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Preference and uptake rates of nitrogen forms by the red seaweed *Asparagopsis armata*

UNIVERSIDADE DO ALGARVE

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Mestrado em Biologia Marinha

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Declaração de auditoria de trabalho

Preference and uptake rates of nitrogen forms by the red seaweed *Asparagopsis armata*

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Raquel Torres Cardenal)

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RESUMO

A indústria da aquacultura é o setor de produção alimentar com maior crescimento a nível mundial. Como consequência, há um aumento de descargas de resíduos orgânicos e inorgânicos nos ecossistemas costeiros. Por forma a manter a atividade do setor de uma forma sustentada, torna-se fundamental promover um tratamento eficaz destas descargas no meio marinho. O conceito de Aquacultura Multi-Trófica Integrada (IMTA) apresenta-se como uma opção de produção com caráter mais sustentável, baseada na conversão eficiente de resíduos e desperdícios em novas matérias-primas, através do cultivo integrado de espécies de diferentes níveis tróficos. Devido à elevada capacidade que a maioria das macroalgas apresenta para absorver nutrientes inorgânicos do meio externo, estas têm sido consideradas candidatas ideais para serem usadas como biofiltros em sistemas IMTA. No entanto, a escolha da espécie de macroalga para biofiltro depende da sua capacidade de remover azoto inorgânico ou orgânico nas suas diferentes formas na água e do tipo de sistema de produção. A alga vermelha *Asparagopsis armata* é uma alga com uma capacidade ímpar para remover azoto de efluentes de sistemas de aquaculturas com fluxo de água contínuo. Os efluentes de sistemas de produção com fluxo de água contínuo são ricos em amónia, o principal produto catabólico dos peixes e a fonte de azoto preferida pela maioria das macroalgas. Em sistemas de aquacultura com recirculação (RAS), a amónia é convertida em nitrato através de processos de nitrificação microbiana. O potencial da *A. armata* como biofiltro para a remoção do nitrato é desconhecido. Uma vez que os custos dos sistemas de biofiltração são elevados, a sua viabilidade depende não só da capacidade de remoção de nutrientes da alga usada como biofiltrador como também do seu valor comercial. As algas do género *Asparagopsis* produzem níveis elevados de compostos halogenados, os quais possuem propriedades antibacterianas e antifúngicas com aplicações na medicina e na cosmética. Existe atualmente um interesse renovado na aquacultura do género *Asparagopsis* em virtude da capacidade demonstrada por estas algas para reduzir drasticamente as emissões de metano provenientes de animais ruminantes, quando incluídas na sua ração diária.

Esta tese teve como objetivos (1) determinar o padrão temporal de absorção de azoto inorgânico (amónia e nitrato) e orgânico (aminoácidos) pela *A. armata*, (2) determinar as taxas de absorção destas três formas de azoto pela *A. armata* e avaliar a sua seletividade relativamente a uma das formas azotadas quando presentes

simultaneamente no meio, e (3) determinar o efeito das diferentes formas de azoto nas taxas de crescimento da espécie.

Por forma a distinguir a fase de rápida absorção inicial de nutrientes (surge phase) da fase de absorção subsequente, caracterizada por taxas mais baixas e relativamente constantes, a absorção das diferentes formas de azoto pela alga foi continuamente monitorizada ao longo do tempo, após um pulso inicial de elevada concentração (~200 μM). Numa outra experiência, as taxas de absorção das diferentes formas de azoto foram quantificadas com base na incorporação de amónia, nitrato e aminoácidos marcados isotopicamente (^{15}N) durante a fase de absorção estabilizada definida na primeira experiência, a diferentes concentrações durante 2 h. As algas foram pré-incubadas durante 4 horas com nutrientes não marcados isotopicamente, por forma a excluir a fase de absorção rápida inicial. Após a pré-incubação, as algas foram incubadas em soluções contendo $^{15}NH_4CL$, $K^{15}NO_3$ ou ^{15}N-aa, a concentrações de 5, 25, 50, 100 e 200 μM, adicionadas separadamente ou em conjunto. As taxas de crescimento de *A. armata* foram determinadas após 14 dias de cultivo em soluções contendo NH_4Cl, KNO_3 ou N-aa, fornecidos individualmente ou em conjunto nas mesmas concentrações testadas na experiência de absorção de ^{15}N.

A. armata tem capacidade para absorver rapidamente qualquer uma das formas de azoto, exibindo um padrão temporal de absorção de azoto caracterizado por taxas iniciais elevadas em relação às taxas da fase subsequente, controlada internamente e caracterizada por taxas de absorção mais baixas e constantes. Na fase inicial após da adição dos nutrientes, as taxas absorção de amónia, nitrato e aminoácidos por *A. armata* foram mais elevadas durante os primeiros 105 minutos de incubação, período após o qual se verificou um decréscimo acentuado das taxas, seguido de uma fase de estabilização das mesmas. No contexto dos sistemas de aquacultura, em que as algas são produzidas sob um fornecimento contínuo de azoto, a fase na qual as taxas se encontram estabilizadas e constantes é mais adequada para avaliar capacidade de absorção das várias formas de azoto pela alga. A experiência de incorporação de ^{15}N, realizada após a fase de absorção rápida inicial, mostrou que a amónia é a forma preferencial de azoto da *A. armata*, a qual foi absorvida a taxas significativamente mais elevadas que as de nitrato e aminoácidos. Para além disso, na presença de múltiplas formas de azoto, as taxas de absorção de amónia não foram afetadas, enquanto que as taxas de absorção de nitrato e aminoácidos diminuíram significativamente. Notavelmente, a absorção de

aminoácidos foi significativamente mais elevada do que a de nitrato, revelando uma capacidade limitada de *A. armata* para absorver nitrato em ambientes com múltiplas fontes de azoto. Esta capacidade significativa para absorver aminoácidos, sugere que a incorporação desta forma de azoto pode contribuir substancialmente para os requisitos totais de azoto da espécie. A presença de várias formas azotadas no meio de crescimento não afetou de forma significativa o crescimento da *A. armata* relativamente a quando apenas um adas formas de azoto (amónia, nitrato ou aminoácidos) foi fornecida. De uma forma geral, as taxas de crescimento apresentaram uma elevada variação entre os replicados.

A preferência da *A. armata* pela amónia demonstrada neste estudo implica que o posicionamento da alga como biofiltro em sistemas de recirculação deverá ser anterior ao biofiltro bacteriano, onde as concentrações de amónia são mais elevadas. A capacidade significativa demonstrada pela *A. armata* para absorver azoto orgânico sugere que a avaliação da incorporação desta forma azotada seja considerada em estudos futuros de aquisição de azoto pela *A. armata*, bem como por outras espécies de algas. Este estudo confirmou *A. armata* como uma excelente espécie de alga como biofiltro de azoto em sistemas de aquacultura integrada

ABSTRACT

The global aquaculture industry continues to grow rapidly, and it is important to improve sustainability through effective treatment of its waste products. Seaweeds have been used in Integrated Multi-Trophic Aquaculture (IMTA) systems as an effective tool to reduce the nutrient burden of the fish farm effluents. Species of the genus *Asparagopsis armata* are successful nitrogen biofilters of flow-through fish aquaculture systems because of their high capacity to take up ammonium, the main metabolic waste product of fish. However, the potential of *A. armata* as a biofilter of recirculating aquaculture systems (RAS), where effluents have higher concentration of nitrate than ammonium, is unknown. In this study, a series of experiments were designed to evaluate the preference and the uptake rates of nitrogen in the forms of ammonium, nitrate and amino acids, as well as the effect of the different nitrogen forms on the growth rates of *A. armata.* Nitrogen uptake rates were quantified based on the incorporation of ^{15}N-labeled ammonium, nitrate and amino acids in the tissues, during the internally controlled phase, when uptake rates are stabilized and relatively constant. Our results revealed that inorganic and organic nitrogen sources can be simultaneously taken up by *A. armata.* The species exhibited a clear preference of ammonium over the two other forms of nitrogen (nitrate and amino acids), either when supplied separately or in combination. Notably, in the presence of multiple sources, amino acids were taken up at faster rates than nitrate by *A. armata*. The species showed a limited capacity to use nitrate when other nitrogen forms were present in the medium. The availability of the different nitrogen forms in the growth medium did not influence the growth rate of *A. armata*, either when these were supplied alone or in combination, and growth rates did not show an increasing pattern with nutrient concentration. This study confirms that *A. armata* is a suitable seaweed to be used as a biofilter in IMTA systems.

Keywords: Ammonium; Aquaculture; *Asparagopsis armata*; Nitrate; Nitrogen uptake; Rhodophyta; Seaweeds; Organic nitrogen

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GENERAL INTRODUCTION

The global aquaculture industry is growing rapidly, and it becomes important to improve sustainability through effective treatment of its waste products. Seaweeds have been proposed as an effective tool to reduce the nutrient burden of the fish farm effluents, mostly because of the high capacity displayed by most seaweeds to remove nutrients from the external environment (Neori et al., 2004; Schuenhoff et al., 2006). Furthermore, seaweeds have the capacity to stabilize the levels of oxygen, pH and CO_2 in these systems (Neori et al., 2004), while generating biomass for multiple economic purposes. The efficiency of seaweeds as nutrient biofilters varies depending on the forms of nitrogen present in the effluents of the aquaculture, which in turn depends on the type of aquaculture practice, as well as on the preference and capacity of the cultivated seaweed to remove the different forms of nitrogen.

