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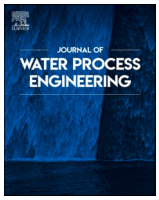

Effect of ammonium/nitrate ratio on microalgae continuous cultures: Species-specificity of nutrient uptake and modelling perspectives

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ABSTRACT

Microalgae-based wastewater treatment is promising in view of circularity as they can uptake most of the nitrogen sources usually present in waste streams: ammonium, nitrite and nitrate as well as some organic species. However, regulation pathways are not fully understood yet. This complexity is a challenge for accurate modelling of these processes, necessary for the design and optimization of wastewater treatment systems. It is generally observed in microalgae that the presence of ammonium inhibits the assimilation of other nitrogen compounds. This was confirmed in this work for *Synechocystis* sp. PCC6803, even in acclimated continuous cultures. A different uptake regulation was instead observed for the species *Auxenochlorella protothecoides*, able to uptake nitrate even when high concentrations of ammonium are present. From a modelling perspective, the Droop model was implemented to account for the exploitation of both ammonium and nitrate: a switch function was used to describe the ammonium inhibition, but this was applicable for *Synechocystis* only. For *A. protothecoides*, a changing nutrient uptake rate is needed, which was found to be function of the dissolved nitrogen concentration. A bioinformatic analysis was performed to infer putative players involved in nitrogen uptake, accounting for the different behavior of the two taxonomic groups.

1. Introduction

The development of sustainable processes that recover resources and preserve natural habitats is one of the main concerns of our time. Human activities produce wastewaters rich in organic and inorganic compounds that must be reintroduced in natural waterbodies without damaging ecosystems. Effluents from domestic, agricultural, and industrial sources contain nitrogen (N) and phosphorous (P), but also heavy metals and other compounds, whose concentration is reduced in multiple-step treatments [1].

A promising alternative to conventional wastewater treatment (WWT) is the application of a diverse group of photosynthetic microalgae, including eukaryotic and prokaryotic species [2]. The potential of these microorganisms consists in the ability to consume pollutants in wastewater for the production of biomass that can be further applied for different purposes depending on its quality, effectively establishing a circular economy process [3]. Microalgae can be applied alone or in consortia with bacteria, establishing a symbiotic relation based on the exchanges of gases and compounds [4–6], and, depending on the species, they can grow in photoautotrophic, mixotrophic and heterotrophic metabolisms, representing a resource to treat different kinds of wastewater. Most of all, microalgae have been demonstrated to be efficient to drastically reduce nitrogen and phosphorus compounds [7,8].

The composition of wastewater widely varies depending on its source. One of the main pollutants is nitrogen, which above certain limits is a threat to both health and ecological environments. Nitrogen in wastewater is present in inorganic forms, such as ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-), and organic compounds such as urea, amino acids and proteins. Ammonium is the preferred nitrogen source for most the eukaryotic microalgae and cyanobacteria [9], and is the most abundant species in domestic, agricultural and aquaculture wastewater. Its presence usually inhibits the assimilation of other nitrogen sources, such as nitrite (NO_2^-), nitrate (NO_3^-), and urea. The preference for ammonium is probably due to energetic reasons, as ammonium does not need to be further reduced or hydrolyzed and can be directly combined with 2-oxoglutarate (2-OG) to form glutamic acid (Glu), in a reaction catalysed by the glutamine synthetase-glutamate synthase (GS-GOGAT) [10]. The preference for ammonium over other nitrogen sources has been observed in various eukaryotic microalgae, such as *Chlorella vulgaris* [11], *Chlamydomonas reinhardtii* [12], and

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Nannochloropsis sp. [13] and cyanobacteria, such as *Synechocystis* [14] and *Anabaena cylindrica* [15]. However, most of these experiments were conducted in batch systems in which the biomass grows consuming an initial supply of nutrients, while the preferred operation mode for WWT is the continuous one, where possible different acclimation regimes can occur. As recently observed [16], the microalgal behavior may be different in chemostats, where nutrients are continuously supplied, and the biomass reaches a steady state condition following an initial acclimation phase. The acclimation phase may in fact play a role in determining the expression of transporters and regulatory proteins involved in nitrogen assimilation.

In cyanobacteria, the assimilation of inorganic nitrogen has long been studied leading to the identification of transporters for ammonium, nitrite and nitrate uptake. Ammonium transporters are classified in the ammonium/methylammonium permeases (AMT/MEP) family. Nitrate/nitrite transporters can be divided in two families: the ABC-type NRT, which comprises four proteins (NrtA, NrtB, NrtC, and NrtD), and the MFS family transporter NrtP. In cyanobacteria, several proteins involved in uptake have been identified, such as PII, a major metabolism sensor responsible for regulating nitrate uptake [17]. In eukaryotic microalgae, AMT are mainly responsible for NH_4^+ uptake, whereas three classes of transporters are responsible for NO_2^- and NO_3^- uptake: the Nitrate Transporter 1 (NRT1) family belonging to the Peptide Transporters (PTR), the Nitrate Transporter 2 family (NRT2), and the family NAR1 (Nitrate Assimilation-Related component 1). The mechanism of ammonium regulation of nitrate assimilation has been studied in *C. reinhardtii*, but the picture is still fragmented, and the regulatory mechanisms remain to be clarified [18].

Unlike most cyanobacterial species, where the inhibition by ammonium overrides the assimilation of nitrite, in eukaryotic microalgae a positive effect of nitrate on its own assimilation may be active in the presence of ammonium. In *C. reinhardtii,* the repression of nitrate assimilation is not simply inhibited by ammonium and appears to be a quantitative process, modulated by the concentrations of both ammonium and nitrate [19,20].

Accordingly, it is clear that a higher complexity should be accounted for when applying microalgae consortia to wastewater with different nitrogen species. The interaction between the various nitrogen species should be of great interest when microalgae are applied as biological WWT since the behavior of microalgae in complex media with multiple nitrogen sources has yet to be defined. Unlike traditional WWT methods, such as the Activated Sludge systems, which have been successfully modelled and validated, microalgae modelling is still in progress [21], and it usually does not consider the complexity of nitrogen uptake. The description through mathematical models is necessary for process design and optimization, in particular to better understand the availability of different nitrogen sources which also affect the bacterial composition (e.g., competition with nitrifying bacteria). In addition, traditional approaches for microalgae modelling apply the Monod model for the nutrient uptake, which is not appropriate to describe the internal quota accumulation of nitrogen and phosphorus [22]. An accurate modelling of microalgal growth and nutrient consumption is a fundamental step for an efficient WWT process.

In this work, we applied the Droop equation [23] to describe biomass growth and nutrients uptake, a model that describes growth as a function of internal nutrient quota. In the simultaneous presence of multiple nitrogen sources in the medium, the approach based on Solimeno model was used [24], and adjusted depending on the species considered. With this work, our aim is to investigate the behavior of two microorganisms, the eukaryotic microalga *Auxenochlorella protothecoides*, which showed a peculiar regulation of uptake, and the cyanobacterium *Synechocystis* sp. PCC 6803, in chemostat reactors when two different nitrogen sources, i.e. ammonium and nitrate, are supplied. We approach this problem from a modelling perspective based on experimental data, and try to link the observed differences to the biological and molecular differences of transport and uptake between the two microorganisms. This work represents the first attempt to better describe the nutrient uptake by microalgae in the presence of different nitrogen sources, in continuous systems. It also applies a multidisciplinary approach that tries to explain the model results with the biological information related to transport and uptake.

2. Material and methods

2.1. Microalgal strains and culture medium

The cyanobacterium *Synechocystis* sp. PCC 6803 and the eukaryotic microalga *Auxenohlorella protothecoides* 33.80 (formerly classified as *Chlorella protothecoides*) were purchased respectively from the Pasteur Culture Collection of Cyanobacteria (France) and SAG Goettingen (Germany). The cultures were axenically maintained in BG11 medium at room temperature in Erlenmeyer flasks, as reported in Marchetto et al., 2021 [25].

