Citation	<p>Gibran Sidney Aléman-Nava, Koenraad Muylaert, Sara Paulina Cuellar Bermudez, Orily Depraetere, Bruce Rittmann, Roberto Parra-Saldívar, Dries Vandamme (2017)</p> <p>Two-stage cultivation of <i>Nannochloropsis oculata</i> for lipid production using reversible alkaline flocculation</p> <p>Bioresource Technology,</p>
Archived version	<p>Author manuscript: the content is identical to the content of the published paper, but without the final typesetting by the publisher</p>
Published version	<p>http://dx.doi.org/10.1016/j.biortech.2016.11.121</p>
Journal homepage	<p>http://www.sciencedirect.com/science/article/pii/S096085241631642X</p>
Author contact	<p>your email dries.vandamme@kuleuven.be your phone number + 32 (0)56 24 6041</p>
IR	<p>url in Lirias https://lirias.kuleuven.be/handle/123456789/558844</p>

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Accepted Manuscript

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PII: S0960-8524(16)31642-X
DOI: <http://dx.doi.org/10.1016/j.biortech.2016.11.121>
Reference: BITE 17367

To appear in: *Bioresource Technology*

Received Date: 13 September 2016
Revised Date: 29 November 2016
Accepted Date: 30 November 2016

Please cite this article as: Sidney Aléman-Nava, G., Muylaert, K., Paulina Cuellar Bermudez, S., Depraetere, O., Rittmann, B., Parra- Saldívar, R., Vandamme, D., Two-stage cultivation of *Nannochloropsis oculata* for lipid production using reversible alkaline flocculation, *Bioresource Technology* (2016), doi: <http://dx.doi.org/10.1016/j.biortech.2016.11.121>

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**Two-stage cultivation of *Nannochloropsis oculata* for lipid production using reversible
alkaline flocculation**

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Graphical abstract

Abstract

Two-stage cultivation for microalgae biomass is a promising strategy to boost lipid accumulation and productivity. Most of the currently described processes use energy-intensive centrifugation for cell separation after the first cultivation stage. This laboratory study evaluated alkaline flocculation as low-cost alternative separation method to harvest *Nannochloropsis oculata* prior to cultivation in the second nutrient-depleted cultivation stage. Biomass concentration over time and the maximum quantum yield of photosystem II expressed as Fv:Fm ratio showed identical patterns for both harvesting methods in both stages. The composition of total lipids, carbohydrates, and protein was similar for biomass harvested via alkaline flocculation or centrifugation. Likewise, both harvest methods yielded the same increase in total lipid content, to 40% within the first 2 days of the nutrient-depleted stage, with an enrichment in C16 fatty acid methyl esters. Centrifugation can therefore be replaced with alkaline flocculation to harvest *Nannochloropsis oculata* after the first cultivation stage.

Keywords: flocculation; algae; nutrient starvation; separation; lipids

Introduction

Microalgae are a promising resource for the production of renewable oils (Sheehan et al., 1998; Wijffels and Barbosa, 2010). The applications of microalgae oils are diverse: They can be used for biofuel production such as biodiesel or jet fuel (Delrue et al., 2012; Greenwell et al., 2010), but they can also be valuable for food industries because of their high content of omega 3, 6, and 9 long-chain polyunsaturated fatty acids (PUFAs) and in the cosmetic industry (Markou and Nerantzis, 2013; Neofotis et al., 2016). Although biofuels from microalgae are not yet commercialized, food and cosmetic applications are already in the market today (Ruiz Gonzalez et al., 2016; Skjanes et al., 2012; Spolaore et al., 2006).

Lipid accumulation in microalgae can be increased by different stress factors such as temperature, light intensity, or nitrogen or phosphorus starvation (Pal et al., 2011). Lipids are synthesized as energy and carbon reserve to stress conditions and accumulated as triacylglycerids (TAG) in the cytoplasm. However, applying any of these stress factors during cultivation to boost lipid accumulation decreases biomass productivity (Guschina and Harwood, 2006). In order to maintain a high biomass productivity, recent studies have proposed two-stage cultivation strategies: optimization of productivity in the first stage and optimization for lipid production in the second stage by application of a stress factor such as pH, nutrient starvation, light intensity, or CO₂ supply (Doan and Obbard, 2014). The advantage of two-stage cultivation is that one can operate at the optimum for growth in the first stage followed by operating at optimal lipid accumulation conditions in the second stage. This strategy was efficient with *Neochloris oleoabundans* cultivation coupled to a pH-shock regime in the second stage, resulting in up to 35% w/w of TAG (Santos et al., 2014). *Scenedesmus obtusus* also responded favorably when its cultivation was coupled to addition

