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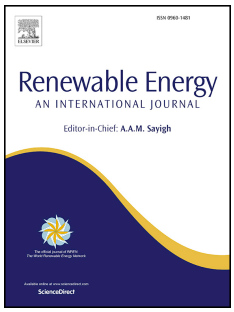


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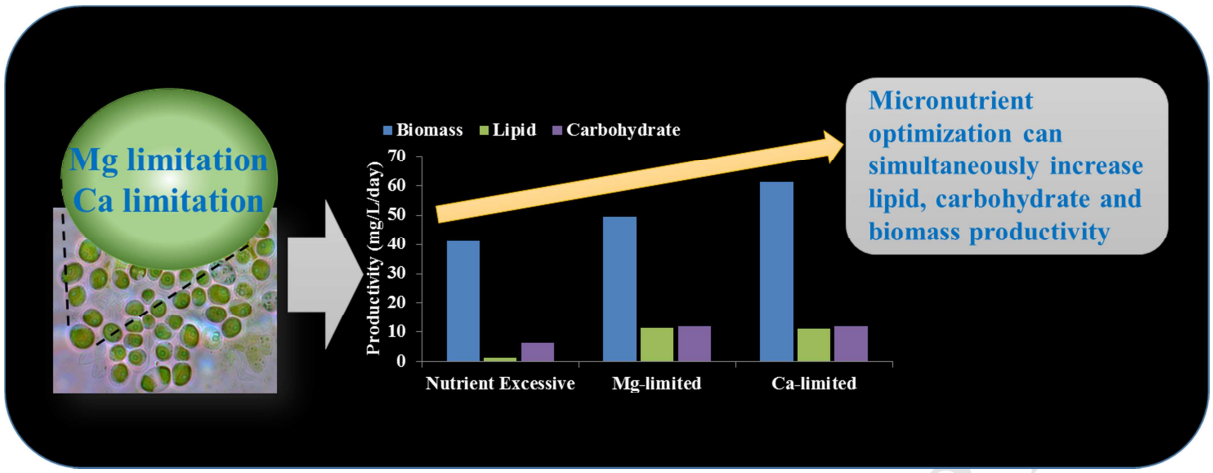

# Production of lipid and carbohydrate from microalgae without compromising biomass productivities: Role of Ca and Mg

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## Abstract

We report the cultivation of *Chlorella sorokiniana* str. SLA-04 in media containing trace amounts of Ca and Mg. The differences in productivities of biomass, lipids and carbohydrates were assessed relative to cultures grown in standard media (BG-11) that contain approximately 8× higher concentration of Ca and 30× higher concentration of Mg. Culture performance in N-limited standard media was also investigated. In addition to growth and accumulation of storage products (lipid and carbohydrate), we measured the utilization of N, Ca and Mg and monitored changes in cell size and photosynthetic activity. Our results showed that limitation of Ca or Mg did not inhibit cell replication and culture growth. On the contrary, Ca-limited ($Ca^L$) limited cultures had ~30% higher biomass productivity relative to the control with excessive nutrients possibly due to improvement in cell wall flexibility and cell division. We also observed that $Ca^L$ and Mg-limited ($Mg^L$) cultures had nearly 3-fold higher lipid concentration (measured as fatty acid methyl ester) and 50% higher carbohydrate concentration than the nutrient excess control cultures. Simultaneous culture growth and lipid accumulation in $Ca^L$ and $Mg^L$ cultures suggest that *de novo* synthesis was the primary mechanism for lipid accumulation in Ca/Mg-limited media. Overall, our study demonstrates that *micro*nutrient optimization, in addition to optimization of macronutrients, could significantly improve microalgal biorefinery yields.


Keywords: *Chlorella sorokiniana*; cultivation; micronutrient; biofuel; nutrient limitation/deficiency; biorefinery

# 1 Introduction

Microalgae are photosynthetic microorganisms that can be used as feedstock for biofuels and biopolymers, in processes for wastewater treatment and as sources of pharmaceutical products and food supplements [1-5]. Microalgae are typically grown in defined media or wastewaters that contain essential macronutrients (N and P) and micronutrients (e.g. Mg and Ca) [6-8]. The roles of N and P are well known – they are used in the synthesis of cellular proteins and nucleotides [9, 10]. Of the essential micronutrients, Mg is a key component in light harvesting pigments and is known to contribute to the structural stability of these molecules [11]. Mg also catalyzes reactions of the $CO_2$ fixing enzyme complex, RuBisCo [12]. Ca is another essential micronutrient and serves to crosslink pectin polymers (i.e. homogalacturonans) to form rigid gels in microalgae cell walls [13]. Pectin structure/chemistry can influence cell properties such as cell expansion, cell-cell adhesion and cell wall permeability/porosity [14-16].

Nutrient deprivation/deficiency is generally expected to inhibit microalgae growth. For instance, N deficiency impedes synthesis of proteins including essential photosynthesis enzymes [17]. Mg deficiency decreases chlorophyll a, b content of microalgae cells followed by reduction of growth rate [18, 19]. In terrestrial plants, Ca deficiency reduces cell integrity and therefore inhibits growth [20] and similar effects can be expected in microalgae. However, to our knowledge, the specific effects of Ca deficiency on microalgae have not been previously reported in the literature. In addition to nutrient deficiency, the excess of *micro*nutrients (i.e. Ca, Mg) in cultivation media could also impede the growth of microalgae. For example, excessive amounts of Mg in media causes increase in the chlorophyll content of cells [21] which can then decrease light diffusion into the culture and thereby lower culture productivity [22]. Although the

effect of high Ca concentration on microalgae growth is unknown, excessive Ca rigidifies the cell walls of terrestrial plants and prevents growth [20].

As cultures grow and uptake nutrients, the transition from nutrient sufficiency to limitation alters the microalgae cell composition – primarily protein, carbohydrate, and lipid content [23, 24]. It is well established that many microalgae species stop replicating and accumulate lipids and/or carbohydrates, when the media becomes depleted in N [25]. However, recent studies indicate that some microalgae can continue to replicate for several hours after N depletion in the external media by relying on intracellularly stored N [26, 27]. During this period, an increase in cellular content of N-free molecules (i.e. carbohydrate and lipid content) was also observed [26].

While effects of macronutrient limitations have been extensively reported in the literature [26, 28-30], the effects of *micro*nutrients on microalgae cultures is less established. Standard media recipes usually contain 2-5× higher concentration of Mg and Ca than microalgae stoichiometric requirements [31, 32]. In this study, the effects of Ca and Mg limitations on cell growth and composition was investigated. Our results suggest that optimization of micronutrient concentrations in the medium can be employed as strategy to improve the overall biomass, lipid and carbohydrate productivity which has important implications for biofuel production.

