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Bioresource Technology 169 (2014) 588–595

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Influence of the N:P supply ratio on biomass productivity and time-resolved changes in elemental and bulk biochemical composition of *Nannochloropsis* sp.

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HIGHLIGHTS

- Effect of N + P supply on *Nannochloropsis* sp. growth dynamics and chemical content.
- Detailed and comprehensive batch culture data series for support of modelling.
- Cellular N main driver of lipid accumulation, with lesser effect of P starvation.
- P usage can be halved (to N:P of 32:1) with no significant effect on productivity.
- Maximum lipid content and concentration of 52% DW and 230 mg L^{-1}, respectively.

ARTICLE INFO

Article history:
Received 21 March 2014
Received in revised form 8 July 2014
Accepted 13 July 2014
Available online 18 July 2014

Keywords:
Microalgae
Nannochloropsis sp.
Phosphorus usage
Lipid accumulation
Nitrogen deficiency

ABSTRACT

This work reports for the first time the detailed impacts of dual nitrogen (N) and phosphorus (P) stress on growth dynamics and biochemical composition in the Eustigmatophyte *Nannochloropsis* sp. P-stress concurrent with N-stress had subtle effects on culture bulk biochemical composition, but negatively influenced biomass productivity. However, the N:P supply ratio can be raised to at least 32:1 without compromising productivity (yielding a maximum lipid content of 52% of dry weight and volumetric lipid concentration of 233 mg L^{-1}). The maximum biomass and lipid yields per unit of cell-P were 1.2 kg DW (g P)$^{-1}$ and 0.54 kg lipid (g P)$^{-1}$. The P concentration of many common media is thus in surplus for optimal *Nannochloropsis* sp. biomass and lipid production, offering potential for significant savings in P usage and improving the sustainability of algal cultivation.

1. Introduction

Balancing nutrient supply is critical for optimising microalgal composition and commercial viability. Single nutrient depletion (typically of nitrogen (N); Adams and Bugbee, 2014) is often used to alter or enhance biochemical content, with other nutrients such as phosphorus (P) added to excess. This practice wastes expensive resources and contributes to pollution. Here, for the first time, we describe how manipulating the N:P supply can simultaneously be used to control production and minimise nutrient wastage in the commonly used microalga, *Nannochloropsis*.

Life cycle analyses have highlighted the significant impact that nutrient demand can have upon the environmental and economic impact of large-scale microalgal cultivation (Handler et al., 2012; Johnson et al., 2013; Sills et al., 2013; Yang et al., 2011). P is an essential macronutrient, being a component of nucleic acids, phospholipids and phosphorylated sugars, as well as being critical in cellular energy metabolism and modulating protein function (e.g., for microalgae, Moseley and Grossman, 2009). In microalgae, P typically accounts for <1% of cell dry weight, but attains ca. 4% under circumstances allowing "luxury uptake" (Powell et al., 2009).

Johnson et al. (2013) recently calculated that N-based fertilizers require between 50 and 113 MJ kg-N^{-1} in fossil energy and produced between 3.9 and 9.2 kg of CO_2 kg-N^{-1} for different products (ammonium nitrate, urea or urea-ammonium nitrate blends). Calculations for P are complex, due to phosphate rock mining and the convoluted production and shipping processes. However, direct energy inputs for P-containing fertilisers such as mono- and diammonium phosphate may be estimated at between 56 and 64 MJ kg-P^{-1} (calculated from Johnson et al., 2013, and

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http://dx.doi.org/10.1016/j.biortech.2014.07.048
0960-8524/

references therein, including EFMA, 2000; Handler et al., 2012). Moreover, the economic if not energetic costs for the supply of P-fertilizers are sure to increase given that readily available mineral deposits of P are forecast to be exhausted within the next 100 years (Cordell et al., 2009). Already the price of phosphorus rock has increased from a stable level of around \$40 t^{-1} in 2006 to above \$100 t^{-1} by the end of 2013 ("Historical Phosphate Rock Prices and Price Chart – InvestmentMine" 19/2/14), with a significant spike in 2008 (>\$400 t^{-1}) due to a sudden increase in oil prices (Cordell et al., 2011). The spatial imbalance in global P reserves and geo-political instability are additional factors likely to affect supply and pricing (Elser and Bennett, 2011).

Nutrient supply is thus expected to become a serious issue over the coming decades, significantly impacting a number of industries and agricultural sectors, and inevitably affecting the commercial viability of microalgal biomass production (Britton and Baur, 2010; Shilton et al., 2012). The National Research Council of the National Academies (U.S.A.) in a 2012 report on the production of algal biofuels in the U.S.A., highlighted the need for more clear indicators of sustainability with regards to resource use, proposing that general indicators of nutrient requirement to produce volumes of dry biomass and volumes of fuel be measured and compared. For example, 0.71 kg P is required to generate 1 kg of biodiesel from microalgae (Yang et al., 2011). It is hence critical to develop strategies where both biomass production and product composition is optimised in relation to nutrient utilisation.

