seagrass ecosystems a hotspot of carbon sequestration in the biosphere (Duarte et al. 2005, UNEP 2009).

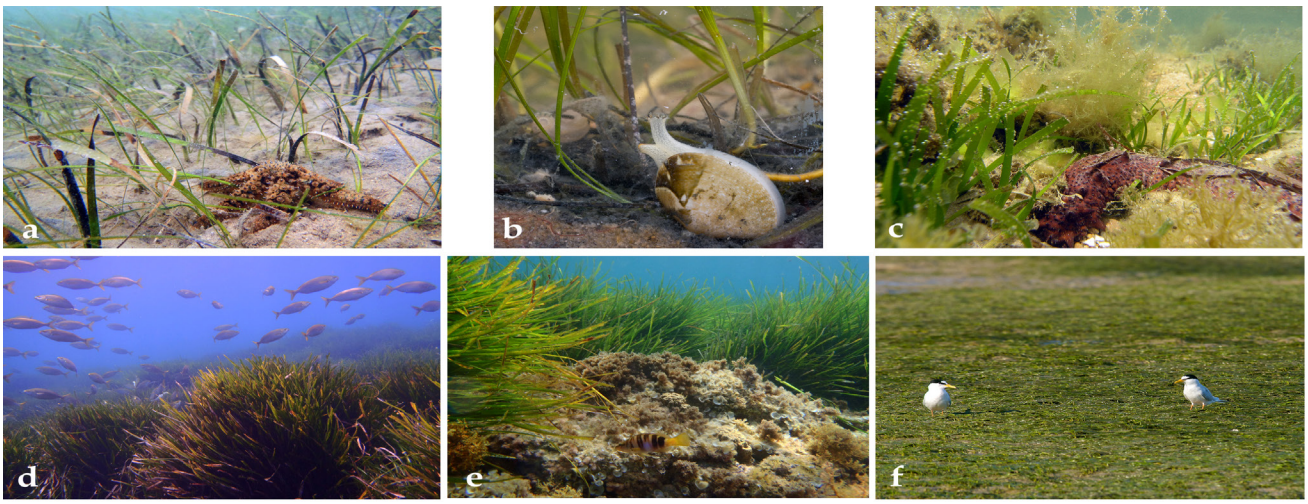


Fig. 2. Seagrass meadows and associated fauna. **a**, *Coscinasterias sp*. in a mixed meadow of *Cymodocea nodosa* and *Zostera noltei*; **b**, *Scrobicularia plana* in *Z. noltei* meadow; **c**, *Holoturia sp.* in *Cymodocea nodosa* meadow; **d**, *Salpa salpa* in *Posidonia oceanica* meadow; **e**, *Serranus scriba*, photophilic seaweeds and *P. oceanica*; **f**, *Sternula albifrons* on an intertidal *Z. noltei* meadow. (**a**, **d**, **e** by Ricardo Bermejo Lacida and **b**, **c**, **f**, photographs by Fernando G. Brun).

Eutrophication: Deterioration of coastal habitats and seagrass decline

In the last century, human activities are generating environmental and ecological changes from local to global scales, altering the climate and producing changes in diversity; these changes have important natural and social consequences that we are just starting to understand (Chapin et al. 2000, UNEP 2006, Thomas et al. 2004, UNEP 2009).

Approximately the 23% of the global human population lives within 100 km from the coastline (Nicholls & Small 2002). Growth of human population along coastal areas has inevitably produced the degradation and loss of coastal ecosystems such as salt marshes, mangroves, coral reefs and seagrasses, and even worse, the rate of loss is increasing in the last decade. Therefore, 50% of salt marshes, 35% of mangroves, 30% of coral reefs, and 29% of seagrasses are either lost or have been degraded worldwide (Valiela et al. 2001, Orth et al. 2006, UNEP 2006, Waycott et al. 2009). Currently, on average, between 2–7% of marine ecosystems are lost annually (UNEP 2009), being the decline in seagrasses estimated from less than 0.9% $year^{-1}$ in the 1970s to 7% $year^{-1}$ since 2000 (Waycott et al. 2009).

Reports on seagrass decline have estimated that more than the 70% of the losses of these crucial ecosystems are attributed to direct or indirect human-related activities along the coastal zone (Short & Wyllie-Echeverria 1996, Orth et al. 2006, Waycott et al. 2009, Short et al. 2011). Nevertheless, natural disturbances such meteorological events (e.g. hurricanes), and biological interactions (e.g. grazing, bioturbation and diseases) have also been responsible for a fraction of these losses (Short & Wyllie-Echevarria 1996).

Among the anthropogenic disturbances, eutrophication caused by nutrient overload (mainly nitrogen and phosphorus), is recognized as one of the most widespread anthropogenic impacts on seagrass ecosystems (Short & Wyllie-Echeverria 1996, Burkholder et al. 2007). Over the last 20 years, the loss of seagrass beds related to eutrophication became a common problem worldwide, including, for example, *Posidonia oceanica* in Europe (Pergent-Martini & Pergent 1996, Marbá et al. 2014), *Zostera marina* in USA (Orth & Moore 1983, Orth et al. 2010) and *Z. noltei* in the Wadden Sea (Philippart & Dijkema 1995) and in the Southern coasts of Spain and Portugal (Hernández et al. 1997, Cardoso et al. 2004, Cabaço et al. 2008).

High nutrient levels affect seagrasses in several ways (Fig. 3). The major and more studied effects are indirectly caused by the proliferation of either phytoplankton, epiphytic microalgae and/or fast-growing drifting macroalgae promoting light attenuation (e.g. Sand-Jensen & Borum 1991, Hernández et al. 1997, Hauxwell et al. 2001, McGlathery 2001, Lyons et al. 2012), or increasing the sediment organic matter load, which may reduce oxygen levels and increase the risk of anoxia (Greve et al. 2003) and promote sulfide intrusion into the plants (Holmer & Bondgaard 2001, Borum et al. 2005, Olivé et al. 2009). Furthermore, enrichment by inorganic nitrogen (N_i), especially NH_4^+, can provoke a direct toxic effect on seagrasses (e.g. Burkholder et al. 1992, van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011), since exposure to high concentrations of NH_4^+ has been acknowledged to be toxic for photosynthetic organisms (e.g. Marschner 1995, Britto & Kronzucker 2002).

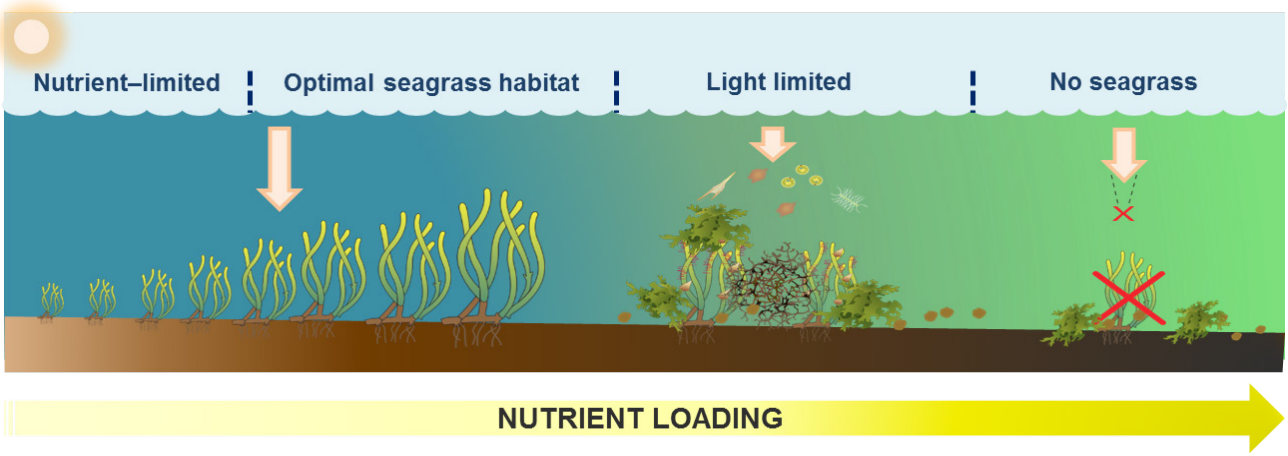


Fig. 3. Conceptual diagram illustrating the effect of increasing nutrient loading on seagrass meadows.

Nutrient over-enrichment in coastal waters is increasing as population grows within coastal zone (Bricker et al. 1999). The major anthropogenic sources of these nutrients are sewage effluents, production and use of fertilizers, combustion of fossil fuels, agricultural run-off and the expansion of fish farming and other aquaculture practices (e.g., shellfish culture) (Nixon 1995, Ralph et al. 2006, Bouwman et al. 2014). A recent study (Beusen et al. 2013) estimated that submarine water discharge input of nitrogen to global coastal water had increased about 38% between 1950 and 2000, and a further 22% increase is projected for the period 2000-2050. Therefore, NH_4^+ release is predicted to increase in a significant manner in shallow marine areas in the near future (Fig. 4) (Glibert et al. 2010, Sobota et al. 2013). For sure, it will affect seagrass populations since elevated NH_4^+ concentrations have negative effects on seagrasses by reducing photosynthesis, growth and survival (e.g. van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011). Thus, a high NH_4^+ availability may produce a loss of seagrass habitats with the associated decline in accompanying fauna (Nixon 1995, Deegan 2002) and economic value (e.g. fisheries, carbon burial, etc.). Therefore, NH_4^+ may arise as a new threat for seagrasses in the near future with important implications for these marine ecosystems.

Ammonium and seagrasses: an asset or a burden

Paraphrasing Hemminga (1998) in its seminal article, ammonium can act as an asset or as a burden for these photosynthetic organisms depending on other environmental factors. Seagrasses are autotrophic organisms, requiring light, nutrients and inorganic carbon in large quantities for growth and survival. Among all the nutrients, nitrogen is a fundamental one for growth, and plays important functions in plant metabolism as a major component of proteins and nucleic acids (Marschner 1995).

The main available forms of inorganic nitrogen (N_i) for terrestrial and aquatic organisms are ammonium (NH_4^+) and nitrate (NO_3^-). At difference with terrestrial plants, seagrasses are capable to incorporate NH_4^+ and NO_3^- both through the leaves and the roots (Stapel et al. 1996, Lee & Dunton 1999, Touchette & Burkholder 2000, Alexandre et al. 2010). Therefore, N_i is available for seagrasses as

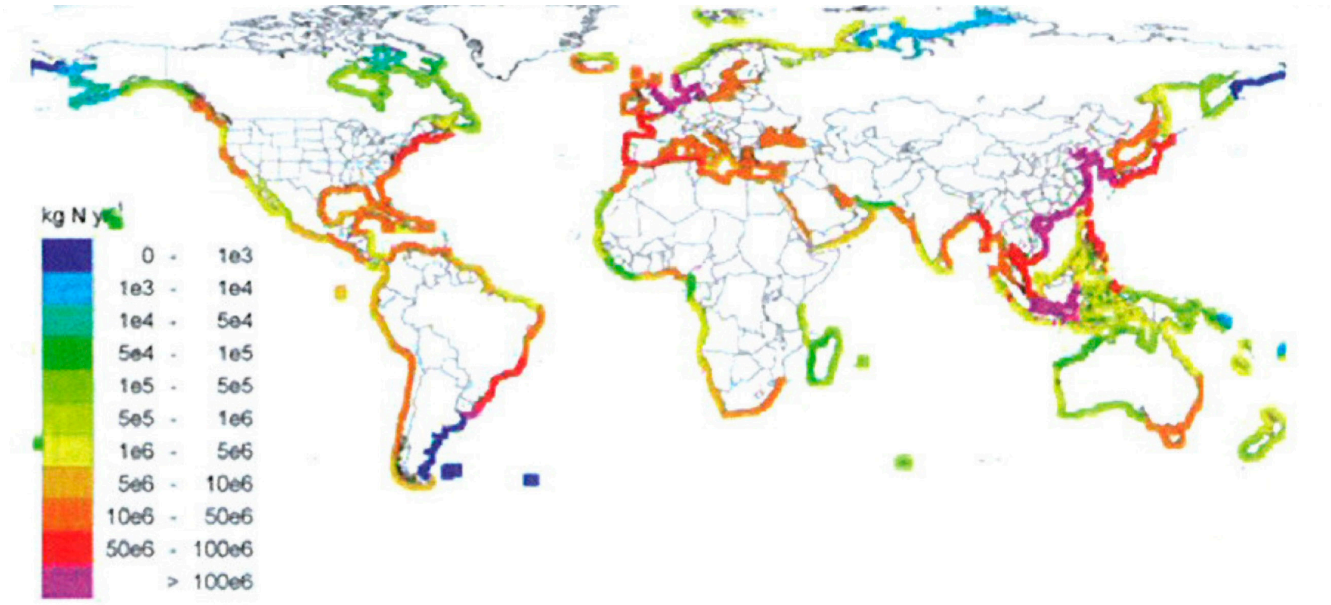


Fig. 4. Ammonium exported to the coastal zone based on modified global news models (LOICZ 2012).

NH_4^+ and/or NO_3^- either in the water column or in the porewater sediment. NH_4^+ is considered to be the dominant form of N_i in the water O_2 saturated, hypoxic or anoxic sediments which characterize seagrass habitats (Stapel et al. 1996, Stumm & Morgan, 1996), ranging from 10 to 300 μM NH_4^+ in sediment pore water (Harlin & Thorne-Miller 1981, Izumi et al. 1982, Kenworthy et al. 1982, Hemminga et al. 1994) and occasionally even above 1000 μM (Short 1983, Pedersen & Borum 1992, Hemminga et al. 1994). Meanwhile, concentrations in the water column are typically quite low (< 5 μM) in habitats lacking appreciable anthropogenic influence (Touchette & Burkholder 2000). However, seagrass meadows growing near wastewater and/or run-off discharge points may be subjected to high NH_4^+ concentrations, ranging from 150 to more than 600 μM (Hernández et al. 1997, Cabaço et al. 2008).

According to previous studies, NH_4^+ is usually preferred over NO_3^- in a broad range of different photosynthetic organisms such as for instance, the microalga *Chlorella sorokiniana* (Tischner & Lorenzen 1979), the seaweed *Fucus vesiculosus* (Pedersen & Borum 1997) or the terrestrial plant *Picea abies* (Gessler et al. 1998). As well, a variety of seagrass species show higher affinities and maximum uptake rates for NH_4^+ than for NO_3^- (Table 1).

This preference for ammonium over nitrate is usually ascribed to the lower energetic cost for the uptake, as well as for the assimilation process in comparison to NO_3^-. Assimilation of nitrogen into amino acids is a complex, energy-requiring process (Fig. 5) (Marschner 1995). Prior assimilation, NO_3^- must be reduced to NH_4^+ with an associated energetic cost of 15 mol of ATP. On the other hand, the redox state of NH_4^+ eliminates the need for its reduction in the cells and thus it can be utilized immediately for the synthesis of amino acids with a lower cost of ATP; only 5 mol of ATP equivalents are required for NH_4^+ assimilation (Turpin 1991, Marschner 1995).

Table 1. Comparison between ammonium and nitrate kinetic parameters of different seagrass species based on the Michaelis-Menten model. The estimated parameters were maximum uptake rate (V_{max}, μmol g^{-1} DW h^{-1}), half-saturation constant (K_M, μM) and affinity coefficient (α, V_{max}/K_M). nd: not determined.

Species	Nutrient	V_{max} (μmol g^{-1} DW h^{-1})	K_M (μM)	α (V_{max}/K_M)	Source
Phyllospadix iwatensis	NH_4^+	2.2-35.5	12.7-133.5	0.12-0.28	Hasegawa et al. (2005)
Phyllospadix torreyi	NH_4^+	95.6-204.3	9.3-33.9	nd	Terrados & Williams (1997)
Thalassia testudinum	NH_4^+	8.3-16.45	7.6-15	0.57-2.82	Lee & Dunton (1999)
Zostera noltei	NH_4^+	28.3-31.9	28.7-34.2	0.93-0.99	Alexandre et al. (2011)
Phyllospadix iwatensis	NO_3^-	1.0-2.1	13.9-21.1	0.05-0.11	Hasegawa et al. (2005)
Phyllospadix torreyi	NO_3^-	24.9-75.4	4.4-17.0	nd	Terrados & Williams (1997)
Thalassia testudinum	NO_3^-	3.7-6.5	2.2-38.5	0.15-1.68	Lee & Dunton (1999)
Zostera noltei	NO_3^-	0.19-0.26	6.48-6.61	0.03-0.04	Alexandre et al. (2011)

The nitrate is reduced to nitrite by nitrate reductase (NR) in the cytosol, using mainly NADH as a reductant. The reduction of NO_2^- to NH_4^+ is catalyzed by the enzyme nitrite reductase (NiR), which is located exclusively in the chloroplasts. To do that, six electrons are required (Fig. 5). This enzyme utilizes ferredoxin as electron donor, which is supplied by photosystem I (Ferrario-Méry et al. 1997, Heldt 2005). The conversion of NH_4^+ into amino acids is catalyzed by the enzyme glutamine synthetase (GS) (localized in the cytosol and chloroplast of photosynthetic tissues, and in the cytosol of roots). This pathway involves initial incorporation of NH_4^+ to glutamate by GS, using ATP to catalyze formation of glutamine (Fig. 5). This process also has a large requirement of energy (i.e. ATP) and carbon skeletons, which are provided directly from photosynthesis or from C-reserves (Turpin et al. 1990, Marschner 1995, Heldt 2005).

On the other hand, NO_3^- is taken up by an active transport system, which requires the energy stored in the membrane potential for its transport inside the cell (Rubio et al. 2007) with an associated ATP cost (Fig. 5). Recent studies in the nitrate transport systems have reported physiological evidences that NO_3^- transport is mediated by a high-affinity Na^+ dependent transport system in roots and leaves of *Zostera marina* (García-Sánchez et al. 2000, Rubio et al. 2005). However, the NH_4^+ uptake mechanism is still matter of controversy. There are at least two distinct NH_4^+ transport systems: a low-affinity (LATS) based on passive influx and efflux of NH_4^+ through membrane channels, and a high-affinity (HATS) system requiring protein synthesis for the formation of membrane transporters (Ocurry 1997). Rubio et al. (2007) suggested that high-affinity NH_4^+ transport in roots and leaves of *Z. marina* is not dependent on sodium, and could be mediated by an uniport membrane transporter depending on both membrane potential and NH_4^+ concentration gradient. So the high-affinity NH_4^+ transports system could be passive and mediated by AMT1 transporter family, in contrast with the sodium-coupled and ATP dependent transport of nitrate (Fig. 5).

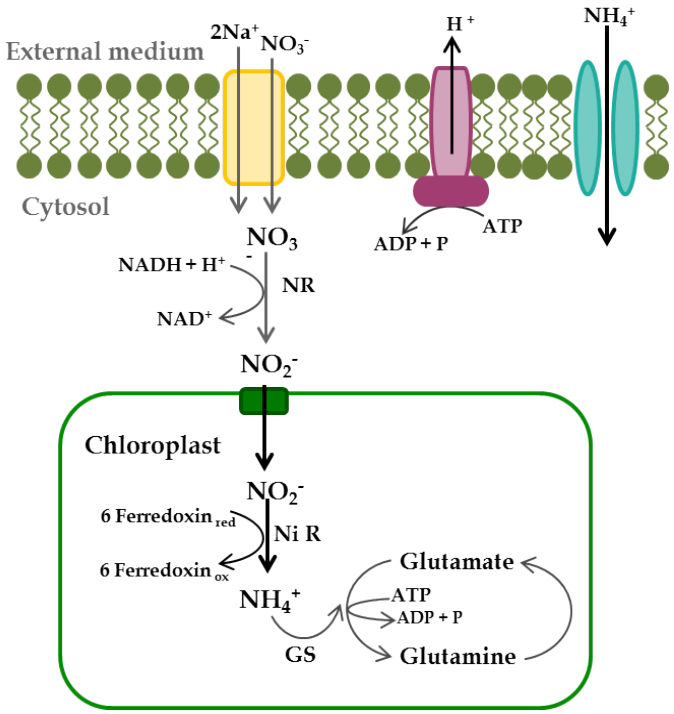


Fig. 5. Conceptual diagram illustrating the uptake and assimilation processes for nitrate and ammonium. NR: Nitrate reductase; NiR: Nitrite reductase; GS: glutamine synthetase.

Previous studies have shown that seagrass leaves take up NH_4^+ in direct proportion to the concentration in the surrounding water (Thursby & Harlin 1982, Iizumi & Hattory 1982, Touchette & Burkholder 2000, Alexandre at el. 2010). This passive movement of NH_4^+ can be not only a problem when plants are growing under high NH_4^+ loading, but also in plants growing under moderate NH_4^+ concentrations with low internal C-reserves and energy (ATP), as they eventually may be not capable to assimilate incorporated NH_4^+. If ammonium is not assimilated, it can accumulate inside the cells and become a toxic substance (Marschner 1995).

The toxic effect of NH_4^+ is mainly related to the uncoupling of ATP production during photosynthesis, although others processes as the increase in plant respiration and the reduction in the uptake of other ions such as potassium, magnesium, calcium, phosphate or nitrate may strength the toxicity (e.g. Ullrich et al. 1984, Pearson & Stewart 1993, Marschner 1995, Smolders et al. 1996, van Katwijk et al. 1997). Other studies indicate that high NH_4^+ concentrations may cause enhanced ethylene synthesis, increased energy consumption related to active efflux of NH_4^+, and reduced photo-protection mechanisms (Britto et al. 2001, Britto & Kronzucker 2002). The negative effect of high NH_4^+ availability on plants may also be related to an imbalance in the carbon economy of the plants, since accumulation of internal NH_4^+ stimulates the synthesis of amino acids in plants decreasing the pool of intermediary organic C compounds (Marschner 1995).

In a recent review, Burkholder et al. (2007) summarized different studies about nutrient enrichment in seagrasses. A disparity of responses, from negative to positive and even no effects were recorded. In some cases, a moderate increase in the availability of inorganic nitrogen (<10 μM) may stimulate growth and biomass of seagrasses when these are growing under nutrient limited conditions (e.g. Orth 1977, Alcoverro et al. 1997, Peralta et al. 2003, Invers et al. 2004). In other studies little or no effect of nutrient enrichment were observed (e.g. Harlin & Thorne-Miller 1981, Dennison et al. 1987, Murray et al. 1992, Pedersen & Borum 1993, Pedersen 1995, Lee & Dunton 2000). However, a growing body of studies suggests that enrichment with inorganic nitrogen (N_i), especially NH_4^+, can have adverse effects on seagrasses reducing photosynthesis, growth and/or survival (e.g. Burkholder et al. 1992, van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011).

These varieties of responses to inorganic nitrogen supply can be altered by factors that change NH_4^+ uptake rates; if they are enhanced, an increment in intracellular NH_4^+ concentration could be expected, intensifying the toxic effects of this nutrient in seagrasses. On the other hand, any environmental stressor draining energy and non-structural carbohydrates (NSC; needed to support NH_4^+ assimilation) will make seagrasses more vulnerable to NH_4^+ enrichment (Fig. 6).

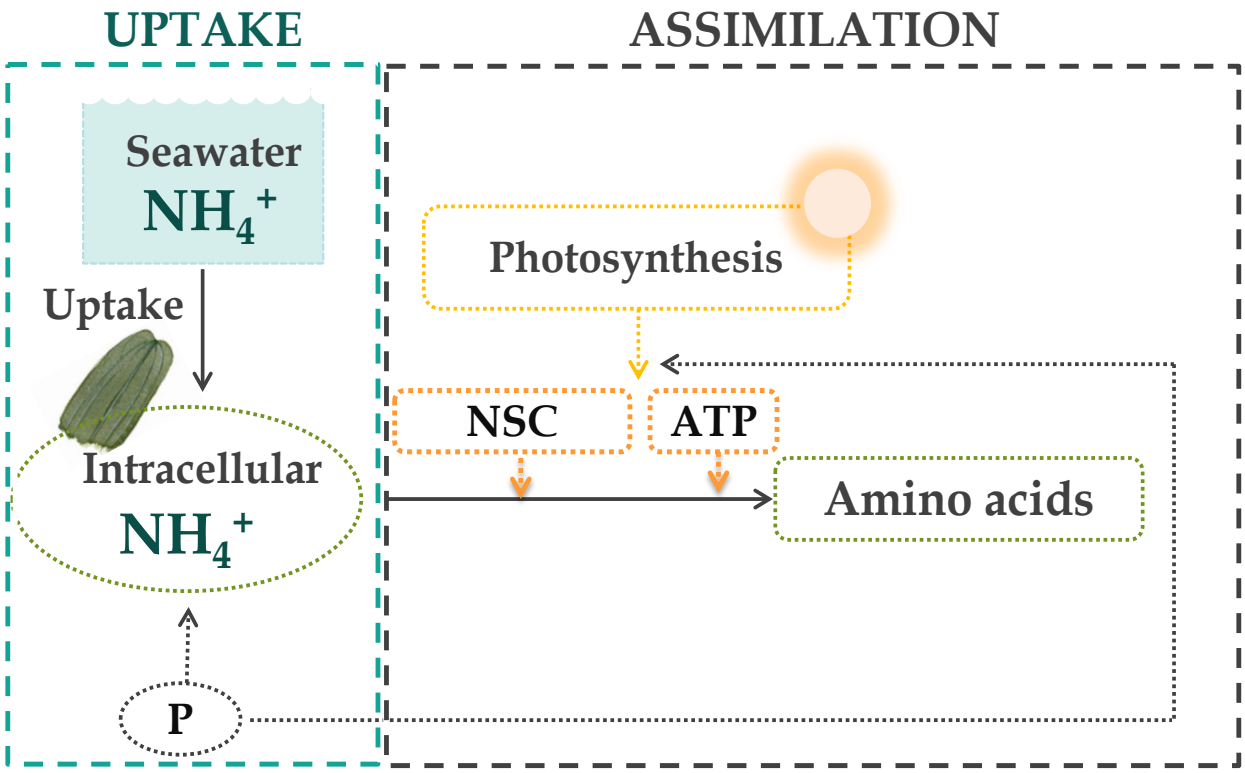


Fig. 6. Conceptual diagram illustrating the key points of ammonium uptake and assimilation processes in seagrasses. In the first part (blue) this diagram shows the uptake process in which seagrasses take up NH_4^+ from the surrounding water. This NH_4^+ can be accumulated inside the cell. The second part (grey) shows the assimilation process. To avoid the harmful effect of this nutrient inside the cell, NH_4^+ has to be assimilated rapidly to amino acids. For this assimilation, non-structural carbohydrates (NSC) as well as phosphorus (P) are needed, which are mainly provided by photosynthesis and plant reserves. NSC: Non-structural carbohydrates (sucrose and starch), P: Phosporus.

Several factors may lead to higher NH_4^+ uptake rates and hence intracellular NH_4^+ concentrations: a higher availability of NH_4^+ in seawater (e.g. Short & McRoy 1984, Stapel et al. 1996, Pedersen et al. 1997, Lee & Dunton, 1999, Alexandre et al. 2011); an increased flow velocity, which may enhance NH_4^+ uptake rates by decreasing the diffusive boundary layer (DBL) thickness in leaves (Cornelisen & Thomas 2004, Morris et al. 2008); meadow density and the presence of other photosynthetic organisms could be also important factors, since higher meadow densities and higher richness of photosynthetic photoautotrophs could decrease the specific uptake rates as well as the accumulation of NH_4^+ inside the cells, reducing the toxic NH_4^+ effects on seagrasses (Van der Heide et al. 2008, Moreno-Marín et al. in preparation).

On the other hand, any environmental condition that decrease the energy (i.e. ATP) and C-skeletons can limit the NH_4^+ assimilation and promote a shift from positive to negative response of seagrasses to high NH_4^+ concentrations. One of the most important factors is light. A sufficient light level that guarantee an adequate photosynthetic production and the synthesis of non-structural carbohydrates (NSC) and ATP levels will make plants more capable to assimilate ammonium. Some studies in *Zostera noltei* plants showed that low light, in contrast, enforced the NH_4^+ toxic effect (Brun et al. 2008), which was correlated with a drop in sucrose levels in leaves as well as in rhizomes and roots (Brun et al. 2002, 2008).

As well, increased temperatures may cause an increase in plant respiration. As a consequence, less NSC and energy will be available for NH_4^+ assimilation. In this way, an increment of temperature from 15 to 20 ^{o}C under high NH_4^+ loading showed a negative effect on *Zostera marina* plants (van Katwijk et al. 1997).

Another important factor is pH, since acidification increases the availability of CO_2 and, as a result photosynthetic rates, NSC and ATP content may also increase, which benefit the assimilation process (Beer & Koch 1996, Zimmerman et al. 1997, Invers et al. 2001). In contrast, if pH levels increase (i.e., a dense an productive meadow with low water renewal), it may cause the opposite trend, as less NSC and ATP may be available to assimilate NH_4^+. Moreover, as pH increases, the concentration of ammonia (NH_3) raises dramatically, which is considered to be more toxic that NH_4^+ (Collos & Harrison 2014). For example, increasing pH from 7.0 to 9.0, increases 60-fold the deprotonated NH_3 concentration (Collos & Harrison 2014). Accordingly, a previous study, *Z. marina* plants growing under high NH_4^+ became necrotic at 9 pH, but no effect were found in plants growing at pH 8 (van der Heide et al. 2008).

Salinity may also alter the photosynthetic rates in seagrasses. Hiposaline stress may reduce net photosynthesis and increase the demand for energy to restore turgor pressure and the osmotic balance in the cell (Touchette 2007). On the other hand, high salinity may also decreases photosynthetic rates, and at the same time, salinity may stress seagrasses because a large amount of energy (i.e. ATP and non-structural carbohydrates) is required to maintain turgor pressure, ionic balance and membrane integrity (Hellebust 1975, Touchette 2007, Karsten 2012). Therefore, high salinities and high NH_4^+ concentrations can therefore cause a synergistic negative effect, because both processes drain C reserves and, thus,

compete with other C-demanding or energy consuming metabolic processes, thereby reducing overall fitness on seagrasses (van Katwijk et al. 1999).

Lastly, the availability of phosphorus (P) is an essential factor in the response of seagrasses to NH_4^+ enrichment, because P plays an important role during NH_4^+ assimilation. Phosphorus limitation can depress photosynthesis, since affect the rate of synthesis and regeneration of substrates in the Calvin-Benson cycle (Dietz & Foyer 1986, Woodrow & Berry 1988). In addition, phosphorus is an important element as metabolic energy transfer (Stitt 1997). Thus, when C turnover increases as a result of N assimilation, P plays an important role and the negative effect of NH_4^+ in *Zostera noltei* was ameliorated in plants growing both with phosphate or precultured under phosphate enrichment (Brun et al. 2002, 2008).

In the next decades, NH_4^+ loading is expected to increase in shallow marine coastal waters (Glibert et al. 2010, Sobota et al. 2013). This potential increment of NH_4^+ concentrations overlaps with the worldwide distribution of seagrasses (Fig. 7). As well, as a consequence of global change, different environmental factors (i.e. hydrodynamics, pH, light and salinity levels) could be modified (IPCC 2007), which may profoundly alter the overall response of seagrasses to NH_4^+ loading in the future. Thus, NH_4^+ could become in a global threat for seagrasses in the future, because of seagrasses will be simultaneously subjected to multiple human-derived stressors, which may drive either synergistic or opposite effects in seagrass populations in response to NH_4^+ loading. For this reason, it is necessary to understand how these different stressors interact and affect the response of seagrasses to high NH_4^+ loading, in order to predict future changes in their populations.

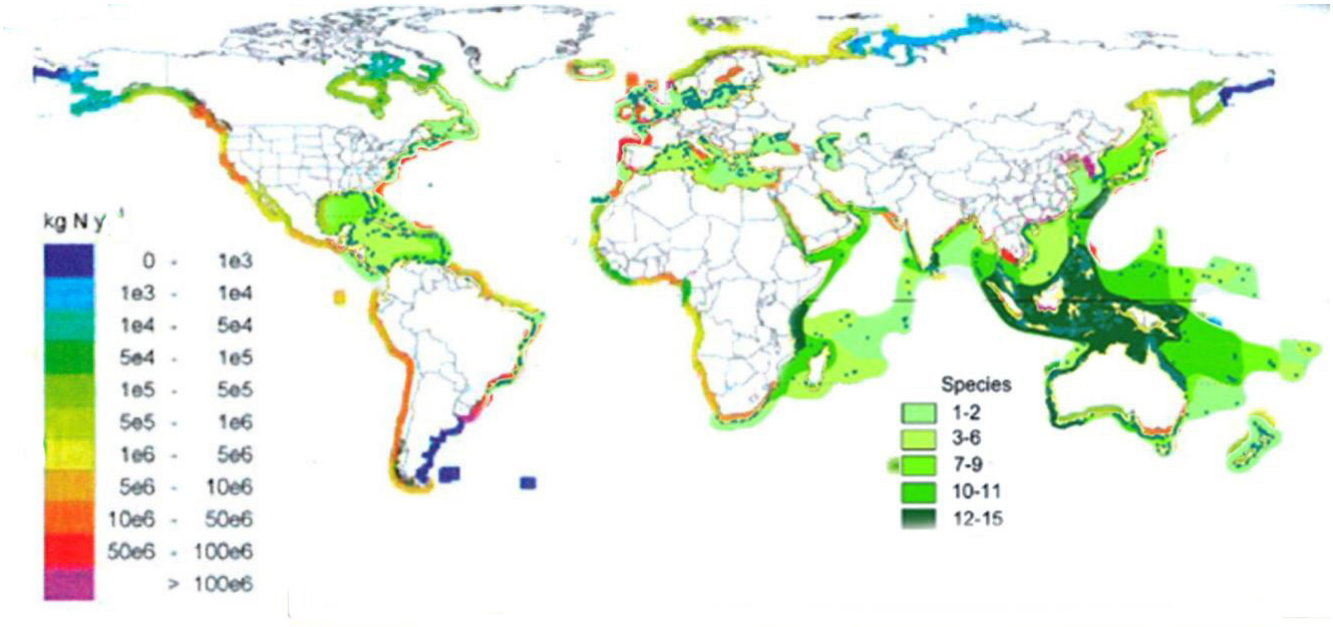


Fig. 7. Map showing the overlapping between the forecast increase in ammonium exportation in coastal areas, and seagrass distribution (LOICZ 2012, Short et al. 2007).

The latter summarizes the main objective of this PhD Thesis was to study the effects and interactions among different environmental factors on ammonium toxicity in two species from the genus *Zostera*, where the interaction between NH_4^+ and phosphate uptake rates and the interactive effect of light, hydrodynamics and NH_4^+ enrichment was studied in *Zostera noltei* plants. Moreover, in the species *Z. marina*, the interactive effect of light and NH_4^+ loading were studied, as well as the response to NH_4^+ enrichment under hyposaline stress.

