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Simultaneous effect of nitrate (NO$_3^-$) concentration, carbon dioxide (CO$_2$) supply and nitrogen limitation on biomass, lipids, carbohydrates and proteins accumulation in *Nannochloropsis oculata*

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HIGHLIGHTS

➢ Novel approach to simultaneously study the effects of culture conditions on *Nannochloropsis oculata.*

➢ Comprehensive experimental study on *N. oculata* lipids production.

➢ Importance of initial NO$_3^-$ concentration, CO$_2$ and N limitation on lipid production.

➢ Importance of biomass, protein and carbohydrates responses was also simultaneously evaluated.

GRAPHICAL ABSTRACT
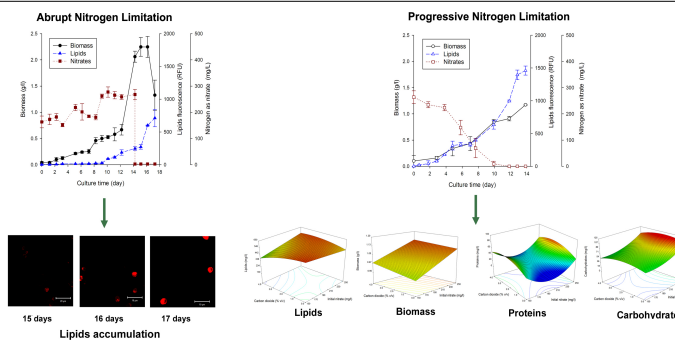
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ABSTRACT

Biodiesel from microalgae is a promising technology. Nutrient limitation and the addition of CO$_2$ are two strategies to increase lipid content in microalgae. There are two different types of nitrogen limitation, progressive and abrupt limitation. In this work, the simultaneous effect of initial nitrate concentration, addition of CO$_2$, and nitrogen limitation on biomass, lipid, protein and carbohydrates accumulation were analyzed. An experimental design was established in which initial nitrogen concentration, culture time and CO$_2$ aeration as independent numerical variables with three levels were considered. Nitrogen limitation was taken into account as a categorical independent variable. For the experimental design, all the experiments were performed with progressive nitrogen limitation. The dependent response variables were biomass, lipid production, carbohydrates and proteins. Subsequently, comparison of both types of limitation i.e. progressive and abrupt limitation, was performed. Nitrogen limitation in a progressive mode exerted a greater effect on lipid accumulation. Culture time, nitrogen limitation and the interaction of initial nitrate concentration with nitrogen limitation had higher influences on lipids and biomass production. The highest lipid production and productivity were at 582 mgL^{-1} (49.7 % lipid, dry weight basis) and 41.5 mgL^{-1}d^{-1}, respectively; under the following conditions: 250 mgL^{-1} of initial nitrate concentration, CO$_2$ supply of 4% (v/v), 12 d of culturing and 2 d in state of nitrogen starvation induced by progressive limitation. This work presents a novel way to perform simultaneous analysis of the effect of the initial concentration of nitrate, nitrogen limitation, and CO$_2$ supply on growth and lipid production of *Nannochloropsis oculata,* with the aim to produce potential biofuels feedstock.

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1. Introduction

The potentials of microalgal biomass as feedstock for renewable fuels is widely known. This is due to the several advantages that microalgae offer compared to other renewable sources, i.e., they present higher growth rate and require less water than terrestrial crops (Balat, 2011); microalgae have a very short harvesting cycle (1–10 d) compared to other land-based feedstock (which are harvested once or twice a year)(Harun et al., 2010); and algae cultivation does not cause competition with food over arable lands and could be well achieved over desert and seashore lands (Demirbas and Demirbas, 2011). Finally, their phototrophic growth has a favorable environmental impact, since the CO_2 released to the atmosphere during hydrocarbons combustion is recycled by microalgae in photosynthetic processes (100 tons of biomass uptakes about 183 tons of CO_2) (Chisti, 2007).

Particularly, biodiesel is one of the most attractive final products derived from microalgae due to their high lipid content; and after the removal of the lipid fraction, the remaining residual biomass (mainly carbohydrates and proteins) can also be used for energy generation or high value by-products (Pienkos and Darzins, 2009).

Phototrophic metabolism of microalgae is mainly influenced by light irradiance, culture temperature and nutrients supply. However, it is almost impossible to control diurnal fluctuations of irradiance and temperature in open large-scale systems (Grima et al., 1999; Borowitzka and Moheimani, 2013). For this reason, the availability of nutrients is the most documented approach to enhance lipid production. Nutrients limitation strategy is based on a two-stage cultivation with the first one focused on cell growth in a rich nutrient medium; while the second stage triggers fatty acids accumulation in cells through nutrients limitation. In this context, there are two different types of nutrients limitation. Progressive limitation occurs due to continuous substrates consumption by cells, which induces the nutrient- limited stage. While abrupt limitation is based on transferring the cells from a rich culture medium to another lacking one or more than one type of nutrients. Certain microalgae species are able to accumulate up to 40-63 % of lipid per dry cell weight (DCW) in media with low nitrogen concentrations (Illman et al., 2000). However, the nitrogen deprivation strategy involves a reduction of cell duplication that results in low biomass productivities during fatty acids accumulation (Rodolfi et al., 2009). One proposed strategy to overcome this problem was to supply inorganic carbon to the nitrogen-limited culture. It was observed that the addition of bicarbonate ($NaHCO_3$) and carbon dioxide (CO_2) to microalgal cultures not only enhanced biomass and fatty acid methyl esters (FAME) productivities, but also promoted nitrogen assimilation (Chiu et al., 2009; Lin et al., 2012). Moreover, the specific growth rate of microalgal cultures could increase up to three folds when low concentrations of CO_2 were supplied during the aeration (2-5% v/v) (Chiu et al., 2009).

Since microalgal cultures depend on multiple factors, multivariate statistic techniques are required to study several variables simultaneously. The Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques developed to model experimental responses. It is based on the fit of a polynomial equation to the experimental data, which describes the behavior of a data set with the aim to find the optimal response within specified ranges of the factors (Montgomery and Myers, 2003). This methodology includes the interactive effects of all the variables studied. The experimental design is a crucial aspect of the RSM, which is a specific set of experiments defined by a matrix composed by the different level combinations of the variables studied (Bezerra et al., 2008).