There have been many studies on the impacts of single nutrient stresses on microalgae, mostly concerning N-stress (Adams and Bugbee, 2014; Hu and Gao, 2006). Fewer studies have investigated the effect of different P regimes on growth and biochemical composition for several species of microalgae (Chu et al., 2013, 2014; Roopnarain et al., 2014; Ruangsomboon et al., 2013; Wu et al., 2012, 2013). These report differing conclusions on the role of P-limitation in algal physiology, and in particular for lipid accumulation. A key factor complicating the interpretation of N vs P stress impacts is that the relationship between stress, as indicated by the elemental quotas, and growth rate differs between these nutrients. Thus the relationship between growth and N:C is linear, while for P:C it is distinctly non-linear, and that while P:C may increase during N-stress, N:C can decrease during P-stress even with excess N-nutrient availability (Flynn, 2008).

There are very few studies of coupled N–P stress in which a suite of biochemical parameters have been measured, a factor that severely limits our ability to understand and thence model microalgal growth (Flynn, 2008) and explore productivity scenarios (Kenny and Flynn, 2014). Accordingly, this study investigates the growth dynamics and biochemical composition of the widely used marine *Nannochloropsis* sp. for the first time under different phosphate supply regimes, in which the N:P nutrient supply ratios ranged from 16:1 to 80:1.

2. Methods

2.1. Algae strain and culture conditions

The marine Eustigmatophyte microalga *Nannochloropsis* sp. (CCAP 211/78) was batch cultivated in 10 L tubular airlift photobioreactors (PBR; acrylic plastic, 0.1 m diameter/light path, 1.2 m height). Reactors were maintained at 22 ± 1 °C, and aerated with filtered (0.2 μm) ambient air (i.e., 0.039% CO_2) at a rate of 0.1 L L^{-1} (v/v) into the base of the tube through a 1 mm i.d. plastic capillary tube. Bioreactors were illuminated at 100 μmol photons m^{-2} s^{-1} PAR (reactor surface) using daylight fluorescent tubes (T8, 58 W) mounted perpendicular to the bioreactors, and operated under a light:dark cycle of 18:6 h. Irradiance was measured using a cosine-corrected light meter (WALZ ULM-500).

Cultures were grown on modified Walnes media (Andersen, 2005), with $NaNO_3$ and KH_2PO_4 as the macro-nutrients (see below for concentrations). The seawater base was natural, pumped from Swansea Bay (U.K.), filtered to 1 μm, UV treated and ozonated. Salinity was 30 (±2). pH was maintained between 7.8 and 8 by addition of 10 mM Tris–HCl (Melford Chemicals). Inocula for the growth experiments were grown in the same PBR set-up and then diluted to give a starting concentration of 2×10^6 cells mL^{-1}. Relative to the final yield, these inocula were ca. 5% of the maximum final cell density, contributing approximately 1.0 mg biomass-N L^{-1} and 0.11 mg biomass-P L^{-1}.

2.2. Experimental design

Nannochloropsis sp. was cultivated in nutrient media with 4 different N:P molar ratios, 16:1, 32:1, 64:1 and 80:1 (1.38, 0.69, 0.35 and 0.028 mg P L^{-1}). The nutrient-N content was fixed at 10 mg N L^{-1} (714 μM N), with the P content adjusted accordingly. Batch culture experiments were run for 10 days, with each treatment repeated at least 3 times. The use of samples grown under a variety of nutrient regimes and harvested at different time points throughout the batch culture, yielded biomass samples of various biochemical composition.

2.3. Analytical techniques

2.3.1. Analysis of growth dynamics

Cell number, total cellular volume and cell size were recorded daily using a Coulter counter (C4 Beckman Coulter GmBH, Drefield, Germany). Dry weight (DW) was determined by filtering a known volume of culture onto precombusted GF/F Whatman filters (GE Healthcare, Germany). Filters were washed with ammonium formate (0.5 M) to remove salts, then dried for at least 18 h at 70 °C, before cooling at room temperature in a desiccator. On a routine basis, DW was estimated from the Coulter counter-derived biovolume (i.e. total cellular volume) using a previously established calibration of 1 mL biovolume = 1 g DW.

Biomass-specific growth rate was calculated from changes in the biomass concentration (mg DW L^{-1}) using the following equation:

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0}$$

where N_0 and N_1 are the biomass concentrations at times t_0 and t_1. The observed exponential (maximum) growth rate (μ^{exp}, d^{-1}) was calculated over the first 4 days of growth (0–4 days).

2.3.2. C, N and P elemental composition

Elemental C and N content was determined using an elemental analyser interfaced with an isotope ratio mass spectrometer, according to the method described in Mayers et al. (2013); all measurements were made in duplicate. Cellular-P content was measured by digesting cells, from a known volume of culture collected upon pre-combusted 13 mm A/E glass fibre filter (Pall Corporation, NY, USA), using the acidic persulfate digestion method (0.015 M $K_2S_2O_6$ plus 0.018 M H_2SO_4, autoclaved at 121 °C for 75 min). Cellular-P was converted to free orthophosphate and measured spectrophotometrically using an ammonium molybdate assay at 880 nm (Strickland and Parsons, 1968).

