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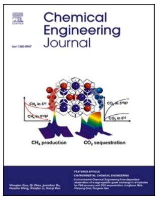

Nitrate and phosphate uptake dynamics in two halotolerant strains of *Chlorella vulgaris* is differentially influenced by carbon, nitrogen and phosphorus supply

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ABSTRACT

Microalgae are attractive for the development of environmentally sustainable processes with the option for carbon fixation, given the photosynthetic capacity of these microorganisms. Nitrogen and phosphorus are key nutrients for microalgal growth as well as carbon fixation and product formations. These are also important nutrients in agriculture, the sustainable supply of which is a challenge. They are often lost in waste streams but can be effectively recovered using microalgae and made available for agriculture. In this investigation, we systematically examined the nitrate and phosphate uptake response of two halotolerant strains of *Chlorella vulgaris* exposed to four carbon and three nitrogen and phosphorus supply regimes to develop an understanding of the relationship between uptake and supply in two microalgae strains from a similar habitat. Markedly differential response to changes in carbon and nutrient supply regimes were noted between the supply regimes and the strains. Changes in carbon supply was found to significantly influence nitrogen and phosphorus uptake in the two strains. Kinetics of nutrient depletion and carbon fixation were observed to be dynamic and non-linear for both the strains, under high carbon and nutrient supply regimes. This investigation provides for the first time a clear evidence base for the role of supply regimes in the management of nutrient uptake in microalgae, including the relationship between the two, and the significance of carbon supply in nitrogen and phosphorus uptake by microalgae. This will enable development of sustainable processes and predictable strategies involving microalgae towards net-zero solutions, as well as nitrogen and phosphorus recovery from waste streams.

1. Introduction

The rapid rise in atmospheric CO_2 levels in recent decades and the resulting environmental consequences of global warming and climate change is increasingly becoming apparent [1]. This is a global challenge that needs to be addressed urgently. This increasing realisation has led to a global drive to address the concern and has prompted countries and multiple stakeholders to adopt strategies not only to reduce CO_2 emissions to the atmosphere, but also to capture CO_2 to arrest the rapid increase.

One of the strategies consists in the use of microalgae, photosynthetic microorganisms that can be cultivated to utilise CO_2 from air as well as industrial point source emissions, in both photoautotrophic and mixotrophic cultivations. In addition to carbon, the primary nutrients required for growth of microalgae include nitrogen and phosphorus in the inorganic form, which can be sourced from waste streams, and this can include water in the form of wastewater or deep/eutrophic seawater to sustain growth. The possibility of sourcing nitrogen, phosphorus and water from process wastes allows for the development of environmentally sustainable cultivation routes to capture CO_2. In addition, nitrogen and phosphorus sequestered within algal biomass can also serve as a source of developing bio-fertilisers for use in agriculture as sustainable replacement for chemical fertilisers that often cause algal blooms and eutrophication in lakes [2–4]. The use of microalgae to reduce

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atmospheric levels of carbon dioxide, then, can be combined with the generation of products of value to develop economically sustainable solutions, and a viable proposition to address the global challenge of climate change, combined with economic growth and environmental sustainability. Whilst this is conceptually well recognised, practical solutions require further research and development. In particular, there is a lack of quantitative relationships between the supply and uptake of the elements and how this differs at species and strain levels. This data is required to develop sustainable processes and strategies with predictable directions.

The capacity to take up nitrogen and phosphorus differs between species of microalgae [5], and it has been shown to be dependent on CO_2 supply in some [6] but independent in others [7]. In the natural environment, location can be a contributing factor towards different nutritional demands and consumption characteristics. Surface ocean waters are often limited in dissolved inorganic nitrogen and phosphorus, potentially leading to the species in this environment to evolve to have more efficient uptake characteristics [8,9]. An estuarine environment with a constant and fluctuating inflow of N and P from rivers may have a rich supply of nitrogen and phosphorus in some locations, albeit seasonal, could encourage a predominance of species with higher uptake capacity [10,11]. The development of sustainable processes for CO_2 sequestration that leads to generation of economic value will ideally require processes where nitrogen and phosphorus consumptions are minimal, so these can be efficiently sourced or supplied. Where sequestration of nitrate and phosphate from waste streams is a key driver in establishing the process, species that have maximal capacity to take up nitrate and phosphate will be desired.

Generally, growth and growth related metabolism is accelerated with increase in supply of carbon, nitrogen and phosphorus [12–14] and phosphate is more rapidly consumed than nitrate [15,16]. However, species and conditions can also dictate the relationships and exceptions to the norm are not uncommon [17–20]. Nitrate and phosphate uptake in microalgae is dynamic and is influenced by several driving factors, among which carbon, nitrogen and phosphorus supply is a key driver [21–26] that can be managed. A better understanding of uptake mechanisms with respect to supply will enable us to develop strategies for effective utilisation of these key nutrients and establish sustainable processes for CO_2 sequestration using microalgae.

The objective of the work presented here is to develop an understanding of the influence of carbon supply, as well as nutrient (nitrate and phosphate) supply on the nitrogen and phosphorus uptake in two halotolerant strains of *Chlorella vulgaris* (BDUG 91771 and CCAP 211/21A) that have been isolated from a similar habitat, albeit from two geographically distinct zones. BDUG 91771 has been isolated from a salt pan in India, and has been shown to have desirable characteristics, such as good lipid accumulation and cultivability in open ponds for developing towards biodiesel productions [27,28], including large scale cultivations [29]. CCAP 211/21A is a salt tolerant strain of *Chlorella* isolated from a brackish ecological niche in England that has been shown to have desirable characteristics with commercial potential [30–32]. The motivation behind this investigation stems from establishing an evidence base for two impact areas (a) development of microalgae based biomanufacturing with associated carbon sequestration strategies to help meet the challenges of removing carbon from the atmosphere towards a net-zero carbon economy, and (b) development of microalgae based strategies to recover nitrogen and phosphorus from waste streams. It is important to understand the relationship between carbon, nitrogen and phosphorus supply and uptake to enable sustainable strategies to be developed in these impact areas.

In order to achieve this objective, the two strains were grown in a seawater medium and exposed to four different carbon supply regimes (resulting in different scenarios of available carbon) and three nitrate and phosphate supply regimes (increasing in nitrogen and phosphorus concentrations). This was done to establish the influence of carbon, nitrogen, phosphorus supply on nitrogen and phosphorus uptake dynamics, and assess the differences in the behaviour of the two strains, if any, given their ecological origins. The findings of the investigation will help form the basis of a framework to be developed in the long run, for establishing economic, environmentally sustainable solutions that employ cultivation of microalgae to address food and energy security and manufacturing solutions of the future.

2. Materials and methods

2.1. Microalgae strains

Two brackish water strains of *Chlorella vulgaris* species (CCAP 211/21A and BDUG 91771 - referred to in the manuscript as CCAP and BDUG, respectively) were investigated. CCAP was obtained from The Culture Collection of Algae and Protozoa, Oban, UK, and BDUG from an Indian repository (NRMC, Bharathidasan University, India).

For molecular confirmation of strains, DNA extraction and PCR amplification were followed as detailed elsewhere [33]. Analysis of similarity with BLAST result showed at least 96% similarity with the referenced sequences of the strains in the NCBI database (Supplemental Figure). This confirmed the identity of microalgae strains used in this study as well as their genotypic similarity, despite the differing geographical isolation background.

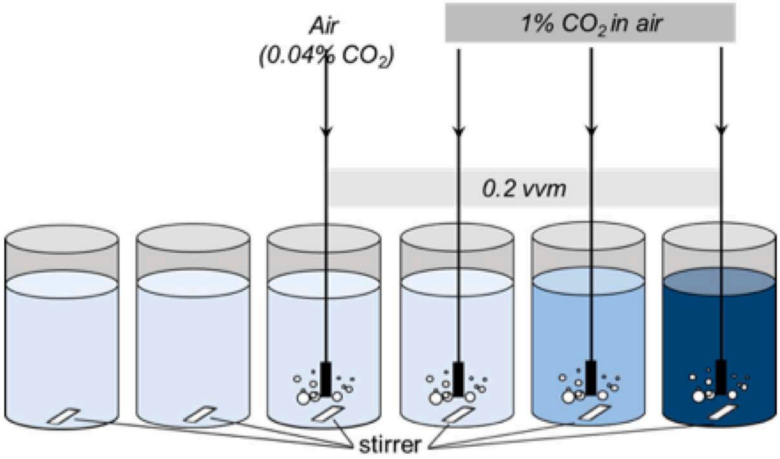
2.2. Cultivation conditions and experimental set-up

Both the strains were cultivated in stirred Duran bottles (1L) for ten days using standard *f*/2 media [34], or *f*/2 media modified for nitrate and phosphate concentrations, at an initial pH of 8.2 (±0.1), and at a room temperature of 23 °C. Four different carbon supply regimes and three nitrate and phosphate concentrations were experimented, as detailed below. The experimental setup is schematically represented in Fig. 1.

