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# pH-shift processing of *Nannochloropsis oculata* microalgal biomass to obtain a protein-enriched food or feed ingredient

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ABSTRACT

Fractionation of plant and animal raw materials by pH-shift processing has been widely applied to purify proteins. The principle is to solubilize proteins at high or low pH, removing debris and precipitating the proteins near their isoelectric point. We here describe the pH-shift process on commercially available *Nannochloropsis oculata*. The partitioning of major nutrients into the various fractions of the process was studied. Proteins were found to exhibit maximal solubility between pH 7 and 10, with a minimal solubility below pH 4. Two process versions were investigated in this study, with solubilization at either pH 7 (native pH) or 10; both versions were precipitated at pH 3. Up to 85% of both the protein and total fatty acids were recovered in the final product, compared to the initial algal slurry. Protein, total fatty acids and carbohydrates were concentrated in the final product, while the ash content was lower compared to the starting material. From a processing point of view, solubilization of *Nannochloropsis* at native pH was found to be preferable, since less chemicals were consumed compared to high-pH solubilization. Owing to its content of protein and (total) fatty acids (23 and 12% of dry weight, respectively), the latter enriched in eicosapentaenoic acid (EPA), the product of the pH-shift process has potential as a functional food ingredient.



## 1. Introduction

Microalgae have potential as human and animal nutrition; marine species can be cultivated without competing with traditional crops for land or fresh water, and furthermore, many species have a favorable nutritional profile [1,2]. Important nutrients found in microalgae include lipids, proteins, vitamins, and trace minerals [3]. The lipid fraction of some species includes the long-chained n-3 polyunsaturated fatty acids (LC n-3 PUFAs) which have been shown to have beneficial effects on cardiovascular diseases and other inflammatory-type diseases [4]. Proteins from microalgae can have balanced amino-acid profiles and good technical functionality [3,5,6], making them good candidates as functional food or feed ingredients. However, most of the microalgae grown on commercial scales are not consumed by humans or terrestrial animals directly. In spite of the microalgae's potential as a food and feed ingredient, most of the biomass is directed towards aquaculture or nutraceuticals.

To isolate the microalgal proteins, we propose using their solubility under strongly alkaline conditions, a method well-established for recovering proteins from animal and plant raw materials [7–10]. The proteins hereby obtain a net negative charge causing repulsions within and between protein molecules, favoring their interaction with water. Lowering the pH to the proteins' isoelectric point (pI) cancels out the charges on proteins, minimizing the interaction with water and rendering them insoluble. These insoluble proteins precipitate and can thus be efficiently recovered. The method, known as the pH-shift process, was developed on whole gutted fish, to remove insoluble parts such as bones and skin while isolating and retaining proteins in a functional state [10,11], see Fig. 1. Likewise, we hypothesize that it may be possible to apply the method on whole algae to remove indigestible cellulosic cell walls while recovering a protein-enriched product. When the method is applied to animal raw tissue, the material is simply ground with five to ten parts water, and the pH of the mixture is adjusted to an extreme acidic or alkaline pH. This suspension is then centrifuged, rendering a pellet of insoluble remains, and a supernatant containing soluble protein. The supernatant is recovered and the proteins are precipitated at a pH close to their iso-electric point. A second centrifugation yields a partially dewatered protein pellet, which can be neutralized before further processing. The pH-shift process is relatively cheap, applicable in large scale, and gentle towards proteins which are otherwise easily and irreversibly denatured. Proteins largely retain their functionality, including the gel-forming capacity, in part because cold processing

*Abbreviations:* EPA, eicosapentaenoic acid; LC n-3 PUFA, long-chained n-3 polyunsaturated fatty acid; P1, pellet from first centrifugation; P2, pellet from second centrifugation; pI, isoelectric point; S1, supernatant from first centrifugation; S2, supernatant from second centrifugation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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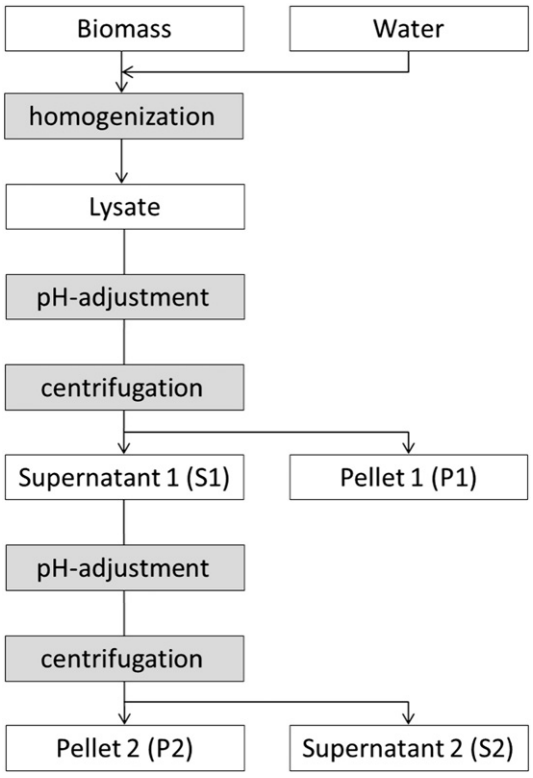


**Fig. 1.** Overview of the pH-shift process. Shaded boxes indicate processing steps while white boxes indicate products of the various steps. After disrupting the cell walls of *N. oculata*, proteins are solubilized either at the native pH or by adjusting the pH into the alkaline region. Soluble proteins are then recovered in the supernatant after centrifugation. The proteins in the supernatant are precipitated by adjusting the pH to 3 in the next step, and recovered in the pellet of the second centrifugation.

conditions are applied. Some studies have even reported on improved protein functionality following pH-shift processing, which has been explained by a favorable protein refolding pattern during precipitation [12].

Variations of the pH-shift process to recover protein from microalgae have been published on *Spirulina platensis* [13,14], and on *Nannochloropsis* [15]. Although their recovery process used pH-adjustments to fractionate the different components of the raw material, the method was applied to algal biomass which had been previously defatted with either hexane [16] or isopropanol [15]. Application of organic solvents risks denaturation of proteins and is therefore questionable from a protein-functionality point of view. More recently, proteins have been recovered from *Tetraselmis* sp. using a process which includes pH-adjustments [17]. The aim of the *Tetraselmis* fractionation was to recover a highly purified protein product, which justified the sophisticated process steps such as dialysis and preparative chromatography.

We believe that residuals of *e.g.* LC n-3 PUFA could improve the nutritional profile and organoleptic properties of a protein product. Therefore, we propose a relatively simple fractionation process, in which the final product can be utilized as a multiple-component food or feed ingredient, containing nutritionally valuable proteins and lipids. This is important due to increased demand of both vegetarian proteins for humans, as well as PUFAs and protein sources high in essential amino acids for humans and animals alike. To reduce processing costs we also suggest using the culture medium directly in the process, instead of adding water: the harvest of microalgae tends to be costly, requiring time and energy to dewater the algae.