To study the effect of multiple nitrogen sources in continuous systems, the composition of BG11 was changed to vary the concentration of ammonium, supplied as $(NH_4)_2SO_4$, respectively, and nitrate, supplied as $NaNO_3$. The concentration of other nutrients was doubled to provide them in excess and avoid limitations. The media were prepared using demineralized water and sterilized in autoclave for 20 min at 121 °C.

2.2. Experimental set up

Continuous experiments were carried out in vertical flat-plate photobioreactors (PBR) with a thickness of 3.5 cm and a working volume of 200 mL. The PBRs were continuously illuminated by a white LED lamp and maintained at a constant temperature of 24 °C for *A. protothecoides* and 30 °C for *Synechocystis*.

Fresh sterilized medium was continuously supplied to the reactor at a constant flowrate through a peristaltic pump (Sci-Q 400, Watson-Marlow 120 U/DM3), while an overflow tube was located on the opposite side to keep the working volume constant. The mixing was ensured by a magnetic stirrer, placed at the bottom, and by a continuous flow of CO_2-enriched air (5 % *v*/v) at a flow rate of 1 L h^{-1}. The system obtained is approximated to a Continuously Stirred Tank Reactor (CSTR).

By maintaining a constant flowrate and working volume, it is possible to operate in chemostat mode and reach the steady state. After inoculation, the reactors showed a transitory period of acclimation (average duration 7–10 days, checked by OD measuring of biomass concentration), before steady state was reached. Steady state was then kept for at least 5 days, during which sampling to determine biomass and nutrient concentration were performed. Subsequently, the culture conditions were changed, in terms of residence time or feed concentration, and another transitory phase was observed before reaching a new steady state. Usually, the timing needed to reach subsequent steady states is lower than the first one (about 7 days). The flowrate was periodically modified to operate at different hydraulic retention time (HRT), calculated as the ratio between the reactor volume and the inlet flowrate.

To study the nitrogen uptake in presence of two different nitrogen sources, i.e. ammonium and nitrate, several experiments have been performed in *Synechocystis* and *A. protothecoides*, as summarized in Table 1. The extent of ammonium stripping by bubbling was measured and accounted for in the modelling section. As the purpose of the work is to apply a modelling approach to describe the regulation of nutrient consumption by the two species, experiments with nitrate and ammonium alone are needed. For *A. protothecoides*, data and model parameters from Barbera et al. 2022 [26] were used to describe the assimilation of NH_4^+ and NO_3^- when provided as individual nitrogen species. On the other hand, for *Synechocystis*, an additional set of experiments (Table 2) was required to determine the values of the kinetic parameters, as described in Section 3.

Table 1
Experimental condition for *Synechocystis* sp. and *A. protothecoides*. Experiments with a mix of ammonium and nitrate.

	Synechocystis			
Experiment number	Incident light intensity	HRT	NH_4-N $_{in}$	NO_3-N $_{in}$
	(μmol photons m^{-2} s^{-2})	(d^{-1})	(mg L^{-1})	(mg L^{-1})
1	150	1.1	15.5	50.3
2	150	1.1	22.0	37.7
3	150	0.9	59.2	35.8
4	150	1.1	44.0	38.0

	A. protothecoides			
Experiment number	Incident light intensity	HRT	NH_4-N $_{in}$	
	(μmol photons m^{-2} s^{-2})	(d^{-1})	(mg L^{-1})	(mg L^{-1})
5	100	0.70	10.0	40.0
6	100	0.70	40.0	10.0
7	100	0.65	30.0	60.0
8	100	0.71	80.0	30.0
9	100	0.72	100.0	100.0

Table 2
Experimental condition for *Synechocystis* for the determination of the kinetic parameters relative to a single nitrogen species.

Experiment number	Incident light intensity	HRT	NH_4-N $_{in}$	NO_3-N $_{in}$
	(μmol photons m^{-2} s^{-2})	(d^{-1})	(mg L^{-1})	(mg L^{-1})
10	150	0.95	19.3	–
11	150	0.95	33.8	–
12	150	0.85	59.4	–
13	150	0.87	69.5	–
14	150	0.88	–	23.3
15	150	0.86	–	55.0
16	150	1.1	–	80.3
17	150	0.86	–	93.8

2.3. Analytical methods

Biomass concentration was monitored daily. Optical density (OD) was measured at 750 nm using a spectrophotometer (Shimadzu). Dry weight was determined by filtering a known volume of culture with nitrocellulose filters (pore size 0.45 μm for *A. protothecoides* and 0.22 μm for *Synechocystis*). The samples were dried at 100 °C in a laboratory oven before weighing.

Ammonia (NH_4^+-N) and nitrates (NO_3^--N) concentration in fresh and exhausted medium was measured using standard test kits based on colorimetric reactions (Hydrocheck Specratest kit by Reasol®, id 6201 and id 6223). The N-quota in biomass was determined based on the external nutrient consumption, and then verified by measurement of nitrate released from biomass following alkaline persulfate digestion.

2.4. Bioinformatics

To investigate the distribution of genes for proteins involved in nitrogen assimilation in cyanobacteria and microalgae, different databases have been consulted to integrate information, including NCBI, Phytozome 13 (https://phytozome-next.jgi.doe.gov/), and Phycocosm (https://phycocosm.jgi.doe.gov/phycocosm/home). Some proteins had already been identified and annotated, and corresponding genomic sequences were retrieved through TBlastN; moreover, BlastN and BlastP were also used to find orthologous genes and proteins. The assembly access codes are listed below as reported in NCBI: *Anabaena cylindrica* PCC 7122 - GCF_000317695.1, *Arthrospira platensis* C1 - GCF_025200965.1 [27], *Microcystis aeruginosa* NIES-843 - GCF_000010625.1 [28], *Nostoc* sp. PCC 7120 (GCF_000009705.1) [29], *Oscillatoria acuminata* PCC 6304 - GCF_000317105.1 [30], *Synechocystis* sp. PCC 6803 - GCF_000009725.1 [31], *Synechococcus elongatus* PCC 7942 - GCF_000012525.1, *Thermosynechococcus vestitus* BP1 - GCF_000011345.1 [32], *Auxenochlorella protothecoides* UTEX 25 - GCA_003709365.1 [33], *Chlamydomonas reinhardtii* - GCF_000002595.2 [34], *Chlorella sorokiniana* SLA-04 - GCA_025917655.1, *Chlorella variabilis* NC64A - GCF_000147415.1 [35], *Dunaliella salina* CCAP 19/18 - GCA_002284615.2 [36], *Haematococcus lacustris* - GCA_030144725.1 [37], *Tetradesmus obliquus* UTEX 3031 - GCA_030272155.1 [38]. Multialignment of protein sequences was performed with CLUSTAW.

3. Mathematical model

From a mathematical modelling point of view, a few models have been published so far to describe microalgae growth in wastewaters, taking into account the presence of multiple nitrogen species. Examples of these studies include the BIO_ALGAE model [24] and the ALBA model [39], both aiming at modelling the behavior of algae-bacteria consortia in WWT applications. Their main assumption is that algae phototrophic growth using NH_4^+ as nitrogen source is favoured over growth using NO_3^-, considering that the latter requires more energy to be assimilated. Therefore, the "two growths" are considered as separate, with growth over nitrate occurring only when ammonium is limiting. Mathematically, this is achieved by means of a *switch function*. However, these models, which are based on well-acknowledged Activated Sludge Models (ASMs), adopt a Monod-type function to describe the kinetic dependence of biological growth on nutrients availability. This assumes a constant yield of biomass-over-nutrients (i.e., a constant elemental composition of the biomass), which has been demonstrated to be inaccurate when dealing with microalgae, who are quite versatile and adapt their internal composition based on the environmental conditions [40]. In this regard, the Droop model, which uncouples microalgae growth from nutrients uptake by considering a variable internal nutrient quota, has proven to be more accurate [23,26,41].