Nitrogen is present in wastewater effluents of aquaculture systems as both dissolved inorganic nitrogen (ammonia or nitrate) and organic nitrogen (mainly urea and amino acids) (Kajimura et al., 2004; Porrello et al., 2005). Ammonium is the main excretion product of fishes and high concentrations of it in the water is negative for fish growth and health, and it is toxic at concentrations above 100 µM (Handy and Poxton, 1993; Neori et al., 2004; Corey et al., 2013). For this reason, the fish water has to be renewed frequently with clean seawater and the wastewater is either discharged to the environment (flow through systems) or recirculated back to the fish (recirculation aquaculture systems; RAS) passing through a microbial nitrification biofilters to convert ammonium into nitrate, a non-toxic form of nitrogen (Chopin et al., 2001; Neori et al., 2004). Thus, in RAS, discharge effluents are mainly composed by nitrate, in contrast to flow-through aquaculture where the ammonium fraction is much higher. In RAS, the decision for the species to be used as biofilter and its position in the system (upstream or downstream of the bacterial biofilter) depends on the seaweed's nutritional preference (ammonium versus nitrate versus organic nitrogen) (Corey et al., 2013). Hence, the knowledge of the nitrogen preferences of the species is fundamental for the analysis of its potential as a biofilter in Integrated Multi Trophic Aquaculture (IMTA) systems.

A review on the nutrient uptake kinetics of seaweeds showed that ammonium is generally taken up more efficiently than nitrate (Rees, 2003). Several seaweeds used as

biofilters in fish farm effluents, such as *Gracilaria spp*. (e.g. Abreu et al., 2011; Liu et al., 2016; Duan et al., 2019), *Palmaria palmata* (e.g. Corey et al., 2013; Grote, 2016) and *Ulva spp.* (e.g. Naldi and Wheeler, 2002; Luo et al., 2012; Li et al., 2016) showed greater affinity for ammonium than for nitrate as a nitrogen source. One possible explanation is that ammonium is energetically less costly compared to nitrate, which must first be reduced to nitrite and then to ammonium before assimilation, in contrast to ammonium which can be immediately used for the amino acids synthesis via glutamine synthetase in the chloroplasts (Lobban and Harrison, 1994). Another possible reason is the interaction between inorganic nitrogen forms, in which the presence of ammonium in solution inhibits or even suppresses the uptake of nitrate in certain macrophytes (Smit, 2002; Grote, 2016, Ross et al., 2018).

Nevertheless, ammonium is not always the preferred nitrogen source of seaweeds. For example, Ahn et al. (1998) showed that two brown seaweeds, *Laminaria saccharina* and *Nereocystis luetkeana*, are able to take up ammonium and nitrate simultaneously in salmon farming environments, but *N. leutkeana* took up nitrate significantly faster than ammonium. Also, the green seaweeds *Caulerpa lentillifera* and *Ulva prolifera* selectively preferred nitrate when other sources of nitrogen were also present in the medium (Liu et al., 2016; Li et al., 2019). Although the assimilation of nitrate requires more energy consumption, the preference for this over other nitrogen sources may be related to a higher nitrate reductase activity (one of the key enzymes involved in nitrate uptake and assimilation) and/or the size of the intracellular nitrate pool of each species (Gordillo et al., 2002). These observations show that the preferential uptake of a particular nitrogen form is species-specific.

Research on the use of seaweeds as a biofiltration treatment of aquaculture effluents focused mainly on the uptake of inorganic nitrogen forms. However, organic nitrogen is also a significant component of the total dissolved nitrogen in aquaculture wastewaters (Porrello et al., 2005; Shpigel et al., 2019), with urea and amino acids the most readily available forms for seaweed uptake (Smith et al., 2018). Compared to ammonium and nitrate, the uptake of organic nitrogen by seaweeds has been much less studied (Hurd et al., 2014). The capacity for urea and amino acid uptake was previously demonstrated in the green seaweed *U. lactuca* and the red seaweed *Gracilaria vermiculophyla* (Tyler et al., 2005). Urea was also found to be an important nitrogen source for kelps, as uptake rates of the giant kelp *Macrocystis pyrifera* were comparable to those of ammonium,

suggesting that this nitrogen form is used to sustain growth throughout the year (Smith et al., 2018). Other recent studies have also shown that seaweeds can take up organic nitrogen as urea and amino acids (Ross et al., 2018; Li et al., 2019; Shpigel et al., 2019), sometimes more effectively than inorganic nitrogen forms as nitrate (Xiu et al., 2019). Overall, these findings show that potential uptake of organic nitrogen should be included in seaweed nutrition studies and in bioremediation of aquaculture wastewater.

The mechanisms of nutrient absorption from the external environment have been studied in many seaweed species (e.g. DeBoer, 1981; Lobban and Harrison, 1994; Gordillo, 2012; Hurd et al., 2014). The movement of nutrients across the cell membrane is controlled, among other factors, by the availability of the external resources and the demand for growth (Gordillo, 2012; Hurd et al., 2014). In seaweeds, nutrients can move across a membrane in two ways, passively and actively, and the distinction between both has to do with whether or not cell energy is used. Passive mechanisms such as diffusion (simple or facilitated) use no energy, while active transport requires energy. In diffusion (passive transport), the uptake rate of the nutrient increases proportionally to the substrate concentration (Abreu et al., 2011). Passive transport occurs down a concentration gradient, but usually external concentrations of inorganic nutrients are much lower than intracellular concentrations (Hurd et al., 2014). Therefore, nutrients enter the cells mostly via active transport. In seawater, inorganic nitrogen occurs mainly as nitrate and ammonium. Since oceanic pH values are around 8.2, and the *pKa* for NH_3/NH_4^+ is 9.4, the proportion of nitrate to ammonium is generally much lower (Gordillo, 2012; Hurd et al., 2014). The uptake of ions such as nitrate and ammonium usually displays saturation kinetics, i.e. the uptake of such ions increases with increasing nutrient concentration until it reaches a maximum (Abreu et al., 2011a). The uptake of these nutrients is usually represented by a hyperbolic function where nutrient uptake rates are plotted against nutrient concentrations. The equation describing this curve is known as the Michaelis-Menten Equation: V = Vmax * S/Ks+S (Lobban and Harrison, 1994) where V is the uptake rate, Vmax is the maximum uptake rate, S is substrate concentration and Ks is the half-saturation constant, the uptake rate when V=Vmax/2. The affinity constant for a specific nutrient is given by Vmax/Ks. The slope of the initial linear portion of the curve (at low S values), α, is useful to compare the uptake affinity for a specific nutrient between species (Harrison et al., 1989). A high α (steep linear slope) indicates a high affinity for the nutrient, i.e. high capacity to take up

the nutrient when available at low concentrations in the seawater, while a high Vmax indicates a high capacity to rapidly take up nutrients when concentration in the external environment is high (Harrison and Hurd, 2001; Hurd et al., 2014). The uptake rates of the different nitrogen sources may be affected by a variety of factors: environmental factors (light, temperature, water motion and tolerance to desiccation), nutritional history of the species, species biology and genetics, and the external nutrient concentration (Lobban and Harrison, 1994).

Red seaweed species of the genus *Asparagopsis* have proven successful as biofilters of fish farm effluents in flow through aquaculture systems rich in ammonium, the main catabolic product from fish and the preferred (less energetic) form of nitrogen of most seaweed species, with biomass productivities well above the average for photosynthetic organisms (Schuenhoff et al., 2006; Mata et al., 2010). Because the cost of biofiltration systems is high, its viability depends not only on the species's capacity to remover nutrients but also on the species' commercial interest. Seaweeds of the genus *Asparagopsis* produce high levels of halogenated organic compounds, mainly brominated, such as bromoform. These secondary compounds have antibacterial and antifungal properties with numerous commercial applications: as antifouling agents, preservatives for products such as medicine and cosmetics, and anti-tumour properties (see review by Pinteus et al., 2018), with a potential high-value market. There has been a renewed interest in the aquaculture of *Asparagopsis* species due to its capacity to dramatically reduce enteric methane emissions from ruminant animals when included in their feed at 0.5-2% of daily ration (Vucko et al., 2017; Machado et al., 2018; Li et al., 2018; Roque et al., 2019). However, to support the supply of biomass for this application, cultivation of *Asparagopsis* will have to be considered in any type of land or sea based IMTA systems or in monoculture systems with artificial nutrition. Considering the *Asparagopsis* economic value and the high capacity demonstrated to remove ammonium from fish farm effluents, seaweeds of this genus are good candidates as efficient biofilters in RAS systems. In RAS, the decision of the position of the seaweed biofilter in the system (upstream or downstream of the bacterial biofilter) depends on the seaweed's nutritional preference (ammonium versus nitrate versus organic nitrogen) (Corey et al., 2013). However, the success of *Asparagopsis* as a nitrogen biofilter in RAS systems, where nitrate accumulates over time, is not yet known because there are no studies on the capacity and efficiency of *Asparagopsis* to

take up this form of inorganic nitrogen. It is also not known the ability of *Asparagopsis* to use organic nitrogen.

The red seaweed genus *Asparagopsis* Montagne (Bonnemaisoniales) is composed by two species with similar morphologies: *A. armata* Harvey, which is common in temperate seas, and *A. taxiformis* (Delile) Trevisan occurring typically along tropical and warm temperate coasts (Bonnin and Hawkes, 1987). *Asparagopsis* exhibit a triphasic, diplo-haplontic, life cycle, with gametophytes (haploid) morphologically distinct from the tetrasporophytes (diploid) (Feldmann and Feldmann, 1942; Chihara, 1961, 1962). Gametophytes thalli are composed of sparsely branched, creeping stolons and erect shots from which numerous side branches develop in all directions with plumose appearance (Børgesen, 1915 in Andreakis et al., 2004). The tetrasporophyte phase is filamentous and is the ideal morphology for tank cultivation (Schuenhoff et al., 2006).

The aim of this thesis was to investigate the potential of *Asparagopsis armat*a as a biofilter in IMTA. To do this, the uptake rates of inorganic (ammonium and nitrate) and organic (amino acids) nitrogen were determined. It was also assessed how the presence of the different nitrogen sources affected the selective uptake of any of the nitrogen forms, as well as its growth rates.

