THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Fractionation of lipids and proteins from the microalga *Nannochloropsis oculata*

pH-shift process characterization and *in vitro* accessibility

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Abstract

To feed Earth's growing population, microalgae have been proposed as a source of protein and long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs). Currently, microalgae are mainly sold either as whole biomass, or the economically valuable LC n-3 PUFA fraction is extracted with organic solvents. As an alternative to solvent extraction, pH-driven protein solubilization in water followed by isoelectric precipitation, known as the pH-shift process, has been proposed to recover both lipids (*i.e.* oils) and functional proteins, while removing insoluble material.

In this project, the pH-shift process was applied to *Nannochloropsis oculata*, a marine microalga containing LC n-3 PUFA and essential amino acids in more than adequate amounts for human nutrition. It was hypothesized that the pH-shift process would render separate lipid and protein fractions. A pH-shift process for *Nannochloropsis* was developed based on documentation of its pH-dependent protein solubility: *Nannochloropsis* proteins were solubilized at pH 7, insoluble material was removed by centrifugation at 4 000×g and the proteins were then recovered by precipitation at pH 3. By using seawater, a process in which algal culture medium was used directly in the pH-shift process was simulated, thus reducing freshwater consumption.

Contrary to the hypothesis, the developed process did not result in two fractions, but in a combined product of 2.3% LC n-3 PUFA and 23% protein per dry weight, compared to 1.9% and 19% respectively in the initial material, suggesting that the product had potential as a functional food ingredient. However, nutrients need to also be accessible for uptake by the gastrointestinal tract. To assess the link between processing and accessibility of *Nannochloropsis* fatty acids and proteins, a static *in vitro* digestion model was applied to whole *Nannochloropsis*, and various products of the pH-shift process. The results indicated that whole *Nannochloropsis* cannot be digested by mammalian enzymes at all, hence the lipids and proteins cannot be absorbed. However, in cell-disrupted *Nannochloropsis ca.* 35% fatty acids and protein were hydrolyzed, with hydrolysis somewhat increased with further pH-shift processing.

This project indicates that the nutritional profile of *Nannochloropsis oculata* is favorable for human consumption, but that cell disruption is paramount to make the lipids and proteins accessible to the digestive enzymes. The pH-shift process applied here provided such cell disruption, slightly increased the concentration of proteins and lipid digestibility, and demonstrated a scalable process.

Keywords: microalgae, pH-shift process, acid, alkaline, solubilization, precipitation, lipids, proteins, n-3 polyunsaturated fatty acids, *Nannochloropsis oculata*, food processing, *in vitro* digestion.

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För att kunna förse jordens växande befolkning med näringsrik mat, så har mikroalger föreslagits som en källa till protein och långkedjiga fleromättade omega-3 fettsyror (LC n-3 PUFA). I dagsläget säljs mikroalger antingen som hel biomassa, eller som LC n-3 PUFA-rik olja efter extraktion med organiska lösningsmedel. Som ett mer miljövänligt alternativ till att använda lösningsmedel har den så kallade pH-skift processen föreslagits, för samtidig isolering av både lipider (olja) och proteiner. Processen är baserad på att proteinerna löses upp i vatten vid ett specifikt pH, varefter olösligt material avlägsnas och proteinerna fälls ut vid dess isoelektriska punkt genom att skifta pH't.

I detta projekt har pH-skift processen applicerats på *Nannochloropsis oculata*, en marin mikroalg som innehåller LC n-3 PUFA-rika lipider och proteiner rika på essentiella aminosyror som gör den väl anpassad som livsmedel. En processversion utvecklades baserat på proteinernas lösningsegenskaper vid olika pH. *Nannochloropsis*proteinerna löstes upp vid pH 7, olösligt material avlägsnades genom centrifugering vid 4 000×g, och proteiner fälldes sedan ut vid pH 3. Genom att använda havsvatten simulerades en process där själva algkulturen användes, vilket minskade sötvattensåtgången. Förhoppningen var att extraktionen skulle resultera i separata lipid- och proteinfraktioner, men extraktionen resulterade istället i en kombinerad produkt med 2.3% LC n-3 PUFA och 23% protein på torrviksbasis, jämfört med 1.9% och 19% i utgångsmaterialet. Denna sammansättning indikerar att produkten har potential som funktionell livsmedelsingrediens.