According to the Droop model, microalgae growth ($r_{x,avg}$, g m^{-3} d^{-1}) is modelled as a function of light and internal nutrients quota, according to [26]:

$$r_{x,avg} = \frac{1}{W}\int_0^W \left[\left(\mu_{max} \bullet \frac{I(z)}{I(z) + K_I \bullet \left(\frac{I(z)}{I_{opt}} - 1\right)^2} \bullet \left(1 - \frac{q_{N,min}}{q_N}\right) \bullet \left(1 - \frac{q_{P,min}}{q_P}\right) - k_d \right) \bullet C_x \right] dz \quad (1)$$

In eq. (1), C_x is the biomass concentration (g_X m^{-3}); q_i and $q_{i,min}$ (i = N, P) represent the internal nutrient quota and the minimum nutrient quota in the biomass for nitrogen and phosphorus (g_i/g_X), respectively; μ_{max} is the maximum specific growth rate of the microorganism (d^{-1}), while k_d is the decay rate (d^{-1}), related to cell respiration and maintenance; K_I and I_{opt} are model parameters related to the effect of light intensity, namely the light half-saturation constant (μmol m^{-2} s^{-1}) and the optimal light intensity (μmol m^{-2} s^{-1}). Since light intensity is not uniform along the reactor depth z, but decreases according to the Lambert-Beer law:

$$I(z) = I_o exp(-k_a C_x z) \quad (2)$$

the local biomass growth rate is calculated and then averaged along the reactor depth, W. In eq. (2), I_0 is the incident light intensity, and k_a is the biomass light absorption coefficient (m^2 g_X^{-1}).

Nutrients uptake rates (g_i m^{-3} d^{-1}), including NH_4-N, NO_3-N, and P, are described according to:

$$r_{NH_4-N} = -\rho_{NH_4-N} \bullet \frac{C_{NH4-N}}{K_{NH4-N} + C_{NH4-N}} \bullet \left(\frac{q_{N,max} - q_N}{q_{N,max}}\right) \bullet C_x \quad (3)$$

$$r_{NO_3-N} = -\rho_{NO_3-N} \bullet \frac{C_{NO_3-N}}{K_{NO_3-N} + C_{NO_3-N}} \bullet \frac{K_{i,NH4}}{K_{i,NH4} + C_{NH4-N}} \bullet \left(\frac{q_{N,max} - q_N}{q_{N,max}}\right) \bullet C_x \quad (4)$$

$$r_P = -\rho_P \bullet \frac{C_P}{K_P + C_P} \bullet \left(\frac{q_{P,max} - q_P}{q_{P,max}}\right) \bullet C_x \quad (5)$$

In eqs. (3)–(5), ρ_i($g_i\ g_X^{-1}\ d^{-1}$) is the maximum uptake rate for nutrient *i*, C_i($g_i\ m^{-3}$) is the nutrient concentration in the medium, K_i($g_i\ m^{-3}$) is the nutrient half-saturation constant, and $q_{i,max}$ is the maximum nutrient quota in the biomass. As it can be seen, in eq. (4) the inhibition term $\frac{K_{i,NH4}}{K_{i,NH4}+C_{NH4}}$ is included as a *switch function*, so that nitrate uptake only occurs when ammonium concentration is almost depleted. It should be noted that, as discussed later in the Section 4.2, the switch function was not applied in the case of *A. protothecoides*, so that the nitrate uptake is written as:

$$r_{NO_3-N} = -\rho_{NO_3-N} \bullet \frac{C_{NO_3-N}}{K_{NO_3-N} + C_{NO_3-N}} \bullet \left(\frac{q_{N,max} - q_N}{q_{N,max}}\right) \bullet C_x \quad (4a)$$

It should be noted that, according to [22], for *Synechocystis* sp. the P uptake rate can be simplified to a first-order kinetics with respect to phosphorus, so that:

$$r_P = -\gamma_P \bullet C_P \bullet C_x \quad (6)$$

with γ_P($m^3\ g_X^{-1}\ d^{-1}$) being the kinetic constant. To avoid possible P limitations, all experiments were provided with excess P (about 10 mg L^{-1} in the inlet, and the outlet concentration was always measured at steady state to check that no limitation occurred).

Finally, the accumulation of internal nutrient quota q_i is modelled, considering that it is the result of a positive contribution related to nutrient uptake from the medium, and a negative one due to consumption for biomass growth. Accordingly, for N and P it is obtained, respectively:

$$r_{q_N} = \left(\rho_{NO_3-N} \bullet \frac{C_{NO_3-N}}{K_{NO_3-N} + C_{NO_3-N}} \bullet \frac{K_{i,NH4}}{K_{i,NH4} + C_{NH4-N}} + \rho_{NH_4-N} \bullet \frac{C_{NH4-N}}{K_{NH4-N} + C_{NH4-N}}\right)\left(\frac{q_{N,max} - q_N}{q_{N,max}}\right) - q_N \bullet \frac{1}{W}\int_0^W \left(\mu_{max} \bullet \frac{I(z)}{I(z) + K_I \bullet \left(\frac{I(z)}{I_{opt}} - 1\right)^2} \bullet \left(1 - \frac{q_{N,min}}{q_N}\right) \bullet \left(1 - \frac{q_{P,min}}{q_P}\right) - k_d\right) dz \quad (7)$$

$$r_{q_P} = \left(\rho_P \bullet \frac{C_P}{K_P + C_P}\right)\left(\frac{q_{P,max} - q_P}{q_{P,max}}\right) - q_P \bullet \frac{1}{W}\int_0^W \left(\mu_{max} \bullet \frac{I(z)}{I(z) + K_I \bullet \left(\frac{I(z)}{I_{opt}} - 1\right)^2} \bullet \left(1 - \frac{q_{N,min}}{q_N}\right) \bullet \left(1 - \frac{q_{P,min}}{q_P}\right) - k_d\right) dz \quad (8)$$

The kinetic models of microalgal growth, NH_4-N, NO_3-N, and P uptake, and N and P quota were used to write the corresponding mass balances, referred to a CSTR reactor, for a total of 6 equations, according to:

$$\frac{dC_{x,out}}{dt} = -\frac{C_{x,out}}{HRT} + r_{x,avg} \quad (9)$$

$$\frac{dC_{i,out}}{dt} = \frac{C_{i,in} - C_{i,out}}{HRT} + r_i,\ i = NO_3 - N, \text{and P} \quad (10)$$

$$\frac{dC_{NH4-N}}{dt} = \frac{C_{NH4-N,in} - C_{NH4-N,out}}{HRT} + r_{NH_4-N} + k_L a \bullet \sqrt{\frac{D_{NH3}}{D_{O2}}} \bullet \left(K_H \bullet p_{NH3} - \frac{C_{NH4-N,out}}{\left(1 + 10^{(pKa-pH)}\right)}\right) \quad (11)$$

$$\frac{dq_i}{dt} = r_{q,i},\ i = \text{N and P} \quad (12)$$

In eq. (9)–(11), *HRT* (d) is the retention time of the culture inside the photobioreactor. Note that the material balance for ammonium (eq. (11)) includes a term related to ammonia loss to the atmosphere. In fact, ammonium ions in solution are in equilibrium with free ammonia (NH_3), which is volatile, and can therefore be transferred to the gaseous phase. This term depends on the overall mass transfer coefficient ($k_L a = 123\ d^{-1}$ measured for the reactors used in this study), corrected by taking into account the diffusivity of ammonia ($D_{NH3} = 2.4\ m^2\ s^{-1}$) with respect to oxygen ($D_{O2} = 2.5\ m^2\ s^{-1}$) and on the difference between the equilibrium ammonia concentration and the actual one. The former is calculated according to the Henry's law, where K_H is the Henry constant at the operating temperature and p_{NH3} is the partial pressure of ammonia in the gas. The actual concentration depends on pH through the acid dissociation constant pK_a.