of sodium chloride in the second stage, yielding a total lipid content of 47.7% w/w; 49.8% w/w when nitrogen starvation was applied in the second stage (Xia et al., 2013). *Nannochloropsis* sp. reached a total lipid content of 40% w/w when adjusting irradiance and temperature in the second stage of cultivation (Mitra et al., 2015), 45% w/w when adjusting salinity (Su et al., 2010), and 48% w/w after addition of sodium acetate (Doan and Obbard, 2014). The strain *Chlamydomonas* sp. exhibits a total lipid content of 59% upon salinity increase and nitrogen starvation in the second cultivation stage (Ho et al., 2014).

The most frequently used and most effective strategy to induce lipid accumulation is nutrient starvation (Pal et al., 2011; Wijffels and Barbosa, 2010). Applying this strategy in a two-stage cultivation approach requires a rapid modification of the medium composition that is achieved by harvesting of the biomass and re-suspending the biomass in a new, nutrient-limited medium. In such a two-stage cultivation approach, the biomass is usually harvested by means of centrifugation (Gruber-Brunhumer et al., 2016; Li et al., 2015; Liu et al., 2015; Mitra et al., 2015; Santos et al., 2014). This method of harvesting, however, requires large capital expenses and is energy intensive, factors that become especially problematic when scaling up. When biomass is harvested to transfer it to a new cultivation medium, complete dewatering is not essential, and a simple primary concentration method, such as flocculation, can be used (Vandamme et al., 2013).

When the biomass is to be transferred to the new medium in a two-stage cultivation process, the flocculation process needs to be reversible so that the cells can be re-suspended. Several approaches have recently been developed for reversible flocculation of microalgae. One attractive approach is alkaline flocculation, which is flocculation induced by precipitation of magnesium ions from the medium at high pH between 10.1 and 10.7 (Castrillo et al., 2013;

Schlesinger et al., 2012; Vandamme et al., 2015b). After primary concentration of the biomass, these magnesium precipitates can be easily removed from the biomass by gentle acidification to pH 8 and the cells can be re-suspended without affecting the quality of the biomass, a process described as reversible alkaline flocculation (Vandamme et al., 2015a). Therefore, alkaline flocculation could be a reliable method for microalgae harvesting in any two-stage cultivation process without the need for any external coagulant.

In this study, we compared reversible alkaline flocculation versus centrifugation as a concentration and separation method for *Nannochloropsis oculata* in a two-stage cultivation process to boost lipid accumulation by nitrogen starvation. We monitored biomass concentration, the maximum quantum yield of photosystem II expressed as Fv:Fm ratio, and composition of the biomass throughout the complete process for both separation methods in order to assess the impact of the harvesting process on biomass production and lipid accumulation.

Materials and methods

Two-stage cultivation of Nannochloropsis oculata

A two-stage cultivation process was used to boost lipid accumulation in the marine microalga *Nannochloropsis oculata* 38.85 (SAG). In the first stage, *Nannochloropsis* was cultured for 7 days in synthetic seawater (30 g L⁻¹ synthetic sea salt; Homarsel, Zoutman Belgium) that was enriched with nutrients according to the formulation of Wright's Cryptophyte medium (10 mM-N, 1 mM-P) (Guillard and Lorenzen, 1972). Prior to the second stage, the biomass was harvested by either centrifugation or flocculation and re-suspended in the same synthetic seawater medium without addition of nitrogen and phosphorus (0 mM-N, 0

mM-P). In this second stage, *Nannochloropsis* was cultured for 8 days. Cultures were grown in 2-L air-bubble-column photobioreactors and irradiated with daylight fluorescent tubes at a light intensity of $60 \mu\text{Einst m}^{-2} \text{s}^{-1}$ measured at the surface of the reactor. Growth of the microalgae was monitored by measuring the absorbance (OD) at 750 nm. Absorbance was calibrated with micro-algal dry weight (DW) between 0 and 0.9 g L^{-1} , which was determined gravimetrically by filtration using Whatman glass fiber filters (Sigma–Aldrich) and drying at 105°C until constant weight ($\text{DW} = 0.65 \cdot \text{OD} - 0.03$; $R^2=0.99$). The specific growth rate (μ ; d^{-1}) was calculated using eq. 1 (Moheimani et al., 2013):

$$\mu = \frac{\ln \frac{N_{t2}}{N_{t1}}}{t_2 - t_1} \quad (1)$$

where N_{t1} and N_{t2} are the biomass densities at time t_1 and t_2 , respectively.