The light intensity was maintained throughout the experiment at an average of 200 $\mu E\ m^{-2}\ s^{-1}$ using cool-white light emitting diodes (LED), in all the reactors. This reading was taken as an average for at least six positions in each reactor. An inoculum of 0.2 (OD_{595} – optical density at 595 nm), maintained in the same cultivation medium, was employed. The lighting period adopted was a 24:0 daylight regime to have a high biomass growth and carbon uptake [35–37]. Illumination was provided by strips of LED attached to the frame of a hollow cylinder, positioned around the reactors. A photosynthesis activated radiance (PAR) irradiance sensor (QSL-2100, Biospherical Instruments Inc.) was used to measure the light intensity.

The composition of the *f*/2 medium per litre is: 33.6 g artificial seawater salts (Ultra Marine Synthetic Sea Salt, Waterlife), 75 mg sodium nitrate ($NaNO_3$), 5.65 mg sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), 1 ml trace elements stock and 1 ml vitamin mix stock. The trace elemental solution (per litre) consisted of 4.16 g Na_2EDTA, 3.15 g iron chloride ($FeCl_3 \cdot 6H_2O$), 0.18 g manganese chloride ($MnCl_2 \cdot 4H_2O$), 10 mg cobalt chloride ($CoCl_2 \cdot 6H_2O$), 10 mg copper sulphate ($CuSO_4 \cdot 5H_2O$), 22 mg zinc sulphate ($ZnSO_4 \cdot 7H_2O$) and 6 mg sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$). The vitamin mix solution (per litre) consisted of 100 mg vitamin B1, 0.5 mg vitamin B12 and 0.5 mg biotin.

In terms of carbon supply, four different regimes (all in *f*/2 media) were examined i.e. (1) without active carbon supply (allowing passive diffusion of atmospheric CO_2) (C_0), (2) with a high enough initial supply of carbon in the form of 0.01 M bicarbonate ($C_{0.01M}$), (3) continuous active supply of atmospheric CO_2 (0.04%) (C_{air}) and (4) continuous active supply of CO_2 at a higher level (1% CO_2 in air) ($C_{1\%}$). In addition, two other modifications of the media with differing nitrate and phosphate concentrations were experimented, by increasing the nitrate and phosphate contents to 4-fold and 8-fold (2*f* and 4*f*), respectively, whilst keeping the other ingredients the same as in *f*/2, in the $C_{1\%}$ carbon supply regime. 2*f* media had 3.6 mM nitrate and 0.15 mM phosphate, whilst 4*f* had 7.2 mM nitrate and 0.29 mM phosphate.



C	C_0	$C_{0.01M}$	C_{air}	$C_{1\%}$		
N and P	f/2				2f	4f

Fig. 1. A schematic of the experimental set-up employed to test the nutrient and carbon supply regimes. The culture medium used in the first four set-up was *f*/2. C_0 – no external supply of carbon, $C_{0.01M}$ – an additional supply of 0.01 M bicarbonate added at the start of the culture, C_{air} – culture aerated with air at a flow rate of 0.2 vvm; $C_{1\%}$ - culture aerated with 1% CO_2 (in air) at a flow rate of 0.2 vvm. In addition, $C_{1\%}$ was operated with 2*f* and 4*f* medium.

All the reactors were stirred at 200 rpm to ensure mixing and minimise mass transfer limitations. For the C_{air} and $C_{1\%}$ regimes, air or 1% CO_2 (v/v) in the air were respectively bubbled continuously, whilst the reactor was stirred at 200 rpm. Aeration was supplied from gas lines (air and/or CO_2 enriched air) in the laboratory through Teflon tubing. The aeration rate was set at 0.2 vvm, for C_{air} and $C_{1\%}$ carbon supply regimes. The flowrate was ensured by connecting a rotameter between the gas supply and the reactor. To monitor the CO_2 concentration, gas supply to the culture was split to create one extra flow to BlueInOne gas analyser (BlueSens, GmbH, Germany) which recorded the CO_2 concentration every hour. All the reactors and media were autoclaved before usage throughout the experiment. Sterile practices were applied when dealing with the culture during the experiment.

2.3. Analytical methods

The biomass concentration was determined based on the optical density (OD), measured using UV/Visible Spectrophotometer at 595 nm [38,39].

The productivity of the cultures was calculated based on the amount of biomass produced per litre per day, calculated on a dry weight basis. The amount produced is obtained based on the difference in the dry weight on the desired day and that at the beginning. The amount of biomass is obtained by converting the optical density to dry weight (mg/l) using calibrations that were established prior to experimentation, as given below Eq. (1):

$$\text{BDUG dry weight (mg/l)} = (157.9 \times \text{Optical Density} - 6.4) \tag{1a}$$

$$\text{CCAP dry weight (mg/l)} = (171.9 \times \text{Optical Density} - 22.9) \tag{1b}$$

Specific growth rate (μ) was calculated based on the following formula:

$$\mu = \frac{\ln(X_i/X_0)}{t_i - t_0}$$

X_0 and X_i represent the concentration of biomass at time t_0 and t_i, respectively.

Biomass yield on nitrogen and phosphorus (g/mg) was calculated using Eq. (2):

$$Y_{X/N} = \frac{X_f - X_i}{N_i - N_f} \tag{2a}$$

$$Y_{X/P} = \frac{X_f - X_i}{P_i - P_f} \tag{2b}$$

X_f and X_i represent the final and initial concentration of biomass respectively. N_i and N_f represent the initial and final concentration of nitrate respectively. P_i and P_f represent the initial and final concentration of phosphate respectively.

pH measurements were made using an Orion Star pH Meter A211 Manual (Thermo Fisher). The pH meter was calibrated with a buffer added with a similar salt concentration to avoid error due to liquid junction potential, which occurs due to difference in ionic strength between the samples and buffers [40].

Dissolved inorganic nitrogen (DIN) and phosphorus (DIP) concentration in the culture were determined spectrophotometrically using the culture supernatants [41,42]. In brief, DIN in the media was determined by measuring the absorbance of 0.5 ml supernatant at 220 nm. DIP was determined by measuring the absorbance at 885 nm after reacting 1.0 ml of the supernatants with 0.1 ml of composite reagent containing molybdic acid, sulphuric acid, ascorbic acid and trivalent antimony in the ratio of 2:5:2:1. The absorbance was read about half an hour after the reaction to allow the blue colour developed to remain stable.

Carbon fixation rate (CFR) is calculated based on a simple photosynthetic stoichiometry as given below [43], similar to the method done by Adamczyk et al. [44].

$5CO_2 + NO_3^- + 0.05H_3PO_4 + 3.93H_2O \rightarrow C_5H_7O_2NP_{0.05} + 7.06O_2 + OH^-$.

2.4. Statistical analysis

Each experiment was conducted as a minimum of three biological replicates. In all the figures, the data points are the mean values of the replicates, whilst the error bars denote the standard errors around the mean. When comparing two data, only significant differences at 95% confidence (*t*-test) or above ($p < 0.05$) were considered. In addition, a two-way ANOVA was performed, where applicable, to evaluate the difference among treatments in which $p < 0.05$ value was considered statistically significant.

3. Results and discussion

The two *Chlorella* strains were cultivated in *f*/2 medium, which is a frequently used saline medium to maintain and cultivate marine algae. The carbon supply regimes were carried out as follows, all in *f*/2 medium, so at fixed initial nitrate and phosphate concentrations: (1) carbon limited – C_0, (2) abundant initial carbon supply – $C_{0.01M}$, (3) continuous limited supply – C_{air}, (4) abundant continuous supply – $C_{1\%}$. The nitrate and phosphate supply regimes were examined for the abundant carbon regime (i.e., in $C_{1\%}$), but with increasing concentrations of nitrate and phosphate that were 4-fold (2*f*) and 8-fold (4*f*) higher than in *f*/2 and were compared to *f*/2. There is thus a gradation in the supply of carbon, nitrogen and phosphorus within the experimental set-up that allows for a systematic examination of uptake dynamics with respect to supply that has not been reported before to the best of the authors' knowledge.

3.1. Biomass accumulation is influenced by carbon and nutrient supply differentially in the two strains

Growth, as reflected by a change in biomass at a given time point compared to that at the start (X_t/X_0), showed significant differences between the carbon and nutrient supply regimes for the two strains, as would be expected, but there were also marked differences in the response between the two strains that could be observed (Fig. 2). The increased response to carbon supply can be seen to be more visible in BDUG (Fig. 2**A**) than in CCAP (Fig. 2**B**). This can also be seen with the specific growth rates (Fig. 2**E**).