För att näringen från algerna ska vara till nytta för konsumenten krävs att de är tillgängliga för upptag i mag- tarmkanalen. Sambandet mellan processning och tillgängligheten av lipider och proteiner från *Nannochloropsis* undersöktes *in vitro* i en digereringsmodell. Resultaten visar att upptaget av dessa näringsämnen från hela algceller är obefintligt, vilket antyder att nedbrytning av *Nannochloropsis* inte sker i mag- tarmkanalen hos människan. Från de sönderdelade cellerna var däremot ca 35% av proteinerna och fettsyrorna hydrolyserade, med en ytterligare ökning efter fortsatt pH-skift process.

Detta arbete visar att den näringsmässiga sammansättningen av *Nannochloropsis oculata* gör att denna alg passar som livsmedel, men att cellerna måste sönderdelas för att tillgängliggöra lipiderna och proteinerna. pH-skift processen som appliceras här inkluderar en sådan sönderdelning och innefattar steg vilka underlättar för uppskalning. Processen ökade koncentrationen av protein och tillgängligheten av lipider från algbiomassa; slutprodukten visar god potential för framtida applikation som livsmedel.

List of publications

This dissertation is based on the work described in the following papers:

Paper I Lillie Cavonius, Eva Albers and Ingrid Undeland. **pH-shift processing of *Nannochloropsis oculata* microalgal biomass to obtain a protein-enriched food or feed ingredient**. *Algal Res,* 2015. **11**: p. 95-102.

Paper II Lillie Cavonius, Eva Albers and Ingrid Undeland. ***In vitro* bioaccessibility of proteins and lipids of pH-shift processed *Nannochloropsis oculata* biomass**. *Submitted manuscript.*

Paper III Lillie Cavonius, Nils-Gunnar Carlsson and Ingrid Undeland. **Quantification of total fatty acids in microalgae: comparison of extraction and transesterification methods**. *Anal Bioanal Chem,* 2014. **406**: p. 7313-1722.

Paper IV Lillie Cavonius and Nils-Gunnar Carlsson. **Fatty acid contaminations originating from commercially available solid-phase extraction columns**. *Chem Sci Rev Lett,* 2015. **16**: p. 1107-1110.

Published papers not included in this dissertation

Karin Larsson, Lillie Cavonius, Marie Alminger, Ingrid Undeland. **Oxidation of cod liver oil during gastrointestinal in vitro digestion**. *J Agri Food Chem,* 2012. **60**: p. 7556-7564.

Lillie Cavonius, Helen Fink, Juris Kiskis, Eva Albers, Ingrid Undeland and Annika Enejder. **Imaging of lipids in microalgae with coherent anti-Stokes Raman scattering microscopy**. *Plant Physiol,* 2015. **167**: p. 603-616.

The author’s contributions

Paper I — The author, LRC, was involved in the study design, carried out the lab work, was involved in the interpretation of the data and was responsible for writing the manuscript.

Paper II — LRC, was involved in the study design, carried out the lab work, was involved in the interpretation of the data and was responsible for writing the manuscript.

Paper III — LRC, was involved in the study design, carried out the lab work, was involved in the interpretation of the data and was responsible for writing the manuscript.

Paper IV — LRC, was involved in the study design and the lab work, interpreted the data and wrote the manuscript.

Abbreviations

DHA	docosahexaenoic acid
EFSA	European Food Safety Authority
EPA	eicosapentaenoic acid
LC n-3 PUFA	long-chain n-3 polyunsaturated fatty acid
SDS	sodium dodecyl sulfate
SPE	solid phase extraction

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1. Introduction

Our planet with its limited resources faces a considerable challenge: in the next decade alone, the global human population is expected to increase by 1 000 000 000 individuals[1]. We humans are challenged to find ways of feeding and increasing the affluence of everyone. To survive, we need energy, building blocks for our own cells and various micronutrients. Fish is an excellent source of energy, proteins and micronutrients including long-chain n-3 polyunsaturated fatty acids (colloquially "omega-3s", here abbreviated "LC n-3 PUFAs")[2]. LC n-3 PUFAs are in demand for their health effects, including maintenance of cardiovascular health[3]. LC n-3 PUFAs are consumed by humans in the form of fish, dietary supplements and functional foods[4]. However, the largest consumer of LC n-3 PUFA-rich fish oil is aquaculture: most aquacultured species require LC n-3 PUFAs in their diet, which originates from captured, wild fish[5]. In 2014, 29% of the marine fish stocks were fished in a non-sustainable manner, further threatening the livelihood of humans[5] and by extension, their very existence.