In this work, a novel and comprehensive experimental design using the microalgae strain *Nannochloropsis oculata* was performed. By RSM the simultaneous effect of initial nitrate concentration, CO_2 supply, and nitrogen limitation on biomass and lipid production, as well as carbohydrates and proteins contents in the residual biomass after oil extraction were analyzed. Furthermore, the effect of progressive and abrupt limitation of nitrogen on biomass and lipids production, as well as carbohydrates and residual proteins was also investigated.

2. Material and method

2.1. Microalgal cultures

The microalgae strain *N. oculata* used in this study was obtained from the Centro de Investigación Científica y de Educación Superior de Ensenada

collection (CICESE, Mexico). The strain was grown in cylindrical glass photobioreactors (1 L), at 25±1 °C, under photoperiods of 12:12 h (light: dark) using cool-white fluorescent light with an intensity of 100 μmol photons $m^{-2}s^{-1}$. Microalgal cultures were aerated continuously (1.6 $Lmin^{-1}$). Cells were first cultured for proliferation until stationary phase; then, algal cultures were inoculated by adjusting the initial cell concentration to 2 x 10^6 cells ml^{-1} approximately. For abrupt nitrogen limitation experiments, cell cultures were daily fed with nitrate ($NaNO_3$) to ensure nitrate excess until the beginning of the nitrogen limitation phase. At that time, cells were centrifuged for 15 min at 3500 ×g, the supernatant was removed and cells were resuspended in a fresh medium lacking nitrate.

2.2. Medium composition

Microalgae cells were cultivated in the modified f/2 medium with artificial sea water using the following composition (gL^{-1}): 29.23 NaCl, 1.105 KCl, 1.21 tris-base, 2.45 $MgSO_4$ ·7H₂O, 1.83 $CaCl_2$ ·2H₂O, 0.25 $NaHCO_3$, and 3 mL of trace elements solution. The trace elements solution contained (gL^{-1}): 5 NaH_2PO_4·H_2O, 4.1 Na_2EDTA, 3.16 $FeCl_3$ ·6H₂O, 0.18 $MnCl_2$ .4H₂O, 0.01 $CoCl_2$.6H₂O, 0.01 $CuSO_4$.5H₂O, 0.023 $ZnSO_4$.7H₂O, and 0.006 Na_2MoO_4. Nitrogen source was $NaNO_3$ and three different initial concentrations of the ion (NO_3^-) were tested (250, 200 and 150 mg L^{-1}). All chemicals were analytical grade.

2.3. Response surface experimental design

An experimental design was established by taking into account initial nitrogen concentration, culture time and CO_2 aeration as independent numerical variables, and nitrogen limitation as a categorical independent variable (Table 1). All the experiments were performed with progressive nitrogen limitation. The dependent response variables were biomass and lipid production, as well as carbohydrates and proteins contents. The experimental results were analyzed using the software Design Expert 8.0.7.1.

Table 1.
Experimental design.

Parameters Range	Initial nitrate concentration (mg/l)	CO_2 supply (% v/v)	Culture time (days)	Nitrogen limitation*
-1	150	0	7	N/A
0	200	2	10	With
1	250	4	14	Without

* Categorical parameter

Analysis of variance (ANOVA) was used for graphical analyses of the data to obtain interactions between the independent and dependent variables. The quality of the fit polynomial model was expressed by the coefficient of determination (R^2) and its statistical significance was checked by the Fisher F-test. Model terms were accepted or rejected based on the p value (probability) with a 95% of confidence level. Three dimensional plots were obtained based on the effect of the levels of two factors.

2.4. Biomass determination

Biomass was determined by: a) dry weight, i.e., culture samples of 5 ml were filtered in pre-weighed glass-fiber filters (Ahlstrom ©, 0.7 μm), excess of salts were removed with 15 ml of NH_4HCO_3 0.5 N, filters were dried completely after 24 h of oven incubation at 48°C and weighed, b) direct cell count was done by using a counting Neubauer hemocytometer and the software Cell C Counting (Selinummi et al., 2005); and c) optical density was obtained at 750 nm with a GENESYS-10S spectrophotometer.

2.5. Lipid extraction

Culture samples of 30 ml each were concentrated by centrifugation (6000 rpm for 15 min), the pellet was washed with 20 ml of deionized water and centrifuged again (6000 rpm for 15 min), and the biomass was dried at 48°C. Dried biomass was milled and resuspended in 4 ml of hexane. Suspensions were sonicated during 30 min (Branson 1510 sonicator, 30 KHz) and then

stored for 12 h at 5°C. Afterwards, the lipid extract was transferred to a pre-weighed vial and two rounds of washing were performed by adding 3 ml of hexane to the residual biomass and the solvent was recovered with the initial extract (10 ml of final lipids extract). Afterwards, hexane was removed with nitrogen gas and the vials were dried until reached a constant weight and finally weighed.

2.6. Neutral lipid analysis by Nile Red (NR) staining and Confocal Laser Scanning Microscopy

One ml samples of fresh algal suspensions were adjusted to cell concentrations in a range between 1×10^4 to 5×10^5 cell ml^{-1}. Cell suspensions were stained with 2 µl of NR (20 µg NR L^{-1} in acetone). The reaction was allowed to occur during 20 min in darkness at room temperature. The relative fluorescence was measured in a Trilogy™ spectrofluorometer using excitation and emission wavelengths of 530 and 575 nm, respectively and non-stained cells and medium without cells were used as control. For image acquisition, 3 µl of the stained mixture were placed on microscope slides and analyzed in a Carl Zeiss LSM710 Confocal Lasser Scanning Microscope (Germany). Photographs with image size of 512×512 pixels were taken using a Plan-Apochromat 63×/1.4 Oil DIC M27 objective. The variable band pass filter for emission was centered at 488 nm with 2.0% of potency and emission light was detected at 539-628 nm. Transmitted light and fluorescent images were merged and colored using the Zen software.

2.7. Analysis of FAMEs

The extracted lipid was transesterified with 750 µl of methanolicchlorhydric acid 0.5 N at 80 °C during 3 h. FAMEs were dissolved in 1 ml of hexane and filtered with a 0.2 µm polytetrafluoroethylene membrane. Two µl samples of FAMEs were analyzed by gas chromatography (GC) on a Clarus ® 500 gas chromatograph (Perkin Elmer). A 30 m column was used for the separation (AT-WAX, Polyethylene glycol (PEG) with internal diameter of 0.25 mm, and film thickness of 0.2 µm). Helium was used as the carrier gas at a flow rate of 0.96 ml min^{-1}. The injector temperature was set at 230 °C and the detector (flame ionization detector, FID) was set at 250 °C. The temperature ramp for the oven was set as follows: 5 min at 140 °C, a temperature rate increase of 8 °C min^{-1} to 240 °C, then 15 min at 240 °C. Retention times of FAMEs components were taken from a standard that was used as reference to identify the FAMEs in the samples (Supelco 37 FAME mix, Sigma Aldrich No. 47885-U).