2.4. Nutrient analysis

2.4.1. Fourier-transform infrared spectroscopy (FTIR) biochemical determination

The content of lipid, protein and carbohydrate in biomass samples was determined using FTIR, using the method described in Mayers et al. (2013). Briefly, freeze-dried algal biomass was finely powdered and spectra measured on a PerkinElmer Spectrum Two instrument equipped with a diamond crystal iATR reflectance cell with a DTGS detector scanning over the wavenumber range of 4000–450 cm^{-1} at a resolution of 4 cm^{-1}. Three replicates (each of 10 scans averaged) for each sample were taken and the results averaged. Background correction scans of ambient air were made prior to each sample scan. Scans were recorded using the PerkinElmer spectroscopic software Spectrum and quantification methods had been previously built in SpectrumQuant (version 10. PerkinElmer, Germany). Ethanol (60% v/v) was used to clean the diamond ATR between samples.

2.5. Statistical analysis

Differences in treatments were assessed by one-way analysis of variance (ANOVA). If ANOVA results were significant ($p < 0.05$), comparisons between means were made using Tukey's Post Hoc Analysis. Statistical analyses were conducted using the software R (version 0.97.551).

3. Results and discussion

3.1. Effect of nutrient supply N:P ratio on batch growth dynamics

The pattern of batch growth is shown in Fig. 1; all treatments starting with the same initial N concentration (10 mg N L^{-1}), but with varied P supplementation (1.38, 0.69, 0.35 and 0.028 mg P L^{-1}). For the first 4 days of cultivation *Nannochloropsis* sp. grew exponentially at similar rates (μ^{exp} ~0.6 d^{-1}; Fig. 1D; Table 1), except those grown at a supply N:P ratio of 80, which grew significantly slower. Cell division had markedly slowed in the 80:1 treatment by day 4, whereas the 64:1 treatment began to slow by day 5 (Fig. 1A). The 16:1 and 32:1 treatments attained similar maximum biomass concentrations, which were significantly greater than the 64:1 and 80:1 treatments ($p < 0.05$; Fig. 1C), with no difference between these latter two treatments. The maximum biomass productivity over this period ranged from 45.4 to 56.6 mg DW L^{-1} d^{-1}, with the 80:1 treatment having a significantly lower productivity then the other treatments (Table 1). Similar trends to those seen for biomass were also seen in cell density, with the exception that the maximum cell density of the 80:1 treatment was significantly lower than those grown 64:1 N:P, as well as the 32:1 and 16:1 treatment ($p < 0.01$; Fig. 1A). These results would suggest that the 64:1 N:P treatment provided adequate P for exponential growth but became P-stressed shortly after exhaustion of the N source, whereas supply N:P ratios $\sim \geqslant$64:1 significantly effected growth.

Rapid depletion of extracellular-P and the subsequent decrease in cellular-P have been previously shown to cause immediate decreases in cell division rates (Wu et al., 2012). Despite the cessation of cell division at earlier time points, both the 64 and 80:1 treatments continued to increase in biomass concentration past these points. Only the 80:1 treatment showed a significant increase in cell size compared to other treatments, becoming apparent from day 6 onwards (Fig. 1B), eventually reaching a cell volume 1.7–1.9-fold greater on day 10. All other N:P supply treatments had cells of approximately the same size. The increase in cell size with P-stress, implicating restrictions in the cell cycle at S-phase, is an important feature of microalgal growth and one which complicates greatly the analysis of cell-quota (as distinct from C-quota) relationships with growth rate (Flynn, 2008). No previous reports in the literature for the increase in cell size due to P-limitation have been seen in *Nannochloropsis* sp., but it is likely to be a result of the accumulation of carbon products. A culture approach that deliberately increased cell size could be beneficial from a processing point of view, were harvesting may be more efficient, particularly by membrane filtration (Gerardo et al., 2014).

N and P uptake were measured as the amount assimilated into biomass over the course of the experiment. The maximum N uptake per unit biomass per day did not vary significantly between treatments and was between 0.027 and 0.029 μg N DW^{-1} d^{-1} (Table 1). The maximum rate of N uptake occurred between days 2 and 4 in the higher P treatments, in which N was >95% removed in the first 4 days, while the maximum uptake in the 80:1 treatment was between days 0 and 2, with these cells not achieving >95% of N removal until day 6 (Supplementary Information Fig. 1A).

The maximum rate of P uptake per unit biomass was positively related to the level of P supplied (Table 1; $r^2 = 0.92$, $p < 0.001$). The lowest P treatment (N:P 80:1) exhausted P by day 6, with a decreased growth rate in the days preceding that time (Fig. 1D) and a rapid cessation of cell division after day 6 (Fig. 1A). The other treatments exhausted P by between day 6 and 10 (Supplementary Information Fig. 1B). P removal in the 16:1 treatment between days 0 and 2 (14.1 μg P DW^{-1} d^{-1}, Table 1) was 1.7 and 1.5-fold greater then that of the other treatments, with no significant difference in biomass concentration, except in the case of the lowest P level. The results were consistent with a "luxury uptake" of P in the highest supplied treatment (Powell et al., 2009), and could represent a potential wasted consumption of a costly fertiliser.