## 2 Materials and methods

### *2.1 Media and culture conditions*

Cultivation experiments were performed with *Chlorella sorokiniana* str. SLA-04 (henceforth referred to as SLA-04) which was originally isolated from Soap Lake (WA, USA) [25, 33]. Inoculum and nutrient replete controls were cultivated in a modified BG-11 medium

that contained following: $NaNO_3$ (14.3 mM), $KH_2PO_4$ (1.5 mM), $NaHCO_3$ (30 mM), $MgSO_4.7H_2O$ (0.3 mM), $CaCl_2.2H_2O$ (0.45 mM), NaCl (0.42 mM), ferric ammonium citrate (10 mg/L) and 1 mL trace metal solution. The trace metal solution comprised - $H_3BO_3$ (9.7 mM), $MnCl_2.4H_2O$ (1.26 mM), $ZnCl_2$ (0.15 mM), $CuCl_2.2H_2O$ (0.11 mM), Na□MoO□.2H□O (0.07 mM), CoCl□.6H□O (0.06 mM), $NiCl_2.6H_2O$ (0.04 mM), $V_2O_5$ (0.01 mM) and KBr (0.08 mM).

Algal growth experiments were conducted at room temperature (20 °C) in ePBR V 1.1 (Phenometrics Inc., MI , USA) photobioreactors [34]. Each photobioreactor had a working volume of 0.5 L, illumination of 1500 µmoles/$m^2$/s and a light-dark cycle of 14 h/10 h. pH of the cultures was adjusted daily to a value of 8.2 using a 2% $CO_2/N_2$ gas mixture. The presence of significant bicarbonate buffer (30mM) in the media allowed the pH to remain below 9 for the entire duration of the experiment. In cultures that were set up to assess the effect of Ca and Mg limitation (designated as $Ca^L$ and $Mg^L$), Ca and Mg were removed from the media by immersing a water softener pillow (Mars Fishcare Inc., PA, USA) in the culture for 2 days (trace amounts of Ca and Mg were present in final solutions). In this process, Ca and Mg were exchanged for Na ions by the ion exchange resin [35]. No external N was added to N-limited ($N^L$) cultures. Samples were taken periodically to measure cell number, cell size, total suspended solid (TSS), photosystem II (PSII) activity, soluble nutrient concentrations (i.e. N, Mg and Ca) and lipid content (measured as FAMEs). At the end of the experiment, biomass was recovered and freeze dried for carbohydrate and elemental analyses.

### *2.2 Analytical methods*

#### 2.2.1 Cell number and size

Cell concentrations (cells/mL) and size were assessed using a portable FlowCAM™ imaging cytometer (Fluid Imaging Technologies, Scarborough, ME, USA) and the associated

data analysis software (VisualSpreadsheet software, version 2.4.10). The instrumental procedure involved pumping 1 mL culture samples (10× diluted) at 0.2 mL/min using 1 mL syringe pump through a flow cell (100 µm × 2mm) where image-data was acquired using a white LED light source under 100× magnification. The system was flushed with de-ionized (DI) water prior to each sample run. The software was set to calibrate the background using the first data frame and define the particles by a segmentation threshold for both dark and light pixels which was set to a value of 20. The distance to nearest neighbor and auto image rate were set to 3 µm and 20 frames per second, respectively. The cell concentrations and sizes (as equivalent spherical diameters) were estimated from image analysis.

#### 2.2.2 Total suspended solids

Cell dry weight was determined as total suspended solid (TSS) using the Laboratory Analytical Procedure developed by the National Renewable Energy Laboratory (NREL) [36] with a slight modification. Briefly, 20 mL culture samples were collected and the pH was adjusted to 6 (using 0.1 mM HCl) to dissolve precipitates, if any. Then, culture samples were filtered through pre-weighed and dried 0.45 µm glass fiber filter discs (Fisher Scientific, Pittsburgh, PA) followed by washing with 20 mL de-ionized (DI) water. The filter discs containing algal biomass were dried at 40 °C in a convection oven until a constant dry weight was obtained (~24 h). TSS values were calculated by subtracting the weight of clean filter paper from the weight of filter holding dried biomass.

#### 2.2.3 Photosynthetic activity

The chlorophyll fluorescence yield of PSII was determined using a Dual-PAM-100 measuring system (Walz, Germany), following the procedure reported in the literature [37]. Briefly, 2 mL of culture was transferred to a quartz cuvette and adapted to dark conditions for 2

min. Subsequently, dark fluorescence yield ($F_0$) of the microalgae culture sample in the cuvette was measured. Then, a saturating light of intensity 10,000 µmol photons/$m^2$/s was applied for a duration of 600 ms to the culture sample to detect the maximum fluorescence ($F_m$). The variable fluorescence ($F_v$) is the difference between $F_m$ and $F_0$ and finally $F_v/F_m$ was assessed as the maximum quantum yield in the dark adapted state.

2.2.4 Soluble nutrient analyses

1.5 mL samples from the culture were titrated to pH 6 using 0.1 M HCl (to allow/promote dissolution of precipitates, if any) followed by filtration through a 0.2 µm filter. The filtered samples were analyzed using an ICS-3000 ion chromatograph (Dionex, USA). The anions (e.g. $NO_3^-$) were separated on a Dionex IonPac$^{TM}$ CS12A column by using a 30 mM KOH eluent. Cations (e.g. $Mg^{+2}$ and $Ca^{+2}$), were separated on a Dionex IonPac$^{TM}$ AS11-HC column by using 30 mM methanesulfonic acid as the eluent. Both columns were maintained at 30 °C. Analytes were detected using a CD20 conductivity detector (Dionex). Peaks were integrated using the Chromeleon$^{TM}$ 7.0 software (Thermo Fisher). Standard solutions (mixtures of $NaNO_3$, $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$) were analyzed to obtain calibration curves for each anion and cation. The concentrations of nutrients in the samples were assessed by comparison of peak areas with calibration standards.

2.2.5 Lipid analysis

15 mL culture samples were harvested by centrifugation, washed twice with DI water and the collected pellet was freeze-dried (Freezone 6 plus, Labconco Inc., USA). An *in-situ* transesterification method was used for quantification of biomass lipids (measured as FAMEs) in the solid samples [38]. 10-20 mg of freeze-dried biomass was accurately weighed and transferred to 5 mL serum vials followed by the addition of 1 mL acidified methanol (containing 5% v/v

$H_2SO_4$ in methanol) and 2 mL of hexane. The vials were sealed with Teflon-lined caps and incubated for 90 min at 90 °C in a shaking water bath. Thereafter, the vials were cooled down to room temperature and the hexane phase (<1 mL) from each vial was transferred to new 2 mL crimp-top gas chromatography (GC) vials for analysis using a Shimadzu 2010 GC. A Restek (Bellefonte, PA) Rtx Bio-diesel column (15 m × 0.32 mm ID × 0.1 µm) was used to separate the analytes and the FAMEs were quantified using a flame ionization detector (FID). Helium was used as the carrier gas with a linear velocity of 50 cm/s. The temperatures of the GC oven, injector and FID were maintained at 370 °C. FAME standards (mixtures of $C_8$-$C_{22}$ FAMEs) were purchased from Sigma Aldrich (St. Louis, MO, USA) and analyzed to obtain the calibration curves. Lipid contents were quantified by comparison of GC-FID peak areas of samples with calibration curves of FAMEs standards.

