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Monocultures vs. polyculture of microalgae: unveiling physiological changes to facilitate growth in ammonium rich-medium

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Abstract

Due to the increasing production of wastewater from human activities, the use of algal consortia for phytoremediation has become well-established over the past decade. Understanding how interspecific interactions and cultivation modes (monocultures vs. polyculture) influence algal growth and behaviour is a cutting-edge topic in both fundamental and applied science. Ammonium-rich growth media were used to challenge the monocultures of *Auxenochlorella protothecoides*, *Chlamydomonas reinhardtii* and *Tetradesmus obliquus*, as well as their polyculture; NO_3^- was also used as the sole nitrogen chemical form in control cultures. The study primarily compared the growth, carbon and nitrogen metabolisms, and protein content of the green microalgae monocultures to those of their consortium. Overall, the cultivation mode significantly affected all the measured parameters. Notably, at 50 mM NH_4^+, the assimilation rates of carbon and nitrogen were at least twice as high as those in the monoculture counterparts, and the protein content was three times more abundant. Additionally, the consortium's response to NH_4^+ toxicity was investigated by observing a linear relationship between the indicator of tolerance to NH_4^+ nutrition and the N isotopic signature. The study highlighted a high degree of acclimation through metabolic flexibility and diversity, as well as species abundance plasticity in the consortium, resulting in a functional resilience that would otherwise have been unattainable by the respective monocultures.

1 | - INTRODUCTION

Over the past two decades, microalgae have received increasing interest due to their unique biological characteristics, positioning them as promising candidates for a multitude of applications across various sectors and industries (Borowitzka and Vonshak 2017; Benedetti et al. 2018; Khan et al. 2018). The biotechnological potential of microalgae lies in their distinctive unicellular form and metabolic versatility: through the variation of growth conditions, such as light and nutrient availability, microalgae can synthesize and accumulate a diverse array of bioactive compounds, e.g. proteins may constitute up to 70% of their total dry weight (Hu 2013; Chandra et al. 2019; Garofalo et al. 2022).

Compared to their photosynthetic counterparts (i.e. plants), microalgae possess a higher productivity and energy conversion into biomass (Benedetti et al. 2018; Bošnjaković and Sinaga 2020; Chai et al. 2021). Additionally, factors contributing to the sustainability of microalgae cultivation include the use of non-arable land and non-drinkable water; algae are indeed able to exploit wastewater as growth media (Gonçalves et al. 2017; Sakarika et al. 2019; Kaloudas et al. 2021).

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Although representing an untapped source of water and nutrients, wastewaters are typically treated and discarded using various eco-unfriendly methods, highly demanding in terms of energy and chemical reagents (Foglia et al. 2020; Marinelli et al. 2021). Growth of microalgae in wastewaters offers a sustainable approach both to remediate these media and to produce valuable algal biomass by incorporating nutrients and organic carbon into new cellular macromolecules thanks to a mixotrophic metabolism (Pang et al. 2019; Fabris et al. 2020; Kumar Patel et al. 2021; Arun et al. 2022; Chieti et al. 2024). A type of wastewater gaining attention is the ammonium (NH_4^+)-rich wastewater (Shen et al. 2020), such as digestate as the liquid by-product of the anaerobic digestion of biomass leftovers (Uggetti et al. 2014; Laiq Ur Rehman et al. 2019; Rude et al. 2022). Despite being rich in N, the N/P ratio must be considered since un-balanced proportions distant from the Redfield ratio could affect the algal productivity and thus the nutrients remediation (Geider and La Roche 2002; Wang et al. 2010).

While N-NH_4^+ may enhance the growth rate of algae in a species-specific manner (Markou et al. 2016), dealing with it poses challenges due to its potential toxicity to algal cells (Britto and Kronzucker 2002; Källqvist and Svenson 2003; Esteban et al. 2016; Gutierrez et al. 2016) which is also dependent on the aqueous equilibrium between NH_4^+ and NH_3 influenced by pH and temperature, (Østergaard 1985; Jiang et al. 2021; Wang et al. 2021).

As reported by Collos and Harrison (2014), there are significant differences among phylogenetically diverse algae with respect to NH_4^+ tolerance, with chlorophytes being those with the highest tolerance (optimal NH_3/NH_4^+ concentration of 7.6 ± 7.6 mM and toxic concentration of 39.2 ± 60.0 mM). Various approaches to increase NH_4^+ tolerance have been proposed; they include pH control to avoid NH_3 formation, supply of organic carbon for increased incorporation of reduced nitrogen (Lu et al. 2018), supply of extra potassium (K^+) to outcompete NH_4^+ for the channel-mediated influx (Mollo et al. 2024). Physio-chemical techniques such as NH_3 stripping, precipitation or electrochemically recovered nutrients have also been employed to pretreat wastewater (Bonmatí and Flotats 2003; Uludag-Demirer et al. 2005; Mahmoud et al. 2022). However, these approaches come with costs, and insufficient data support their efficacy.

Recently, the exploitation of algal consortia has emerged as a successful approach for wastewater remediation and bio-metabolite production. Indeed, although monocultures are commonly used due to easier monitoring and manipulation (e.g. *Tetradesmus* sp. and *Chlorella* sp.), they are prone to contamination (Padmaperuma et al. 2018; Gururani et al. 2022) and have a low resilience toward fast changes in growth conditions as outdoor cultivation systems (Mandal and Corcoran 2022). On the contrary, consortia replicate natural conditions, enhancing resilience to environmental changes, such as the variability of nutrient content in wastewater streams (Rashid et al. 2019; Mugnai et al. 2023), and demonstrating higher productivity and tolerance to high ammonium concentrations (Britto and Kronzucker 2008; Szczerba et al. 2009; Mollo et al. 2024).

With the aim to better understand the effect of algal co-cultivation, the three chlorophytes (*Auxenochlorella protothecoides*, *Chlamydomonas reinhardtii*, *Tetradesmus obliquus*) forming the consortium or cultivated individually were challenged to grow in the presence of ammonia. The species were previously selected based on growth and remediation performances in a synthetic nutrient-rich digestate (Mollo et al. 2024) to investigate if algae grown in polyculture showed a synergistic behaviour as compared to monocultures. Seven concentrations of ammonium chloride (NH_4Cl) were tested in the range between 1.5–50 mM in comparison with the same concentrations of sodium nitrate ($NaNO_3$). The tested N concentrations fall in the concentration range of NH_4^+-rich wastewater such as digestate (Salbitani and Carfagna 2021). The algal response was evaluated both during the exponential and stationary phases. The analysis focused on (1) the consortium growth and composition in terms of species abundance and analysis of cell size and shape, (2) the photosynthetic apparatus, which is one of the main targets of NH_3/NH_4^+ toxicity, (3) protein content, C and N quotas and their isotopic fractionation.

2 | MATERIALS AND METHODS

2.1 | Algal species and consortium

Three algal species, *Auxenochlorella protothecoides* (CCAP 211/8D https://www.ccap.ac.uk/), *Tetradesmus obliquus* (CCAP 276/3A), *Chlamydomonas reinhardtii* (RCC125 https://roscoff-culture-collection.org) and their consortium were chosen for the experiments. The consortium was already established and previously used to assess its growth and N-remediation in artificial digestate containing 4.7 mM of N-NH_4^+ (Mollo et al. 2024). Single species and consortium axenic cultures were maintained in BG11 until the beginning of the experiment at 20°C and 100 µmol photons m^{-2} s^{-1} under a 24-h light regime. This photoperiod allowed the experiment to be reduced in complexity since the physiology and metabolism of the algae were not determined by the changes in day/night (Krupinska and Humbeck 1994).

2.2 | Experimental design

Algal growth was carried out using a modified BG11 medium with $NaNO_3$ or NH_4Cl as the N source. Hereafter, the two culture conditions will be reported as NO_3^- cultures or nitrate-supplied cultures and NH_4^+ cultures or ammonium-supplied cultures. The consortium was exposed to seven N concentrations (1.5, 3, 5, 10, 15, 30, 50 mM) while single species to only three concentrations (1.5, 15, 50 mM).