2.8. Carbohydrates and proteins determination

After lipids extraction, 5 mg of the exhausted biomass were placed in glass tubes for carbohydrates and proteins quantification. For carbohydrates, 3 ml of HCl 2 N were added and biomass was hydrolyzed at 100°C during 2 h in a Major Science dry bath block (USA). The hydrolyzate was analyzed according to the Dubois method (Dubois et al., 1951). For protein determination, 3 ml of NaOH 1 N were added to the exhausted biomass and hydrolyzed at 100°C during 1 h in a Major Science dry bath block (USA). The hydrolyzate was analyzed using the Bradford method (Bradford, 1976).

2.9. Nitrate quantification

Nitrate concentration in the medium was measured according to a modified version of the Keeney Nelson method (Keeney and Nelson, 1982). Briefly, 2 ml of the culture were centrifuged at 17000 ×g for 5 mins, 500 µl of supernatant were transferred into glass tubes and dried in a Major Science dry bath block (USA). Then, 500 µl of 2-4 biphenyl sulfonic acid were added to the dried samples, followed by gentle addition of 2.2 ml of KOH 12 N. The reaction was allowed to occur during 5 min and 100 µl of the resulting supernatant were placed in plastic cuvettes containing 900 µl deionized water. The samples were analyzed at 410 nm in a GENESYS-10S spectrophotometer.

3. Results and discussion

3.1. Response Surface analysis

Microalgal cultures showed a regular cellular growth behavior. Biomass was accumulated while nutrients were consumed. This progressive consumption induced a nitrogen-limited state at different culture times based on the initial nitrate concentration. Thus, nitrogen limitation was achieved after 6, 8 and 8 d in cultures with initial nitrate concentrations of 150, 200 and 250 mg L^{-1}, respectively (Fig. 1). Cellular growth was affected by nitrogen starvation, showing a stationary phase after 10 d in cultures with initial nitrate concentrations of 150 and 200 mg L^{-1}, whereas stationary cell growth was observed after 13 d in cultures with initial nitrate concentration of 250 mg L^{-1} after 5 d of nitrogen starvation.
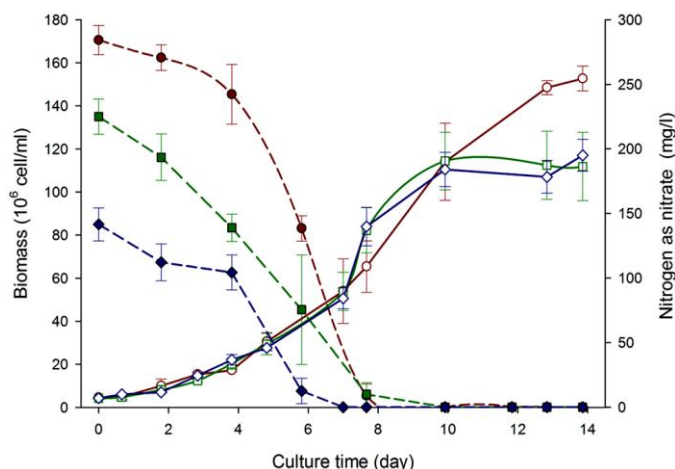
Fig.1. Progressive nitrate limitation and biomass production in *N. oculata* cultures at different initial nitrate concentrations. Continuous lines indicate cellular growth and dashed-lines represent initial nitrate (NO_3^-) consumption of 150 (diamond), 200 (square), and 250 (circle) mg L^{-1}.

A statistically significant difference at the 95% level ($p = 0.0024$) was observed on final cells concentration between the highest level of initial nitrogen concentration (250 mg L^{-1}) and the other two levels (200 and 150 mg L^{-1}). Although nitrogen limitation plays an important role in cell growth, the presence of other macronutrients (e.g. Fe, K, and Mg) also affects biomass generation (Converti et al., 2009; Chen et al., 2011), which might be one explanation for the cell growth observed during nitrogen restriction.

An experimental design was performed in *N. oculata* cultures considering initial nitrate concentration, CO_2 supply, culture time and state of nitrogen limitation as independent variables (Table 1). Lipid content, biomass, carbohydrates and proteins were analyzed as response variables due to their importance as a raw material for food supplement or bioenergetics feedstock.

ANOVA results were assessed with various descriptive statistic tools, such as the p-value, that refers to the probability of the test to be distributed under the null hypothesis; probability of lack of fit (PLOF); and the adequate precision (Table 2).

Table 2.
Experimental ANOVA and regression analysis of the experimental design.

Response	PLOF	p	Adequate precision	R^2	Model equation
Lipids (mg/L)	0.3348	<0.0001	16.97	0.9325	$286.71 + 198.50$ D $+ 0.63$ AB $- 9.06$ BC $- 55.59$ BD
Biomass (g/L)	0.3833	0.0003	8.14	0.7436	$0.93 + 0.263$ C $+ 0.109$ D $- 0.0954$ AD
Carbohydrates (mg/L)	0.1030	<0.0001	18.20	0.9349	$23.03 - 17.9$ A $- 2.39$ B $+ 9.86$ C $- 19.95$ D $- 10.01$ AC $- 14.79$ AD $+ 15.29$ $A^2 - 14.28$ $B^2 + 27.01 C^2$
Proteins (mg/L)	0.8835	<0.0001	11.44	0.8681	$72.05 - 12.61$ A $- 7.68$ B $+ 16.17$ C $+26.77$ D $- 67.80$ AC $- 82.72$ AD $- 15$ BD $+ 90.35$ CD $+ 36.78$ $A^2 - 17.95$ $B^2 + 56.75$ C^2

PLOF = probability of lack of fit   p = probability of error
A = Initial (NO_3^-) nitrate concentration (mg/l)   B = CO_2 supply (% v/v)
C = Culture time (day)   D = Nitrogen limitation (categorical factor)

Adequate precision measures the signal to noise ratio and numerical values greater than 4 indicate that the models can be used to navigate the designed space. The terms of the equations were selected based on their significance of the p-values <0.05. The fit of the model yielded R^2 values up to 0.9325 for lipids and 0.9349 for carbohydrates, but not good values were obtained for proteins and biomass (**Table 2**). The independent factors that showed higher influence on the response variables were listed hierarchically in **Table 3**, based on their parameter estimation.