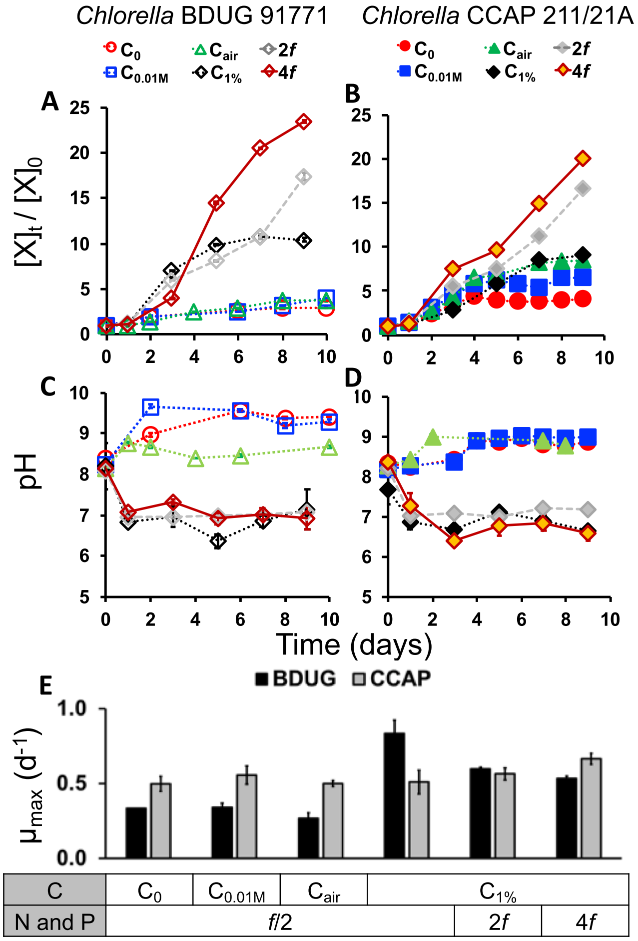


Fig. 2. Growth response differs markedly for the two strains at different carbon (C), nitrogen (N) and phosphorus (P) supply regimes. Growth profiles of BDUG (A) and CCAP (B) measured as relative change in biomass (OD_{595nm}) at each time point relative to the start. Time profile of pH during growth in the cultivation of the respective organism, BDUG (C) and CCAP (D). Maximum specific growth rate for the two strains under the different carbon, nitrogen and phosphate supply regimes is plotted in E. Results plotted are mean of biological triplicates with error bars indicating standard error about the mean.

In BDUG, the $C_{1\%}$ carbon supply regime led to a marked difference in growth (>10-fold increase in final biomass), compared with the other carbon supply regimes (<5-fold increase). This difference in response is less obvious in CCAP, where the increase ranged between 4 and 9-fold, for all conditions. Biomass production is markedly low in BDUG under carbon supply regimes other than $C_{1\%}$, suggesting carbon supply has a significant influence on growth and biomass production in this strain. This is not seen as much in CCAP, where the difference is more marked with C_0, but seems comparable otherwise in *f*/2. Since the difference within *f*/2 is with carbon supply, the observed differences can be attributed to CO_2 availability and/or uptake. Increased supply of CO_2 is widely acknowledged to result in increased growth up to a certain level of CO_2 in the medium [6,7,45,46], beyond which decrease in pH inhibits growth.

In addition to carbon supply, nitrogen and phosphorus supply regimes have been shown to influence growth [47–51]. For CCAP, the difference is more marked with nutrient supply, a progressively greater biomass production seen at higher nutrient supply (2*f* and 4*f*), compared to *f*/2. Biomass production appears saturated at high carbon ($C_{1\%}$), in *f*/2, for BDUG, with a concomitant and progressive increase in growth with increase in nutrient supply (2*f* and 4*f*), suggesting increased capacity to convert excess nutrients to biomass. This is not the case with CCAP, where the response is highest between *f*/2 and 2*f* with no further increase of note seen with 4*f*.

The pH of the culture media turned more alkaline in C_0, $C_{0.01M}$ and C_{air}, whilst it reduced and plateaued at a more neutral pH in $C_{1\%}$, for both the strains (Fig. 2**C, D**). This is along expected lines. pH is expected to increase in microalgae cultivation due to the loss of CO_2 during photosynthesis since carbon consumption during this process results in an accumulation of hydroxide ions [52–54]. Higher CO_2 supply (as in $C_{1\%}$) tends to acidify the medium due to the increased formation of the weak carbonic acid on CO_2 dissolution that exceeds carbon uptake by algae.

Overall, a more marked response to biomass production was seen with increase in carbon, nitrogen and phosphorus supply in BDUG than in CCAP. This suggests that even for species isolated from similar habitats there can be differences in the response to carbon, nitrogen and phosphorus supply regimes, and that these responses should be characterised sufficiently for the strain of microalgae cultivated to maximise carbon, nitrogen and phosphorus sequestration potentials.

3.2. Carbon supply shows greater influence on nitrate and phosphate depletion in BDUG, whilst nutrient supply has a greater influence in CCAP

Nitrate and phosphate consumption, as inferred respectively from the residual dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphates (DIP) left in the medium is plotted as nitrate and phosphate removed from the medium. This mirrored changes in nutrient and carbon supply for both the strains (Figs. 3, 4).

C_0 had the least depletion in *f*/2, for both the strains, where < 20% of the supplied nitrate was consumed (Fig. 3), suggesting mass transfer limitations of dissolved inorganic carbon and a resultant poor availability of carbon (Table 1) leading to lower consumption of nitrate, although phosphate is more readily taken up. A marked difference in nutrient consumption can be noticed in $C_{0.01M}$, between the two strains. The nitrate consumption was higher in BDUG (60% of the supplied nitrate was consumed), compared to CCAP, where the consumption was poor and equivalent to what was observed in C_0, at ~20%, despite the complete utilisation of the supplied carbon in both the strains (Table 1). However, phosphate consumption was slower in BDUG compared to CCAP, in this condition (Fig. 4). Higher biomass accumulation in CCAP under this condition (Fig. 2), suggests that CCAP is more efficient in

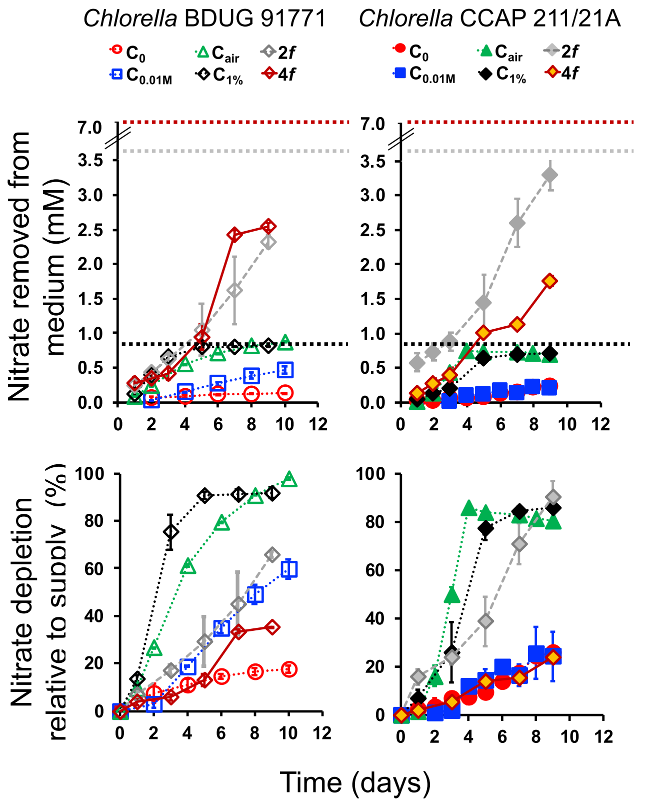


Fig. 3. Nitrate removed (top panels) and nitrate depleted as a percent of supply (bottom panels) in the microalgal culture medium for each of the *Chlorella* strains, in the different carbon and nutrient supply regimes. The black, grey and red dotted lines in the top panels indicate the amount of nitrogen supplied at the beginning of the cultivation for *f*/2 (0.9 mmoles/kg), 2*f* (3.5 mmoles/kg) and 4*f* culture (7.0 mmoles/kg), respectively.

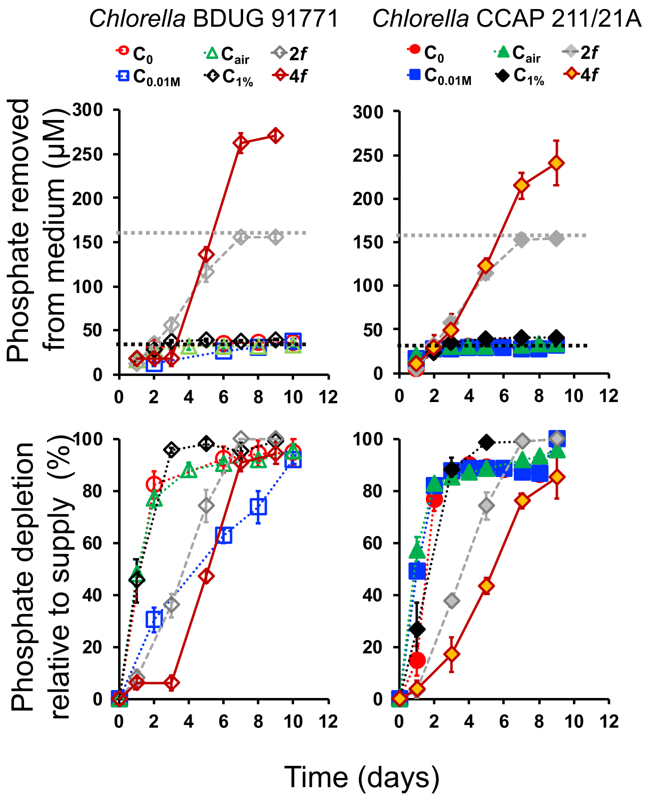


Fig. 4. Phosphate removed (top panels) and phosphate depleted as a percent of supply (bottom panels) in the microalgal culture medium for each of the *Chlorella* strains, in the different carbon and nutrient supply regimes. The black, grey and red dotted lines in the top panels indicate the amount of phosphorus supplied at the beginning of the cultivation for *f*/2 (36 μmoles/kg), 2*f* (144 μmoles/kg) and 4*f* culture (288 μmoles/kg), respectively.