An initial cell concentration of 5×10^5 cells mL^{-1} was used as inoculum and washed twice with a BG11 N-free medium to remove all traces of extracellular nitrogen. Hence, the inoculum was transferred to 100 mL glass tubes filled with 30 mL of N-free BG11 added with a sterile solution of $NaNO_3$ or NH_4Cl to achieve the desired N concentration. Growth was followed for a total of 10 days at the experimental condition of 20°C, 100 µmol photons m^{-2} s^{-1} under a 24 h light regime. Physiological and biochemical analyses were carried out on samples collected at the onset of the stationary phase.

Moreover, a few parameters in consortia cultures were also measured in the exponential phase.

Cell density and size were assessed using an automatic cell counter (Casy TT, Innovatis AG). Together with cell concentration (cell mL^{-1}), average cell diameter and volume (μm and fL) were also recorded.

The β-function (Yin et al., 2003), recently implemented for physiological studies (Mollo et al. 2023, 2024; Petrucciani et al. 2023; Chieti et al. 2024), was used as a non-linear regression model to determine maximum growth rate (μ_{max}), achieved at the inflection point of the curve (T_m), and maximum density (Nt_e), achieved at the end of growth (T_e).

2.3 | Species proportion

Algal samples collected from the consortium cultures were analysed by imaging flow cytometry (IFC) (FlowSight®, Amnis Corp.). INSPIRE software package (Amnis Corp.) was used to assess morphological characteristics and proportion among species. Acquisition and post-acquisition data analysis were performed, as illustrated in Petrucciani et al. (2023). Based on the morphological features inferred from the images generated by the instrument, the algal species of the consortium were classified into two categories: circular species and elongated species. *A. protothecoides* and *C. reinhardtii* belonged to the first group, while *T. obliquus* was in the second. Due to morphological similarities, it was impossible to clearly distinguish the two circular species, so they were grouped together. On the contrary, *T. obliquus* always maintained an elongated shape (even in mono-cultures as observed through an optical microscope), allowing for proper identification. The two groups were then reported as % of the total living species, and species proportion variability was evaluated according to the N source and concentration.

2.4 | Photosynthetic efficiency and pigment concentration

The F_v/F_m ratio was measured only in consortium cultures during the exponential and stationary phase using a Dual Pulse Amplitude Modulation (PAM) 100 fluorimeter (Heinz Walz GmbH), and the Dual PAM v1.8 software (Walz GmbH) was used to obtain data. Microalgal cells were collected by centrifugation (1500 *g*, 5 min) and resuspended in 2 mL fresh growth medium at a concentration of 3×10^6 cells mL^{-1}. Algae were then acclimated in the dark for 30 min, and an analysis was carried out, as reported by Baker (2008), in glass cuvettes under continuous stirring.

The same samples were used to quantify chlorophyll *a*, chlorophyll *b* and carotenoid contents in the consortium cultures. Pigment content was also assessed in single-species cultures. Concentrations were expressed as a function of the average cell volume (fg fL^{-1}). The samples were centrifuged at 1500 *g* for 8 min the supernatant discarded, and the pellet re-suspended in an equal volume of methanol. The samples were stored at −20°C overnight; thus, they underwent a second centrifugation, and the supernatant (pigments methanol extract) was analysed spectrophotometrically and quantified based on the equations reported by Ritchie (2006).

2.5 | Protein quantification

Samples of consortium and single species cultures were collected to quantify the protein content expressed in function of the average cell volume (fg fL^{-1}). An aliquot of 1 mL algal sample was centrifuged (1500 g for 5 min), and the cell pellet was kept at −20°C until analysis. The protein content was quantified spectrophotometrically using the Lowry colourimetric method, as reported by Peterson (1977). A volume of 500 μL 1% sodium dodecyl sulfate (SDS) and 0.1 mol L^{-1} of NaOH was added to each pellet and vortexed for 60 s. Thus, 500 μL of reagent A (25% H_2O, 25% SDS 10%, 25% NaOH 0.8 M, 25% CTC reagent) was added, and the samples were immediately vortexed. After 10 min, the samples were added with 250 μL of reagent B (83.3% H_2O, 16.7% Folin and Cicalteau's phenol reagent), vortexed and incubated in the dark for 30 min. Absorbance at 750 nm was measured through a Beckman DU 640 Spectrophotometer (Beckman Coulter). Quantification was calculated by interpolating absorbance data in a standard curve made with known concentrations of bovine serum albumin (BSA).

2.6 | Elemental composition and isotopic signature

C and N quotas, C/N ratio and isotopic fractionation ($\delta^{13}C$ and $\delta^{15}N$) of algal samples were determined as reported by Petrucciani et al. (2022). An elemental analyser (ECS 4010, Costech) connected to the ID Micro EA isotope ratio mass spectrometer (Compact Science Systems, Lymedale Business Centre) was used. Acquisition and data analysis were performed using the EAS-Clarity software (Costech Analytical Technologies Inc.). C and N assimilation rates of each experimental condition were calculated by multiplying the N and C content per unit of biovolume (fg fL^{-1}) by the μ_{max} (Giordano et al. 2022).

2.7 | NH_4^+ *versus* NO_3^- supplied algae comparison

Algal tolerance to NH_4^+ was evaluated by calculating the ratios between cell abundances at the 2 different N regimes and those between biomass at the same N concentration (Equation 1–2). During the stationary phase, 1 mL of each N regime sample was collected: cell concentration (cell mL^{-1}) was initially measured (see 2.2) in the sample, then the sample was dried at 80°C to quantify the dry weight (mg mL^{-1}).

$$\text{Cell ratio} = \frac{Cells\,mL^{-1}\,NH_4Cl}{Average\,Cells\,mL^{-1}\,NaNO_3} \quad (1)$$

$$\text{Biomass ratio} = \frac{\text{mg mL}^{-1}\ NH_4Cl}{\text{Average mg mL}^{-1}\ NaNO_3} \quad (2)$$

A ratio close to 1 meant similar cell or biomass density between culture couples (same N concentration). Values for each ratio, which were close to 0 meant a high difference between the culture couples and the presence of ammonium toxicity.

A linear regression between $\delta^{15}N$ of NH_4Cl-supplied algae and biomass ratio was also performed, according to Ariz et al. (2011).

2.8 | Statistical analysis

All experiments were performed in at least 3 independent biological replicates. Data are reported as mean followed by standard deviation (±SD). Principal Component Analysis (PCA) was carried out using Quasar 1.9.1 (Toplak et al. 2021). PCA was performed on growth parameters (μ_{max}, T_e, T_m, Nt_e) (dependent variables) among different growth conditions (independent variables). Data were normalised using z-values [(n-mean)/SD].

GraphPad Prism 9.5.0 (GraphPad Software) and IBM SPSS Statistics were used to perform statistical analysis. Two-way ANOVA was used to analyse parameters as a function of N source and N concentration (single species parameters and isotopes analysis). Three-way ANOVA was used to analyse parameters as a function of N source, N concentration and growth phase (consortium parameters) and to analyse parameters as a function of cultivation mode, N source and N concentration (mono-culture vs. co-cultures parameters). All statistical analyses were performed with a significance level of $\alpha = 0.05$. Asterisk (*) were used in figures to distinguish significantly different groups ($p < 0.05$). The results of the statistical analysis are reported in detail in the supplementary material.

3 | RESULTS

3.1 | Mono-cultures and consortium by comparison: effects of N source and concentration on growth and biochemistry

The mode of cultivation (single species or consortium) and the N supplied to the cultures had a great impact on the μ_{max} of microalgae (Table 1, Table S1). On average, μ_{max} was higher in nitrate-supplied cultures compared to the ammonia-supplied cultures; moreover, μ_{max} values decreased with increasing N concentration, a phenomenon emphasized in NO_3^- cultures. The significance of the cultivation mode was confirmed by statistical analysis ($p = 0.041$, Table S1), but the other factors did not explain the variability of the system. Nonetheless, the N source did explain variability in μ_{max} in combination with the cultivation mode ($p = 0.002$). Even though the μmax of consortia was generally lower than that of monocultures, it was consistently higher in NH_4^+ cultures compared to NO_3^- cultures. This was not the case in monocultures. (Table 1).