Objectives

Structure of the thesis

Un viaje de mil millas

comienza con el pirmer paso

Albert Lao-tsé

The main objective of this PhD Thesis was to study the toxicity mechanism promoted by ammonium from a multifactorial perspective. Therefore, the effects and interactions among different environmental factors that can co-occur in nature and affect ammonium toxicity process were explored in two species from the genus *Zostera*, a seagrass group that has declined worldwide over the last decades in part as a consequence of eutrophication processes. This aim was undertaken by analyzing ammonium uptake, assimilation and physiological, dynamic, and morphological responses in *Zostera noltei* and *Z. marina* species.

This main objective was reached through four specific objectives:

1. To study the interaction between ammonium and phosphate uptake in *Zostera noltei*, since previous studies in this species demonstrated that phosphate ameliorates ammonium adverse effects (**Chapter 1**).

2. To explore the interactive effect of light and hydrodynamics on morphological and dynamic variables in *Zostera noltei* plants growing under ammonium enrichment conditions (**Chapter 2**).

3. To evaluate the interaction between light levels and ammonium concentrations in the physiological and morphological responses, and in the nitrogen assimilation of *Zostera marina* (**Chapter 3**).

4. To test the interaction between high ammonium availability and hiposalinity on physiological and morphological variables of *Zostera marina* (**Chapter 4**).

The first two chapters were performed in *Zostera noltei,* which is one of the most abundant species in the European coasts, particularly in Cádiz Bay; previous studies from our group showed toxicity promoted by ammonium, affecting in this species to growth, survival and morphological variables. The last two chapters were focused in *Zostera marina*, a congeneric species, which is the main seagrass species in Northern Europe. In this species also some previous studies have demonstrated the existence of toxicity effects promoted by ammonium.

Chapter 1

Interaction between ammonium and phosphate uptake rates in the seagrass *Zostera noltei*

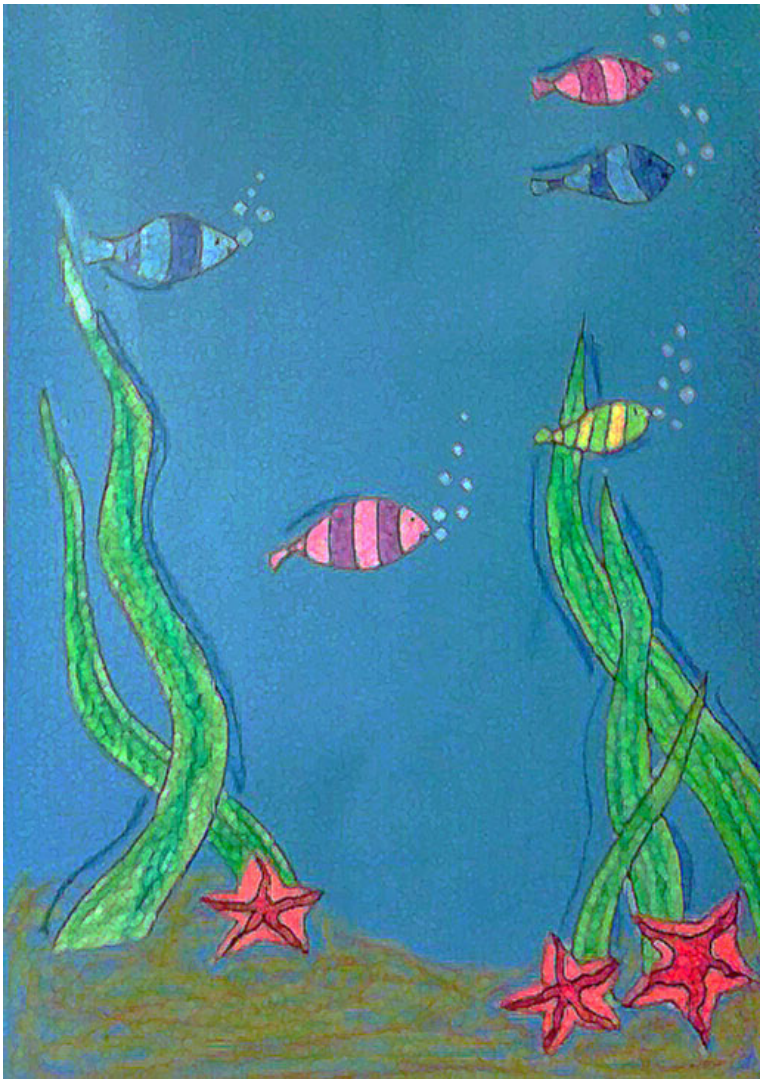
Villazán B, Brun FG, Jiménez-Ramos R, Pérez-Lloréns JL, Vergara JJ (2013)

Marine Ecology Progress Series 488:133-143

Elige un trabajo que te guste y

no tendrás que trabajar ni un solo día de tu vida

Confucio



Ilustrated by Miriam Villazán Rodríguez

Interaction between ammonium and phosphate uptake rates in the seagrass *Zostera noltei*

Beatriz Villazán, Fernando G. Brun, Rocío Jiménez-Ramos, J. Lucas Pérez-Lloréns, Juan J. Vergara

Departamento de Biología (Área de Ecología), Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Campus de Excelencia Internacional del Mar (CEIMAR), Puerto Real 11510, Cádiz, Spain

ABSTRACT: Foliar ammonium and phosphate uptake rates and their interactions were assessed in whole seagrass *Zostera noltei* plants incubated in 2-compartment transparent chambers. This method allowed the calculation of nutrient uptake rates by leaves and by roots and rhizomes independently, avoiding plant breakage. Overall, a direct linear relationship between foliar uptake rates and seawater nutrient concentrations (phosphate or ammonium) was found, with uptake rates much higher in the first 5 min (nutrient adsorption). This faster adsorption was followed by slower uptake rates (nutrient absorption) in the next time intervals. When both nutrients were supplied separately, foliar ammonium uptake rates were 3-fold higher than those of phosphate in the range of the nutrient concentrations assayed for the whole incubation interval (120 min). When both nutrients were added simultaneously (10 μM phosphate and 50 μM ammonium, final concentrations), ammonium uptake rates were similar to those values recorded when ammonium was provided alone, whereas phosphate uptake rates were about 55% lower than those measured when phosphate was added alone. This study reveals for the first time the inhibitory effect of ammonium on phosphate uptake in seagrasses.

INTRODUCTION

Seagrasses are aquatic angiosperms confined to marine environments (den Hartog & Kuo 2006). Such flowering plants provide numerous and important ecological services to marine ecosystems (Costanza et al. 1997, Waycott et al. 2009). However, seagrass ecosystems are declining worldwide mainly as a consequence of human-driven actions (Short & Wyllie-Echeverria 1996, Orth et al. 2006, Waycott et al. 2009). Although nutrient enrichment can foster seagrass growth and biomass in oligotrophic environments (Short 1983, Alcoverro et al. 1997, Peralta et al. 2003, Invers et al. 2004, Brun et al. 2008), it frequently results in negative physiological responses and even in growth inhibition (Hauxwell & Valiela 2004, Orth et al. 2006, Burkholder et al. 2007). This negative response has been mostly attributed to nutrient-driven indirect effects, such as light reduction caused by proliferation of fast-growing species (phytoplankton, epiphytes and opportunistic macroalgae) (Sand-Jensen & Borum 1991, Hernández et al. 1997, McGlathery 2001, Brun et al. 2003, Lyons et al. 2012) or sediment anoxia and sulphide intrusions into the plants caused by enhanced organic matter sedimentation (Holmer &

Bondgaard 2001, Pérez et al. 2007, Olivé et al. 2009). Moreover, high availability of inorganic nitrogen, especially ammonium, can have a direct effect on seagrasses (Burkholder et al. 1992, van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011).

Ammonium toxicity has been documented in a wide range of photosynthetic organisms (see review by Britto & Kronzucker 2002 and references therein). This toxic effect is mainly related to the uncoupling of ATP production and photosynthetic electron transport (Britto et al. 2001), enhanced respiratory demands (Goyal et al. 1982, Marschner 1995) and decreased potassium, magnesium, calcium or nitrate uptake (Ullrich et al. 1984, Lee & Drew 1989, Pearson & Stewart 1993, Marschner 1995, Smolders et al. 1996, van Katwijk et al. 1997, Wang et al. 2010). To minimize this harmful effect, ammonium must be rapidly assimilated into amino acids and other N-organic compounds (Marschner 1995), generating a strong depletion of organic carbon skeletons (Marschner 1995, Brun et al. 2002, 2008). In addition, ammonium could be pumped out of the plant, which in turn increases respiratory demands by 40% (Britto et al. 2001). Phosphate is required in these processes as a part of the metabolic energy transfer coin (i.e. ATP) and as a major component of organic compounds (Stitt 1997). Thus, when carbon turnover and ATP consumption increase as a result of ammonium assimilation, an enhancement of phosphate demands would be expected (Brun et al. 2002). Consequently, phosphate may play a key role in reducing ammonium toxicity, as previously suggested for the seagrass *Zostera noltei* (Brun et al. 2002, 2008). Therefore, any process limiting phosphate availability and acquisition might make seagrasses more vulnerable to ammonium toxicity. Phosphate uptake is an active transport process that depends upon ATP and membrane potential (García-Sánchez et al. 2000, Palmgren 2001, Rubio et al. 2005). Since ammonium uptake produces a large membrane depolarization in seagrass leaf mesophyll cells (Rubio et al. 2007), phosphate uptake could decline under high ammonium concentrations even if the external phosphate levels are adequate to maintain seagrass growth.

To date, studies on seagrass nutrient uptake are rather limited (Touchette & Burkholder 2000) (Table 1) and interactions between nutrient uptake rates were only tested for ammonium and nitrate (Short & McRoy 1984, Paling & McComb 1994, Terrados & Williams 1997, Lee & Dunton 1999, Alexandre et al. 2011) (Table 1). Overall, these studies reported that seagrass leaves had higher affinity for ammonium than for nitrate, and it was generally ascribed to the lower energetic cost associated with the uptake and assimilation of ammonium (Turpin 1991). However, there are no studies dealing with the interaction between any inorganic nitrogen source and phosphate, even though phosphorus is one of the 3 major elements of seagrass tissues (Duarte 1990) and a basic component for the energetic metabolism (Stitt 1997).

Over the last few decades, ammonium loads in coastal areas have been increasing and further increases are expected (Glibert et al. 2010, Sobota et al. 2013). This highlights the relevance of ammonium toxicity, which is likely to increase for species inhabiting coastal areas, and also species growing near discharge points (i.e. rivers, wastewater and/or run-off discharge points) or in phosphate-limited environments (i.e. carbonate sediments).

Table 1. Estimated phosphate and ammonium kinetic parameters of seagrass species based on the Michaelis-Menten model and on the linear regression model for uptake kinetics. The estimated parameters were maximum uptake rate (V_{max}, μmol g^{-1}DW h^{-1}), half-saturation constant (K_M, μM), incubation time (t, h) and slope (S, g^{-1} DW h^{-1}) of the curves ($0.5 \times V_{max}/K_M$). nd: not determined because of linearity.

Nutrient	Species	V_{max}	K_M	Slope	Reference
NH_4^+	*Amphibolis antarctica*	n.d.	n.d.	0.68	Paling & McComb (1994)
	Amphibolis antarctica	604	1041	0.29	Pedersen et al. (1997)
	Phyllospadix iwatensis	2.2-35.5	12.7-133.5	0.06-0.14	Hasegawa et al. (2005)
	Phyllospadix torrey	95.6-204.3	9.3-33.9	3.01-5.36	Terrados & Williams (1997)
	Ruppia maritima	9.7-14.1	8.1-9.2	0.77-0.60	Thursby & Harlin (1984)
	Thalassia hemprichii	32-37	21-60	0.31-0.76	Stapel et al. (1996)
	Thalassia hemprichii	37.36-37.93	67.89-76.89	0.25-0.28	Zhang et al. (2011)
	Thalassia testudinum	8.3-16.4	7.6-15	0.28-1.14	Lee & Dunton (1999)
	Halophila stipulacea	9.79	57.95	0.17	Alexandre et al. (2014)
	Zostera marina	19.4-20.5	8.5-9.2	1.11-1.14	Thursby & Harlin (1982)
	Zostera marina	n.d.	n.d.	0.8	Short & McRoy (1984)
	Zostera noltei	28.33-31.91	28.73-34.1	0.47-0.49	Alexandre et al. (2011)
PO_4^{3-}	*Amphibolis antarctica*	n.d.	n.d.	0.50	Paling & McComb (1994)
	Thalassia hemprichii	2.2-3.2	7.7-15	0.11-0.14	Stapel et al. (1996)
	Thalassia hemprichii	3.63-4.7	4.7-13.17	0.18-0.25	Zhang et al. (2011)
	Thalassia testudinum	0.5-1.9	1.2-11.3	0.08-0.31	Gras et al. (2003)
	Zostera noltei	0.9-3	7-7.1	0.06-0.21	Pérez-Lloréns et al. (1995)

For instance, some intertidal seagrass species (such as the temperate *Zostera noltei)* usually occur near wastewater and/or run-off discharge points where ammonium concentrations can range from 158 to 663 μM (Hernández et al. 1997, Cabaço et al. 2008) and tropical species such as *Thalassia testudinum* thrive in carbonate sediments known to be phosphate limited (Short et al. 1990, Touchette & Burkholder 2000, Gras et al. 2003). Thus, a better understanding of the ammonium toxicity process, especially those issues related to its interaction with phosphate, is needed to improve our management capacity. Consequently, the main objective of this study was to test the existence of a short-term interaction between ammonium and phosphate uptake on leaves of *Zostera noltei* as a first step involved in ammonium toxicity mechanisms. Most studies report that both nutrient uptake and affinity rates are higher in leaves than in roots (Thursby & Harlin 1984, Stapel et al. 1996, Pedersen et al. 1997, Terrados & Williams 1997, Gras et al. 2003, Alexandre et al. 2011); hence, our study was performed only in leaves of *Z. noltei* plants.

To address this objective, a set of short-term nutrient uptake experiments (ammonium and phosphate assayed both separately and simultaneously) was performed using transparent incubation chambers with two independent compartments (for above and belowground plant parts), which allowed the independent study of leaf nutrient uptake in entire *Zostera noltei* plants.

MATERIALS AND METHODS

Plant collection and preparation for uptake experiments

Zostera noltei specimens were collected by hand from February to March 2010 from an intertidal muddy bed at Los Toruños, a salt-marsh of 773 ha situated in the Cádiz Bay Natural Park, Spain (36°30′ N, 6°10′ W). The area is surrounded by a spit 12 km long and has a maximum depth of 4 m. Mean seawater temperature is ~18°C (range 10°C to 29°C) and the average annual salinity is 38. The system can be regarded as meso eutrophic, with nutrient concentrations in the sampling site varying widely throughout the year (Pérez-Lloréns et al. 2004). In the water column, nutrient peaks usually occur in winter, with values up to 1.4 μM NO_2^-, 12 μM NO_3^-, 25 μM NH_4^+ and 1.5 μM PO_4^{3-} (Tovar et al. 2000).

Experimental plant units (EPUs) of *Zostera noltei* consisted of individual shoots (e.g. short shoots) characterized by a high aboveground/belowground biomass ratio (AG:BG) (Brun et al. 2006). EPUs were individually selected in the field and collected from different patches in a large area (~100 × 100 m), to ensure the genetic independence of plants. They were transported to the laboratory within 120 min of collection. Harvesting of EPUs took place during 2 different days for logistic and experimental reasons; the first set was used to estimate phosphate and ammonium uptake rates of plants separately, whereas the second set was used to test the interaction between ammonium and phosphate uptake. Upon arrival, plants were rinsed in seawater and any epiphytes were wiped off with a soft tissue paper. At the beginning of each assay, morphometric measurements (width, length and number of leaves, aboveand belowground biomass) were conducted in 10 EPUs haphazardly taken from the pool of collected plants (Table 2). A Student's t-test for independent samples revealed no significant differences in morphometric variables for plants collected at different dates (Table 2).

Before starting the incubations, EPUs were maintained for 24 h in identical conditions to the nutrient uptake experiments for temperature (20°C) and light (150 μmol photons m^{-2} s^{-1}, measured with a LICOR spherical quantum sensor Li-1000 Data Logger). To keep basal nutrient concentrations at a minimum and to maintain the same conditions in all the uptake assays, aerated artificial seawater (ASW) and modified marine culture medium (Woelkerling et al. 1983) without any inorganic nitrogen and phosphorus was used. Dissolved inorganic carbon and salinity were set at 2.2 mM and 33.2 ± 0.08 respectively, while pH (8.23 ± 0.01) was adjusted by using concentrated sodium hydroxide (0.1 N) and hydrochloric acid (1.2 N) solutions. Under these experimental conditions the ratio of total NH_4^+-N to NH_3-N was 17.3 (Whitfield 1974).

Feature (units)	EPUs	p-value
Aboveground (AG) biomass (g DW)	0.18 ± 0.004	0.62
Belowground (BG) biomass (g DW)	0.08 ± 0.004	0.15
AG/BG biomass ratio	2.40 ± 0.19	0.27
Leaf abundance (no. leaves shoot^{-1})	3.15 ± 0.13	0.054
Mean leaf length (cm)	24.65 ±1.37	0.73
Leaf width (mm)	0.93 ± 0.03	0.23
Leaf thickness (mm)	0.16 ± 0.01	0.28
Leaf cross-section (mm^2)	0.11 ± 0.01	0.052
Leaf area (m^2)	0.0005 ± 0.003	0.76
Aboveground biomass /leaf area (g DW m^{-2})	17.36 ± 0.79	0.91

Table 2. *Zostera noltei*. Initial morphometric features of the experimental plant units (EPUs) collected at the beginning of the experiment (mean ± SE; n = 20) and p-value obtained for the Student's t-test for independent samples to compare the differences in morphometric features for the 2 pools of plants collected on different days (α = 0.05)

Nutrient uptake experiments

Foliar nutrient uptake was measured using a combination of the multiflask and the perturbation techniques (Pedersen 1994, Martínez & Rico 2004) in partitioned Plexiglas chambers (Pérez-Lloréns et al. 1993). Each chamber (diameter 7 cm) contained 400 ml ASW in the upper compartment and 250 ml in the lower compartment (Fig. 1). Six EPUs per chamber were randomly selected from an initial pool of plants and positioned across the holes located in the central piece of PVC used to split the chamber; roots and rhizomes were in the lower compartment and leaves in the upper one. Holes were sealed with petroleum jelly based product (Vaseline®) to avoid any leakage between compartments. Prior to incubations, the watertightness of the chambers was tested by mounting the EPUs and adding a dye (uranine) to the top compartment. After 24 h, water samples were collected from the bottom compartment by inserting a syringe needle in the sampling port (Fig. 1) and measured with a fluorometer (Turner TD-700) to detect any presence of the dye. The analysis did not reveal any sign of uranine, indicating that the chamber design prevented leakage between compartments.

To keep temperature constant, chambers were placed into an aquarium (0.5 × 0.5 × 0.35 m) filled with distilled water and connected to a thermostatic bath (20°C) in a closed circuit. Light was supplied from the top by cool fluorescent tubes (Sylvania Gro-Lux F18W/Gro), providing about 150 µmol photons m^{-2} s^{-1} to the upper compartment. Bubbling was provided individually to upper compartment of chambers to provide water mixing throughout the incubation period, reduce the thickness of the leaf boundary layer (Pérez-Lloréns et al. 1993) and ensure that leaf nutrient uptake is mostly dependent on the nutrient concentration in the water (Stapel et al. 1996). Although belowground parts were incubated in darkness to mimic buried conditions as much as possible, the water of the lower compartment was oxygenated which can deviate from natural sediment conditions. However, previous studies in the same species reported no effects on foliar uptake rates (ammonium and nitrate) when roots were incubated either in anoxic or in oxygenated medium (Alexandre et al. 2010).

At the onset of the incubations specific amounts of ammonium (NH_4Cl) or phosphate (KH_2PO_4) from stock solutions were added to the upper compartment (i.e. initial phosphate and ammonium concentrations). Subsequently, the time course of nutrient concentrations in seawater was monitored during 120 min. Incubation time was selected according to the existing literature on seagrass nutrient uptake (see Table 1): in those studies, 120 min was the average time interval used to avoid nutrient depletion in the culture medium.

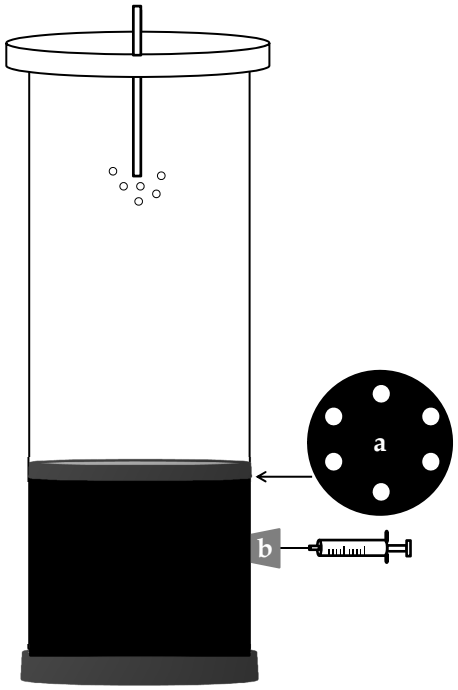


Fig. 1: Schematic drawing of the two-compartment chamber used for incubations (upper-compartment 0.4 L, lower compartment 0.25 L). The lower compartment was darkened during the incubations. (a) Black acrylic disc connecting aboveground (leaves) and belowground parts (roots/rhizomes) of the experimental plants, sealed with petroleum jelly based product (vaseline®) where plants were placed. (b) Lower compartment sampling port.

Four nutrient treatments were assayed in triplicate: (1) P, phosphate concentrations ranging from 0 to 20 μM (0, 0.5, 1, 2, 3, 5, 10, 15, 20 μM) without ammonium; (2) N, ammonium concentrations ranging from 0 to 100 μM (0, 1, 3, 5, 10, 20, 30, 50, 100 μM) without phosphate; (3) P+Nc, variable concentrations of phosphate (1, 5, 10, 20 μM) and a constant concentration of ammonium (50 μM); (4) N+Pc, variable concentrations of ammonium (1, 5, 10, 50, 100 μM) and a constant concentration of phosphate (10 μM).The selected ammonium and phosphate concentrations ranged from values usually measured in seawater (typically less than 5 μM of NH_4^+ and 2 μM PO_4^{3-}; Touchette & Burkholder 2000) to those recorded in *Zostera noltei* meadows growing in eutrophic waters or near wastewater discharge points (Hernández et al. 1997, Cabaço et al. 2008). In addition, to test the interactions between the uptake rates of both nutrients, a saturating concentration of both nutrients was added (i.e. avoiding the possible depletion of nutrients added as a background level). Moreover, 50 μM of ammonium was selected as constant concentration because this concentration has a negative effect on *Z. noltei* plants (Brun et al. 2002, 2008).

Nutrient uptake experiments were carried out in 2 different sets. In each set 9 chambers were used simultaneously and randomly placed in the aquarium. In a first set (treatments P and N), uptake rates were measured on 3 consecutive days (temporal replication). One replicate of each treatment (P and N) with 9 different concentrations per nutrient was carried out in the same day. The second set was carried out to test the interaction between ammonium and phosphate uptake (P+Nc, N+Pc). In this case, one replicate of each treatment (P+Nc, N+Pc, in total 9 different concentrations; see aforementioned concentrations) was also performed over 3 consecutive days (temporal replication).

Once incubations started, water samples (3 samples of 2 ml each) were taken from the upper compartment at 0, 5, 30, 60 and 120 min following the nutrient addition according to each treatment. In the lower compartment, water samples (3 samples of 2 ml each) were taken at the beginning (0 min) and the end (120 min) of the incubation to detect any presence of nutrients (Fig. 1). After collection, water samples from both compartments were frozen and kept at –20°C until analysis. Phosphate and ammonium analysis were made according to Murphy & Riley (1962) and Solorzano (1969), respectively. At the end of each experiment, EPUs were split into above and belowground biomass (cut at the point of the sealer), dried at 60°C for 72 h and weighed.

Estimation of uptake kinetic parameters

Foliar uptake rates (V, μmol g^{-1} DW h^{-1}) were estimated from changes in nutrient concentration (S, μM) at each sampling time (t = 0, 5, 30, 60 and 120 min). The change in nutrient concentration of the upper compartment was corrected by the change in the total volume after each water collection (Pedersen 1994):

$$V=\frac{(S_0 \times vol_0) - (S_t \times vol_t)}{t \times B}$$

where S_0 and vol_0 are the nutrient concentration (μM) and the water volume (l), respectively at the beginning of a sampling interval; S_t and vol_t are the nutrient concentration and the water volume, respectively at the end of a sampling interval; t is the time elapsed between two successive sampling events (h); and B is the foliar dry weight (DW, g). Uptake rates (n = 3) were plotted against the initial concentration of each treatment each time interval.

Conductance (μm s^{-1}) was calculated to analyze the permeability of *Zostera noltei* to nutrients. Values were computed from the initial slope of nutrient uptake rates versus nutrient concentration curves by expressing the nutrient uptake rate on an areal basis. Dry weight per surface area ratio (g DW m^{-2}) was estimated by measuring the surface area in an initial pool of EPUs (Table 2), which were subsequently dried at 60°C for 72 h and weighed. Leaf area was calculated as 2 × length × width, i.e. the area of both sides of the leaf.

Statistical analyses

Uptake rates from each time interval (n = 3) were plotted against nutrient concentrations at the beginning of each time interval. Outlier values were identified following Grubb's (n > 25) and Dixon's (n < 25) tests (Fry 1993). This analysis determined that 4 phosphate uptake rates and 2 ammonium uptake rates (one combined with phosphate) could be considered as outliers and therefore removed from the data set. Excluding these outliers, the data were fitted by least-squared regression analysis (Ricker 1984, Martínez & Rico 2004).

A 1-way analysis of variance (ANOVA) test was used to determine the differences among conductances within the same treatment (P, N, P+Nc and N+Pc). When significant differences were found a post-hoc Tukey test was performed (Zar 1984). Similarly, a Student's t-test was used to examine significant differences between conductances in the same time interval among different treatments. Homoscedasticity and normality of the data were checked before conducting ANOVAs and t-tests; only phosphate uptake rates were log-transformed due to the detection of heterocedasticity. Data are shown as mean ± standard error (SE). Significance level was set at a probability of 5% ($\alpha = 0.05$).

RESULTS

No release of nutrients by roots or rhizomes was recorded in any treatment, since no significant concentrations were detected in the lower compartment (which contained artificial seawater without nutrients). Percentages (referred to the initial concentration) of nutrient disappearance in the upper compartment at the end of incubations (120 min) showed no signs of nutrient depletion in any treatment (34.1 ± 3.0% for P; 48.9 ± 3.5% for N; 16.2 ± 1.8% for P+Nc and 54.5 ± 3.9% for N+Pc; these values represent the mean ± SE of all concentrations assayed per treatment). The regression lines of phosphate uptake rates (either with or without ammonium addition) against phosphate concentration rendered a positive and near to zero intercept in most of the time intervals tested. However, for ammonium assays (either with or without phosphate), the intercept was negative (but close to zero) during the first 5 min (Table 3).

Foliar phosphate uptake

Phosphate foliar uptake was a linear function of phosphate concentration at each sampling interval (0–5, 5–30, 30–60 and 60–120 min; Fig. 2). Conductance was significantly higher (9.11 ± 0.82 $\mu m\ s^{-1}$; ANOVA, $p < 0.05$) during the first 5 min, followed by a decline in the rest of sampling intervals. There were no significant differences between the 2 last time intervals (Fig. 2, Table 3).

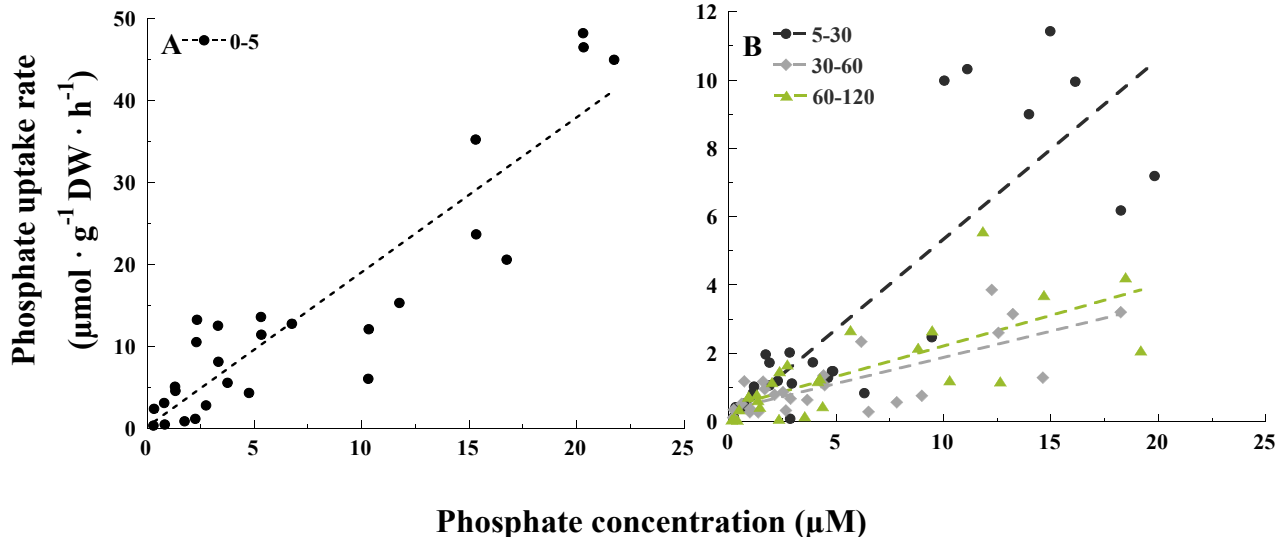


Fig. 2. *Zostera noltei.* Foliar phosphate uptake rates (µmol g^{-1} DW h^{-1}) versus phosphate concentration (µM) at the assayed time intervals, when phosphate was the only nutrient present (P treatment) in the water of the upper compartment. (A) Phosphate uptake rate in the first 5 min. (B) Phosphate uptake rate in the remaining time intervals (5–30, 30–60, 60–120 min). Note different y-axis scales in (A) and (B).

Foliar ammonium uptake

Foliar ammonium uptake versus ammonium concentration fitted to a linear function (Fig. 3), as observed also for phosphate uptake. Conductance was similar (i.e. no significant differences, ANOVA, $p > 0.05$) for all time intervals except the first 5 min, when values were significantly higher (ANOVA, $p < 0.05$). When uptake rates were plotted against nutrient concentrations for the whole incubation interval (0–120 min), the conductance for ammonium was approximately 3× higher (2.75 ± 0.19 µm s^{-1}) than that estimated for phosphate (0.87 ± 0.10 µm s^{-1}) (Table 4, Fig. 4).

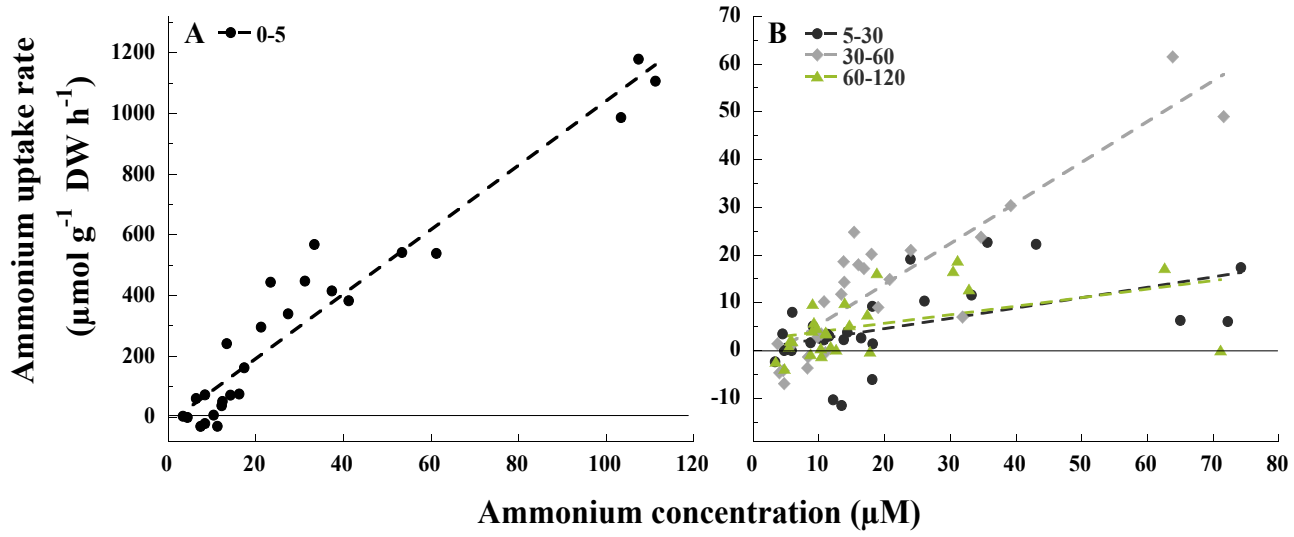


Fig. 3. *Zostera noltei.* Foliar ammonium uptake rates (µmol g^{-1} DW h^{-1}) versus ammonium concentration (µM) at the assayed time intervals, when ammonium was the only nutrient present (N treatment) in the water of the upper compartment. (A) Ammonium uptake rates in the first 5 min. (B) Ammonium uptake rates in the remaining time intervals (5–30, 30–60, 60–120 min). Note different y-axis scales in (A) and (B).

Table 3. *Zostera noltei*. Foliar phosphate and ammonium uptake rates expressed as mean (± SE) conductance (μm s^{-1}) and intercept value in each treatment (P, N, P+Nc, N+Pc) at each sampling interval (0–5, 5–30, 30–60, 60–120 min). See text for further information on calculations and treatments. Letters indicate significant differences among sampling intervals in each treatment (1-way ANOVA and post-hoc Tukey test; $p < 0.05$). # Significant differences either be tween P and P+Nc, or N and N+Pc treatments (t-test, $p < 0.05$). n: number of observations.