During the course of the experiments, the maximum quantum yield of photosystem II, determined by ratio of variable versus maximal fluorescence; $F_v:F_m$, was measured using an AquaPEN PAM fluorometer (Photon Systems Instruments). This parameter is a sensitive indicator of the stress experienced by microalgae and was used to monitor the potential impact of the harvesting/resuspension process as well as nutrient stress on the microalgae (Maxwell and Johnson, 2000). Measurements were conducted in triplicate after 20 min of dark adaptation of the microalgae.

Biomass harvesting and re-suspension

Between the first and the second cultivation stages (day 7), the biomass was harvested and re-suspended in the nutrient-depleted medium. Harvesting using reversible alkaline flocculation followed by sedimentation was compared with centrifugation. Alkaline flocculation was induced by addition of 0.5 M NaOH (Vandamme et al., 2015a). The optimal dose of NaOH was determined in a preliminary jar test using a magnetic plate stirrer (IKA RT 15). To

standardize the dosage of NaOH, the pH of the culture medium was adjusted to pH 6 prior the experiment by addition of 0.5 M HCl. In the jar test, the microalgal suspension was mixed intensively during dosing of NaOH (3 min, 1000 rpm), following by a period of gently mixing (20 min, 250 rpm) and 30 min of settling. The separation efficiency, or the percentage of microalgae biomass removed from suspension, was calculated based on cell count prior to pH adjustment (CC_i) and after settling (CC_f):

$$\text{Separation efficiency (\%)} = \left(1 - \left(\frac{CC_f}{CC_i} \right) \right) * 100 \quad (2)$$

The optimal dosage of NaOH to induce alkaline flocculation in this preliminary experiment was found to be 2.5 mM, which was used to harvest the biomass in all subsequent flocculation experiments (Suppl. Figure 1). These experiments were carried out in cylindrical jars containing 1 L of microalgae broth, and mixing was achieved by magnetic stirring (same stirring time and speed and settling time as in jar tests). The separation efficiency was calculated using eq. 2. After settling, the volume of the settled sludge was 101 ± 7 mL. Based on this result, a fixed volume of 100 mL for all the settled biomass samples was used for further treatment to ensure accurate comparison between all flocculation and centrifuged samples. The settled biomass was acidified to pH 6 using 4 ± 0.7 mL of 0.5M HCl to allow dissolution of magnesium (Vandamme et al., 2015a), followed by centrifugation at 10,000g for 10 min at 21°C. The biomass pellet was then resuspended in 30 mL of ammonium formate (0.5 M) followed by centrifugation at 10,000g for 10 min at 21°C. This washing step was repeated 3 times to eliminate salt accumulation in the second cultivation stage. The biomass pellet was subsequently washed three times in an identical manner using 30 mL of Mili-Q water to remove any residual nitrogen. Finally, the concentrated biomass was re-suspended in 900 mL marine medium without major nutrients (N, P), as described in the previous section, prior to the start of the second cultivation stage.

In the second treatment, 1 L of *Nannochloropsis* was centrifuged at 10,000 g for 10 min at 21°C (SORVAL ultracentrifuge, ThermoFisher Scientific). To use the same volumetric split as in the flocculation treatment, 900 mL of supernatant was removed by decantation, and the remaining 100 mL was identically treated as the flocculated biomass samples: pH was adjusted until 6 and the biomass pellet was washed as previously described. The biomass pellet was subsequently resuspended in nutrient-free Wright's Cryptophyte medium prior to the start of the second cultivation stage.