The aim of this paper is to demonstrate the feasibility of applying pH-shift processing to *Nannochloropsis oculata*, a commercially available marine microalga, in order to concentrate its proteins. To achieve this, mapping of the protein solubility at a range of pH-values was essential, as was also an investigation of the partitioning of lipids and carbohydrates into the different fractions obtained during the two centrifugation steps. Considering application as a food or feed ingredient, amino acid profiles as well as color changes of the algae throughout the process were also monitored.

## 2. Material and methods

### 2.1. Chemicals and special materials

Microalgae, *N. oculata* "Phytobloom ice" for aquaculture, were purchased partly dewatered from Necton in March 2012 and delivered frozen. After arrival in the laboratory, they were stored at −80 °C. Seawater was collected from the Swedish marine research station of Tjärnö, filtered to remove visible particles and autoclaved. Fatty acid standards used were: i) GLC reference standard 463 (Nu-Chek Prep, Inc., USA) ii) PUFA-3 from menhaden oil (Supelco, USA), iii) tricosanoic acid (C23:0, Larodan, Sweden).

### 2.2. Adaption of the pH-shift processing to N. oculata

#### 2.2.1. Homogenization of algal biomass with seawater

One part frozen algal biomass was mixed with four parts of cold (3 °C) seawater and shaken until the algae had dissociated, to mimic a microalgae culture which had been harvested by partial de-watering to a dry weight of 10%. Cell disruption was carried out with a FastPrep®-24 instrument (MP Biomedicals, France): glass beads (425–600 μm) and microalgal suspension (1:4 ratio) were vigorously shaken (6.5 m/s, 7 cycles á 60 s). Cell suspension was held on ice between cycles. Phase-contrast microscopy confirmed that very few cells remained intact [Axiostar plus, Carl Zeiss, Germany, at 400× magnification with an A-plan 40×/0.65 objective (∞/0.17) with the appropriate phase plate]. Since most of the cells had been broken, the suspension will be referred to as lysate in the following. Glass beads were removed from the iced lysate either by sedimentation or by passing the lysate through a sieve (200 μm mesh size).

#### 2.2.2. Determination of pH for protein solubilization and precipitation

To determine the pH at which the proteins of *N. oculata* have their maximum solubility, corresponding to the first pH-adjustment of the pH-shift process, the following experiment was carried out: Small aliquots of lysate (2–4 ml) were adjusted to pH values between 1.0 and 12.0 with 1.0 unit steps with additional half-unit steps in regions of interest (pH 4.5, 5.5, 9.5, 10.5, 11.5; pH meter: PHM210, Radiometer analytical, BergmanLabora, Sweden, with a Double Pore electrode from Hamilton, Christian Berner, Sweden). The lysate's initial pH was 7. Acidification was carried out with 1.0 M HCl, alkalization with 1.0 M NaOH. Due to buffering effects of the suspension, the measured pH tended to change slightly over time, making it necessary to re-measure the pH of each aliquot prior to centrifugation. After centrifugation at 4000× *g* and 4 °C for 10 min (swinging bucket centrifuge, Heraeus multifuge 1 S-R, Kendro Laboratory Products, Germany), supernatant (S1) was recovered, freeze-dried and stored at −20 °C, awaiting protein analysis (described in Section 2.3.1).

Protein solubility was defined as the protein concentration in S1 ($[\text{protein}]_{S1}$), divided by the protein concentration in the lysate ($[\text{protein}]_{\text{lysate}}$), expressed in percent:

$$\text{Protein sol., solubilization step} = \frac{[\text{protein}]_{S1}}{[\text{protein}]_{\text{lysate}}} * 100. \quad (1)$$

The pH at which solubilized *N. oculata* proteins are least soluble, and thus could be efficiently concentrated in the second centrifugation of the pH-shift process, was determined as follows: since it was unclear if the solubilization step had an impact on the precipitation pH, two

parallel precipitation experiments were performed. In one trial, lysate was left at its native pH (*ca.* 7), and in the other trial lysate was adjusted to pH 10. Both of these pH-values were in the high solubility range according to the trial above (see Fig. 2A). After centrifugation of both lysates, S1 was recovered and divided into aliquots (2–4 ml). These aliquots were adjusted in duplicate with 1.0 M HCl to pH values between 1.0 and 4.5, in 0.5 unit steps. After the second centrifugation (4000× *g*, 4 °C, 10 min) the product (P2) was recovered and treated as S1 above, before the protein content was analyzed.

Protein solubility after the second centrifugation was defined according to the same principle as above:

$$\text{Protein sol., precipitation step} = \frac{[\text{protein}]_{S2}}{[\text{protein}]_{S1}} * 100, \quad (2)$$

where $[\text{protein}]_{S2}$ is the protein concentration in S2.

Protein solubilities in the solubilization and precipitation steps were then used to adapt the pH-shift process to *N. oculata* (see Section 2.2.3 below and the overview in Fig. 1).

### 2.2.3. pH-shift process adapted to N. oculata

*N. oculata* was mixed with seawater to a dry weight of 10% and the cells disrupted by bead-beating. In the simplest process version, the lysate was then centrifuged at its native pH (*ca.* 7) at 4000× *g* and 4 °C for 10 min. The first centrifugation's supernatant (S1), containing the majority of the proteins and lipids, was transferred to a fresh test tube and adjusted to pH 3 by addition of 1.0 M HCl. The centrifugation was repeated (4000× *g* and 4 °C for 10 min), yielding a pellet (P2) with concentrated proteins and lipids. In an alternative process, the lysate was adjusted to pH 10 with 1.0 M NaOH prior to the (first) centrifugation, with precipitation and protein concentration steps as above. S2 and P2 samples from the two process versions are referred to as pH 7/3, or pH 10/3.