3.2. Biochemical composition under different nutrient regimes

Changes in the bulk biochemical composition of *Nannochloropsis* strains, with regards to the accumulation of lipid under severe nitrogen stress, have been reported previously (Adams and Bugbee, 2014; Hu and Gao, 2006). However, no studies have investigated the effect of N:P supply stoichiometry on the dynamics of cellular C:N:P and thence on bulk biochemical composition shifts in detail. Here, growth in a batch mode with different initial nutrient-P concentrations but a set nutrient-N concentration reveals the subtle effect of P-limitation on biochemical composition.

All treatments had an initial lipid content of between 31% and 34% DW (Fig. 2B), consistent with the start inocula having recently entered into N-stress. In the first 2 days, when N was readily available and the cell N-status thus became replete, there was an increase in biomass protein content, with a corresponding decrease in carbohydrate and lipid content (expressed as % DW). Protein content subsequently dropped during N-stress to values between 17% and 20% DW on day 10 across treatments. Overall, there was no significant difference in the volumetric concentration (mg L^{-1}) of protein between treatments (Fig. 2D). This pattern was expected due to the same level of N provided in the culture media (10 mg N L^{-1}), effectively limiting the maximum size of the protein pool. Once media N was exhausted and net protein synthesis ceased, the protein pool was subsequently split between cells upon division, up until a critical threshold was reached and growth stopped. Protein as a% DW is hence effectively diluted by continued accumulation of carbon fixation products. In the context of N-quota kinetics, growth halts when the N-quota (here described as protein as % DW) attains its minimum value (Flynn, 2008). However, the degree of P-stress affected the dynamics of that event.

From day 4, following N depletion, all treatments showed a characteristic increase in lipid content (1.6–1.9-fold increase per

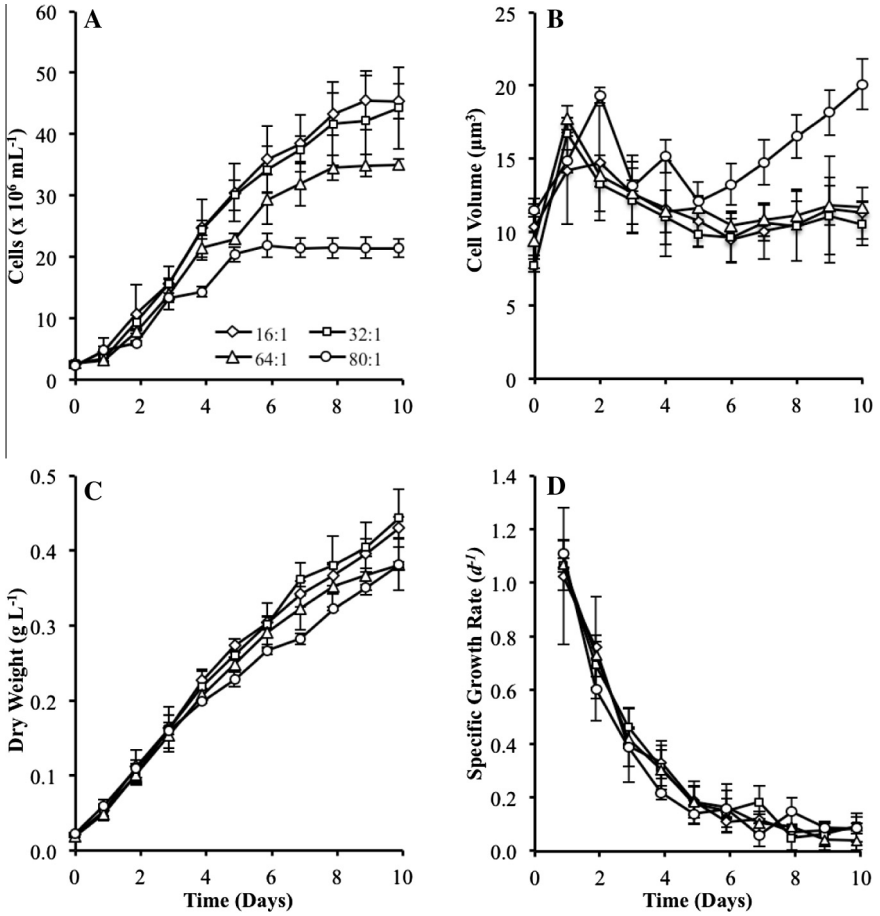

Fig. 1. Cell number (A), cell volume (B), culture dry weight (C) and specific growth rate (D) of *Nannochloropsis* sp. grown in 10-day batch cultures under different N:P supply ratios (n = 3, error bars = 1 SD).

Table 1
Effect of media N:P ratio on exponential (μ^{exp}) specific growth rate, maximal biomass productivity (DW_{max} Prod.) and the maximum N and P uptake rates (0–2 days) of *Nannochloropsis* sp. during 10-day batch cultures.