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Preference and uptake rates of nitrogen forms by the red seaweed *Asparagopsis armata*

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ABSTRACT

The global aquaculture industry continues to grow rapidly, and it is important to improve sustainability through effective treatment of its waste products. Seaweeds have been used in Integrated Multi-Trophic Aquaculture (IMTA) systems as an effective tool to reduce the nutrient burden of the fish farm effluents. Species of the genus *Asparagopsis armata* are successful nitrogen biofilters of flow-through fish aquaculture systems because of their high capacity to take up ammonium, the main metabolic waste product of fish. However, the potential of *A. armata* as a biofilter of recirculating aquaculture systems (RAS), where effluents have higher concentration of nitrate than ammonium, is unknown. In this study, a series of experiments were designed to evaluate the preference and the uptake rates of nitrogen in the forms of ammonium, nitrate and amino acids, as well as the effect of the different nitrogen forms on the growth rates of *A. armata.* Nitrogen uptake rates were quantified based on the incorporation of ^{15}N-labeled ammonium, nitrate and amino acids in the tissues, during the internally controlled phase, when uptake rates are stabilized and relatively constant. Our results revealed that inorganic and organic nitrogen sources can be simultaneously taken up by *A. armata.* The species exhibited a clear preference of ammonium over the two other forms of nitrogen (nitrate and amino acids), either when supplied separately or in combination. Notably, in the presence of multiple sources, amino acids were taken up at faster rates than nitrate by *A. armata*. The species showed a limited capacity to use nitrate when other nitrogen forms were present in the medium. The availability of the different nitrogen forms in the growth medium did not influence the growth rate of *A. armata*, either when these were supplied alone or in combination, and growth rates did not show an increasing pattern with nutrient concentration. This study confirms that *A. armata* is a suitable seaweed to be used as a biofilter in IMTA systems.

Keywords: Ammonium; Aquaculture; *Asparagopsis armata*; Nitrate; Nitrogen uptake; Rhodophyta; Seaweeds; Organic nitrogen

1. INTRODUCTION

The aquaculture industry is the fastest growing animal food production sector worldwide (Ottinger et al., 2016; Liu et al., 2018). The global rise and intensification of the production systems generates an increasing output of organic and inorganic nutrients, with environmental consequences to the coastal ecosystems (Chopin et al., 2001; Ottinger et al., 2016). Therefore, it is important to find effective means to treat aquaculture waste products to improve its environmental sustainability. Integrated Multi-Trophic Aquaculture (IMTA) systems has been proposed as a practice to reduce the environmental impact of the aquaculture industry (Chopin et al., 2001; Troell et al., 2003; Neori et al., 2004; Huo et al., 2012; Shpigel, 2019). The principle of IMTA consists in using the waste products from one aquatic species (e.g. fish, shrimp) as inputs (fertilizers, food) for the production of other organisms (e.g. seaweed, shellfish), creating a more balanced system and bioremediation services.

Seaweeds display high capacity to uptake and remove nutrients from the external environment (Neori et al., 2004; Schuenhoff et al., 2006). This characteristic makes seaweeds ideal candidates to be used as extractive organisms coupled with animal production in IMTA systems, reducing the inorganic nutrient burden of the fish farm effluents to the environment (Chopin et al., 2001). Furthermore, seaweeds also have the capability to stabilize the levels of oxygen, pH and CO_2 in the system (Neori et al., 2004), while generating biomass for multiple economic purposes. The efficiency of seaweeds as a nutrient biofilter will vary depending on the forms of the nitrogen present in the effluents of the aquaculture, which depends on the type of aquaculture practice, as well as on the preference and capacity of the cultivated seaweed to remove the different forms of nitrogen.

Nitrogen compounds are present in wastewater effluents of aquaculture systems as both dissolved inorganic nitrogen (ammonia or nitrate) and organic nitrogen (mainly urea and amino acids) (Kajimura et al., 2004; Porrello et al., 2005). Most fish excrete nitrogenous waste as ammonia. Consequently, ammonia is the main dissolved inorganic nitrogen form present in the effluents of water flow through (single pass) fish

aquaculture systems (Chopin et al., 2001; Neori et al., 2004). Conversely, in recirculation aquaculture systems (RAS), ammonia is converted into nitrate by microbial nitrification biofilters, and the effluents have higher concentration of nitrates than ammonia. The choice for a seaweed species as an effective biofilter in flow through or RAS systems, as well as the position of the biofilter within the system depends on the seaweed preferential form of nitrogen and uptake capacity (Corey et al., 2013). The preference for a specific nitrogen source by seaweeds is species-specific. However, most seaweed species prefer to take up ammonium over other forms of nitrogen (Smit, 2002; Naldi and Wheeler, 2002; Abreu et al., 2011; Corey et al., 2013; Wang et al., 2014). The presence of ammonium in solution may inhibit or even suppress the uptake of nitrate in macrophytes (Smit, 2002; Grote, 2016, Ross et al., 2018). But a preference for nitrate over other sources of nitrogen has also been reported for some seaweed species (Liu et al., 2016; Li et al., 2019). The growth of the seaweeds is also expected to vary depending on the nitrogen forms available (Luo et al., 2012; Wang et al., 2014; Grote, 2016; Han et al., 2018). For example, higher growth rates were reported for the red seaweed *Gracilaria tenuistipitata* when cultured with ammonium over nitrate (Wang et al., 2014), whereas nitrate supported higher growth rates of *Palmaria palmata* (Grote, 2016). Other species have similar growth rates on either ammonium or nitrate as a nitrogen source (Corey et al., 2013). Research on the use of seaweeds as a biofiltration treatment of aquaculture effluents focused mainly on the uptake of inorganic nitrogen forms. However, organic nitrogen is also a significant component of the total dissolved nitrogen in aquaculture wastewaters (Porrello et al., 2005; Shpigel et al., 2019). Recent studies have demonstrated that seaweeds can take up organic nitrogen such as urea and amino acids (Ross et al., 2018; Li et al., 2019; Shpigel et al., 2019), sometimes more effectively than inorganic nitrogen forms as nitrate (Xiu et al., 2019). This shows that organic nitrogen contributes significantly to the total nitrogen budget of seaweeds.

The red seaweed *Asparagopsis armata* is an effective biofilter of total ammonia nitrogen (TAN) of fish farm effluents produced in flow through systems, with biomass productivities well above the average for photosynthetic organisms (Schuenhoff et al., 2006; Mata et al., 2010). There is a renewed interest in the aquaculture of *Asparagopsis* species due to its capacity to dramatically reduce enteric methane emissions from ruminant animals when included in their feed at 0.5-2% of daily ration (Vucko et al.,

2017; Machado et al., 2018; Li et al., 2018; Roque et al., 2019). To support the supply of biomass for this application, cultivation of *Asparagopsis* will have to be considered in any type of land or sea based IMTA systems or in monoculture systems with artificial nutrition. However, the nitrogen preference or acquisition strategies of *A. armata* have not been investigated in detail so far.

The objectives of this study are (1) to determine the time course of inorganic (ammonium and nitrate) and organic (amino acids) nitrogen uptake by *A. armata,* 2) to assess the uptake rates of ammonium, nitrate and amino acids by *A. armata*, and whether it selectively takes up any of the nitrogen forms when they are simultaneously present, and 3) to determine how growth rates are affected by the different nitrogen sources.

2. MATERIALS AND METHODS

2.1. Sampling and cultivation conditions

The filamentous tetrasporophyte phase of *Asparagopsis armata* (previously known as the "*Falkenbergia rufolanosa"*) was used for this study as it is a good candidate for tank cultivation (Schuenhoff et al., 2006). Thalli were collected in the intertidal zone in Praia de Arrifes, Albufeira, South Portugal (37º 07'62''N, 8º 27'67''W), in March 2019, and immediately transported to the laboratory. Upon arrival, the seaweeds were rinsed with autoclaved seawater and visible epiphytes were removed by hand under a dissecting microscope.

Seaweeds were maintained under constant light (30 µmol photons m^{-2} s^{-1}) and temperature (20 ºC), with a light:dark cycle of 12:12 h, in autoclaved natural seawater (salinity of 33 and pH of 8.2) enriched with a quarter-strength (VS_5) medium, modified from von Stosch's (Guiry and Cunningham's, 1984). The VS medium is composed of equal volumes of the following stock solutions (all in 500 ml Milli-Q water): 21.26 g $NaNO_3$; 2.68 g Na_2 DL-beta-glycerophosphate; 0.139 g FeSO· $7H_2O$; 0.98 g Mn $C1_2 \cdot 4H_2O$; 1.86 g Na_2 EDTA· $2H_2O$; 0.05 g Biotin; 0.1 g Aneurine HCl and 0.2 mg Vitamin B12. Quarter-strength (VS_5), corresponded to 5 ml of stock solution per litre of sterile seawater. The medium was changed on a weekly basis. The seaweeds were kept under constant movement through bottom aeration.

2.2. Species identification

The filamentous tetrasporophic phase of the two species of *Asparagopsis* (*A. armata* and A. *taxiformis*) is morphologically similar (but see Zanolla et al., 2014). This renders the need of molecular tools to identify the *Asparagopsis* species. A small portion of cleaned thalli was collected from the culture vial and stored dehydrated in silica-gel for species identification following DNA sequencing. The mitochondrial marker cytochrome oxidase subunit 2 – subunit 3 (*cox2 – 3*) spacer was chosen to obtain the phylogenetic information (Zuccarello et al., 1999). Genomic DNA was extracted using NucleoSpin Plant II kits (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Extraction results were checked by electrophoresis in 1% agarose gel stained with GelRed. Polymerase chain reactions (PCRs) were performed in a 20 µL solution containing 1x GoTaq Flexi buffer (Promega, Madison, WI, USA), 2.5 mM of $MgCl_2$, 0.5 mM of dNTPs, 0.5 µM of each primer and 1:500 diluted DNA. The amplification programme consisted in an initial denaturation step (94 ºC, 4 min) followed by 35 cycles of 93 ºC 1 min, 48 ºC 1 min and 72 ºC 1.5 min, and a final extension step (72 ºC, 5 min), described in Zuccarello et al. (1999). PCR products were checked afterwards by electrophoresis in a 1% agarose gel stained with GelRed. DNA amplifications were sequenced in an automated capillary sequencer (Applied Biosystems – CCMAR, Portugal) using the forward primer from the PCR amplification. Sequences were proofread, edited and aligned in GENEIOUS v.2019.2.1 (http://www.geneious.com). Sequences of *Asparagopsis* species (Andreakis et al., 2016) downloaded from GenBank were used for the specie identification. Phylogenetic relationships were reconstructed in MEGA X (Kumar et al., 2018) using the Maximum Likelihood method and Tamura-Nei model the Neighbor-Joining method. The *cox2 – 3* sequence of the tetrasporophyte used in this study is identical to those obtained from French and Spanish specimens and fell within the *A. armata* cluster (Fig. 1).