	Interval (min)	Conductance (μm s^{-1})	Intercept (μmol·g^{-1} DWh^{-1})	r^2	n
P	0-5	[a] 9.11 ± 0.82	0.09 ± 1.66	0.83	27
	5-30	[b] 2.56 ± 0.34#	0.07 ± 0.50	0.70	26
	30-60	[c] 0.74 ± 0.14#	0.35 ± 0.19	0.57	25
	60-120	[c] 0.86 ± 0.14#	0.42 ± 0.26	0.53	26
N	0-5	[a] 51.89 ± 3.18	-33.31± 29.28	0.91	27
	5-30	[b] 1.06 ± 0.33	0.29 ± 0.33	0.24	27
	30-60	[b] 3.13 ± 0.48	-0.46 ± 2.77	0.64	27
	60-120	[b] 1.59 ± 0.29	-0.29 ±1.32	0.55	26
P+Nc	0-5	[a] 11.38 ± 1.54	-0.27 ± 3.81	0.83	12
	5-30	[b] 0.34 ± 0.24	0.66 ± 0.48	0.17	12
	30-60	[b] 0.29 ± 0.29	0.08 ± 0.54	0.11	12
	60-120	[b]-0.04 ± 0.04	0.31 ± 0.10	0.07	12
N+Pc	0-5	[a] 46.05 ± 3.57	-30.10 ±38.08	0.92	15
	5-30	[b] 1.11 ± 0.46	1.00 ± 2.61	0.52	14
	30-60	[b] 1.44 ± 0.39	1.00 ± 2.47	0.50	15
	60-120	[b] 1.64 ±0.05	-0.21 ± 0.37	0.98	15

Interactions between foliar ammonium and phosphate uptake

In treatments were nutrients were assayed independently (i.e. P and N), a linear relationship between uptake rates and nutrient concentrations was recorded when both nutrients were added together (i.e. P+Nc and N+Pc). A high conductance (11.38 ± 1.54 and 46.05 ± 3.57 μm s^{-1} for phosphate (P+Nc) and ammonium (N+Pc), respectively) was recorded in the first 5 min (ANOVA, $p < 0.05$) followed by a substantial reduction in the rest of the sampling intervals (Table 3). Except the first interval (0–5 min), the conductance for phosphate in the presence of ammonium (P+Nc) was significantly lower than

that measured in the absence of ammonium (P) (Fig. 4, Table 4) (Student's t-test, $p < 0.05$), which would indicate that ammonium restricted phosphate uptake after the first 5 min. Contrastingly, phosphate addition did not affect ammonium conductance in any of the sampling intervals (Tables 3 & 4) when compared to values measured when ammonium was assayed alone (Table 4, Fig. 4B). Thus, phosphate did not cause any effect on the ammonium uptake rates. When uptake kinetics were analyzed for the whole incubation period (120 min), conductance for ammonium was similar regardless of the presence or absence of phosphate; however, phosphate conductance decreased by 55% in the presence of ammonium (Table 4, Fig. 4A).

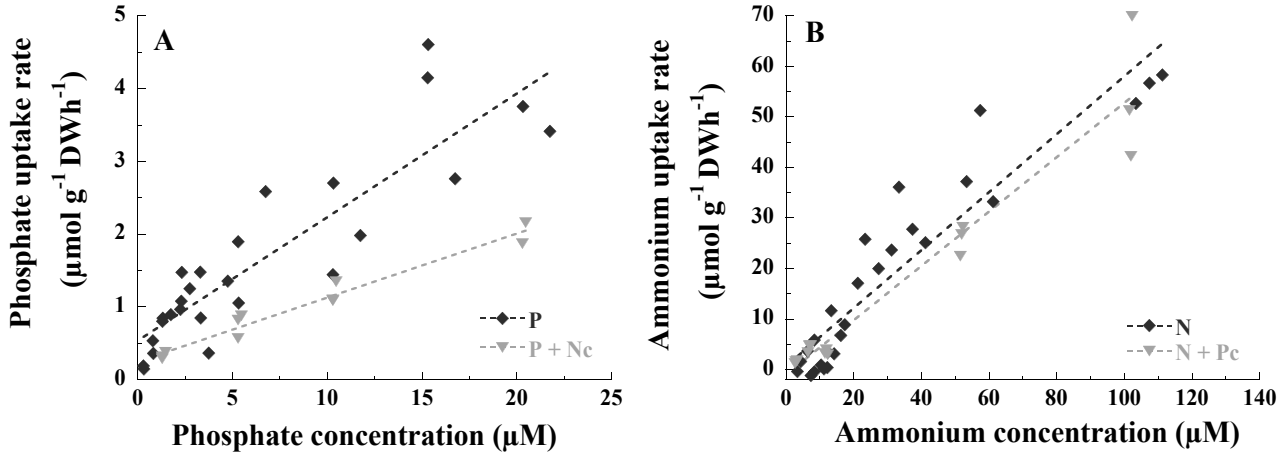


Fig. 4. *Zostera noltei.* Foliar (A) phosphate and (B) ammonium uptake rates (µmol g^{-1} DW h^{-1}) versus phosphate and ammonium concentration (µM) in the whole culture interval assayed (120 min). (A) Phosphate uptake rates (µmol g^{-1} DW h^{-1}) with (P+Nc) and without (P) the presence of ammonium in the water of the upper compartment (50 µM). (B) Ammonium uptake rates (µmol g^{-1} DW h^{-1}) with (N+Pc) and without (N) the presence of phosphate in the water of the upper compartment (10 µM).

Table 4. *Zostera noltei.* Foliar phosphate and ammonium uptake rates expressed as mean (± SE) conductance (µm s^{-1}) during the whole incubation interval (120 min). # Significant differences between P and N+Pc or N and P+Nc treatments (t-test, $p < 0.05$). See 'Materials and methods' for further information on these calculations and treatments.

	P	N	P+Nc	N+Pc
Conductance	0.87 ± 0.10#	2.75 ± 0.19	0.39 ± 0.20#	2.60 ± 0.19
Intercept	0.52 ± 0.17	0.68 ± 1.95	0.26 ± 0.06	-1.02 ± 2.04
r^2	0.78	0.87	0.96	0.93

DISCUSSION

This study showed that high ammonium concentrations (50 μM) reduced foliar phosphate uptake in the seagrass *Zostera noltei* by more than 50% while foliar ammonium uptake remained unaffected by phosphate enrichment (10 μM) in seawater. Consequently, this is the first time that this inhibitory effect of ammonium has been recorded in seagrasses, and it could constitute an important first-step mechanism related to the widely-observed ammonium toxicity process in these marine plants (van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011).

Phosphate transport depends on the high negative potential of the membrane and on the electrochemical gradient of sodium (García-Sánchez et al. 2000, Rubio et al. 2005). Thus, the reduction in phosphate uptake recorded in our study could be related to the rapid and strong depolarization of the membranes (~100 mV) observed in *Zostera marina* (Rubio et al. 2007) and in terrestrial plants (Smith & Walker 1978, Felle 1980, Ullrich et al. 1984) under ammonium enrichment. Moreover, the plasma membrane potential is maintained by the activity of the H^+-ATPase (Fernández et al. 1999), thus changes in the membrane potential driven by ammonium uptake may increase the ATP consumption to reestablish the membrane potential. Considering the high turnover of ATP, the active ATP-dependent phosphate uptake (García-Sánchez et al. 2000) will be affected in the short term (minutes to hours), as ammonium addition decreased ATP levels within 5 min of ammonium exposure (Turpin et al. 1990). Apart from adverse effects in membrane transport processes, ammonium is a natural uncoupler of ATP synthesis (Lawlor 1993, Marschner 1995); therefore, if a massive entrance of ammonium to the cells is not accompanied by amino acid synthesis, ATP synthesis will also be strongly affected in the chloroplasts.

Additional insights into the negative effect of ammonium on phosphate uptake arise from kinetic curves. During the first 5 min ('surge uptake' according to Harrison et al. 1989), phosphate uptake rates were very high and unaffected by ammonium addition (Table 3). This could be explained because the main underlying process accounting for the surge uptake is related to the adsorption of ions into the plant tissue (e.g. cell wall, periplasmic space) and consequently independent of membrane transporters. Beyond this surge uptake, active mechanisms through specific phosphate transporters are the main areas responsible of the phosphate uptake ('internally controlled uptake'; Harrison et al. 1989) and hence these transporters were affected by the presence of ammonium (Table 3).

In the present study, ammonium uptake rates were higher than those for phosphate (Tables 3 & 4). For comparison purposes, the initial slope of the saturation uptake curves was calculated through Michaelis-Menten parameters such as $0.5 \times V_{max} / K_M$, re-analysing published data when necessary (Table 1). In our study, slopes for ammonium uptake (0.6 l g^{-1} DW h^{-1}) were within the range found in other seagrasses (0.06–5.35 l g^{-1} DW h^{-1}; Table 1), as were those for phosphate uptake (our study: 0.18 l g^{-1} DW h^{-1}; previous studies: 0.08–0.50 l g^{-1} DW h^{-1}; Table 1), although phosphate uptake studies are scarcer than ammonium ones (Touchette & Burkholder 2000).

Although saturation kinetics have been reported for foliar phosphate uptake in seagrasses (Pérez-Lloréns & Niell 1995, Stapel et al. 1996, Zhang et al. 2011; see Table 1), other studies revealed linear kinetics (e.g. seedlings of *Amphibolis antarctica*, Paling & McComb 1994; *Thalassia testudinum* under low phosphate concentrations [<2 μM], Gras et al. 2003). The observed disparity in *Zostera noltei* kinetics between our study (linear), and those of Pérez-Lloréns & Niell (1995) (saturation) for the same range of concentrations, could be caused by differences in the nutritional history of the plants (e.g. specimens harvested at different seasons and sampling sites) and/or by different experimental conditions (e.g. different starvation periods: 24 h in this study versus 72 h in Pérez-Lloréns & Niell 1995) which could affect the nutrient uptake response of the plants (Touchette & Burkholder 2000).

Our results bring into awareness 2 important aspects that should be considered for experimental and management issues. Firstly, the passive uptake of ammonium (in contrast to nitrate) may affect nutrient enrichment experimental set-ups, where the recorded effects can be opposite depending on the nitrogen source used (i.e. ammonium versus nitrate). For instance, factors such as hydrodynamics (e.g. reduced boundary layers; La Nafie et al. 2012), temperature (van Katwijk et al. 1997, Brun et al. 2002) or pH (van der Heide et al. 2008) can enhance ammonium uptake and promote toxicity in plants if ammonium is used as the nitrogen source instead of nitrate. Secondly, the inhibition of phosphate uptake in the presence of ammonium should be more relevant in P limited environments, as for instance, in seagrasses inhabiting carbonate sediments (Touchette & Burkholder 2000). At these locations ammonium could promote a higher toxicity on plants, since phosphate alleviates ammonium toxicity to some extent (Brun et al. 2002, 2008). Additionally, the presence of high levels of ammonium in these areas can intensify the phosphate limitation suffered by seagrass species, affecting the actual rate of phosphate uptake and nutrient stoichiometry.

In summary, this study showed for the first time that there was a short-term negative effect of ammonium on phosphate uptake in the leaves of the seagrass *Zostera noltei*, which can be considered as a first-step mechanism in the ammonium toxicity process. This result has implications for plant nutrient stoichiometry, experimental design and in the implementation of managing policies in these crucial ecosystems.

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Chapter 2

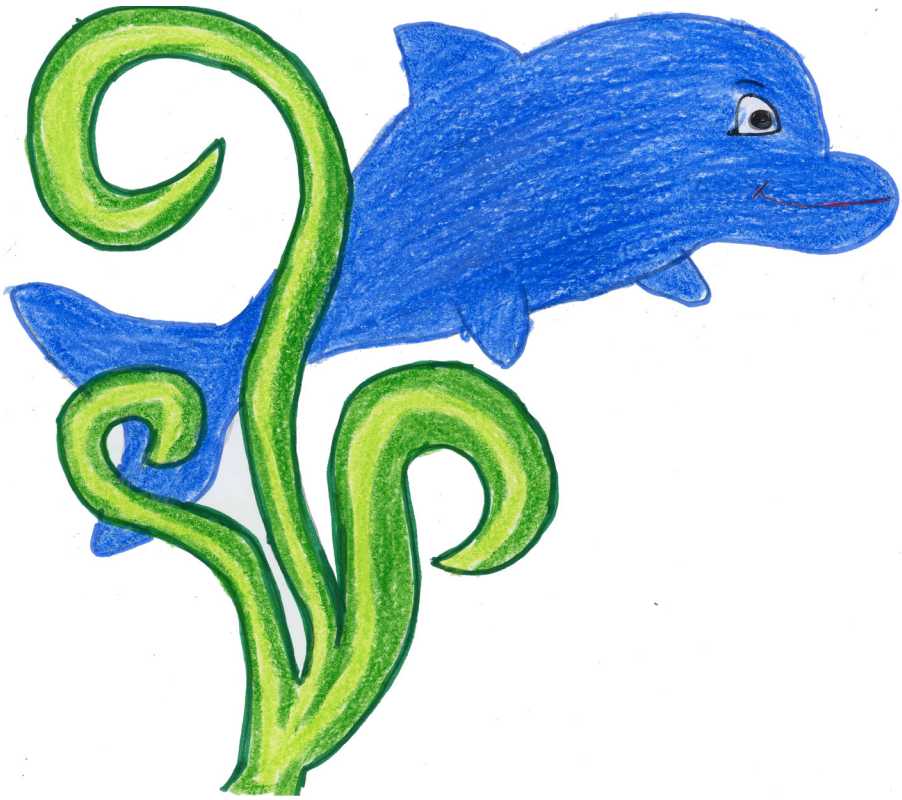
Flow velocity and light levels drive a non-linear response on the seagrass *Zostera noltei* under ammonium enrichment

Villazán B, Brun FG, González-Ortiz V, Moreno-Marín F, Bouma TJ, Vergara JJ

Submited to Limnology and Oceanography

What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what´s going on

Jacques Yves Cousteau



Ilustrated by Patricia Villazán Gamonal

Flow velocity and light levels drive a non-linear response on the seagrass *Zostera noltei* under ammonium enrichment

Beatriz Villazán [1], Fernando G. Brun[1], Vanessa González-Ortiz[1], Francisco Moreno-Marín[1], Tjeerd J. Bouma[2], Juan J. Vergara[1]

[1]Departamento de Biología (Área de Ecología). Facultad de Ciencias del Mar y Ambientales. Universidad de Cádiz. Campus de Excelencia Internacional del Mar (CEIMAR), Puerto Real E-11510. Cádiz. Spain

[2]Netherlands Institute for Sea Research (NIOZ), PO Box 140, 4400 AC. Yerseke. The Netherlands

ABSTRACT: We investigated the interactive effects of light (low and saturating light levels) and flow velocity (3 levels) under NH_4^+ enriched conditions on *Zostera noltei* plants, by measuring the response at morphological and dynamic levels during a 5 weeks flume experiment. Our results showed a non-linear response of *Z. noltei* plants to such factorial design, recording the strongest negative effect of NH_4^+enrichment at intermediate flow velocities for almost all the dynamic variables (i.e. survival, net production, shoot appearance rate). This negative effect of NH_4^+ enrichment was intensified under low light conditions, where net production was only positive in those plants growing at high flow velocity. This positive effect of flow velocity was ascribed to the more horizontal position of the leaves, allowing higher levels of light capture. Whereas enhancing current velocity increase NH_4^+ uptake rates till adverse levels, which may potentially trigger NH_4^+ toxicity, this negative effect of flow velocity appear to be modulated by the higher light quantities captured at high flow, making intermediate current velocities most harmful. In summary, our results highlighted the importance of studying the complexity of the interaction between multiple stressors that frequently co-occur in nature, to improve, for instance, our forecast capacity on the response of seagrass populations to global change scenario.

INTRODUCTION

Seagrasses are clonal marine plants that inhabit intertidal and subtidal areas, extending from tropical to temperate latitudes (den Hartog & Kuo 2006). These species constitute one of the most productive coastal ecosystems (Mann 2000) and provide significant ecological, social and economical values (Costanza et al. 1997). Although many factors have been acknowledged to contribute to the decline of seagrass ecosystems (Waycott et al. 2009), nutrient enrichment is considered as one of the most important drivers (Burkholder et al. 2007).

Nutrients are needed for photosynthetic organisms, and its availability usually stimulates growth and biomass production in seagrasses (e.g. Orth 1977, Alcoverro et al. 1997). However, high levels of nutrients under some specific circumstances (e.g. low light levels, low water renovation time in the basin, etc.) may produce adverse effects on seagrasses due to algal overgrowth. Such large algae

accumulation may cause light reduction (Burkholder et al. 2007), enhance fluxes of organic matter to the sediment (e.g. rising the risk of sediment anoxia; Greve et al. 2003) and promote sulphide intrusion into the plants (Borum et al. 2005, Pérez et al. 2007), which may reduce photosynthesis, growth and survival in seagrasses. In addition, if nitrogen is mainly available in its reduced form (i.e. NH_4^+) seagrasses are also prone to experience a direct toxic effect (van Katwijk et al. 1997, Brun et al. 2008, Villazán et al. 2013b).

Adverse effects of high NH_4^+ concentrations have been traditionally explained by internal accumulation of NH_4^+ (Marschner 1995), which may affect internal pH and enzyme kinetics, uncoupling the photosynthetic production of ATP, increasing respiration rates and reducing the uptake of some ions (e.g. potassium, magnesium, calcium or phosphate) (van Katwijk et al. 1997, Villazán et al. 2013a). To prevent internal NH_4^+ accumulation and to minimize harmful effects, plants must rapidly assimilate NH_4^+ into amino acids and other N-organic compounds, generating strong internal demands of ATP, phosphorous and organic carbon skeletons (Brun et al. 2002, 2008, Villazán et al. 2013b), which must be supplied directly from photosynthesis or mobilized from internal C-reserves. Therefore, any factor that increases the uptake rates of NH_4^+ and/or reduces photosynthetic rates or internal C reserves (i.e. less carbon and energy available for the plant) may enhance NH_4^+ toxicity on plants.

NH_4^+ release into shallow marine areas is increasing and will continue rising in the near future (Glibert et al. 2010), potentially affecting seagrass populations. In addition hydrodynamic conditions in coastal areas are changing worldwide, due to anthropogenic engineering activities changing tidal flows (Kennis 2001), while global change processes are expected to increase the frequency and intensity of storms and wave stress (Young et al. 2011). Previous studies have described a consistent relationship between hydrodynamic conditions and NH_4^+ uptake or photosynthetic rates in seagrasses due to the influence of hydrodynamics on the mass transfer of resources to and from the seagrass beds (Koch et al. 2006, Morris et al. 2008). Increased current velocity or waves may enhance NH_4^+ uptake rates on seagrasses by decreasing the diffusive boundary layer (DBL) thickness in leaves (Koch et al. 2006). At the same time, photosynthetic rates may be a compromise among its reduction caused by enhanced sediment resuspension (e.g. decreasing light levels; Koch 2001), and its increase caused by higher carbon uptake rates due to the reduction of the DBL (Koch 2006), and because of the higher light levels captured by leaves due to its reconfiguration at high flow velocities (e.g. a more horizontal arrangement; Zimmerman 2003, de los Santos et al. 2010). Therefore, in shallow areas, different environmental factors (i.e. NH_4^+ concentration, hydrodynamics, light levels), may drive either synergistic or opposite effects on seagrass health. Thus, the outcome of the interaction among NH_4^+ enrichment, light and hydrodynamic conditions is far from being simple, and interactions between negative and positive effects may lead to a non-linear response in seagrasses under natural conditions, where multiple factors may act at the same time. Therefore, this study is aimed to test the complex interacting effect among NH_4^+ enrichment, hydrodynamic conditions and light levels on the survival, morphometric and dynamic variables of the seagrass *Zostera noltei*. This was accomplished by growing *Z. noltei* plants under a chronic high NH_4^+ concentration at three different flow velocities and at two different light intensities

MATERIAL AND METHODS

Zostera noltei plants were collected in June from an intertidal bed at Los Toruños salt marsh (Cádiz Bay Natural Park; 36º30´N, 6º10´W, Cádiz, Southern Spain). Plants were rinsed in seawater and sent to the NIOZ laboratory (The Netherlands). Upon arrival (less than 48 hours after collection), apical shoots formed by 2 rhizome internodes with 1 apical shoot and 1 lateral shoot, and the associated roots were selected as experimental plant unit (EPU). Prior transplantation, epiphytes were wiped out with a soft tissue paper. The EPUs were kept 5 days in a tank filled with aerated natural seawater from Oosterschelde estuary under saturating light dose (ca. 18 mol photons m^{-2} d^{-1}) in a 16 light: 8 h dark cycle at 20°C before the experiment.

Twelve flume tanks (Peralta et. al 2006) were used to expose *Zostera noltei* plants from June to August (36 days) to 3 contrasting current velocities (0.01, 0.10 and 0.35 m s^{-1}, thereafter low, medium and high velocity, LV, MV, HV) and two light levels (4.1 ± 0.1 and 14.3 ± 0.1 mol photons m^{-2} d^{-1}), corresponding to sub-saturating (low light, LL) and saturating (high light, HL) light doses for this species (Peralta et al. 2002). Four replicates (flume tanks) within each velocity treatment were used.

Velocity treatments were supplied by individual water pumps for each flume and measured with an ADV Doppler Nortek at 25 Hz (see Peralta et al. 2006 for further information). These velocity treatments were selected according to the range observed on tidal flats bordering intertidal vegetation, and the velocity range registered in *Zostera noltei* beds in Cádiz Bay: from low values of 0.02 m s^{-1} to exceptionally high flow velocity values of 0.25 m s^{-1} (Lara et al. 2012; Morris et al. 2013). Moreover, for comparison purposes, velocity levels were selected according to previous hydrodynamic experiments performed with this species (Peralta et al. 2006, de lo Santos et al. 2010).

Each flume tank was filled with natural seawater from Oosterschelde estuary (south-west Netherlands, salinity 30 and nutrient concentrations of 0.27 μM NH_4^+, 1.59 μM NO_3^-, 0.22 μM PO_4^{3-}). Water was renewed twice per week to avoid excessive growth of phytoplankton. Photoperiod was set at 18 h light: 6h dark cycle. Temperature was kept constant at 20°C using independent cooling units for each flume, to obtain optimal growth conditions for the plants (Peralta et al. 2002).

Five EPUs were haphazardly transplanted into a pot (12x12x25cm) filled with a homogenous mixture of clay, sand and gravel. Subsequently, 4 pots were randomly positioned within each flume tank (i.e. a total of 20 EPUs per flume tank) and kept one week under saturating light and at the same temperature and seawater conditions as those used for the experimental set-up. After this acclimation period, experiment started: 2 pots were kept at high light while other 2 pots per flume tank were shaded with a neutral light-quality screen (low light), and different flow velocities (LV, MV and HV) were applied to the tanks. Seawater was enriched with NH_4^+ (50 μM) and phosphate (5 μM) in each flume from a NH_4Cl and KH_2PO_4 stock-solution every 2 days. Water samples were collected coinciding with water renewal (twice weekly) and nutrients were measured using a San Plus segmented flow Skalar Autoanalyser® model 8805. The average nutrient concentrations measured just after nutrient supply

through the whole experimental period in all the flumes was 53.6 ± 0.8 μM for NH_4^+ and 5.0 ± 0.1 μM for PO_4^{3-} (n=80). Average remaining nutrient concentrations through the whole experimental period before each water renovation (i.e., 1 week after nutrient supply) in all the flumes was 6.4 ± 0.9 μM for NH_4^+ and 0.6 ± 0.1 μM for PO_4^{3-} (n=80). The concentration of NH_4^+ selected (i.e. 50 μM), was demonstrated in previous experiments (Brun et al. 2002, 2008) to promote a chronic toxicity in *Zostera noltei* plants, and has ecological relevance since it can be found in nature when meadows are growing in eutrophic waters or nearby wastewater discharge points (Burkholder et al. 2007). Phosphate was added simultaneously to NH_4^+ in the flumes to keep nutrient stoichiometry, and since previous studies suggested that any process limiting phosphate availability may make seagrasses more vulnerable to NH_4^+ toxicity (Brun et al. 2002, 2008).

Biological measurements

At the beginning of the experiment, morphometric measurements were conducted on 10 experimental plant units (EPUs) taken from the pool of collected plants (width, length and number of leaves, above and belowground biomasses were measured). Prior to transplantation into the pots, each EPU was weighed (initial fresh weight, FW) and each rhizome was individually tagged. At the end of the experiment, all surviving plants were carefully harvested and each tagged plant was weighed (FW) to estimate net production per plant (g FW $plant^{-1}$ d^{-1}), from the net change in individual plant weight along the experiment; as well, morphometric measurements were carried out in all the plants. Furthermore, each harvested EPU was divided into leaves (aboveground, AB) and roots/rhizomes (belowground, BG), freeze-dried and weighed to calculate the AG/BG ratio. Morphometric information was used to estimate plant dynamic properties, according to Peralta et al. (2006) and de los Santos et al. (2010) (Table 1). Leaf necrosis was quantified as the area with brown-black discolouration of the leaves on each shoot in relation to the total leaf surface (%).

Total carbon and nitrogen content

Total C and N content in tissues were determined in four freeze-dried, ground samples of leaves and roots/rhizomes from each treatment using a Carlo-Erba NA-1500 CHNS analyzer.

Estimation of phosphate and ammonium uptake rates

Foliar $NH_4^+$and PO_3^- uptake rates (V, μmol g^{-1} DW h^{-1}) were calculated on each flume tank along the experiment. It included plants growing at LL and HL within the same flume. Foliar uptake rates at each velocity treatment were estimated from changes in nutrient concentration (S, μM) in each flume, which was measured two times per week along the experiment. Uptake rates were computed as follow:

$$V=\frac{(S_0 \times vol_0) - (S_t \times vol_t)}{t \times B}$$

where S_0 and S_f are nutrient concentrations (μM) at the beginning and at the end of every sampling interval, t is the time elapsed between 2 successive samplings (h), B is the estimated foliar dry weight (DW, g), and V is the volume of each flume. NH_4^+ and PO_4^{3-} uptake rates (n = 10, twice weekly during 5 weeks) were plotted against time (36 days), being the calculated uptake rate of each day the mean ± SE value of each velocity treatment (n = 4 flumes per velocity regime).

Dry weight in each sampling interval was estimated by assuming linear growth fitted between the initial and the final foliar dry weight of each flume tank. Foliar dry weight was calculated according to the ratio total FW: foliar FW (1.56 ± 0.10, n= 10) and fresh weight was converted in dry weight according to the ratio of 4:1 g FW/g DW (n= 10).

Table 1. *Zostera noltei.* Morphometric properties and dynamic variables measured or calculated in this study. D_1 and D_2: main rhizome diameters. Subscripts i and f: initial and final conditions, respectively. M_0: meristems activated initially, M_A: meristems activated during experimental period, experimental time (d).

Variables	Units	Description
Morphometric properties		
Aboveground biomass (AG)	g DW plant^{-1}	Leaves dried biomass
Belowground biomass (BG)	g DW plant^{-1}	Rhizome and roots dried biomass
Aboveground/belowground biomass (AG/BG)	Dimensionless	$AG/BG=\frac{AG}{BG}$
Foliar necrosis	Dimensionless (%)	Mean values for all the shoots in an EPU
Leaf abundance (LA)	nº EPU^{-1}	Mean values for all the shoots in an EPU
Leaf length (LL)	cm EPU^{-1}	Mean values for all the shoots in an EPU
Leaf width (LW)	mm EPU^{-1}	Mean values for all the shoots in an EPU
Leaf thickness (LTh)	mm EPU^{-1}	Mean values for all the shoots in an EPU
Leaf cross section (L_{sec})	mm^2 EPU^{-1}	$L_{sec}=\frac{LW}{2}\cdot\frac{LTh}{2}\cdot\pi$
Internode abundance (IA)	nº internodes EPU^{-1}	Mean values in an EPU
Internode length (IL)	cm EPU^{-1}	Mean values in an EPU
Internode cross section (I_{sec})	mm^2 EPU^{-1}	$I_{sec}=\frac{D_1}{2}\cdot\frac{D_2}{2}\cdot\pi$
Root lenght (RL)	cm EPU^{-1}	Mean values for all the shoots in an EPU
Dynamics variables		
Survival	Dimensionless (%)	$SR=\frac{LiveEPU_s}{Initial\ EPU_s}\cdot 100$
Net production (NP)	g FW d^{-1} EPU^{-1}	$NP=\frac{Biomass_f - Biomass_i}{t_f-t_i}$
Shoot appearance rate (SAR)	nº shoots d^{-1} EPU^{-1}	$SAR=\frac{\sum M_A}{\sum M_0}\cdot\frac{1}{(t_f-t_i)}$
Internode appearance rate (IAR)	nº internodes d^{-1} EPU^{-1}	$IAR=\frac{IA_f-IA_i}{t_f-t_i}$

Statistical analysis

We used a nested permutational MANOVA (PERMANOVA) to test the overall effects of the treatments (velocity and light levels) for morphometric and dynamic plant variables (i.e. survival, net growth rate, shoot and internode appearance rates, necrosis, aboveground/belowground biomass, leaf abundance, leaf length, leaf cross section, internode cross section, internode length and root length). The experimental design included one random factor (tank) nested within velocity, and 2 fixed factors: light (two levels, LL and HL) and velocity (3 levels; LV, MV and HV). The multivariate approach was chosen because plant variables were measured in plants coming from the same experimental unit (tank) and because many of the plant variables were likely inter-correlated (Quinn & Keough 2002). Data were normalized (subtract means and divide by standard deviation) to minimize scale differences among morphometric and dynamic variables before analysis, and PERMANOVA was executed using Type III sum of squares on geometric (Euclidean) distances and unrestricted permutation of raw data (Anderson et al. 2008).

Univariate nested PERMANOVA (3 or 4 factorial) was subsequently used to test the effect of the factors on each variable separately as suggested by Quinn & Keough (2002). All factors (light and velocity) were considered fixed except the nested factor (tank), which was assumed like a random factor. For carbon and nitrogen total content, tissue was included like a fixed factor (two levels; leaves and roots/rhizomes). These tests were also conducted using Type III sum of squares on geometric (Euclidean) distances and unrestricted permutation of raw data.

All tests (permutational MANOVA and ANOVA) were carried out using a significance level of α = 0.05. We used the estimation of P values obtained by Monte Carlo sampling (Anderson et al. 2008). To test the interactions among factors, we used pairwise comparisons when significant interaction terms were detected (Anderson et al. 2008). Permanova tests were performed using the software PRIMER v6.1.13 and PERMANOVA+ v1.0.3 statistical package.

Uptake rates from each velocity (n= 4) were plotted against experimental time. The data were fitted by least-squared regression analysis. To test differences in NH_4^+ and PO_3^- uptake rates, a one-way analysis of variance (ANOVA) test was used to check differences among slopes in each velocity treatments. When significant differences were found a post-hoc Tukey test was performed (Zar 1984). Homoscedasticity and normality of the data were checked before conducting ANOVA tests; NH_4^+ and phosphate uptake rates were log-transformed due to detection of heterocedasticity. Data are shown as mean ± standard error (SE). Significance level was set at a probability of 5% (α= 0.05).

RESULTS

Dynamic variables and morphometric properties

Overall, the response of dynamic and morphometric variables was affected by light and velocity treatments separately (Permanova, p<0.001; Table 2), but no significant interactions were detected (Permanova, p= 0.284, Table 2). In addition the nested design showed that the tanks (flumes) had no significant effects within velocity treatments (Permanova, p= 0.893; Table 2). Low light negatively affected all variables, independently of the velocity regime (Table 2). In addition, the negative effect of NH_4^+ was stronger at medium than at low and high velocity regimes (Table 2, Fig. 1 and 2).

Table 2. *Zostera noltei.* Statistical results of the MANOVA and ANOVA analyses examining the effects of light, velocity and flume tank on dynamic and morphometric properties. Significant results (p<0.05) in bold letter.

Variable/factors	*df*	MS	F	p
MANOVA				
Light	1	54.29	0.81	**<0.001**
Velocity	2	27.73	3.28	**<0.001**
Tank	9	8.36	0.81	0.893
Light x Velocity	2	12.21	1.18	0.284
ANOVA				
Survival rate (SR)				
Light	1	6.11	8.59	**0.021**
Velocity	2	3.21	5.71	**0.015**
Tank	9	0.55	0.56	0.831
Light x Velocity	2	0.42	0.59	0.564
Net growth rate (NP)				
Light	1	8.95	5.88	**0.038**
Velocity	2	3.66	4.40	**0.043**
Tank	9	0.83	1.00	0.449
Light x Velocity	2	0.76	0.50	0.619
Shoot appearance rate (SAR)				
Light	1	7.82	28.19	**<0.001**
Velocity	2	3.32	4.49	**0.039**
Tank	9	0.73	0.75	0.665
Light x Velocity	2	0.78	2.96	**0.086**
Internode appearance rate (IAR)				
Light	1	13.49	49.04	**<0.001**
Velocity	2	1.49	1.88	0.203
Tank	9	0.78	0.84	0.577
Light x Velocity	2	1.26	4.78	**0.028**

Table 2 *(continued)*

Variable/factors	*df*	MS	F	p
Necrosis				
Light	1	1.08	1.19	0.288
Velocity	2	2.45	6.14	**0.015**
Tank	9	0.37	0.37	0.941
Light x Velocity	2	2.87	3.46	0.073
Aboveground/belowground biomass (AG/BG)				
Light	1	3.30	8.22	**0.017**
Velocity	2	7.37	4.23	**0.045**
Tank	9	0.78	0.89	0.538
Light x Velocity	2	1.28	1.43	0.294
Leaf abundance (LA)				
Light	1	10.45	32.27	**<0.001**
Velocity	2	7.31	15.20	**0.001**
Tank	9	0.46	0.54	0.841
Light x Velocity	2	1.38	4.39	**0.038**
Leaf length (LL)				
Light	1	0.63	0.35	0.577
Velocity	2	0.23	0.43	0.669
Tank	9	0.52	0.55	0.832
Light x Velocity	2	1.57	0.87	0.445
Leaf cross section (L_{sec})				
Light	1	1.69	0.99	0.342
Velocity	2	1.99	2.80	0.106
Tank	9	0.70	0.77	0.642
Light x Velocity	2	1.15	0.67	0.533
Internode cross section (L_{sec})				
Light	1	1.79	4.42	0.057
Velocity	2	0.90	0.81	0.475
Tank	9	1.11	1.06	0.392
Light x Velocity	2	0.19	0.83	0.831
Internode length (I_{sec})				
Light	1	3.60×10^{-4}	3.45×10^{-4}	0.985
Velocity	2	2.13	2.37	0.142
Tank	9	0.89	0.90	0.518
Light x Velocity	2	0.77	0.74	0.501
Root length (RL)				
Light	1	1.02	0.77	0.406
Velocity	2	0.96	0.82	0.469
Tank	9	1.18	1.23	0.292
Light x Velocity	2	0.32	0.24	0.789

Survival rate (SR), net production (NP) and shoot appearance rate (SAR) were significantly affected by light and velocity but not by their interaction (Table 2). However, internode appearance rate (IAR) was affected by light and by the interaction between light and velocity, but not by velocity conditions alone (Table 2).

Under HL, survival decreased from ca. 75% both at HV and LV to ca. 60% at MV (Fig. 1a). At HL levles, survival declined in comparison to LL in all the velocity treatments (Fig. 1a). The net production of plants under LL was only positive at HV, whereas negative values were recorded in EPUs grown at LV and MV. At HL levels, net production reached its minimum at MV, while higher and similar values were recorded at LV and HV (Fig. 1b).