translating nutrient consumption to biomass accumulation, under this carbon supply regime, compared to BDUG, which appears to have been better at sequestering the nitrate, with little translation to equivalent increase in biomass. BDUG's higher nitrate consumption is perhaps reflective of a higher photosynthetic requirement, as nitrogen is a key constituent of photosynthesis. It is also possible that a sharper increase in pH in BDUG compared to CCAP may have had an influence in suppressing growth. It should be noted that the dissolved inorganic carbon available at the start of the cultivation in this regime is 3–11-fold higher than that available in the other regimes (Table 1), suggesting that this regime is more useful in assessing nitrate and phosphate consumption under carbon excess conditions at the initial stages of growth. It is also possible that BDUG is adopting a strategy of luxury storage of nitrate. Luxury uptake and/or storage of nitrogen [55–58] and phosphorous [22,31,59–63] is known to occur in microalgae.

Nitrate consumption increases on continuous supply of carbon (C_{air} and $C_{1\%}$), where carbon mass transfer limitations are expected to be minimal. In cultures of *Chlorella vulgaris* and other microalgae, the dynamics of carbon uptake can significantly influence nitrate and phosphate uptake to different degrees [21–25,64]. Similarly, nitrate and phosphate supply can also influence growth and nutrient uptake capacities differently [22,26].

Nitrate consumption relative to supply also approaches 100% in C_{air} and $C_{1\%}$ regimes, within 4 days. Whilst this can be seen to be translated to a relative increase in biomass for both the conditions in CCAP, there is a marked difference in translation to relative increase in biomass between C_{air} and $C_{1\%}$ in BDUG, where the decreased carbon (in C_{air}) (Table 1) appears to have limited growth in this strain. This trend is not seen in the case of CCAP, where the relative increase in biomass is comparable for the two conditions. For BDUG, nitrate consumption can be seen to be faster in $C_{1\%}$ than in C_{air}, whilst the trend is reversed for CCAP. Phosphate was rapidly depleted in two days for both the strains in these two carbon supply regimes. This again suggests that BDUG is adopting a strategy of luxury storage of nutrients in these carbon supply regimes, as well, and/or has a higher photosynthetic requirement than CCAP.

Table 1
Dissolved inorganic carbon (DIC - mM) at the start (Initial) and end (Final) of the cultivation under the different carbon (C), nitrogen (N) and phosphorus (P) supply regimes, for the two strains of *C. vulgaris*.

C/N/P supply		BDUG		CCAP	
		Initial	Final	Initial	Final
C_0	*f*/2	3.7 ± 0.1	0.1 ± 0.0	1.8 ± 0.0	0.1 ± 0.0
$C_{0.01M}$	*f*/2	11.3 ± 0.7	0.4 ± 0.1	12.1 ± 0.2	0.3 ± 0.0
C_{air}	*f*/2	1.6 ± 0.0	0.5 ± 0.0	1.4 ± 0.1	0.1 ± 0.0
$C_{1\%}$	*f*/2	1.6 ± 0.2	3.1 ± 0.1	1.7 ± 0.3	2.9 ± 0.2
$C_{1\%}$	2*f*	1.5 ± 0.0	4.4 ± 0.4	1.5 ± 0.0	5.0 ± 0.4
$C_{1\%}$	4*f*	1.0 ± 0.1	6.1 ± 0.3	1.1 ± 0.2	5.2 ± 0.4

Further increase in the supply of nitrate and phosphate (2*f* and 4*f*) results in concomitant increase in nutrient consumption, but the efficiency drops in BDUG, where nitrate consumption reaches 60% in 2*f* and < 40% in 4*f*, and complete phosphate consumption takes 4 more days. In CCAP, the nitrate consumption is greater in 2*f*, reaching 90%, suggesting a greater capacity by this strain to sequester nitrate, in this condition. However, this does not necessarily translate to a relative increase in biomass, which is comparable for both the strains in 2*f*. Further increase in supply (4*f*) results in a drastic drop in consumption in CCAP, reaching 20%, suggesting peak nitrate consumption capacity has been reached for this strain at 2*f*, and perhaps there is a regulatory mechanism that senses nitrogen availability to be in excess of requirements at 4*f* that is not seen at 2*f* that inhibits nitrate uptake. Phosphate consumption relative to supply was slower for both the strains, compared to *f*/2, with a lag in 4*f* for BDUG. Nevertheless, unlike nitrate, the phosphate consumption relative to supply was above 80% in 4*f* for both the strains. Luxury uptake and storage of nitrogen appears to operate in CCAP with nutrient supply regimes, akin to that seen with carbon supply regimes for BDUG. There is evidence of microalgae showing continued growth in media where nitrate and phosphate have apparently been depleted, thanks to luxury uptake of nitrates and phosphates that are stored in vacuoles or as reserves in the cells for later usage [48,60,63].

The overall molar biomass yield on nitrogen and phosphorus is plotted in Fig. 5, for the two strains under the different regimes. A two-factor ANOVA (strain and regime being the two factors) showed that the nutrient supply regimes (*f*/2, 2*f*, 4*f*) influence biomass yield on nitrogen that is statistically more significant ($p < 0.001$) than the carbon supply regimes (C_0, $C_{0.01M}$, C_{air}, $C_{1\%}$) ($p < 0.01$), with no statistical difference between the strains, indicating that the behaviour is similar for the two strains. This was also noted with biomass yield on phosphorus, where both the carbon and nutrient supply regimes showed an influence on a similar statistically significant extent ($p < 0.001$).

A two tailed *t*-test between the conditions for each strain indicated that the molar yield on nitrogen in C_{air} is seen to be higher in CCAP compared to BDUG ($p < 0.001$) and 4*f* ($p < 0.01$) conditions, whilst this difference is seen only for C_{air} ($p < 0.01$), for the yield on phosphorus. For BDUG, the molar yield on nitrogen and phosphorus is lower in C_{air} and increases significantly ($p < 0.01$) in $C_{1\%}$, indicating that the carbon supply regime influences biomass yield on both nitrogen and phosphorus in this strain. This is not observed with CCAP. For the CCAP strain, C_{air} showed a comparable biomass yield on both nitrogen and phosphorus to $C_{1\%}$, but the yield was lower with increased nutrient supply ($Y_{X/N}$ - Cair > 2*f* ($p < 0.01$); $Y_{X/P}$ - C_{air} > 2*f* ($p < 0.05$), C_{air} > 4*f* ($p < 0.001$)); the biomass yield on nitrogen for CCAP increases on further nutrient supply ($Y_{X/N} - 4f > 2f$ ($p < 0.01$)), which is not seen with phosphorus uptake, whilst for BDUG $Y_{X/N}$ decreases on increasing nitrogen and phosphorus supply (2*f* and 4*f*), as has been noticed with *Chlorella sorokiniana* [56].

It is also noteworthy that for both the strains biomass yield on phosphorus is the lowest in C_0, and that for BDUG, $Y_{X/P}$ in $C_{0.01M}$ is higher than in C_0 ($p < 0.01$), C_{air} ($p < 0.01$), 2*f* ($p < 0.01$) and 4*f* ($p < 0.05$), but is lower than in $C_{1\%}$ ($p < 0.01$), suggesting that phosphate consumption is more effectively translated to biomass in bicarbonate than in CO_2 supply regimes. This can also be seen to an extent for CCAP, where $Y_{X/P}$ in 4*f* is lower than in $C_{0.01M}$ ($p < 0.001$) and C_{air} ($p < 0.001$). These observations suggest that an optimal balance between carbon and nutrient supply can influence translation of nutrient uptake to biomass in this strain. Overall, it can be seen that both carbon and nutrient supply regimes can influence nutrient uptake differently and that nutrient uptake by BDUG is influenced more by carbon supply and CCAP by nutrient supply, both showing evidence of luxury uptake in these respective cases. As was noted earlier with respect to biomass accumulation, nutrient uptake can also be influenced by supply regimes, to different degrees, even within species isolated from a similar habitat. This suggests that strategies can be developed to maximise carbon, nitrogen and phosphorus sequestrations, based on supply.