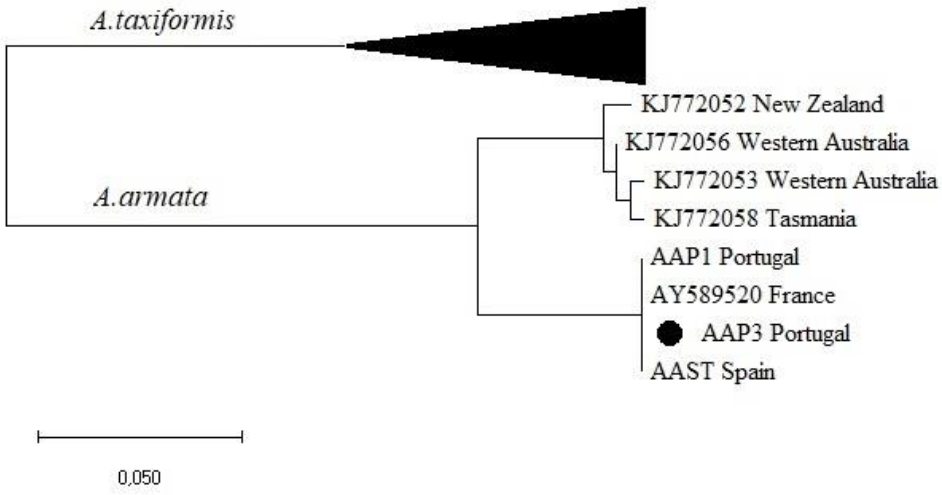

Fig. 1. Cluster in Maximum likelihood phylogenetic hypothesis inferred from eight *Asparagopsis* lineages (Mata et al., 2017) and the *Asparagopsis* sample used in this study (●)

2.3. Pre-treatment for the experiments

Six days before nutrient experiments, the seaweeds were transferred to artificial seawater (ASW) (salinity of 33 and pH of 8.2) enriched with VS_5 without nitrogen to decreased and equalize the nitrogen content in the tissues (Abreu et al., 2011; Liu et al., 2016). ASW was prepared by dissolving 4.7g $MgCl_2 \cdot 6H_2O$; 1.4 g $CaCl_2 \cdot 2H_2O$; 24.7 NaCl; 0.7 KCl; 6.3 g Na_2SO and 0.2 g $NaHCO_3$ per 1 L of fresh water purified using Millipore Elix 10 UV water purification system. The pH of the ASW was adjusted to 8.2 using NaOH.

2.4. Time course of nitrogen uptake

The time course of nutrient uptake by nutrient-limited seaweeds when exposed to high nutrient concentration is usually described by three successive phases: 1) a phase of surge uptake, characterized by high and transient uptake rates, 2) a phase of internally controlled uptake rates, characterized by lower, stabilized and constant rates, followed by 3) a phase of declining uptake rates, which are controlled by the depletion of the nutrient in the medium (Pedersen, 1994). We used the perturbation method to identify the internally controlled uptake phase of *A. armata* for ammonium, nitrate and amino acids. In an aquaculture scenario where seaweeds are grown under continuous nitrogen supply, this phase is useful to evaluate the long-term nitrogen uptake rates (Ghaly et al., 2005). We followed the time course of the uptake of the different nitrogen sources by measuring the depletion of the added nutrients over time.

Pre-treated tetrasporophytes of *A. armata* (see section 2.3) were incubated in 200 ml of ASW (salinity of 33.0, pH of 8.2) enriched with either NH_4Cl, KNO_3 or algal amino acid mixture (ULM-2314-1, Cambridge Isotope Laboratories) at initial concentrations of 245, 180 or 135 µM, respectively. Two different biomass to volume ratios were tested (0.1 and 0.5 g fresh weight in 200 ml) because no previous information of the nitrogen uptake rates of *A. armata* was available. The media were constantly stirred during incubations using an orbital shaker (125 rpm).

The depletion of each nutrient from the medium was followed by collecting 10 mL water samples at 45, 105, 225, 345 and 465 min after the beginning of the incubation. Water samples were filtered (cellulose acetate filter, 0.45 µm pore size) and analysed immediately for ammonium and nitrate. Samples for amino acid determination were stored at -20 ºC until analysis. The pH of the seawater was recorded on each treatment at the beginning and at the end of the experiment. Inorganic nutrient concentrations (ammonium and nitrate) were analysed in a loop-flow analyser (lMAC-1000; Systea, Anagni, Italy). Ammonium concentration was determined using the phenol-hypochlorite method and nitrate concentration was determined using the Cd-Cu column reduction method. Total free amino acid N was determined fluorometrically according to Jones et al. (2002), using glycine as a standard because it is the most predominant amino acid and its relative fluorescent intensity is similar to that of other dominant amino acids (Parsons et al. 1984). Briefly, 20 µl of sample or standard and 200 µl of working reagent (0.5 ml of OPAME concentrate + 20 ml of borate buffer 0.02 M pH 9.5) were combined and read after 1 min on a multi-mode microplate reader (Biotek Instruments, Synergy ^{TM}4) with an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The OPAME reagent was obtained by dissolving 5 mg of o-phthaldialdehyde in 0.5 ml of methanol and adding 10 µl of β-mercaptoethanol.

The uptake rates of each N source were derived from the depletion of the nutrient in the medium at each time interval using the following equation:

$$V = \frac{(Si - Sf) * vol}{t * B}$$

where V = uptake rate (µmol g FW^{-1} h^{-1}), Si = substrate concentration at the beginning of time interval (µM), Sf = substrate concentration at the end of time interval (µM), vol = water volume at the end of time interval (l), t = time (h), and B = biomass of incubated tissue (g fresh weight).

2.5. Nitrogen uptake rates

Nitrogen uptake rates were determined during the internally controlled uptake phase, i.e. when rates are stabilized. For *A. armata*, the uptake rates of ammonium, nitrate and amino acids stabilized and remained constant after four hours of the nutrient addition, according to the results obtained in the time course experiment (see section 2.3.). To reach the stabilized uptake phase, pre-treated seaweeds (see section 2.2.) were pre-incubated in non-labelled NH_4Cl, KNO_3 and amino acids for four hours, after which they were transferred into the incubation media containing ^{15}N-labelled substrates (Table 1). The ^{15}N stable isotope technique is based on the incorporation of ^{15}N-labelled substrates present in the incubation medium by the seaweed tissues. This is a sensitive and robust technique, which minimises sources of error (Glibert and Capone, 1993), and allows to differentiate the uptake of one nitrogen form when multiple N sources are present.

^{15}N uptake rates were assessed by incubating 0.1 g of fresh weight of pre-incubated *A. armata* in 1 L solutions of artificial seawater (salinity of 33.0, pH of 8.2) containing one single isotopically labelled N form ($^{15}NH_4Cl$, $K^{15}NO_3$ or ^{15}N algal amino acid mixture, atom % = 98, Sigma) and in solutions containing the three nitrogen forms but with only one nitrogen form isotopically labelled. The ^{15}N algal amino acid mixture contained 16 amino acids extracted from a blue-green algal source, with dominance of alanine (14.7 %). Uptake rates were assessed at a range of concentrations (5, 25, 50, 100 and 200 μM). Each nitrogen source (labelled and non-labelled) was provided at equal concentration, originating a total nitrogen concentration of 15, 75, 150, 300 and 600 μM in the media containing multiple N sources (Table 1). Incubations were performed in triplicate at constant light (30 μmol photons m^{-2} s^{-1}) and temperature (20 ºC) for two hours, in a total of 90 incubations (6 treatments x 5 concentrations x 3 replicates). To overcome feasibility constraints derived from the setup of a large number of incubation chambers necessary to test all treatments and replicates simultaneously, incubations were run simultaneously for all treatments and concentrations, whereas replicate incubations were done sequentially (n = 3). The media were constantly stirred during incubation with an orbital shaker (125 rpm) to prevent local depletion of substrates and ensure a homogeneous concentration of the labels. Nutrient concentration in the media did not vary noticeably during incubations, i.e. nutrient concentrations remained fairly constant throughout the experiment.

Table 1. Experimental design of the pre-incubation and the ^{15}N uptake experiments with *Asparagopsis armata*, showing the different nitrogen treatments and the range of concentrations used

Pre-Treatment (four hour incubations)	N concentrations (µM)	Total N concentrations (µM)
NH_4Cl	5, 25, 50, 100, 200	5, 25, 50, 100, 200
$NH_4Cl + KNO_3 +$ N-aa	5, 25, 50, 100, 200	15; 75; 150; 300; 600
KNO_3	5, 25, 50, 100, 200	5; 25; 50; 100; 200
$NH_4Cl + KNO_3 +$ N-aa	5, 25, 50, 100, 200	15; 75; 150; 300; 600
N-aa	5, 25, 50, 100, 200	5; 25; 50; 100; 200
$NH_4Cl + KNO_3 +$ N-aa	5, 25, 50, 100, 200	15; 75; 150; 300; 600
^{15}N uptake rates (two hour incubations)	^{15}N concentrations (µM)	Total N concentrations (µM)
$^{15}NH_4Cl$	5, 25, 50, 100, 200	5, 25, 50, 100, 200
$^{15}NH_4Cl + KNO_3 +$ N-aa	5, 25, 50, 100, 200	15, 75, 150, 300, 600
$^{15}KNO_3$	5, 25, 50, 100, 200	5, 25, 50, 100, 200
$NH_4Cl + {}^{15}KNO_3 +$ N-aa	5, 25, 50, 100, 200	15, 75, 150, 300, 600
^{15}N-aa	5, 25, 50, 100, 200	5, 25, 50, 100, 200
$NH_4Cl + KNO_3 + {}^{15}$N-aa	5, 25, 50, 100, 200	15, 75, 150, 300, 600

At the end of the ^{15}N incubations, the seaweeds were removed from the media and the tissues were rinsed with deionized water to remove salts and adherent label. Tissues were dried at 60 ºC for 48 h and reduced to a fine powder using TissueLyser II (QUIAGEN, Hilden, Germany). The nitrogen content and the ^{15}N atom % of the dried tissues were analysed using a Elementar Vario EL Cube or Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Precision of the $\delta\,^{15}N$ analysis was higher than 0.03 ‰. ^{15}N background levels in the tissues were measured in five replicate samples.