The effect of N-NH_4^+ form on maximum cell density (Nt_e) was observed both in monospecific cultures and in consortium where, regardless of the concentration, the Nt_e of NH_4^+ supplied algae was always significantly lower than the respective value of nitrate-supplied ones ($p = 0.021$). As for μ_{max}, the culture condition (mono- or co-culture) highly affected the Nt_e values ($p < 0.001$, Table S1), with cellular densities usually higher in consortium cultures compared to the ones of single species. Among the species, *T. obliquus* showed the lowest Nt_e but less difference between the two N-form groups as compared to the other species; nevertheless, the consortium formed by the three species achieved a similar value to those of *A. protothecoides* and *C. reinhardtii* monospecific cultures (Table 1).

The protein pool was highly different between monocultures and consortium ($p < 0.001$, Table 1): consortium cultures showed two to four times the content of the single species cultures. According to what was observed in monocultures, protein abundance was species-specific, *T. obliquus* having the highest content and *A. protothecoides* the lowest. All the factors and their interactions significantly affected the protein content (Table S1).

The C content did not change due to different N concentration with the only exception of *A. protothecoides* and consortium cultures: while the C content in *A. protothecoides* decreased along with increasing N concentration (especially in NO_3^- cultures), the consortium one significantly increased up to 449.5 ± 48.7 fg fL^{-1}, the highest value among all the tested conditions. On the other hand, the content was generally higher in ammonium-supplied cultures than in nitrate ones ($p < 0.001$), as reported in Table 1. The observed difference was also related to a variation in cellular volume when exposed to the different N sources (Figure S1): indeed, consortium cultures had lower average biovolume when supplied with ammonium than when supplied with nitrate. On the contrary, *T. obliquus* and *A. protothecoides* had higher cellular volume when exposed to ammonium. *C. reinhardtii* was the only species whose average volume was not significantly affected by the N source.

A significant difference between monocultures and the consortium was observed in the C and N assimilation rate (Tables 1, S1): both the assimilation rates increased with increasing N concentration in the consortium cultures, while the same trend was observed only in ammonium-supplied monocultures of *T. obliquus*. When supplied with NH_4Cl, algae grown in consortium (together with *A. protothecoides*) had higher assimilation rates than when supplied with $NaNO_3$; moreover, the C and N assimilation rates of consortium supplied with 50 mM NH_4Cl were also the highest recorded (Table 1).

The C to N ratio significantly decreased both in monocultures and consortium cultures with increasing N concentration (Tables 1, S1). As for the C content, even the C to N ratio of *C. reinhardtii* cultures did not change due to N source and concentration (Table 1).

The above-mentioned data and the statistical analysis reported in Table S1 and in Table S3 proved that the cultivation mode (cultivation of single species or co-cultivation of multiple species) was the most principal factor responsible for the observed variability since it always significantly explained the observed variability.

TABLE 1 Growth (μmax, Nt_e), C content, C to N ratio, C and N assimilation rate, protein content of A) single species and B) consortium divided per nitrogen concentration and nitrogen source. For the consortium, data of growth conditions corresponding to single species (1.5, 15, 50 mM of N) are reported only. Data are reported as mean ± SD. Results of the statistical analysis are reported in detailed in the supplementary material (Table S1).

(A)

Species	N-conc	N-source	μmax (d^{-1})	Nte (cells mL^{-1})	C content (fg fL^{-1})	C/N	C assimilation rate (fg fL^{-1} d^{-1})	N assimilation rate (fg fL^{-1} d^{-1})	Protein (fg fL^{-1})
A. protothecoides	1.5 mM	$NaNO_3$	0.75 ± 0.04	1.01E+07 ± 3.83E+05	175.4 ± 22.3	5.2 ± 0.0	32.4 ± 4.4	6.2 ± 0.8	33.1 ± 11.1
		NH_4Cl	0.21 ± 0.01	3.38E+06 ± 2.19E+05	224.9 ± 57.6	5.5 ± 0.0	54.3 ± 15.3	9.9 ± 2.7	26.1 ± 7.7
	15 mM	$NaNO_3$	0.64 ± 0.06	8.45E+06 ± 1.30E+06	74.7 ± 11.9	5.8 ± 0.3	24.0 ± 2.1	4.7 ± 0.4	26.6 ± 7.7
		NH_4Cl	0.21 ± 0.01	3.87E+06 ± 3.72E+04	253.5 ± 49.6	5.0 ± 0.2	42.7 ± 1.9	8.5 ± 0.6	41.7 ± 4.3
	50 mM	$NaNO_3$	0.60 ± 0.08	1.02E+07 ± 1.73E+06	65.2 ± 12.8	5.8 ± 0.3	12.6 ± 2.4	2.2 ± 0.3	30.6 ± 7.3
		NH_4Cl	0.21 ± 0.00	3.41E+06 ± 4.66E+04	212.1 ± 6.1	5.1 ± 0.1	45.4 ± 3.6	8.8 ± 0.7	40.6 ± 7.5
T. obliquus	1.5 mM	$NaNO_3$	0.87 ± 0.08	5.24E+06 ± 3.17E+05	187.9 ± 23.1	5.1 ± 0.0	183.9 ± 55.5	36.4 ± 11.0	40.5 ± 15.9
		NH_4Cl	0.58 ± 0.11	2.96E+06 ± 2.95E+05	184.5 ± 32.3	4.9 ± 0.1	91.1 ± 13.8	18.5 ± 3.0	51.6 ± 6.7
	15 mM	$NaNO_3$	0.66 ± 0.08	6.24E+06 ± 5.25E+05	189.6 ± 43.5	5.1 ± 0.1	118.5 ± 34.8	23.3 ± 6.4	59.3 ± 16.5
		NH_4Cl	0.54 ± 0.06	4.35E+06 ± 6.59E+05	193.1 ± 30.7	5.3 ± 0.2	103.7 ± 25.7	19.7 ± 4.3	41.7 ± 8.8
	50 mM	$NaNO_3$	0.77 ± 0.05	6.73E+06 ± 1.35E+05	184.2 ± 8.1	5.0 ± 0.1	130.9 ± 8.1	26.3 ± 1.2	54.8 ± 5.4
		NH_4Cl	0.68 ± 0.11	4.31E+06 ± 3.00E+05	229.3 ± 50.9	5.6 ± 0.0	139.2 ± 28.7	25.0 ± 5.2	48.7 ± 9.1
C. reinhardtii	1.5 mM	$NaNO_3$	1.01 ± 0.17	4.64E+06 ± 1.54E+05	204.0 ± 26.0	5.5 ± 0.4	159.5 ± 18.5	29.1 ± 1.6	44.6 ± 1.4
		NH_4Cl	0.90 ± 0.00	2.94E+06 ± 6.31E+04	155.0 ± 39.7	5.2 ± 0.1	128.2 ± 24.7	24.9 ± 5.2	47.2 ± 5.8
	15 mM	$NaNO_3$	0.80 ± 0.15	9.16E+06 ± 2.13E+06	185.8 ± 29.7	4.4 ± 0.1	143.1 ± 51.2	32.5 ± 11.1	27.5 ± 0.4
		NH_4Cl	0.53 ± 0.09	3.49E+06 ± 2.08E+05	217.2 ± 42.5	5.0 ± 0.1	105.0 ± 22.2	20.8 ± 4.2	46.8 ± 10.5
	50 mM	$NaNO_3$	0.69 ± 0.07	8.73E+06 ± 1.31E+06	201.8 ± 39.5	5.4 ± 0.4	130.0 ± 22.2	24.5 ± 5.5	44.4 ± 7.5
		NH_4Cl	0.69 ± 0.01	4.00E+06 ± 5.27E+04	184.9 ± 5.3	5.2 ± 0.3	119.6 ± 17.0	22.9 ± 2.5	40.0 ± 13.1