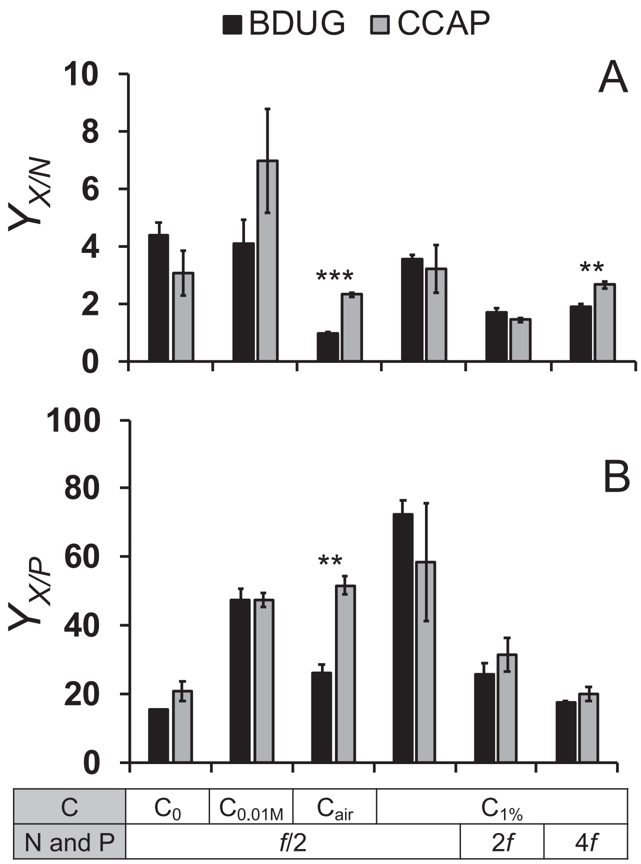


Fig. 5. Overall molar biomass yield on nitrogen depleted (A) and phosphorus depleted (B) for the two *Chlorella* strains. Results plotted are mean of three biological replicates (error bars indicate standard error about the mean). Statistically significant difference between the species is indicated as *** ($p < 0.001$) and ** ($p < 0.01$).

3.3. *Nutrient depletion and biomass accumulation show a nonlinear relationship for both the strains in all regimes.*

The relationship between nitrate or phosphate consumption (inferred from depletion of the respective nutrient from the media) and biomass accumulation (Fig. 6) is predominantly polynomial for most of the conditions, for both the species, a quadratic or cubic equation sufficiently fitting the relationship (Table 2).

For BDUG, a logarithmic relationship describes better phosphate depletion and biomass accumulation, under C_0, $C_{0.01M}$ and $C_{1\%}$ in *f*/2, indicating initial rapid consumption of available phosphate from the medium and continued biomass accumulation after phosphate depletion from media. This suggests cellular adsorption/storage before metabolism, perhaps luxury storage discussed earlier. Interestingly, the same strain demonstrated an apparent linear relationship at similar conditions to C_0, but at a higher nitrate concentration and a lower light intensity, although only 3 data points were available to establish that relationship [28]. For CCAP, a logarithmic relationship is observed for phosphate removal only under the C_0 regime. Even here, the uptake of phosphate is the slowest among the conditions and lagging behind BDUG. A polynomial relationship describes better the relationship of phosphate uptake and biomass accumulation for the other conditions, for CCAP.

For CCAP, in C_0, an exponential relationship suggests a lag in uptake of nitrate. This exponential relationship is also observed in BDUG in the 4*f* condition. These could be related to carbon uptake by both the species, or slower acclimatisation to the conditions, despite pre-acclimatisation of the cultures in the respective conditions before experimentation.

For both the strains, a polynomial relationship is noted for all the other conditions. More biomass is accumulated for a minimal uptake of nitrate, in $C_{0.01M}$, compared to the other conditions, in both the strains, suggesting a more efficient biomass accumulation in $C_{0.01M}$. This also perhaps suggests that both the species are most effective in translating nutrient uptake to biomass, under this carbon supply regime. It can also be seen that in C_{air}, both the strains remove nitrogen from the medium most with minimal translation to biomass, compared to the other conditions, as has also been noted with biomass yield on nitrogen (Fig. 5**A**).

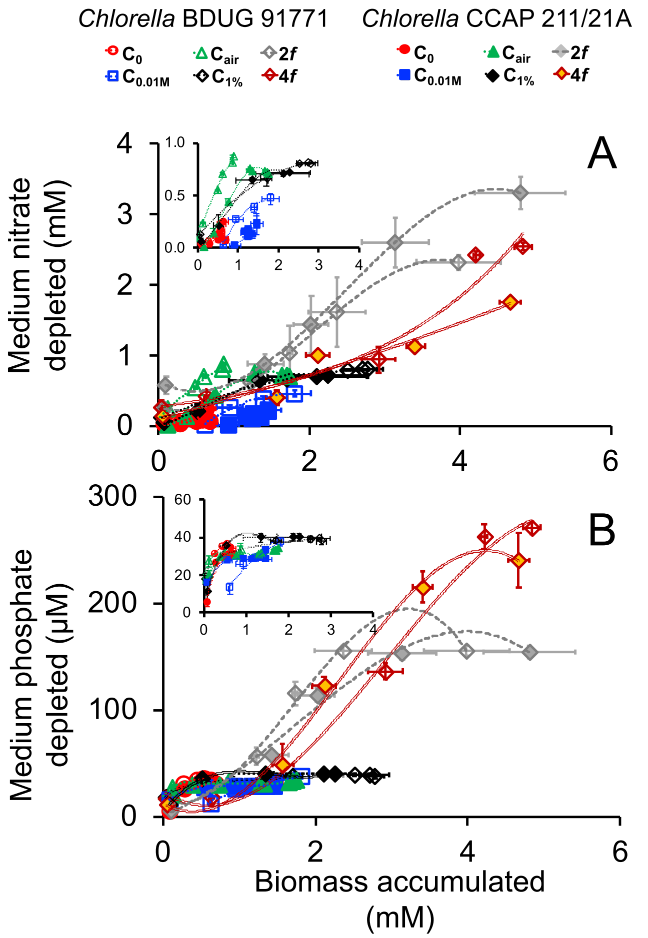


Fig. 6. Relationship of biomass accumulation with nitrate (A) and phosphate (B) depletion, under the different carbon and nutrient supply regimes for the two *C. vulgaris* strains.

Table 2a
Relationship between biomass accumulation (*x*) and nitrate depletion (*y*) in the two *Chlorella vulgaris* strains for the different carbon (C), nitrogen (N) and phosphate (P) supply regimes.

C/N/P supply		BDUG 9171		CCAP 211/21A	
		Correlation	R^2	Correlation	R^2
C_0	f/2	$y = 0.10\ln(x) + 0.19$	1.00	$y = 0.02e^{3.13x}$	0.33
$C_{0.01M}$	*f*/2	$y = -0.32x^2 + 1.14x - 0.55$	0.98	$y = 0.22x^2 - 0.17x$	0.91
C_{air}	*f*/2	$y = -0.27x^2 + 1.09x + 0.11$	0.99	$y = -0.36x^3 + 0.69x^2 + 0.32x - 0.05$	1.00
$C_{1\%}$	*f*/2	$y = -0.07x^2 + 0.45x + 0.10$	1.00	$y = -0.11x^3 + 0.23x^2 + 0.36x$	0.99
$C_{1\%}$	2*f*	$y = -0.10x^3 + 0.61x^2 - 0.31x + 0.27$	1.00	$y = -0.09x^3 + 0.67x^2 - 0.59x + 0.63$	1.00
$C_{1\%}$	4*f*	$y = 0.28e^{0.47x}$	0.96	$y = 0.02x^2 + 0.28x + 0.11$	0.94

This effect is seen to be more for BDUG compared to CCAP. It is possible that the excess nitrate is required for photosynthesis, and points to a possible difference in photosynthetic rates between the two strains in air, as alluded to earlier.

Although an increase in biomass productivity is achieved with increase in CO_2 supply, it has been shown that this increased CO_2 supply does not significantly affect nitrate or phosphate removal rates in a freshwater strain of *C. vulgaris* [7], whilst increase in CO_2 supply has been shown to increase nitrate and phosphate removal rates in a *C. kessleri* grown in synthetic wastewater [6], suggesting that the relationship is species specific and needs to be established independently. Here we show that differences exist even between two halotolerant strains of *C. vulgaris,* in view of carbon and nutrient supply.

Table 2b
Relationship between biomass accumulation (*x*) and phosphate depletion (*y*) in the two *Chlorella vulgaris* strains for the different carbon (C), nitrogen (N) and phosphate (P) supply regimes.