^{15}N uptake rates of *A. armata* were calculated using the following equation:

$$^{15}N\ incorporation\ (g) = (^{15}N_f - {}^{15}N_i) * N_t / DW$$

where ^{15}Nf is the post-incubation ^{15}N level, $^{15}N_i$ is the initial background level, N_t is the total nitrogen content in the tissue (%) and *DW* is the sample dry weight (g). ^{15}N uptake rates were expressed in µmol N g^{-1} DW h^{-1} and plotted against substrate concentration (µM).

Because data did not displayed saturation kinetics, they were fitted with linear regression model ($V = a + bx$), where V is the uptake rate (µmol g^{-1} DW h^{-1}), a is the intercept and b is the slope of the regression (which has the same meaning as α, i.e. the nutrient affinity coefficient, in the Michaelis-Menten saturation model).

2.6. Growth rates

Growth trials were performed using the apical (actively growing) section of the *A. armata* filaments. Parent fronds were placed in a petri dish with sterile seawater under a dissecting microscope and healthy (unfouled) tips (~10 – 20 cells in length) were excise with forceps. *A. armata* was grown in solutions containing one single nitrogen form (NH_4Cl, KNO_3 or amino acids) and in solutions containing the three nitrogen forms at a range of concentrations (5, 25, 50, 100, 200 µM) (n = 3). For each nitrogen treatment, one tip was placed into a 60 mm petri dish (90 in total) filled with 15 ml sterilized seawater (salinity of 33 and pH of 8.2), enriched with VS_5. Each nitrogen source was provided at equal concentration, originating a total nitrogen concentration of 15, 75, 150, 300 and 600 µM (n=3) in the media containing multiple nitrogen sources. Image analysis was used to determine the growth rates to avoid weighing errors from weighting very small filamentous thalli. *Asparagopsis* filaments display apical growth and regular branching, which can be measured by stereomicroscopy analysis of photographs (Paul et al., 2014; Mata et al., 2017). Each replicate was photographed under a dissecting microscope (Leica S8 APO) at the start of the experiment and the length of the filamentous thalli was determined using the software Leica Application Suite V4. 12. The growth media were changed after 7 days and the experiment lasted 14 days. At the end of the experiment, each filament was photographed once again and the length determined as described above.

The very small biomass to volume ratio in the petri dishes ensured that the nutrient concentrations in the media remained fairly constant throughout the experiment.

Specific growth rates (SGR) were measured as the difference between the size of the thalli at the beginning of the experiment and after 14 days following the equation:

$$SGR\ (\%\ day^{-1}) = (\ln S_0 - \ln S_t) / (T_t - T_0) * 100$$

where S_0 and S_t are the size (mm) at the initial (T_0) and at the end (T_t) of the experiment. Seven out of the total 90 experimental filaments did not survive and were excluded from the analysis.

2.7.Statistical analyses

Two-way analysis of variance (ANOVA) was used to test the effect of the presence of multiple nitrogen sources and concentration on the ^{15}N uptake and growth rates of each nitrogen source, and also to test differences in the uptake rates of $^{15}NH_4Cl$, $K^{15}NO_3$ and ^{15}Naa in the presence of other non-labelled nitrogen sources at different concentrations. The significance level of the test was $\alpha = 0.05$. Statistical analyses were performed using SigmaPlot 14.0 software.

3. RESULTS

3.1. Time course of nitrogen uptake

The concentrations of ammonium, nitrate and amino acid in the culture medium decreased over time (Fig. 2, inset graphs). Two phases were apparent when nitrogen uptake rates were plotted against time: a surge uptake phase, with higher rates within the first 105 minutes of incubation, followed by an internally controlled uptake phase from this time onwards, when rates stabilized to values 4, 7 and 5-fold lower than the initial levels for ammonium, nitrate and amino acids respectively (Fig. 2). The uptake rates of amino acids were more variable than those of ammonium, nitrate, and with some cases of negative uptake rates that were considered as zero (or no uptake) (Fig. 2C).

The pH of the seawater in the incubation media at the end of the experiment was around 8.7 in the culture vials with 0.5 g of biomass and 8.3 in those with 0.1 g FW. *Asparagopsis* is carbon limited at pH levels above 8.5 (Mata et al., 2007) and, consequently, biomass to volume ratios of 0.1 g FW to 200 ml were chosen in the ^{15}N uptake experiments.

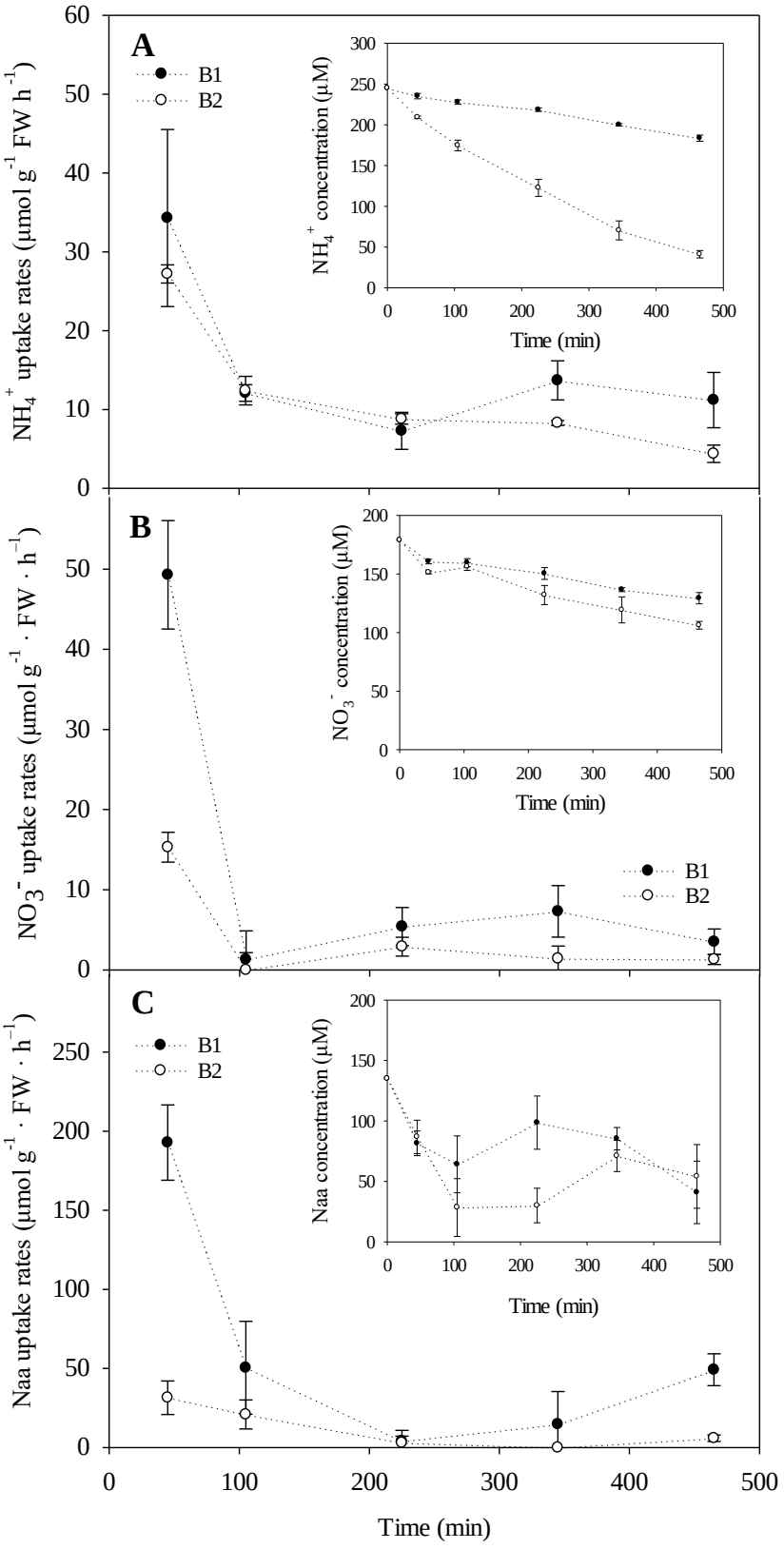

Fig. 2. Time course of the uptake rates of ammonium (NH_4^+) (A), nitrate (NO_3^-) (B) and amino acids (Naa) (C) by *Asparagopsis armata*. Inset graphs represent the pattern of nitrogen depletion over time. Values are mean ± SD (n = 3). FW, fresh weight. B1 = 0.1 g FW; B2 = 0.5 g FW

3.2. Nitrogen uptake rates

The uptake rates of ammonium and amino acids by *A. armata* increased linearly and significantly with nitrogen concentration, whereas the uptake rates of nitrate were relatively constant and were not affected by the different concentrations tested (Fig. 3, Tables 2 and 3).

The ammonium uptake rate by *A. armata* was not significantly affected by the presence of other nitrogen sources in the medium, whereas the uptake rates of both nitrate and amino acids were significantly lower (10 and 2-fold, respectively) when the other nitrogen sources were present (Fig. 3, Table 2). Nitrate uptake rates showed a decreasing pattern with increasing nitrate concentration in the presence of other nitrogen sources (Table 3).