#### 2.2.6 Elemental analysis

Analysis of elemental carbon, nitrogen and hydrogen was carried out using a CHN analyzer (Flash 2000 series, CE Elantech Inc, NJ, USA) equipped with an autosampler and a thermal conductivity detector (TCD). The combustion temperature was set to 950 °C and helium was used as both carrier (140 mL/min) and reference gas (100 mL/min). 3-7 mg of freeze-dried biomass were analyzed. The CHN standard (2.5-Bis(5-tert-butyl-benzoxazol-2-yl) thiophene (BBOT)) was purchased from CE Elantech Inc. (NJ , USA) and used for calibrating the instrument.

#### 2.2.7 Carbohydrate analysis

Total carbohydrate analysis was performed using the analytical procedure described by Van Wychen et al. [39]. Briefly, 0.5 mL of $H_2SO_4$ was added to 30 mL autoclavable crimp-top vials containing 50 mg of freeze-dried biomass. The reaction mixtures were then incubated for

60 min at 30 °C. Samples were continuously mixed using magnetic stir bars during incubation. Then, the acid solutions were diluted with 13.89 mL DI water to reach a final concentration of 4% $H_2SO_4$ (v/v), the vials were sealed using Teflon-lined caps and autoclaved for 60 min at 121 °C. The samples were then cooled down to room temperature, neutralized by $CaCO_3$ and filtered using 0.2 μm filters. Finally, the filtered samples were analyzed by an Agilent 1100 (Agilent Technologies Inc., Santa Clara, CA) high performance liquid chromatograph (HPLC) equipped with a refractive index detector (RID). Analytes were separated on a Shodex™ SH1101 column (Showa Denko America Inc., New York, NY) which was maintained at 55 °C under isocratic conditions. 5 mM $H_2SO_4$ was applied as the mobile phase with a flow rate of 0.6 mL/min. HPLC-RID peak areas were compared with calibration curves of known standards (solution mixtures of glucose, galactose, arabinose and mannose) to estimate the carbohydrate content of samples.

2.2.8 Ca and Mg content analysis by ICP-MS

Freeze-dried biomass was digested in $HNO_3$ using a CEM Mars microwave. Digestion was performed for 15 min at 1600 W, 250 °C and 800 psi. Following digestion, the sample was cooled, filtered (to remove particulate material) and diluted to 3.5% (v/v) $HNO_3$ for analysis by inductively coupled plasma mass spectrometry (ICP-MS, Xseries 2, Thermo Scientific, MA, USA). For quantitative analysis, standards and internal standards were prepared in 3.5% $HNO_3$ by using certified ICP-MS standards (Inorganic Ventures). Correlation coefficients for calibration curves were above 0.999.

# 3 Results and discussion

## *3.1 The effects of Mg, Ca and N concentrations in media on SLA-04 growth*

Fig. 1a shows that cell concentrations increased in the nutrient-excess control as well as in the $Ca^L$ and $Mg^L$ cultures throughout the 32 d duration of the cultivation experiment (Fig. 1a). Observations from monitoring the change in cell size during cultivation (Fig. 1b) indicated that $Ca^L$ and $Mg^L$ cultures had larger cells than the nutrient-excess control which suggests that the nutrient-limited cultures could have higher cellular content of carbon storage compounds (lipid and carbohydrate) [40]. Measurements of biomass concentrations (Fig. 1c) showed that biomass productivity of $Mg^L$ and nutrient-excess control were similar while $Ca^L$ cultures accumulated significantly higher amounts of biomass. Taken together, the results shown in Fig. 1 show that micronutrient limitations did not appear to inhibit growth.

N-limited ($N^L$) cultures also grew for a short period but cell replication appears to have ceased after 8d (Fig. 1a). In the literature, while N deficiency in media is widely reported to arrest growth [17, 41], Msanne et al. (2012) have made similar observations as us and have reported microalgae growth under external N deficiency for a short period of time [40]. They speculated that microalgae growth in the absence of external N is sustained by internal "recycling" of cell proteins and/or pigments. Consistent with the hypothesis of Msanne et al., in our experiments, $N^L$ cultures showed a 3-fold decrease in the N-content of biomass (Fig. 2a) over the duration of the cultivation experiment. Correspondingly, the biomass concentrations showed an approximately 3× increase (Fig. 1c) which indicated N mass balance closure in the N-limited culture.

Since $Ca^L$ and $Mg^L$ cultures grew well, we measured the cellular Ca- and Mg- content to assess the possibility of internal storage of the micronutrients and subsequent utilization. After 8 d of cultivation, the $Ca^L$ and $Mg^L$ biomass had nearly half the Ca and Mg content relative to control (Fig. 2b). During the same period, the biomass concentrations approximately doubled in these cultures (Fig. 1c). While the mechanism for storage of *micro*nutrients in microalgae cells is not studied well in the literature, Tang et al. (2017) reported the internal storage of Mg and Ca in higher plants which can be released through regulation mechanisms [42]. Similar to plants, we hypothesize that the micronutrient storage and "recycling" mechanisms might also exist in microalgae that could have allowed cultures to grow even in the absence of an external supply of these micronutrients.

Interestingly, we also observed that growth was, in fact, enhanced (~10% higher cell numbers, ~8% larger cell diameter and 30% greater biomass concentrations) in the $Ca^L$ cultures. Hepler et al. (2010) report that when the concentration of Ca in plant cells is high, the accumulated Ca binds with negatively charged pectin molecules, resulting in aggregation of pectin chains, increase in cell wall rigidity and ultimately inhibition in growth [20]. Similarly for microalgae, Domozych et al. (2011) show Ca-crosslinked pectin polymers (i.e. homogalacturonans) form rigid gels in the microalgae cell wall [13]. Inversely, the lower content of such rigid gels in the cell wall could improve cell expansion and cell wall porosity. While our study did not include a precise analysis of Ca in the cell wall, we measured an increase in cell size (Fig. 1b) and cell number (Fig. 1a) in the $Ca^L$ treatment, suggesting the possibility of improved cell wall flexibility due to lower cellular Ca content [13].

### *3.2 Photosynthetic performance of SLA-04 in $Mg^L$, $Ca^L$ and $N^L$ cultures*

The $F_v/F_m$ factor, which represents the PSII maximum quantum yield at dark adapted state, for the tested cultivation conditions is shown in Fig. 3. A decrease in $F_v/F_m$ is commonly ascribed to environmental or nutritional stresses that damage PSII [43]. Fig. 3 shows that $F_v/F_m$ for cultures under nutrient replete conditions (control) remained largely unchanged during cultivation, indicating that the nutrient-rich cultures were not under environmental stress. Fig. 3 also indicates that $Ca^L$ cultures had similar $F_v/F_m$ values as the nutrient-replete controls. Since the primary role of Ca is related to cell wall structure rather than photochemical reactions, its limitation in the medium likely did not negatively impact PSII activity [44].