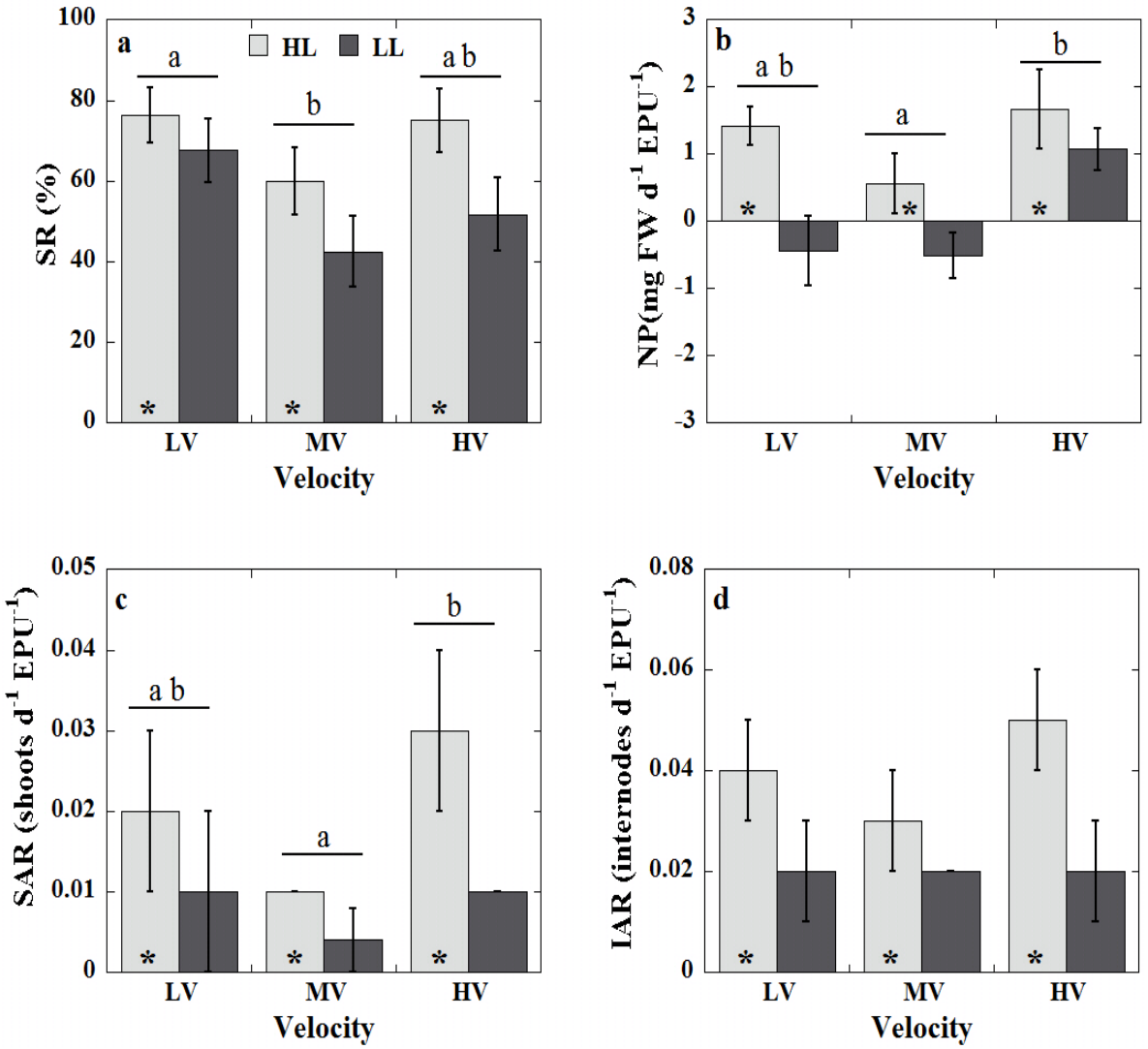


Fig. 1: *Zostera noltei.* Dynamic variables under each velocity and light treatment with a constant NH_4^+ addition (50 µM). (a) Survival rate (SR), (b) net growth production (NP), (c) shoot appearance rate (SAR), (d) internode appearance rate (IAR). Lines with letters indicate significant differences among velocity treatments and asterisks in bars show significant differences between low light (LL) and high light (HL) treatments. LV, MV, HV: low, medium and high velocity, respectively. Data are presented as means ±1 SE.

The shoot appearance rate was affected negatively under LL conditions in all the velocity regimes, reaching the lowest values at MV, meanwhile higher and similar values were recorded at LV and HV treatments (Fig. 1c). Under HL levels, the highest value of shoot appearance rate was found at HV (Fig. 1c).

Internode appearance rate was affected negatively by LL in all the velocity treatments. The reduction was stronger under HV, decreasing from 0.05 to 0.02 internodes d^{-1} EPU^{-1}. However, internode appearance rate was unaffected by velocity both under LL and under HL levels (Fig. 1d).

In contrast to dynamic variables no significant differences were detected in most of the morphometric variables measured. Only necrosis, aboveground/belowground ratio and leaves abundance responded significantly to the treatments (Table 2).

Necrosis was significantly affected by velocity conditions (Table 2), reaching the highest value at MV under both LL and HL conditions (70 and 60% respectively; Fig. 2a). Necrosis was unaffected by light treatments in all the velocity regimes. Aboveground/belowground biomass ratio (AG/BG) was affected by light and velocity treatments (Table 2), but not by their interaction (Table 2). AG/BG ratio was higher in plants cultivated under HL (Fig. 2b) in all the velocity treatments. The lowest value was recorded at MV, being higher at HL than at LL (Fig. 2b).

Leaf abundance was affected by light, velocity and their interaction (Table 2). The number of leaves per EPU was reduced from ca. 5 in LV and HV to 3 in MV at HL levels (Fig. 2c). The effect of LL was stronger in LV and HV, decreasing from ca. 5 to ca. 3 in LV and HV, while similar values were found in MV under LL and HL conditions (Fig. 2c).

However, the rest of the morphometric properties, that is, leaf length, leaf and internode cross sections, internode and root lengths were unaffected by the treatments (Table 2; Fig. 2d, 2e, 2f, 2g, 2h).

Total carbon and nitrogen content

Total carbon and nitrogen content were higher in leaves than in the roots/rhizomes in all the assayed treatments. Leaf carbon content did not show any significant difference among light and velocity treatments, with the mean value being approximately 27% DW (Table 3, Fig. 3a).

In roots/rhizomes total C content showed a slight increase with current velocity at HL (Fig. 3a). Under LL levels, the lowest value of C-content in leaves was recorded in MV (19.5%), while leaves of EPUs cultivated at LV and HV had similar values (21.4 and 22.0%, respectively). On the other hand, total N-content decreased at MV in leaves and roots/rhizomes at both light levels (Fig. 3b).

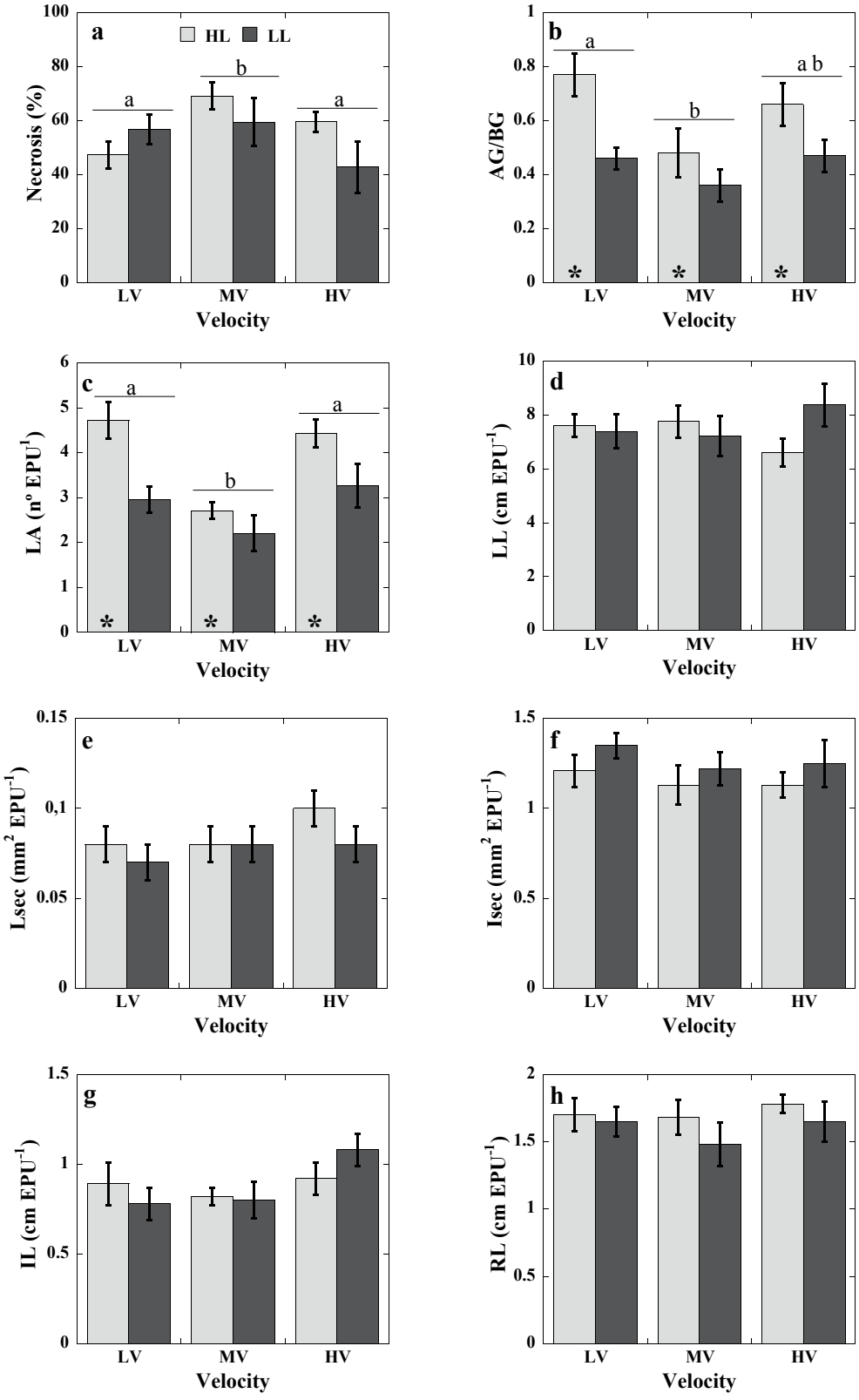


Fig. 2: *Zostera noltei*. Morphometric properties under each velocity and light treatment with a constant NH_4^+ addition (50 μM). (a) Necrosis, (b) aboveground/belowground biomass ratio (AG/BG), (c) leaf abundance (LA), (d) leaf length (LL), (e) leaf cross section (Lsec), (f) internode cross section (Isec), (g) internode length (IL), (h) root length (RL). Lines with letters indicate significant differences among velocity treatments, and asterisks inside bars indicate significant differences between low light (LL) and hihigh light (HL) treatments (low (LL). LV, MV, HV: low, medium and high velocity respectively. Data are presented as means ± 1 SE.

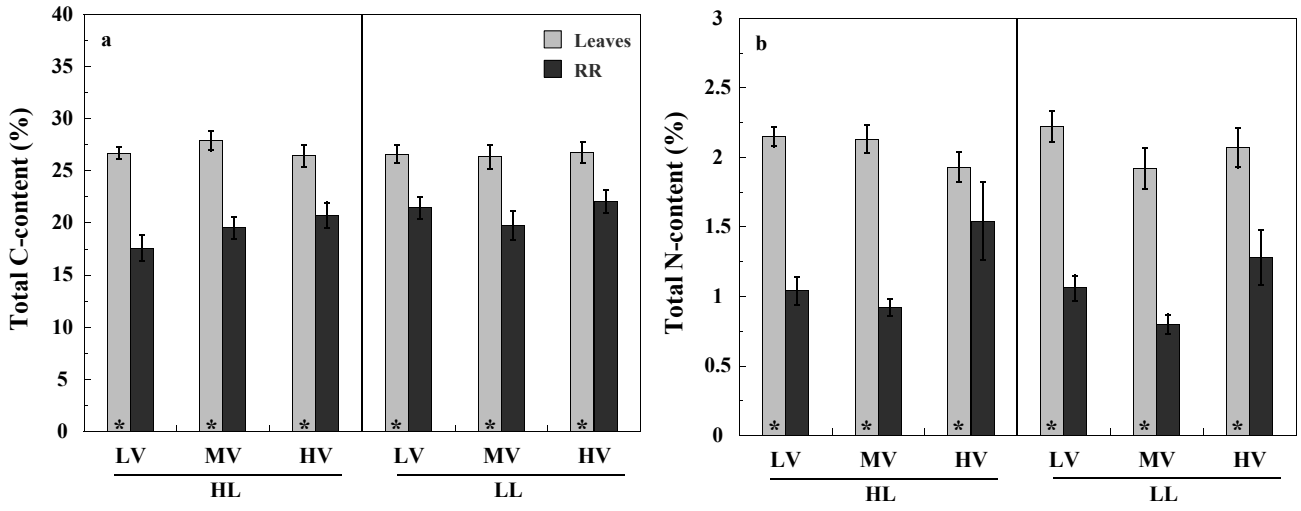


Fig. 3. *Zostera noltei.* Total carbon (a) and nitrogen (b) content in above- (leaves) and belowground (RR) tissues under low light (LL) or high light (HL) and different velocity regimes (LV, MV, HV: low, medium and high velocity respectively). Asterisks inside bars indicate significant differences between above and belowground tissues in each light and velocity treatments. Data are presented as means ± 1 SE.

Table 3. Statistical results of the ANOVA tests examining the effects of light, velocity, flume tank and plant tissue, in total carbon and total nitrogen content in plants of *Zostera noltei*. Significant results ($p<0.05$) in bold letter.

Variable/factors	*df*	MS	F	P
ANOVA				
Total Carbon				
Light	1	1.44	2.64	0.133
Velocity	2	0.30	0.34	0.731
Tissue	1	20.01	116.22	**<0.001**
Tank	9	0.89	1.39	0.206
Light x Velocity	2	0.49	0.89	0.448
Light x Tissue	2	5.95	28.18	**<0.001**
Velocity x Tissue	2	12.72	74.67	**<0.001**
Light x Velocity x Tissue	2	12.35	58.95	**<0.001**
Total Nitrogen				
Light	1	0.77	1.99	0.191
Velocity	2	1.26	1.49	0.275
Tissue	1	33.78	64.93	**<0.001**
Tank	9	0.85	1.66	0.104
Light x Velocity	2	0.29	0.76	0.489
Light x Tissue	2	17.46	34.32	**0.001**
Velocity x Tissue	2	9.11	17.51	**0.001**
Light x Velocity x Tissue	2	11.75	23.10	**<0.001**

Ammonium and phosphate uptake rates

The uptake rates of both nutrients increased with incubation time and velocity treatments (ANOVA, p-value< 0.001 for NH_4^+ and PO_4^{3-} uptake rates; Fig. 4). Uptake rates of NH_4^+ were higher than PO_4^{3-} uptake rates under all velocity conditions. In comparison to LV, the slope of PO_4^{3-} uptake was almost 4 and 16 times higher in MV and HV, respectively. Moreover, the uptake rate for NH_4^+ was ca. 2 fold-higher in MV and 4 fold-higher in HV when compared to LV.

The net uptake ratio of NH_4^+/PO_4^{3-} was unaffected by velocity conditions, (11.17 ± 0.44 μmol NH_4^+/ μmol PO_4^{3-}). This value was similar to the ratio of concentrations supplied throughout the experiment (10 μmol NH_4^+/ μmol PO_4^{3-}).

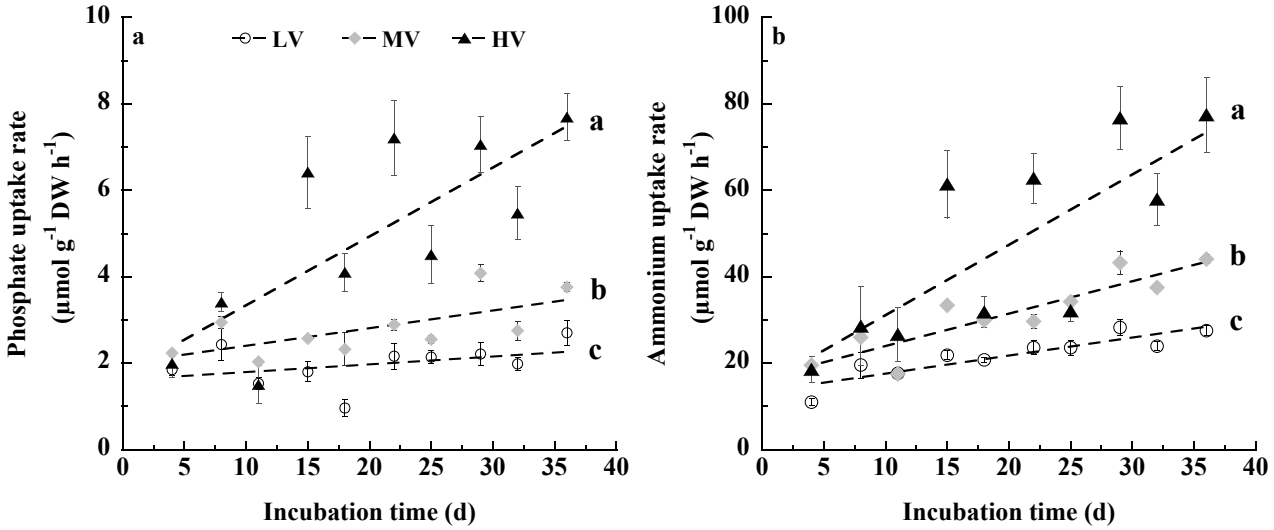


Fig. 4. *Zostera noltei.* Foliar phosphate (a) and ammonium (b) uptake rates (μmol g^{-1} DW h^{-1}) in each velocity condition versus incubation time (36 d). Letters indicate significant differences among velocity treatments (ANOVA, α= 0.05). LV, MV, HV: low, medium and high velocity respectively. Data are presented as means ± 1 SE (n= 4).

Discussion

Our results showed that adverse effects of high NH_4^+ availability were intensified when plants were cultured under moderate current velocities and low light levels. In spite that the effects of multiple factors are often assumed to be additive (Halpern et al. 2007), our results pointed a non-linear response of the seagrass *Zostera noltei* to NH_4^+ enrichment, when it was subjected to contrasting flow velocities and light levels. Such non-linear response is important, as enhanced current velocities and low light levels are factors that will often co-occur under natural conditions.

Previous studies combined flow velocity and NH_4^+ to analyze how flow velocity affects NH_4^+ uptake rates in different seagrass species (Cornelisen & Thomas 2004, Morris et al. 2008, 2013). However, to our knowledge, there are no studies specifically dealing with the effects that such factors may produce in seagrasses at the plant level (e.g. production, growth or survival), in spite that it is well established that high NH_4^+ concentrations could be toxic for these organisms (van Katwijk et al. 1997, Brun et al. 2002, Villazán et al. 2013b). Only one single study showed some indirect evidences of a negative effect in the seagrass *Z. noltei* of high nutrients (including NH_4^+) availability when waves were present (La Nafie et al. 2012).

Adverse effects of NH_4^+ are usually related to its intracellular accumulation (Marschner 1995). Therefore any process increasing NH_4^+ uptake and/or its intracellular accumulation should make seagrasses more prone to NH_4^+ toxicity. In this way, uptake rates of NH_4^+ in seagrasses can be strongly influenced by water flow, being NH_4^+ transfer limited at velocities below 0.3 m s^{-1} (Cornelisen & Thomas 2004). A higher flow velocity reduces the thickness of the diffusive boundary layer, which has been proved to increase the uptake rate of NH_4^+ (Cornelisen & Thomas 2004, Morris et al. 2008). This is consistent with our results, in which increased NH_4^+ uptake rates with water velocity (Fig. 4b) and, as a consequence higher NH_4^+ uptake rates and a major accumulation of intracellular NH_4^+ could be expected when flow velocity increases. Thus, under high NH_4^+ concentrations, NH_4^+ toxicity should increase with flow velocity in seagrasses. However, a non-linear response with enhancing water velocity was recorded, with a stronger toxic effect at medium than at high velocity. This result may be partly explained because of the positive effect that flow velocity has on photosynthetic rates, which may increment carbon skeletons and energy (i.e. ATP) reserves in the plants.

This positive effect can be procured both from a higher inorganic carbon uptake due to the reduction in the thickness of the diffusive boundary layer (Koch 1994), and from the higher light capture due to the hydrodynamic enforced reconfiguration of the shoot (Zimmerman 2003, de lo Santos et al. 2010). In our experiment, at the highest velocity the bending angle could increase so that the leaves were positioned horizontally, while they were almost vertical at lower velocities. Such horizontal arrangement of the leaves might increase light capture, since the projected leaf surface area is larger in HV than in MV and LV conditions, thereby benefiting photosynthesis, as previously demonstrated by de los Santos et al. (2010). As a result, plants growing under HV conditions could be more capable to assimilate NH_4^+ into amino acids than under MV, preventing a toxic intracellular accumulation of this nutrient, since a direct relationship between plant carbon skeletons and energy reserves with NH_4^+ toxicity effects was previously observed in seagrasses (Brun et al. 2002, 2008, Villazán et al. 2013b).

In addition, phosphate uptake rates in our experimental design followed a positive relationship with flow velocity, recording at HV four-fold higher uptake rates than at MV. Since phosphate plays an important role buffering the toxicity of NH_4^+ in *Zostera noltei* plants (Brun et al. 2002, 2008), the higher phosphate uptake rates recorded in those plants subjected to HV conditions could also contribute to the alleviation of the toxic effect recorded under HV.

In previous studies when the same factors were analyzed individually (i.e. flow velocity, light levels and NH_4^+ concentration), *Zostera noltei* plants showed a linear response to increasing levels of such variables (Brun et al. 2008, Peralta et al. 2006, de los Santos et al. 2010). However, our results showed a clear non-linear response, indicating the existence of synergistic and/or negative feedbacks acting at the same time. Thus, this study highlights the importance of untangling the interaction of multiple stressors that can co-occur in nature, as assuming linear additive responses is quite unrealistic (Darling & Côté 2008, Koch et al. 2009), and can yield inaccurate predictions for an effective management of these ecosystems in a scenario of future global change. For instance, NH_4^+ enrichment in coastal areas is expected to increase in the next years (Glibert et al. 2010), and hydrodynamic conditions may change in coastal zones worldwide due to anthropogenic engineering activities changing tidal flows (Kennis 2001) and the increase in storms frequency and waves stress (Young et al. 2011).

An increment in NH_4^+ may stimulate light attenuation as a consequence of the proliferation of phytoplankton, epiphytic microalgae and fast-growing drifting macroalgae (Burkholder et al. 2007). In addition, increased storm frequency and wave stress may also produce a reduction in light conditions by promoting sediment resuspension (Koch 2001). Given this framework of ongoing and expected changes in environmental drivers, it is utmost important to understand how the combined effects of enhanced NH_4^+ concentrations, increased hydrodynamics and reduced light availability may drive future changes in seagrass populations.

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Chapter 3

Elevated ammonium concentration and low light form a dangerous synergy for eelgrass *Zostera marina*

Villazán B, Pedersen MF, Brun FG, Vergara JJ

Marine Ecology Progress Series 493:141-154

¿Piedras en el camino? Las voy guardando

todas para construirme un castillo

Fernando Pessoa

Ilustrated by Carla Villazán Rodríguez

Elevated ammonium concentrations and low light form a dangerous synergy for eelgrass *Zostera marina*

Beatriz Villazán[1], Morten F. Pedersen[2], Fernando G. Brun[1], Juan J. Vergara[1]

[1]Departamento de Biología (Área de Ecología), Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Campus de Excelencia Internacional del Mar (CEIMAR), Puerto Real 11510, Cádiz, Spain

[2]Department of Environmental, Social and Spatial Change (ENSPAC), Roskilde University, PO Box 260, 4000 Roskilde, Denmark

ABSTRACT: We studied the effect of ecologically relevant ammonium concentrations and light on several morphological and physiological properties, nitrogen metabolism and carbon reserves of eelgrass *Zostera marina* L. Eelgrass was grown under mesocosm conditions at 3 levels of ammonium enrichment (target concentrations of 0, 10 and 25 μM) and 2 levels of light (low and high light). High ammonium supply combined with low light had a negative effect on several morphological and physiological response parameters, while no such effects were found when ammonium was supplied under high light. N enrichment caused an increase in the content of total N, intracellular ammonium, free amino acids and residual N in the plants and this response was more pronounced under low-light conditions than under high light. The soluble proteins content decreased, in contrast with external ammonium enrichment. The accumulation of free amino acids and residual N in NH_4^+ enriched plants was followed by a substantial drop in carbohydrate reserves (sucrose and starch), which was larger in plants grown under low-light conditions. Our results indicate that N enrichment increases the demand for C-skeletons and energy, and that photosynthesis cannot supply enough C and energy to cover that demand under low-light conditions. Eelgrass plants exposed to reduced light conditions, for example close to their depth limit or when covered by drift macroalgae, may thus be especially susceptible to enhanced ammonium concentrations. Our study demonstrates that ammonium toxicity may explain why eelgrass and other seagrasses deteriorate under nutrient-rich, low-light conditions.

INTRODUCTION

Seagrasses are the dominant benthic primary producers in many coastal areas and they provide many ecologically and economically important services to marine ecosystems (Costanza et al. 1997, Duarte 2000, Waycott et al. 2009). Seagrass ecosystems have declined worldwide over the last 4 to 5 decades (Orth et al. 2006, Waycott et al. 2009, Short et al. 2011) as a consequence of increasing anthropogenic nutrient loading and subsequent eutrophication (Short et al. 1995, Short & Wyllie-Echevarria 1996, Burkholder et al. 2007). High nutrient availability affects seagrasses in several ways. The major effects are indirectly caused by the proliferation of phytoplankton, epiphytic microalgae and

fast-growing drifting macroalgae promoting light attenuation (Sand-Jensen & Borum 1991, Hernández et al. 1997, Valiela et al. 1997, Hauxwell et al. 2001, McGlathery 2001, Bryars et al. 2011, Lyons et al. 2012) or increasing the sediment organic matter load, which may reduce oxygen levels and increase the risk of anoxia (Greve et al. 2003) and sulfide intrusion into the plants (Holmer & Bondgaard 2001, Borum et al. 2005, Pérez et al. 2007, Olivé et al. 2009). Furthermore, there may be a direct effect of high nutrient availability on seagrasses since exposure to high concentrations of NH_4^+ can be toxic to higher plants (e.g. Marschner 1995, Britto & Kronzucker 2002, Brun et al. 2002, 2008).

A moderate increase in the availability of inorganic nitrogen (<10 μM) may stimulate growth and biomass of seagrasses when these are growing under nutrient limited conditions (e.g. Orth 1977, Alcoverro et al. 1997, Peralta et al. 2003, Invers et al. 2004). However, some studies have shown little or no effect of nutrient enrichment (e.g. Harlin & Thorne-Miller 1981, Dennison et al. 1987, Murray et al. 1992, Pedersen & Borum 1993, Pedersen 1995, Lee & Dunton 2000), most likely because these studies were carried out in areas with relatively high ambient availability of nutrients where the plants under study were nutrient replete. A growing body of evidence suggests that enrichment by inorganic nitrogen (N_i), especially NH_4^+, can have an adverse effect on seagrasses by reducing photosynthesis, growth and survival (e.g. Burkholder et al. 1992, van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011).

Adverse effects of high NH_4^+ concentrations on seagrasses and other higher plants have traditionally been explained by internal accumulation of NH_4^+, which may affect internal pH and enzyme kinetics, uncouple the production of ATP during photosynthesis, increase respiration and reduce the uptake of other cations (e.g. Marschner 1995). Other studies indicate that high NH_4^+ concentrations may cause enhanced ethylene synthesis, increased energy consumption related to active efflux of NH_4^+ and reduced photo-protection (Britto et al. 2001, Britto & Kronzucker 2002). The negative effect of high NH_4^+ availability on plants may also be related to an imbalance in the carbon (C) economy of the plants since accumulation of internal NH_4^+ stimulates the synthesis of amino acids in plants (Marschner 1995). This synthesis requires C-skeletons and energy, which must be provided directly from photosynthesis or be mobilized from C reserves within the plant. Continuous uptake and assimilation of NH_4^+ can therefore drain the C reserves and, thus, compete with other C-demanding or energy consuming metabolic processes.

The aims of this study were to test whether elevated, but ecologically relevant, levels of NH_4^+ affect eelgrass fitness and to study the underlying mechanisms behind this toxicity in terms of N metabolism and the possible consequences for the C reserves in the plant. We cultivated *Zostera marina* plants under 3 different NH_4^+ concentrations (0, 10 and 25 μM) at 2 different light levels (low and high) for 5 wk. We hypothesized that increasing concentrations of NH_4^+ in the growth media would cause increasingly negative effects in *Z. marina*, because C reserves may be drained in order to support the assimilation of NH_4^+. We expected that low light would enforce and high light alleviate the potential negative effects of NH_4^+.

MATERIALS AND METHODS

A 2-factorial culture experiment was conducted from October to November 2011 (ca. 5 wk) to test how NH_4^+ concentrations and light levels affected eelgrass *Zostera marina*. Individual shoots of *Z. marina* were collected from Isefjorden, Denmark, at a depth of 1–2 m in late September 2011. Healthy looking shoots with intact rhizomes (6–9 internodes) were transferred to the laboratory where they were held in aerated water from the sampling site under sub-saturating light (ca. 30 µmol photons m^{-2} s^{-1}) in a 16 h light: 8 h dark cycle at 15°C until used in the experiment (ca. 1 wk). Shoots were first 'standardized' to have 4 (visible) leaves and 4 rhizome internodes (by removing older leaves and internodes) before being used in the experiment. Each of 18 aquaria (volume = 20 l) was filled with ca. 2–3 l of sediment from the sampling site and 15 l of filtered water from the North Sea. The salinity of the seawater was adjusted to 20‰ by dilution with tap water and the temperature was kept constant at 15°C to obtain optimal growth conditions for the plants (Nejrup & Pedersen 2008). The water in the aquaria was aerated to ensure mixing and changed weekly to avoid nutrient limitation and excessive growth of phytoplankton. Light above the aquaria was provided by lamps with halogen spots (12 V, 35 W) in a 16 h light: 8 h dark cycle.

Fourteen eelgrass plants were planted in each of the 18 aquaria, which were then subjected to 3 target concentrations of NH_4^+ (0, 10 and 25 µM; treatments called C, +N and +NN, respectively) and 2 levels of light (26 ± 3 and 70 ± 9 µmol photons m^{-2} s^{-1} PAR; treatments called LL and HL, respectively) with 3 replicate aquaria within each treatment combination. The light intensity provided in the LL treatment was low, but above the light compensation point (I_C) of *Zostera marina*, while that provided in the HL treatment was close to saturating levels (I_K) (Marsh et al. 1986, Olesen & Sand-Jensen 1993).

The water added to the aquaria contained low levels of ammonium (ca. 1 µM) and nitrate (2–3 µM), and ammonium was added to the aquaria (in the +N and +NN treatments) from a NH_4Cl stock solution every day to keep the concentrations as close to the target concentrations as possible. The NH_4^+ addition corresponded to 150 µmol aquaria^{-1} d^{-1} in the +N treatment and 375 µmol aquaria^{-1} d^{-1} in the +NN treatment. The concentration of ammonium was monitored twice weekly in all aquaria. Water samples were collected just before and right after addition of ammonium. The concentrations before adding new ammonium averaged 0.8 ± 0.2 µM in the control treatment, 0.7 ± 0.2 µM in the +N treatment, and 1.2 ± 0.2 µM in the +NN treatment (mean ± SE across 3 replicate aquaria and over 10 sampling dates in each treatment). The concentration of ammonium just after adding ammonium averaged 0.8 ± 0.2 µM in the control treatment, 11.2 ± 0.3 µM in the +N treatment, and 24.7 ± 0.4 µM in the +NN treatment. All water in the aquaria was changed once weekly to prevent accumulation of ammonium (especially in the +NN treatment) and to reduce the risk of limitation by phosphorus or micronutrients.

Physiological and morphological responses

Prior to transplantation into the aquaria, each plant was weighed (initial fresh weight biomass) and marked for measuring leaf elongation rate. At the end of the experiment, all surviving plants were harvested and each plant was weighed (fresh weight, FW) and the number of leaves per shoot was counted. Net production (g FW $plant^{-1}$ d^{-1}) was estimated from the net change in individual plant weights over the course of the experiment while the production of new leaves (plastochrone interval) and leaf elongation rate was measured using the leaf-marking technique (Sand-Jensen 1975). The appearance of new side shoots per original shoot was recorded. Survival rate was estimated from the number of surviving plants in each aquarium at the end of the experiment. Leaf necrosis was quantified as the area with brown-black discoloration of the 3 youngest leaves on each shoot (van Katwijk et al. 1997).

Maximum net photosynthetic rate (P_{max}) and dark respiration were measured as O_2 production or consumption under saturating light conditions (ca. 150 μmol photons m^{-2} s^{-1} PAR) or in darkness. Four randomly chosen eelgrass shoots were collected from each aquarium at the end of the experiment and incubated in a 800 ml gas-tight, transparent chamber equipped with a circulation pump (Aqua Bee, 300 l h^{-1}) used to ensure circulation within the chamber. Two shoots were fixed in each chamber, which was filled with natural seawater without ammonium enrichment (salinity 20‰) having an O_2 concentration corresponding to ca. 70% of air saturation to prevent supersaturation of O_2 in the chamber during incubations. The chamber was finally submerged into a water bath with constant temperature (15°C). The chamber was equipped with a Clark-type O_2 microelectrode (OX-500, Unisense) that was connected to a pico-amperemeter (Picoammeter PA2000, Unisense) and a Pico Technology ADC-16 data logger. A lamp with 8 halogen spots (OSRAM Decostar 51; 12 V, 35 W) illuminated the set-up. The water bath held two replicate chambers at a time. The O_2 concentrations were recorded every minute throughout the incubations and rates of O_2 release or uptake were calculated from periods with constant changes in O_2 concentration over a minimum of 10–15 min.

Biochemical responses

Total C and N

Total C and N content were determined on duplicate freeze-dried, ground samples of leaves and roots/rhizomes from each aquarium using a Carlo-Erba NA-1500 CHNS analyzer.

Intracellular inorganic N

Intracellular concentrations of NH_4^+ and NO_3^- were measured on duplicate leaf and rhizome samples from each aquarium. Samples were rinsed in deionized water and ca. 0.5 g (FW) was ground in 20 ml of boiling deionized water (Dortch et al. 1984). Samples were sonicated for 10 min and then

centrifuged for 20 min at 5000 × g. The concentration of NH_4^+ and NO_3^- was finally measured in the supernatant according to Bower & Holm-Hansen (1980) and Grasshoff et al. (1983).