The model parameters for *Synechocystis* sp. and *A. protothecoides* were retrieved by fitting the model to the experimental data using Matlab® fmincon function to minimize the sum of square errors between the experimental and calculated values of outlet biomass, nitrate and ammonium concentrations. The latter were determined as the steady-state values obtained from the solution of the dynamic material balances using ode23.

4. Result and discussion

4.1. Microalgae have species-specific nutrient uptake regulation in complex media

Several nitrogen species are commonly present in wastewater, especially ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) as well as some organic species, such as urea and amino acids. Such a complexity represents a challenge for an accurate modelling of the processes, which is necessary for the design and optimization of WWT systems. As mentioned above, it is generally observed that in most species, the presence of ammonium inhibits the assimilation of other nitrogen compounds. This phenomenon has been observed previously in *Synechocystis* sp. in batch reactors [14,42]. In this section, results of continuous experiments are reported, aimed at assessing the effect of long-term acclimation on the nutrient consumption. Chemostat experiments conducted with *Synechocystis* sp. (Table 3) confirm that ammonium is the preferred nitrogen source for such a species, while nitrate is consumed only when ammonium is growth-limiting (Fig. 1). In fact, as reported in Fig. 1A, nitrate consumption is significant in experiments 1 and 2, and occurs only when the complete ammonium depletion is reached. On the opposite, when ammonium is present in excess (Exp 3–4) nitrate is barely consumed. This suggests that inhibition of nitrate uptake due to ammonium excess occurs in *Synechocystis,* which is a model organism for cyanobacteria, and it is confirmed even in continuous system, after acclimation.

The same approach was used in the case of *A. protothecoides,* cultivated in continuous system under the supply of mixture of nitrate and

Table 3
Experimental results for *Synechocystis* in a mix of ammonium (NH_4^+) and nitrate (NO_3^-).

Experiment number	NH_4-N in (mg L^{-1})	NO_3-N in (mg L^{-1})	NH_4-N out (mg L^{-1})	NO_3-N out (mg L^{-1})	C_X (mg L^{-1})
1	15.5	50.3	0.0 ± 0.0	30.0 ± 1.9	437.5 ± 16.6
2	22.0	37.7	0.3 ± 0.3	24.4 ± 1.3	351.3 ± 29.9
3	59.2	35.8	19.7 ± 3.7	31.4 ± 0.9	350.1 ± 37.2
4	44.0	38.0	9.16 ± 1.1	31.6 ± 0.7	505.0 ± 32.4

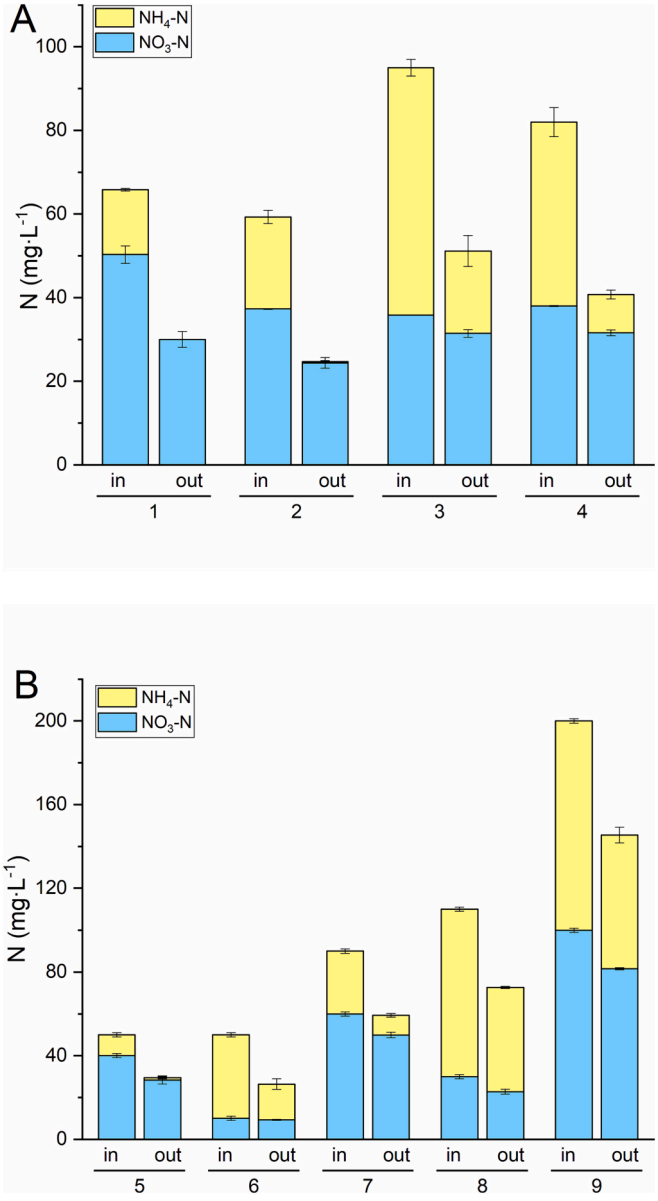

Fig. 1. Inlet and outlet nitrogen concentration as ammonium (yellow) and nitrate (blue) for steady state continuous experiments with *Synechocystis* (A) and *A. protothecoides* (B). Experimental conditions are identified by numbers, as listed in Table 1.

Table 4
Experimental results for *A. protothecoides* in a mix of ammonium (NH_4^+) and nitrate (NO_3^-).

Experiment number	NH_4-N in (mg L^{-1})	NO_3-N in (mg L^{-1})	NH_4-N out (mg L^{-1})	NO_3-N out (mg L^{-1})	C_X (mg L^{-1})
5	10.0	40.0	1.1 ± 0.5	28.4 ± 1.9	176.4 ± 46.6
6	40.0	10.0	17.1 ± 2.6	9.3 ± 0.2	256.6 ± 20.5
7	30.0	60.0	9.4 ± 0.9	49.9 ± 1.3	173.1 ± 5.1
8	80.0	30.0	49.7 ± 0.5	22.8 ± 1.2	266.2 ± 2.8
9	100.0	100.0	68.5 ± 3.7	81.6 ± 0.5	275.3 ± 41.3

ammonium (Table 4). Even though the inhibition on nitrate uptake by ammonium has been observed in this *A. protothecoides* strain [16] and several *Chlorella* species in batch conditions [11,43] (former taxonomic name of the genus *Auxenochlorella*), a different result was obtained in continuous system (Fig. 1B): if *A. protothecoides* is constantly supplied with both sources and is monitored following a period of acclimation, the situation appears more complex. In *A. protothecoides*, the consumption of nitrate does not only occur because of ammonium depletion (Exp 5) and in certain conditions (Exp 9) the consumption of the two species is surprisingly comparable suggesting that a different uptake regulation takes place in this genus. In particular, nitrate is always consumed even when ammonium is the more concentrated form. These results suggest that nitrate assimilation in *A. protothecoides* is not always subjected to ammonium inhibition, but the regulation of the two species is more complex and interconnected. These outcomes provide an interesting new perspective to consider in modelling these processes for future application, as the genuses *Chlorella* and *Auxenochlorella* are often found in WWT facilities.

4.2. Modelling of ammonium and nitrate assimilation in Synechocystis and A. protothecoides

For the design of WWT systems, mathematical modelling is necessary in order to efficiently describe microalgae growth and nutrient assimilation. Understanding the behavior of microalgae and cyanobacteria in complex media in continuous systems is a crucial step to apply these organisms in WWT.

Based on the qualitative considerations described in the previous Section 4.1, the mathematical model described in Section 3 was first applied to describe the growth and nitrogen uptake by *Synechocystis*. In particular, the model parameters were fitted based on the experimental results of Table 3. In the case of *Synechocystis*, additional experimental points of continuous cultivation under nitrate and ammonium alone were used and reported in Table 5.