Biomass analysis

Total carbohydrates, total protein, total lipids, and fatty acid methyl esters (FAME) were analyzed for samples before and after reversible alkaline flocculation and centrifugation treatments. Carbohydrates were extracted and measured with the phenol-sulfuric acid method using glucose as the standard monosaccharide (Dubois et al., 1956). Total protein was analyzed using a Lowry assay with bovine serum albumin as the standard (Lowry et al., 1951). Total lipids were analyzed based on a colorimetric reaction using sulfo-phosphovanillin with canola oil (Supelco) as the standard (Mishra et al., 2014). The FAME content was analyzed using direct transesterification, and the obtained FAMES were separated by gas chromatography (GC) with cold on-column injection and flame ionization detection (FID) (Thermo Scientific Trace GC Ultra) using a Grace EC Wax column (length, 30 m; ID 0.32 mm; film, 0.25 µm) (Ryckebosch et al., 2012). The time-temperature program was 70–180°C (5°C/min), 180–235°C (2°C/min), and 235°C (9.5 min). Fatty acid identification was performed using standards containing a total of 35 different FAMES (Nu-check). Peak areas were quantified with Chromcard for Windows software (Interscience).

Statistical analysis

The two-stage cultivation process was repeated in three separate photobioreactors for each treatment. All mean values are reported with one standard deviation around the mean. The separation efficiency for alkaline flocculation versus centrifugation was evaluated using a paired t-test with a level of significance of 0.05. The FAME analysis was statistically evaluated using a three-way analysis of variance (ANOVA) test among FAME content, cultivation stage, and separation method, with a level of significance of 0.05. ANOVA was followed by Tukey's *post-hoc* test to analyze pairwise differences. Normality of the data was determined with the Shapiro-Wilk normality test (Sigmaplot 11, Systat Software Inc.).

Results and discussion

N. oculata was cultivated first in a nutrient-replete conditions followed by a nutrient-depleted cultivation stage. Figure 1 presents biomass concentration and the Fv:Fm ratio as function of cultivation time. At day 7, the biomass was harvested via alkaline flocculation or centrifugation and resuspended in nutrient-depleted medium. Figure 2 shows that the harvesting efficiency for alkaline flocculation was almost the same as for centrifugation after the nutrient-replete (N+; $p=0.062$) and the nutrient-depleted stages (N-; $p=0.277$). After settling, the volume of the settled sludge was 101 ± 7 mL, which was equal to a concentration factor of 10. The sludge volume is very much dependent on the dosage of base. This could be reduced by optimizing the dosage which would lead to higher concentration factors (Vandamme et al., 2013). This is however beyond the scope of this study, which aims at demonstrating the potential of alkaline flocculation as a low-cost method for transferring microalgae from stage 1 to stage 2 in a two-stage cultivation process.

Biomass growth (Fig. 1A) was the same for all experiments in the first stage (N+), and alkaline flocculation led to slightly greater growth in the second stage (N-). In N+, the

biomass concentration increased from 0.05 to 0.5 g L⁻¹ over 7 days. In N-, the biomass concentration continued to increase in a roughly linear manner up to ~1 g L⁻¹. The computed specific growth rate (μ) was ~3 times lower in nutrient-free medium ($\mu = 0.09$ d⁻¹) when compared to nutrient-rich medium ($\mu = 0.3$ d⁻¹). Although N concentrations in the medium were not measured, we assume that the medium in N- did not contain any residual nitrogen because the biomass was washed multiple times with MiliQ water prior to resuspension in the nutrient-deplete medium. It is not unusual to observe continued growth of microalgae in nitrogen-depleted medium in two-stage cultivation experiments. Su et al. (2010) reported an increase in dry weight biomass concentration from 0.66 to 0.85 g L⁻¹ for *Nannochloropsis* over the first four days in the nutrient-depleted phase and attributed this to the production of C metabolites. Gruber-Brunhumer (2016) observed similar effect for *Acutodesmus obliquus*.

The Fv:Fm ratio showed virtually identical patterns for both harvesting methods in both stages (Fig 1B). After inoculation, the Fv:Fm ratio increased sharply once the initial lag phase in the growth curve was passed (about day 1). However, the Fv:Fm ratio stabilized at ~0.6 at about day 4. While the Fv:Fm ratio decreased slightly to 0.55 at the beginning of the second stage, it returned to ~0.6 for both harvesting methods. This observation is in line with previous published work for centrifugation-based two-stage cultivation of *Desmodesmus* sp., *Nannochloropsis* sp. and *Tetraselmis* sp. (Gruber-Brunhumer et al., 2016; Su et al., 2010). Our results show additionally that replacing centrifugation with alkaline flocculation leads to similar biomass productivities in the second, nutrient-depleted stage. Thus, pH changes involved in reversible alkaline flocculation (pH 11 to 6) did not affect the biomass productivity nor the viability of the microalgae compared to centrifugation for this microalgal strain.