Free amino acids

Intracellular concentrations of free amino acids (FAA) were measured on duplicate leaf and rhizome samples from each aquarium. Leaves or rhizome internodes were cut from the plants and wiped with a piece of cloth to remove attached epiphytes and debris. Samples were transferred to a 20 ml glass vial with 10 µl 96% ethanol for extraction. The extract was then transferred to a 1.5 ml HPLC vial with 70 µl 10 mM borate buffer at pH 8.8. Primary and secondary amines in the sample were derivatized with 20 µl 10 mM 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Liu et al. 1995) using an AccQ Tag kit (Waters Corp.). The derivatives were heated to 55°C for 10 min to degrade a tyrosine side product that interferes with the chromatographic separation of amino acids. The derivatives were separated on a Waters Alliance 2695 separation module with a 3.9°−150 mm Nova-Pak C-18 column. The solvents used for the separation were (1) 98.9 mM sodium acetate and 6.34 mM triethylenamine at pH 5.70, (2) 98.9 mM sodium acetate and 6.34 mM triethylenamine at pH 6.80, (3) acetonitrile, and (4) water. The separated amino acid derivatives were quantified by fluorescence (250 nm excitation and 395 nm emission) using a Waters 474 scanning fluorescence detector. The detection limit of the method was about 1 pmol of each amino acid. The amount of N bound in FAA was finally estimated using the specific C:N ratio of each of the identified amino acids.

Soluble proteins

The content of soluble proteins was determined on duplicate leaf and rhizome samples from each aquarium using a modification of the Bradford method (Jones et al. 1989). Fresh plant material (ca. 0.1 and 0.5 g for leaf and rhizome samples, respectively) was ground and transferred to a centrifuge tube with 1 ml 0.1 M NaOH (pH 12.8). The mixture was shaken on a vortex mixer and then sonicated for 1−2 min. Samples were left to extract for 30− 60 min at room temperature before shaking once again. Samples were centrifuged for 5 min at 5000 ×g and the supernatant was subsequently transferred to a test tube. Aliquots (0.1 ml) of each sample were mixed with 5 ml of Bradford reagent and soluble polyvinylpyrollidone (concentration: 3 mg PVP ml^{-1} reagent). The absorbance was read using a spectrophotometer at 595 nm after 5 and within 10 min after addition of the reagent. Blanks (aliquots of 0.1 M NaOH) and standards (0.1 ml aliquots of bovine serum albumin dissolved in 0.1 M NaOH) were treated as the samples. The amount of N bound in soluble proteins was finally estimated assuming an average C:N ratio of 6.1:1.

Chlorophyll-bound N

Chlorophyll $_{a+b}$ concentrations were determined on duplicate leaf samples from each aquarium using the method of Wintermans & De Mots (1965). Samples were freeze-dried, ground and extracted overnight in 96% ethanol. The extract was filtered and the chlorophyll concentrations were determined spectrophotometrically at wavelengths of 649, 665 and 750 nm. The amount of chlorophyll-bound N was estimated assuming that N constituted 6.23% of the molar weight of chlorophyll a (Stryer 1981).

Residual N

The amount of N not accounted for by the aforementioned analyses was termed residual N. This pool was likely made up by a mixture of structural proteins, cyclic amino acids and other low molecular weight N compounds, and was estimated as the total amount of N minus the N bound in intracellular NH_4^+, NO_3^-, chlorophyll, FAA and soluble proteins.

Sucrose and starch

The concentrations of sucrose and starch were measured on duplicate leaf and rhizome samples from each aquarium. Samples were freeze-dried and ground prior to analysis. Total non-structural carbohydrates were measured following Brun et al. (2002). Sugars (sucrose and hexoses) were first solubilized by 4 sequential extractions in 96% (v/v) ethanol at 80°C for 15 min. The ethanol extracts were evaporated under a stream of air at 40°C and the residues were then dissolved in 10 ml of deionized water for analysis. Starch was extracted from the ethanol insoluble residue by keeping it for 24 h in 1 N NaOH. The sucrose and starch content of the extracts was determined spectrophotometrically using a resorcinol and anthrone assay with an absorbance of 486 and 640 nm, respectively, with sucrose as a standard.

Statistical treatment

We used 2-factorial (for physiological and morphological response variables) or 3-factorial (for biochemical response variables) permutational MANOVA (PERMANOVA) to test for effects of the treatments (NH_4^+ enrichment, light level and plant part, i.e. leaves and roots/rhizomes) and their interactions. All treatment factors were considered fixed. The multivariate approach was chosen because all response variables were obtained from plants originating from the same experimental unit (aquarium) and because many of the response variables were likely intercorrelated. Data were normalized to minimize scale differences among response variables before analysis and PERMANOVA was executed using Type III sum of squares on geometric (Euclidean) distances and unrestricted permutation of raw data (Anderson et al. 2008).

Univariate permutational ANOVA (2- or 3-factorial) was subsequently used to test the effect of the treatment factors and their interactions on each response variable separately as suggested by Quinn & Keough (2002). These tests were also conducted using Type III sum of squares on geometric (Euclidean) distances and unrestricted permutation of raw data. All tests (permutational MANOVA and ANOVA) were carried out using an α-level of 0.05.

RESULTS

Physiological and morphological properties

The composite response of all physiological and morphological parameters was affected by the interaction between NH_4^+ addition and light (PERMANOVA, $p = 0.007$; Table 1). Enrichment with NH_4^+ affected the composite response variable negatively at low light, but not at high light.

High NH_4^+ levels affected most of the individual response variables negatively under low-light conditions, whereas no clear or even positive effects of NH_4^+ were recorded under high-light conditions. Maximum photosynthetic and respiration rates (Fig. 1a) were not affected significantly by NH_4^+, light or their interaction ($p > 0.05$, Table 1), although P_{max} in plants cultivated in low light tended to decrease with increasing NH_4^+ loading and the opposite trend was recorded in plants cultivated in high light.

Net production (i.e. net changes in plant biomass) was affected by the interaction between NH_4^+ and light (Fig. 1b, Table 1): NH_4^+ enrichment caused a marked reduction in net production at low light, decreasing from ca. 15 mg FW $shoot^{-1}$ d^{-1} in the control to almost −10 mg FW $shoot^{-1}$ d^{-1} under the highest NH_4^+ loading. NH_4^+ enrichment had, in contrast, no effect on net production under high light conditions (mean across N levels was ca. 22 mg FW $shoot^{-1}$ d^{-1}).

Leaf elongation rate (Fig. 1c) was affected by both NH_4^+ loading and light, but not by their interaction (Table 1). Leaf elongation decreased from 2.6 to 2.1 cm $shoot^{-1}$ d^{-1} with increasing NH_4^+ concentration at low light, but increased from 2.6 to 3.1 cm $shoot^{-1}$ d^{-1} with increasing NH_4^+ concentration at high light. The plastochrone interval (Fig. 1d) was only affected significantly by light (Table 1), being 25–30% higher in plants cultivated under low light than in those exposed to high light. The plastochrone interval tended to increase with increasing NH_4^+ addition in plants held at low light.

The production of side-shoots (Fig. 1e) was affected by the interaction between NH_4^+ and light (Table 1). New side-shoots were produced at a rate of 0.018 $shoot^{-1}$ d^{-1} without NH_4^+ enrichment in low light, but this rate was reduced to 0.007 $shoot^{-1}$ d^{-1} in the high NH_4^+ treatment. In contrast, enrichment with NH_4^+ stimulated the production of new shoots (from 0.012 to 0.022 $shoot^{-1}$ d^{-1}) in high light. Leaf abundance (Fig. 1f) was affected by the interaction between NH_4^+ and light (Table 1): the number of leaves per shoot was reduced from 5.2 in the control to ca. 2.5 at high NH_4^+ addition in low light.

The degree of necrosis (Fig. 1g) was affected by the interaction between NH_4^+ and light (Table 1). In low light, necrosis increased from ca. 0% in the control treatment to more than 40% in the +NN treatment. A similar pattern occurred under high light, although at much lower levels (max. ca. 5%). Survival (Fig. 1h) was unaffected by all treatment factors and remained close to 100% in all the treatment combinations.

Table 1. Statistical results of the MANOVA (composite response) and ANOVA (individual responses) analyses examining the effect of light level and ammonium supply on various morphological and physiological properties of *Zostera marina.* Bold values indicate significant results at p <0.05.

Variable/factors	*df*	MS	F	P
MANOVA				
Ammonium supply (N)	2	11.79	2.30	**0.029**
Light (L)	1	38.88	7.60	**0.002**
L × N	2	14.56	2.84	**0.007**
ANOVA				
Photosynthetic rate (P_{max}):				
Ammonium supply (N)	2	0.217	0.22	0.817
Light (L)	1	0.005	0.01	0.823
L × N	2	2.235	0.23	0.139
Respiration rate:				
Ammonium supply (N)	2	0.560	0.51	0.604
Light (L)	1	0.051	0.05	0.832
L × N	2	1.380	1.27	0.310
Net production (NP):				
Ammonium supply (N)	2	1.363	7.20	**0.008**
Light (L)	1	9.077	47.90	**0.001**
L × N	2	1.461	7.71	**0.005**
Leaf elongation rate (LER):				
Ammonium supply (N)	2	2.77	7.04	**0.010**
Light (L)	1	6.69	17.01	**0.001**
L × N	2	0.024	0.06	0.940
Plastochrone interval (PI)				
Ammonium supply (N)	2	0.658	2.47	0.132
Light (L)	1	11.921	44.74	**0.001**
L × N	2	0.282	1.06	0.388
Side-shoot appearance rate:				
Ammonium supply (N)	2	0.068	0.09	0.935
Light (L)	1	0.551	0.70	0.432
L × N	2	3.402	4.29	**0.031**
Leaf abundance:				
Ammonium supply (N)	2	2.401	15.04	**0.001**
Light (L)	1	6.865	43.02	**0.001**
L × N	2	1.709	10.71	**0.001**
Necrosis:				
Ammonium supply (N)	2	3.285	26.71	**0.001**
Light (L)	1	3.671	29.85	**0.001**
L × N	2	2.641	21.48	**0.001**

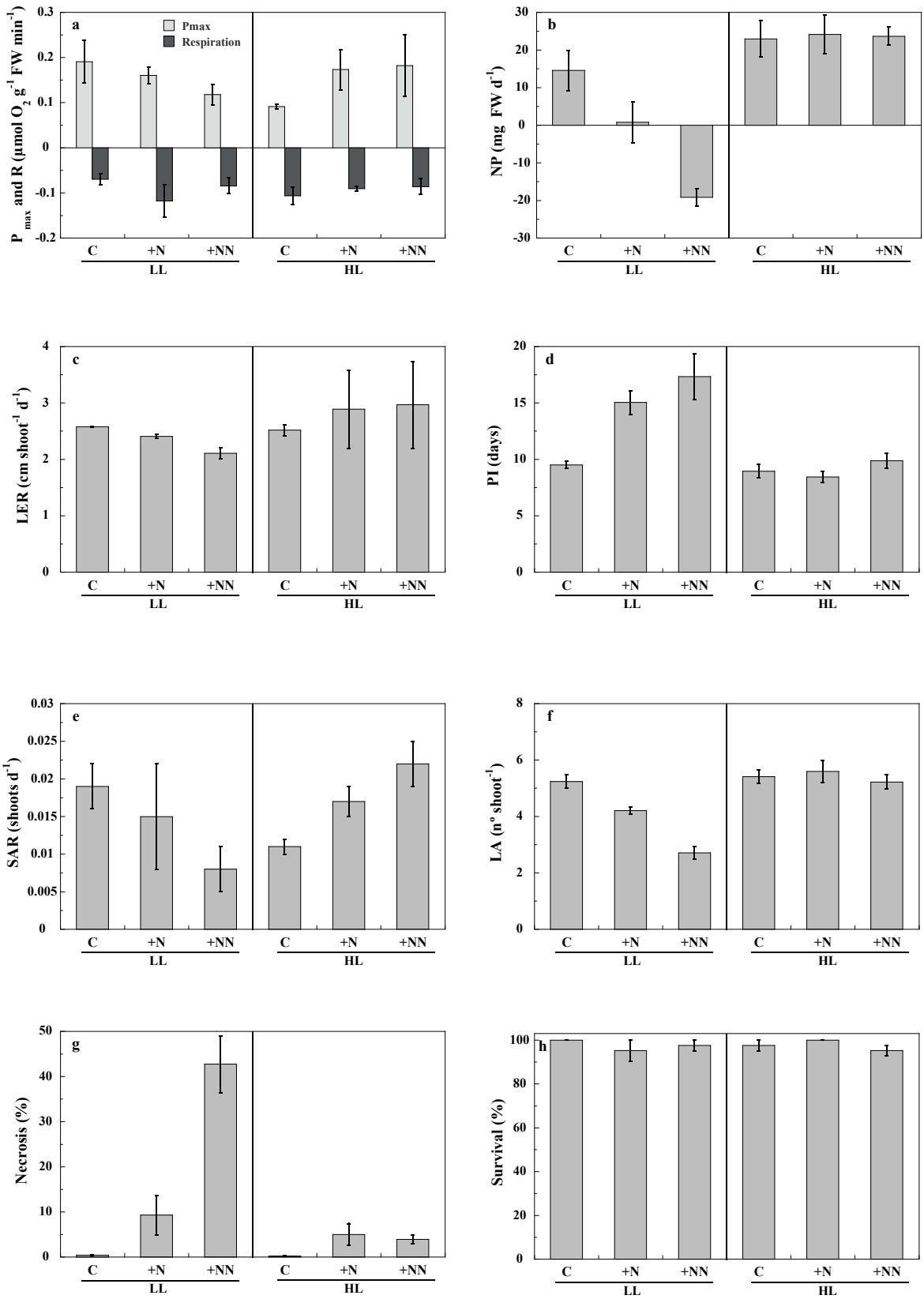

Fig. 1. *Zostera marina.* Dynamics and physiological features of plants under each ammonium and light treatment(means ± SE across 3 replicate aquaria): (a) maximum photosynthetic (P_{max}) and respiration rate (R), (b) leaf elongation rate (LER), (c) plastochrone interval (PI), (d) shoot appearance rate (SAR), (e) leaf abundance (LA), (f) net production (NP), (g) degree of necrosis, and (h)survival rate (SR). C, + N, +NN: 0, 10, 25 µM ammonium concentration, respectively; LL: low light; HL: high light.

N pools

The composite response of all N-related response parameters was affected by light and by the NH_4^+ × tissue interaction (PERMANOVA, p = 0.002 and p = 0.006, respectively; Table 2). The effect of NH_4^+ enrichment was stronger in leaves (all three treatment levels different from each other, p <0.05) than in the roots/rhizomes (C treatment only different from the +NN treatment, p = 0.007). Total N and most of the N species within the plants (i.e. intracellular inorganic N, FAA and residual N) increased substantially with NH_4^+ enrichment, although the content of soluble proteins showed the opposite pattern. All N species were typically more abundant in plants grown under low light than under high light, and levels were also higher in leaves than in the roots/rhizomes.

Total N (Fig. 2a) was affected by plant part and the interaction between NH_4^+ and light (Table 2). Total N was 2-fold higher in leaves than in the roots/rhizomes, and increased about 30–50% with NH_4^+ addition, being ca. 2.5% of DW in the +NN treatment. The relative increase in total N with NH_4^+enrichment was larger in plants cultivated under low light than under high light.

Intracellular NH_4^+ (Fig. 2b) constituted less than 1‰ of total N, but was affected significantly by NH_4^+ enrichment, light and plant part, but not by any of the interactions (Table 2). Intracellular NH_4^+ increased substantially with NH_4^+ enrichment and levels were higher in plants grown in low light than in high light. Leaves contained always more NH_4^+ than the roots/rhizomes.

Intracellular NO_3^- made up less than 1‰ of total N (Fig. 2b) and was only affected by light (p = 0.046); levels were higher in plants cultivated under high light.

Nitrogen bound in free amino acids (FAA-N) made up between 4 and 12% of total N depending on treatment (Fig. 2c). FAA-N was affected by the interactions between NH_4^+ and light and NH_4^+ and plant part (Table 2). FAA-N increased more with NH_4^+ enrichment in the leaves than in the roots/rhizomes and more in low light than in high light.

The amount of N bound in soluble proteins (TSP-N) made up 25–60% of total N (Fig. 2d) and was affected by light and by the interaction between NH_4^+ and plant part (Table 2), but responded quite different than the other N species. TSP-N in leaves decreased markedly with NH_4^+ enrichment, being 30–60% lower in plants from the +NN treatment than in those from the control treatment. TSP-N in the roots/rhizomes was relatively unaffected by NH_4^+ treatment. TSP-N was higher in plants grown in high than in low light.

The amount of N bound in chlorophyll $_{a+b}$ (Chl-N) made up 1–2% of total N in the leaves (Fig. 2e). Chl-N was only affected by the light (Table 2), being ca. 30% higher in plants grown in low light than in high light.

The amount of residual N compounds made up 30–63% of total N depending on treatment and plant part (Fig. 2f). Residual N was affected by the highest order interaction (i.e. NH_4^+ × light × plant part); the amount increased with NH_4^+ enrichment, but more so in the leaves than in the roots/rhizomes and more so in low light than in high light.

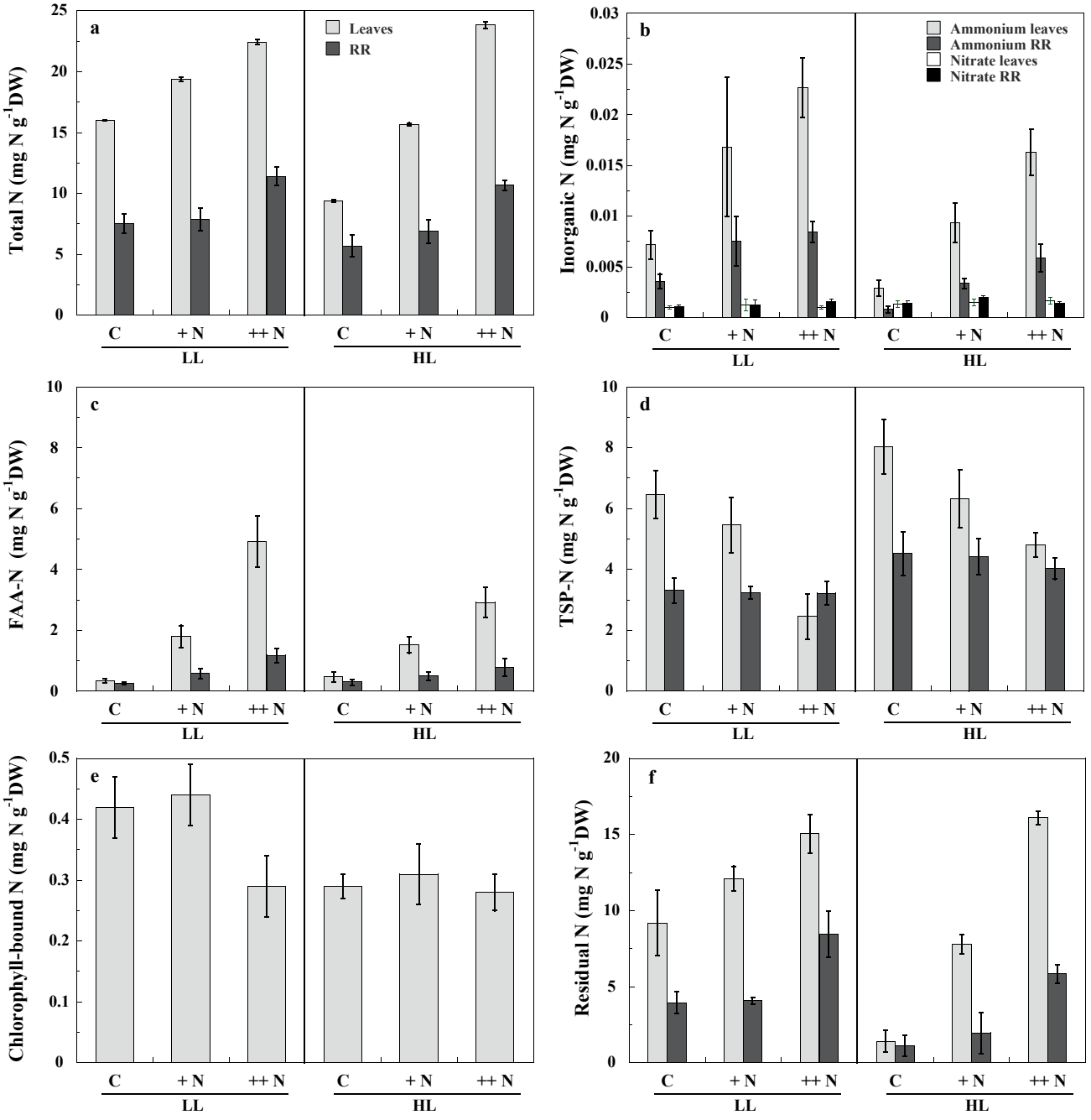

Fig. 2. *Zostera marina.* Nitrogen pools in aboveground (leaves) and belowground (root/rhizomes, RR) tissues under each ammonium and light treatments (means ± SE across 3 replicate aquaria): (a) total nitrogen content, (b) intracellular nitrogen content (ammonium and nitrate), (c) free amino acid nitrogen (FAA-N), (d) total soluble protein nitrogen (TSP-N), (e) chlorophyll-bound N, and (f) residual nitrogen. C, + N, +NN: 0, 10, 25 μM ammonium concentration, respectively; LL: low light; HL: high light.

Table 2. Statistical results of the MANOVA (composite response) and ANOVA (individual responses) analyses examining the effect of light level, ammonium supply and plant tissue on various N pools (total N, ammonium-N, nitrate-N, free amino acid-N, soluble protein-N, chlorophyll-bound N and residual N) in *Zostera marina.* Bold values indicate significant results at $p < 0.05$.

Variable, factors	*df*	MS	F	P
MANOVA				
Ammonium supply (N)	2	27.431	11.63	**0.001**
Light (L)	1	18.181	7.71	**0.002**
Tissue (Ti)	1	86.517	36.69	**0.001**
N × L	2	1.961	0.83	0.509
N × Ti	2	8.809	3.74	**0.006**
L × Ti	1	1.933	0.82	0.441
N × L × Ti	2	5.367	1.14	0.319
ANOVA				
Total N content:				
Ammonium supply (N)	2	4.946	66.10	**0.001**
Light (L)	1	1.315	17.58	**0.001**
Tissue (Ti)	1	19.450	259.96	**0.001**
N × L	2	0.263	3.51	**0.049**
N × Ti	2	0.572	7.65	**0.003**
L × Ti	1	0.084	1.13	0.311
N × L × Ti	2	0.397	5.31	**0.018**
Ammonium content:				
Ammonium supply (N)	2	4.563	9.11	**0.003**
Light (L)	1	2.992	5.98	**0.026**
Tissue (Ti)	1	8.279	16.53	**0.001**
N × L	2	0.053	0.11	0.888
N × Ti	2	1.081	2.16	0.138
L × Ti	1	0.287	0.57	0.471
L × N × Ti	2	0.016	0.03	0.969
Nitrate content:				
Ammonium supply (N)	2	1.032	1.00	0.375
Light (L)	1	4.559	4.42	0.059
Tissue (Ti)	1	1.140	1.10	0.329
N × L	2	0.107	0.10	0.895
N × Ti	2	0.090	0.09	0.925
L × Ti	1	0.099	0.10	0.755
L × N × Ti	2	0.983	0.95	0.424

Table 2 (*continued*)

Variable, factors	df	MS	F	P
Free amino acids:				
Ammonium supply (N)	2	6.834	49.78	**0.001**
Light (L)	1	0.830	6.05	**0.024**
Tissue (Ti)	1	8.789	64.02	**0.001**
N × L	2	0.684	4.98	**0.017**
N × T	2	3.034	22.10	**0.001**
L × Ti	1	0.367	2.68	0.107
L × N × Ti	2	0.307	2.24	0.141
Soluble proteins:				
Ammonium supply (N)	2	3.238	8.39	**0.002**
Light (L)	1	4.448	11.52	**0.002**
Tissue (Ti)	1	8.315	21.54	**0.001**
N × L	2	0.045	0.12	0.883
N × T	2	2.792	7.24	**0.006**
L × Ti	1	0.276	0.72	0.362
L × N × Ti	2	0.274	0.71	0.502
Chorophyll $_{a+b}$				
Ammonium supply (N)	2	0.434	2.41	0.132
Light (L)	1	1.416	7.85	**0.009**
N × L	2	0.277	1.54	0.250
Residual N:				
Ammonium supply (N)	2	6.601	48.14	**0.001**
Light (L)	1	3.329	24.28	**0.001**
Tissue (Ti)	1	10.545	76.91	**0.001**
N × L	2	0.669	4.88	**0.012**
N × T	2	1.024	7.47	**0.003**
L × Ti	1	0.112	0.82	0.391
L × N × Ti	2	0.568	4.14	**0.024**

C pools

The composite response of all C-related response parameters was affected by all main factors, i.e. N treatment, light and plant part (all $p < 0.001$; Table 3), but not by any of the interactions. Total C content (Fig. 3a) averaged 350.6 ± 10.4 and 309.7 ± 8.8 mg C g^{-1} DW in leaves and roots/rhizomes, respectively, and was only affected by plant part (Table 3).

Table 3. Statistical results of the MANOVA (composite response) and ANOVA (individual responses) analyses examining the effect of light level, ammonium supply and plant tissue on various carbon pools (total carbon, sucrose and starch) in *Zostera marina*. Bold values indicate significant results at $p < 0.05$.

Variable, factors	*df*	MS	F	P
MANOVA				
Ammonium supply (N)	2	11.922	8.75	**0.001**
Light (L)	1	9.551	7.01	**0.001**
Tissue (Ti)	1	26.527	19.46	**0.001**
N × L	2	2.901	2.13	0.079
N × T	2	2.045	1.52	0.203
L × Ti	1	0.415	0.30	0.790
L × N × Ti	2	0.996	0.73	0.615
ANOVA				
Total carbon:				
Ammonium supply (N)	2	0.030	0.08	0.916
Light (L)	1	0.320	0.83	0.370
Tissue (Ti)	1	21.725	56.21	**0.001**
N × L	2	0.438	1.13	0.349
N × T	2	1.013	2.62	0.090
L × Ti	1	0.387	1.00	0.308
L × N × Ti	2	0.165	0.43	0.655
Sucrose:				
Ammonium supply (N)	2	7.231	41.18	**0.001**
Light (L)	1	8.274	47.12	**0.001**
Tissue (Ti)	1	4.796	27.31	**0.001**
N × L	2	0.060	0.34	0.736
N × T	2	0.783	4.46	**0.014**
L × Ti	1	0.020	0.11	0.721
L × N × Ti	2	0.774	4.41	**0.026**
Starch:				
Ammonium supply (N)	2	4.661	5.82	**0.007**
Light (L)	1	0.957	1.19	0.304
Tissue (Ti)	1	0.007	0.01	0.939
N × L	2	2.403	3.00	0.073
N × T	2	0.279	0.35	0.719
L × Ti	1	0.008	0.01	0.916
L × N × Ti	2	0.057	0.07	0.920

The concentration of sucrose (Fig. 3b) was affected by the highest order (NH_4^+ × light × plant part) interaction (Table 3). Sucrose decreased substantially with NH_4^+ enrichment and the decrease was largest in low light plants where the content in leaves decreased to ca. 16% of that in plants from the control treatment. The decrease in sucrose content with NH_4^+ enrichment was more pronounced in leaves than in the roots/rhizomes.

The starch content was always one order of magnitude lower than that of sucrose (Fig. 3c). Starch was only affected significantly by NH_4^+ treatment (Table 3). The content of starch was rather similar in leaves and roots/rhizomes and NH_4^+ enrichment caused a significant drop in starch in both plant parts. Plants cultivated under high light had similar contents of starch across NH_4^+ treatments.

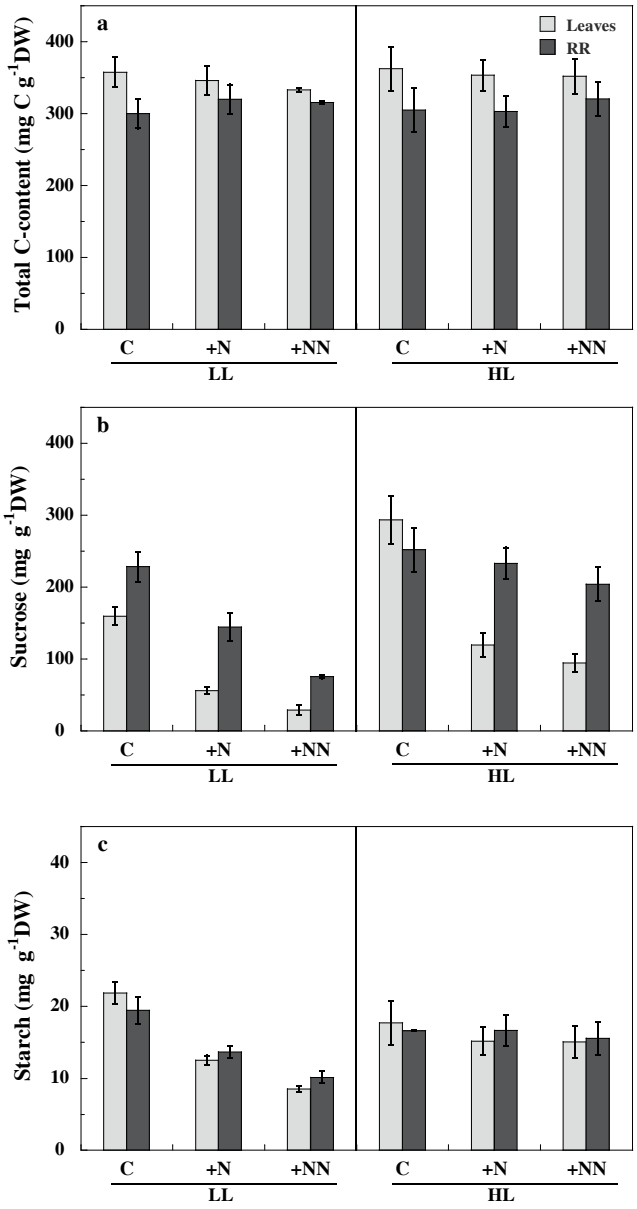

Fig. 3. *Zostera marina.* (a) Carbon content, (b) sucrose and (c) starch concentration in aboveground (leaves) and belowground (roots/rhizomes, RR) tissues under low light (LL) or high light (HL) and different ammonium supply (C, +N, +NN: 0, 10, 25 μM, respectively) as treatments. Data are means ± SE across 3 replicate aquaria.

Ratios of C:N, sucrose-C:total C and sucrose-C:FAA-N were typically higher in the root/rhizomes than in leaves (Table 4). The C:N ratio mainly reflected variations in total N and declined with NH_4^+ enrichment. The sucrose-C:total C ratio mainly reflected changes in the sucrose content and was strongly influenced by NH_4^+ enrichment and light, reaching its lowest values in the +NN treatment under low light. The sucrose-C:FAA-N ratio was affected by NH_4^+ enrichment and light, being lowest at high NH_4^+-enrichment combined with low light.

Table 4. *Zostera marina.* C:N, sucrose-C:total C and sucrose-C:FAA-N ratios under each ammonium and light treatment in aboveground (leaves) and belowground (roots/rhizomes, RR) tissues (mean ± SE). C, + N, +NN: 0, 10, 25 μM, respectively; LL: low light; HL: high light.

Treatments		C:N (mg C/ mg N)		Sucrose:C (%)		Sucrose:N-FAA (mg C/ mg N)	
Light	NH_4^+	Leaves	RR	Leaves	RR	Leaves	RR
LL	C	26.6 ± 2.7	46.6 ± 1.0	18.8 ± 1.4	32.0 ± 1.5	202.0 ± 37	370.3 ± 37.9
	+N	20.8 ± 0.3	47.3 ± 1.1	6.8 ± 0.7	18.9 ± 0.9	14.0 ± 2.6	122.8 ± 37.3
	+NN	17.4 ± 1.0	28.6 ± 3.9	3.7 ± 0.8	10.8 ± 0.6	2.9 ± 1.1	29.2 ± 2.9
HL	C	45.4 ± 2.4	62.6 ± 2.7	34.0 ± 3.4	33.1 ± 5.5	282.7 ± 68.1	448.6 ± 229.9
	+N	26.4 ±1.3	52.1 ± 3.1	14.2 ± 2.0	29.6 ± 1.8	34.2 ± 7.1	205.3 ± 59.7
	+NN	17.3 ± 0.6	36.0 ± 4.0	11.4 ± 1.7	27.3 ± 4.1	13.6 ± 0.3	135.5 ± 37.3

DISCUSSION

Our study demonstrated that relatively high, but ecologically relevant, concentrations of NH_4^+ (i.e. in the range of 0−10 and 0−25 μM) in the water had significant negative effects on the composite and on several individual physiological responses that represented plant fitness. Exposure to 10 and 25 μM NH_4^+ for 5 wk lead to leaf necrosis, and slowed down the leaf growth rate, the production of side-shoots, the leaf abundance and the net growth rate, but did not affect photosynthesis, respiration, plastochrone interval or survival. The adverse effects of NH_4^+ were intensified when plants were cultured under relatively low light.

Toxic effects of high NH_4^+ concentrations are well studied among terrestrial plants, including crop plants (Britto & Kronzucker 2002). High water concentrations of NH_4^+ can stimulate leaf necrosis and reduce the photosynthetic performance, leaf elongation rate, shoot size, biomass and survival in several seagrass species (e.g. van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008). The negative responses reported in these studies show a great deal of variability depending on the experimental set-up (i.e. applied N concentrations, pulsed versus constant enrichment, duration) and seagrass species involved. Most of these studies have, however, exposed plants to rather high concentrations of inorganic N, e.g. 100−200 μM NH_4 (van Katwijk et al. 1997, Brun et al. 2002, van der

Heide et al. 2008, Christianen et al. 2011). Dissolved inorganic N concentrations undergo considerable seasonal variations in eutrophic estuaries, but rarely exceed 100–150 μM. A review on nutrient concentrations in 33 Danish estuaries (all considered eutrophic) revealed that the average (across estuaries) concentration of inorganic N ranges from ca. 100 μM in winter (October to March) to a few μM in summer and that the bulk of this nitrogen is in the form of NO_3^-, whereas NH_4^+ typically makes up less than 10–20% of the total inorganic N (Conley et al. 2000). Only 2 studies have so far investigated the effect of lower and more ecologically relevant NH_4^+ concentrations. Brun et al. (2002) found that leaf-elongation, plastochrone interval and net plant growth in *Zostera noltei* were affected negatively when exposed to a constant concentration of 16 μM NH_4^+, while Brun et al. (2008) reported that ca. 15 μM NH_4^+ had a negative effect on net shoot growth and photosynthetic performance (F_v/F_m) in *Z. noltei.* Brun et al. (2008) further documented that the adverse effect of elevated NH_4^+ was correlated to a reduction in sucrose within the plants and that the negative effects of NH_4^+ were alleviated by high light. These results indicate that the adverse effect of NH_4^+ may be related to in creased competition for C-skeletons between NH_4^+ assimilation and other metabolic processes (Brun et al. 2008). Uptake of NH_4^+ by seagrasses depends on the external concentration in the medium (Thursby & Harlin 1982, Rubio et al. 2007, Villazán et al. 2013a) and may be passive at high concentrations where low-affinity systems tend to operate (Britto & Kronzucker 2002). In order to avoid intracellular accumulation of toxic levels of ammonium, this compound is quickly assimilated into amino acids, which are used for the synthesis of proteins or stored if the assimilation of inorganic N exceeds the requirements needed for growth (e.g. Marschner 1995).