Table 3.
Hierarchical influence of equations terms of the response variables i.e. lipids, biomass, residual carbohydrates and residual proteins.

Response variables	Equation terms (descendant order of influence)
Lipids	D, BD, BC, AB
Biomass	C, AD, D
Carbohydrates	C^2, D, A, A^2, AD, B^2, AC, C, B
Proteins	CD, AD, AC, C^2, A^2, D, B^2, C, BD, A, B

A = Initial nitrate (NO_3^-) concentration (mg/l)   B = CO_2 supply (% v/v)
C = Culture time (day)   D = Nitrogen limitation (categorical factor)

Concerning the enhancement of lipid accumulation in *N. oculata,* the effect of nitrogen limitation on lipid content in microalgal cultures has been previously reported (Pruvost et al., 2009; Rodolfi et al., 2009). Additionally, in this study simultaneous effect of different factors interaction i.e. nitrogen limitation with carbon dioxide, culture time with carbon dioxide, and initial nitrate concentration with carbon dioxide were observed. The highest lipid concentration obtained was at 582 mg/l (**Fig. 2A**), corresponding to 49.7% of lipid DCW. This was achieved under the following conditions: 250 mg L^{-1} of initial nitrate concentration, supply of 4% CO_2 (v/v) and 14 d of cultivation. Two days out of these 14 d, the cells were exposed to nitrogen starvation.
The terms with higher influence on biomass production were: culture time, nitrogen limitation and the interaction of initial nitrate concentration with nitrogen limitation (**Table 3**). The highest biomass concentration was recorded at 1.17g/l (**Fig. 2B**) under the same optimal conditions for lipid accumulation. Compared to the work of Chiu et al.(2009), where they observed higher biomass concentration in cultures of *N. oculata* enriched with 2% of CO_2 (v/v) and reported growth inhibition at CO_2 concentrations higher than 5% (v/v), in this work the addition of CO_2 did not have a statistically significant effect ($p = 0.2871$) in the levels of the experimental design. In general, the higher the initial concentration of nitrate, the larger the concentration of biomass obtained. This emphasizes the more important role of initial nitrate concentration over CO_2 supply on biomass generation.

The highest concentration of carbohydrates was measured at 108.9 mg/l, and was obtained using 250 mg L^{-1} of initial nitrate concentration, 4% CO_2 supply (v/v) and a period of 14 d cultivation including 2 d nitrogen limitation (**Fig. 2C**). Carbohydrate accumulation is inversely proportional to the lipids production, since the lipid precursor glycerol-3-phosphate is produced by glucose catabolism (Chen et al., 2013). However, microalgal glucose polymers produced via cellulose/starch are the predominant components in the cell walls, and both, starch and most of the cell wall polysaccharides can be converted into fermentable sugars for subsequent bioethanol production via microbial fermentation (Wang et al., 2011).

The highest concentration of proteins was at 84.1 mg/l and it was achieved in cultures with 250 mg L^{-1} of initial nitrate, without CO_2 supply, and after 10 d of cultivation; however, the final concentration of proteins under the optimal conditions for lipids and biomass production was 81.6 mg/l (**Fig. 2D**). Carbohydrates and proteins models showed two similar terms with interaction of factors: initial nitrate concentration with nitrogen limitation, and initial nitrate concentration with culture time. This implied the more significant effect of nitrate addition over CO_2 supply (**Table 3**).

Despite the fact that low concentrations of carbohydrates and proteins were obtained after lipids extraction (9.2 and 6.9% of DCW, respectively), these molecules would play an important role in sustainable microalgae-based bioprocesses at large scale.
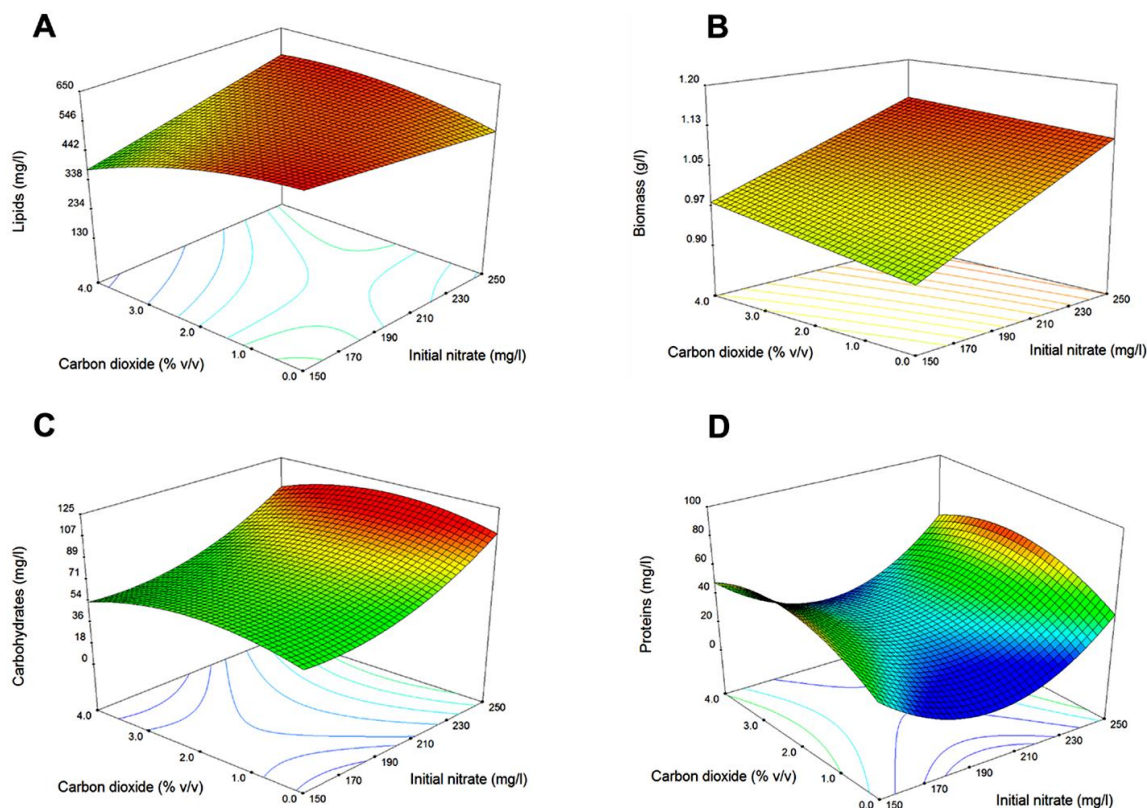
Fig.2. Response surface graphs for the effect of initial nitrate concentration and CO_2 supply on lipids (A), biomass (B), residual carbohydrates (C) and residual proteins (D) in *N. oculata*.