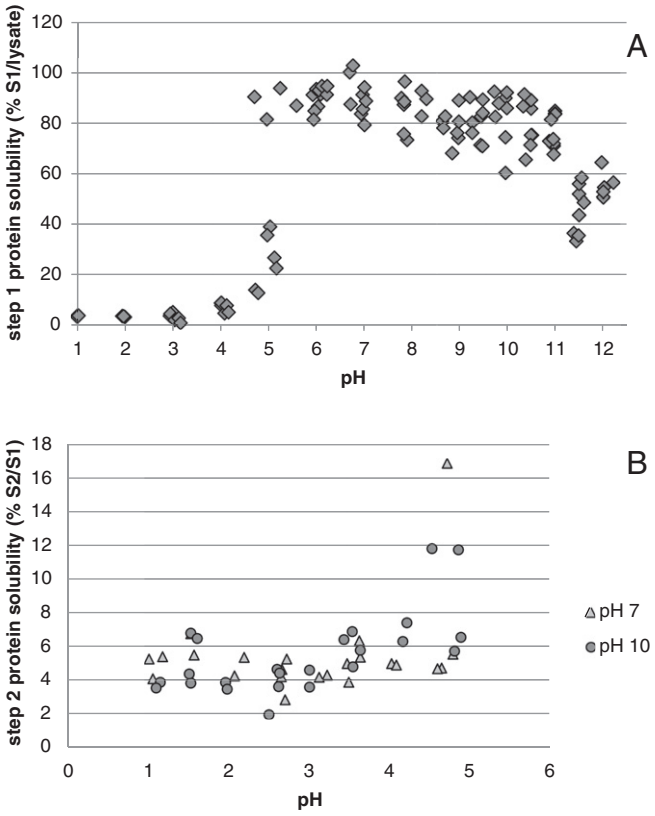


**Fig. 2.** (A) Solubility of *N. oculata* protein in four parts seawater in the first step of the pH-shift process, in the pH range 1–12. (B) Solubility of *N. oculata* protein in the second step of the pH-shift process in which solubilization was carried out at either pH 7 or 10, and S1 was then adjusted to pH 1–5. Note the difference in scale to panel A. Points show individual replicates, with experiments from 5 separate days in A and 2 separate days (for both pH 7 and 10) in B.

## 2.3. Characterization of the pH-shift process's fractions

### 2.3.1. Protein, polypeptides and amino acids

Protein content in all freeze-dried fractions was determined according to [18]. The method is based on that of Lowry [19] and extracts proteins using trichloroacetic acid precipitation and spectrophotometric quantification (Cary Win UV 50, Varian, Australia, with either a quartz suprasil flow-cuvette, Hellma, Germany, or 0.5 ml disposable plastic cuvettes, Kartell, Italy, for samples with volumes < 3 ml). Bovine serum albumin (Bio-Rad Laboratories AB, Sweden) was used as standard.

Protein recovery was calculated as follows: masses of P2 and S2 were summed to calculate a theoretical mass of S1. The mass of P1 was added to S1 to calculate the theoretical initial mass of the lysate. This back-calculation was done in order to compensate for losses of sample from centrifuge tubes, pipettes, *etc.* during the pH-shift process. Due to the high water content, the density of the wet fractions was assumed to be 1.0 g/ml, giving the theoretical volume of each of the fractions. This volume was multiplied with the measured protein concentration (mg/ml) to obtain a theoretical protein mass for each of the fractions. The protein masses of the fractions were then expressed as percentage of the total proteins in the lysate (*i.e.* the starting material) or S1 (giving the yield over the second centrifugation step).

For polyacrylamide gel electrophoresis analysis of the polypeptide profile, samples of lysate pH 7, lysate pH 10, S1 pH 7, S1 pH 10, P2 pH 7/3, P2 pH 10/3, S2 pH 7/3, and S2 pH 10/3 were collected from a single pH-shift process, mixed with an equal volume of Laemmli sample buffer, prepared as follows: Laemmli 1:1 sample buffer was purchased from Bio-Rad and 1.0 ml augmented with 78.5 mg sodium dodecyl sulfate (Sigma-Aldrich) and 479 mg urea (Sigma-Aldrich). Samples were then incubated at room temperature for 30 min and promptly frozen at −20 °C for storage. After thawing them, samples were centrifuged at 15,000× *g* for 5 min (Heraeus Fresco 17, Thermo Fisher Scientific, Sweden) and supernatant corresponding to 20 μg of protein loaded onto the gel. The ladder used was Bio-Rad's Precision Plus Protein Dual Color Standard (10–250 kD). The gel used was mini-protean TGX 12% polyacrylamide gel (Bio-Rad) in a Tris/Glycine/SDS running buffer (Bio-Rad). Voltage (220 V) was constant throughout the run (22 min). The gel was fixed in a 2:5:13-solution of acetic acid (Scharlau), 2-propanol (Fluka), and deionized water for 30 min, stained in 0.025% (w/v) Coomassie blue C25 dissolved in deionized water with 10% acetic acid for 120 min, and destained in 10% acetic acid in deionized water overnight. For imaging purposes, gels were scanned on a GS-800 Calibrated Densitometer (Bio-Rad).

The amino acid composition was determined using high pressure liquid chromatography (HPLC) by an external, accredited lab (Eurofins) for the initial material (lysate), product P2 pH 7/3 and P2 pH 10/3. Single measurements were done for lysate pH 7 and lysate pH 10, while P2 pH 7/3 and P2 pH 10/3 were analyzed in duplicates.

### 2.3.2. Fatty acids: content and yields

Lipid content was analyzed as total fatty acids following direct transmethylation with HCl as described elsewhere [20]. The following freeze-dried fractions were analyzed: lysate, S1 pH 7, S1 pH 10, P1 pH 7, P1 pH 10, S2 pH 7/3, S2 pH 10/3, P2 pH 7/3, and P2 pH 10/3. The pH-shift process and analysis was repeated on separate days, giving at least duplicates of each measurement. Yields for total fatty acids were calculated as for protein (see Section 2.3.1).

### 2.3.3. Carbohydrate content

Total carbohydrates were determined using a method based on Herbert, Phipps & Strange [21], adapted for 96-well plates. 30 μl of either

of the following was pipetted into a 96-well plate (Nunclon Delta Surface, Thermo Scientific, Denmark) in triplicate: sample (diluted with MQ water, if necessary), blank or standard. To this, 30 μl of 5% phenol in MQ water was added and mixed, where after 150 μl of concentrated sulfuric acid was added and mixed. The plate was incubated at room temperature for 30 min and the absorbance at 488 nm was measured in a plate reader (Safire II, Tecan, Austria). Standard curves were constructed from glucose. The following freeze-dried fractions were analyzed from two pH-shift processes (run on the same day): lysate, S1 pH 7, S1 pH 10, P1 pH 7, P1, pH 10, S2 pH 7/1.0–4.5, in 0.5 unit steps, S2 10/1.0–4.5, in 0.5 unit steps, P2 pH 7/1.0–4.5, in 0.5 unit steps, and P2 10/1.0–4.5, in 0.5 unit steps. On a separate day, the experiment was repeated partially, again with two parallel processes, with analysis of the following fractions: lysate, S1 pH 7, S1 pH 10, P1 pH 7, P1, pH 10, S2 pH 7/1.0–4.5, in 1.0 unit steps, S2 10/1.5–4.5, in 1.0 unit steps, P2 pH 7/1.5–4.5, in 1.0 unit steps, and P2 10/1.0–4.5, in 1.0 unit steps.

#### 2.3.4. Water, ash and sodium content

Water content in all fractions was determined by weighing samples before and after freeze-drying (at −50 °C and a minimal pressure of 0.05 mPa) to constant pressure. Water content was also confirmed by the overnight drying step of the ash content-determination.