Instead of emptying the oceans of fish, microalgae have been proposed as a way of converting sunlight and carbon dioxide into biofuels, feed, and food ingredients[6], including proteins and LC n-3 PUFAs. However, few species of algae are currently consumed as they are: many species are surrounded by cell walls which cannot be degraded by human digestive enzymes, leaving nutrients in the interior of the algae inaccessible[7]. The microalgae which are currently cultured for their LC n-3 PUFAs *e.g.* for use in infant formulas, are usually extracted to render a pure oil fraction, rich in LC n-3 PUFAs[8]. Although industrial extraction procedures are proprietary, it is likely that methods rely heavily on organic solvents. In keeping with the aim of finding more sustainable solutions, the herein presented work investigates a water-based separation process – known as the pH-shift process – on a microalga, enabling the recovery of a functional food or feed ingredient.

The pH-shift process is patented for the recovery of animal protein: i) proteins are solubilized in water at either high or low pH, ii) insoluble material (such as skin and bones) is removed by centrifugation and iii) proteins are precipitated at their isoelectric point[9, 10]. One of the advantages of the pH-shift process is that it is mild and leaves the protein in a state where it has good techno-functional properties, which is to say that it *e.g.* has good gelling properties or good emulsification properties[9]. In contrast, when hot organic solvent is applied to biomass to extract oils, proteins tend to denature irreversibly, resulting in poor techno-functional properties. The pH-shift process may also be applied to biomass to recover crude oil: when the biomass contains enough fat, the first centrifugation may render a floating oil layer[9].

Thus, we hypothesized that the pH-shift process could be applied to microalgae to recover oil rich in LC n-3 PUFA and then a separate protein fraction.

This work details the development of a pH-shift process on *Nannochloropsis oculata* (in the following referred to simply as *Nannochloropsis*), the characterization of the process's product and the accessibility of the nutrients in an *in vitro* digestion model. An overview of the four studies included in this thesis is given in **Figure 1** and the specific aims follow in chapter 2. Chapter 3 presents a background to microalgae: their celebrated diversity and proposed applications including human food, with special emphasis on microalgae as a source of dietary lipids and proteins. Chapter 4 introduces the pH-shift process in general and explains the pH-shift process as developed on *Nannochloropsis* and published in Paper I. Having developed a pH-shift process for *Nannochloropsis*, the product was characterized in regard to lipids, proteins, carbohydrates, water, ash and color. Although the product showed potential as a functional food ingredient, the question remained whether the nutrients were accessible for uptake by the human digestive system. Chapter 5 details the *in vitro* digestion model applied to *Nannochloropsis* in various stages of the pH-shift process, as reported in Paper II. In this study, the necessity of breaking open the algal cells was demonstrated: when whole algae passed through the model, neither lipids nor proteins were digested, while the lipids and proteins of pH-shift processed cells (broken) showed good digestibility. For the reader interested in methods, the analytical tools used to assess lipids and proteins in Papers I and II are described in chapter 6 and include those published in Papers III and IV. For the impatient reader, the major findings of Papers I-IV can be found in chapter 7, and the future outlook in chapter 8.