(B)

Species	N-conc	N-source	μmax (d^{-1})	Nte (cells mL^{-1})	C content (fg fL^{-1})	C/N	C assimilation rate (fg fL^{-1} d^{-1})	N assimilation rate (fg fL^{-1} d^{-1})	Protein (fg fL^{-1})
Consortium	1.5 mM	$NaNO_3$	0.47 ± 0.03	8.61E+06 ± 1.48E+06	85.3 ± 38.4	6.2 ± 0.4	40.1 ± 16.5	6.6 ± 3.1	109.4 ± 39.8
		NH_4Cl	0.61 ± 0.09	3.55E+06 ± 1.64E+05	218.6 ± 30.9	6.1 ± 0.3	154.1 ± 46.7	25.0 ± 6.9	150.3 ± 39.8
	15 mM	$NaNO_3$	0.43 ± 0.03	9.97E+06 ± 1.80E+06	201.4 ± 1.1	5.9 ± 0.5	88.6 ± 26.6	15.3 ± 5.8	223.3 ± 35.6
		NH_4Cl	0.49 ± 0.03	4.51E+06 ± 1.33E+05	285.6 ± 43.0	5.2 ± 0.2	142.5 ± 13.5	27.2 ± 1.9	109.8 ± 5.8
	50 mM	$NaNO_3$	0.56 ± 0.10	1.05E+07 ± 1.15E+06	125.6 ± 20.3	5.4 ± 0.5	76.6 ± 5.2	14.1 ± 0.4	111.5 ± 7.1
		NH_4Cl	0.61 ± 0.04	4.13E+06 ± 1.20E+05	449.5 ± 48.7	5.5 ± 0.2	313.6 ± 20.7	56.9 ± 2.2	119.5 ± 12.0

3.2 | Variability of consortium cultures under different nutritional regimes

3.2.1 | Growth and species composition

Growth parameters of the consortium cultures (μ_{max}, Nt_e, T_m and T_e) were statistically analysed by PCA and results are graphically reported in Figure 1A. The principal component one (PC1) accounted for 83.91% of the variance, while the principal component two (PC2) accounted for 8.93% of the variance, cumulatively explaining 92.84% of the total variance. All variables contributed to separating the treated and control groups by PC1 (Figure 1B), which described most of the variance: NH_4^+ supplied samples were grouped on the left side of the graph, while the NO_3^- supplied ones were on the opposite side. The highest μ_{max} values were achieved in cultures treated with 3 to 10 mM of N-NH_4^+ (Figure S2A). Concurrently, cultures supplied with reduced N reached far lower Nt_e values than the respective NO_3^- supplied counterparts (Figure S2B). The μ_{max} and T_m were also inversely related since faster growth usually led to a shorter time at which μ_{max} was achieved or the inflection point. While Nt_e separated the two treatment groups, it could not help to discriminate samples within these groups since Nt_e remained similar at N concentration above 10 mM (Figure S2B).

N source and concentration strongly influenced species proportion, too (Figure 2). Circular species (*A. protothecoides* and *C. reinhardtii*) (Figure 2B, D) were promoted at the expense of *T. obliquus* (Figure 2C) as N concentration increased. The trend was emphasized when NH_4^+ was the N source. Indeed, *T. obliquus* represented about 50 and 25% of the total cells at the lowest N concentration (1.5 mM) in NO_3^- and NH_4^+ supplied groups, respectively. The percentage dropped to 11 and 3% at the highest tested N concentration (50 mM), respectively (Figure 2A).

3.2.2 | Functional changes of photosynthetic apparatus

Photosynthetic apparatus was greatly affected by the chemical form of N supplied to the cultures and by the growth phase (Figure 3, Table S2). In the exponential phase, photosynthetic efficiency and pigments of the consortia exposed to NO_3^- or NH_4^+ were almost identical (Figure 3). The values of Chl *b* and F_v/F_m remained independent

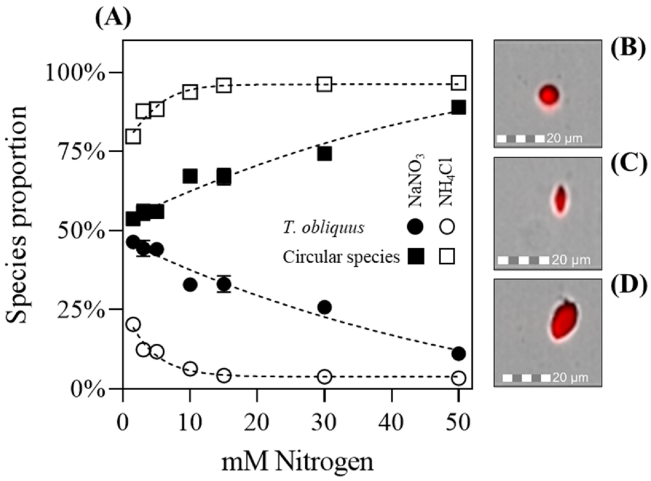


FIGURE 2 (A) Species proportion in the consortium as a function of N concentration in each experimental condition; circular species is the cell-cluster grouping both *A. protothecoides* and *C. reinhardtii*. Cytofluorimeter picture of each species belonging to the consortium are also reported: (B) *Auxenochlorella protothecoides*, (C) *Tetradesmus obliquus*, (D) *Chlamydomonas reinhardtii*.

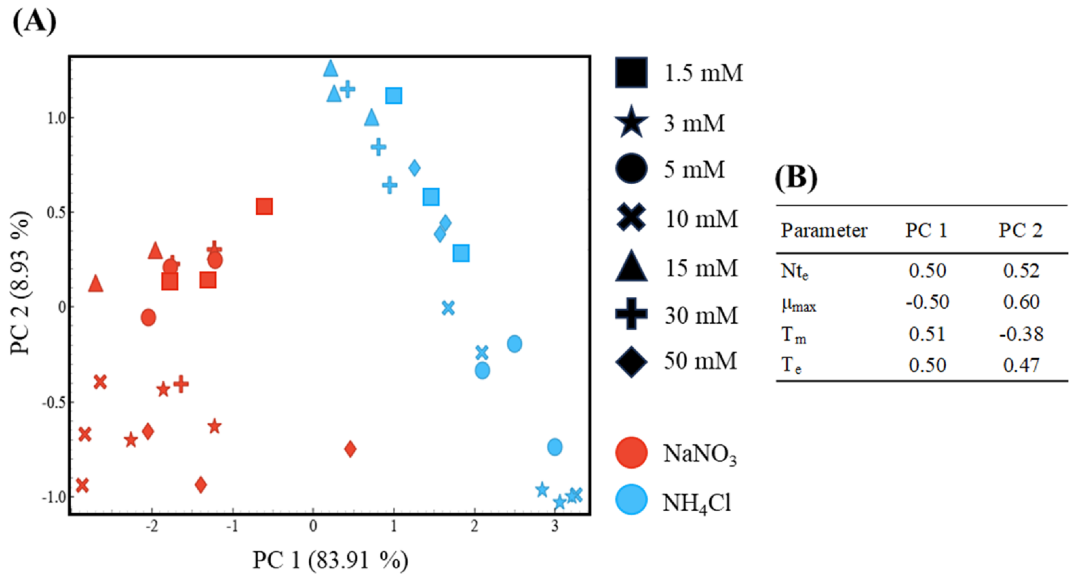


FIGURE 1 Principal component analysis of consortium based on growth parameters (µmax, Nt_e, T_m, T_e). (A) Scatter plot with the percentage of variance explained by each PC and (B) the loadings values of each parameter are reported. Different symbols indicate different experimental conditions as reported in the legend.