For $N^L$ cultures, the $F_v/F_m$ values decreased from 0.65 to 0.26 indicating a clear stress in these cultures and is consistent with the observations of Jiang et al. [45]. $Mg^L$ cultures also showed a decrease in $F_v/F_m$ values (from 0.7 to 0.51), but the decrease was less drastic than the $N^L$ cultures. However, growth of $Mg^L$ and initial growth of $N^L$ cultures (until day 8) did not seem to be inhibited relative to nutrient replete conditions (Figs. 1a and 1c) despite the decrease in $F_v/F_m$ (from 0.7 to 0.51 for $Mg^L$ and from 0.65 to 0.55 for $N^L$ (Fig. 3)). Similar observations have been previously made by Simionato et al. [28] who observed a decrease in $F_v/F_m$ (from 0.6 to 0.49) in cultures that were N-limited, but also observed that growth rates were similar to cultures that were nutrient sufficient and maintained a high $F_v/F_m$ ratio. From their observations, Simionato et al. (2013) suggested that the available antenna complexes possibly remained energetically coupled with PSII reaction centers left in microalgae cells which resulted in continued efficient transfer of light energy to PSII in the low $F_v/F_m$ cultures. In our experiments, $N^L$ and $Mg^L$ cultures also likely experienced modifications in the PSII system since Mg is integral to the light harvesting chlorophyll. However, the energy coupling with the PSII reaction

centers appears to have remained efficient for a short period in $N^L$ cultures (8 days) and for longer duration (32 days) in the $Mg^L$ cultures.

### *3.3 Nutrient and micronutrient utilization by SLA-04 cultures*

N, Ca and Mg concentrations measured during culture growth are shown in Fig. 4 and the concentrations of Mg and Ca in abiotic control are shown in Fig. 1S (supporting information). From the biomass growth and nutrient consumption data, stoichiometric yield coefficients (Y) were calculated using Eq. 1

$$Y_{X/S} = \frac{X_{final} - X_{initial}}{S_{initial} - S_{final}} \quad (1)$$

where, $X$ represents biomass concentrations (measured as TSS) and $S$ represents substrate (or nutrient/micronutrient) concentration. The initial (day 0) and final (day 32) measurements were used for these estimates.

From Fig. 4a, N consumption rate for $Mg^L$ cultures was lower than the control, although biomass growth rates for these cultures were similar (Fig. 1c). Correspondingly, the $Y_{X/N}$ (Table 1) values for $Mg^L$ cultures were higher compared to the nutrient replete cultures. Since the nutrient utilization values are in the denominator, a larger Y value indicates a lower nutrient use. It is possible that the lower N utilization in $Mg^L$ cultures was due to down-regulation of cell constituents that contain both N and Mg (e.g., chlorophyll and RuBisCo) [46, 47]. Msanne et al. (2012) also showed the reduction in N uptake is positively correlated with a reduction of chlorophyll and RuBisCo content of microalgae cells [40]. $Ca^L$ cultures utilized higher N than the control (Fig. 4a), but also produced more biomass (Fig. 1c) such that $Y_{X/N}$ for $Ca^L$ cultures was similar to the control (Table 1).

Abiotic controls (Fig. 1S) showed no measurable precipitation of Ca and Mg in the media indicating that the decrease in micronutrient concentrations in the cultures was as a result of biological uptake. Consumption of Mg was higher in the $Ca^L$ cultures (Fig. 4b), but calculated $Y_{X/Mg}$ values (Table 1) indicate that the $Ca^L$ cultures consumed similar Mg as the nutrient excess control and the $N^L$ cultures. Consumption of Ca relative to biomass produced was also statistically similar for all cultures (Fig. 4c and Table 1). From Table 1, it can also be observed that overall N utilization was 30-fold higher than the use of the micronutrients Ca and Mg since N is quantitatively the most important nutrient (after C) for microalgae production [48].

### *3.4 Lipid and carbohydrate production in $Mg^L$, $Ca^L$ and $N^L$ cultures*

Lipid content measurements (measured as FAMEs) during cultivation are shown in Fig. 5a. To determine the total lipid concentration in the cultures, results from biomass concentration (Fig. 1c) were multiplied by lipid content of the biomass (Fig. 5a) and these values are shown in Fig. 5b. The results for total lipid concentration (Fig. 5b) indicated a 4-fold higher lipid concentration for $Ca^L$ and $Mg^L$ (0.39 g/L) in comparison to the nutrient-excess control (0.09 g/L). $N^L$ cultures accumulated 32% higher lipid (0.58 g/L) compared to $Mg^L$ and $Ca^L$.

Two mechanisms are generally hypothesized for lipid accumulation in microalgae: (1) conversion of cellular organic materials, such as proteins and carbohydrate, to lipid [49], and (2) *de novo* synthesis of lipid from inorganic carbon [27]. Interconversion is shown to occur, likely via the Kennedy pathway, when N deficiency impairs growth and sustained carbon fixation due to inadequate re-synthesis of proteins in the photosystem reaction pathway [50]. On the other hand, Klok et al. (2013) observed that cell replication and lipid accumulation can occur simultaneously in N-limited cultures [26]. They suggest that the imbalance in energy demand and supply causes the formation of energy-dense lipids for storage of excessive energy.

Simionato et al. (2013) have also made observations similar to Klok et al. (2013) and have shown a 15% (wt/wt) increase in total lipid content for N-limited cultures, while growth (measured as the increase in cell concentration) was similar to N-replete conditions [28]. Their analysis shows that the majority (>85%) of lipid accumulation originated from *de novo* synthesis. Msanne et al. (2012) have suggested that both mechanisms may occur within the same organism – *de novo* lipid synthesis during growth followed by inter-conversion of cellular carbohydrate to lipid as protein and pigment concentrations (e.g. chlorophyll and RuBisCo) diminish under N stress [40].

In our study, a comparison of the lipid analyses (Fig. 5) with cell growth data (Fig.1.a) indicates that the increase in cellular lipid and cell numbers occurred simultaneously for $Mg^L$ and $Ca^L$ cultures throughout the 32 d cultivation period. These observations indicate that lipid accumulation in the $Ca^L$ and $Mg^L$ SLA-04 cultures occurred through *de novo* fatty acid biosynthesis as suggested previously [26, 28]. However, cultivation under N-limitation resulted in cessation of cell replication after day 9 (Fig. 1a) which also coincided with the onset of lipid accumulation (Fig 5a) indicating that a majority of the lipid accumulation likely occurred from inter-conversion [27, 40].