Parameter	16:1	32:1	64:1	80:1
μ^{exp} (d^{-1})	0.62 ± 0.01 [a]	0.62 ± 0.02 [a]	0.60 ± 0.01 [a]	0.56 ± 0.01 [b]
DW_{max} productivity (mg L^{-1} d^{-1})	56.6 ± 2.21 [a]	52.3 ± 0.44 [b]	50.5 ± 1.52 [b]	45.4 ± 0.48 [c]
N uptake rate (µg N mg DW^{-1} d^{-1})	28.9 ± 2.82 [a]	27.5 ± 1.50 [a]	26.5 ± 2.96 [a]	26.5 ± 1.13 [d]
P uptake rate (µg P mg DW^{-1} d^{-1})	1.41 ± 0.05 [a]	0.93 ± 0.06 [b]	0.84 ± 0.09 [b]	0.70 ± 0.04 [c]

Significant differences between treatments are represented by different letters (One-way ANOVA with post hoc LSD, $p < 0.05$).

unit DW, Fig. 2B) concurrent with a (i.e., balanced by) a halving of protein per unit DW (Fig. 2A). Lipid accumulation slowed by day 9 and 10, attaining a plateau of 46–53% DW as lipid. On a volumetric basis the 64:1 and 80:1 N:P treatments had a significantly lower lipid concentration from day 8 onwards (Fig. 2E), with the 16:1 and 32:1 regimes attaining a maximum of approximately 230 mg lipid L^{-1} on day 10. This increase in lipid concentration reflects the continuing accumulation of lipids per unit biomass coupled with the continuing increase of biomass per unit culture.

The 16:1 and 32:1 N:P treatments did not vary significantly with respect to lipid production rates, with both reaching a maximum on day 8, of 22–23 mg L^{-1} d^{-1}. The higher N:P treatments achieved lower levels of productivity. A cellular N:P ratio of 16:1 is typically held as being optimal (Geider and La Roche, 2002). If the aim of cultivation is lipid production, then the 32:1 N:P ratio treatment may represent a move favourable option for production of *Nannochloropsis* sp., as there is no increase in productivity at higher supply levels, whereas ratios approaching 64:1 begin to show decreased productivities. Additionally, from a process optimisation point of view, harvesting a dense culture (g DW L^{-1}) is a more attractive scenario than harvesting a low density equivalent when considering many common harvesting techniques. Subsequently, at large scales of cultivation, even small differences in biomass concentration (or in cell size; Fig. 1C) may result in changes in costs and energy usage for downstream processing (Greenwell et al., 2010). To achieve greater biomass concentrations and further decrease downstream processing costs, greater quantities of N and P can be added at these ratios. However, the effects of decreased light per cell/unit biomass (assuming the same light energy input) may affect bulk biochemistry, in particular decreasing the rate of lipid accumulation under N-stress due to decreased carbon fixation rates. This affects bioreactor design and operation (Kenny and Flynn, 2014).

Carbohydrate content followed the same pattern as lipids, being accumulated concurrently as protein content decreased, albeit to lower levels (Fig. 2C). Cells in the 80:1 N:P treatment had a significantly greater carbohydrate content than those in the 16:1