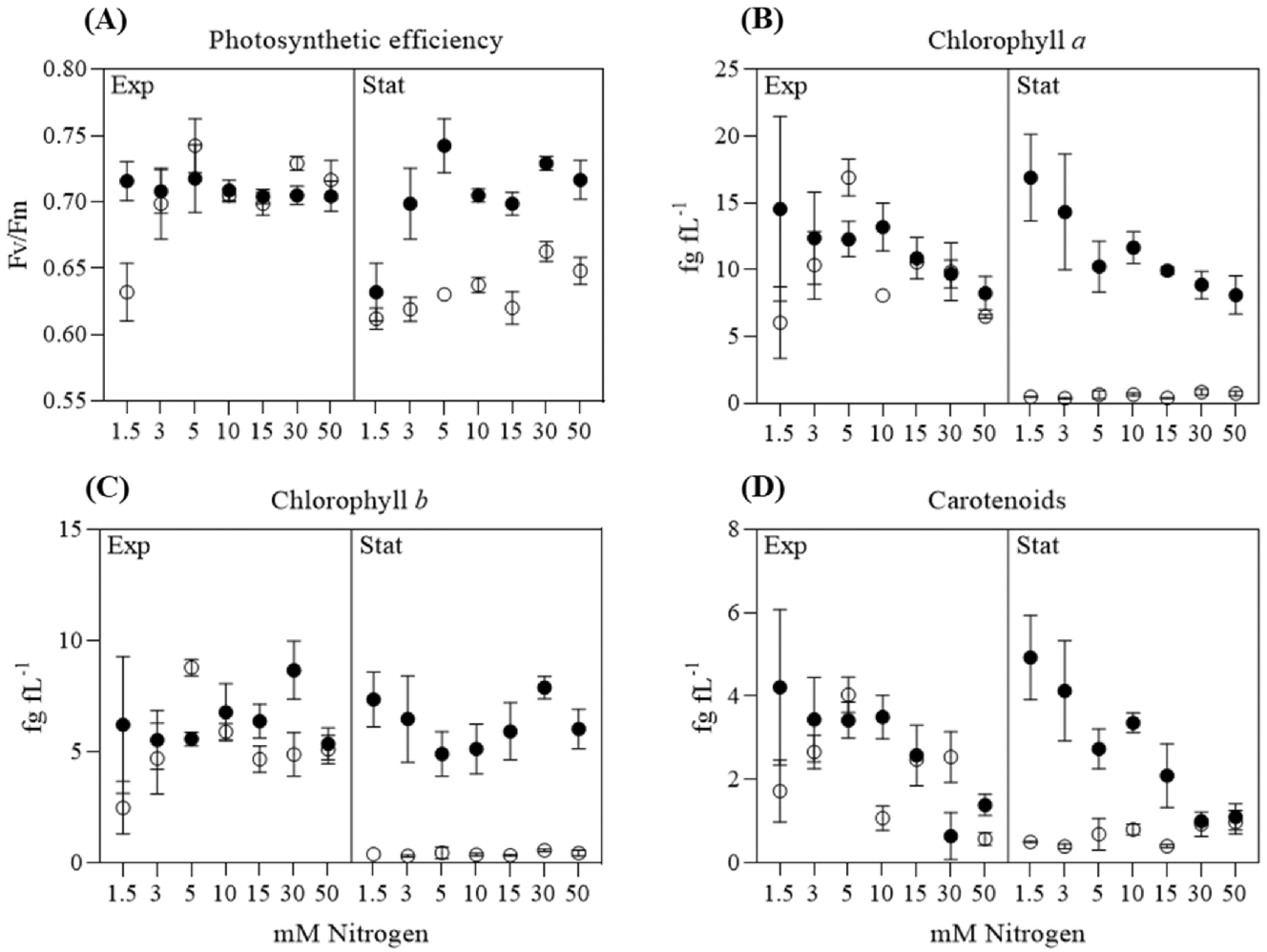


FIGURE 3 Changes in photosynthesis-related parameters in NH_4^+ (white dots) and NO_3^- (black dots) consortium cultures as a function of N concentration and growth phase. Values of (A) photosynthetic efficiency (F_v/F_m), (B) chlorophyll *a*, (C) chlorophyll *b*, (D) carotenoids, are displayed separately and grouped by growth phase (on the left being the exponential phase, on the right the stationary phase). Pigments concentration is expressed per unit of cell volume. Data are represented as mean ± SD.

from the N concentration, while Chl *a* and carotenoids showed an inverse relationship with increasing N concentration.

Major differences between the two N source groups occurred once cultures reached the stationary phase (Figure 3, Table S2): in NO_3^- growth condition, photosynthetic efficiency and pigment content were constant once cultures entered the stationary phase. On the contrary, pigments of the NH_4^+ grown cultures in the stationary phase were ten times less abundant as compared to values in the exponential phase (both chlorophylls and carotenoids, Figure 3B, C), while photosynthetic efficiency averagely was 15% lower.

3.2.3 | C and N abundances and protein content

The C to N ratio slightly decreased (from 6.2 to 5.5, Figure 4B) with increasing N concentration but did not respond to the N source ($p = 0.084$, Table S2). Regarding C and N assimilation, a linear increase with increasing N concentration was observed in NO_3^- cultures but only in the range of 1–10 mM for NH_4^+ cultures.

C and N amounts per biovolume (fg fL^{-1}) were always higher in NH_4^+ cultures than in NO_3^- cultures, and they increased, with different intensities, in both N source conditions with increasing N concentrations (Figure 4A, Figure S3); indeed, N source and concentration drastically changed both the species composition within the consortium and the average cell volume which led to a different accumulation of these elements in the cell. While C content increased from 84.8 ± 29.8 fg fL^{-1} (1.5 mM) to 140.8 ± 33.6 fg fL^{-1} (50 mM) in NO_3^- cultures, it increased from 247.0 ± 44.9 fg fL^{-1} (1.5 mM) to 514.0 ± 24.0 (50 mM) in NH_4^+ cultures.

The protein content was generally higher in NH_4^+ -grown consortia than in their counterparts during the exponential phase, while the opposite occurred in the stationary phase; it was difficult to find other trends. Anyhow, the N concentration was the main factor explaining the variance among the data (3-way ANOVA, $p < 0.001$, Table S2).

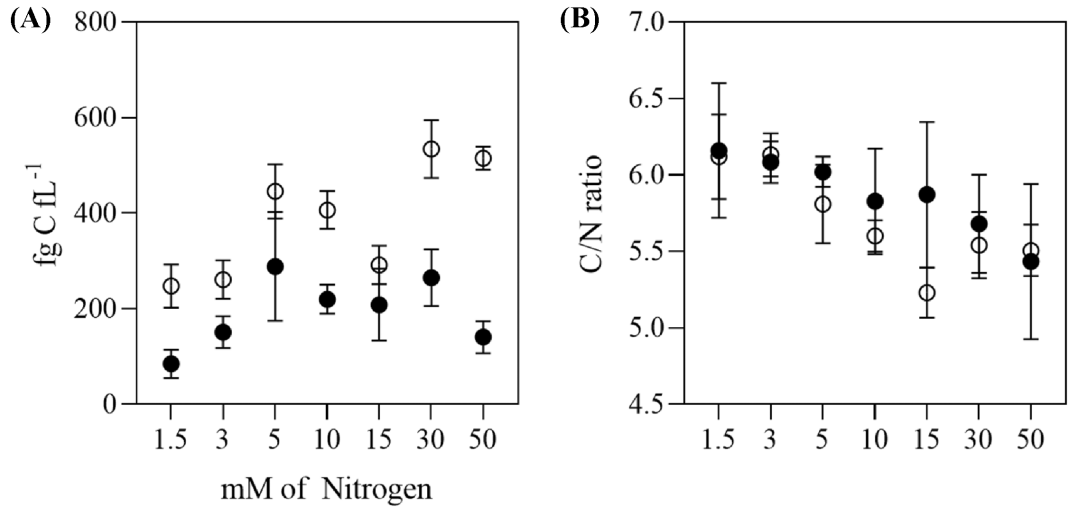


FIGURE 4 C content (A) and C/N ratio (B) in NH_4^+ (white dots) and NO_3^- (black dots) consortium cultures as a function of N concentration. Data are represented as mean ± SD.