3.2. Comparison of abrupt and progressive nitrogen limitations

Since nitrogen limitation was the common representative term for all the response variables in the experimental design (**Table 3**), it was decided to compare two different types of nitrogen limitation under the optimal conditions led to the highest biomass, lipids and carbohydrates production using the experimental design. Thereby, abrupt nitrogen limitation was performed by maintaining nitrate concentration (roughly 250 mg L^{-1}) for 14 d, and after this period of time, the cells were transferred to another culture medium lacking nitrate, while maintaining a continuous CO_2 supply (4% v/v).

The cultures that were maintained with sufficient amounts of nitrate reached the highest biomass concentration (i.e., 2.25 g L^{-1}) after 14 d (**Fig. 3A**); two folds the highest value obtained under progressive nitrate consumption (i.e., 1.17g L^{-1}) (**Fig. 3B**). Three days after the abrupt nitrate limitation, the biomass concentration reduced to 40.9% from the highest value, and a slight reduction in cells concentration was also observed (data not shown). While in cultures with progressive nitrate consumption, the trend of biomass accumulation was to increase despite of nitrogen limitation conditions.

In both abrupt and progressive limitation modes, relative fluorescence of lipids bodies stained with NR dye revealed increased lipid accumulation. The highest lipid concentrations for abrupt and progressive limitations were 284 mg L^{-1} after 16 d and 582 mg L^{-1} after 14 d of cultivation, respectively. The lower lipids content (12–20% DCW) coupled to biomass diminution in cultures under abrupt limitation was also observed in other studies involving *N. oculata* (Van Vooren et al., 2012). In fact, cell growth diminution was a result of photo-oxidative stress due to nitrogen source scarceness. Surprisingly, higher concentrations of residual protein were achieved in the present experiments with abrupt nitrogen limitation reaching 1.31 g L^{-1} (58% DCW) 16 d after cultivation; just 2 d after the abrupt limitation.

With regards to lipids accumulation, qualitative analysis was conducted by Confocal Laser Scanning Microscopy of cells stained with NR dye after 24, 48 and 72 h of abrupt nitrogen limitation (**Fig. 4**). Total lipids quantification showed a consistent correlation with triacylglycerol measurements (relative fluorescence). In better words, higher lipids quantities led to stronger relative fluorescence observed in the NR-stained cells.
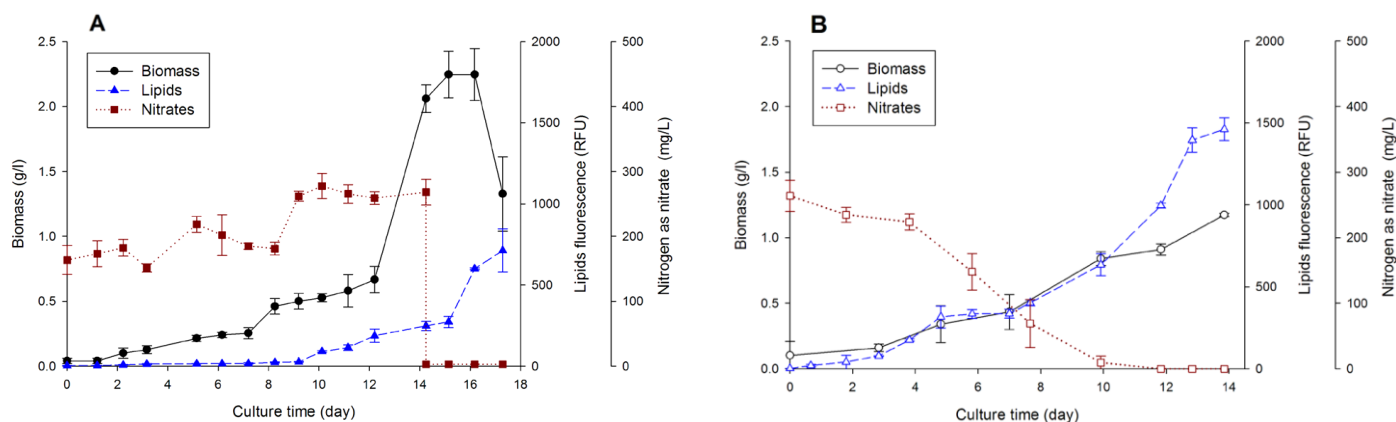
Fig.3. Effect of abrupt (A) and progressive (B) nitrate limitation on biomass (g L^{-1}) and lipids accumulation (relative fluorescence).

Fig.4. Confocal Laser Scanning Microscopy images of *N. oculata* cultures at (I) 24, (II) 48, and (III) 72 h after the abrupt nitrate limitation. In red (A): lipids stained with NR dye, in green (B): chlorophyll auto-fluorescence, in yellow (C): lipids and chlorophyll co-localization, and in gray (D) optical images.