The ash content was determined by pipetting 2 ml of sample into a pre-weighed crucible, drying the crucible overnight at 90 °C, re-weighing it, and heating the crucible to 550 °C for 3 h. After cooling the crucible in a desiccator, the mass of the remaining sample was determined. The following fractions were analyzed: lysate, S1 pH 7, S1 pH 10, P1 pH 7, P1 pH 10, S2 pH 7/3, S2 pH 10/3, P2 pH 7/3, and P2 pH 10/3. The pH-shift process and analysis were repeated on separate days, giving at least duplicates of each measurement (except for P2 pH 10, where triplicates from one pH-shift process are present).

Sodium content of the lysate, P2 pH 7/3 and P2 pH 10/3 was analyzed in duplicate by high-pressure ion chromatography: samples were diluted accurately in MQ water, centrifuged at 16,000× *g* for 10 min to sediment particles and 20 μl injected on an IonPac CS14 column equipped with a CG14 guard column, a Dionex GS50 Gradient Pump BioLC, CMMS3 suppressor and Dionex CD20 Conductivity Detector. The elution was isocratic with 9 mM methanesulfonic acid as the mobile phase at 0.90 ml/min. Peaks were integrated manually with Chromeleon Software v. 6.80. A standard was prepared from sodium chloride (Sigma). In the calculation, it was assumed that every sodium ion in the sample was paired with one chloride ion.

#### 2.3.5. Color measurements

Color was measured with a colorimeter (CR-400, Konica Minolta Sensing, Japan) in the CIE L*a*b* color space by holding a probe directly against the bottom of the round-bottom centrifuge tubes (clear polypropylene round-base centrifuge tubes, TPP, Switzerland) that held 3.0 ml of the suspensions, taking five measurements of L*, a* and b*. From the results, the mean was calculated and used to calculate whiteness according to the formula:

$$\text{whiteness} = 100 - \sqrt{(100-L)^2 + a^2 + b^2}. \quad (3)$$

The following fractions were analyzed from two pH-shift processes run on separate days with solubilization at either pH 7 or 10 (with analysis duplicates): lysate, S1, S2, and P2. The pH-shift process and the color analysis were repeated on separate days, giving at least duplicates of each measurement.

To determine how color changed over time, one experiment was carried out on P2 pH 7/3 and P2 pH 10/3: samples were held on ice and the color measured as above at time 0 (right after pellet and supernatant had been separated), after 0.5 h, 1.1 h and 3.3 h. At 4 h the samples were frozen (−20 °C). The frozen sample was measured after 27 h in its frozen state. Samples were prepared in duplicate.

### 2.4. Statistics

In order to determine if groups were significantly different, 2-tailed independent sample T-tests were applied (SPSS, version 19, IBM). P-values < 0.05 were considered significant.

## 3. Results

### 3.1. Adaption of the pH-shift process to N. oculata

To establish suitable pH settings for the various steps of the pH-shift process, the protein solubility for *N. oculata* in seawater was initially determined over a wide pH-span. Protein solubility was high (>60%) between pH 5.5 and 10.5, see Fig. 2A. In spite of some variation caused by experiments being repeated on separate days, there was a plateau between pH 5.5 and 8.5 where 73 to 103% of the protein concentration in the lysate could be detected in supernatant 1. As the pH increased, the solubility dropped slowly to an average of 51% at pH 11.5–12. At the other end of the interval, when the pH was decreased, solubility dropped sharply below pH 5.5, and was 9% or lower at pH ≤ 3.5. Based on this result, pH 7 and 10 were chosen as the solubilization-pH in further investigations.

The second part of the pH-shift process aims to precipitate as much of the protein in S1 as possible, *i.e.* as little protein should remain soluble as possible. As can be seen in Fig. 2B, the protein concentration of S2 was only 2−7% of S1 below pH 3.5. The solubilization pH (7 or 10) did not appear to have a major impact on the protein precipitation; however, after the centrifugation, it was noted that S2 pH 7 did not separate as distinctly from P2, as S2 pH 10. Although solubility was very constant in the pH range 1–3.5, the volume of P2 decreased as the amount of added acid increased (see Fig. 3), which reflects a denser protein conformation and/or reduced water-holding capacity of the proteins remaining in the pellet [22]. As a compromise between protein solubility, amount of added acid, and volume of P2, pH 3.0 was chosen as the precipitation pH for further investigations of the pH-shift process.

### 3.2. Characterization of pH-shift process's fractions: macronutrients

#### 3.2.1. Concentration of macronutrients

Table 1 gives an overview of the macronutrients of the starting material and fractions of the various steps of the pH-shift process. The process resulted in an enrichment of protein, fatty acids and carbohydrates, whereas the amount of ash and water was reduced, as can be seen by comparing lysate and P2 (*i.e.* starting material and product). The increase in protein content was minor for both processes, from 19% to *ca.* 24% protein *per* dry mass; the difference was significant ($p < 0.05$) only for the pH 7-process. The increase in total fatty acid content was only significant for the pH 10-process: from 10% to 13% total fatty

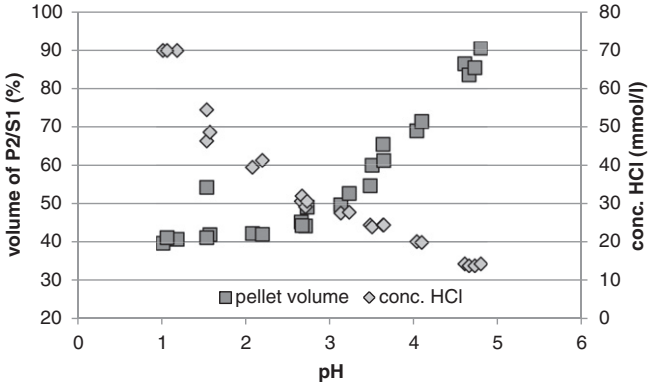


**Fig. 3.** Volume of P2 as percentage of S1 for the pH-shift process with solubilization at pH 7 (left axis) and the hypothetical concentration of HCl in the algal lysate (right axis). Individual points are plotted. See Fig. 2B for the protein solubility curve.