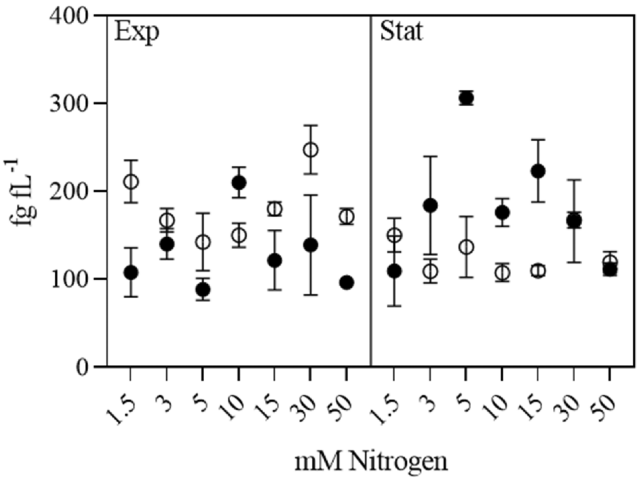


FIGURE 5 Protein abundance in NH_4^+ (white dots) and NO_3^- (black dots) consortium cultures as a function of N concentration. Protein concentration is expressed per unit of cell volume; on the left values observed in exponential phase, on the right values in stationary phase. Data are represented as mean ± SD.

3.2.4 | C and N isotopic fractionation

In general, on the base of the natural isotopic signature data, C fractionation tended to increase with increasing N concentration (Figure 6A, Table S2) for both N source groups. Similarly, N fractionation was significantly different due to the N concentration in the external medium but not to the source of N. Indeed, a depletion of the heavier isotope (^{15}N) was observed with increasing N concentration within both N source groups, where $\delta^{15}N$ decreased from ~ −7.5 (at 1.5 mM N) to ~ −11.2 (at 50 mM N) (Figure 6A).

The cell and biomass ratios between NH_4^+ and NO_3^- cultures in the stationary phase were used as indicators of tolerance to NH_4^+ nutrition (Figure 6B). The cell ratio was always less than or equal to 0.5, meaning a cell density of NH_4^+ cultures in stationary phase less or equal to half of NO_3^- culture ones (Figure 6B, Figure S2B). On the contrary, the biomass ratio slowly decreased from a maximum of 0.87 ± 0.01 at 1.5 mM of N to a minimum of 0.25 ± 0.02 at 50 mM N in the growth medium, meaning a lower biomass production in NH_4^+ cultures than in NO_3^- ones.

The biomass ratio was always higher than the cell ratio at 1.5, 3, 5 and 10 mM N. Equal values were achieved at 15 mM N; from that point on, the cell ratio was statistically higher than the biomass ratio (Figure 6B). A biomass ratio higher than the cell ratio indicates the presence of bigger or heavier cells in ammonium-supplied consortia than nitrate-supplied ones. At 30 and 50 mM N, cells supplied with reduced N were smaller or lighter than cells in NO_3^- cultures since the biomass ratio was lower than the cell ratio.

A strong relation ($R^2 = 0.6109$) between the $\delta^{15}N$ of NH_4^+ supplied cultures and the biomass ratio was observed (Figure 6C). Thus, the lower biomass ratio observed at 30 and 50 mM N was associated with a lower $\delta^{15}N$, meaning a lower tolerance to NH_4^+ (higher stress) at the cellular level. The observed behaviour did not belong to the algal cells grown in monocultures except somehow for *C. reinhardtii* (Figure S4).

4 | DISCUSSION

4.1 | Monocultures versus consortium in response to N

All tested N regimes promoted the growth of all species both in monocultures and in consortium: in particular, monospecific cultures grown in NH_4^+ tended to have a lower μ_{max} than in NO_3^- grown counterparts (Table 1). Among the species, *A. protothecoides* supplied with NH_4^+ showed one-third of the growth rate in NO_3^- and was the species most affected; nevertheless, its presence in the consortium

(A)

Parameter	N-source	N concentration						
		1.5 mM	3 mM	5 mM	10 mM	15 mM	30 mM	50 mM
$\delta^{13}C$	$NaNO_3$	-29.94 ± 1.20	-29.51 ± 0.90	-32.6 ± 0.71	-34.64 ± 1.66	-38.40 ± 2.31	-38.1 ± 0.82	-35.38 ± 1.90
	NH_4Cl	-29.42 ± 0.67	-30.82 ± 0.78	-31.2 ± 0.72	-29.87 ± 0.00	-41.61 ± 4.06	-35.67 ± 0.36	-36.12 ± 0.57
$\delta^{15}N$	$NaNO_3$	-7.35 ± 0.68	-8.36 ± 0.53	-8.98 ± 0.38	-9.00 ± 0.68	-9.21 ± 0.07	-11.87 ± 0.43	-11.17 ± 0.38
	NH_4Cl	-7.66 ± 1.05	-9.50 ± 0.06	-7.50 ± 0.28	-9.95 ± 0.49	-9.40 ± 0.73	-10.57 ± 0.50	-11.26 ± 1.75

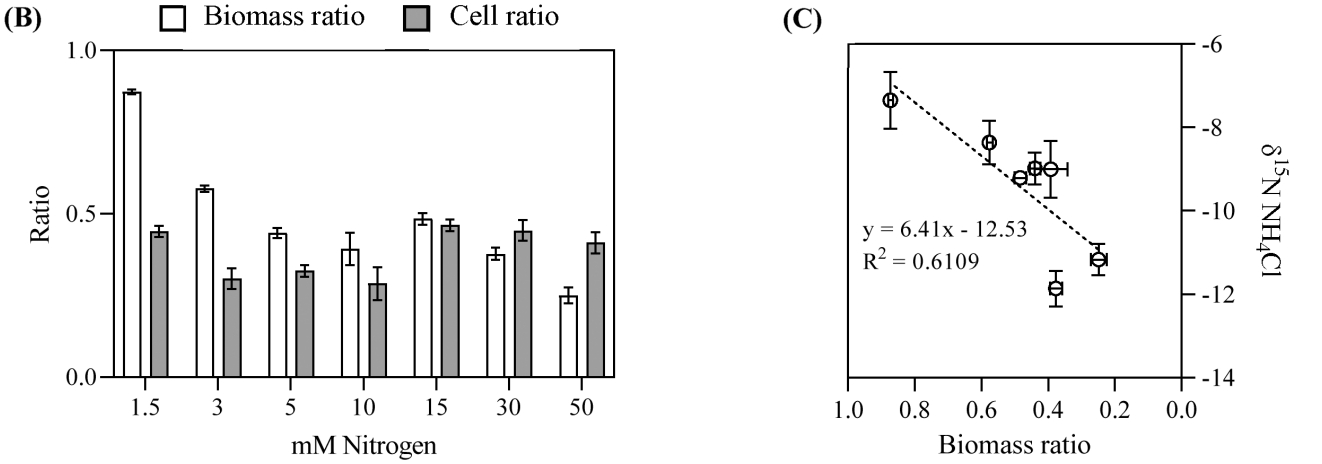


FIGURE 6 (A) C and N isotopic fractionation of consortium cultures grown in $NaNO_3$ or NH_4Cl as the only N source from 1.5 to 50 mM. Data are reported as mean ± SD. (B) biomass and cell ratios. (C) Relationship between the $\delta^{15}N$ of ammonium-supplied cultures and biomass ratio. Simple linear regression was carried out, and the coefficient of determination is reported in the graph. The dataset displayed represents the average values ± SD.

did not prevent NH_4^+ grown consortia from showing a similar rate to NO_3^- grown ones at the same N concentrations. The positive effect of co-cultivation on growth rate was not observed for maximal cell density (Nt_e), which resembled that of monocultures.