C/N/P supply	BDUG 9171			CCAP 211/21A	
		Correlation	R^2	Correlation	R^2
C_0	*f*/2	$y = 6.6\ln(x) + 40$	0.99	$y = 12\ln(x) + 37$	0.94
$C_{0.01M}$	*f*/2	$y = 22\ln(x) + 24$	0.96	$y = 19x^3 - 56x^2 + 54x + 12$	0.94
C_{air}	*f*/2	$y = 100x^3 - 162x^2 + 81x + 19$	0.99	$y = 15x^3 - 49x^2 + 50x + 15$	0.97
$C_{1\%}$	*f*/2	$y = 4.9\ln(x) + 34$	0.99	$y = 16x^3 - 70x^2 + 92x + 3.7$	0.99
$C_{1\%}$	2*f*	$y = -13x^3 + 65x^2 - 21x + 14$	0.99	$y = -3.8x^3 + 19x^2 + 27x + 1$	0.99
$C_{1\%}$	4*f*	$y = -5.3x^3 + 46x^2 - 49x + 24$	0.99	$y = -8.6x^3 + 60x^2 - 42x + 12$	0.99

3.4. *Kinetics of nutrient depletion and carbon fixation is dynamic and non-linear for both the strains, under high carbon and nutrient supply regimes*

In both the strains, nitrate depletion rate (NDR) was consistently the lowest throughout the cultivation period in C_0 and $C_{0.01M}$ (Fig. 7**A, B**). A higher NDR is observed for BDUG in C_{air} that is mostly constant over the cultivation period (Fig. 7**A**). However, in $C_{1\%}$, there appears to be a peak at day 3; NDR drops at later time points in *f*/2 compared to 2*f* and 4*f*, where there is sufficient nitrate and phosphate in the medium to consume, compared to the *f*/2 conditions. NDR in CCAP can be seen to be drastically improved in 2*f* even at the start of the cultivation that is significantly different to the case in 4*f*, where it is lower, suggesting a peak uptake capacity in 2*f* (Fig. 7**B**). This behaviour is not observed in BDUG (Fig. 7**A**). Peak nitrogen and phosphorous removal with the increase of nutrients has been observed in *C. vulgaris* (freshwater strain) [26] and in *Neochloris oleoabundans* [65].

Phosphate depletion rate (PDR) decreased with time for both the strains in *f*/2 conditions, suggesting that as cultivation progresses, a limiting phosphate concentration is reached in *f*/2 (Fig. 7**C, D**). PDR is the lowest in $C_{0.01M}$, for BDUG (Fig. 7**C**). For CCAP, change in PDR over time is similar in all carbon supply regimes in *f*/2 (Fig. 7**D**). Increase in nutrients (2*f* and 4*f*) results in an increase in PDR with time, for both the strains, being highest at later time points in 4*f*, suggesting that these conditions are sufficiently replete to allow for an increase in phosphate uptake. Thus, unlike with nitrogen, increased phosphate availability results in increased uptake in both the strains.

Carbon fixation rate (CFR) (inferred from biomass formed) is the lowest in C_0 for both the strains, followed by $C_{0.01M}$ (Fig. 7**E, F**). For BDUG, an increased CFR is noted in C_{air} at Day 4 that then decreases at later time points, and $C_{1\%}$ displayed the highest CFR in *f*/2 that peaks at day 3 (Fig. 7**E**). 2*f* and 4*f* regimes resulted in marginal gains in CFR, seen at later time points. For CCAP, 2*f* and 4*f* resulted in higher CFR in day 3 that remained high until the end of cultivation, whilst the behaviour in $C_{0.01M}$, C_{air} and $C_{1\%}$ was similar, being higher than C_0 but lower than the nutrient replete regimes, 2*f* and 4*f* (Fig. 7**F**). Clearly, both carbon supply as well as nutrient supply regimes can be seen to influence CFR differently in the two strains.

Changes in carbon supply (C_0, $C_{0.01M}$, C_{air}, $C_{1\%}$) significantly influences overall maximum NDR for both the species (ANOVA, $p < 0.001$), that is seen to be more significant for a given nutrient regime (*f*/

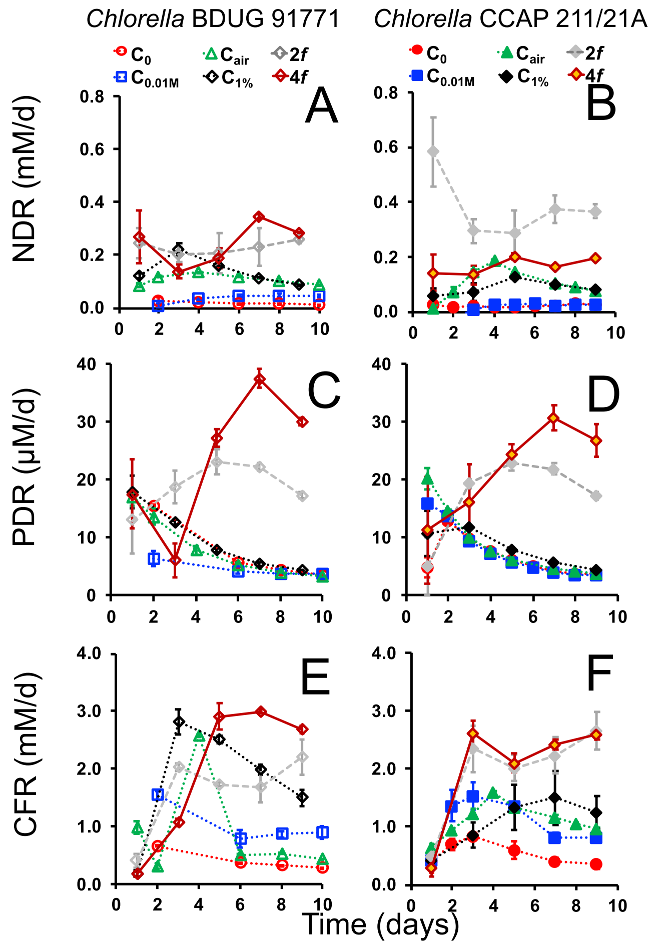


Fig. 7. Time profiles of nitrogen depletion rate (NDR) (A, B), phosphate depletion rate (PDR) (C, D) and carbon fixation rate (CFR) (E, F), the latter inferred from biomass, for the two strains BDUG 91771 (A, C, E) and CCAP 211/21A (B, D, F). Mean of triplicate biological runs is plotted with error bars indicating the standard error about the mean.

2) (ANOVA, $p < 0.01$) than changes in nutrient supply (*f*/2, 2*f*, 4*f*), for a given carbon supply regime ($C_{1\%}$), and this is species dependent (Fig. 8**A**). For both the species, C_0 and $C_{0.01M}$ conditions resulted in the lowest maximum NDR that showed little difference between the two conditions, but was statistically lower ($p < 0.05$, at least) to all the other regimes. For BDUG, C_{air} resulted in a maximum NDR that was statistically higher than in C_0 and $C_{0.01M}$, but less than in 2*f* ($p < 0.05$) and 4*f* ($p < 0.01$) (Fig. 8**A**). No statistically significant difference was observed between C_{air} and $C_{1\%}$, suggesting that beyond a certain point, increase in carbon supply elicits a change in NDR only if there is also a concomitant change in nutrient supply, in this strain. Increasing the nutrients (4*f*) resulted in a concomitant increase in NDR for BDUG that is statistically different to the other conditions, except 2*f*, which was only statistically higher compared to C_0, $C_{0.01M}$ and C_{air} (Fig. 8**A**), suggesting that a higher increase in nutrient supply (4*f*) is needed to elicit an increase in NDR for comparable carbon supply ($C_{1\%}$). This influence of carbon and nutrient supply on NDR was even weaker for CCAP, which only showed a statistically higher NDR when moving from C_0 or $C_{0.01M}$ to the other regimes. A statistically significant difference in maximum NDR between the strains is noted only in C_{air} ($p < 0.001$) and 4*f* ($p < 0.01$) conditions. The median specific NDR (Fig. 8**B**) is the lowest for both species in C_0 and $C_{0.01M}$, whilst it is seen to be the highest in C_{air} with no further significant increases with increase in either carbon or nutrient supply.

Changes in nutrient supply (*f*/2, 2*f*, 4*f*), on the other hand, influences overall maximum PDR for both the species more significantly (ANOVA, $p < 0.001$), compared to the carbon supply regimes (ANOVA, $p < 0.01$), with no significant difference between the strains (Fig. 8**C**). The maximum PDR can be seen to be statistically different between the two strains only in C_0 and $C_{0.01M}$ ($p < 0.01$ for both). It is the lowest in $C_{0.01M}$ for BDUG compared to other regimes ($p < 0.05$), with no further statistically significant differences seen within the carbon supply regimes. Nutrient supply regimes, on the other hand, enhance maximum PDR to a statistically significant extent ($p < 0.05$), for BDUG. For CCAP, $C_{1\%}$ results in a PDR value that is statistically lower than C_{air}, but comparable to C_0 and $C_{0.01M}$ within the carbon supply regimes, higher nutrient supply regimes (2*f*, 4*f*) leading to statistically significant increases as seen for BDUG (Fig. 8**C**). For BDUG, $C_{0.01M}$ resulted in the least PDR that was statistically lower than the response in all other conditions (Fig. 8**C**). Only an increase in nutrient supply appears to elicit a statistically significant increase in PDR, otherwise for both the species (Fig. 8**C**). No clear trend can be noticed with respect to specific PDR (Fig. 8**D**), and the values for C_0 itself is fairly high for both species, indicating that mass transfer limitations are not as significant a factor for P assimilation (for a unit of biomass) as it might be for N assimilation (for a unit of biomass) (Fig. 8**D**, compared to Fig. 8**B**).