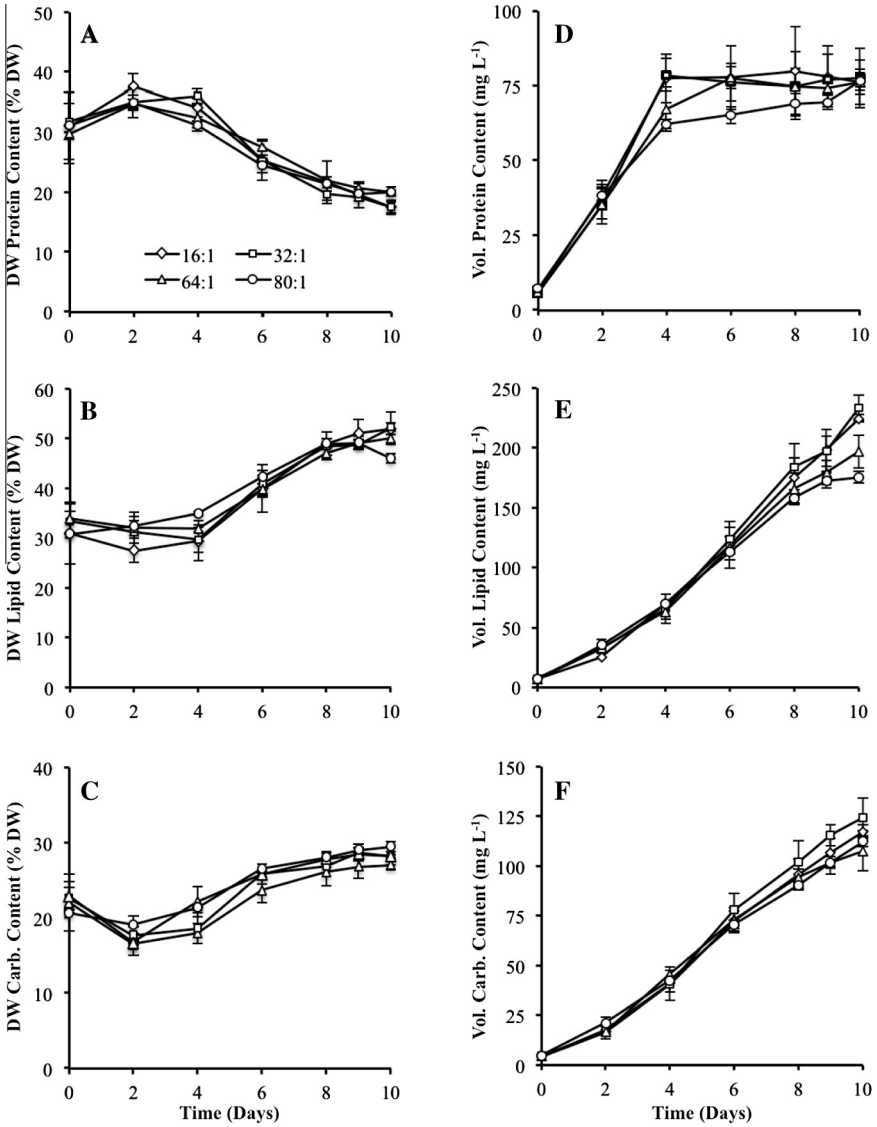

Fig. 2. Change in bulk biochemical composition (% DW) and volumetric content (mg L^{-1}) (A and D – Protein, B and E – Lipids, C and F – Carbohydrates) of *Nannochloropsis* sp. grown in 10-day batch cultures under different N:P supply ratios, as determined by FTIR spectroscopy (n = 3, error bars = 1 SD).

N:P treatment on days 4, 6 and 10 ($p < 0.05$) and also achieved the greatest carbohydrate content, of 29.5% DW as carbohydrate by day 10. There was no difference between the 16:1, 32:1 and 64:1 treatments (% DW; $p > 0.05$); furthermore, the volumetric concentration of carbohydrate did not vary significantly between treatments (Fig. 2F; $p > 0.05$).

Overall, the effect of P-limitation concurrent with N-stress did not significantly effect bulk biochemical composition on a (DW) biomass basis, however, on a per cell basis, the subtle effects of nutrient stress can be seen. The N $cell^{-1}$ did not vary significantly between the 16:1, 32:1 and 64:1 treatments on day 10 of cultivation (Supplementary Table 1; 27–31 μg N $cell^{-1}$), but was much greater in the 80:1 ratio (53 μg N $cell^{-1}$), suggesting that P-deplete cells did not experience as great a level of N-stress as those supplied at high N:P. This would suggest that P-limitation did in fact cause an increase in lipid content per cell; under these conditions *Nannochloropsis* sp. continued to photosynthetically fix carbon, which was directed towards storage products rather then structural compounds, despite not having decreased N content per cell to the same level as those in other treatments. This is consistent with the behaviour of *Selenastrium minutum* (Elrifi and Turpin, 1985, as presented in Flynn, 2008). The larger lipid content per cell also explains the significant increase in cell size seen in the 80:1 treatment compared to the others (Supplementary Table 1).

3.3. Biomass and lipid yield on phosphorus supply

The relationship between the quantity of biomass and lipids produced (g L^{-1}) after 10 days and the amount of P supplied are plotted in Fig. 3A; both show a decreased production at the lower P supply concentrations at N:P ratios 64:1 and 80:1. There were no increases in either the biomass or lipid concentration at N:P supply ratios lower than 32:1, suggesting that lower ratios provided a surplus of P and did not affect production of biomass and lipids, relative to the amount of N supplied (here, 10 mg N L^{-1}). There was a high degree of correlation between the supply of P to cultures and the yield of volumetric lipid production (g lipid L^{-1}; Fig. 3A; $r^2 = 0.889$, $p \gg 0.001$) allowing for predictions to be made based on the P supply under fixed N concentrations in this system. At higher N-supply concentrations, where light will become increasingly limiting due to biomass self-shading, the optimal supply N:P is expected to be greater again, as the N-nutrient itself is less likely to be fully consumed (Kenny and Flynn, 2014).