Similar to assessments of lipid, we measured carbohydrate content (Fig. 6a) and concentration in the cultures (Fig. 6b). Our results for carbohydrate analysis indicated that the carbohydrate content of $Mg^L$, $Ca^L$ and $N^L$ cultures was about 20-25% higher than the control (Fig. 6a). However, after accounting for the increase in biomass during growth, the carbohydrate concentration for both $Mg^L$ and $Ca^L$ cultures (0.45 g/L) was about 50% higher compared to the nutrient-excess control and $N^L$ cultures (Fig. 6b). Other studies also show that nutrient limitation in media induces carbohydrate accumulation in microalgae cells [51, 52]. Moreover, prolonged

nutrient stress was found to result in carbohydrate degradation and conversion to lipid [53]. Breuer et al. (2015) showed that the cultivation of *Scenedesmus obliqus* under N starvation first leads to a simultaneous increase in biomass concentration and carbohydrate content and is subsequently followed by a decrease in growth and conversion of accumulated carbohydrate to lipid [54]. From the literature, it is now widely understood that cultivation under N limitation causes metabolic changes in microalgae cells that results in the synthesis of storage compounds (e.g. carbohydrates and lipid) [55]. From our results, we speculate that Ca/Mg limitations similarly redirect the carbon flux towards synthesis of storage lipids and carbohydrates (starch).

### *3.5 Productivity estimates under micronutrient limitation and implications for microalgae cultivation*

A comparative evaluation of biomass, lipid and carbohydrate productivities (calculated over the 32 d cultivation period using Eq. 2) is summarized in Table 2.

$$Productivity = \frac{(Concentration)_{final} - (Concentration)_{initial}}{Cultivation\ period} \quad (2)$$

$Ca^L$ cultures show 32% higher biomass productivity whereas $Mg^L$ had similar biomass productivity as the nutrient replete control. Moreover, the $Mg^L$ and $Ca^L$ cultures showed a 10-fold higher lipid productivity and up to 2-fold higher carbohydrate productivity in comparison to control cultures with excessive nutrients (Table 2). The higher carbohydrate productivity under micronutrient limitations suggests that subsequent N-limitations could trigger higher lipid production from carbohydrate reapportionment.

The results for lipid profile analysis of samples from $Ca^L$, $Mg^L$ and $N^L$ cultures after completion of the cultivation experiment (32 d) are summarized in Table 3 which also shows the proportion of poly unsaturated fatty acids (PUFAs), saturated fatty acids (SFAs) and mono

unsaturated fatty acids (MUFAs) in the lipid extracted from each sample. Our results show that $Ca^L$ cultures produced significantly higher concentrations of PUFAs than $Mg^L$ and $N^L$ cultures, although the total lipid produced in $N^L$ cultures was the highest. From the data, it appears that most of the "excess" lipid produced by $N^L$ cultures was in the form of SFAs. In fact, SFA concentration in $N^L$ cultures was up to 2-fold higher than micronutrient-limited cultures. Consistent with these observations, the PUFA content of micronutrient-limited cells was up to 2-fold higher than $N^L$ cultures. In addition to lipid content, lipid quality is an important consideration for end-use applications. PUFA-rich lipid is desirable for nutraceutical and pharmaceutical applications and also improves the cold flow properties, cetane number and viscosity of biodiesel [56, 57]. Micronutrient limitations can thus be a useful strategy for obtaining PUFA-rich microalgae biomass.

## 4 Conclusions

Our findings show that micronutrient optimization should be an important consideration, along with optimization of macronutrients (N and P), to maximize biomass, lipid and carbohydrate productivity. Our results suggest that microalgae cells may accumulate intracellular reserves of Ca and Mg when cultivated in traditional media that contain excess micronutrients. In micronutrient-limited media, microalgae cultures show significant improvements in biomass, lipid and carbohydrate productivities. A potential strategy for further improving lipid productivity could be to first grow cultures in a micronutrient-optimized medium followed by N starvation.

## 5 Acknowledgements

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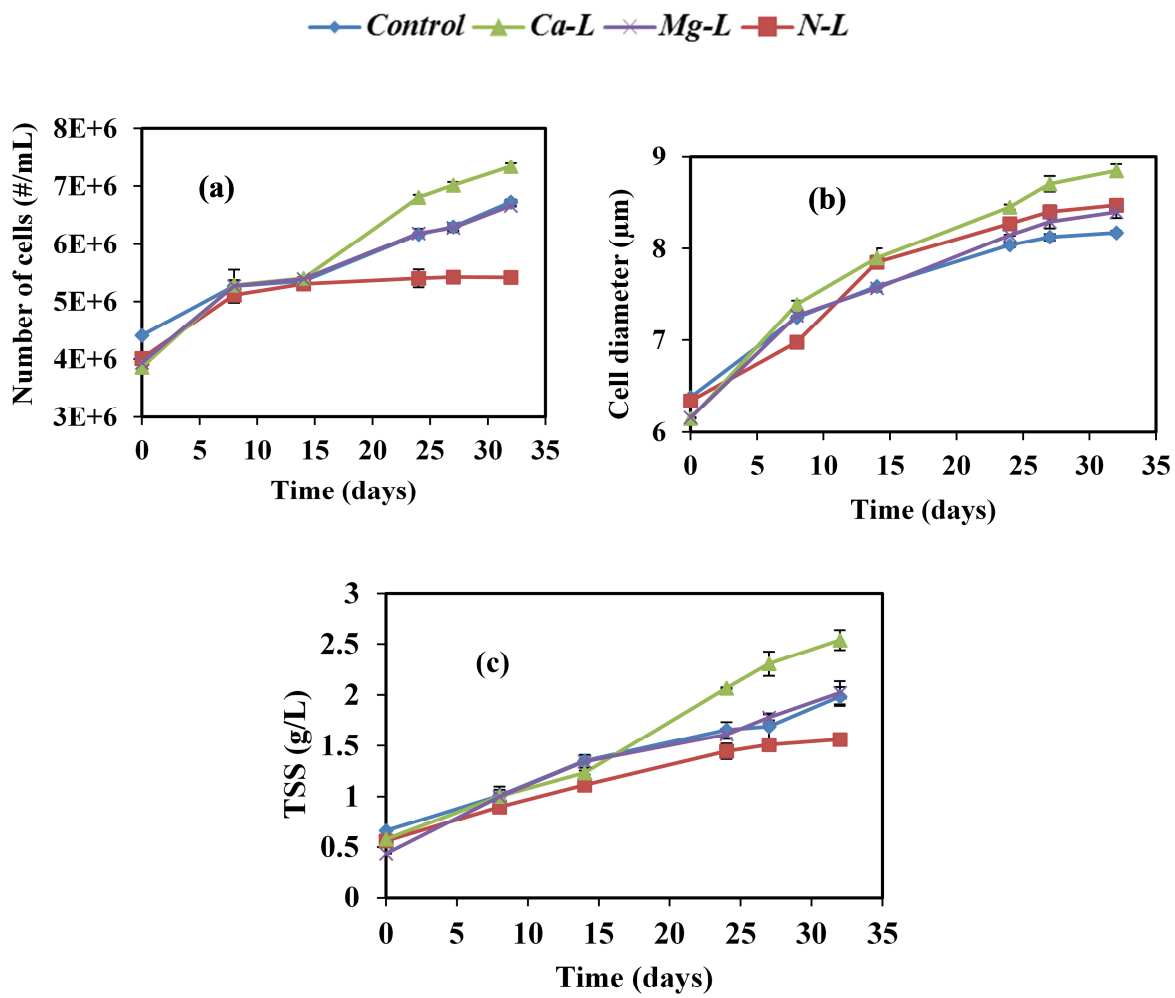