Table 2. Results of the two-way analysis of variance (ANOVA) of the effect of the presence of multiple nitrogen sources and nutrient concentration on the uptake rates of ammonium, nitrate and amino acids by *Asparagopsis armata*

Source	$^{15}NH_4Cl$			$^{15}KNO_3$			^{15}Naa		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Treatment	1	0.0134	0.0134	1	92.380	<0.001	1	164.947	<0.001
Concentration	4	155.66	<0.001	4	0.211	0.929	4	43.698	<0.001
Interaction	4	2.713	0.059	4	0.273	0.892	4	4.629	0.008
Residual	20			20			20		

Table 3. Equations of the linear regression model fitted to the uptake rates of ammonium, nitrate and amino acids when supplied alone or combined with other N sources (*V* = uptake rate, *S* = substrate concentration, r^2 = coefficient of determination, P = level of significance)

	Single N source			Multiples N sources		
	Equations	r^2	*P*	Equations	r^2	*P*
$^{15}NH_4Cl$	$V=0.12*S+10.45$	0.99	0.0004	$V=0.14*S+8.79$	0.98	0.001
$^{15}KNO_3$	-	-	-	$V=(-0.001)*S+0.45$	0.84	0.029
^{15}Naa	$V=0.007*S+1.69$	0.73	0.066	$V=0.006*S+0.88$	0.99	0.0001

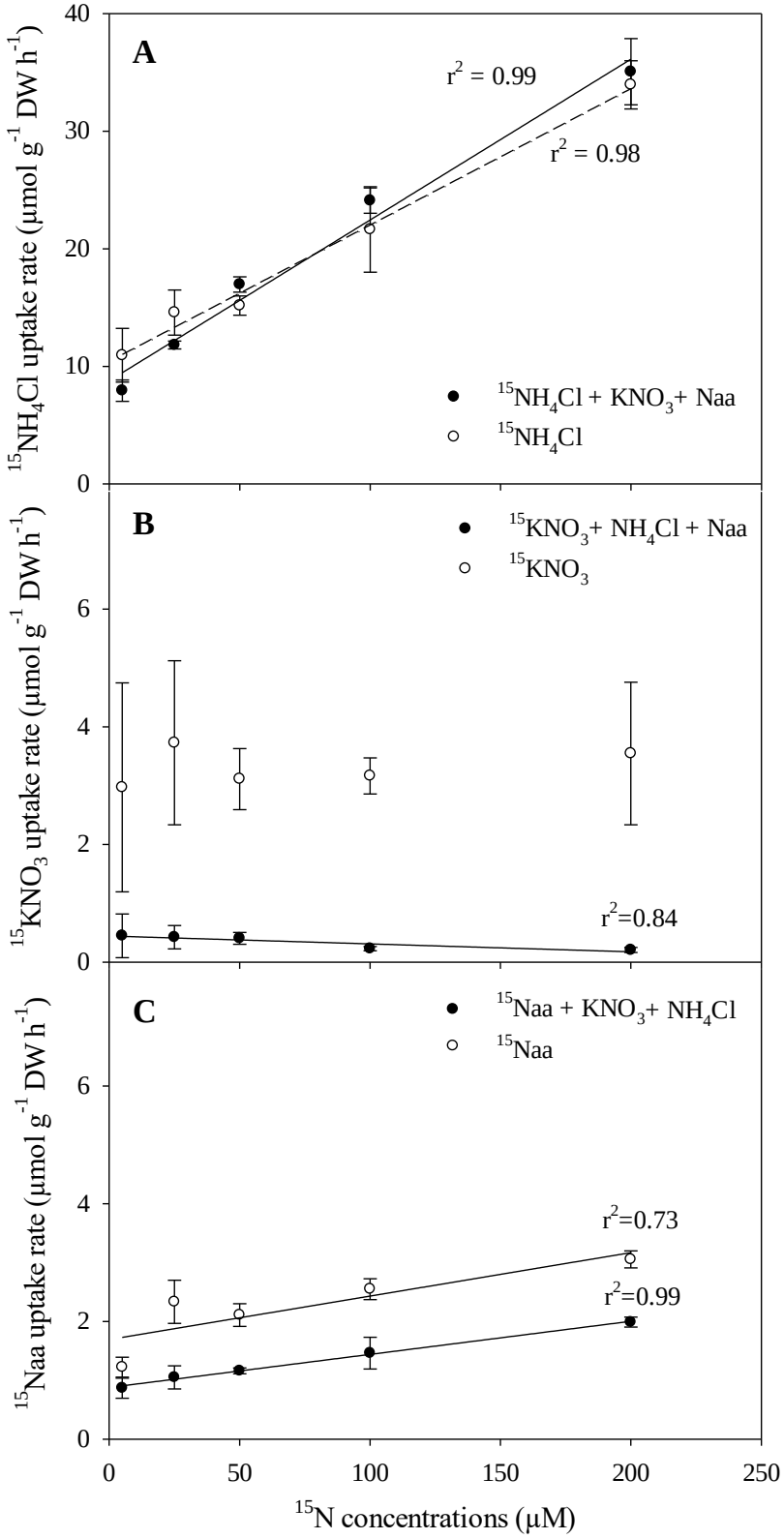

Fig. 3. Uptake rates (µmol g^{-1} DW h^{-1}) of ammonium (A) nitrate (B) and amino acids (C) by *Asparagopsis armata* as a function of labelled nitrogen concentrations (µM) incubated with a single nitrogen source (○) and with multiples nitrogen sources (●). The lines represent the linear regression model fitted to the data. Values are mean ± SD (n = 3). DW, dry weight

In the presence of multiple nitrogen sources, the uptake rates of ammonium were significantly higher than those of nitrate and amino acids, showing that ammonium is the preferential nitrogen source of *A. armata* (Fig. 4, Table 4). Ammonium uptake rates reached 35.1 μmol g^{-1} DW h^{-1} at 200 μM, the highest concentration tested, and were, on average, 56 and 15-fold higher than those of nitrate and amino acids, respectively. The uptake of organic nitrogen, as amino acids, was significantly higher (on average 4-fold higher) than the uptake of nitrate (Table 4).

Table 4. Results of the two-way analysis of variance (ANOVA) of the effect of nitrogen source and concentration on uptake rate of *Asparagopsis armata*

	df	*F*	*p*
Treatment	2	3690.896	<0.001
Concentration	4	190.808	<0.001
Interaction	8	89.562	<0.001
Residual	30		

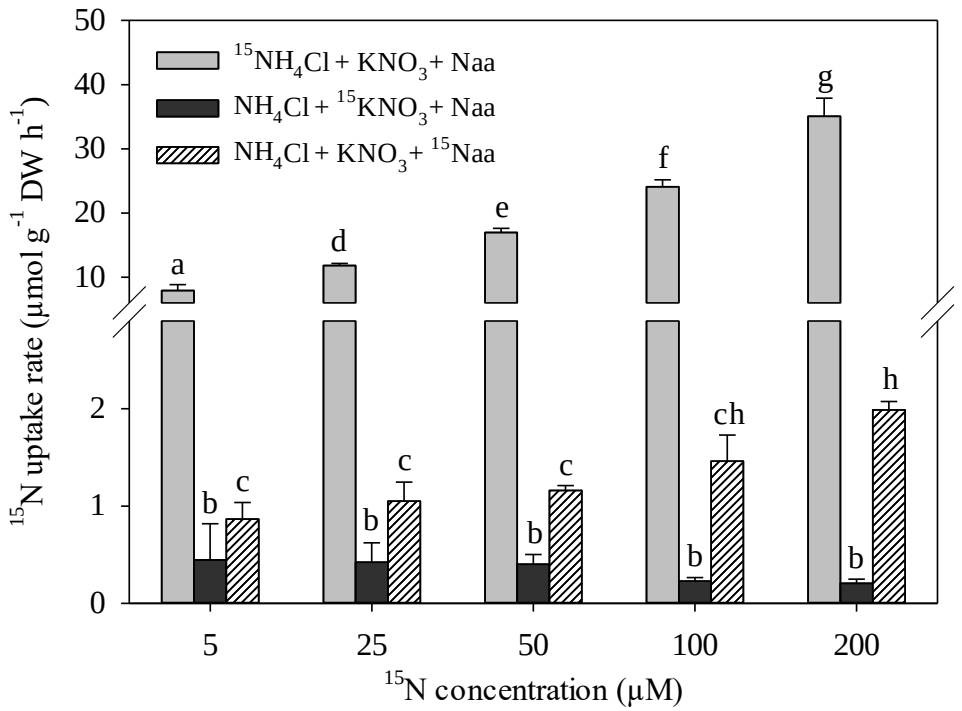

Fig 4. ^{15}N uptake rates (μmol g^{-1} DW h^{-1}) of the different N sources ($^{15}NH_4Cl$, $^{15}KNO_3$ and ^{15}N-amino acids) of *Asparagopsis armata* in the presence of other non-labelled nitrogen sources as a function of nutrient concentration (μM). Data are mean ± SD (n = 3); different letters represent significant differences among the treatments (P < 0.05)

3.3. Growth rates

There was no evident pattern of growth of *A. armata* and nutrient concentration for any of the nitrogen sources (Figs. 5 and 6). The presence of multiple nitrogen sources in the culture medium did not affect significantly the growth rate of *A. armata* relative to when only one single nitrogen source (ammonium, nitrate or amino acids) was present (Fig. 5, Table 5). Similarly, there were no significant differences in the specific growth rate of *A. armata* when only one single nitrogen form was present in the incubation medium (Fig. 6, Table 6). There was a high variability in the growth rates among replicates for all nitrogen sources and concentrations. Average specific growth rates varied from 3.16 ± 3.23 % day^{-1} in cultures containing multiple nitrogen sources (NH_4^+ + NO_3^- + N-aa) at 200 μM each (total N = 600 μM) to 12.02 ± 4.98 % day^{-1} in cultures containing ammonium at 100 μM as single nitrogen source of the average specific growth rates of *A. armata* when ammonium, the preferential nitrogen source, was individually supplied was, on average, 1.5-fold higher compared to when ammonium was supplied with nitrate and amino acids (Fig. 5A).