Five weeks of NH_4^+ enrichment led to a doubling of total N in the plants. All investigated N pools (with the exception of NO_3^- and soluble proteins) increased in response to NH_4^+ enrichment. Intracellular NH_4^+ increased almost 4-fold, but made up less than 1‰ of total N in all treatments, suggesting rapid assimilation or an active efflux of NH_4^+ (Britto & Kronzucker, 2002). Rapid assimilation seems most feasible since the amount of FAA increased almost 7-fold in the +NN treatment relative to that in the control treatment. The amount of N bound in the residual N pool, i.e. aromatic and structural amino acids, structural proteins and other N compounds not accounted for in the chemical analyses, increased by a factor of 3. The pools of FAA-N and residual N were both rather large, making up 12.5% and 62.5% of total N in the +NN treatment, respectively. The large size and substantial increase of these N-pools during N-enrichment indicate that these N-compounds constitute the major storage compounds in eelgrass. Rapid assimilation and synthesis of amino acids and other N compounds were able to keep intracellular concentrations of NH_4^+ low in our plants despite a relatively high external concentration in the medium.

Soluble proteins decreased by almost 50% with increasing N enrichment, which was somewhat unexpected given the increase in total FAA and total N. Similar patterns have been observed in terrestrial plants exposed to high NH_4^+ concentrations and it has been suggested that high NH_4^+ availability either causes a higher turnover rate of proteins, or that energy and C-skeletons are diverted from protein synthesis to NH_4^+ assimilation (e.g. Domínguez-Valdivia et al. 2008). This would explain why the

concentration of soluble proteins was inversely related to the concentration of FAA. It would also explain why concentrations of soluble proteins were higher while concentrations of FAA were lower in high-light plants where more C and energy derived from photosynthesis were available.

Sustained synthesis and storage of amino acids may constitute a problem for seagrasses under low light conditions since these processes require C-skeletons and energy, both of which must be provided from photosynthesis or through mobilization of C reserves. Amino acids have C:N ratios ranging from 6:1 to 5:3, which means that 6 to 1.7 mol C are required for each mol N assimilated. Extended periods with high DIN availability and low light may therefore lead to competition between N assimilation and other metabolic processes for C and energy.

Ammonium enrichment caused the concentration of sucrose in the leaves to drop 68 and 84% (in high and low light, respectively) over the course of the experiment, whereas the concentrations in the roots/rhizomes decreased by 19 and 67%. The starch concentration in the leaves was also reduced, although less than sucrose (15 and 61% for high and low light plants, respectively). Because enrichment with NH_4^+ did not affect net photosynthesis and respiration significantly, the drop in sucrose and starch cannot be explained by a lower net gain of inorganic C in plants enriched with NH_4^+. We suggest that the depletion in sucrose and starch resulted from mobilization of C reserves to cover the demands related to enhanced assimilation of NH_4^+. A simple mass balance shows that this is indeed possible. The net uptake of NH_4^+-N over 35 d in the +N and high light treatment amounted to ca. 250 μmol N $plant^{-1}$ (taking growth and changes in total N into account). If all that NH_4^+-N was assimilated it would correspond to a C requirementof ca. 625 μmol C $plant^{-1}$ assuming that glutamine (having a C:N ratio of 5:2) was the major amino acid being synthesized. Using the observed rates for photosynthesis and respiration (Fig. 1a), net photosynthesis should yield ca. 622 μmol C $plant^{-1}$ over 35 d (using a 16 h light: 8 h dark cycle), while mobilization of the sucrose and starch could provide 112 μmol C $plant^{-1}$. Photosynthesis and mobilization of C could thus cover the C demand needed for assimilation of the acquired N. A similar estimate for plants in the +NN, high light treatment shows that photosynthesis and mobilization together could provide ca. 1070 of the 1088 μmol C $plant^{-1}$ needed for assimilation of the acquired N.

We were unable to carry out the same sort of estimate for plants grown under low light and N enrichment due to the large amount of biomass lost by these plants over the course of the experiment. However, these plants were exposed to a light level close to their compensation irradiance and nearly all the C needed for N assimilation must therefore have been provided from mobilization of sucrose and starch. A larger importance of sucrose and starch mobilization in low light plants is indicated from the larger drop in both these compounds compared with the high light plants. Thus, all the metabolic and catabolic processes in plants grown under low light and elevated NH_4^+ concentrations may have undergone tougher competition for C-skeletons and energy, which may have affected growth and fitness of the plants. This hypothesis is supported by studies where addition of α-ketoglutarate (i.e. C-skeletons) to N-enriched plants can stimulate N assimilation and the synthesis of amino acids (e.g. Magalhaes et al. 1992).

We found that that high, but ecologically relevant, concentrations of NH_4^+ can have an adverse effect on *Zostera marina*, especially under low light conditions. Several measures for growth, but not survival, were affected negatively by the combination of elevated NH_4^+ concentrations and low light. Our experiment lasted only for 5 wk, but the sucrose reserves were almost completely depleted in low light plants by the end of the experiment. We suggest that continued exposure to these conditions would have reduced survival substantially. The most vulnerable plants will therefore be those living in deeper waters close to their depth limit or those shaded by phytoplankton, epiphytes or drifting macroalgae

Light attenuation in the water column is the main predictor of eelgrass depth limits, but studies on the relationship between Secchi depth, light attenuation and seagrass depth limits often tend to overestimate predicted depth limits in eutrophic areas with a high turbidity (Duarte et al. 2007). Krause-Jensen et al. (2011) showed that sediment characteristics such as a high content of organic matter, total N, total P and hydrogen sulphide could partly explain why observed depth limits of eelgrass were lower than predicted in Danish coastal waters. Elevated concentrations of NH_4^+ near the bottom may also explain why the depth limits are lower than predicted from the light environment alone. Although the concentrations of NH_4^+ in the water column are typically low (<2 μM) during summer, little is known about the concentrations in the bottom water close to the sediment. Fast decomposition of sediment organic matter and anoxia may stimulate the release of sediment NH_4^+ into the water during summer. Conley et al. (2007) showed that the net flux of NH_4^+ from sediment to the bottom water could reach ca. 300 μmol m^{-2} h^{-1} during mid-summer in shallow Skive Fjord (Denmark). This efflux caused the concentration of NH_4^+ in the bottom water to increase from <5 μM to 50–100 μM for 1 mo, while no increase was detected in the surface waters. The NH_4^+ concentration in the bottom water surrounding eelgrass plants may thus be significantly higher than indicated from water samples taken further up in the water column, and they may reach concentrations at which the performance of eelgrass is affected.

Coastal eutrophication is often followed by accumulation of drifting macroalgae that may cover entire seagrass meadows (e.g. Rasmussen et al. 2013). Mass accumulation of macroalgae in seagrass meadows typically occurs in summer and may impair light availability, but it also may cause an increase in the concentrations of NH_4^+ within and below the mat. Field studies by Bierzychudek et al. (1993) and Hauxwell et al. (2001) demonstrated that the NH_4^+ concentration increased from a few μM in the water above the algal mats to more than 100 μM at the bottom of mats with a thickness of 20–30 cm. Similar results have been obtained in laboratory experiments using mats of the green alga *Chaetomorpha linum* (e.g. Krause Jensen et al. 1999, McGlathery et al. 1997). These studies show that seagrasses can be exposed to conditions of low light and very high NH_4^+ concentrations in summer when more optimal conditions (i.e. high insolation and low NH_4^+ concentration) otherwise are expected. Whether algal mats may cause a serious impact on the seagrasses may to a large extent depend on the duration of the algal cover.

In summary, high, but ecologically relevant NH_4^+ concentrations had a negative effect on eelgrass performance. Net photosynthesis was not affected by NH_4^+ enrichment, but other measures of growth were affected negatively by elevated NH_4^+ concentrations. The negative effects were much more apparent in plants cultivated under low light than under high light and the adverse effects were correlated to a substantial decrease in sucrose and starch reserves. The negative effect of elevated NH_4^+ concentrations on eelgrass thus seems to be related to an imbalance in the C economy of the plant.

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Chapter 4

Low salinity amplifies the adverse effect of high ammonium availability on estuarine eelgrass, *Zostera marina*

Villazán B, Salo T, Brun FG, Vergara JJ, Pedersen MF

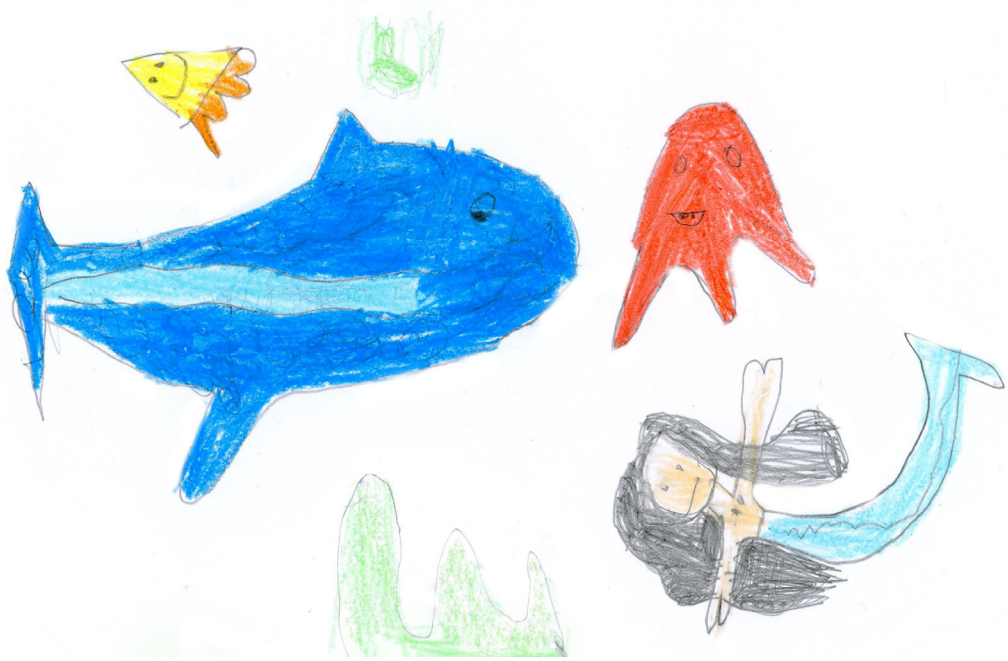
In preparation

Dear ocean,

thank you for making us feel tiny,

humble, inspired and salty… all at once

Anonymous



Ilustrated by Laura Villazán Gamonal

Low salinity amplifies the adverse effect of high ammonium availability on estuarine eelgrass, *Zostera marina*

Beatriz Villazán[1], Tiina Salo[2, 3], Fernando G. Brun[1], Juan J. Vergara[1]. Morten F. Pedersen[2]

[1]Departamento de Biología, Área de Ecología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain

[2]Department of Environmental, Social and Spatial change (ENSPAC), Roskilde University, PO Box 260, DK-4000, Roskilde, Denmark

[3]Department of Biosciences, Åbo Akademi University, Artillerigatan 6, 20520 Åbo, Finland

ABSTRACT: Eelgrass often inhabits estuarine systems affected by freshwaters inputs, which may lead to increased nitrogen load and decreased salinity. The interactive effects of ammonium loading and low salinity were studied on eelgrass (*Zostera marina*). Plants were grown for 4 weeks under mesocosm conditions at 3 levels of ammonium enrichment (0, 10 and 25 μM) and 3 levels of salinity (5, 12.5 and 20) and in all possible combinations of these. Our results pointed out that high ammonium loading and hyposalinity had negative and synergistic effects on eelgrass performance. High ammonium supply combined with low salinity affected morphological and physiological responses negatively, while ammonium had positive effects on eelgrass performance at ambient salinity (20). Decreased salinity had negative effects on chlorophyll content, chlorophyll fluorescence, net photosynthesis, growth, plant size, survival, and C-reserves (sugar and starch), while both ammonium enrichment and low salinity led to increase in total nitrogen and intracellular ammonium concentration. The results indicate that while ammonium enrichment increases demands for C-skeletons and energy, hyposalinity decreases production, leading to a situation where photosynthesis cannot supply enough C and energy to meet the higher demand, leading to increased stress effects. The results emphasize that the expected future changes in precipitation and ammonium loading in coastal areas due to the climate change are likely to have serious impacts for seagrass ecosystems in the future.

INTRODUCTION

One of the most cosmopolitan seagrass species throughout the world is eelgrass (*Zostera marina*), which is the dominating seagrass in the Northern Hemisphere where it grows at salinities ranging from 5 to 35 (den Hartog 1970). Eelgrass meadows provide a broad range of relevant ecological functions for maintaining healthy estuarine and coastal ecosystems (Duarte 2000, Moore & Short 2006). Estuaries are typically affected by variations from natural (precipitation, rivers and surface run-off) and/or anthropogenic (agricultural run-off and water discharges) freshwater inputs, which may increase

nitrogen loads and decrease salinity (Thorhaugh et al. 2006). In addition, climate change is expected to increase the frequency and intensity of rainfall events in temperate coastal areas (Meier et al. 2012, IPPC 2013), which may promote the discharge of nutrient rich freshwater to estuarine systems (Waycott et al. 2007, Faxneld et al. 2010). Thus, the discharge of low-saline, nutrient rich water will be frequent in coastal zones in the near future (Waycott et al. 2007).

Both processes (increased nutrient loading and altered salinity regimes) may contribute to the loss of seagrass meadows (Rudnick et al. 2005, Kahn & Durako, 2006, Jiang et al. 2013). High nutrient loads may cause accumulation of drift macroalgae, epiphytes and phytoplankton, which may lead to reduced light conditions for seagrasses (Sand-Jensen & Borum 1991, Hernández et al. 1997, McGlathery 2001, Lyons et al. 2012). Nutrient enrichment may also lead to higher fluxes of organic matter into the sediment enhancing the risk of sediment anoxia (Greve et al. 2003) and sulphide toxicity (Holmer & Bondgaard 2001, Borum et al. 2005, Pérez et al. 2007). High concentrations of ammonium (NH_4^+) may finally have a direct toxic effect on seagrasses since accumulation of NH_4^+ within the plants can affect intracellular pH and enzyme kinetics, uncouple the photosynthetic production of ATP, increase respiration rates and reduce the uptake of other ions (e.g. phosphate; Marschner 1995, Britto et al. 2001, van Katwijk et al. 1997, Villazán et al. 2013a). The negative effect of high NH_4^+ availability on plants may be also related to an imbalance in the carbon economy of the plants, since the accumulation of NH_4^+ stimulates the synthesis of amino acids in plants (Brun et al. 2002, 2008 Villazán et al. 2013b).

On the other hand, exposure to low salinity is a stress factor that can alter important physiological processes through a reduction of photosynthetic rates (Touchette 2007), which can influence plant metabolism, growth, development, reproduction and cause increased mortality in seagrasses (Zieman 1975, Irlandi et al. 2002, Kahn & Durako 2006, Chollet et al. 2007, Nejrup & Pedersen 2008). Moreover, elevated energetic cost is required to maintain an adequate internal ionic balance due to changes in intracellular ionic concentrations and to counteract the influx of water associated with hyposaline conditions (Hellebust 1976, Kirst 1989, Karsten 2012).

The potential effects of low salinity and NH_4^+ enrichment on seagrasses are generally well known, but studies on the combined effects of multiple stressors, such as low salinity and high NH_4^+ availability, are an understudied issue. Organisms are often expected to be more sensitive to a stressor if they are affected simultaneously by another stressor (Crain et al. 2008, Darling & Côté 2008). For instance, Khan & Durako (2006) and Jiang et al. (2013) showed that *Thalassia hemprichii* and *T. testudinum* were more negatively affected by NH_4^+ enrichment when salinity was low. The synthesis of amino acids in plants is stimulated under NH_4^+ rich conditions to avoid accumulation of intracellular NH_4^+ (Marschner 1995). This synthesis requires energy, which must be provided directly from photosynthesis or be mobilized from C-reserves within the plant. At the same time, exposure to low salinity may reduce net photosynthesis and increase the demand for energy to restore turgor pressure and the osmotic balance of the cells (Touchette 2007, Karsten 2012). A reduction in salinity could therefore enforce the toxic effect of NH_4^+ in seagrasses by increasing the competition for energy.

The aim of this study was to test if lowered salinity enhances the adverse effects of NH_4^+ enrichment on eelgrass. We cultivated *Zostera marina* plants under a full-factorial design with three different NH_4^+ concentrations (0, 10, 25 μM) and three levels of salinity (5, 12.5, 20) for 4 weeks, using a range of physiological and biochemical response variables (e.g. photosynthesis, chlorophyll content, growth, survival, nutrient uptake rates, tissue nitrogen and NH_4^+, non-structural carbohydrates and content of different ions). We hypothesized that increasing concentrations of NH_4^+ would cause increasingly negative effects in *Z. marina* under increasing hyposaline conditions

MATERIAL AND METHODS

A 4-week 2-factorial aquaria experiment was conducted during October and November 2012 to test how NH_4^+ concentration and salinity affected eelgrass response. Eelgrass shoots were collected from Isefjorden (N 55°42'44, E 011°47'35), Denmark, at a depth of 0.5-1 m at the beginning of October 2012. Healthy looking shoots with intact rhizomes were transferred to the laboratory where they were kept in aerated seawater under sub-saturating light (ca. 30 μmol photons m^{-2} s^{-1}) at 15°C before the experiment (ca. 2 days). Prior to the experiment, shoots were standardized to have 3-4 (visible) leaves and 3 rhizome internodes (by removing older leaves and internodes). 27 aquaria (volume = 20 L) were filled with ca. 2-3 L of sediment from the Isefjord and 15 L of 20 salinity water mixed from seawater (salinity 30) and tapwater with naturally high DIC-levels.

Twelve eelgrass shoots were planted in each of the 27 aquaria, which were then subjected to 3 target concentrations of NH_4^+ (0, 10 and 25 μM; treatments called C, +N and +NN, respectively) and 3 levels of salinity (salinities 5, 12.5, 20) with 3 replicate aquaria in each treatment combination. Prior to the start of the experiment, target levels of salinity in the aquaria were obtained by decreasing the salinity by ca. salinity 3 every second day. Three days after the target levels of salinity were reached, the first pulse of NH_4^+ was added.

The water used in the experiment contained low levels of NH_4^+ (ca. 0.5 μM) and additional NH_4^+ was added to the aquaria (in the +N and +NN treatments) from a NH_4Cl stock-solution every day. The NH_4^+ concentrations were selected to correspond to those in a previous study on NH_4^+ effects on eelgrass (Villazán et al. 2013b). The daily loading of NH_4^+ to the aquaria corresponded to 0 μmol $aquaria^{-1}$ in the control treatment, 150 μmol $aquaria^{-1}$ d^{-1} in the +N treatment and 375 μmol $aquaria^{-1}$ d^{-1} in the +NN treatment. The NH_4^+ concentration was measured twice weekly in all aquaria; water samples were collected before and after nutrient additions and analysed according to Bower & Holm-Hansen (1980). The NH_4^+ concentration before adding a new pulse of NH_4^+ was close to zero in almost all of the treatments (Table 1), although accumulation of NH_4^+ was observed in aquaria from salinity 5 and high N treatment (Table 1).

Table 1. Average ammonium concentrations (μM) in aquarium tanks over the course of the experiment, just before and right after adding new ammonium in each treatment combination (mean ± 1 SE across 3 replicated aquaria and over 8 sampling dates in each treatment).

		NH_4^+ (μM)		
Sampling interval	Salinity	C	+N	+NN
Before adding	20	0.09 ± 0.07	0.42 ± 0.13	0.35 ± 0.12
	12.5	0.39 ± 0.20	0.70 ± 0.29	1.28 ± 0.44
	5	2.58 ± 1.22	4.84 ± 1.41	15.81 ± 3.20
After adding	20	0.35 ± 0.12	0.23 ± 0.22	0.10 ± 0.07
	12.5	9.95 ± 0.48	10.82 ± 0.30	12.52 ± 0.92
	5	25.90 ± 1.45	29.10 ± 1.68	41.62 ± 4.09

DIC concentrations in the water were measured twice (2nd and 4th week) over the course of the experiment in all aquaria by Gran Tritation method. DIC concentrations in the water averaged 3.37 ± 0.06 mM (mean ± SE, across 3 replicate aquaria and over 2 sampling dates in each of the 9 treatments), which was high enough to saturate DIC uptake and photosynthesis in eelgrass (Sand-Jensen & Gordon 1984). Light intensity above the aquaria was close to the saturating level (ca. 80 μmol photons m^{-2} s^{-1}) (Marsh et al. 1986, Olensen & Sand-Jensen 1993) and was provided by lamps with Halogen spots (12V, 35 Watt) in a 16:8 hour light-dark cycle. Water temperature was kept constant at 15°C to obtain optimal growth conditions (Nejrup & Pedersen 2008). About half of the water in each aquarium was changed weekly to reduce the risk of carbon, phosphorous and/or micronutrient limitation, and to prevent the growth of epiphytes.

Physiological and morphological responses

Maximum net photosynthetic rate (P_{max}) and dark respiration was measured as O_2 production or consumption under saturating light and in darkness. By the end of the experiment two randomly chosen eelgrass shoots from each aquarium were incubated in a 800 mL gas tight, transparent chamber equipped with a circulation pump (AquaBee, 300 L h^{-1}) to ensure circulation within the chamber. The chamber was filled with seawater (salinity corresponding to that in the respective aquaria). To prevent O_2 saturation in the chambers during incubations, the O_2 level of the water was reduced to ca. 70% of air saturation by bubbling with N_2. The chamber was finally submerged into a water bath at constant temperature (15°C) and equipped with a Clark-type O_2 microelectrode (model OX-500, Unisense, Denmark) connected to a pico-amperemeter (model Picoammeter PA2000, Unisense, Denmark) and a Pico Technology ADC-16 data logger. A lamp with 8 halogen spots (OSRAM Decostar 51; 12V, 35W) illuminated the set-up (375 μmol photons m^{-2} s^{-1}). The O_2 concentrations were recorded every minute and rates of O_2 release or consumption were calculated from periods with a constant change in O_2

concentration over a minimum of 15 min.

Chlorophyll fluorescence was measured using PAM fluorometry (Hansatech instruments Ltd.). The maximum quantum yield (F_v/F_m) of photosystem II was measured on 3 random plants in each aquaria. Prior to the measurements the plants were dark-adapted for 15 minutes.

At the end of the experiment, all surviving plants were harvested and each plant was weighed (fresh weight, FW) and the number of leaves per shoot was counted. Net production (g FW plant^{-1} d^{-1}) was estimated from the net change in individual plant weights over the course of the experiment.

Leaf elongation rates were measured using the plastochrone interval technique described by Sand-Jensen (1975). The appearance of new internodes (IAR) per original shoot was recorded, and survival rate was determined from the final number of surviving plants in each aquarium at the end of the experiment. Leaf senescence was estimated as the proportion of dark brown or black coloured leaf tissue in the first 3 leaves of each shoot according to van Katwijk et al. (1997).

Nutrient uptake rates

Phosphate (PO_4^{3-}) and ammonium (NH_4^+) uptake rates were measured on whole eelgrass shoots with attached roots and rhizomes by using the perturbation method (Harrison et al. 1989, Pedersen 1994) in the last week of the experimental period. Two plants from each aquarium in each of the 9 treatments (n=3) were randomly selected and introduced in a glass chamber containing 1 L of seawater with the same salinity as in the respective treatment. The chambers were kept at 15°C and the water in each chamber was bubbled with air to ensure water mixing and to reduce the thickness of the leaf boundary layer (Koch 1994).

At the onset of each incubation, NH_4^+ or PO_4^{3-} was added to the chambers from stock solutions (NH_4Cl or KH_2PO_4, respectively) to obtain initial concentrations equal to 25 µM of NH_4^+ or 8 µM of PO_4^3. Triplicate water samples were taken at the beginning and at the end of the incubation (at 0 and 120 min), and the NH_4^+ and PO_4^{3-}samples were analyzed immediately according to Bower & Holm-Hansen (1980) and Murphy & Riley (1962), respectively. At the end of each uptake measurement, plants were weighed (FW) and returned to their aquaria.

Nutrient uptake rates (V, µmol g^{-1} FW h^{-1}) were estimated from changes in nutrient concentration (S) over the course of the incubation using Equation 1, where S_0 and vol_0 are the initial nutrient concentration and water volume, and S_f and vol_f are the final nutrient concentration and water volume, respectively. t is the time elapsed between sampling events and FW is the fresh weight of the plants.

$$V = \frac{(S_0 \times vol_0) - (S_f \times vol_f)}{(t \times FW)}$$

Equation 1

Biochemical responses

Biochemical responses were analyzed at the end of the incubation time. Chlorophyll$_{a+b}$ concentrations were determined on duplicate leaf samples from each aquarium. Samples were freeze-dried, ground and extracted overnight in 96% ethanol; the extractant was homogenized and filtered before determining the chlorophyll concentrations spectrophotometrically (Wintermann & DeMotts (1965).

Total C and N content was determined on duplicate freeze-dried, ground samples of leaves and roots/rhizomes from each aquarium using a Carlo-Erba NA-1500 CNS analyzer.

Inorganic ions (NH_4^+, Na^+, K^+ and Cl^-) were determined in the leaf tissue of one random plant in each aquarium according to Marín-Guirao et al. (2013). Leaves were cleaned (to remove any epiphytes and salts from the leaf surface) using MilliQ water, dried to constant weight at 60°C for 48 h and ground. The plant material was dissolved in 15 ml of 3.5 mM HNO_3, stirred for 30 min and subsequently centrifuged at 2000 g for 5 min. The supernatant was passed through a 0.45 μm filter and the concentrations of NH_4^+, Na^+, K^+ and Cl^- were finally analyzed using an ion chromatograph (Metrohm 850 ProfIC AnCat-MCS) with chemical suppression and conductimetric detection.

The concentrations of sucrose and starch were measured on triplicate, freeze-dried and ground leaf and roots/rhizomes samples from each aquarium. Total non-structural carbohydrates (TNC) were measured following Alcoverro et al. (1999), based on Yemm and Willis (1954). Sugars (sucrose and hexoses) were first solubilized by four sequential extractions in 96% (v/v) ethanol at 80°C for 15 min. The ethanol extracts were evaporated and the residues were dissolved in deionized water for analysis. Starch was extracted from the ethanol-insoluble residues by incubating 24 h in 1N NaOH. Sucrose and starch contents of extracts were determined spectrophotometrically using resorcinol and anthrone assays at 486 and 640 nm, respectively, with sucrose as a standard.

Statistical analysis

We used 2-factorial permutational multivariate analysis of variance to test the overall effect of NH_4^+ enrichment and salinity on physiological and morphological variables (i.e. P_{max}, respiration, F_v/F_m, Chl-content, net production, leaf elongation rate, internode appearance rate, leaf abundance, necrosis and survival) and nutrient uptake rates (PO_4^{3-} and NH_4^+ uptake rates). The multivariate approach was chosen because some of the measured response variables were likely inter-correlated.

To test the effect of the treatment factors on each response variable more specifically, the multivariate analyses were followed by univariate PERMANOVA (2 or 3-factorial), as suggested by Quinn & Keough (2002). For sucrose, starch and total-N we used 3-factorial univariate PERMANOVA including plant parts (i.e. leaves and roots/rhizomes) as a third factor, while all the remaining analyses were conducted using a 2-factorial PERMANOVA. All treatment factors were considered fixed. Data

were normalized to minimize scale differences among response variables before analysis and all the resemblance matrixes were based on Euclidean distances. MDS plots and PERMDISP were used to inspect the dispersion of the data (Anderson et al. 2008). All test were carried out using $\alpha = 0.05$.

RESULTS

Physiological and morphological variables

The multivariate responses of all physiological, morphological variables and nutrient uptake rates were affected by the interaction between NH_4^+ and salinity (Table 2). Enrichment with NH_4^+ affected the multivariate response at salinity 5, but not at 12.5 and 20. High NH_4^+ loading affected most of the individual response variables negatively at salinity 5, whereas there was no clear or even a positive effect of NH_4^+ enrichment at higher salinities.

Table 2. Statistical results of the MANOVA and ANOVA analyses examining the effecst of salinity levels and ammonium supply on physiological and morphological responses. Bold values indicate significant results at $p<0.05$.

Variable, factors	*df*	MS	F	P
MANOVA:				
Ammonium supply (N)	2	25.21	5.22	**0.001**
Salinity (S)	2	68.60	14.20	**0.001**
N × S	4	9.36	1.94	**0.007**
Residual	18	4.83		
ANOVA				
Photosynthetic rate (P_{max}):				
Ammonium supply (N)	2	2.30	5.79	**0.009**
Salinity (S)	2	3.02	7.62	**0.005**
N × S	4	2.05	5.17	**0.006**
Residual	18	0.40		
Respiration rate:				
Ammonium supply (N)	2	0.80	0.72	0.501
Salinity (S)	2	1.09	0.99	0.393
N × S	4	0.60	0.55	0.700
Residual	18	1.10		
Fv/Fm				
Ammonium supply (N)	2	0.11	0.14	0.886
Salinity (S)	2	5.35	6.79	**0.005**
N × S	4	0.22	0.28	0.897
Residual	18	0.79		

Table 2 *(continued)*

Variable, factors	*df*	MS	F	P
Chlorophyll concentration				
Ammonium supply (N)	2	2.40	4.50	**0.028**
Salinity (S)	2	4.71	8.88	**0.002**
N × S	4	0.56	1.07	0.405
Residual	18	0.53		
Net production (NP):				
Ammonium supply (N)	2	0.63	5.35	**0.015**
Salinity (S)	2	9.39	80.32	**<0.001**
N × S	4	0.97	8.28	**<0.001**
Residual	18	0.12		
Leaf elongation rate (LER):				
Ammonium supply (N)	2	1.35	24.80	**<0.001**
Salinity (S)	2	9.86	181.69	**<0.001**
N × S	4	0.65	12.07	**<0.001**
Residual	18	0.05		
Internode appearance rate (IAR):				
Ammonium supply (N)	2	0.09	0.18	0.837
Salinity (S)	2	6.90	14.35	**<0.001**
N × S	4	0.84	1.75	0.179
Residual	18	0.48		
Leaf abundance (LA):				
Ammonium supply (N)	2	0.03	0.18	0.844
Salinity (S)	2	9.88	53.78	**<0.000**
N × S	4	0.72	3.89	**0.008**
Residual	18	0.18		
Necrosis:				
Ammonium supply (N)	2	0.39	3.18	0.067
Salinity (S)	2	10.25	83.79	**<0.000**
N × S	4	0.63	5.12	**0.005**
Residual	18	0.12		
Survival (SR):				
Ammonium supply (N)	2	2.78	5.85	**0.013**
Salinity (S)	2	3.55	7.46	**0.005**
N × S	4	1.19	2.50	0.080
Residual	18	0.48		
NH_4^+ uptake rate:				
Ammonium supply (N)	2	6.32	19.22	**<0.001**
Salinity (S)	2	3.29	10.01	**0.002**
N × S	4	0.21	0.64	0.638
Residual	18	0.33		
PO_4^{3-} uptake rate:				
Ammonium supply (N)	2	8.03	31.80	**<0.001**
Salinity (S)	2	1.29	5.12	**0.018**
N × S	4	0.70	2.79	0.060
Residual	18	4.54		

P_{max} was affected by the interaction between NH_4^+ and salinity (Fig. 1a ; Table 2). P_{max} was unaffected by NH_4^+ at salinity 5 and 12.5, but increased from 0.64 to 2.63 mg O_2 g^{-1} DW h^{-1} with increasing NH_4^+ concentration at salinity 20. Respiration rate was unaffected by the NH_4^+ and salinity treatments (Table 2, Fig. 1a), although rates tended to be higher in all hyposaline treatments (salinity 5). Maximum quantum yield (Fv/Fm; Fig. 1b) was only affected by salinity (Table 2), being lower at salinity 5 than at 12.5 and 20.

$Chlorophyll_{a+b}$ content were affected by ammonium and salinity, but not by their interaction (Fig. 1c; Table 2). A reduction in salinity lead to lower values of $Chlorophyll_{a+b}$. In contrast, higher NH_4^+ loading caused an increment in the $Chlorophyll_{a+b}$ content at salinities 20 and 12.5; however, similar values were observed at salinity 5.

Net production and leaf elongation rates were both affected by the NH_4^+ × salinity interaction (Table 2). Net production at salinity 5 was low and decreased with increasing NH_4^+-load (from 8 in the control to ca. 2 g FW $plant^{-1}$ d^{-1} in the +NN treatment), but increased slightly with increasing NH_4^+-load at salinities 12.5 and 20 (Fig. 1d). Leaf elongation rates followed the same pattern (Fig. 1e); they were reduced by ca. 50% with increasing NH_4^+ at salinity 5, but increased with increasing NH_4^+-loading at salinity 12.5 and 20.

The internode appearance rate was only affected by salinity (Table 2), being lower at salinity 5 than at 12.5 and 20 (Fig. 1f). The leaf abundance was affected by the interaction between NH_4^+ and salinity (Table 2). Plants had less leaves per shoot at salinity 5 and the number decreased with increasing NH_4^+ load. No effect of NH_4^+ was evident at salinities 12.5 and 20 (Fig. 1g).

Necrosis was affected by the interaction between NH_4^+ and salinity (Table 2). Necrosis was more widespread at salinity 5 than at 12.5 and 20, and it increased with increasing NH_4^+-loading at salinity 20 (Fig. h). Survival was affected by NH_4^+-treatment and salinity (Table 2). Survival was close to 100% at salinity 20 across all NH_4^+-treatments, but decreased with increasing NH_4^+-loading at lower salinities (12.5 and 5) (Fig. 1i).

Nutrient uptake rates

Uptake rates of NH_4^+ and PO_4^{3-} were affected by both factors (NH_4^+ and salinity), but not by their interaction (Table 2). Decreasing salinity had a negative effect on NH_4^+ and PO_4^{3-} uptake rates (Fig. 2). The NH_4^+ uptake rates (Fig. 2a) decreased in general with increasing NH_4^+ treatments, being ca. 50% lower in the +NN treatment than in the control one. In contrast, PO_4^{3-} uptake rates (fig. 2b) increased with NH_4^+ enrichment and decreased with decreasing salinity.