Fig. 1: Change in (a) cell concentration, (b) cell diameter and (c) total suspended solids (TSS) of $Ca^L$, $Mg^L$ and $N^L$ cultures. TSS represents cell dry weight. Control indicates cultivation under excessive amount of nutrients. Error bars indicate one standard deviation from mean values of duplicate experiments.

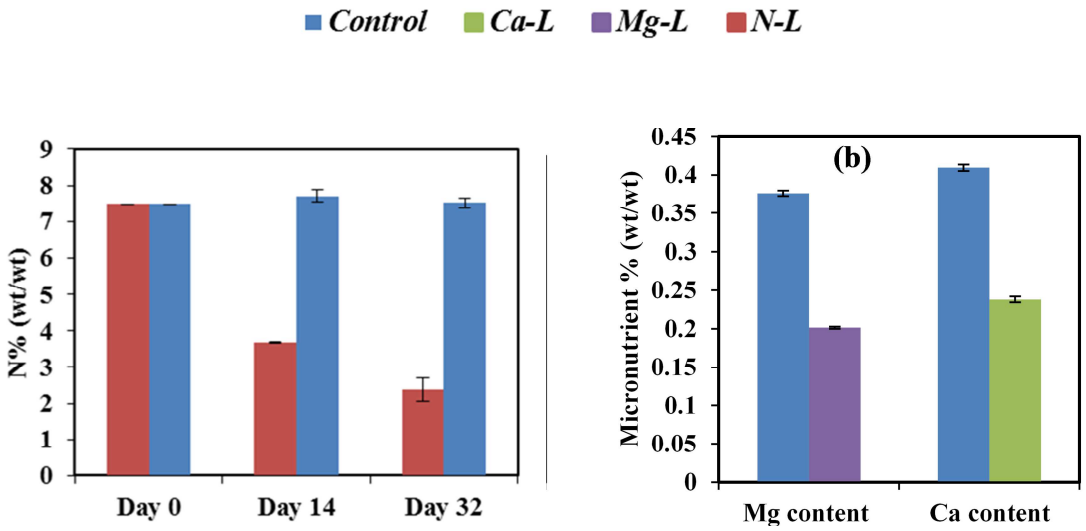
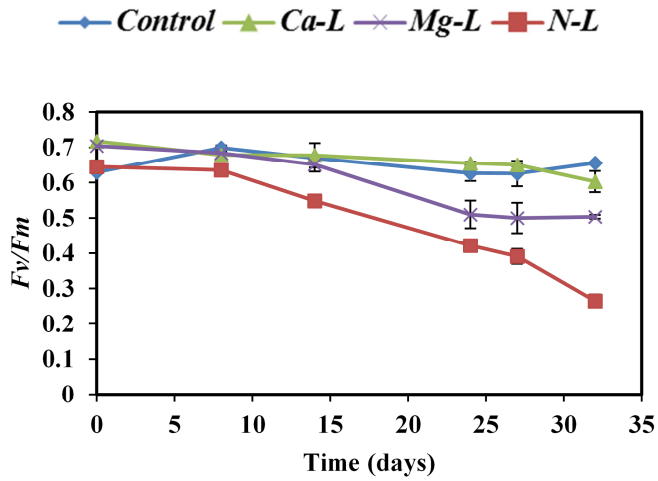



Fig. 2: (a) N content analysis in biomass and (b) *micro*nutrient content analysis (t=8 d) for cultures under limited and excessive amount of nutrients (The elemental analysis for nutrient-excess control showed that N , Ca and Mg contents were 7.5%, 0.4% and 0.37%, respectively, which is consistent with the reported range of nutrient content for microalgae in literature [58]). Error bars indicate one standard deviation from mean values of duplicate experiments.




1

2 Fig. 3: $F_v/F_m$ values for nutrient-excess controls as well as $Ca^L$, $Mg^L$ and $N^L$ cultures. Error bars indicate

3 one standard deviation from mean values of duplicate experiments.