Table 6 summarizes the model parameters (distinguishing between those taken from the literature, and those retrieved in this work), while Fig. 2 shows the parity plot for biomass, NO_3-N, and NH_4-N concentrations at the reactor outlet.

It can be observed that the model is able to well represent the experimental data in a wide variety of nitrogen concentrations, including conditions in which nitrate or ammonium were supplied as single N sources as well as simultaneously (Fig. 2). Interestingly, the values of the maximum uptake rates (ρ_{NO_3-N} and ρ_{NH_4-N}) and of the half-saturation constants (K_{NO_3-N} and K_{NH_4-N}) are similar for nitrate and ammonium, indicating that when only one of the two nitrogen species is present, the overall behavior will be the same, regardless of the actual nitrogen source. This is also in accordance with previous observations [26]. Moreover, the value of the inhibition constant of ammonium over nitrate ($K_{i,NH4} = 0.803\ g_N\ m^{-3}$) indicates that even small concentrations

Table 5
Experimental results for *Synechocystis* with a single nitrogen species.

Experiment number	NH_4-N in (mg L^{-1})	NO_3-N in (mg L^{-1})	NH_4-N out (mg L^{-1})	NO_3-N out (mg L^{-1})	C_X (mg L^{-1})
10	19.3	–	0.8 ± 1.3	–	305.0 ± 29.0
11	33.8	–	1.3 ± 1.0	–	305.0 ± 17.5
12	59.4	–	17.7 ± 1.1	–	314.4 ± 14.8
13	69.5	–	28.3 ± 1.9	–	268.2 ± 31.1
14	–	23.3	–	0.9 ± 0.8	198.8 ± 15.0
15	–	55.0	–	17.0 ± 2.0	237.8 ± 11.1
16	–	80.3	–	28.7 ± 6.2	361.0 ± 22.9
17	–	93.8	–	51.9 ± 2.4	285.4 ± 23.0

Table 6
Summary of model parameters for *Synechocystis*.

Parameter	Value	Units	Reference
μ_{max}	2.8	d^{-1}	[22]
K_I	100	μmol m^{-2} s^{-1}	[22]
I_{opt}	350	μmol m^{-2} s^{-1}	[22]
k_a	0.1866	m^2 g_X^{-1}	[22]
γ_P	0.0014	m^3 g_X^{-1} d^{-1}	[22]
$q_{P,min}$	0.0012	g_P g_X^{-1}	[22]
$q_{P,max}$	0.1	g_P g_X^{-1}	This work
k_d	0.1	d^{-1}	This work
ρ_{NO_3-N}	1.27	g_N g_X^{-1} d^{-1}	This work
ρ_{NH_4-N}	1.05	g_N g_X^{-1} d^{-1}	This work
K_{NO_3-N}	12.67	g_N m^{-3}	This work
K_{NH4-N}	14.22	g_N m^{-3}	This work
$K_{i,NH4}$	0.803	g_N m^{-3}	This work
$q_{N,min}$	0.0044	g_N g_X^{-1}	This work
$q_{N,max}$	0.175	g_N g_X^{-1}	This work

of ammonium can drastically reduce the nitrate uptake, which is what was experimentally observed.

When considering *A. protothecoides*, the kinetic parameters related to ammonium and nitrate assimilation and to biomass growth in the presence of these nutrients (when they are supplied as single sources) have already been determined [26], so that experimental data reported in Table 4 were used to calculate the value of the ammonium inhibition constant $K_{i,NH4}$ for this microalga under combined presence of both sources. However, as also highlighted from experimental observations, *A. protothecoides* appeared to behave differently from *Synechocystis*, with nitrate uptake occurring even in the presence of ammonium and, in general, suggesting a more complex interaction between the two nitrogen sources. In fact, the model described in Section 3 was not suitable to represent the behavior of this species under combined presence of nitrate and ammonium. Assuming that the kinetic parameters related to the maximum and minimum nitrogen quota ($q_{N,max}$ and $q_{N,min}$) and the half-saturation constants (K_{NH4-N} and K_{NO3-N}) should not be affected by the co-presence of different nitrogen species, in order to better understand the behavior the values of the maximum uptake rates (ρ_{NH_4-N} and ρ_{NO_3-N}) were fitted individually for each experiment, in a new model equation which does not apply the switch function. Table 7 summarizes the results, while Fig. 3 A and B shows the parity plots of experimental vs simulated data.

Table 7 shows that the uptake rate values vary depending on the corresponding nitrate or ammonium concentration. It can be noted that the values obtained from experiments with individual nitrogen species were $\rho_{NH_4-N} = 0.62$ g_N m^{-3} and $\rho_{NO_3-N} = 0.60$ g_N m^{-3}, respectively.

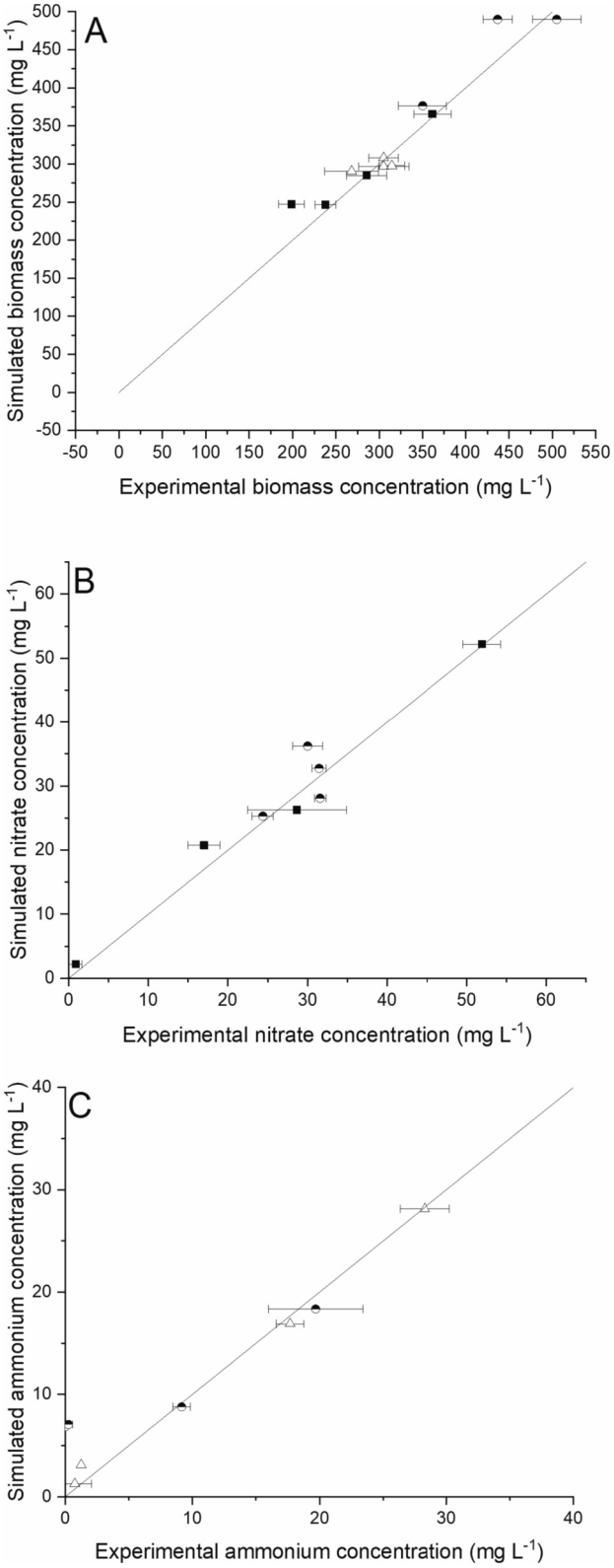

Fig. 2. (A) Parity plot showing experimental vs simulated data for *Synechocystis* biomass obtained in continuous cultures with nitrate (square), ammonium (triangles) and a combination of the two as nitrogen source (circles). (B) Parity plot showing experimental vs simulated data of outlet concentration for NO_3-N when supplied alone (square) and in the presence of ammonium (circles). (C) Parity plot showing experimental vs simulated data of outlet concentration for NH_4-N when supplied alone (triangles) and in the presence of nitrate (circles).