Total lipids, carbohydrates and protein were monitored during the entire cultivation process, and the results are summarized in Figure 3. The three parameters were similar for biomass harvested by alkaline flocculation or by centrifugation. Total lipid content (Fig. 3A) increased to 10% in the nutrient-replete stage, and then it increased rapidly to 40% during the first 2 days of the nutrient-depleted stage. Su et al. (2010) also reported a comparable rapid increase in total lipid content for *Nannochloropsis oculata*, from 10% to 38% after 48 hours in nitrogen-depleted medium. However, the light irradiation during cultivation was 5-fold higher compared to this study.

Carbohydrates (Fig. 3B) decreased gradually from 28% to 15% in the nutrient-replete stage. However, after the first day in the nutrient-depleted stage, the carbohydrate content dropped significantly, from 15% to 4.5%, but stayed stable afterwards. Protein content (Fig. 3C) increased from 60% at day 0 to 70% at day 2, decreased back to 60% at day 4, and remained constant until day 9. At day 11, protein content decreased to 40%.

Under nitrogen starvation, the photosynthetically fixed carbon flow is turned from the protein synthesis metabolic pathway towards lipid or carbohydrate synthesis pathways (Markou et al., 2012). However, nitrogen and/or phosphorus starvation only caused lipid accumulation for *Nannochloropsis sp.*, and not carbohydrate accumulation. Moreover, (Rodolfi et al. (2009) reported in laboratory trials that lipid synthesis may continue in *Nannochloropsis sp.* during N-starvation not only at the expense of other cellular components, but also starting from newly fixed carbon, which is generally coupled with enhanced lipid productivity (Rodolfi et al., 2009). In summary, the present results showed a distinct shift in biochemical composition over cultivation time. In the nutrient-repleted stage, proteins were accumulated while total carbohydrates gradually decreased. In the nutrient-depleted stage, lipid accumulation was boosted within 48 hours at the expense of total carbohydrate content,

and this to an equal degree for cells harvested via alkaline flocculation or centrifugation. These present results show that the proposed two-stage culture strategy using alkaline flocculation was effective for increasing lipid production from *Nannochloropsis oculata*.

Table 1 summarizes the total FAME content of the biomass and its composition. In parallel the 4-fold increase in the total lipid content during the nutrient-depleted phase (Fig 3A, N-), the total FAME content increased from ~10% at the end of the nutrient-replete stage to >25% in the nutrient-depleted phase. C16:0 and C16:1 significantly increased in the replete phase, while the content of long chain FAMES C18:2; C20:4 n6; C20:5 and C22:0 remained constant. This means that the microalgal biomass was enriched in C16, in accordance with previous studies on the impact of nitrogen limitation on FAME profiles for *Nannochloropsis oculata* (Huang et al., 2012). The most important trend is that the FAME content and composition were not affected by flocculation versus centrifugation; Supplemental Table 1 shows no significant interaction between FAME content and separation method ($p = 0.0090$). Both methods allowed a similar boost in FAME content and relative biomass enrichment in C16 FAME.

Alkaline flocculation was induced in this study by addition of 2.5 mmol of sodium hydroxide and re-acidification with 2 ± 0.4 mmol of hydrochloric acid; re-acidification allows magnesium recycling. The acid and bases additions correspond with doses of 0.20 ton sodium hydroxide ton^{-1} biomass and 0.16 ton hydrochloric acid ton^{-1} biomass, which is in line with previously published results for reversible alkaline flocculation of *Phaeodactylum tricornutum* (Vandamme et al., 2015a). This would lead to a slight increase in medium salinity, which is only 0.12 g L^{-1} , or 04% of the salt content in marine conditions. However, base and acid addition would increase overall costs by minimum of US\$ 150 ton^{-1} biomass.

Alternatively, slaked lime could be used to induce alkaline flocculation which could decrease costs by 50%, while using CO₂ for re-acidification could potentially eliminate the need for hydrochloric acid.

Conclusions

Centrifugation can be replaced by alkaline flocculation in a two-stage cultivation process of *Nannochloropsis oculata*. Biomass growth, separation efficiency, and lipid content and composition were the same in the nutrient-depleted stage, irrespective of the harvesting method. This means that the economic benefits eliminating centrifugation in a two-stage cultivate based on nutrient depletion can be realized without affecting biomass productivity and composition.