Combining the observations from Figs. 7 and 8, it can be noted that: (a) continuous supply of carbon (C_{air} and $C_{1\%}$) is needed for a higher NDR for both strains (and a higher median specific NDR in C_{air}), but carbon supply influences NDR more significantly than nutrient supply in BDUG compared to CCAP; CCAP shows a significant increase in NDR on increasing nutrient supply (2*f*), but that is not sustained with further increase in supply (4*f*), whilst BDUG requires this higher nutrient supply (4*f*) to show a statistically significant increase in maximum NDR that is not reflected in the median specific NDR; (b) PDR steadily decreased with cultivation time in all carbon supply regimes (C_0, $C_{0.01M}$, C_{air}, $C_{1\%}$) in *f*/2, but steadily increased on supply of nutrients (2*f* and 4*f*) for both the strains, suggesting nutrient supply influences PDR to a greater extent than carbon supply (also seen with maximum PDR); (c) both carbon supply as well as nutrient supply regimes can be seen to influence carbon fixation rates differently in the two strains. Changes in carbon supply

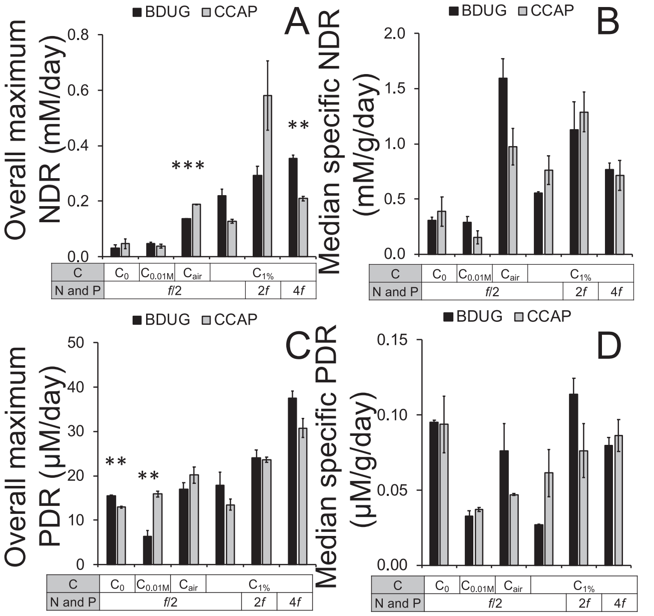


Fig. 8. A comparison of nitrogen and phosphorus depletion rates for the two species. Overall maximum depletion rates for nitrogen (A) and phosphorus (C) are plotted alongside the median specific uptake rates for nitrogen (B) and phosphorus (D), for the two species. The values plotted are mean of three biological replicates (error bars indicate standard error about the mean). Statistically significant differences between species are indicated as *** ($p < 0.001$) and ** ($p < 0.01$).

have been shown to influence nitrate and phosphate uptake in other species, such as *C. kessleri* [6].

Nitrate depletion relative to phosphate depletion is higher at later time points in all *f*/2 conditions, for both strains (Fig. 9). This is more clearly seen in $C_{0.01M}$, C_{air} and $C_{1\%}$ carbon regimes. This suggests that phosphate is taken up more readily at the initial stages of the growth than nitrate. Such a behaviour has been reported with other strains of *C. vulgaris* [15]. Under nutrient replete conditions (2*f* and 4*f*), the trend reverses, as sufficient phosphate is still available in the medium for uptake.

Differences in changes in nitrate with respect to changes in phosphate can clearly be observed between the carbon supply regimes in *f*/2, suggesting that the dynamics of carbon uptake can significantly influence nitrate and phosphate uptake to different degrees, as has been shown in *Chlorella* sp. [6,24].

For both the strains, a better correlation ($R^2 > 0.8$) was seen for carbon fixation rate with phosphate depletion rate than with nitrate depletion rate, in general (except for $C_{0.01M}$ for BDUG) (Table 3, Fig. 10).

CFR can be seen to correlate linearly with both NDR and PDR, for BDUG, in C_0 and $C_{0.01M}$ (Fig. 10, Table 3). However, in the $C_{0.01M}$ regime, an inverse relationship is observed between CFR and NDR, for BDUG, suggesting that nitrate uptake is lagging behind carbon uptake. It is possible that the ready availability of high bicarbonate at the start of the culture induced a faster uptake of carbon at the start that was then followed by nitrate uptake. This behaviour is not observed with CCAP, where a polynomial fit describes the correlations under most conditions; the correlation is however weaker for $C_{1\%}$ regime in *f*/2 and 4*f* (Table 3).

The highest nitrate and phosphate depletion rates can be seen in $C_{1\%}$ carbon regime, with NDR being maximum for both strains in 2*f* and 4*f*, suggesting nutrient replete conditions that allow for a higher uptake. Clearly, the nutrient absorption and corresponding carbon fixation happens at a higher rate under replete conditions, the other regimes suggesting limited or deplete environments. This can also be seen in the NDR and PDR correlations (Fig. 10, bottom panels), where the curves for C_0 and $C_{0.01M}$ regimes are at the lower end of the correlation, distinctly away from the response at the other regimes. This can be seen to be more stark in BDUG than in CCAP.

The negative correlation between NDR and PDR for both strains in $C_{0.01M}$ regime suggests that nitrate consumption is lagging behind phosphate consumption in this condition. For BDUG, nitrate consumption was also noted to lag behind carbon fixation. This negative correlation between NDR and PDR can also be seen for CCAP in C_{air} and $C_{1\%}$ (*f*/2) regimes. For both the strains, $C_{1\%}$ regime clearly shows a higher CFR, NDR and PDR, suggesting better availability of carbon in this regime, resulting in a higher corresponding uptake of carbon, nitrogen and phosphorus. The increased availability of nitrate and phosphate also leads to a corresponding increase in depletion rates, for both the strains.

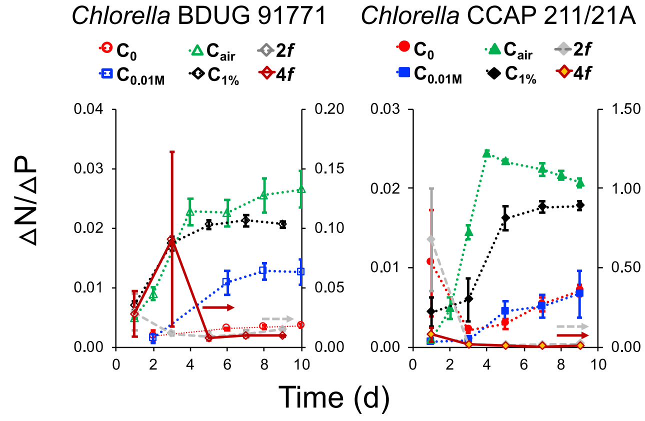


Fig. 9. Relative changes in nitrogen with respect to phosphorus over the cultivation period, in the different carbon and nutrient supply regimes, for the two strains.

Table 3a
Relationship between rates of carbon fixation (*x*) and nitrate depletion (*y*), for the two *Chlorella vulgaris* strains, under the different carbon (C), nitrogen (N) and phosphorus (P) supply regimes tested.

C/N/P supply		BDUG 9171		CCAP 211/21A	
		Correlation	R^2	Correlation	R^2
C_0	$f/2$	$y = 0.04x + 0.00$	0.99	$y = 0.11x^2 - 0.14x + 0.06$	0.87
$C_{0.01M}$	$f/2$	$y = -0.05x + 0.09$	0.97	$y = -0.09x^2 + 0.20x - 0.08$	0.96
C_{air}	$f/2$	$y = 1.42x^2 - 1.22x + 0.36$	0.40	$y = 0.03x^2 + 0.14x - 0.09$	0.97
$C_{1\%}$	$f/2$	$y = 0.05x^2 - 0.11x + 0.14$	0.99	$y = 0.04e^{0.59x}$	0.61
$C_{1\%}$	$2f$	$y = 0.04x^2 - 0.12x + 0.29$	0.42	$y = 0.12x^2 - 0.49x + 0.79$	0.92
$C_{1\%}$	$4f$	$y = 0.07x^2 - 0.22x + 0.30$	0.52	$y = -0.05x^2 + 0.14x + 0.10$	0.46

Table 3b
Relationship between rates of carbon fixation (*x*) and phosphate depletion (*y*), for the two *Chlorella vulgaris* strains, under the different carbon (C), nitrogen (N) and phosphorus (P) supply regimes tested.