4

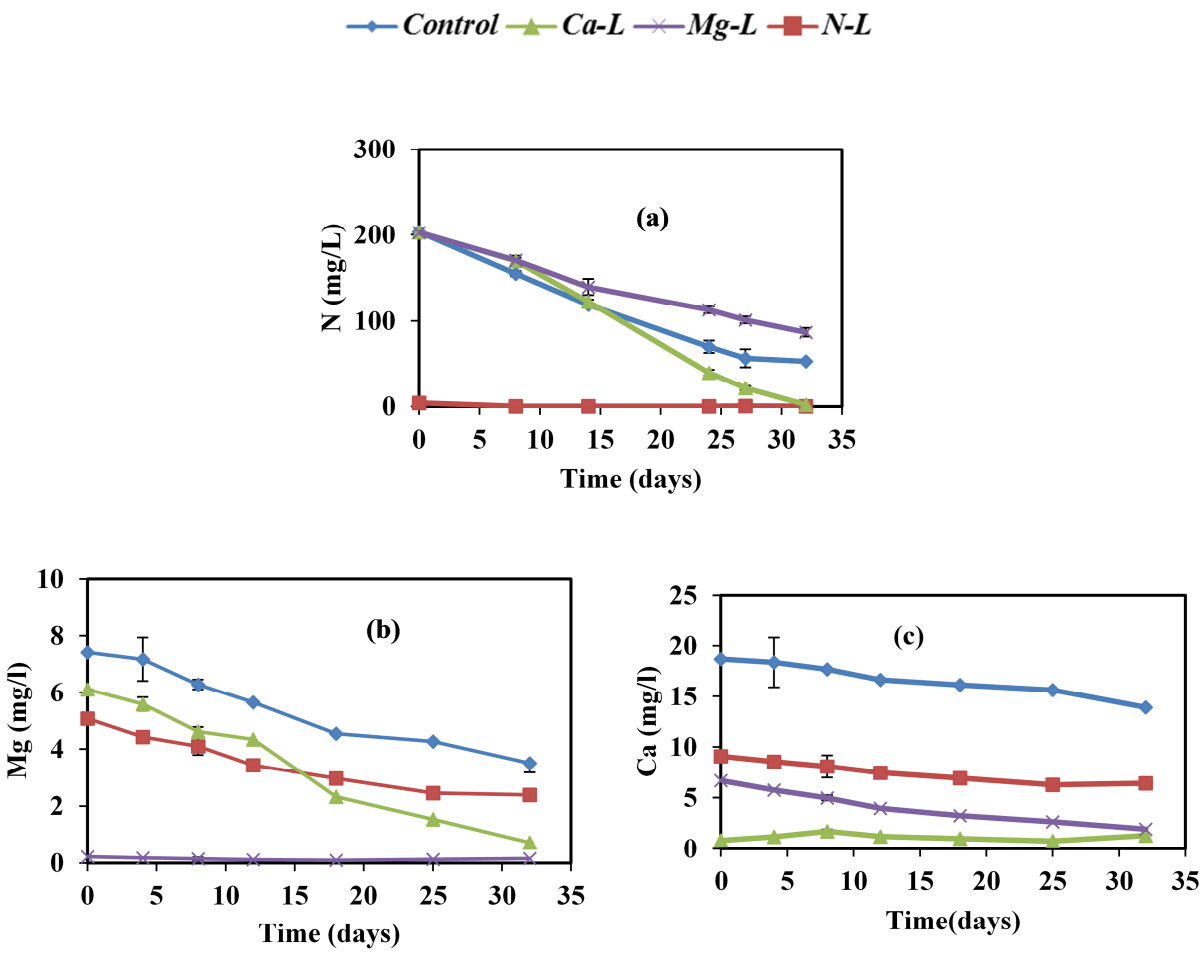
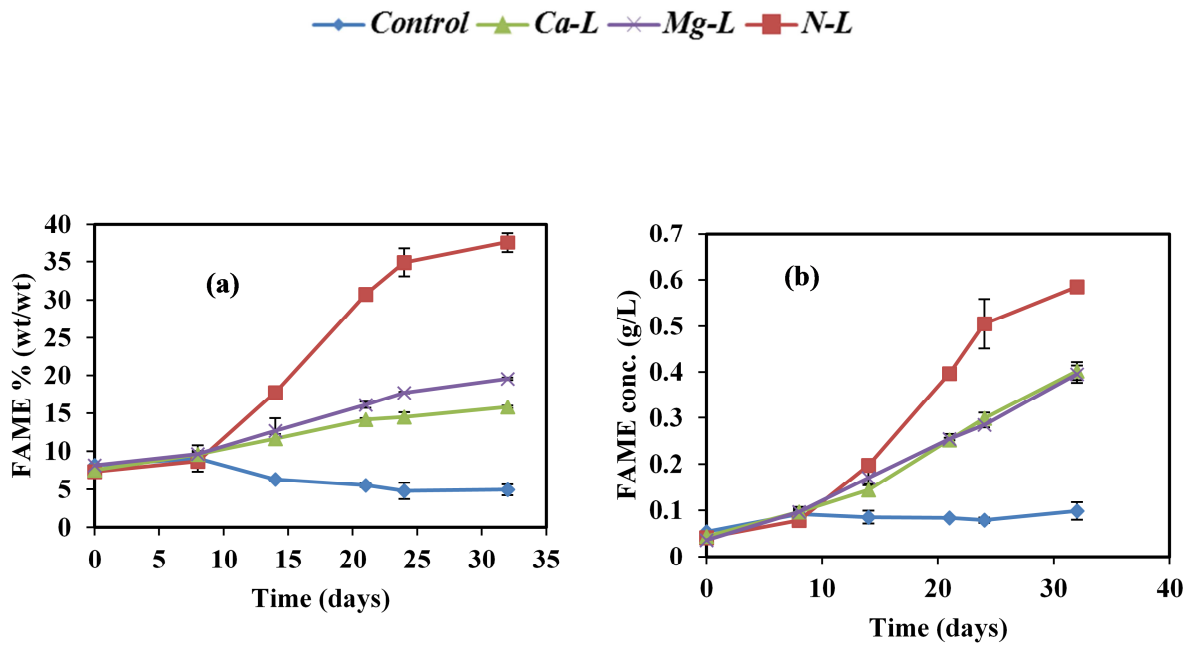



Fig. 4: Change in (a) N, (b) Mg and (c) Ca concentrations during cultivation of nutrient excess controls as well as $Ca^{L}$, $Mg^{L}$ and $N^{L}$ cultures. Error bars indicate one standard deviation from mean values of duplicate experiments.

1




2

3 Fig. 5. (a) Lipid content of cultures (measured as FAME wt/wt %) and (b) lipid concentration of culture
4 during cultivation of nutrient excess controls as well as $Ca^{L}$, $Mg^{L}$ and $N^{L}$ cultures. Error bars indicate one
5 standard deviation from mean values of duplicate experiments.

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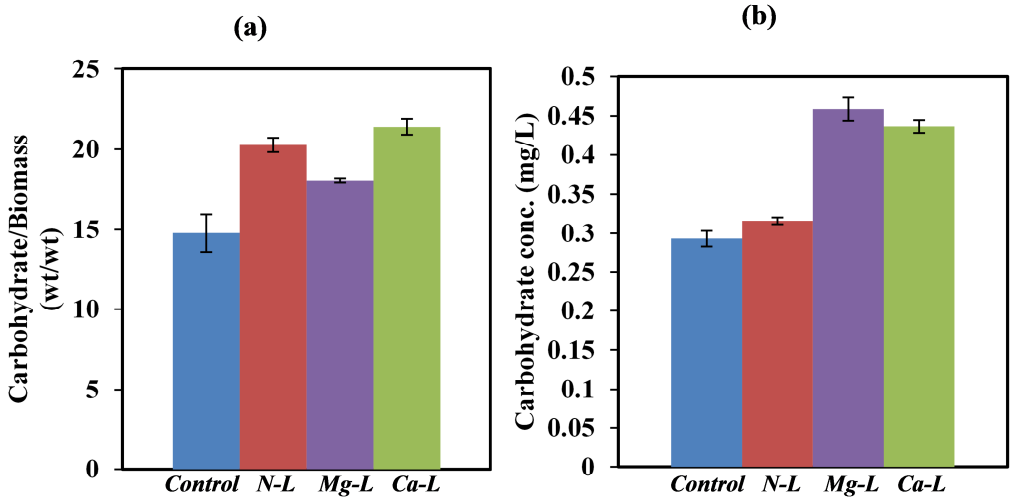


Fig. 6. (a) Total carbohydrate content and (b) total carbohydrate concentration of nutrient excess controls as well as $Ca^{L}$, $Mg^{L}$ and $N^{L}$ cultures after completion of cultivation experiment (32 d). Error bars indicate one standard deviation from mean values of duplicate experiments.