Acknowledgements.

D. V. is a postdoctoral Researcher funded by the Research Foundation – Flanders (FWO) (12D8914N). O.D. is a postdoctoral Researcher funded by the KU Leuven – Postdoctoral mandate IF (PDM/15/109). The authors would like to thank Coimbra Group Programme for Latin America for the scholarship provided to Gibrán Alemán-Nava during his research stay at KU Leuven.

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Figures and Tables

Figure 1: (A) Biomass concentration and (B) maximum quantum yield of PSII expressed as Fv:Fm ratio as function of cultivation time for *Nannochloropsis oculata* in a two-stage cultivation process (N+ = nitrogen replete; N- = nitrogen depleted) using reversible alkaline flocculation versus centrifugation as separation processes ($\mu \pm 1\sigma$; n = 3).

Figure 2: Separation efficiency (%) for alkaline flocculation and centrifugation of *Nannochloropsis oculata* at the end of the nutrient-replete (N+) and the nutrient-depleted cultivation stages (N-).

Figure 3: (A) Total lipids, (B) total carbohydrate, and (C) total protein contents of *Nannochloropsis oculata* in a two-stage cultivation process (N+ = nutrient-replete; N- = nutrient-depleted) using reversible alkaline flocculation versus centrifugation as separation process ($\mu \pm 1\sigma$; n = 3).

Table 1: FAME profile (w/w %) of *Nannochloropsis oculata* in a two-stage cultivation process (replete N+/deplete N-) using reversible alkaline flocculation (F) versus centrifugation (C) as separation process

447 **Supplementary Data**

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449 **Suppl. Fig 1:** Reversible alkaline flocculation dose-response curve for *Nannochloropsis*

450 *oculata* ($\mu \pm 1\sigma$)

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452 **Suppl. Table 1:** Summary of 3-way Analysis of Variance between FAME, cultivation stage

453 and separation method.

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Table 1: FAME profile (w/w %) of *Nannochloropsis oculata* in a two-stage cultivation process (replete N+/deplete N-) using reversible alkaline flocculation (F) versus centrifugation (C) as separation process

Fatty acid	N+ (day 7)			N- (day 16)			N+ vs N-
	F	C	p	F	C	p	
C14:0	0.6±0.1	0.6±0.0	0.999	1.7±0.2	1.8±0.2	0.973	0.034*
C16:0	1.7±0.1	1.7±0.0	0.858	9.1±0.2	8.6±1.3	0.608	<0.001*
C16:1	2.2±0.2	2.2±0.1	0.720	7.3±0.9	7.0±1.1	0.732	<0.001*
C18:1 oleic	0.4±0.0	0.4±0.0	0.936	2.3±0.8	2.2±0.4	0.891	<0.001*
C18:2	0.3±0.0	0.3±0.0	0.936	0.4±0.1	0.6±0.2	0.864	0.703
C20:4 n6	0.5±0.1	0.6±0.0	0.873	0.8±0.2	0.9±0.1	0.945	0.612
C20:5	3.9±1.1	4.5±0.2	0.155	4.0±0.7	4.5±0.2	0.633	0.949
C22:0	0.1±0.0	0.1±0.0	0.936	0.1±0.0	0.1±0.0	1.00	0.975
Total (%)	9.7±1.6	10.3±0.4	0.155	25.7±2.7	25.6±3.5	0.918	<0.001*

* significantly different for a level of significance of 0.05

467 **Highlights:**

- 468 • **Alkaline flocculation could replace centrifugation in two-stage cultivation**
- 469 • **Total lipid content increased >3-fold during the nitrogen-depleted cultivation**
- 470 • **Biomass was enriched in C16 fatty acids methyl esters after two-stage cultivation**

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Suppl. Table 1: Summary of 3-way Analysis of Variance between FAME, cultivation stage and separation method.

Source of Variation	Degrees of Freedom	p-value
FAME	8	<0.001
Cultivation stage	1	<0.001
Separation method	1	0.775
FAME x cultivation stage	8	<0.001
FAME x separation stage	8	0.990
Cultivation stage x separation method	1	0.619
FAME x cultivation stage x separation method	8	1.000

Suppl. Fig 1: Reversible alkaline flocculation dose-response curve for *Nannochloropsis oculata* (± 1)