Table 5. Results of the two-way analysis of variance (ANOVA) of the effect of the effect of the presence of multiple nitrogen sources and nutrient concentration on the specific growth rate (% day^{-1}) of *Asparagopsis armata*

Source	NH_4Cl			KNO_3			Naa		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Treatment	1	3.428	0.079	1	0.382	0.544	1	0.083	0.776
Concentration	4	4.432	0.010	4	2.005	0.133	4	0.836	0.518
Interaction	4	0.700	0.601	4	0.636	0.643	4	1.017	0.147
Residual	20			20			20		

Table 6. Results of the two-way analysis of variance (ANOVA) of the effect of nitrogen source and concentration on specific growth rate (% day^{-1}) of *Asparagopsis armata*

	df	*F*	*P*
Treatment	2	1.811	0.185
Concentration	4	0.323	0.860
Interaction	8	0.819	0.594
Residual	24		

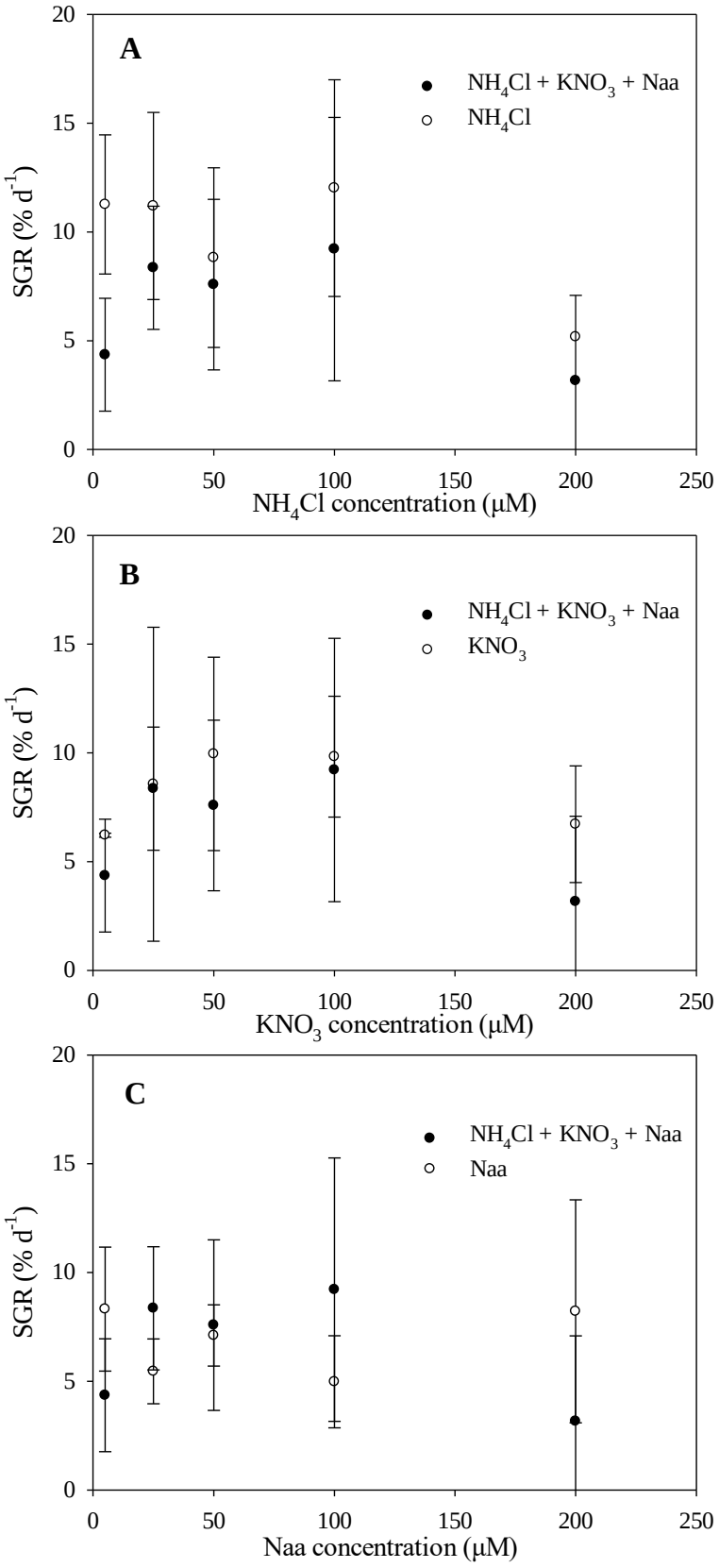

Fig 5. Specific growth rate (% day-1) of *Asparagopsis armata* in the presence of ammonium (A) nitrate (B) and amino acids (C), supplied alone (○) or in combination (●).Values are mean ± SD (n = 3)

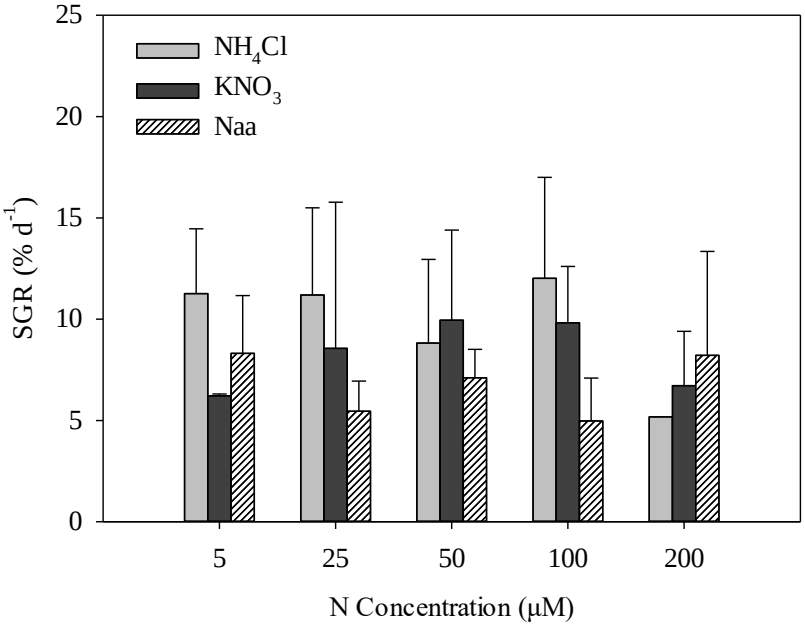

Fig 6. Specific growth rate (% day^{-1}) of *Asparagopsis armata* in the presence of different N sources, supplied alone in the incubation media. Values are mean ± SD (n=3)

4. DISCUSSION

We report in this study significant differences in the ability of *A. armata* to take up two forms of inorganic nitrogen, with a clear preference for ammonium over nitrate, as well as the ability to take up organic nitrogen in the form of amino acids. The differences in the uptake of the various nitrogen forms were not reflected in the growth rate of the seaweed. These results have implications for both researchers and *Asparagopsis* commercial cultivation.

4.1. Nitrogen uptake

The uptake of the three forms of nitrogen (ammonium, nitrate and amino acids) by *A. armata* described a typical temporal pattern, with enhanced initial uptake rates (the surge uptake phase), when uptake rates exceeded by several-fold those of the subsequent uptake phase, the internally controlled phase, characterized by lower but relatively constant uptake rates. This temporal pattern is characteristic of nitrogen-limited seaweeds when supplied with a pulse of nitrogen (Pedersen, 1994; Dy and Yap, 2001; Raikar and Wafar, 2006; den Haan et al., 2016). The uptake rates of ammonium, nitrate and amino acids by *A. armata* stabilized 105 minutes after an initial exposure to nutrient pulses of high concentration (~200 µM). The magnitude and duration of the

surge uptake phase depends on the nitrogen status and the intracellular pools of the individuals (Fujita et al., 1985) and is highly influenced by the concentration of the nitrogen pulse (e.g. Dy and Yap, 2001). Shorter surge uptake phases (<60 minutes) are usually reported in the literature for seaweed species, but incubated at lower initial pulses of nitrogen (<100 µM). For example, in the red seaweed *Kappaphycus alvarezii* the surge uptake phase of ammonium lasted only 30 min after an initial pulse of 30 µM NH_4Cl (Dy and Yap, 2001). Similarly, in the brown seaweed *Dictyota menstrualis*, the ammonium surge uptake phase was shorter (20 to 30 min) in response to a pulse of ammonium of low concentration (5 µM) compared to a pulse of 50 µM (up to 60 min) (den Haan et al., 2016). The uptake of organic nitrogen (as amino acids) by *A. armata* also displayed a much longer surge phase when compared to that measured for the uptake of urea (up to 5 min) by *Gracilaria vermiculophylla* and *Ulva lactuca* after an initial pulse of 8 µM (Tyler et al. 2005). Conversely, the uptake of organic nitrogen by *U. prolifera* did not show a surge phase when a pulse of 80 µM of total nitrogen (including 20 µM of urea and 20 µM of amino acids) was supplied (Li et al., 2019). Another characteristic of the *A. armata* nitrogen uptake temporal pattern is the high variability observed in the uptake rates during the initial phase. . High variability in the uptake rates during the first minutes/hours of incubation has also been observed in other seaweeds (Dy and Yap, 2001) and in seagrasses (Alexandre et al., 2016). This suggests that the internal content of nitrogen in *A. armata* is highly variable. Despite the transient nature of the surge uptake phase, it is evident that *A. armata* has a capacity for a rapid accumulation of nitrogen. This is an important mechanism to sustain growth in areas where nutrient availability is sporadic and occurs in the form of pulses. In the context of aquaculture, it suggests that growth rate of *A. armata* can be maintained using nitrogen pulses as a feeding strategy (Smit et al., 1996). However, in most cases of the seaweed tank aquaculture, seaweeds are produced under a constant flow of fish effluents loaded with nitrogen (Ghaly et al., 2005). In this case, the nitrogen uptake rates determined during the internally controlled phase reflect more adequately the long-term capacity of *A. armata* as a seaweed biofilter. I ^{15}N uptake rates of the different forms of nitrogen by *A. armata* were assessed at a range of concentrations after the surge uptake phase, i.e. during the internally controlled phase, when rates were stabilized.