**Table 1**
Dry matter, macronutritional composition and ash content of the different fractions obtained in the pH-shift process run with solubilization either at pH 7 or 10. Macronutrients (protein, total fatty acids, carbohydrates) and ash are shown as percentage of dry matter. SD = standard deviation, n, the number of samples, Rn = range (max–min). Each measurement is the result of at least two pH-shift experiments from different days, with 1 to 4 four replicates.

| Sample | Dry/wet mass (%) | | | Total dry (%) | Protein/dry mass (%) | | | FAs/dry mass (%) | | | Carbohydrate/dry mass (%) | | | Ash/dry mass (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | Mean | SD | n | | Mean | Rn | n | Mean | Rn | n | Mean | Rn | n | Mean | Rn | n |
| Lysate, pH 7 | 10 | 0.4 | 11 | 100 | 19 | 0.7 | 4 | 11 | 0.6 | 3 | 37 | 3.3 | 2 | 34 | 4.8 | 3 |
| S1, pH 7 | 9 | 0.6 | 9 | 103 | 17 | 1.9 | 2 | 10 | 0.4 | 4 | 37 | 0.9 | 2 | 38 | 7.7 | 3 |
| P1, pH 7 | 20 | 0.2 | 4 | 97 | 17 | 2.0 | 2 | 11 | 2.5 | 2 | 40 | 3.0 | 2 | 29 | 13.3 | 2 |
| S2, pH 7/3 | 6 | 1.0 | 12 | 79 | 1 | 0.1 | 2 | 2 | 4.6 | 7 | 32 | 1.1 | 2 | 44 | 3.1 | 3 |
| P2, pH 7/3 | 13 | 1.2 | 7 | 103 | 23 | 1.0 | 2 | 12 | 1.5 | 2 | 42 | 4.5 | 2 | 25 | 2.4 | 3 |
| Lysate, pH 10 | 10 | 1.1 | 6 | 103 | Not measured[a] | | | 10 | 0.6 | 3 | Not measured[a] | | | 37 | 1.0 | 3 |
| S1, pH 10 | 9 | 0.4 | 10 | 102 | 16 | 0.1 | 2 | 10 | 0.5 | 4 | 34 | 2.1 | 2 | 41 | 8.6 | 3 |
| P1, pH 10 | 17 | 0.9 | 4 | 98 | 18 | 1.6 | 2 | 13 | 1.0 | 2 | 40 | 3.9 | 2 | 27 | 2.9 | 2 |
| S2, pH 10/3 | 6 | 1.5 | 12 | 76 | 1 | 0.1 | 2 | 1 | 0.8 | 7 | 30 | 0.3 | 2 | 44 | 22.5 | 3 |
| P2, pH 10/3 | 13 | 1.3 | 7 | 123 | 24 | 2.2 | 2 | 13 | 0.5 | 2 | 58 | 4.6 | 2 | 28 | 14.5 | 3 |

[a] pH 10-lysate was not measured, but since the only difference to pH 7-lysate is a small amount of added base, pH 10-lysate is assumed to be as pH 7.

acids/dry weight. In both processes the increase in carbohydrate content was significant when comparing the lysate with P2 in the interval between pH 2.5 and 3.5. At pH 3, the carbohydrates/dry weight increased from 37% to 42% for the pH 7-process and from 37% to 58% for the pH 10-process. The ash content decreased over the process from 34% to 25% dry weight for the pH 7-process and from 37% to 28% for the pH 10 process, representing a 25% average decrease in ash, though the decrease was only statistically significant for the pH 7-process. Concurrent with this decrease, a small net removal of water was seen through the process: While the starting material contained 10% dry matter, the final product contained 13% dry matter; this increase in dry matter was significant for both processes. The sodium chloride concentration did not change measurably during the process: in lysate and P2 (irrespective of solubilization pH) it was 2.5% on a wet basis.

### 3.2.2. Yields: protein and total fatty acid

The pH-shift process was found to recover the major part of the protein and total fatty acids initially present in *N. oculata*. Protein recovery over the pH-shift process was 86% for the pH 7-process and 72% for the pH 10-process (see Table 2). Loss of protein occurred mainly in the first step (*i.e.* solubilization), where 15% of the protein remained unaccounted for, though some of this loss is thought to be sampling and analysis error.

Fatty acids present in the original lysate were mainly recovered in S1 in the first step. However, during the precipitation step, only traces remained in S2, indicating that the fatty acids accumulated in the protein-rich product of the pH-shift process. As can be seen in Table 2, 85% of the total fatty acids present in the lysate were recovered in P2 when the homogenate was solubilized at pH 7, while 73% was recovered in P2 when the homogenate was solubilized at pH 10.

**Table 2**
Percentage of recovered protein and fatty acids for the various fractions of the pH-shift process, expressed as percentage of total proteins and fatty acids in either lysate or S1.

| | Protein | | Total FAs | |
|---|---|---|---|---|
| | % lysate | % S1 | % lysate | % S1 |
| S1, pH 7 | 80 | – | 90 | – |
| P1, pH 7 | 5 | – | 7 | – |
| S2, pH 7 | 2 | 2 | 3 | 3 |
| P2, pH 7 | 86 | 107 | 85 | 95 |
| S1, pH 10 | 70 | – | 81 | – |
| P1, pH 10 | 14 | – | 21 | – |
| S2, pH 10 | 2 | 2 | 3 | 3 |
| P2, pH 10 | 72 | 103 | 73 | 90 |

## 3.3. Further characterization of pH-shift process's isolates

### 3.3.1. Amino acid and polypeptide profile

The amino acid pattern for the lysate and P2 is shown in Table 3. Glutamic acid and proline were especially abundant in all fractions, accounting for 11 and 10% of the total amino acids, respectively. The percentage of proline in the product was significantly lower than in the lysate in both processes: 10.3% of the amino acids in the lysate were proline, while P2 contained 8.5% and 7.7% proline for the pH 7-process and pH 10-process, respectively.[1] In the pH 10-process, this was offset by a slight, but significant increase in leucine and phenylalanine (from 8.7 to 9.2% and 5.1 to 5.4%, respectively).

The polypeptides of lysate, S1, P2 and S2 were separated by gel electrophoresis into near-identical band patterns, see Fig. 4, indicating that the polypeptide compositions were barely affected by the pH-shift process, no matter if it was carried out with solubilization at pH 7 or 10. Prominent bands in all samples had apparent molecular weights of 50 kDa and *ca*. 21 kDa. The 21 kDa band was slightly enriched in S2 and P2 compared to lysate and S1, particularly after solubilization at pH 10.

### 3.3.2. Fatty acid profile

The main fatty acids of *N. oculata* in the lysate at pH 7 were palmitoleic acid at 31% of total fatty acids, palmitic acid at 28% and the LC n-3 PUFA eicosapentaenoic acid (EPA) at 18% (see Table 4); these percentages were also typical for products precipitated at pH 3, irrespective if the initial lysate was solubilized at pH 7 or 10. Other fatty acids identified in lysate and final product were oleic acid, arachidonic acid, myristic acid and linoleic acid, present at 3–8% of total fatty acids.

### 3.3.3. Color

All fractions appeared dark green to the naked eye (roughly the color of fresh spinach slurry), which was reflected in a*-values between −5.7 and −8.4. P1, which was not measured because sample volume was too small, was also dark green, but it was noted that it was the only fraction that did not appear homogenous: instead a thin gray layer bisected the otherwise dark green pellet. The lysate and S1 were indistinguishable in color with respect to L*-(lightness), a*-(redness) and b*-(yellowness) values, with very little difference between the solubilization pH, see Fig. 5. The final fractions of the precipitation step, S2 and P2, had a significantly increased L*-, b*- and W-values when compared to the lysate for both the pH 7 and the pH 10-process. Concurrently, a* decreased significantly in P2 when compared to the lysate, reflecting increased green

[1] Note that in the statistical analysis, the single measurements of lysate pH 7 (n = 1) and lysate pH 10 (n = 1) were treated as duplicates of either pH value (n = 2).