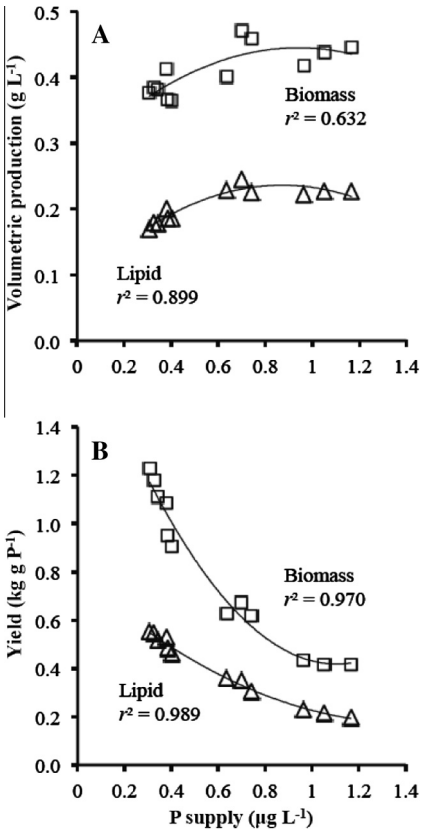

Fig. 3. Relationship between (A) volumetric biomass (squares) and lipid (triangles) production and P supply and (B) the maximum yields of biomass and lipid per unit of P for different P supplies of *Nannochloropsis* sp. grown in 10-day batch cultures. Curves were fitted using a second order polynomial regression. Each data point represents an individual experimental sample taken at day 10.

In this study, P-specific production was between 0.43 and 1.17 kg biomass $(g\ P)^{-1}$, and 0.21 and 0.54 kg lipid $(g\ P)^{-1}$ across the range of treatments tested (Fig. 3B). Generally high levels of P supply resulted in the lowest yields, as there is little increase in biomass for a higher level of P uptake and accumulation, suggestive of "luxury uptake". These results are comparable with the study of Wu et al. (2013) that achieved yields in the region of 2.1–3.1 kg biomass $(g\ P)^{-1}$ and 0.37–0.83 kg lipid $(g\ P)^{-1}$ in different *Chlorella* species, but poorer in comparison to a yield of 6.1 kg biomass $(g\ P)^{-1}$ and 1.83 kg lipid $(g\ P)^{-1}$ in *Scenedesmus*. However, there are two differences in experimental design between the current study and that by Wu et al. (2013): (i) the current study utilised a lower N media enabling significant N-depletion while concurrently testing P-starvation; (ii) Wu et al. did not include the P content of the original inoculum in their estimation of P-specific production, leading to possible yield overestimations. Calculations of the biomass yield per unit of P need to account (as they did here) for the total P used throughout the process, and not just during batch growth in the final large-scale reactor. In the current study, the inocula contained a molar biomass N:P of ~18, a value close to the Redfield ratio (16) considered optima (Geider and La Roche, 2002). For the highest supply N:P (80:1), the residual biomass-P in the inocula made a significant contribution to the final biomass P.

3.4. Linking elemental content to biochemical composition

The minimum and maximum N content of *Nannochloropsis* sp. was approximately 2.6% and 5.7% of DW and had a corresponding lipid content of 58% and 26%, respectively (Fig. 4A). These values were comparable to those in the literature (Adams and Bugbee, 2014). Higher N and correspondingly lower lipid contents have been recorded by the authors (data not shown) when *Nannochloropsis* sp. has been grown for longer under more N replete conditions. The P content of *Nannochloropsis* sp. in this study ranged from 0.08% to 0.60% of DW, resulting in cellular N:P ranging from 18 to 79, depending upon treatment and the sampling time point, showing significant deviation from the Redfield ratio of 16 (Geider and La Roche, 2002). Biomass N:P ratios shifted to reflect that of the media N:P after 10 days batch culture as would be expected (Flynn, 2010).

Fig. 4A shows the relationship between cellular-N content (% DW) and the corresponding lipid and carbohydrate content for the different P regimes. The relationship between N content and lipid content showed a strong inverse linear relationship for each treatment (Fig. 4A, closed symbols; Table 2; $r^2 > 0.90$, $p < 0.001$), with no significant difference between treatments. When N:P treatments were disregarded and the data taken as a single group, there was also a significant correlation between N and lipid content (Table 2; $r^2 > 0.95$, $p < 0.001$). The carbohydrate content of the biomass was also found to correlate highly with N content (Fig. 4A, open symbols; Table 2; $r^2 > 0.90$, $p < 0.001$), as was also the case with the grouped data (Table 2; $r^2 > 0.90$, $p < 0.001$). The

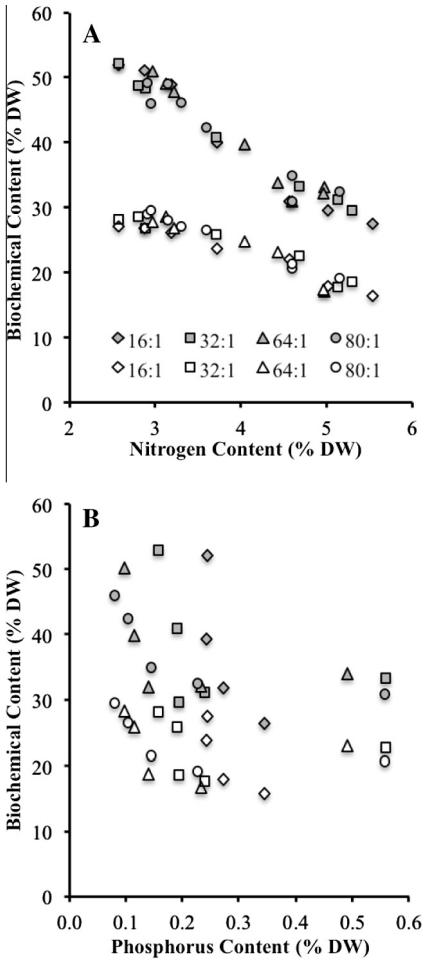

Fig. 4. Relationship between cellular (A) nitrogen or (B) phosphorus content (% DW) and lipid content (closed symbols) and carbohydrate content (open symbols) for *Nannochloropsis* sp. grown under different N:P supply ratios and grouped. Each data point represents the average of three repeats at different days of cultivation, representing all growth phases.