During the internally controlled phase, *A. armata* took up ammonium preferentially over the two other forms of nitrogen (nitrate and amino acids), either when supplied

separately or in combination. This is probably due to the lower energetic cost associated with the uptake and readily assimilation of ammonium into amino acids, compared to the uptake of nitrate that must first be reduced to nitrite and then to ammonium prior assimilation (Turpin, 1991; Hurd et al., 2014). This preference pattern is a common feature of other seaweeds species (Smit, 2002; Naldi and Wheeler, 2002; Cohen and Fong, 2004; Abreu et al., 2011; Luo et al., 2012; Grote, 2016; Han et al., 2017; Ross et al., 2018; Xi et al., 2019) and even seagrasses (Alexandre et al., 2011 and references therein). Nevertheless, nitrate was reported the preferred nitrogen form of *U. prolifera* when it was supplied simultaneously with other nitrogen sources (Li et al., 2019). In a similar study, where uptake rates were assessed during the internally controlled phase, the ammonium uptake rates by *G. lichenoides* and *Caulerpa lentillifera* were 13.4 and 5.8 μmol g DW^{-1} h^{-1} respectively, when ammonium and nitrate were simultaneously supplied at concentrations of 71 μM of each source. Comparing with these species, the ammonium uptake rate of *A. armata* was higher (18.73 μmol g DW^{-1} h^{-1}) when exposed to the same nutrient concentration.

Notably, the high affinity and capacity for ammonium by *A. armata* were not affected by the presence of nitrate and amino acids in solution. Conversely, the uptake of nitrate and amino acids by *A. armata* decreased significantly in the presence of other nitrogen sources. The inhibition of nitrate uptake rates in the presence of ammonium is common amongst seaweeds (Naldi and Wheeler, 2002; Rees et al., 2007; Grote, 2016; Ross et al., 2018), seagrasses (Alexandre et al., 2010) and aquatic plants (Thursby and Harlin, 1984, Dudley et al., 2001). An inhibition mechanism mediated by a product of ammonium assimilation (e.g. glutamine) or a repression of the active transport of nitrate across the membrane roots have been suggested to explain the reduced uptake of nitrate in the presence of ammonium in seagrasses and higher plants (Iizumi and Hattori, 1982; Flynn, 1991). An alternative hypothesis suggests that the presence of ammonium leads to cessation of the induction of nitrate reductase and may inhibit the biosynthesis of nitrate/nitrite reductase, two key enzymes involved in the assimilation of nitrate with consequent effects on the uptake rates (Syrett and Leftley, 2016). Interactions between inorganic and organic nitrogen sources during uptake are less studied but have been reported in terrestrial plants (Henry and Jefferies, 2003; Thornton and Robinson, 2005; Gioseffi et al, 2012). In these studies, the uptake of inorganic nitrogen was downregulated by the presence of organic nitrogen in solution and vice-versa. On the

other hand, in the green seaweed *Cladophora parriaudii*, the presence of both ammonium and urea in the medium enhanced the uptake of the other co-existing inorganic nitrogen forms (Ross et al., 2018). In *A. armata*, the uptake of inorganic nitrogen as ammonium was not affected by the presence of organic nitrogen, but the uptake of organic nitrogen as amino acids decreased in the presence of inorganic nitrogen. Although it is not possible to isolate the effect of ammonium from that of amino acids on the uptake rates of nitrate, our results provide evidence for the existence of a combined effect of both on the uptake rates of nitrate. First, we showed that ammonium affected negatively the nitrate uptake of *A. armata*. In addition, *A. armata* showed a preference for amino acids over nitrate, suggesting a potentially negative effect of organic nitrogen on the nitrate uptake rates. These results indicate that the removal efficiency of nitrate and amino acids by *A. armata* is reduced in the presence of multiple nitrogen sources.

Interestingly, amino acids were taken up at faster rates than nitrate by *A. armata*, suggesting that the uptake of small molecules of organic nitrogen can substantially contribute to the overall nitrogen demand of the species, particularly under conditions of low inorganic nitrogen availability. The uptake of amino acids by seaweeds is poorly investigated and has been reported only in a few studies (Tyler et al., 2005; Li et al., 2019; Xiu et al., 2019). In contrast to *A. armata*, *G. vermiculophylla* and *U. lactuca* showed saturation kinetics for the uptake of a mixture of six amino acids within the range 0-12 μM (Tyler et al., 2005). Compared to these seaweeds, *A. armata* showed a higher capacity for uptake of amino acids, since the uptake rate increased linearly with nutrient concentration (5–200 μM), without reaching saturating within this concentration range. The high uptake capacity for organic nitrogen demonstrated by *A. armata* represents a competitive advantage over other species which lack or have a lower capacity for the uptake of organic nitrogen in environments where the concentrations of this form of nitrogen are high.

The uptake of nitrogen by macroalgae is usually described by a saturating function of substrate concentration according to the Michaelis–Menten equation ($V = Vmax * S / Km + S$), where the affinity for the substrate is given by the α ($Vmax / Km$) (Pedersen 1994; Hurd et al., 2014). However, the uptake of ammonium, nitrate and amino acids by *A. armata* did not follow the Michaelis-Menten saturation kinetics, as saturation was not reached for ammonium and amino acid uptake even at 200 μM, the highest

concentration tested. The uptake of ammonium and amino acids was, therefore, best described by a linear model, increasing linearly with nutrient concentration. On the other hand, the uptake of nitrate did not follow an increasing pattern with concentration, showing that *A. armata* has a limited capacity to use nitrate, even when nitrate was the only N source available. This may represent a disadvantage in areas were other nitrogen sources are scarce or inexistent, as growth and survival may be compromised. Non-saturable kinetics for ammonium uptake has been shown for other seaweeds (Taylor et al., 1998; Smit, 2002; Abreu et al., 2011; Wang et al., 2014; den Haan et al., 2016). One possible explanation for the observed linear uptake of ammonium and amino acids in *A. armata* may be that the highest concentration tested (200 μM) was not sufficient to induce a saturating uptake response in *A. armata*. We showed that *A. armata* is able to take up ammonium and amino acids at concentrations as high as 200 μM without reaching saturation.

4.2. Growth rate

The availability of the different nitrogen sources in the growth medium had no influence on the growth rate of *A. armata*, either when these were supplied alone or in combination, and growth rates did not show an increasing pattern with nutrient concentration. Given the high capacity and affinity of *A. armata* for the uptake of ammonium demonstrated by the ^{15}N uptake experiments a higher growth rate of the seaweed was expected when ammonium was available in the growth medium. However, the higher uptake rates of ammonium did not translate directly into higher growth rates of *A. armata*. Similarly, Corey et al. (2013), also found no differences in the growth rates of the red seaweeds *P. palmata* and *C. crispus* exposed at different ammonium to nitrate ratios for two weeks, regardless of their preference for ammonium as a nitrogen source.

Although not significantly different, the growth rates of *A. armata* were generally slightly slower when nitrogen was supplied as multiple sources (ammonium, nitrate and amino acids) compared to when only one single nitrogen source was present, and particularly at 200 μM, where the total nitrogen concentration is 600 μM. One possible explanation may be that such high nitrogen concentration may have reached a toxic level, with effect on the growth rates. For example, the growth of seedlings of *S. hemiphyllum* decreased with concentrations of ammonium, nitrate and urea higher than 50 μM (Han et al., 2018). The hypothesis behind these observations was that nutrient

uptake and accumulation in the tissues required most of the cells' energy and resources, with a reduced amount available for growth (Fong et al., 2004; Wilson et al., 2015).

The methodological approach used to measure growth of the filamentous *A. armata* was adapted from Paul et al., (2014) and Mata et al., (2017). The SGRs of *A. armata* varied from 3.16 to 12.02 % d^{-1} after 14 days, similar to those reported for *A. taxiformis* grown under different temperatures over 42 days (7.17 to 13.59 % d^{-1}) (Mata et al., 2017). However, we experienced that this method of cutting small tips of the filaments is very invasive and prone to physically damage the individuals. Some of the tips in our study did not develop the normal growth pattern for the species, and that may have influence our results We suggest a different approach for future studies using seaweed cultures with a higher initial density and measure growth using reticulated spinner (Ross et al., 2017). Furthermore, this method is more representative of the conditions at which seaweeds are cultured within tank systems.

4.3. Implications to aquaculture

Determining the nitrogen preferences and uptake rates for seaweed species are key factors to determine their suitability as biofilters in different animal aquaculture systems and to improve their efficiency by selecting the location for biofiltration within the aquaculture system (Corey et al 2013; Rodela and Hurd, 2019). From this study, it is clear that ammonium is the nitrogen form preferred of *A.armata*, which takes it up at a much higher degree than any other nitrogen from present in the medium and it and is not affected by their presence in the medium. These results explain well the high ammonium biofiltration ability of *A. armata* in fish farm flow through effluents (Schuenhoff et al., 2006; Mata et al., 2010). In a recirculation aquaculture system, where ammonium is transformed into nitrate in the bacterial biofilter, the A. armata cultivation system should be placed prior to the bacterial biofilter to be exposed to the highest concentrations of ammonium in the system. This study demonstrates for the first time the ability of *A. armata* to uptake organic forms of nitrogen. Dissolved organic nitrogen represents a significant fraction (40–50% of the total dissolved nitrogen) of the waste components of marine fish pond effluents (Shpigel et al., 2019). The biofiltration of this large component of total nitrogen should be taken more into account in future studies on bioremediation of aquaculture effluents using seaweeds. The ability demonstrated by *A. armata* for the uptake of amino acids, combined with its high

ammonium uptake capacity confirms this species as an excellent candidate as a nitrogen biofilter in animal aquaculture systems.

5. CONCLUSIONS

We demonstrated that *A. armata* is capable of take up simultaneously both inorganic and organic nitrogen, showing a clear preference for ammonium over the two other forms of nitrogen (nitrate and amino acids), either when supplied separately or in combination. However, the presence of ammonium in the medium reduced the uptake of the other nitrogen forms present. Because interactions between the different nitrogen sources occurred, this study highlights the importance of assessing uptake rates of *A. armata* in incubations using multiple nitrogen sources. On the other hand, amino acids were taken up at faster rates than nitrate by *A. armata*, showing that uptake of organic nitrogen should be included in future studies assessing total nitrogen budgets.

ACKNOWLEDGMENTS

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