Again, a synergistic effect of co-cultivation was observed in the presence of NH_4^+ (not NO_3^-) by calculating the C and N assimilation rates (Tables 1, S1). Indeed, at each NH_4Cl concentration and especially at the highest one, the three species grown in consortium assimilated more C and N per time unit than in mono-cultures, with assimilation rates up to 2 times higher than those of mono-cultures (Table 1). The pattern was less evident but still true in the case of the C (and N) content per unit of volume, mostly at 50 mM NH_4^+. The extra energy required to boost C and N metabolisms was likely saved from the high energetic costs of cell maintenance under NH_4^+ nutrition when algae grew in polyculture. Indeed, a higher energetic burden is reported in NH_4^+ sensitive phototrophs (Britto et al. 2001); the consortium cultivation mode did eventually lower the burden and increase the energy availability of the entire crop.

Regarding proteins, cocultivation was beneficial for both NO_3^- and NH_4^+ grown cells ($p < 0.001$, Table S1): indeed, cells in polycultures had a much higher protein content per unit of biovolume than cells in monocultures as if algae mainly allocated N to proteins (Table 1). A wider protein pool could be consistent with a more complex environment requiring interspecific interactions within the consortium (Poulson-Ellestad et al. 2014; Roccuzzo et al. 2020; Zhao et al. 2024). On the opposite, algae in monocultures allocated only part of the cellular N quota into proteins (Table 1), likely storing the remaining in specific N reserves (e.g. crystalline guanine, polyamines and inorganic N pool; Lavín and Lourenço 2005; Lin and Lin 2018; Mojzeš et al. 2020; Liang et al. 2023).

The cultivation mode also significantly influenced the C isotopic signature ($p < 0.001$, Table S1). Isotopic fractionation is already known to be dependent on several factors, mostly related to the source of nutrients and growth parameters (Popp et al. 1998; Eek et al. 1999). Evidence of relationship among ε_p (C isotope fractionation factor), μ_{max} and $[CO_2(aq)]$ was observed in the microalga *Phaeodactylum tricornutum* by Popp et al. (1998); in our results, $\delta^{13}C$ was not dependent on μ_{max} with only a few exceptions (i.e. *A. protothecoides*, NH_4^+ *C. reinhardtii*, NO_3^- consortium, Figure S3). Although C fractionation in monocultures was quite constant, $\delta^{13}C$ of consortia changed in response to N concentration ($p < 0.001$) but independently from its chemical form ($p = 0.258$, Table S1). Similar findings have already been reported by Eek et al. (1999), Swart et al. (2014) and Thompson and Calvert (1994).

Based on our results, the cultivation mode was the primary factor influencing the change in the use of inorganic carbon as a source for photosynthetic fixation at lower N concentrations (1.5 and 3 mM), as suggested by the $\delta^{13}C$ fractionation data in consortium cultures (Figure 5A). The activation of a CO_2 concentrating mechanism (CCM) would cause a smaller fractionation as observed: indeed, CCM is an

energy-demanding mechanism promoting HCO_3^- pumping and conversion into CO_2 to increase CO_2 local concentration around the enzyme ribulose bisphosphate carboxylase-oxygenase (Rubisco) (Giordano et al. 2005, 2017). Thus, CCM facilitates C fixation by Rubisco and hampers Rubisco discrimination in favour of the light ^{12}C-CO_2, resulting in a more positive $\delta^{13}C$ (Korb et al. 1996; Keller and Morel 1999; Vuorio et al. 2006). Among the consortium-forming species, only *C. reinhardtii* is well known to possess a CCM (Giordano et al. 2003; Wang et al. 2015; Mackinder 2018), while in *T. obliquus* is only partially confirmed (Basu et al. 2014; Huang et al. 2020) and in *A. protothecoides* no CCM was ever reported (Gris et al. 2014). Nevertheless, no variation of the isotopic signature in response to N concentration was found in the *C. reinhardtii* monoculture ($p = 0.9718$), suggesting CCM either had no role in the different values of the consortium $\delta^{13}C$ or played a role only when species were co-cultivated.

Another possible explanation for the smaller biomass fractionation consists of stimulation of β-carboxylation by anaplerosis during the synthesis of amino acids and proteins. Anaplerotic carboxylation, indeed, provides C- skeletons for N assimilation and is generally believed to be initiated in the cytosol with the involvement of a mitochondrial carbonic anhydrase (mtCA) in supplying HCO_3^- to phosphoenol pyruvate carboxylase (PEPc) for NH_4^+ assimilation in air-grown cells (Giordano 2001). In green algae, a high percentage of fixed C passes from PEP into the tricarboxylic acid cycle metabolites and amino acids (i.e. malate, succinate, aspartate, glutamate) (Treves et al., 2021), supporting a high growth rate and metabolic flexibility (Treves et al., 2021) and thus significantly affecting the C fractionation.

The carbon fixation by PEPc, which results in much lower discrimination compared to the one of Rubisco (0.5‰ against 37‰) (O'Leary et al. 1981; Roeske and O'Leary 1985), may temporarily exceed that of Rubisco (Guy et al. 1989) thus leading to more positive values of $\delta^{13}C$. However, as reported in Table 1 and in detail in Table S2 and Figure S5, the rate of N assimilation in consortia supplied with 1.5 mM N concentration was far lower compared to the one at 50 mM N, thus the role of the anaplerotic pathway could not explain the observed values at first glance.

The effect of co-culturing algae, thereby introducing interspecific interactions (e.g. competition) in addition to the intraspecific one, might be a game changer for $\delta^{13}C$ and overall C metabolism compared to the simple sum of the effects of individual components or monocultures.

Interactions among species in the consortium could explain the different N fractionation observed in comparison with the monocultures. The difference is supported by the results displayed in Table S1 from the analysis of variance, where a significative effect of the cultivation mode explained most of the $\delta^{15}N$ variation from the mono-culture conditions ($p = 0.005$). The N concentration also had a significant effect on the $\delta^{15}N$ ($p < 0.001$, Table S2) and, similarly to C fractionation, N fractionation increased at increasing N concentration (Figure 5A, Table S2). The result was in line with what was reported by Liu et al. (2013), who observed a concentration-dependent N isotopic fractionation in phytoplankton. As for $\delta^{13}C$, $\delta^{15}N$ fractionation of consortia was not dependent on the N source. Our data agree with those on green algae by Chieti et al. (2024) and Mollo et al. (2024) and with those by Signa et al. (2020), which investigated the effects of anthropogenic-derived N on macroalgal $\delta^{15}N$. On the opposite, plants show positive $\delta^{15}N$ values when supplied with NO_3^- (while) due to N loss in the form of root efflux and exudates or NH_3 loss from through the stomata, as reported by Ariz et al. (2011).

In algae, since the primary step in $\delta^{15}N$ fractionation is the transamination process, where the NH_2 group is transferred from an amino acid to an α-keto acid (Giordano 2001; Gris et al. 2014; Huang et al. 2020), it can be expected that $\delta^{15}N$ would not be influenced by the N form *per sè*.

To summarize what has been said so far, it can be deduced that, although experimental consortia were just exposed to different nutritional conditions, neither acclimated nor adapted, their superior fitness in terms of growth ability, C fixation and N assimilation, and metabolic flexibility, confirmed what reported by several authors about the enhanced resilience of consortia in response to external perturbations and not optimal growth condition (i.e. cultures supplied with NH_4^+ rich medium) (Mandal and Mallick 2009; Rashid et al. 2019; Gururani et al. 2022; Mugnai et al. 2023).

Greater resilience was demonstrated by the rearrangement of species relative abundances, achieving the same Nt_e within the consortium under varying growth conditions (Figure 2): *T. obliquus* cells were outclassed by cells of *A. protothecoides* and *C. reinhardtii* with increasing N concentration; the process was accelerated when NH_4^+ was the N source despite the fact that *T. obliquus* as single species was quite tolerant to reduced N. Indeed, its μ_{max} was equal to the rate of *C. reinhardtii* and much higher than the rate of *A. protothecoides* when NH_4^+ was 50 mM (Table 1A).