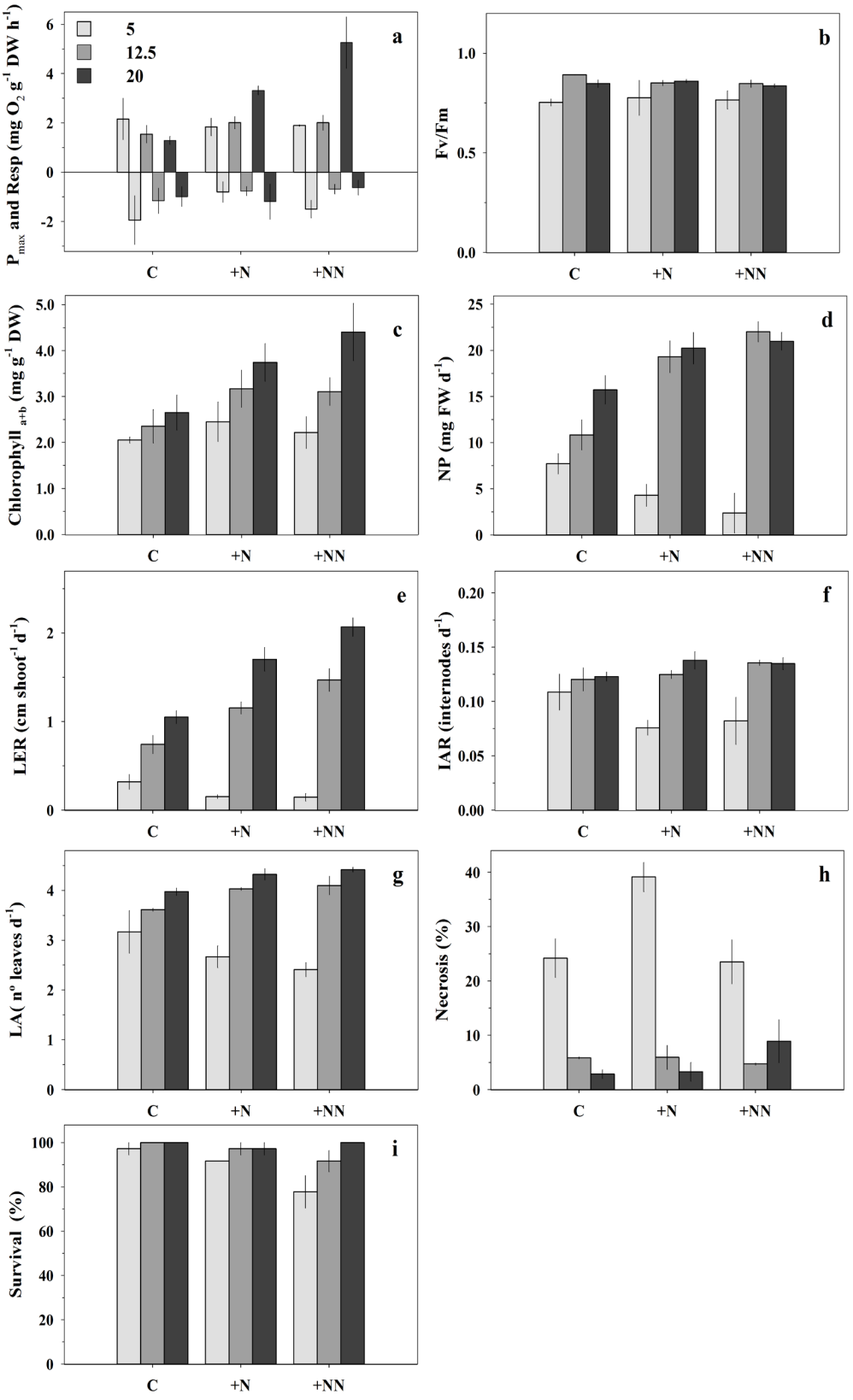


Figure 1. *Zostera marina*. Physiological and morphological variables of eelgrass plants in the ammonium and salinity treatments (a) maximum photosynthetic (P_{max}) and respiration rate (Resp), (b) maximum quantum yield (Fv/Fm), (c) chlorophyll$_{a+b}$ content in leaves. (d) net production (NP), (e) leaf elongation rate (LER), (f) internode appearance rate (IAR), (g) leaf abundance (LA), (h) degree of necrosis (necrosis) and, (i) survival rate (SR). Treatments C, +N and +NN correspond to the target levels of ammonium: 0, 10 and 25 μM, respectively. Data are mean values (across replicated aquaria, n=3) ± 1 SE.

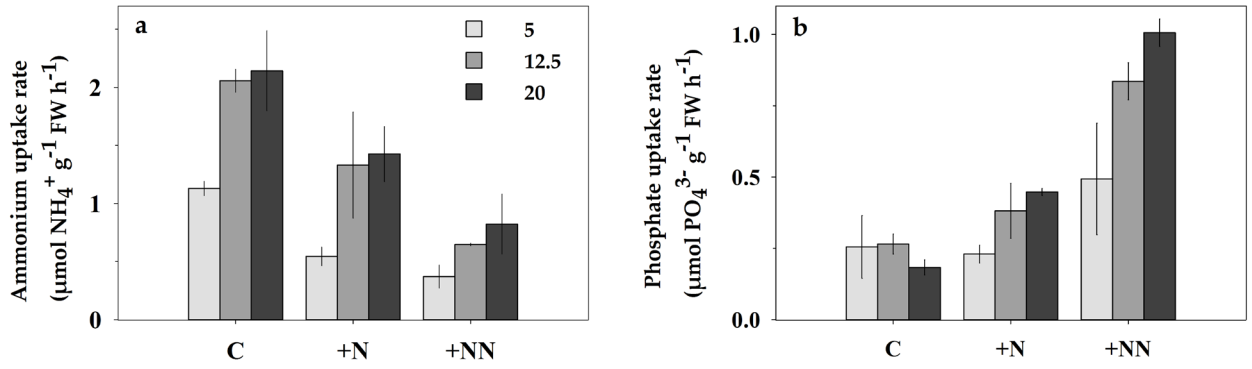


Figure 2. *Zostera marina.* (a) Ammonium (NH_4^+) and (b) phosphate (PO_4^{3-}) uptake rates measured in the last week of the culture experiment on *Zostera marina* plants cultivated under different levels of ammonium availability and salinity. Treatments C, +N and +NN correspond to the target levels of ammonium: 0, 10 and 25 µM, respectively. Data are mean values (across replicated aquaria, n=3) ± 1 SE.

Biochemical responses

Total N was affected by the main factors (NH_4^+, salinity and plant part) and by the interaction between NH_4^+ and tissue (leaves, root/rhizomes) (Table 3). Total-N was 1.5-2 fold-higher in leaves than in roots/rhizomes, but it increased more with NH_4^+ loading in the leaves than in the root/rhizomes. (Fig. 3a). Exposure to salinity 5 led to higher levels of N in all tissues. The concentration of intracellular NH_4^+ was significantly affected by NH_4^+ and salinity, but not by their interaction (Table 3). The concentration of intracellular NH_4^+ increased substantially with NH_4^+-enrichment and levels were higher in plants grown at salinity 5 than at 12.5 and 20 (Fig. 3b).

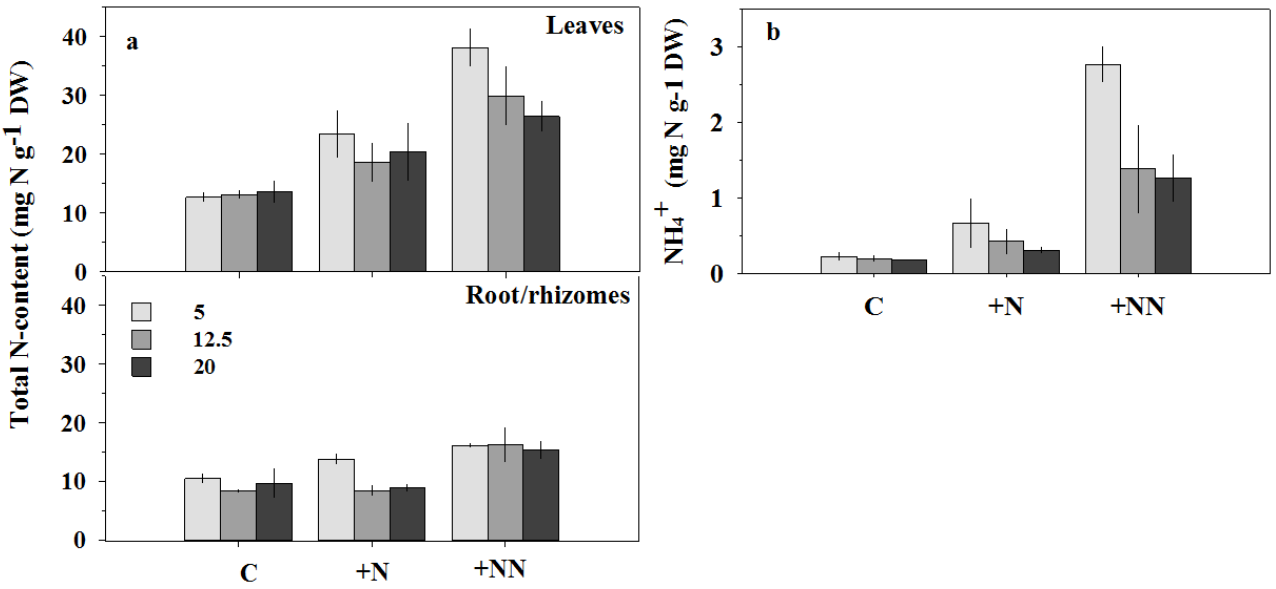


Figure 3. *Zostera marina.* Total nitrogen content and intracellular ammonium in eelgrass plants from the ammonium and salinity treatments: (a) total nitrogen content in aboveground (leaves) and belowground (root/rhizomes, RR) tissues and, (b) intracellular ammonium content in leaves. Treatments C, +N and +NN corresponds to the target levels of ammonium: 0, 10 and 25 µM, respectively. Data are mean values (across replicated aquaria, n=3) ± 1 SE.

Table 3. Results of ANOVA analyses examining the effects of ammonium supply, salinity levels and plant tissues on N pools (total N, ammonium-N) and the internal content of different ions (Na^+, K^+, Cl^-) and K^+:Na^+ molar ratio in leaves of Zostera marina. Bold values indicate significant results at $p<0.05$.

Variable, factors	*df*	MS	F	P
ANOVA:				
N pools:				
Total N content:				
Ammonium supply (N)	2	10.40	50.10	**<0.001**
Salinity (S)	2	1.35	6.50	**0.004**
Tissue (T)	1	14.70	70.86	**<0.001**
N × S	4	0.18	0.84	0.504
N x T	2	2.53	12.21	**<0.001**
S x T	2	0.37	1.78	0.183
N x S x T	4	0.21	1.00	0.413
Residual	36	0.21		
Ammonium content:				
Ammonium supply (N)	2	8.05	31.76	**<0.001**
Salinity (S)	2	1.30	5.12	**0.014**
N × S	4	0.68	2.69	0.061
Residual	18	0.25		
Ions				
Na^+:				
Ammonium supply (N)	2	0.91	10.21	**0.001**
Salinity (S)	2	8.21	91.65	**<0.001**
N × S	4	1.53	17.13	**<0.001**
Residual	18	0.09		
K^+:				
Ammonium supply (N)	2	3.28	14.79	**<0.001**
Salinity (S)	2	1.65	7.42	**0.005**
N × S	4	3.04	13.71	**<0.001**
Residual	18	0.22		
Cl^-:				
Ammonium supply (N)	2	3.46	23.03	**<0.001**
Salinity (S)	2	4.99	33.21	**<0.001**
N × S	4	1.60	10.66	**<0.001**
Residual	18	0.15		
K^+:Na^+:				
Ammonium supply (N)	2	0.93	3.62	**0.030**
Salinity (S)	2	8.89	34.50	**<0.001**
N × S	4	0.43	1.67	0.202
Residual	18	0.26		

The concentrations of Na^+, K^+ and Cl^- were affected by the NH_4^+ × salinity interaction (Fig. 4; Table 3). The concentration of Na^+ (Fig. 4a) and K^+ (Fig. 4b) increased with increasing NH_4^+-loading at salinity 20, were unaffected by NH_4^+ at salinity 12.5 and decreased with increasing NH_4^+-loading at salinity 5. The concentration of Cl^- (Fig. 4c) increased with increasing NH_4^+ at salinity 12.5 and 20, but was unaffected by NH_4^+ treatment at salinity 5. The molar ratio K^+:Na^+ was affected by the two main factors (NH_4^+ and salinity) (Table 3). K^+:Na^+ ratio was lower with decreasing salinity and increased with NH_4^+ enrichment at salinities 12.5 and 20, but remained almost similar at salinity 5 (Fig. 4d).

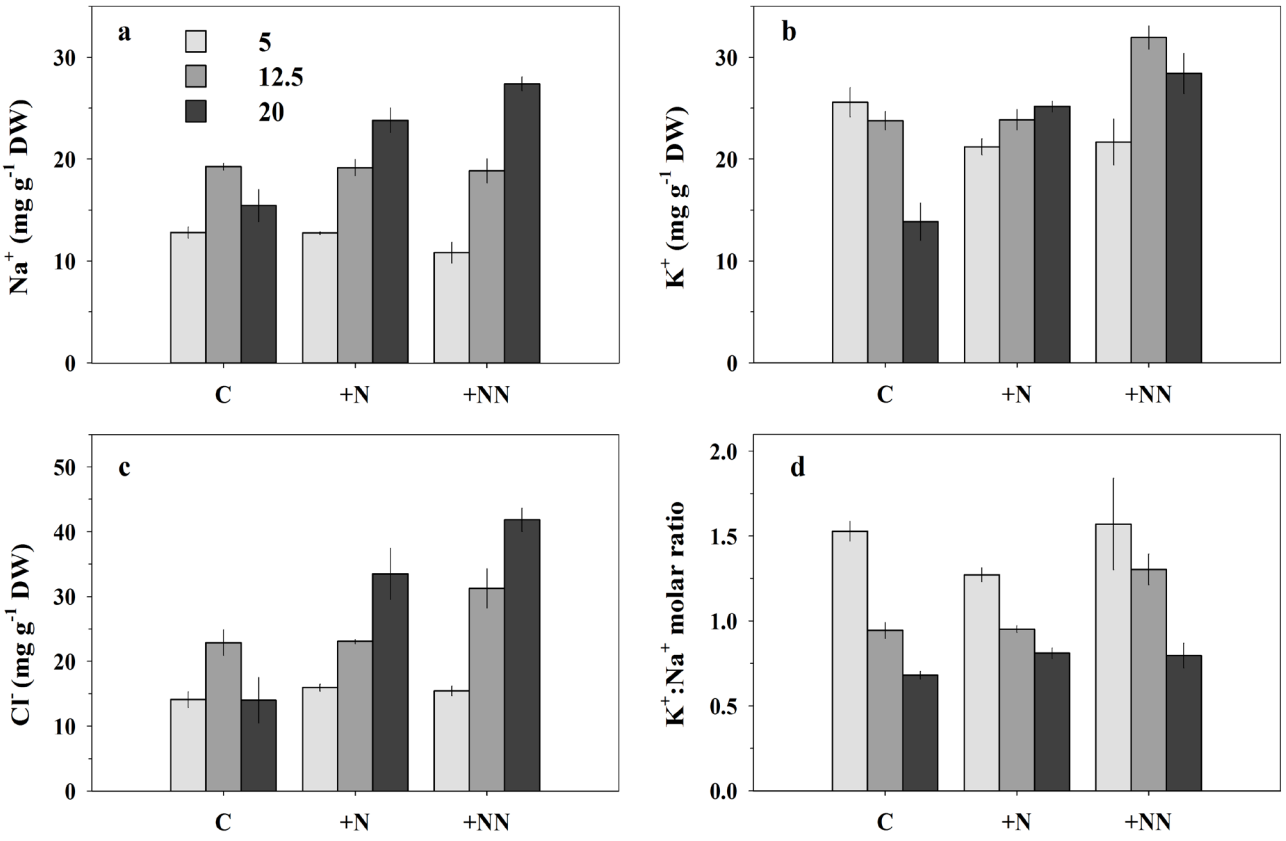


Figure 4. *Zostera marina*. Intracellular concentrations of (a) Na^+, (b) K^+, (c) Cl^- and (d) K^+:Na^+ molar ratio in eelgrass (leaves) cultivated under different levels of ammonium and salinity. Treatments C, +N and +NN corresponds to the target levels of ammonium: 0, 10 and 25 μM, respectively. Data are mean values (across replicated aquaria, n=3) ± 1 SE.

Sucrose concentration was affected by the interactions NH_4^+ × salinity and NH_4^+ × plant part (Table 4). The sucrose content was generally higher in leaves than in the roots/rhizomes and it decreased with decreasing salinity and increasing N loading(Fig. 5a). The starch content was always one order of magnitude lower than the sucrose content (Fig. 5b) and was affected by the interaction between NH_4^+ and plant part (Table 4). The starch content was higher in leaves than in roots/rhizomes and was substantially lower with increasing N-load and decreasing salinity (Fig. 5b).

Table 4. *Zostera marina.* Statistical results of the MANOVA and ANOVA analyses, examining the effects of salinity levels, ammonium supply and plant tissues on total carbon, sucrose and starch concentrations. Bold values indicate significant results at $p<0.05$.

Variable, factors	*df*	MS	F	P
Sucrose:				
Ammonium supply (N)	2	10.29	62.15	**<0.001**
Salinity (S)	2	8.40	50.72	**<0.001**
Tissue (Ti)	1	2.51	15.16	**<0.001**
N × S	4	0.64	3.89	**0.011**
N × T	2	1.57	9.50	**<0.001**
S × Ti	2	0.33	1.98	0.156
S × N × Ti	4	0.20	1.18	0.336
Residual	36	0.17		
Starch:				
Ammonium supply (N)	2	6.12	18.69	**<0.001**
Salinity (S)	2	5.74	17.51	**<0.001**
Tissue (Ti)	1	10.51	32.06	**<0.001**
N × S	4	0.18	0.55	0.701
N × T	2	1.38	4.21	**0.022**
S × Ti	2	1.00	3.06	0.056
S × N × Ti	4	0.37	1.13	0.366
Residual	36	0.33		

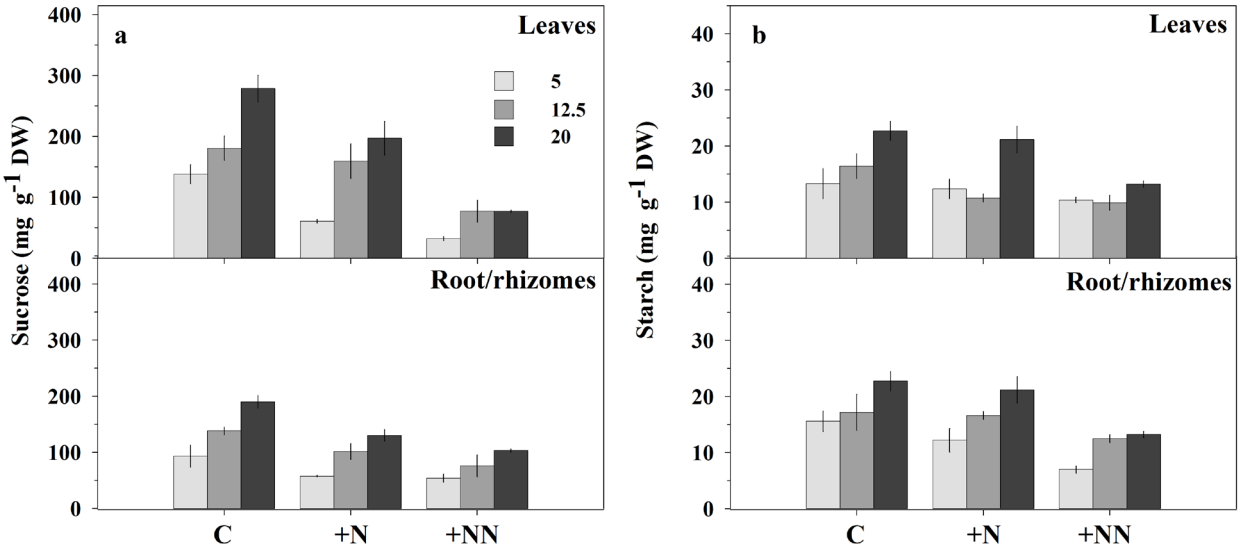


Figure 5. *Zostera marina.* (a) sucrose and (b) starch concentrations in aboveground (leaves) and below ground (root/rhizomes) tissues in eelgrass plants from the ammonium and salinity treatments. Treatments C, +N and +NN correspond to the target levels of ammonium: 0, 10 and 25 μM, respectively. Data are mean values (across replicated aquaria, n=3) ± 1 SE.

DISCUSSION

Seagrasses live in estuarine habitats that might be affected by short and long term variations in salinity and nutrient concentrations due to natural and anthropogenic fresh water inputs. Our results show that in addition to the singular negative effects of these stressors, high ammonium loading and lowered salinity have negative and synergistic effects on eelgrass performance.

Previous studies have shown that relatively low concentrations of NH_4^+ can have adverse effects on eelgrass and other seagrass species (Brun et al. 2002, 2008, Villazán et al. 2013b). These adverse effects can partly be explained by the need of an increased assimilation of reduced nitrogen into amino acids to prevent toxic effect levels of NH_4^+ within the cells (Brun et al. 2002, 2008, Villazán et al. 2013b). Amino acid synthesis requires energy and C-skeletons, which are either provided by photosynthesis or mobilized from C-reserves within the plant (Marschner 1995). A recent study by Villazan et al. (2013b) demonstrated that exposure of eelgrass to increased NH_4^+ concentrations can result in high intracellular NH_4^+ concentrations, reduced net photosynthesis, lower growth and higher mortality when plants were held under low light conditions (ca. 26 μmol photons m^{-2} s^{-1}), compared to high light conditions (ca. 70 μmol photons m^{-2} s^{-1}). Increased energy requirements due to increased NH_4^+ concentration can thus augment the negative impacts of low light levels (Villazán et al. 2013b). Acclimation to other environmental stressors, such as high or low temperatures or unfavourable levels of salinity may also require extra energy and C-skeletons and, may thus compete with other processes for these resources (van Katwijk 1997, 1999, Kahn & Durako 2006). In this way, simultaneous exposure of eelgrass to high NH_4^+ concentrations and to an additional stressor is likely to enhance the negative effects of NH_4^+ (van Katwijk 1997, 1999, Brun et al. 2002, 2008, Van der Heide et al. 2008, Christianen et al. 2011).

In the present study, exposure to high levels of NH_4^+ led to high intracellular NH_4^+ levels, and neutral or positive effects on eelgrass performance as long as salinity was kept at the ambient level (salinity 20; Nejrup & Pedersen 2008). Positive effects are likely due to the low N concentration in the seawater used in the experiment, as the total-N content in plants from the control treatment was close to the limiting level for seagrasses (i.e. 1.2-1.3 % N of DW in the leaves; Short 1987, Duarte 1990). C-reserves were, however, reduced considerably (by 50-60%) when eelgrass plants were exposed to high levels of NH_4^+ for 4 weeks.

Low salinity impacts most seagrasses negatively and typically results in impaired net photosynthesis, slower growth and higher mortality (Kirst 1989, Touchette 2007, Salo & Pedersen 2014). Similar results were recorded in the present study, where decreased salinity resulted in lower production and survival. Hypoosmotic conditions also led to decreased tissue K^+, Na^+ and Cl^- as plants attempt to avoid negative effects of osmotic stress and adjustments of turgor pressure by actively exporting inorganic ions and breaking down organic osmolytes, such as sugars and proline (Hellebust 1976, Kirst 1989, Ritchie 1988, Karsten 2012). Intracellular changes in ion concentrations and composition may further affect intracellular pH, membrane integrity and disturb metabolism (Hellebust 1976, Dickson

et al. 1982, Kirst 1989, Karsten 2012). Hyposalinity also led to increased K^+:Na^+ ratio, which might indicate an unbalanced ionic equilibrium (Touchette 2007). Changes in ion composition were followed by decreased chlorophyll content, chlorophyll fluorescence, net photosynthesis, growth, plant size, survival, C-skeletons (sugar and starch) and nutrient uptake rates, while the intracellular concentration of NH_4^+ and total N increased. These are changes in response to hyposaline conditions reported in macroalgae and seagrasses (Biebl & McRoy 1971, Bejamin et al. 1999, Khan & Durako 2006, Touchette 2007, Karsten 2012). The loss of chlorophyll and the drop of Fv/Fm at low salinity may indicate impaired photosystems; it may result in a lower photosynthetic capacity and, finally, in a reduced growth. It is known for other seagrass species that hyposaline conditions can decrease photosynthetic capacity (Biebl & McRoy 1971, Kahn & Durako 2006, Touchette 2007) by reducing chlorophyll content, changing chloroplast structure and/or inhibiting key photosynthetic enzymes (Touchette 2007). In spite of that, our results showed a slight decrease in Fv/Fm, with values within those reported for healthy plants (0.7-0.8; e.g. Ralph 1999, Durako & Kunelman 2002, Cayabyab & Enríquez 2007). It suggests that the negative symptoms observed on seagrass performance and survival at low salinity, was not mainly caused by the loss of the integrity in the photosynthetic apparatus. Respiration rates tended to be higher under hyposaline conditions, suggesting an increase in plant stress. However, respiratory responses to low salinity stress are usually not as consistent as photosynthetic responses (Ralph 1998, Khan & Durako 2006).

The observed increase in total N may be due to the slower growth rates in plants exposed to low salinity, and therefore, larger amounts of N can accumulate in plant tissues. Large intracellular pools of NH_4^+ and total N may cause a negative feedback on nutrient uptake rates (e.g. Pedersen 1994, McGlathery et al. 1996), explaining why the NH_4^+ uptake rates decreased with decreasing salinity. Moreover, PO_4^{3-} uptake rates may involve a Na^+ dependent transport system (Rubio et al. 2005). This allows seagrasses to take advantage of the inwardly driving force for Na^+ (generated by high saline environments) (Touchette 2007). Therefore, hyposaline conditions could be a disadvantage for PO_4^{3-} uptake rates, and this hypothesis could explain the decrease in the uptake of this nutrient with decreasing salinity. In contrast, PO_4^{3-} uptake rates were higher with increasing NH_4^+ loading. PO_4^{3-} plays an important role in reducing ammonium toxicity, since is required in the assimilation process as a part of the metabolic energy transfer coin (i.e. ATP); as it has been previously reported in the seagrass *Zostera noltei* (Brun et al. 2008). Thus, an enhancement of PO_4^{3-} demands could be expected under ammonium enrichment (Brun et al. 2008)

While impacts of the singular effects of NH_4^+ and/or salinity have been described in a number of previous papers (e.g. Zieman 1975, van Katwijk et al. 1997, Brun et al 2002, 2008, Touchette 2007, Nejrup & Pedersen 2008, Villazán et al. 2013b, Salo & Pedersen 2014), the combined effect of these stressors is an understudied issue. The present study demonstrates that simultaneous exposure to NH_4^+ loading and low salinity can have negative synergistic effects on eelgrass performance and survival. NH_4^+-enrichment has, as stated above, no negative or even a positive effect on eelgrass as long as the salinity was kept at standard values (salinity 20). This neutral or positive effect of high NH_4^+ level was, however,

turned into a rather strong negative effect in plants exposed to lower salinity (salinity 5). Synergistic interactions of NH_4^+ and low salinity have also been observed in *Thalassia testitudinum* seedlings, where low salinities (0-10) and high NH_4^+ loadings impacted plant growth negatively (Kahn & Durako 2006). Van Katwijk et al. (1999) observed a similar interaction between N availability and salinity, although they studied the combined effect of high and increasing salinity and nutrient enrichment on eelgrass; exposure to hipersaline conditions (i.e. salinity 30 as opposed to 23 or 26) lead to increasing negative effects of high nutrient loading on eelgrass. The mechanisms behind the interaction between NH_4^+ enrichment and low or high salinity are not well understood, but we suggest that these interactive effects are due enhanced competition of ammonium assimilation and osmoregulatory processes for energy and carbon skeletons. Low salinity may therefore increase C-skeletons demands of a plant by increasing its requirement of energy or by reducing photosynthetic rate. As high NH_4^+ loading also requires large amounts of energy to cope with the assimilation of NH_4^+ to face with the toxic effects of this nutrient, continuous uptake and assimilation under hyposaline conditions may impair carbon balance within plants and consequently reduce plant growth and increase mortality. Therefore, factors that increase energy demand and interact with low salinity and/or NH_4^+ enrichment, e.g. high temperature (van Katwijk et al 1997, Salo & Pedersen 2014), low irradiance (Villazán et al. 2013b, Jiang et al. 2013) or low oxygen levels (Raun & Borum 2013), may amplify the negative effects of these stressors and can increase mortality in seagrass meadows.

While our results demonstrate physiological responses of eelgrass to hyposaline conditions and NH_4^+ enrichment, we only tested these interactions in constant level of stress. The amount of stress is the product of its intensity and duration (Schulze et al. 2005), thus shorter stress could possibly lead to less severe stress responses. Future studies should evaluate the response to unfavourable levels of salinity as a function of stress characteristics (i.e. frequency and amplitude) and include a recovery phase after stress exposure. Also, more multifactorial experiments are needed to reveal potential interactive effects between natural stressors that might interact with salinity stress and NH_4^+ enrichment.

Eelgrass habitats with low salinity are common in the inner parts of estuaries and/or close to river mouths. Climate models predict increased frequency and intensity of rain-fall along the Atlantic coast of Europe as a result of the ongoing climate changes (Meier et al. 2012). Furthermore, the population growth around the coastal areas and the increase in the use of fertilizers in in the fields is leading to NH_4^+ enrichment in coastal zones (Glibert et al. 2010, Meier et al. 2012). As low salinity and NH_4^+ enrichment can have negative synergistic effects on seagrasses, coastal seagrass populations are in a risk of experiencing increased stress levels in the future. Thus, the existence of synergistic responses in seagrasses when facing with multifactorial co-occurring stresses in nature should be taken into account in the management of coastal areas and in the design of new experimental approaches.

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General discussion

La ciencia se compone de errores, que a su vez,

son los pasos hacia la verdad

Julio Verne

This PhD Thesis focuses on the effects of the interactions among different environmental factors (phosphate availability, hydrodynamics, light and salinity) and ecologically relevant high ammonium (NH_4^+) concentrations in morphological, dynamic and physiological responses of two seagrass species from the genus *Zostera*. It recorded a large spectrum of possible effects of NH_4^+ depending in the factors assayed, from positive to negative ones. For instance, positive and no clear effects were found in *Z. marina* plants subjected to high NH_4^+ loading and high light conditions, while synergistic negative effects were observed when plants were growing under light limitation and hiposaline stress; non-linear responses were even registered in *Z. noltei* plants cultivated under different hydrodynamic conditions. Therefore, the response to NH_4^+ enrichment was not unique, and depended on the interaction among different factors that may alter the uptake (i.e. NH_4^+ availability and water flow) and/or the assimilation of NH_4^+ (i.e. light, low salinity levels and concentration of non-structural carbohydrates). Thus, the present PhD thesis highlights the importance of the study of multiple factors to our best knowledge on NH_4^+ toxicity process in seagrasses.

Ammonium effects in seagrasses

NH_4^+ is a paradoxical nutrient ion. On one hand, it is an essential nutrient playing a crucial role in plant metabolism (Marschner 1995), but on the other hand our results indicated that high, but ecologically relevant, NH_4^+ concentrations also produced toxicity phenomena in seagrasses, by reducing photosynthetic performance, growth and survival in *Zostera noltei* and Z. *marina* plants. This is in agreement with previous studies in seagrasses (van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011), land plants (Britto & Kronzucker 2002) and micro- and macroalgae (Peckol & Rivers 1995, Collos & Harrison 2014), where NH_4^+ also showed this harmful potential.

Toxic effects of high NH_4^+ concentrations reported in previous studies showed a great degree of variability depending on the applied concentrations of NH_4^+, and/or in the interactions with different environmental conditions such us light, temperature, hydrodynamics, pH, etc. (Santamaría et al. 1994, van Katwijk et al. 1997, 1999, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011, Netten et al. 2013, Villazán et al. 2013a, 2013b). However, this Thesis demonstrates that all these factors share similar actuation ways, and thus they can be divided in those that directly or indirectly affected the uptake and/or the assimilation processes of NH_4^+.

The first factor altering NH_4^+ uptake process is the concentration of NH_4^+ in the surrounding water, since NH_4^+ uptake is expected to follow a passive mechanism, being dependent on the NH_4^+ concentration gradient between inside and outside the cells (Stapel et al. 1996, Pedersen et al. 1997, Lee & Duntos 1999, Hasegawa et al. 2005, Alexandre et al. 2011). This linear NH_4^+ uptake was demonstrated in chapter 1, where the increase in external concentrations of NH_4^+ caused linear uptake rates in *Zostera noltei* plants. Thus, passive transport of NH_4^+ may favor that intracellular NH_4^+ accumulates at high external NH_4^+ concentrations. This direct relationship between external and intracellular NH_4^+ concentrations

was demonstrated in chapters 3 and 4, in *Z. marina* plants cultivated under high NH_4^+ concentrations, where it accumulated. This may explain why increasing external NH_4^+ concentrations caused stronger negatives effects on photosynthesis, survival and growth in *Z. marina* and *Z. noltei* plants (van Katwijk et al. 1999, Brun et al. 2002, 2008, Villazán et al. 2013b), since higher NH_4^+ intracellular concentration may produce toxic effects in plants (Marschner 1995).

However, intracellular NH_4^+ usually made up less than 1-5% of total N (Invers et al. 2004, Villazán et al. 2013b), suggesting a rapid assimilation to prevent toxic effects. This may explain the limited response of the intracellular NH_4^+ pools under enrichment conditions (Invers et al. 2004, Villazán et al. 2013b) and the high free amino acids (FAA) concentration recorded under such conditions (Smolder et al. 1996, Invers et al. 2004, Villazán et al. 2013b, Setién et al. 2013). In particular amino acids with high N:C ratio such as glutamine (N:C ratio 0.40), were the most abundant FAA found under NH_4^+ enriched conditions in *Z. marina* (Table 1). This response is typically found in some species of higher plants (Marschner 1995, Smolders et al. 1996, Invers et al. 2004). Although our data can suggest that the low intracellular NH_4^+ levels are due to a fast and efficient assimilation of NH_4^+ into FAA, the possible existence of an active efflux mechanism of NH_4^+, like the one described in some terrestrial plant species (Britto et al. 2001), must not be discarded, since there are no studies dealing with this issue in seagrasses.