**Table 3**
Amino acid composition for *N. oculata* lysate and product (P2) from the pH-shift process with solubilization at either pH 7 or 10. Essential amino acids are marked with asterisks. The first column indicates the mean mg amino acids/g dry mass; the second column shows the range (Rn = max–min), n = 2, where † indicates that P2 is significantly different ($p < 0.05$) from the lysate. The third column shows the percentage of individual amino acids of total amino acids.

| | Lysate | | | P2, pH 7 | | | P2, pH 10 | | |
|---|---|---|---|---|---|---|---|---|---|
| | mg/g | Rn | % total | mg/g | Rn | % total | mg/g | Rn | % total |
| Sum | 235 | 24 | | 265 | 10 | | 340 | 8.2 | |
| Sum non-essential | 137 | 14 | 59 | 151 | 5 | 57 | 193 | 5 | 57 |
| Sum essential | 97 | 11 | 41 | 114 | 5.1 | 43 | 147 | 3.7 | 43 |
| Alanine | 16 | 2.2 | 7.0 | 18 | 0.6 | 7.0 | 24 | 1.1 | 7.0 |
| Arginine | 15 | 1.2 | 6.2 | 17 | 0.7 | 6.4 | 22 | 0.5 | 6.3 |
| Aspartic acid | 21 | 2.2 | 8.9 | 24 | 1.4 | 9.0 | 31 | 1.4 | 9.2 |
| Cystein + cystine | 2.5 | 0.1 | 1.1 | 2.7 | 0.0 | 1.0 | 3.0 | 0.1 | 0.9 |
| Glutamic acid | 26 | 2.6 | 11 | 29 | 1.6 | 11 | 38 | 0.8 | 11 |
| Glycine | 14 | 1.6 | 5.8 | 15 | 1.0 | 5.7 | 20 | 0.7 | 5.8 |
| Histidine* | 4.4 | 0.8 | 1.9 | 5.5 | 0.4 | 2.1 | 7.1 | 0.3 | 2.1 |
| Isoleucine* | 10 | 1.0 | 4.5 | 12 | 0.9 | 4.5 | 16 | 0.4 | 4.6 |
| Leucine* | 20 | 2.1 | 8.7 | 24 | 1.5 | 9.0 | †31 | 1.0 | 9.2 |
| Lysine* | 15 | 1.4 | 6.5 | 18 | 1.1 | 6.7 | 23 | 0.7 | 6.8 |
| Methionine* | 4.3 | 0.2 | 1.8 | 5.5 | 0.6 | 2.1 | 7.0 | 0.5 | 2.1 |
| Phenylalanine* | 12 | 1.3 | 5.1 | 14 | 1.0 | 5.3 | †18 | 0.6 | 5.4 |
| Proline | 24 | 2.0 | 10 | †22 | 0.4 | 8.5 | †26 | 0.8 | 7.7 |
| Serine | 11 | 1.5 | 4.6 | 12 | 0.5 | 4.6 | 16 | 0.7 | 4.7 |
| Threonine* | 12 | 1.4 | 5.0 | 13 | 0.6 | 5.0 | 17 | 0.9 | 5.1 |
| Tryptophane (total)* | 4.1 | 0.7 | 1.7 | 4.8 | 0.7 | 1.8 | 6.2 | 0.1 | 1.8 |
| Tyrosine | 8.8 | 0.5 | 3.7 | 10 | 0.7 | 3.9 | 13 | 0.6 | 3.9 |
| Valine* | 15 | 2.2 | 6.3 | 17 | 0.4 | 6.4 | 21 | 0.7 | 6.3 |

color. Over time, a* increased in P2, *i.e.* the product became less green (see Fig. 6). This change was seen both during storage on ice, and after subsequent frozen storage at −20 °C.

## 4. Discussion

### 4.1. Process

Two versions of the pH-shift process were evaluated in this study, one with the first separation step carried out at the native pH of the algal lysate, and the second after alkalization of the lysate to pH 10; both versions comprised seawater (the main constituent of algal growth media) as a way to reduce process-water consumption. Alkalization has been used in a long series of studies on muscle protein isolation [10,11,23,24], and also in studies of vegetable protein isolation [7,8], including microalgae [15–17,25]. One reason for the alkalization step has been that it improves solubilization due to negative repulsion between

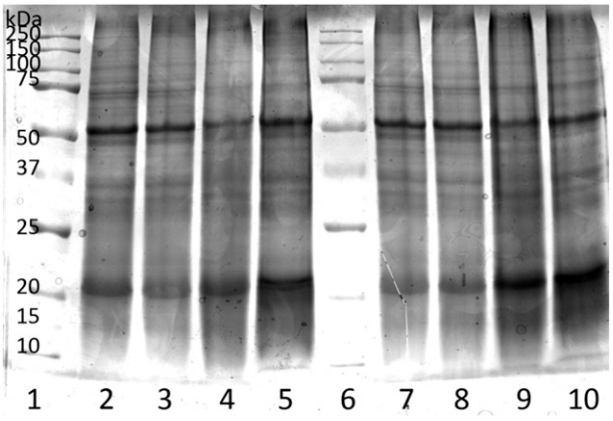


**Fig. 4.** SDS-PAGE of various fractions of the pH-shift process, with solubilization at either pH 7 (lanes 2–5) or pH 10 (lanes 7–10). Lanes 1 and 6 contain protein ladder, with the molecular mass (in kD) indicated at the far left. The sample fractions are: lysate (lanes 2, 7), S1 (lanes 3, 8), P2 (lanes 4, 9), S2 (lanes 5, 10).