Chlorophyll fluorescence was constant while the cell exhibited higher lipids fluorescence over the process time. The fact that lipid content was duplicated from 24 to 72 h under abrupt nitrogen limitation can b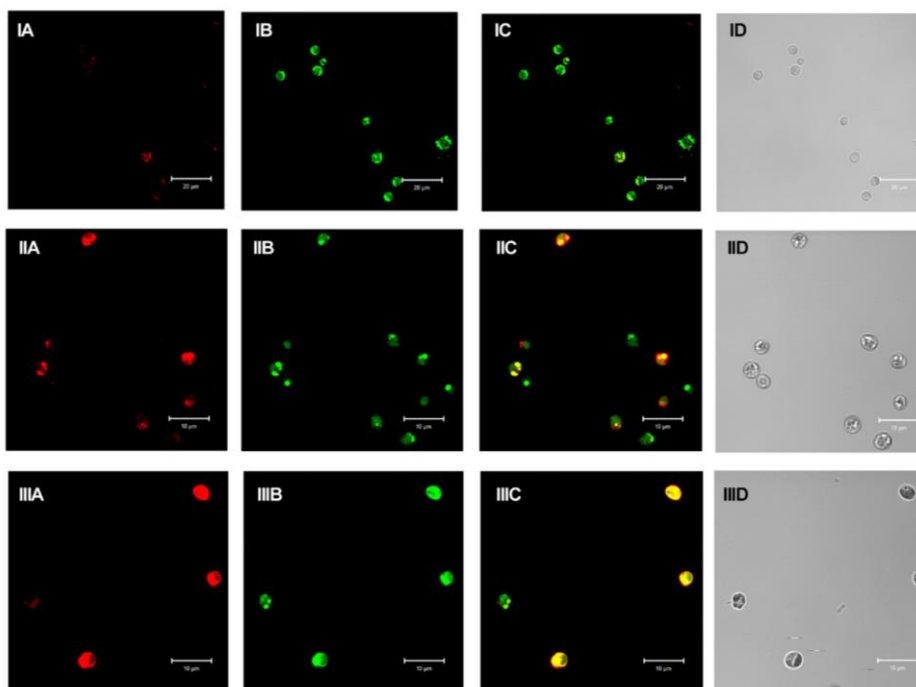e attributed to the simultaneous phenomena of biomass diminution and high lipids expression in nitrate-stressed cells. In general, these results are in agreement with those reported previously (Pruvost et al., 2009; Van Vooren et al., 2012)

progressive nitrate consumption. Maintaining nitrate concentration at 250 mg L^{-1} coupled to 4% CO_2 supply allowed higher biomass concentrations (2.25 g L^{-1}) and higher protein content (1.31 g L^{-1}). FAMEs profile did not differ between the abrupt and progressive limitation. In this work, it was shown that the lipid production was mainly influenced by the combination of nitrogen limitation and carbon dioxide concentration, culture time and carbon dioxide

Table 4.
FAMEs profile in the abrupt and progressive nitrogen (N-NO$_3^-$) limitation experiments.

Type of (N-NO$_3^-$) limitation	FAME (% abundance)								
	C14:0	C16:0	C16:1Δ9	C18:0	C18:1 Δ9	C18:2 Δ9,12	C18:3 Δ9,12,15	C20:3 Δ11,14,17	C20:5 Δ5,8,11,14,17
(A) Progressive limitation	8.09 ± 0.17	41.23 ± 3.09	21.14 ± 3.50	10.19 ± 3.92	5.79 ± 1.46	2.01 ± 0.45	0.76 ± 0.26	3.23 ± 1.03	6.08 ± 1.49
(B) Abrupt limitation	8.28 ± 0.44	38.52 ± 3.58	21.53 ± 6.86	8.26 ± 4.25	7.24 ± 0.39	2.24 ± 0.44	0.95 ± 0.14	4.23 ± 1.88	7.33±2.81

A: 14 d of cultivation
B: 2 d of limitation, 16 d of cultivation

In this work, the highest lipid productivity achieved in cultures of *N. oculata* was recorded at 41.5 mg $L^{-1}d^{-1}$ under progressive nitrogen limitation (N-NO$_3^-$) under the optimal conditions previously described. Compared to the other studies on *Nannochloropsis* species, this value was slightly higher. For instance, Rodolfi et al. (2009) reported lipid productivity of 37.6 mg $L^{-1}d^{-1}$ in their experiments performed in 250 ml culture systems, and the value reported by Gouveia and Oliveira (2009) was 25.8 mg $L^{-1}d^{-1}$ in cultures grown in wall panel systems of 100 l.

Several studies concerning the effect of nitrogen limitation on lipid and biomass have been carried out in diverse microalgae species (Converti et al., 2009; Xin et al., 2010; Chen et al., 2011), pointing out the importance of the lack of nitrogen source for lipids accumulation triggering. In this work, we observed the same effect on *N. oculata* cultures under progressive and abrupt nitrogen limitation. Since nitrogen restriction decreases cellular growth, the addition of inorganic carbon sources has been studied to overcome this issue (Gordillo et al., 1998; Chiu et al., 2009; Chai et al., 2012; Lin et al., 2012). In general, higher biomass concentrations were achieved with the addition of CO to the cultures. However, there still exists the inhibiting effect of high CO_2 concentration i.e. 5% (v/v) on cell growth in *N. oculata* (Chiu et al., 2009). In this work, the highest level of CO_2 supply (4% v/v) applied in the experimental design for the RSM did not show growth inhibition effects in the cultures. Overall, most of the previous studies on lipids and biomass production in microalgae analyzed only one variable at a time, but in the present investigation, we showed the simultaneous effect of nitrogen restriction, initial nitrate concentration and CO_2 supply on lipids accumulation, biomass production, as well as residual carbohydrates and residual proteins content information that would provide a complete outlook on *N. oculata* in the framework of biofuels feedstock production.

3.3. FAME composition

Lipid extracts composition was analyzed by GC-FID in order to identify FAMEs profile for biodiesel production. No significant differences in FAMEs profiles were observed between abrupt and progressive limitation experiments (Table 4). The most abundant components were: palmitic acid (C16:0), palmitoleic acid (C16:1), and stearic acid (C18:0) with abundance values of 39.87, 21.34 and 9.23%, respectively. Saturated fatty acids (SFA) content was recorded at 55.1 and 59% for abrupt and progressive limitation, respectively.

4. Conclusion

Response surface analysis allowed observing the simultaneous effect of different interactions among important factors promoting lipid accumulation such as the combination of nitrogen limitation and carbon dioxide concentration, culture time and carbon dioxide, as well as initial nitrate concentration and carbon dioxide concentration. The optimal conditions for biomass, lipids and carbohydrates production were: 250 mg L^{-1} of initial nitrate concentration, supply of 4% CO_2 (v/v) and 14 d of cultivation under

concentration, as well as initial nitrate concentration and carbon dioxide concentration.