Table 7
Values of nitrate and ammonium maximum uptake rates for *A. protothecoides* obtained by fitting the experimental data with equations that not include the switch function in the case of nitrate uptake (eq. 4a).

Experiment number	NH_4-N $_{in}$ ($g_N\ m^{-3}$)	NO_3-N $_{in}$ ($g_N\ m^{-3}$)	ρ_{NH_4-N} ($g_N\ g_X^{-1}\ d^{-1}$)	ρ_{NO_3-N} ($g_N\ g_X^{-1}\ d^{-1}$)
5	10.0	40.0	0.62	0.46
6	40.0	10.0	0.62	0.60
7	30.0	60.0	0.62	0.35
8	80.0	30.0	0.48	0.19
9	100.0	100.0	0.26	0.38

Thus, it appears that, to properly model the acclimation phenomena of nutrient uptake, the maximum uptake rate, rho, plays a major role. To better understand if a correlation between the adjusted rho values and nitrogen concentration exists, its trend as a function of the outlet dissolved NH_4-N and NO_3-N concentrations was observed (Fig. 3C): interestingly, it appears that the value of ρ_{NH_4-N} remains constant and equal to the maximum value when NH_4-N concentration is low, and then it decreases at higher levels of dissolved NH_4-N. A similar trend is observed for ρ_{NO_3-N}, although that is less clear, probably due to the influence of ammonium. So, to be able to fit the data of experimental absorption of nitrogen, internal quota accumulation and biomass growth, the model suggests that a changing uptake rate ρ_N should be considered, and, generally, the value of this parameter should decrease with nitrogen concentration in the external medium. To explain such an observation with physical biological meaning, the variation of the uptake rate ρ_N could be justified by a different affinity transport that changes depending on the external nutrient concentration: when the concentration of the nutrient in higher, a low affinity transporter is sufficient, while an increased affinity is needed when the concentration is low. These results certainly deserve a biological interpretation, which we attempt to discuss in the following Section 4.3. In any case, they make it clear that a different modelling approach should be used for prokaryotic and eukaryotic microalgae. Indeed, cyanobacterium *Synechocystis* actually exhibits a net preference for ammonium over nitrate, whereas in *A. protothecoides*, the maximum uptake rate for nutrient seems to be a function of the external nitrogen concentration, almost independently with respect to the chemical form of the N species.

4.3. Discussion on nitrogen uptake regulation in microalgae and cyanobacteria

The cyanobacterium *Synechocystis* sp. 6803 and the microalga *A. protothecoides* showed different behaviors in the presence of different nitrogen sources in continuous systems. In *Synechocystis,* the assimilation of nitrate is strongly inhibited by ammonium, whereas in *A. protothecoides* the uptake is influenced by the concentrations of the two nutrients, and a significant proportion of nitrate is also assimilated in the presence of ammonium.

In particular, in A. *protothecoides* the model could not fully describe the experimental data using a single nitrate (ρ_{NO_3-N}) and ammonium (ρ_{NH_4-N}) uptake rate. Although the half-saturation constant (K_{NO_3-N} and K_{NH_4-N}) remained the same, it was necessary to adjust the nitrogen uptake rate to better describe the experimental data.

From a molecular point of view, the uptake rate depends on the type and number of available transporters and their affinity for the substrate. Accordingly, the regulatory mechanisms of transporters influence the maximum assimilation rate described by the uptake rate constant. Understanding the regulation of nitrogen assimilation and the differences between organisms is crucial for a better explanation of macroscopic phenomena (Table 8).

Although nitrogen assimilation follows the same steps in cyanobacteria and *Chlorophyta,* the two taxonomic groups to which these strains belong, most of the players involved in the pathway are different. Ammonium

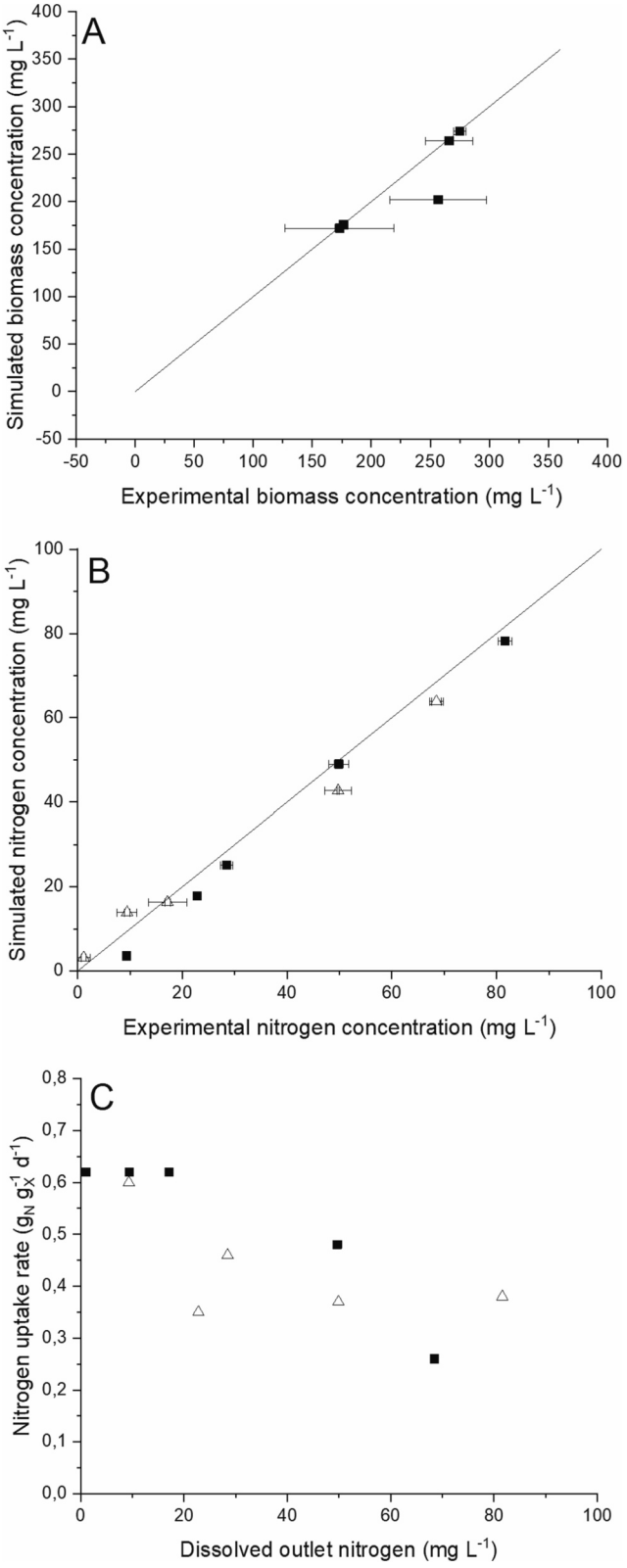

Fig. 3. (A) Parity plots showing experimental vs simulated data for *A. protothecoides* biomass outlet concentration at steady state when both nitrate and ammonium are supplied, using eq. 4a for nitrate uptake, and by changing the value of rho for each data series. (B) NO_3-N (squares) and NH_4-N (triangles) outlet concentrations for *A. protothecoides*. (C). Fitted values of ammonium (ρ_{NH_4-N}, triangles) and nitrate (ρ_{NO_3-N}, squares) maximum uptake rates as a function of corresponding dissolved concentration in the medium are reported.