‘		BDUG 9171		CCAP 211/21A	
		Correlation	R^2	Correlation	R^2
C_0	$f/2$	$y = 32x - 5.5$	1.00	$y = 12x - 0.3$	0.97
$C_{0.01M}$	$f/2$	$y = 3.3x + 1.17$	0.89	$y = 34x^2 - 73x + 40$	0.99
C_{air}	$f/2$	$y = 364x^2 - 344x + 85$	0.95	$y = -245x^3 + 806x^2 - 864x + 307$	0.93
$C_{1\%}$	$f/2$	$y = 6.1x^2 - 20x + 21$	0.99	$y = 34x^3 - 102x^2 + 89x - 11$	0.78
$C_{1\%}$	$2f$	$y = -10x^2 + 29x + 2.8$	0.98	$y = -9.4x^2 + 35x - 10$	0.99
$C_{1\%}$	$4f$	$y = -2.9x^3 + 22x^2 - 36x + 23$	0.93	$y = -7.5x^2 + 27x + 4.1$	0.61

Table 3c
Relationship between rates of nitrate depletion (*x*) and phosphate depletion (*y*), for the two *Chlorella vulgaris* strains, under the different carbon (C), nitrogen (N) and phosphorus (P) supply regimes tested. $k = 1000$

C/N/P supply		BDUG 9171		CCAP 211/21A	
		Correlation	R^2	Correlation	R^2
C_0	$f/2$	$y = 0.8kx - 8.0$	0.98	$y = -94kx^2 + 4kx - 33$	0.44
$C_{0.01M}$	$f/2$	$y = -67x + 7.0$	0.98	$y = -344x + 12$	0.92
C_{air}	$f/2$	$y = 0.18e^{32x}$	0.51	$y = 2kx^2 - 0.4kx + 25$	0.95
$C_{1\%}$	$f/2$	$y = 51kx^3 - 24kx^2 + 4kx - 0.2\ k$	0.43	$y = 3kx^2 - 0.7kx + 41$	0.54
$C_{1\%}$	$2f$	$y = 925kx^3 - 647kx^2 + 150kx - 12\ k$	0.99	$y = -0.2kx^2 + 87x + 10$	0.91
$C_{1\%}$	$4f$	$y = 21kx^3 - 16kx^2 + 4kx - 0.3\ k$	0.85	$y = -9kx^2 + 3kx - 0.3\ k$	0.72

However, for CCAP, there appears to be a saturation effect with 2*f*, where a higher NDR is seen compared to 4*f* that is not really translated to any increase in CFR. But, this is not seen with PDR for this strain.

Except for C_0 and $C_{0.01M}$ carbon supply regimes, which show a clear linear relationship between the carbon fixation and nutrient depletion rates, the relationship is dynamic and nonlinear for all the other regimes.

The findings from this investigation lead us to summarise the following:

(a) Nitrate and phosphate depletion dynamics can be different even

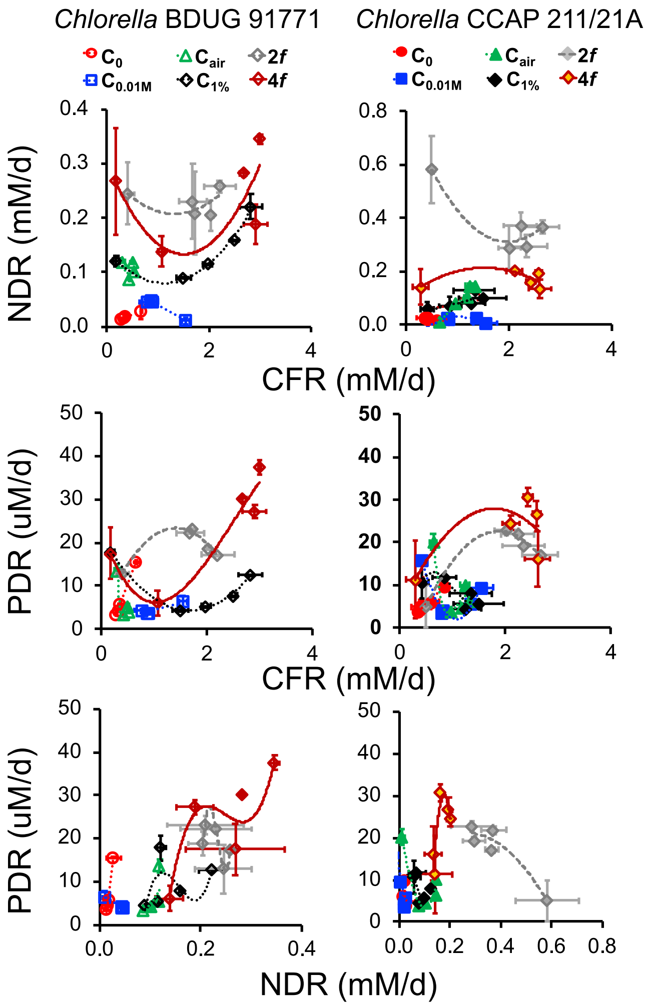


Fig. 10. Relationship between carbon fixation rate (CFR), nitrate depletion rate (NDR) and phosphate depletion rate (PDR), for the two Chlorella strains, under the different carbon and nutrient supply regimes tested.

within species isolated from a similar habitat. This would imply that the uptake characteristics should be studied carefully for the specific strain being used to enable effective design and implementation of strategies for carbon, nitrogen or phosphorus sequestrations.

(b) Nitrogen and phosphate depletion is dependent not only on the supply of nitrate and phosphate, but can also be significantly influenced by how carbon is supplied. This latter aspect is not given due importance in most investigations, where carbon is supplied usually as air sparged into the medium. However, this influence may not be the same for different isolates of the same species. As can be noticed in this investigation, CCAP did not respond to changes in carbon as much as BDUG did. However, a continuous supply of carbon (C_{air} and $C_{1\%}$ regimes in this investigation) was needed to maintain a higher nitrate removal (seen as NDR here), as opposed to bicarbonate supply at the start ($C_{0.01M}$ regime here).

(c) Whilst biomass accumulation can be expected to proportionately increase in response to increase in carbon, nitrogen and phosphorus supply, the degree of influence varies between strains. In this study, a marked difference was seen in BDUG when carbon supply was increased (from C_{air} to $C_{1\%}$) that was not seen with CCAP, which showed a greater increase when nitrogen and phosphorus supply was increased (*2f* compared to *f/2*) that was not seen with BDUG. Nevertheless, both strains showed a more efficient translation of nutrient depletion to biomass accumulation, when bicarbonate was supplied in the medium at start ($C_{0.01M}$ regime here) compared to continuous supply of carbon. This suggests carbon limiting conditions lead to an efficient translation of nutrient depletion to biomass accumulation and carbon replete conditions are more favourable to maximise nitrogen and phosphorus sequestration.

(d) Nitrate and phosphate depletion do not follow similar trends. Whilst both are required to different degrees, nitrate depletion lagged behind phosphate depletion in most supply regimes. Carbon supply appeared to influence nitrate depletion more than phosphate depletion, whilst nutrient supply influenced phosphate depletion more than nitrate depletion.

(e) Luxury storage of nitrogen and phosphorus appears to take place in both the strains, but in different supply regimes. If specific supply regimes can be identified when such luxury storage takes place for a given strain, it is possible to develop these towards employment for effective sequestration purposes.

(f) In all cases, the carbon limiting conditions (C_0 and $C_{0.01M}$ regimes in this investigation) showed poor carbon, nitrogen or phosphorus sequestration, suggesting this to be the least effective for the purpose. The C_0 regime adopted here is close to the conditions normally experienced in raceway ponds, suggesting that this is a baseline condition that can be improved upon for the purposes of carbon fixation and nitrogen and phosphorus sequestrations, using better supply regimes.

These observations clearly indicate that carbon and nutrient supply regimes significantly influence nutrient uptake (and carbon fixation) by microalgae that is differentially perceived, even between two strains isolated from a similar habitat (brackish water, in this instance). This suggests that there is considerable value in pursuing detailed studies on nutrient uptake by microalgae under different regimes not only to understand, but also to develop it as a successful strategy to maximise not only nitrogen and phosphorus uptake by microalgae, but also carbon fixation. We believe this investigation provides us with the evidence base that emphasises the significance of carbon, nitrogen and phosphorus supply regimes, which need to be carefully investigated for specific strains of microalgae to make the most of strategies that can be developed to maximise carbon fixation, as well as nitrogen and phosphorus sequestration from wastewaters. Future investigations into molecular mechanisms will help strengthen the findings from this work.

4. Conclusion

In this study, nutrient and carbon supply regimes have been shown to influence nitrate and phosphate uptake dynamics differently in two halotolerant strains of *Chlorella vulgaris*. Carbon supply was found to have a greater influence on nitrate and phosphate depletion in BDUG, whilst nutrient supply had a greater influence in CCAP. Nutrient depletion and biomass accumulation showed a nonlinear relationship for both the strains in all regimes. It has also been shown that the kinetics of nutrient depletion and carbon fixation is dynamic and nonlinear for both the strains, under high carbon and nutrient supply regimes. These findings suggest that it is important to study carbon and nutrient uptake behaviour in microalgae at species and strain level to identify candidates that will fit the purpose more accurately. In particular, we have highlighted the relationship between supply and uptake of nitrogen and phosphorus, and more importantly the role of carbon supply in nutrient uptake, in two halotolerant strains of *C. vulgaris*. We believe this data to lead to the development of sustainable biomanufacturing routes that employ carbon capture using microalgae, and in the employment of microalgae based processes for efficient sequestration of nitrogen and phosphorus from waste streams, and develop appropriate strategies that enable sustainable use of microalgae in establishing net zero (or even carbon negative) strategies within a circular bioeconomy.

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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