Therefore, when plants are growing under environmental conditions where photosynthetic C flux may be sufficient to meet increased internal C demands arising from N assimilation, a positive or no clear response to high NH_4^+ concentrations have been recorded in segrasses (Brun et al. 2002, Villazán et al. 2013b). These positive effects were found by Brun et al. (2002) in *Zostera noltei* plants in spring, with higher light and temperature levels than in winter, and in our case when *Z. marina* plants were cultured under optimal experimental conditions and high light levels (temperature 20° C and salinity 20, Nejrup & Pedersen 2008).

Although the concentration of NH_4^+ in the water determines the intracellular NH_4^+ concentration, it should be noted that seawater NH_4^+ concentration may be also down-regulated by plants, since the continuous uptake of NH_4^+ reduces water concentration. Thus, NH_4^+ toxicity in the field could be highly dependent in shoot density as previously demonstrated by van der Heide et al. (2008). This may cause a feedback mechanism, since in those conditions where NH_4^+ favors the growth of seagrasses (e.g. more shoots or biomass production), the increase in biomass can produce a lower accumulation of NH_4^+ in the surrounding water, decreasing the specific uptake rate of NH_4^+ and hence the levels of the intracellular NH_4^+ within the plants and its toxic effect. Going deeper into this hypothesis, if different seagrass species have different levels of sensitivity to NH_4^+ toxicity, the presence of higher levels of richness in the community may be beneficial for the more sensitive species to NH_4^+, since the uptake of NH_4^+ by other species may reduce NH_4^+ concentration in seawater and thus toxicity.

Table 1. Tissue concentration of specific free amino acids (mg N g^{-1} DW) and contribution of each in the total amount of FAA (percentage values in parenthesis) in leaves of *Zostera marina* grown under ammonium gradient enrichment and different light intensities (low light (LL) in grey and high light (HL) in white) HL, for 5 weeks. Numbers under the names of FAA indicate the N:C ratio of the compound. Values higher than 70% in bold letter. Data are presented as mean ± SE (n= 3 replicates); from experiments of chapter 3).

FAA	Light	LL			HL		
	NH_4^+	0	10	25	0	10	25
Asparagine		0.001	0.029	0.062	0.001	0.022	0.041
(0.50)		(0.42)	(1.61)	(1.25)	(0.30)	(1.41)	(1.39)
Glutamine		0.048	**1.411**	**3.983**	0.040	**1.118**	**2.488**
(0.40)		(13.82)	**(78.39)**	**(80.95)**	(8.32)	**(73.07)**	**(85.19)**
Alanine		0.033	0.062	0.336	0.033	0.064	0.099
(0.33)		(9.55)	(3.46)	(6.83)	(6.95)	(4.20)	(3.41)
Arginine		0.002	0.006	0.010	0.003	0.003	0.004
(0.66)		(0.66)	(0.35)	(0.20)	(0.63)	(0.18)	(0.13)
Aspartate		0.001	0.002	0.005	0.002	0.003	0.003
(0.25)		(0.49)	(0.12)	(0.10)	(0.48)	(0.35)	(0.10)
Serine		0.013	0.037	0.033	0.015	0.032	0.019
(0.33)		(3.74)	(2.07)	(0.75)	(3.25)	(2.12)	(0.67)
Threonine		0.006	0.023	0.022	0.010	0.010	0.012
(0.20)		(1.69)	(1.28)	(0.44)	(1.99)	(0.67)	(0.40)
Glycine		0.038	0.042	0.051	0.063	0.061	0.030
(0.50)		(10.74)	(2.33)	(1.04)	(13.40)	(3.01)	(1.04)
Histidine		0.070	0.070	0.044	0.152	0.064	0.051
(0.50)		(20.11)	(3.88)	(0.89)	(31.76)	(4.00)	(1.73)
GABA		0.047	0.041	0.126	0.037	0.051	0.052
(0.25)		(13.46)	(2.30)	(2.56)	(7.67)	(3.36)	(1.77)
Tyrosine		0.023	0.023	0.155	0.030	0.034	0.061
(0.11)		(6.51)	(1.28)	(3.15)	(6.17)	(2.22)	(2.07)
Valine		0.001	0.001	0.004	0.001	0.002	0.002
(0.20)		(0.42)	(0.07)	(0.09)	(0.28)	(0.12)	(0.06)
Leucine		0.004	0.002	0.015	0.004	0.005	0.008
(0.33)		(1.17)	(0.11)	(0.30)	(0.91)	(0.36)	(0.23)
Isoleucine		0.002	0.003	0.004	0.004	0.004	0.005
(0.17)		(1.67)	(0.16)	(0.89)	(0.76)	(0.28)	(0.18)
Ornithine		0.001	0.001	0.002	0.001	0.001	0.001
(0.40)		(0.15)	(0.04)	(0.03)	(0.17)	(0.04)	(0.03)
Lysine		0.006	0.006	0.008	0.008	0.008	0.006
(0.33)		(1.67)	(0.35)	(0.09)	(1.69)	(0.50)	(0.20)
Total FAA		0.35	1.80	4.92	0.48	1.53	2.92

This Thesis demonstrates that an important factor affecting NH_4^+ uptake and toxicity are hydrodynamic conditions. Previous studies already showed that increased water velocity reduces the thickness of the diffusive boundary layer leading to higher rates of NH_4^+ uptake (Thomas et al. 2000, Koch et al 2006, Morris et al. 2008). However, none of these studies explored whether this higher NH_4^+ uptake scales up to the physiology of the plant (e.g. growth, production, mortality, etc). Chapter 2, went beyond and demonstrated that the higher uptake rates promoted by flow velocity correlated well with stronger adverse effects promoted by NH_4^+ toxicity. However, a non-linear response with

enhancing velocity was recorded, with the strongest toxic effect at medium ones. This result highlighted the importance of analyzing the complete set of trade-offs caused by the alteration of some important factors such as flow velocity, because of a higher flow velocity enhanced NH_4^+ uptake, but at the same time improved light capture as leaves were positioned horizontally at high velocities (Zimmerman 2003, de lo Santos et al. 2010). Thus, the more horizontal position of the leaves, the higher light capture by the leaves, thereby benefiting photosynthesis, as previously demonstrated by de los Santos et al. (2010). In consequence, plants may increase its content in C-skeletons and energy (i.e. non-structural carbohydrates and ATP) growing under HV conditions and then are more capable to assimilate NH_4^+ into amino acids, preventing high intracellular concentrations of this nutrient.

In nature, increasing water flow may also enhance sediment resuspension, which decreases light levels and photosynthetic rates (Koch 2001), which may promote the opposite effect. Light can be considered as the most important factor affecting the assimilation of NH_4^+, since light is the driving force allowing the production of ATP and carbohydrates biosynthesis (Gerendás et al. 1997), which are considered essential metabolic energy coins to face the toxicity of NH_4^+. Adverse effects of NH_4^+ are mainly observed under low light conditions (e.g. low ATP and non-structural carbohydrates production), correlated to a marked reduction in sucrose and starch in *Zostera marina* (chapter 3) and in *Z. noltei* (Brun et al. 2002, 2008).

Inorganic phosphate plays an important role in reducing NH_4^+ toxicity. This beneficial effect seems to be related to its role in the assimilation process as a part of the metabolic energy transfer coin (i.e. ATP) (Brun et al. 2008*).* Thus, higher phosphate demands could be expected under NH_4^+ enrichment (Brun et al. 2008). This hypothesis was corroborated in chapter 4 in which phosphate uptake rates were higher with increasing NH_4^+ loading in *Z. marina* plants, keeping internal N:P stoichiometry. However, when both nutrients (i.e. phosphate and NH_4^+) were available in the medium, high NH_4^+ concentrations caused a cutoff in foliar phosphate uptake in *Z. noltei*. This may constitute an important first-step mechanism related to the NH_4^+ toxicity process, which has not been previously described. Moreover, this cutoff in phosphate uptake in the presence of NH_4^+ should be more relevant in P limited environments, as for example, in tropical and subtropical areas where phosphate used to be bound to the carbonate sediments in a non-bioavailable form (Short et al. 1990, Touchette & Burkholder 2000).

Nevertheless, light limitation and hydrodynamic conditions are not the only environmental stressors that can affect the assimilation process leading to more remarkable adverse effects under high NH_4^+ availability. Seagrass acclimation to other environmental stressors requires energy and C-skeletons (Marsh et al. 1986, Touchette 2007) and, may thus compete with the NH_4^+ assimilation. For example, exposure to low salinity had a marked negative effect on *Zostera marina* growth and survival (chapter 4). This synergistic interaction between NH_4^+ and low salinity was also observed in seedlings of *Thalassia testudinum* (Kahn & Durako 2006). As well, Van Katwijk et al. (1999) reported a similar interaction between N availability and salinity, although these authors studied the combined effect of high salinity and nutrient enrichment on eelgrass. The mechanisms behind the interaction between

NH_4^+ enrichment and varying low or high salinity are not well understood, but the additive effects of high NH_4^+ and extreme salinity must be related to the enhanced competition for energy and C-skeletons between osmoregulation and N assimilation processes. This argument must explain the remarkable reduction in non-structural carbohydrates found in *Z. marina* plants subjected to the additive effect of NH_4^+ enrichment and low salinity (chapter 4). Furthermore, under such experimental conditions a decrease of chlorophyll content, fluorescence and net photosynthesis was recorded (chapter 4), which matched with the observed decrease in photosynthetic capacity (Biebl & McRoy 1971, Kahn & Durako 2006, Touchette 2007) mediated by the reduction in chlorophyll content, changes in chloroplast structure and/or inhibition of key photosynthetic enzymes under hyposaline conditions (Touchette 2007). Moreover, plants spent large amounts of energy to restore turgor pressure through active export of inorganic ions, and the break-down of organic osmolytes, such us sugars and proline (Shafer et al. 2011, Touchette 2007).

Lastly, previous studies showed that the increase in temperature (van Katwijk et al. 1997) and pH (Netten et al 2013) also caused negative and synergetic effects with NH_4^+ enrichment. An increment in temperature from 15 to 20°C increased mortality in *Zostera marina* plants (van Katwijk et al. 1999). High temperatures increase respiration, draining the reserves of non-structural carbohydrates (York et al. 2013). Thus, less organic C-skeletons will be available to assimilate NH_4^+ (van Katwijk et al. 1997). Regarding to pH, an increment from 7 to 9 enhanced necrosis in *Elodea Canadensis*. As pH basifies, the concentration of unprotonated ammonia increased dramatically (Whitfield 1974). Unprotonated ammonia is considered to be the most toxic form of reduced nitrogen, as it crosses lipid soluble membranes by passive diffusion (Collos & Harrison 2014).

In summary, the response of seagrasses to NH_4^+ enrichment differ when NH_4^+ is assayed as a single stressor or when different factors interplay at the same time, altering the NH_4^+ uptake and/or the N assimilation processes. In the field seagrasses will be simultaneously subjected to multiple human-derived stressors; it may drive either synergistic, non-linear or opposite responses to NH_4^+ loading. For this reason, this PhD Thesis highlights the importance of applying multi-factorial approaches to untangle how different factors affects the response of seagrass populations to NH_4^+ enrichment to improve the management of this crucial ecosystem.

Differential sensitivity among seagrass species

The response of seagrasses to NH_4^+ enrichment depends on the uptake and/or the assimilation of this nutrient. Thus, differences in sensitivity among seagrass species may be caused by differences in the uptake and/or assimilation processes. Different levels of sensitivity to ammonium toxicity may be beneficial for the more sensitive species to NH_4^+, since the uptake of NH_4^+ by other more resistant species may reduce NH_4^+ concentration in the water and thus toxicity.

In spite of these possible interactions, studies on different seagrass species are scarce. Up to date, studies dealing with NH_4^+ toxicity have focused mainly on 2 seagrass species (*Zostera noltei* and *Z. marina)* (van Katwijk et al. 1997, 1999, Brun et al. 2002, 2008, van der Heide et al. 2008, Villazán et al. 2013a, 2013b), and only one study up to date tested the different sensitivity between two different species, *Halodule uninervis* and *Thalassia hemprichii* (Christianen et al. 2011).

The studies carried out in *Zostera noltei (*Brun et al 2002, 2008*)* and *Z. marina* (van Katwijk et al. 1999, Villazán et al. 2013b) showed that *Z. noltei* is more sensible to NH_4^+ enrichment than *Z. marina*. In *Z. noltei* toxic effects of NH_4^+ were found in plants subjected to 16 μM for two weeks (Brun et al. 2002, 2008). In contrast, 25 μM of NH_4^+ during 5 weeks caused from positive to negative effects on *Z. marina* plants, suggesting that these two species have some different mechanism to face NH_4^+ toxicity process.

An unpublished study measuring survival rates showed that *Cymodocea nodosa* is less vulnerable that *Zostera noltei* to NH_4^+ enrichment after 5 weeks under 25 μM NH_4^+ (Fig. 1) (Villazán et al. in preparation a). The response of *C. nodosa* to high NH_4^+ availability is more comparable to *Z. marina* plants, since the NH_4^+ concentrations assayed and experimental time is almost similar in both cases.

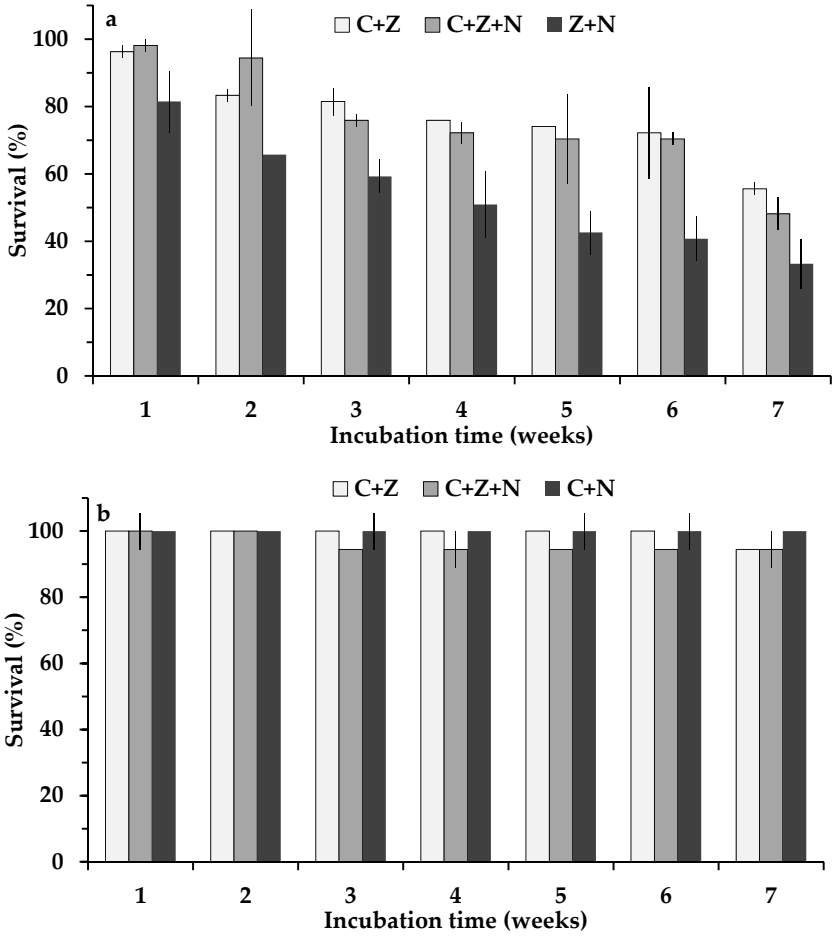

Figure 1. Survival rates (%) of *Zostera noltei* (a) and *Cymodocea nodosa* (b) under 7 weeks of NH_4^+ enrichment experiment (pulses of 25 μM each 2 days). C+Z, mixed culture of *Z. noltei* and *C. nodosa* without NH_4^+ enrichment; C+Z+N, a mixed culture of *Z. noltei* and *C. nodosa* under NH_4^+ enrichment; Z+N, a single culture of *Z. noltei* plants under NH_4^+ enrichment; C+N, single culture of *C. nodosa* under high NH_4^+ enrichment. Data are presented as mean ± SE (n= 3 replicates).

The differences in sensitivity between *Zostera noltei* and *Cymodocea nodosa* may be caused by differences at the uptake or assimilation processes. Recent experiments (Fig. 2) (López-Pulido 2013) showed that NH_4^+ uptake in *C. nodosa* seems to be more regulated than in *Z. noltei* plants. The latter showed a clear linear diffusive uptake pattern with no saturation at high NH_4^+ concentrations (Villazán et al 2013a, López-Pulido 2013). Moreover, uptake curves showed the same shape after 1 week of NH_4^+ enrichment. In contrast, a saturated curve was found in *C. nodosa* and a remarkable downregulation in NH_4^+ uptake rates were found after 1 week of acclimating plants to NH_4^+ loading (López-Pulido 2013).

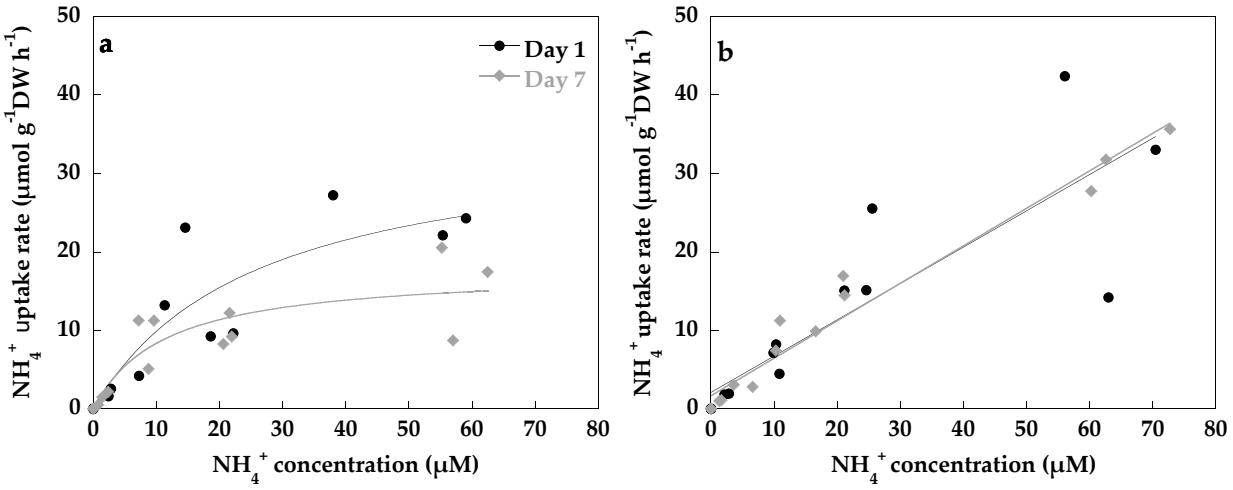

Figure 2. Ammonium (NH_4^+) foliar uptake rates against NH_4^+ concentrations in (a) *Cymodocea nodosa* and (b) *Zostera noltei* plants in the first day of the incubation period (day 1; black) and after 7 days of culture (day 7; grey), in which plants were subjected to NH_4^+ enrichment (pulses each two days). NH_4^+ uptake rates were calculated at intervals of 2 hours.

Similar findings were also recorded in a foliar nutrient uptake experiment in *Zostera marina* plants, in which plants cultivated during 10 days under 50 µM of NH_4^+ showed a decrease in the NH_4^+ foliar uptake rates in comparison with plants kept without NH_4^+ loading (Fig. 3) (Villazán et al. in preparation b). Therefore, *C. nodosa* and *Z. marina* showed a more regulated NH_4^+ uptake than *Z. noltei*.

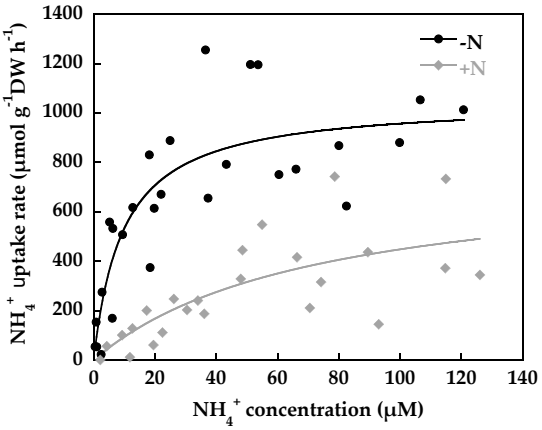

Figure 3. Ammonium (NH_4^+) foliar uptake rates against NH_4^+ concentrations in *Zostera marina* plants cultivated during 10 days with NH_4^+ (+ N, in black) and without NH_4^+ (-N, in grey). NH_4^+ uptake rates were calculated at intervals of 2 hours.

On the other hand, different sensibilities in survival rates maybe ascribed to the different total content in non-structural carbohydrates levels due to both the higher concentration and pool, with its bigger size, in *Cymodocea nodosa* and *Zostera marina* in comparison to *Z. noltei* plants (Brun et al. 2002, 2008, Villazán et al. 2013b, Villazán et al. in preparation a). It may make larger species less vulnerable to NH_4^+ toxicity.

This variety of specific responses to high NH_4^+ loading is an interesting finding, since the co-existence of several species with different sensibilities, could benefit the persistence of the more sensible one. This was found in a mixed culture experiment of *Zostera noltei* and *Cymodocea nodosa*, in which the survival of *Z. noltei* was higher when this species was cultured with *C. nodosa* than when *Z. noltei* was cultured alone (Figure 1) (Villazán et al. in preparation a). Additionally, the presence of other photosynthetic organisms also might decrease the toxic NH_4^+ effects in seagrasses (Moreno-Marín et al. in preparation). The presence of a moderate biomass of *Ulva* sp. may reduce the levels of NH_4^+ in the medium because of their high uptake capacity (Fujita 1985, Pedersen 1994, Luo et al. 2012), and, therefore, it can reduce the toxic effect of NH_4^+ on seagrasses. In stressful environments, positive interactions among species occur frequently, and a high diversity of plant species and traits may ameliorate harsh conditions through a facilitative mechanism (Bertness & Callaway 1994, Mulder et al. 2001, Brooker et al. 2008, Gustafsson & Boström 2013). In the case of NH_4^+ enrichment, a higher diversity might make the ecosystems more resistant and resilient to environmental perturbation.

Could NH_4^+ become a global threat for seagrasses?

Nutrient enrichment in coastal waters is increasing as population grows within the coastal zone (Bricker et al. 1999). In particular, NH_4^+ loads in coastal areas have been increasing and further increases are expected in shallow coastal zones (Glibert et al. 2010, Sobota et al. 2013). In addition, human activities are changing land use, habitats, the chemistry of the Earth's atmosphere and water, rates and balances of biogeochemical processes, and diversity of life on the planet (Short & Necless 1999, Vitousek et al. 1997). Therefore, changes in important environmental conditions, which determine the distribution and structure of seagrass communities are expected as a consequence of the global change (Short & Neckles, 1999). A primary effect is the increase in global temperature, which is expected to raise 1-6.4^{o}C by year 2100 (IPCC 2007). This increase in global temperature will affect the frequency and intensity of extreme weather events modifying hydrodynamic conditions in coastal areas (Byrnes et al. 2011, Young et al. 2011). Climate change will promote also an increase in the frequency and intensity of rainfall events (IPCC 2007), and in consequence freshwater runoffs may alter the salinity of coastal waters (Irlandi et al. 2002).

It also expected that atmospheric CO_2 concentration doubles by the end of this century, causing also the rising of oceanic CO_2 levels (IPCC 2007). This will provoke a decrease of 0.3–0.4 units of pH relative to present values (Caldeira & Wickett 2003, Feely et al. 2004). The lower external pH affects NH_4^+ uptake because the higher content of H^+ may reduce the activity of H^+-ATPases, which are involved

in cation uptake (Marschner 1995). Moreover, CO_2 enrichment may also affect nitrogen uptake and assimilation processes, as growth enhancement at high-CO_2 concentrations is expected to increase the nitrogen demand of plants (Stitt & Krapp 1999). So, the increase in NH_4^+ loading could be a benefit for plants growing under low pH, since nitrogen may become a limiting factor in enriched CO_2 waters (Alexandre et al. 2012).

In addition, climate change is thought to influence seagrass meadows by rising the sea level, which may affect coastal areas in different ways: increasing water depths could reduce the amount of light; changing tidal regimes may affect salinity and enhance water motion and tidal circulation, which may produce light attenuation by increasing turbidity (Short & Neckles 1999).

Thus, in natural conditions where multiple factors may act at the same time (Halpern et al. 2007), NH_4^+ enrichment could be considered as a potential global threat. Firstly, because all these potential changes derived from the global change and the future increase in NH_4^+ loading may co-occur in seagrass habitats. Secondly, because as shown in the present PhD Thesis, most of the interactions among high NH_4^+ availability and the future changes in environmental conditions will cause negative and synergistic effects on seagrass performance and survival, shifting the response of seagrasses to NH_4^+ enrichment from positive or no effects to negative ones.

However, the toxic effects of NH_4^+ enrichment on seagrass populations will be not spatially or temporally homogeneous. For example, increase NH_4^+ loading in oligotrophic areas may benefit seagrass growth (Orth 1977, Alcoverro et al. 1997, Peralta et al. 2003, Invers et al. 2004). In contrast, the potential toxic effect of NH_4^+ loading could be intensified in P-limited areas such as tropical and subtropical ones dominated by carbonate sediments (Touchette & Burkholder 2000). On the other hand, in seagrass communities of high diversity and/or high meadow densities, the negative NH_4^+ effects might be less remarkable (van der Heide et al. 2008, Villazán et al. in preparation a).

In this summary, our results showed that not a single factor, but often a combination of those, is the better explanation deterioration of seagrass habitats. Therefore, a deeper insight into the effects of the interaction of multiple stressors in seagrasses is necessary for a better understanding of seagrass meadow dynamics and a better management of these relevant ecosystems in a scenario of future global change.

Conclusions

Conclusiones/Conclusões

Si consigo ver más lejos es porque

he conseguido auparme a hombros de gigantes

Isaac Newton

Ilustrated by Vanessa González Ortiz

1. The presence of high ammonium concentrations in seawater decreased phosphate uptake rates in *Zostera noltei*. In contrast, high phosphate availability unaffected ammonium uptake. This is a non-described first-step mechanism related to the ammonium toxicity process in seagrasses.

2. High availability of ammonium for 5 weeks led to a marked increment in the total nitrogen content in *Zostera marina*, as well as in the intracellular ammonium concentration. However, intracellular ammonium content made up less than 5 %, suggesting either a rapid assimilation into amino acids or an active efflux of ammonium.

3. Ammonium enrichment caused a strong increment in the amino acids pool in *Zostera marina*, mainly in those bearing high N:C ratio. In contrast, the concentration of soluble proteins decreased when ammonium concentration increased in the medium.

4. The strongest negative effects of ammonium on *Zostera marina* were associated with a severe drop in the concentration of non-structural carbohydrates (sucrose and starch). This result highlights the essential role played by these organic carbon reserves in the response of seagrasses to ammonium enrichment.

5. Light is a crucial factor in the response of seagrasses to ammonium enrichment, since photosynthesis provides both the energetic metabolic coins and the carbon skeletons (ATP and non-structural carbohydrates) needed to cope with the ammonium toxicity process. Thus, a reduction in light levels and ammonium enrichment promoted synergistic negative effects on morphological, dynamic and physiological properties in *Zostera noltei* and *Z. marina* plants.

6. Regarding the effects of hydrodynamic conditions, the strongest adverse effects of ammonium were recorded at medium velocity in *Zostera noltei*, while this negative effect was less noticeable under low and high velocities. This was mainly due to the balance between higher ammonium uptake because of the reduced leaf boundary layer, and the higher light levels captured by leaves due to bending at high flow conditions.

7. Hiposaline stress strengthened the toxic effect of ammonium in *Zostera marina*, since osmoregulation processes competed with the ammonium detoxification process for carbon skeletons and energy.

8. The present Thesis highlighted that the response of seagrasses to ammonium enrichment depends on different stressors that alter ammonium uptake and compete for the same metabolic compounds (ATP and non-structural carbohydrates) to assimilate ammonium. These interactions may provoke antagonistic, synergetic or even non-linear responses on seagrasses. Therefore, a better knowledge among ammonium enrichment and its interaction with multiples stressors is necessary to reach an accurate management of these ecosystems.

9. It is necessary to explore the effects of ammonium enrichment in a broader range of seagrass species, since most of the studies are just focused in *Zostera* genus. Likewise, the study of ammonium effects at population and community levels could be necessary for a better understanding of the relevance of ammonium effects in natural populations.

1. La presencia de elevadas concentraciones de amonio en el agua disminuyó la tasa de incorporación de fosfato en *Zostera noltei.* Por el contrario, la presencia de fosfato no tuvo efecto sobre la incorporación de amonio. Esto supone por tanto, un primer mecanismo no descrito de toxicidad por amonio en angiospermas marinas.

2. Tras un periodo de 5 semanas de cultivo bajo altas concentraciones de amonio, *Zostera marina* mostró un notorio incremento en el contenido total de nitrógeno, así como en la concentración intracelular de amonio. Sin embargo, las concentraciones de amonio intracelulares supusieron menos del 5% del nitrógeno interno total, sugiriendo una rápida asimilación o la existencia de mecanismos de expulsión activa de amonio al exterior celular.

3. El enriquecimiento por amonio produjo un marcado incremento en el contenido de aminoácidos en *Zostera marina*, principalmente en aquellos que poseen una alta relación N:C. Por el contrario, la concentración de proteínas solubles disminuyó con el incremento en la concentración de amonio.

4. En *Zostera marina* los efectos tóxicos más acusados por amonio estuvieron relacionados con una disminución drástica de hidratos de carbono no estructurales (sacarosa y almidón), poniendo de manifiesto su papel esencial en la toxicidad de amonio en angiospermas marinas.

5. La luz juega un papel clave en los procesos de toxicidad por amonio en angiospermas marinas, ya que tanto el ATP como los hidratos de carbono no estructurales necesarios para la asimilación de amonio, provienen del proceso fotosintético. De este modo, una reducción en los niveles de luz y una elevada concentración de amonio producen efectos sinérgicos negativos en términos de crecimiento y supervivencia en las angiospermas marinas *Zostera noltei* y *Z. marina.*

6. En cuanto a las condiciones hidrodinámicas, los efectos más acusados de toxicidad por amonio se registraron a velocidades intermedias de flujo en *Zostera noltei.* Dichos efectos fueron menos evidentes a baja velocidad debido a una menor incorporación de amonio debido al mayor grosor de la capa límite, y a alta velocidad debido a una mayor captación de luz por parte de las plantas al tener sus hojas posiciones más inclinadas (horizontales).

7. El estrés hiposalino potenció el efecto tóxico en *Zostera marina*, debido a una disminución en las tasas fotosintéticas, y a una mayor demanda de energía e hidratos de carbono no estructurales requeridos tanto por los procesos de osmorregulación como de asimilación de amonio.

8. Los efectos producidos por el enriquecimiento de amonio en angiospermas marinas dependen de la interacción entre diferentes factores que afecten tanto a los procesos de incorporación, como también a aquellos que compitan por los mismos recursos necesarios para su asimilación (ATP e hidratos de carbono no estructurales). Esto puede dar lugar a respuestas antagónicas, sinérgicas o incluso no lineales en fanerógamas marinas frente a altas concentraciones de amonio. Por lo tanto, es necesario tener un mejor conocimiento de los procesos que determinan la toxicidad por amonio y la interacción con múltiples estresores, para una correcta gestión de los ecosistemas dominados por angiospermas marinas.

9. Es conveniente realizar estudios sobre los efectos del amonio en diferentes especies de angiospermas marinas ya que la mayoría de los estudios que hay en la actualidad se centran en el género *Zostera*. Asimismo, es necesario dar un salto a nivel de población y comunidad para la entender la relevancia del fenómeno de toxicidad por amonio en poblaciones naturales.

1. As taxas de assimilação de amónio em *Zostera noltei* diminuíram na presença de elevadas concentrações de amónio . Em oposição, a alta disponibilidade de fosfato não teve efeito na assimilação de amónio. Este é um importante mecanismo para entender a toxicidade do amónio em ervas marinhas que não tinha sido descrito anteriormente.

2. A alta disponibilidade de amónio durante 5 semanas implicou um incremento acentuado do conteúdo total em nitrogénio de *Zostera marina*, assim como um aumento da concentração intracelular de amónio. Não obstante, o conteúdo intracelular em amónio representou unicamente um 5% do amónio total, o que sugere uma rápida assimilação em forma de aminoácidos ou uma exsudação de amónio ativa.

3. O enriquecimento em amónio ocasionou um destacado incremento de aminoácidos em *Zostera marina*, especialmente naqueles que apresentavam um baixo ratio N:C. Em contraste, a concentração de proteínas solúveis diminuiu quando a concentração de amónio aumentou no meio.

4. Os maiores efeitos negativos do amónio em *Zostera marina* associam-se a uma considerável (sucrose e amido). Este resultado demonstra o papel essencial das reservas de carbono na resposta das ervas marinhas ao enriquecimento em amónio.

5. A luz é um factor crucial na resposta das ervas marinhas ao enriquecimento em amónio, uma vez que a fotossíntese proporciona tanto a energia metabólica como os esqueletos de carbono (ATP e carboidratos não-estruturais), que são necessários para lidar com o processo de toxicidade do amónio. Assim, a redução nos níveis de luz e o enriquecimento em amónio efeitos negativos sinérgicos nas propriedades morfológicas, dinâmicas e fisiológicas de *Zostera noltei* e *Z. marina*.

6. Com respeito aos efeitos das condições hidrodinâmicas, os efeitos adversos do amónio foram mais pronunciados em velocidades de fluxo médias em *Zostera noltei*, mas este efeito negativo não foi tão destacado com velocidades de fluxo baixas e altas. Isto deveu-se ao balanço entre uma assimilação de amónio mais elevada devido a uma redução da capa limite, e os altos níveis de luz captados pelas folhas devido ao seu dobramento em condições de alto fluxo hidrodinâmico.

7. O stresse hiposalino agravou os efeitos tóxicos do amónio em *Zostera marina*, devido à competição entre os processos de osmorregulação e os de desintoxicação para a obtenção de esqueletos de carbono e energia.

8. Esta tese de doutoramento reafirmou que a resposta das ervas marinhas ao enriquecimento em amónio depende de vários factores stressantes que alteram a assimilação de amónio e competem pelos mesmos compostos metabólicos para a sua assimilação (ATP e carboidratos não-estruturais).

9. É necessário explorar os efeitos do enriquecimento em amónio em mais espécies de ervas marinhas, dado que os estudos apresentados incluem unicamente o género *Zostera*. Assim sendo, o estudo dos efeitos do amónio a níveis de população e comunidade são necessários para um melhor entendimento da relevância dos efeitos do amónio na natureza.

References

No te rindas, que la vida es eso,

continuar el viaje, perseguir tus sueños,

destrabar el tiempo, correr los escombros

y destapar el cielo

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