**Table 4**
Fatty acid composition for *N. oculata* lysate and product (P2) from the pH-shift process with solubilization at either pH 7 or 10. The first column indicates the mean mg fatty acids/g dry mass; the second column shows SD (standard deviation) or Rn (range = max–min) with the number of replicates (n) of pH-shift experiments done on two different occasions; the third column shows the percentage of total fatty acids.

| | Lysate, pH 7 | | | P2, pH 7 | | | P2, pH 10 | | |
|---|---|---|---|---|---|---|---|---|---|
| | mg/g | SD, n = 3 | % total | mg/g | Rn, n = 2 | % total | mg/g | Rn, n = 2 | % total |
| Sum | 105 | 2.9 | | 123 | 15 | | 129 | 4.9 | |
| C12:0 | 0.3 | 0.0 | 0.3 | 0.4 | 0.1 | 0.3 | 0.4 | 0.0 | 0.4 |
| C14:0 | 5.5 | 0.2 | 5.2 | 6.7 | 1.1 | 5.4 | 7.0 | 0.4 | 5.7 |
| C15:0 | 0.4 | 0.0 | 0.3 | 0.4 | 0.1 | 0.4 | 0.5 | 0.0 | 0.4 |
| C16:0 | 29 | 1.1 | 28 | 34 | 4.5 | 28 | 36 | 1.7 | 29 |
| C16:1 n7 | 32 | 1.2 | 31 | 37 | 4.3 | 30 | 39 | 1.4 | 31 |
| C17:0 | 0.3 | 0.0 | 0.3 | 0.4 | 0.1 | 0.3 | 0.4 | 0.0 | 0.3 |
| C17:1 | 0.3 | 0.0 | 0.3 | 0.4 | 0.1 | 0.3 | 0.5 | 0.0 | 0.4 |
| C18:0 | 0.4 | 0.0 | 0.4 | 0.5 | 0.1 | 0.4 | 0.5 | 0.0 | 0.4 |
| C18:1 n9 | 7.9 | 0.2 | 7.5 | 9.5 | 1.4 | 7.7 | 9.9 | 0.6 | 8.0 |
| C18:1 n7 | 0.4 | 0.0 | 0.4 | 0.5 | 0.1 | 0.4 | 0.5 | 0.1 | 0.4 |
| C18:2 n6 | 3.5 | 0.1 | 3.3 | 4.2 | 0.6 | 3.4 | 4.5 | 0.2 | 3.6 |
| C18:3 n6 | 0.4 | 0.0 | 0.4 | 0.5 | 0.1 | 0.4 | 0.6 | 0.0 | 0.5 |
| C20:4 n6 | 5.2 | 0.2 | 4.9 | 6.2 | 0.7 | 5.0 | 6.5 | 0.1 | 5.3 |
| C20:5 n3 | 19 | 0.6 | 18 | 23 | 2.1 | 18 | 24 | 0.1 | 19 |

proteins. In this solubilized state, separation between proteins and non-soluble compounds has been facilitated. However, we found the pH in the range of 5.5 to roughly 11 to have little impact on the solubility and yield of *N. oculata* proteins. Therefore, we suggest omitting the initial pH-adjustment, saving processing time and chemicals. However, it was noted that the final product, P2, of the pH 7-process did not pack

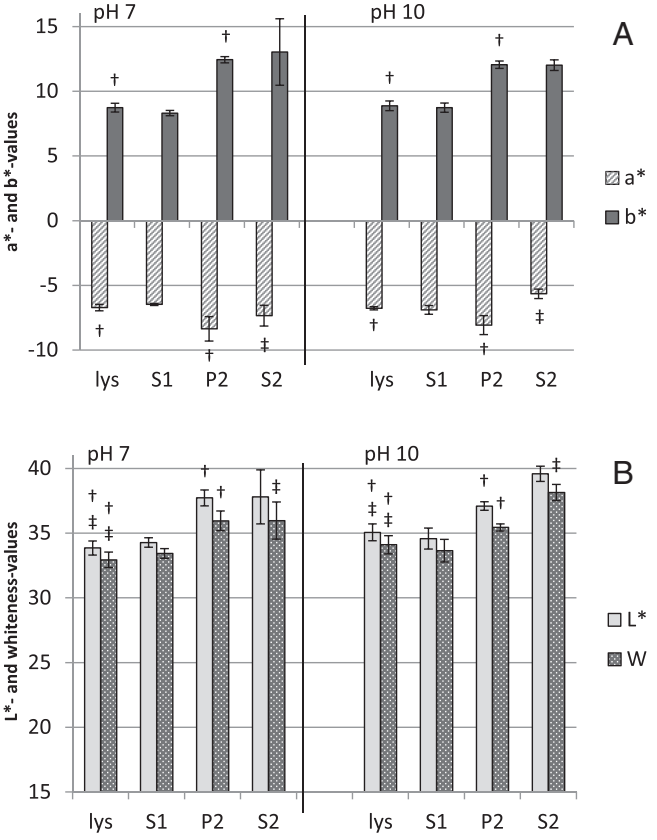


**Fig. 5.** Color of the lysate ("lys"), S1, P2 and S2 of the pH-shift process with solubilization at pH 7 (left) and pH 10 (right). (A) shows b* (yellow/blue color-axel), and a* (red/green color-axel). (B) shows L* (lightness) and W (whiteness) of the sample. Error bars indicate standard deviation, n = 4 from duplicates on two separate occasions; †indicates where lysate is significantly ($p < 0.05$) different from product, ‡ indicates where pH 7 process is significantly different from pH 10 process.

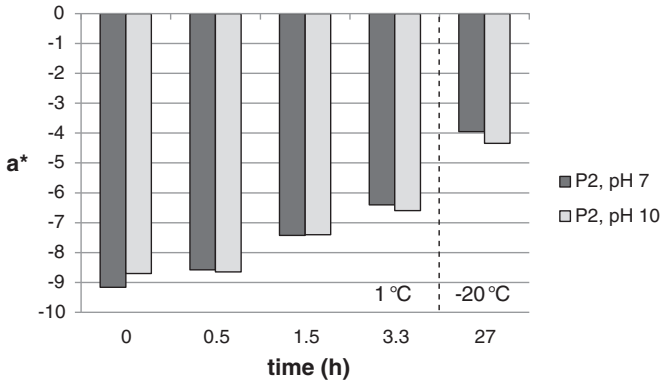


**Fig. 6.** Changes in a*-values over 3.3 h of ice storage of P2 from the pH-shift process with solubilization either at pH 7 or 10; note that the sample was frozen after the fourth measurement; mean of n = 2 is plotted.

as densely as that of the pH 10-process. In other words, P2 and S2 were less distinct. Although the supernatant with a hazy boundary was more difficult to separate from the pellet, no significant difference of the final product was noted from the pH 7-process compared with the pH 10-process in respect to the moisture content, macronutritional composition or color.

P1 was small in volume compared to S1, irrespective of the solubilization pH. It is unclear exactly what was being removed in the first centrifugation: the macronutrient composition of P1 was similar to the lysate, S1 and P2 suggesting considerable similarity to these fractions. We suggest that the green part of P1 consisted of unbroken *N. oculata* cells and thus may have been eliminated by extending the bead-beating time. The gray band in P1 may be invasive organisms and other contaminants, considering that the commercial algal biomass used in our study was not food grade, but rather intended for aquaculture.