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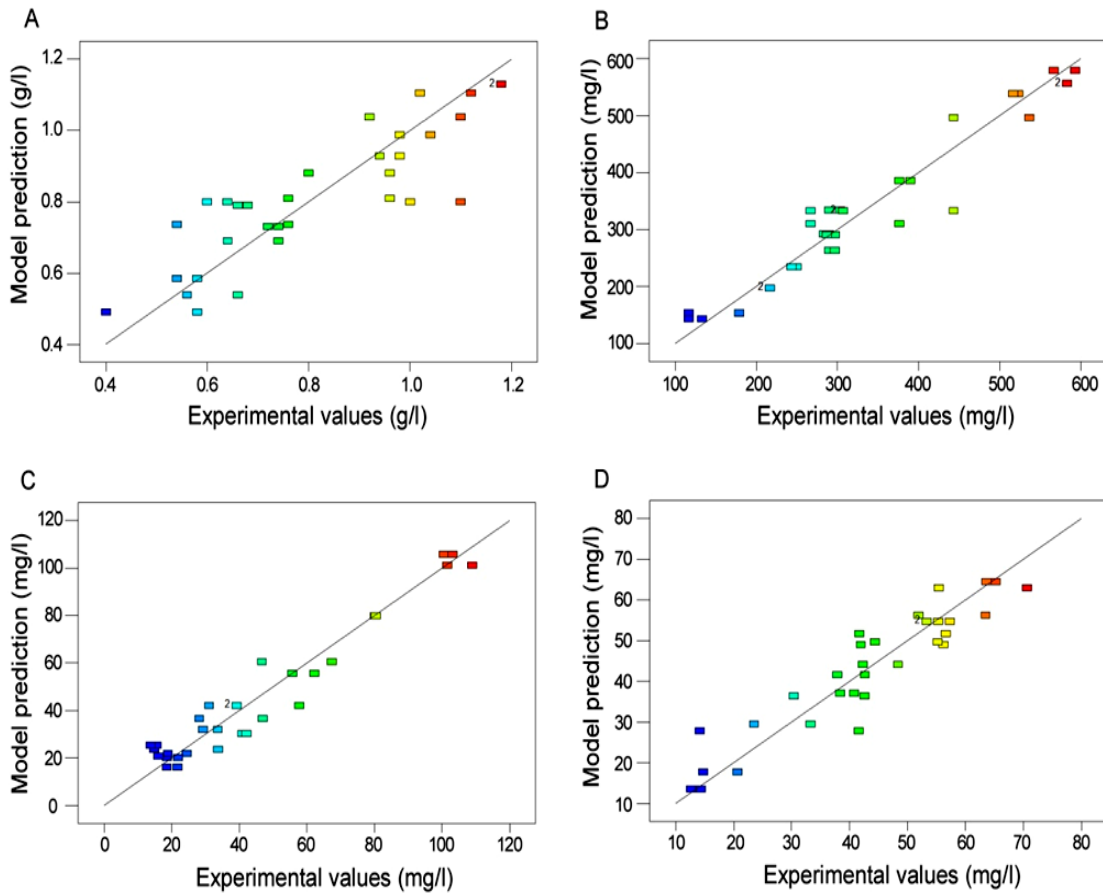
Supplementary Data

Fig. S.1. Experimental values vs. model prediction of: (A) biomass $R^2 = 0.7436$; (B) lipids $R^2 = 0.9325$; (C) residual carbohydrates $R^2 = 0.9349$ and (D) residual proteins $R^2 = 0.8681$
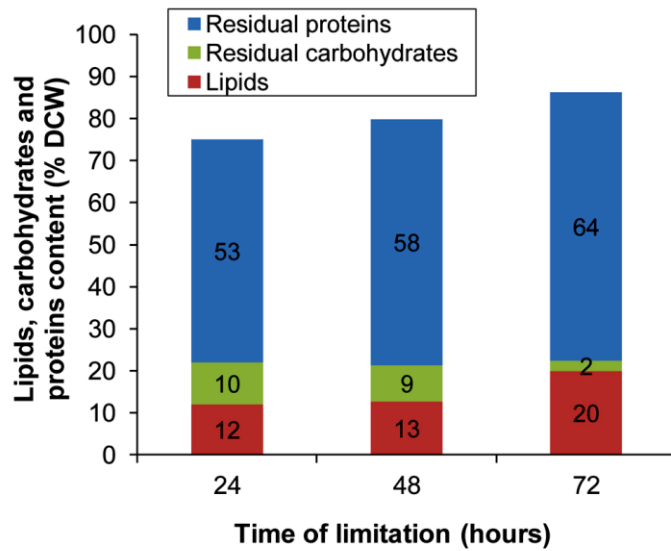

Fig. S.2. Lipid, carbohydrates and proteins content after: 24, 48 and 72 h of abrupt nitrogen limitation in cultures of *N. oculata*.

Table S.1.
ANOVA analysis of the biomass response.

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F
Model	1.09411743	10	0.10941174	6.09321869	0.0003
A-Initial nitrate	0.00420205	1	0.00420205	0.23401514	0.6336
B-Carbon dioxide	0.00979072	1	0.00979072	0.54525215	0.4684
C-Culture time	0.25746318	1	0.25746318	14.33831	0.0011
D-Nitrogen limitation	0.03052348	1	0.03052348	1.69987454	0.2064
AB	0.00073443	1	0.00073443	0.04090116	0.8417
AC	0.00100207	1	0.00100207	0.05580587	0.8155
AD	0.02579629	1	0.02579629	1.43661394	0.2440
BC	0.00645884	1	0.00645884	0.35969772	0.5551
BD	0.00429467	1	0.00429467	0.23917303	0.6299
CD	0.00635706	1	0.00635706	0.35402943	0.5582
Residual	0.37708257	21	0.01795631	__	__
Lack of Fit	0.07818257	4	0.01954564	1.11166257	0.3833
Pure Error	0.2989	17	0.01758235	__	__
Cor Total	1.4712	31	__	__	__

df = degrees of freedom

Table S.2.
ANOVA analysis of the lipids response.

Source	Sum of squares	df	Mean square	F value	p- value Prob > F
Model	571798.618	10	57179.8618	29.938123	< 0.0001
A-Initial nitrate	7985.20599	1	7985.20599	4.18087892	0.0536
B-Carbon dioxide	2278.60514	1	2278.60514	1.19302773	0.2871
C-Culture time	37.0484046	1	37.0484046	0.01939773	0.8906
D-Nitrogen limitation	16598.679	1	16598.679	8.69070468	0.0077
AB	35303.3898	1	35303.3898	18.4840815	0.0003
BC	34586.9582	1	34586.9582	18.1089736	0.0004
BD	61769.1813	1	61769.1813	32.3409901	< 0.0001
CD	4899.79059	1	4899.79059	2.56542301	0.1242
B^2	5723.86347	1	5723.86347	2.99688952	0.0981
C^2	3615.14506	1	3615.14506	1.89281075	0.1834
Residual	40108.63	21	1909.93476	__	__
Lack of Fit	9007.61191	4	2251.90298	1.23090345	0.3348
Pure Error	31101.0181	17	1829.47165	__	__
Cor Total	611907.248	31	—	__	__

df = degrees of freedom

Table S.3.
ANOVA analysis of the residual carbohydrates response.