Table 8
Main proteins for nitrogen assimilation in cyanobacteria and *Chlorophyta* species with a sequenced genome. AMT, ammonium transporter, NrtABCD, NrtP, NRT1, NRT2, and NAR1, nitrate/nitrite transporters; NAR2, accessory component for high affinity nitrate transport; NR, Nitrate Reductase; NiR, Nitrite Reductase; PII, PipX regulatory protein; NtcB, NtcA, NIT2, transcription factors. The dot indicates that at least one copy of the corresponding encoding gene is present in the organism's genome. Some of these proteins have already been investigated by Ohashi et al., 2011 [17] and Sanz-Luque et al., 2015 [18].

A	Transporters			Enzymes		Regulators			
	AMT	NrtABCD	NrtP	NR	NiR	PII	NtcB	NtcA	PipX
Anabaena cylindrica PCC 7122	●	●	●	●	●	●	●	●	●
Arthrospira platensis C1	●	●	●	●	●	●	●	●	●
Microcystis aeruginosa NIES-843	●	●		●	●	●	●	●	●
Nostoc sp. PCC 7120	●	●		●	●	●	●	●	●
Oscillatoria acuminata PCC 6304	●	●		●	●	●	●	●	●
Synechococcus elongatus PCC 7942	●	●		●	●	●	●	●	●
Synechocystis sp. PCC 6803	●	●		●	●	●	●	●	●
Thermosynechococcus vestitus BP1	●	●		●	●	●	●	●	●

B	Transporters					Enzymes		Regulators	
	AMT	NRT1	NRT2	NAR1	NAR2	NR	NiR	PII	NIT2
Auxenochlorella protothecoides UTEX 25	●	●		●		●	●	●	
Chlamydomonas reinhardtii	●	●	●	●	●	●	●	●	●
Chlorella variabilis NC64A	●	●	●	●	●	●	●	●	
Chlorella sorokiniana SLA-04	●	●	●	●	●	●	●		●
Dunaliella salina CCAP 19/18	●	●	●	●		●	●	●	
Haematococcus lacustris	●	●	●	●		●	●	●	●
Tetradesmus obliquus UTEX 3031	●	●	●	●		●	●	●	●

transporters (AMTs) are highly conserved (Fig. 4), but different families have been identified for nitrate and nitrite transport. In eukaryotic microalgae, there are three different types of NO_2^-/NO_3^- transporters: NRT1, NRT2 and NAR1. In addition, NAR2 accessory proteins are often necessary for the full functionality of NRT2 [18]. In cyanobacteria, assimilation occurs mainly through the NrtABCD transporter, an ABC importer bispecific for nitrate and nitrite composed of four subunits. In marine cyanobacteria, the NrtP transporter is also common and interestingly shares few similarities with the eukaryotic NRT2 [17].

More importantly, the transcription factors responsible for nitrate assimilation are different between the two taxonomic groups, and they appear to be susceptible to different regulatory mechanisms. In cyanobacteria, nitrate assimilation is mainly regulated by two transcription factors, NtcA and NtcB, which sense 2-oxoglutarate (2-OG) and nitrite concentrations, respectively. NtcA is defined as a global nitrogen regulator and its interaction with 2-OG and two proteins, PII and PipX, is crucial in regulating its activity. The signal for the negative regulation promoted by ammonium is the intracellular depletion of 2-OG, which, in presence of ammonium, is consumed for the synthesis of glutamate [44,45]. In *Chlorophyta*, nitrogen assimilation involves a network of proteins scarcely known in most species. *C. reinhardtii* is the most studied organism and has proven to be a good model to unravel this process. An important role in nitrate assimilation is certainly played by the transcription factor NIT2, essential for the up regulation of the main genes for nitrate assimilation [46]. Interesting studies in this organism revealed how ammonium repression might to be a quantitative process that isn't solely reliant on ammonium concentration, but rather on the ratio of nitrate to ammonium. The expression of several genes seems susceptible to the balance of the two species, i.e., NR and PII gene, and the transcription factor NIT2 may play a role in the integration of signals [19,20,47].

Concerning the regulation of different signals, the signal transduction protein PII is particularly interesting. PII is a family of signalling proteins widespread in nature and well conserved among cyanobacteria and microalgae (Fig. 5). In cyanobacteria *S. elongatus* PCC 6972 and *Synechocystis* sp. 6803, PII has been shown to have a role in ammonium inhibition of nitrate uptake by interacting with the NrtC and NtrD subunits of the nitrate transporters [14,42]. In *C. reinhardtii*, PII gene expression seems to be affected by the balance of different forms of nitrogen and its pattern resembles those of other nitrate assimilation genes [47].

The results observed in chemostats for *Synechocystis* are consistent with the current picture of nitrogen regulation in cyanobacteria as described in literature. A value of the inhibition constant of ammonium over nitrate ($K_{i,NH4}$) of 0.803 $g_N\ m^{-3}$ indicates that *Synechocystis* is very sensitive to the presence of ammonium, which is strongly preferred over nitrate. On the contrary, in *A. prototechoides*, the general mechanisms of nitrogen regulation could be more sensitive to the NH_4^+/NO_3^- ratio and the expression of several genes could be susceptible to such a balance, as already observed in *C. reinhardtii* [18,19].

In *A. prototechoides*, besides the intricate interplay among different nitrogen sources, empirical evidence revealed a variation in the uptake rate constant. As shown in Fig. 3C, the value of ρ_{NH_4-N} remains constant and equal to the maximum value up when NH_4-N concentration is low, and then it decreases at higher levels of dissolved NH_4-N. One possible explanation for this phenomenon is the engagement of different transporters depending on the nutrient concentration.

Two systems for ammonium transport have been described in literature: a high affinity transport system (HATS) and a low affinity transport system (LATS) [48,49]. The HATS is saturable, regulated by the nitrogen status of the cell and subject to negative feedback mechanisms. The AMT family proteins belong to this category. LATS appears to be a non-saturable system that is expressed constitutively, it exhibits a linear increase in response to increases in ammonium concentration and it is insensitive to nitrogen regulation [49,50]. The LATS may consist of broad-specificity cation channels that can transport NH_4^+, like passive K^+ channels. The HATS activity is predominant at low (submillimolar) concentrations of NH_4^+, whereas the LATS becomes significant at higher external NH_4^+ concentrations (above 1 mM) [51]. Transporters with different affinity for the substrate have also been identified for nitrate and nitrite [52,53]. Therefore, it is evident how extracellular nutrient concentrations, and the subsequent intracellular regulation, affect both the number and type of transporters involved.

Accordingly, it appears reasonable that, from a modelling perspective, this adjustable number of nitrogen transporters is reflected into the value of rho, with the physical meaning of a maximum uptake rate including the changing concentration of the protein, as well described in the Michaelis Menten enzymatic model. In particular, from Fig. 3C it appears that by increasing the dissolved nitrogen concentration, the