What is observed is consistent with small algal species (with a high surface-to-volume ratio) thriving at low nutrient concentration thanks to a favoured nutrient uptake but having no longer advantage as compared to bigger species as nutrient limitation ends (Reynolds 2006; Ryabov et al. 2021). A concentration of 1.5 mM N is higher than in many natural freshwater bulks (European Environment Agency 2023) but still the lowest in this experimental design, which wanted to mimic N-rich wastewater effluents (Ye et al. 2018); therefore, at such a concentration *T. obliquus* did present the highest percentage of total cells, around 50% of the consortium. With increasing N concentration, the abundance of bigger species increased at the expense of *T. obliquus* (being 11% at 50 mM). In any case, co-cultivation caused the algae to grow differently than in monocultures in response to external nitrogen availability, leading to the establishment of a unique pattern of dividing cells, as shown by PCA (Figure 1).

Co-cultivation implies interspecific interaction, such as competition for resources, as already stated. Therefore, resource availability necessarily influences algal cocultivation. Moreover, communication within an algal consortium or natural communities is an important factor in changing community size, structure and composition (Mugnai et al. (2023); Bacellar Mendes and Vermelho 2013), but also in shaping single species cell growth, morphology and organic composition (Pohnert et al. 2007; Rigby and Selander 2021; Petrucciani et al. 2024). While interspecific allelopathic interaction is still to be fully comprehended, an increased resilience and growth performance

have been observed already from the co-cultivation of microalgae (Chieti et al. 2024; Mollo et al. 2024) as occurred in this study.

4.2 | NH_4^+ effect

Toxic NH_4^+ concentration for Chlorophyta is proposed to be around 40 mM (Collos and Harrison 2014); nevertheless, even the lower NH_4^+ concentrations had a strong negative effect on Nt_e both in monocultures and consortium (Table 1). Availability of the other elements (i.e. P, S, trace metals) being equal, the halved number of cells per mL at each N concentration when supplied as NH_4^+ instead of NO_3^- was the most traceable sign of toxicity both in monoculture and co-culture (Table 1, Figure 1).

Moreover, the drastic decline of F_v/F_m values and chlorophyll contents found in NH_4^+ exposed consortia were consistent with NH_4^+ hampering photosynthesis under nutrient limitation.

A similar experiment on the differential effects of nitrate and ammonium on the cyanobacteria *Nostoc* sp. and *Microcystis aeruginosa* was carried out by Yang et al. (2023). While increasing NH_4^+ concentration was detrimental only to the cell density of *Nostoc* sp., the authors did not observe any change in photosynthetic efficiency in any of the species. No effect on chlorophylls and F_v/F_m was also observed in *Chlamydomonas acidophila* exposed to NO_3^- or NH_4^+ (Lachmann et al. 2019). It is important to note that most research has concentrated on cells in the exponential phase (or in continuous culture). Consequently, the effects on the photosynthetic apparatus once the stationary phase was reached might not have been observed, as reported here.

Moreover, consortia exposed to NH_4^+ nutrition showed a lower protein content per biovolume in the stationary phase as compared to their content in the exponential phase (Figure 5) when the NH_4^+ data set is analysed by 2-way ANOVA ($p < 0.0001$).

Therefore, our data suggest that NH_4^+ nutrition required higher energetic costs for cell division and maintenance, thus hampering cell density under replete nutrient conditions (during the exponential phase); subsequently, it impacted photosynthesis under nutrient-limited conditions (stationary phase) since PSII turnover and proton gradient across thylakoids altered by NH_4^+ ions (Enser and Heber 1980; Alencar et al. 2019) were no longer balanced in stationary phase. Indeed, the stationary phase did not cause the *per sè* effect since NO_3^- grown cells did not trigger the change (Figure 3).

It is known that to avoid or to reduce cell toxicity, the intracellular NH_4^+ ions must be incorporated into proteins, the major pool of assimilated N (Giordano et al. 2003; Chai et al. 2021). While NH_4^+ can directly enter the organic matter as ion or NH_3, oxidized forms of N require the conversion to the reduced form through enzymes bearing Fe cofactors, nitrate reductase (NR), nitrite reductase (NiR) and reduced power consumption (Su 2021). Once reduced, N is allocated into biomolecules through the glutamine synthetase (GS)/glutamine 2-oxo-glutarate aminotransferase (GOGAT) pathway, which requires ATP, NADPH and the provision of C skeletons via TCA cycle and anaplerosis (Giordano et al. 2003; Treves et al. 2021); therefore, in parallel, C metabolism must provide C skeletons not to limit N assimilation in the plastid (Lu et al. 2018). Alternatively, algae may activate an energy-dependent efflux of NH_4^+ ions to lower their intracellular amount (Collos and Harrison 2014).

Therefore, both N source and concentration are expected to affect the N assimilation rate and protein abundance in algae. Among the experimental single species, only *A. protothecoides* showed a higher N (and C) assimilation rate when exposed to the reduced N form, even if not dependent on its concentration. When algae were grown in consortium, higher N (and C) assimilation rates were observed in response to NH_4^+ as the N form and to its increasing concentration (Table 1), suggesting these cultures could finely tune N cell quota and better tolerate NH_4^+.

Conversely, no strong modulation of the protein pool in response to N source and concentration as the sole variation source was observed in both monocultures and consortia (Table 1). A limiting resource (e.g. energy) or inhibiting factor (e.g. cytosolic alkalinization due to NH_3 uptake plays a role in protein N-glycosylation, Qin et al. 2008) may have prevented the incorporation of intracellular N into the protein pool.

The biomass ratio in Figure 6B confirms the altered capacity to produce biomass algae were experiencing when supplied with reduced rather than oxidised N source; the biomass ratio as proposed by Ariz et al. (2011) in higher plants can be considered as an indicator of NH_4^+ tolerance/sensitivity also in algae. *A. protothecoides* showed the highest ratio (Figure S6), again proving NH_4^+ tolerance.

Moreover, a clear linear relationship ($R^2 = 0.6109$) was found for the first time in algae between $\delta^{15}N$ of the NH_4^+ supplied consortium and the biomass ratio for each N concentration: a lower biomass ratio corresponded to a lower value of $\delta^{15}N$ (Figure 5C) pointing out the higher stress condition of algae grown at 30 and 50 mM NH_4^+. The finding resembles what is observed in the roots of different plants (Ariz et al., 2011). Nonetheless, when the isotopic signature of NH_4^+ supplied single species was compared with the NH_4^+ tolerance indicator, instead, no linear relationship was observed (Figure S7). As already mentioned, a co-cultivation effect should be primarily taken into account: the $\delta^{15}N$ profile can be utilized to assess NH_4^+ toxicity experienced by algae when cultivated in a consortium. This consortium may undergo changes in species abundance while maintaining the same functional diversity as various higher plants.

5 | CONCLUSION

Based on the results presented here, algal growth under NH_4^+ exposure and nutrition resulted in a lower maximal cell density compared to the NO_3^- regime, indicating a clear sign of toxicity. The energetic burden on algae growing in NH_4^+-rich media was mitigated by cultivating the three single species in consortium. Consortium cultures fixed more C and, assimilated more N and synthesized more proteins than monocultures; they showed the capacity to modulate growth, species abundance, and metabolic pathways to better tolerate NH_4^+ and be functional. For instance, consortium biomass supplied with NH_4^+ was depleted of ^{15}N in a concentration-dependent manner. Consortium cultures were, therefore, more resilient than monocultures.

Overall, the cultivation mode affected all tested parameters: the interspecific competition and communication might play an underestimated role in algal physiology and plant science. Understanding these factors also represents the basic step necessary for the development of crucial applications in the field of biotechnology.

AUTHOR CONTRIBUTIONS

Conceptualization: LM, AN; Investigation: LM, AP; Formal analysis: LM, AP; Methodology: LM, AN; Visualization: LM, AP; Supervision: AN; Writing original draft: LM, AP, AN; Review and editing: LM, AP, AN; Visualization; Project administration and funding acquisition: AN.

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Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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