In accordance with earlier work on semi-fatty and fatty fish [10,24,26], it may be possible to retrieve a layer of floating fat in the first centrifugation: *N. oculata* can accumulate triacylglycerols when grown under nitrogen-limited conditions [27–29]. It may be possible to use the pH-shift process to recover such triacylglycerols which could be expected to coalesce and float as an oil or emulsion layer depending on the emulsification capacity of the other lysate constituents. At low lipid content, such as the *N. oculata* used in the present study (10% lipid per dry weight), fatty acids are mainly present in the form of polar lipids, *e.g.* phospholipids and glycolipids associated with membranes [28]. These lipid classes can be expected to associate with proteins through the cytoskeletal lattice [30] and would therefore not be separated at the relatively low centrifugal forces applied here ($4000\times g$). Earlier work on the pH-shift process has also illustrated how cellular membranes can sediment upon application of centrifugal forces in contrast to the triacylglycerols that float [10].

The protein solubility of *Nannochloropsis* obtained in this study is comparable to that of similar studies: here, solubility was high (>80%) in the range pH 6 to pH 11, while it was low below pH 4. Previous studies have found that *Scenedesmus acutus* protein was maximally soluble at pH 11.5 with the pI at pH 3.5 [31], *S. platensis* protein was maximally soluble at pH 8.0 with the pI at pH 3.0 [13], and *Tetraselmis* sp. protein was highly soluble above pH 6 with minimal solubility below pH 4 [17]. Recent work on *Nannochloropsis* is somewhat difficult to interpret in relation to our own data, since defatted biomass was used in mapping of pH-dependent solubility [15]. However, the same general pattern emerged, with higher solubility at high pH and low solubility below pH 5; pI was determined to pH 3.2. Overall, [15] found lower protein solubility for defatted than non-defatted *Nannochloropsis* which they ascribed to *e.g.* partial denaturation of the proteins by the defatting solvent. In our study, pI was not as distinct as in the study by [15]; solubility instead remained low from pH 3 down to 1, which is most likely explained by the use of seawater in our study. It is well-known that anions such $Cl^-$ can interact with positively charged groups on proteins, shifting the pI towards lower pH-values [32]. This is further supported by the work of [17], who investigated how the protein solubility curve of *Tetraselmis* sp. responded to the ionic strength (0.03, 0.2 or 0.5 M, achieved by different NaCl-levels) of the aqueous medium; they demonstrated that at low pH, solubility was lower at higher ionic strength, while also no distinct pI could be seen [17].

In the previously mentioned study [15] where defatted and non-defatted *Nannochloropsis* was compared in a pH-shift process similar to the one we present here, some differences were that i) P1 was re-extracted and ii) centrifugation was carried out at $20{,}000\times g$. Since P1 was very small in our case, *i.e.* it did not retain a lot of solubilized proteins, we did not feel it was necessary to re-extract P1. We also chose a lower centrifugal force to facilitate scale-up. In spite of the lower centrifugal force applied here, we managed to recover ≥ 72% of the protein, suggesting that $4000\times g$ is sufficient.

### 4.2. Analytical limitations

For the macronutrient composition of the various fractions, the total did not add up to 100% in all cases. In S2 pH 7/3 and S2 pH 10/3 some macronutrients were present at trace amounts only, making quantification uncertain. Since S2 was a clear solution, it seems unlikely that much lipid is present which could be expected to form light-scattering micelles. It also seems unlikely that S2 contained more proteins than detected, considering that all protein present in S1 was recovered in P2 (see Table 2). The measurement of ash content is a robust method, and furthermore a considerable amount of ash was expected in the product, owing to the seawater used in the method. However, we noted considerable variation in the carbohydrate method, which may partially explain why the macronutrients did not add up to 100% in S2 or P2 pH 10/3.

The method for detecting total protein gave a lower result than that for amino acids (*cf.* Tables 1 and 3): Lysate contained 19% protein with the spectrophotometric method and 24% with the chromatographic method. Although the difference was slightly less for P2, pH 7 (23% and 27%, respectively), it was large for P2, pH 10: 24% and 34%, respectively. It is not fully clear to us why the spectrophotometric analysis gave lower results, though it is possible that some protein was lost in manipulation or that the initial weighing of dried sample was erroneous. It has also been described how the Lowry analysis principle can give underestimations for proteins with low percentage of tyrosine, tryptophan, or cysteine residues [33].

### 4.3. Color

The product's (P2's) color was initially dark green, reflected by a negative a*-value, though the green color diminished over time. Presumably, the reduction in green color was a result of $Mg^{2+}$ leaving the porphyrin ring of chlorophyll, a reaction known as the pheophytinization reaction [34]. The pheophytinization takes place at low pH. $Mg^{2+}$ is replaced by $2H^+$ and this reaction results in a shift in the absorption wavelength [35]. If a strongly green product is desired, it is likely that increasing the pH again to neutral would stabilize the chlorophyll. If, on the other hand, the green color is considered undesirable, it may be possible to degrade it by storing the product at pH 3 for prolonged time, though further investigations should address this issue specifically. The storage stability of proteins, fatty acids and other nutrients in the pH-shift-produced algal product is not known at this point.

### 4.4. Sensory and nutritional considerations

Since seawater was used in our process, the sodium chloride content in the final (wet) product was 2.5%. This salt content is slightly less than in other salty foods such as pickled herring (~3.5%), smoked ham

(~3.6%), feta cheese (~2.7%), and pickled olives (~5.6%) [36], and suggests that the algae isolate could bring flavor/saltiness to composite foods while at the same time delivering valuable protein and LC n-3 PUFA.

The amino acid pattern for the lysate and P2 agreed with that previously published by Brown for *N. oculata* [37]. *N. oculata* was found to be a good source of protein, as it exceeds the amounts of indispensable amino acids *per* total amino acids as recommended by the WHO for humans [38]. Particularly tryptophan and threonine were abundant: WHO recommends these proteins to be present at least at 0.6 and 2.3% of the total dietary amino acids, respectively [38]. Although tryptophan comprised only 1.8% of the total amino acids in all fractions and threonine 5.0%, this corresponds to triple and double the recommended percentages, respectively. Likewise, the fatty acid pattern agreed reasonably well with that determined previously for *N. oculata* [39]. With roughly 10% fatty acids per dry weight, of which almost 20% was the LC n-3 PUFA EPA, *N. oculata* is an excellent source of both high-value proteins and n-3 fatty acids. As part of the pH-shift process, the cell walls are disrupted, a step we believe greatly increases the bioavailability of nutrients, though future studies should investigate this issue closer. We see potential for using the product of the pH-shift processed *N. oculata* as a functional food and feed ingredient, although local legislation must be taken into consideration. We chose *N. oculata* not only because it is cultivated in large scale, but also because it is a safe organism to the best of our knowledge.

## 5. Conclusion

We have demonstrated that it is feasible to apply the pH-shift process to *N. oculata*. The product contained slightly elevated concentrations of proteins, total fatty acids and carbohydrate, while water and ash content were reduced. For the neutral and alkaline-process versions tested, the protein and fatty acid yields were over 80% and 70%, respectively. Thus, we propose the product of the pH-shift process on *N. oculata* as a functional ingredient high in protein and LC n-3 PUFA for both food and feed applications.

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