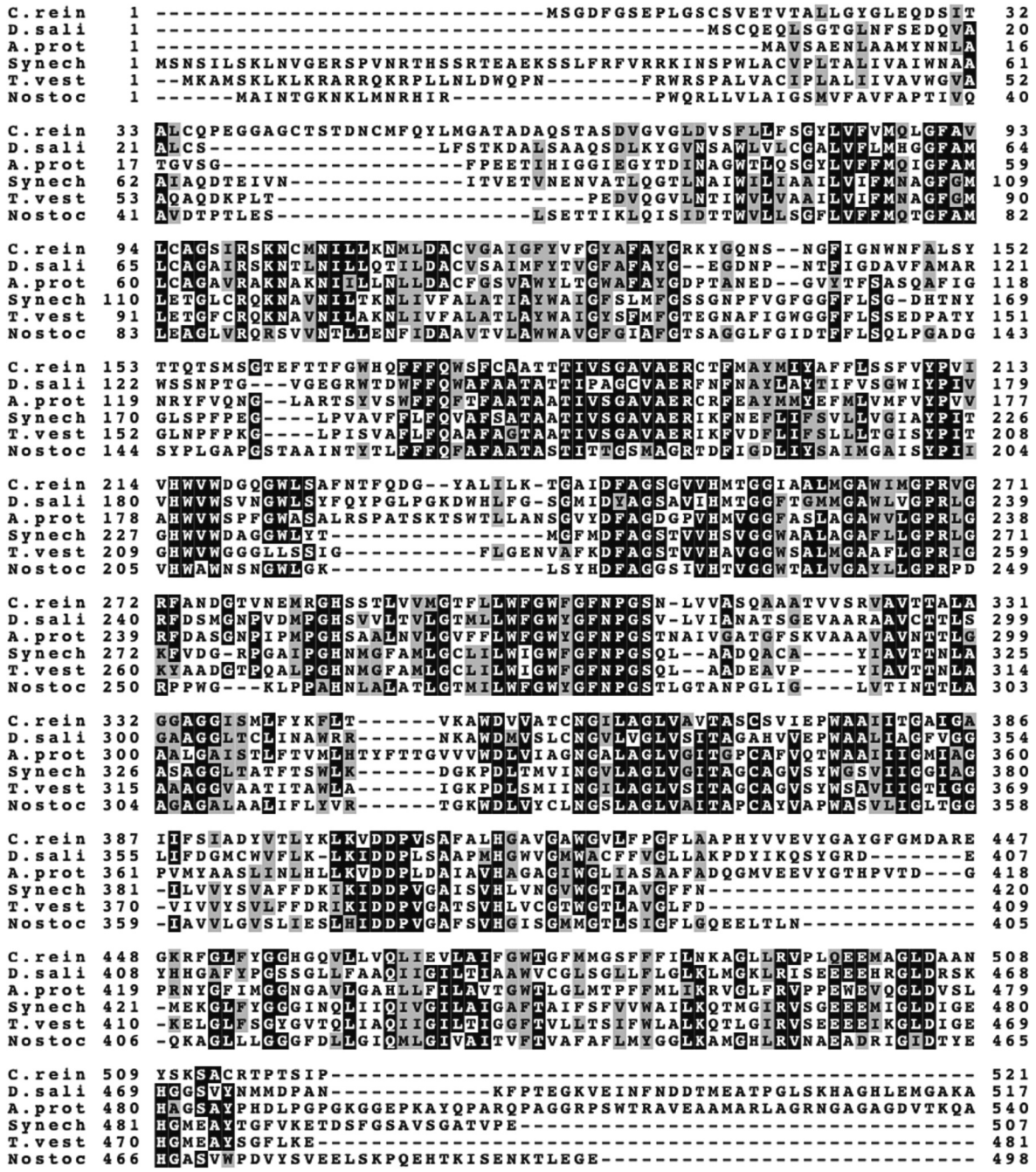

Fig. 4. Multi-alignment of AMT sequences of *C. reinhardtii* (XP_042925794.1), *D. salina* CCAP 19/18 (KAF5828947.1), *A. protothecoides* (RMZ53119.1), *Synechocystis* sp. PCC 6803 (BAA10631.1), *Termosynechococcus vestitus* BP1 (BAC09537.1) and *Nostoc* sp. PCC 7120 (BAB72947.1). The protein code is reported as indicated in NCBI. Identical residues are colored in black, similar ones in grey.

value of rho is proportionally reduced, suggesting that a general rule can be drawn to include this feature. These findings underscore the importance comprehending cellular-level phenomena when advancing modelling efforts. Whether the results obtained are widespread behavior or a peculiarity of this strain of *A. protothecoides* remains an open question given the lack of continuous studies in microalgae in the presence of multiple nitrogen sources. Certainly, a genome sequencing of the specific *A. protothecoides* strain would eventually highlight some possible specificities of the organisms, clarifying if these features related to the uptake of nitrogen are peculiar or more generalized for the taxonomic category considered.

5. Conclusions

A. protothecoides and *Synechocystis* sp. PCC6803 were cultivated in media where both nitrate and ammonium were supplied, and the nutrient consumption was measured at steady state in continuous systems, to account for the acclimation of the species in a constant environment. A different behavior in the uptake was observed within the two taxonomic groups, and while a net preference for ammonium was confirmed in the case of *Synechocystis*, *A. prototothecoides* showed to be able to consume nitrate even in the presence of higher concentration of ammonium. The Droop formulation was applied to account for the nitrogen consumption, but the *switch function* that is usually applied for describing the preference for ammonium in microalgae was not

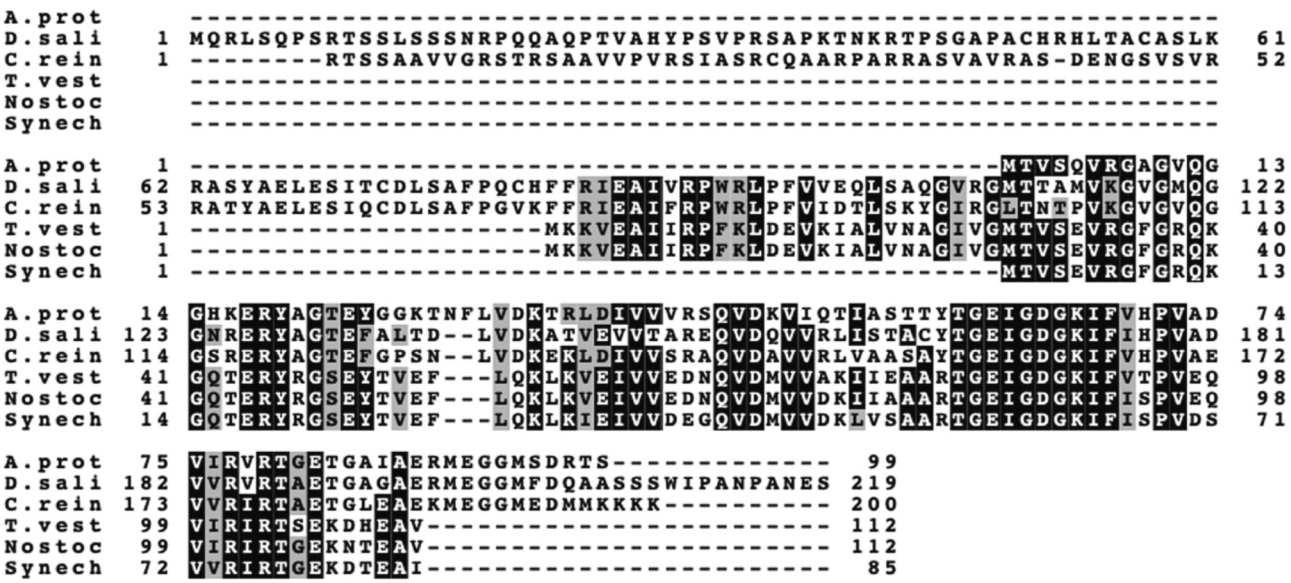

Fig. 5. Multi-alignment of PII sequences of *C. reinhardtii* (PNW81471.1), *D. salina* (KAF5833890.1), *A. protothecoides* (RMZ53731.1), *Synechocystis* sp. PCC 6803 (BAA18533.1), *Termosynechococcus vestitus* (BAC08143.1) and *Nostoc* sp. PCC 7120 (BAB74018.1). The protein code is reported as indicated in NCBI. Identical residues are colored in black, similar ones in grey.

applicable for *A. protothecoides*. However, it was shown that the varying uptake rate of both nitrate and ammonium can be mathematically described by a changing maximum uptake rate rho, which was found to be a function of the external nitrogen concentration in the medium. The evidence collected suggests that such a species can change the affinity for the substrate when considering both expression and regulation of transporters. From a modelling perspective, this can be reasonably accounted for by adjusting the value of the rho.

CRediT authorship contribution statement

Marta Carletti: Data curation, Investigation, Validation, Visualization, Writing – original draft. **Elena Barbera:** Data curation, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. **Francesco Filippini:** Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing. **Eleonora Sforza:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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