Source	Sum of quares	df	Mean square	F value	p-value Prob > F
Model	24625.7225	9	2736.19139	35.0887695	< 0.0001
A-Initial nitrate	1001.73898	1	1001.73898	12.8462461	0.0017
B-Carbon dioxide	114.37602	1	114.37602	1.46675185	0.2387
C-Culture time	369.020491	1	369.020491	4.73229869	0.0406
D-Nitrogen limitation	1045.27481	1	1045.27481	13.4045473	0.0014
AC	332.876698	1	332.876698	4.26879267	0.0508
AD	336.4311	1	336.4311	4.31437412	0.0497
A^2	377.596888	1	377.596888	4.84228195	0.0386
B^2	583.618644	1	583.618644	7.48429374	0.0121
C^2	2427.92192	1	2427.92192	31.1355386	< 0.0001
Residual	1715.54065	22	77.9791206	—	—
Lack of Fit	572.792288	5	134.558458	2.19371601	0.1030
Pure Error	1042.74837	17	61.3381391	—	—
Cor Total	26341.2631	31	—	—	—

df = degrees of freedom

Table S.4.
ANOVA analysis of the residual proteins response.

Source	Sum of quares	df	Mean square	F value	p-value Prob > F
Model	6955.76521	11	532.342292	11.9642879	< 0.0001
A-Initial nitrate	424.019227	1	424.019227	8.02269305	0.0103
B-Carbon dioxide	754.170043	1	754.170043	14.2693406	0.0012
C-Culture time	562.092573	1	562.092573	10.6351219	0.0039
D-Nitrogen limitation	1002.6363	1	1002.6363	18.9704682	0.0003
AC	2297.06542	1	2297.06542	43.4618281	< 0.0001
AD	2423.34314	1	2423.34314	45.8510768	< 0.0001
BD	2879.08559	1	2879.08559	54.4739919	< 0.0001
CD	2729.89239	1	2729.89239	51.6511688	< 0.0001
A^2	1202.18174	1	1202.18174	22.7459853	0.0001
B^2	644.480059	1	544.480059	12.1939416	0.0023
C^2	2779.4837	1	2779.4837	52.5894657	< 0.0001
Residual	1057.04961	20	52.8524804	—	—
Lack of Fit	38.9118876	3	12.9706292	0.21657256	0.8835
Pure Error	1018.13772	17	59.8904542	—	—
Cor Total	8012.81482	31	—	—	—

df = degrees of freedom

Table S.5.
Parameter estimation for the lipid model equation.

Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High
Intercept	286.706957	193.108286	-114.883708	688.297623
A-Initial nitrate concentration	-0.82238544	0.40219959	-1.65880527	0.01403438
B-Carbon dioxide	-39.143468	35.8371967	-113.670999	35.3840624
C-Culture time	-5.76532963	41.3950731	-91.8510968	80.3204376
D-Nitrogen limitation	198.501716	67.334365	58.4722381	338.531193
AB	0.63030551	0.14660614	0.32542135	0.93518968
BC	-9.06135733	2.1293465	-13.4895758	-4.63313886
BD	-55.5930929	9.77561706	-75.9226015	-35.2635843
CD	-11.8786036	7.4162762	-27.3015942	3.5443871
B^2	-8.52965187	4.92715177	-18.7762249	1.71692116
C^2	2.78159806	2.02181204	-1.42299024	6.98618636

Table S.6.
Parameter estimation for the biomass model equation.

Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High
Intercept	0.93473627	0.09382155	0.73962367	1.12984887
A-Initial nitrate concentration	-0.03023344	0.06249792	-0.16020497	0.0997381
B-Carbon dioxide	-0.03496409	0.04735039	-0.13343461	0.06350643
C-Culture time	0.26383107	0.069675	0.11893398	0.40872816
D-Nitrogen limitation	0.10964432	0.08409646	-0.06524384	0.28453249
AB	-0.00909116	0.04495226	-0.10257449	0.08439217
AC	0.01428607	0.06047459	-0.11147772	0.14004987
AD	-0.09548049	0.07966079	-0.26114416	0.07018319
BC	0.02662707	0.0443971	-0.06570174	0.11895589
BD	-0.02931768	0.05994783	-0.15398602	0.09535067
CD	0.05054952	0.08495665	-0.1261275	0.22722654

Table S.7.
Parameter estimation for the residual carbohydrates model equation.

Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High
Intercept	23.0252243	4.78909833	13.0932422	32.9572063
A-Initial nitrate concentration	17.8977805	4.99356905	7.54175218	28.2538089
B-Carbon dioxide	-2.39140147	1.97457743	-6.48642442	1.70362149
C-Culture time	9.8611089	4.53304135	0.46015653	19.2620613
D-Nitrogen limitation	-19.9469631	5.44817073	-31.2457777	-8.64814857
AC	-10.0082486	4.84401581	-20.0541225	0.03762536
AD	-14.7885063	7.11976091	-29.5539867	-0.02302591
A^2	15.2859475	6.94652056	0.87974554	29.6921494
B^2	-14.2798791	5.21974299	-25.1049635	-3.45479473
C^2	27.0114394	4.84082671	16.9721793	37.0506996

Table S.8.
Parameter estimation for the residual proteins model equation.

Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High
Intercept	72.0460147	6.28644486	58.9327205	85.1593089
A-Initial nitrate concentration	-12.6098061	4.45192994	-21.8963692	-3.323243
B-Carbon dioxide	-7.67590611	2.03201871	-11.9146229	-3.43718936
C-Culture time	16.1670358	4.95745937	5.82595676	26.5081148
D-Nitrogen limitation	26.7747073	6.1473184	13.9516259	39.5977888
AC	-67.8034835	10.2848512	-89.2573071	-46.3496599
AD	-82.7219655	12.2164796	-108.205096	-57.2388356
BD	-14.9976185	2.03201871	-19.2363353	-10.7589018
CD	90.3469274	12.5711009	64.1240705	116.569784
A^2	36.7757319	7.71096884	20.6909328	52.8605311
B^2	-17.9510454	5.14064589	-28.6742448	-7.22784593
C^2	56.7472732	7.82520311	40.4241856	73.0703608