Table 2
Correlation coefficients (r^2) between biomass N or P content (% DW) and biomass lipid and carbohydrate content (% DW) for individual N:P supply ratios and grouped data.

N:P supply ratio	Nitrogen		Phosphorus	
	Lipid[a]	Carb.[b]	Lipid[b]	Carb.[b]
16:1	0.96	0.96	0.67	0.77
32:1	0.98	0.93	0.71	0.83
64:1	0.99	0.92	0.65	0.70
80:1	0.93	0.99	0.94	0.94
Grouped	0.96	0.91	0.29	0.43

Carb. = carbohydrate
[a] Values were obtained from linear regression.
[b] Values were obtained from 2nd order polynomial regression.

increase in carbohydrate content from N replete to N deplete conditions was lower then that for lipids (1.5–1.7 vs 1.6–1.9-fold; Fig 4A), suggesting that for every unit change in cellular-N, there was a correspondingly greater change in lipid content over carbohydrate. When lipid and carbohydrate content was related to cellular-P concentration (% DW) there were no significant relationships for individual treatments, except in the case of the 80:1 using a 2nd order polynomial regression, however for grouped data this relationship was lost (Fig. 4B, Table 2).

There were no correlation between P and lipid content on a per cell basis. However, there were significant relationships found between N cell^{-1} and lipid cell^{-1} for the 16:1 and 32:1 treatments ($r^2 > 0.90$, $p < 0.001$), but not in the 64:1 and 80:1 treatments. This suggests an interactive effect between N and P stresses and the lipid content of *Nannochloropsis* sp.

Overall, these results represent one of the most detailed appraisals of the effect of N- and P-limitation on *Nannochloropsis* sp. bulk biochemical composition, suggesting that intracellular-N was the most significant factor impacting large shifts in composition when grown in batch culture. Despite a decrease in the intracellular-P by up to 75% through different external concentrations, the same total volume of lipids and carbohydrate are produced by the culture (mg L^{-1}), albeit with significant differences in the cellular levels of both lipids and carbohydrates, suggesting that P limitation can also bring about an increase in carbon product accumulation (Elrifi and Turpin, 1985; Gong et al., 2012; Liang et al., 2013).

The above mentioned results contrasts with studies that propose that during batch cultivation, plentiful P is required for lipid accumulation under N-starvation in *Chlorella* and *Scenedesmus* species (Chu et al., 2013, 2014). An explanation might be that plentiful P is required in some species to fully exploit nutrient-N and eventually deplete their cellular-N pool resulting in lipid accumulation. This further highlights the importance of always measuring the intracellular nutrient concentrations (C:N:P) in studies of algal lipid production in fully understanding the degree of nutrient stress and the interactive effect of particular stresses, in this instance N and P.

The current study would suggest that in *Nannochloropsis* sp., the P supply could be plentiful or limiting to achieve similarly high levels of lipid accumulation, but more critically that the primary impact of an inadequate P supply is seen in the negative influence on cell division and biomass productivity, which may then ultimately affect overall lipid productivity. The accumulation of lipid (and changes in biochemical content in general) are thus functions of species and strain, and also of the nutrient history and detail of the experimental protocols. Careful experimentation must be made in order to accurately interpret lipid accumulation strategies, ideally with statement of the intracellular nutrient status of the culture. In addition, understanding these relationships is imperative in development of detailed *in silico* models that can aid identification of optimal conditions and production strategies for growth and desirable biochemical composition (Kenny and Flynn, 2014).

4. Conclusion

Nutrient supply is a well-known and critical factor in determining both biomass and lipid productivity in microalgal cultures. Here, it was found that P-limitation alongside N-starvation had a subtle but minimal effect on bulk biochemical composition, but negatively influenced cell division and biomass productivity of *Nannochloropsis* sp. However, it is also apparent that an N:P ratio of 16:1 (the Redfield ratio, generally considered as "optimal") is in surplus of P for biomass production and can be decreased possibly beyond 32:1 without compromising lipid productivity, while offering considerable savings and improving sustainability of microalgal biomass production.

Acknowledgements

The authors would like to gratefully thank Dr Naomi Ginnever, Dr Alla Silkina, Gemma Webb and all of the CSAR technical team for their help, time and support. The work of Joshua Mayers is funded by the European Structural Funds via a Knowledge Economy Skills Scholarship. The authors would also like to acknowledge the financial support of the INTEREG IVB Northwest Europe programme, via the EnAlgae project.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.07.048.

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