1

2

3 Table 1- Estimated stoichiometric yield coefficient values (Y) for biomass (represented as X in the
4 subscript for Y) relative to consumption of Mg, Ca and N. Errors indicate one standard deviation from
5 mean values of duplicate experiments.

| **Stoichiometric yield coefficients** | **Control (g/g)** | **$N^L$ (g/g)** | **$Ca^L$ (g/g)** | **$Mg^L$ (g/g)** |
|---|---|---|---|---|
| **$Y_{X/N}$** | 9.1±0.6 | | 9.8±0.5 | 13.7±0.5 |
| **$Y_{X/Mg}$** | 338±16 | 363±14 | 365±15 | |
| **$Y_{X/Ca}$** | 274±16 | 287±17 | | 332±12 |

6

7

8 Table 2- Biomass, lipid and carbohydrate productivities for nutrient-excess, $Ca^L$, $Mg^L$ and $N^L$ cultures.
9 Errors indicate one standard deviation from mean values of duplicate experiments.

| **Condition** | **Biomass productivity (mg/L/day)** | **Lipid productivity (mg/L/day)** | **Carbohydrate productivity (mg/L/day)** |
|---|---|---|---|
| **Nutrient excessive** | 41.25±2.8 | 1.38±0.59 | 6.33±0.32 |
| **Ca-limited** | 61.25±3.1 | 11.19±0.62 | 7.51±0.14 |
| **Mg- limited** | 49.53±3.5 | 11.23±0.59 | 11.8±0.25 |
| **N- limited** | 31.25±1.7 | 17.00±0.04 | 11.88±0.46 |

10

11

12

Table 3- Lipid profile analysis including proportion of PUFA, MUFA and SFA, for $Ca^L$, $Mg^L$ and $N^L$ after completion of cultivation experiment (32 d). Errors indicate one standard deviation from mean values of duplicate experiments. Fatty acid composition data in percentage values is shown in Supplementary Information, Table S1.

| Fatty acid/fatty acid class | Concentration (mg/L) in | | |
|---|---|---|---|
| | $Ca^L$ cultures | $Mg^L$ cultures | $N^L$ cultures |
| C16:3 | 6.4±1.4 | 4.1±0.2 | 2.3±0.1 |
| C16:2 | 25.3±4.0 | 15.3±0.1 | 28.2±1.7 |
| C16:1 | 51.7±3.7 | 46.2±0.7 | 57.2±1.8 |
| C16:0 | 81.6±7.1 | 90.3±7.7 | 144.7±1.0 |
| C18:3 | 18.6±4.9 | 12.5±0.8 | 9.1±0.7 |
| C18:2 | 92.0±10.4 | 86.1±9.8 | 86.1±3.26 |
| C18:0 | 120.3±13.4 | 137.4±8.2 | 258.01±5.0 |
| Total PUFA | 142.3±0.2 | 118.0±8.9 | 125.6±5.7 |
| Total SFA | 202.0±20.5 | 227.7±15.9 | 402.7±4.0 |
| Total MUFA | 51.7±3.7 | 46.2±0.7 | 57.2±1.8 |
| *Total lipid* | *396.0±17.0* | *392.0±25.5* | *585.5±3.5* |
| | Fraction of total lipid (% wt/wt) in | | |
| | $Ca^L$ cultures | $Mg^L$ cultures | $N^L$ cultures |
| PUFA | 36.0±1.5 | 30.1±0.3 | 21.4±0.8 |
| SFA | 50.9±1.0 | 58.1±0.3 | 68.8±1.1 |
| MUFA | 13.1±1.5 | 11.8±0.6 | 9.8±0.2 |

**Supplementary material**

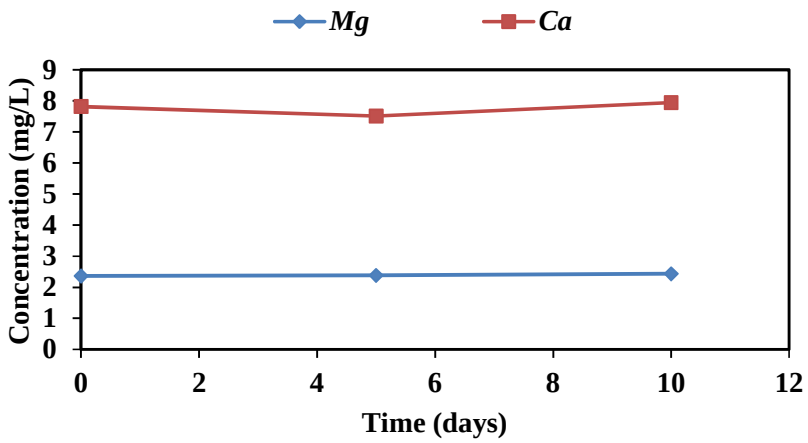


Fig. S1. Change in concentration of Mg and Ca (mg/L) during abiotic experiment

Table S1- Fatty acid composition data in percentage values, for $Ca^L$, $Mg^L$ and $N^L$ after completion of cultivation experiment (32 d). Errors indicate one standard deviation from mean values of duplicate experiments.

| | $Ca^L$ | $Mg^L$ | $N^L$ |
|---|---|---|---|
| Percentage in biomass (%wt/wt) | | | |
| C16:3 | 0.26±0.07 | 0.20±0.02 | 0.15±0.01 |
| C16:2 | 1.01±0.21 | 0.76±0.02 | 1.81±0.17 |
| C16:1 | 2.07±0.26 | 2.30±0.03 | 3.66±0.25 |
| C16:0 | 3.25±0.11 | 4.49±0.25 | 9.27±0.39 |
| C18:3 | 0.75±0.23 | 0.62±0.06 | 0.58±0.06 |
| C18:2 | 3.66±0.23 | 4.28±0.36 | 5.52±0.40 |
| C18:0 | 4.79±0.29 | 6.84±0.21 | 16.52±0.26 |
| Total PUFA | 5.68±0.29 | 5.87±0.27 | 8.05±0.65 |
| Total SFA | 8.05±0.40 | 11.33±0.47 | 25.78±0.66 |
| Total MUFA | 2.07±0.26 | 2.30±0.03 | 3.66±0.25 |
| Percentage in the lipid (%wt/wt) | | | |
| Total PUFA | 35.96±1.49 | 30.10±0.3 | 21.45±0.85 |
| Total SFA | 50.94±0.99 | 58.09±0.29 | 68.78±1.1 |
| Total MUFA | 13.10±1.5 | 11.81±0.59 | 9.77±0.25 |

Highlights

1. Micronutrient limitation increases lipid (10×) and carbohydrate (2×) productivity
2. De novo lipid synthesis occurs during micronutrient limitations
3. Ca limitation improved biomass productivity by 30%
4. *Chlorella* cultures were shown to grow using intracellularly stored micronutrients
5. Micronutrient optimization followed by N-starvation can enhance lipid productivity