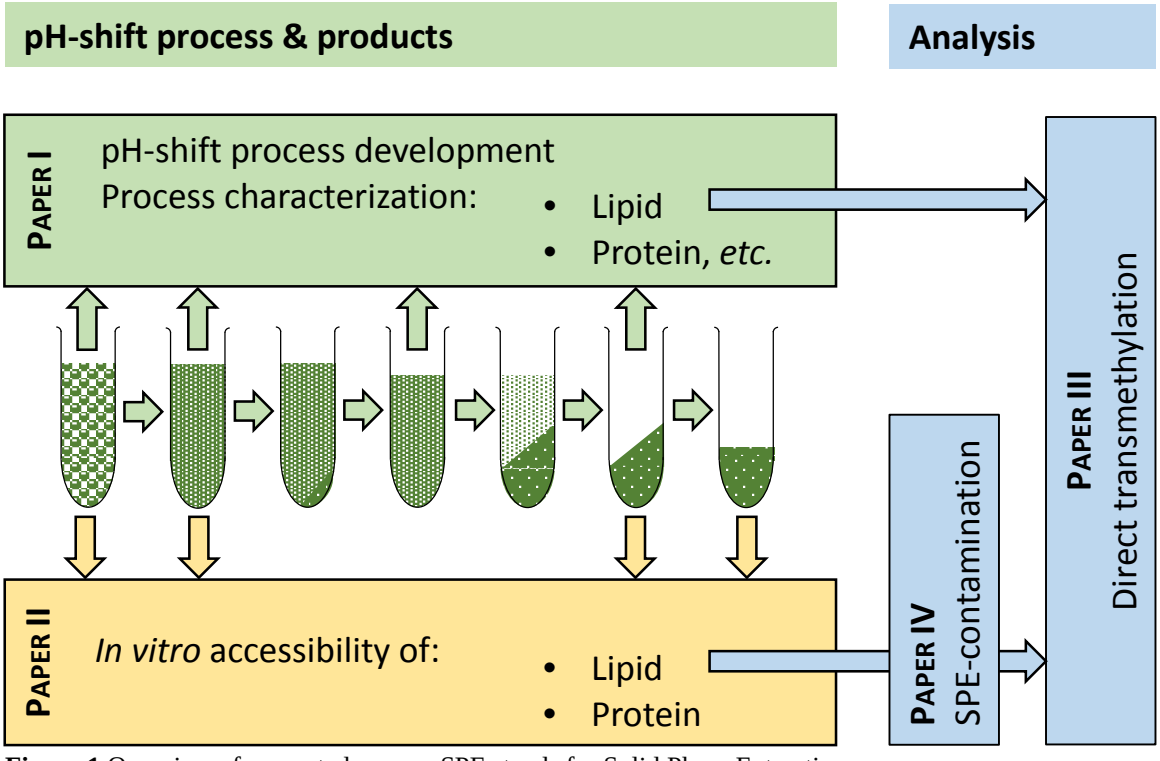

Figure 1 Overview of presented papers. SPE stands for Solid Phase Extraction.

2. Aims

The overall aim of this project was to process microalgal biomass into a nutritious and digestible food ingredient, without the application of organic solvents. Considering the retail value of food and feed, processing steps need to be scalable, *i.e.* it is an advantage if the applied unit operations are already present in large-scale food processing. One process fitting the requirements of using non-toxic solvents, economic viability, scalability and nutritional feasibility, is the pH-shift process. The pH-shift process was applied and modified in the present work to fractionate whole *Nannochloropsis oculata* biomass. To achieve the overall objective, investigations were carried out to:

- Map the pH-dependent solubility of *Nannochloropsis* proteins in seawater (addressed in Paper I).
- Use the protein solubility curves to establish a pH-shift process for *Nannochloropsis* biomass (Paper I).
- Characterize the fractions obtained by the pH-shift process in respect to total fatty acids, protein, total carbohydrates and residual ash (Paper I).
- Investigate how the pH-shift process influences the *in vitro* accessibility of *Nannochloropsis* proteins and lipids (Paper II).
- Establish an analytical approach to quantify fatty acids in different LC n-3 PUFA-containing microalgal strains (Paper III).
- Determine the source of analytical contamination noted during solid phase extraction-based separation of lipid classes (Paper IV).

3. Microalgae and their potential in human nutrition

3.1. Microalgae: general definition and potential applications

The term “microalgae” can be drawn wide enough to include both cyanobacteria and several divisions of eukaryotic organisms[11-14]. Generally, microalgae are in the size range of several µm[15] and grow as single cells or form simple colonies[11, 16]. Here, microalgae will be defined as small organisms, which are either capable of photosynthesis or closely resemble photosynthetic organisms[12, 14]. The first photosynthetic microalgae, cyanobacteria, appeared roughly 2.8 billion years ago[14, 17] and – in the time since – have evolved to inhabit virtually every ecosystem on the planet: seawater and freshwater, hot springs, desert sands, snow and ice have all been found to harbor microalgae[14]. Although their small size implies that microalgae usually go unnoticed by humans in everyday life, these organisms have profound impact on the planet, contributing roughly half of the atmospheric oxygen and forming the prolific base of the aquatic food web, while ancient microalgae provide fossil fuels and thus a cornerstone of the economy[15].

Microalgae are a diverse group of organisms. They exist in unicellular form or simple colonies but do not form differentiated organs (*e.g.* the roots and leaves of land-based plants), though some cyanobacteria grow in simple chains which contain cells dedicated to nitrogen-fixation[14, 16]. Some microalgae appear as single-cell plants, complete with a cell wall and chloroplast, harnessing light energy to fix carbon (**Figure 2**), while other microalgae are motile and ingest organic material to supply their main energy and carbon requirements[14]. To harvest light, the various species of microalgae employ a variety of pigments consisting of different types of chlorophyll, phycobilins, carotenes and xanthophylls[14]. Once the light energy has been stored in chemical bonds, microalgae may store excess energy in different molecules including various polysaccharides[14] and lipids[11, 18]. To add to the already bewildering diversity of microalgae, these organisms may go through different morphologies according to their life cycle and environmental conditions[19, 20] and surround themselves with a variety of different barriers: naked cell membranes, mucilages, scales of organic or inorganic material, silica frustules, or rigid, multi-layered cell walls[16]. Given their diversity and the fact that they are adapted to many different conditions, microalgae have been proposed as solutions to various challenges.

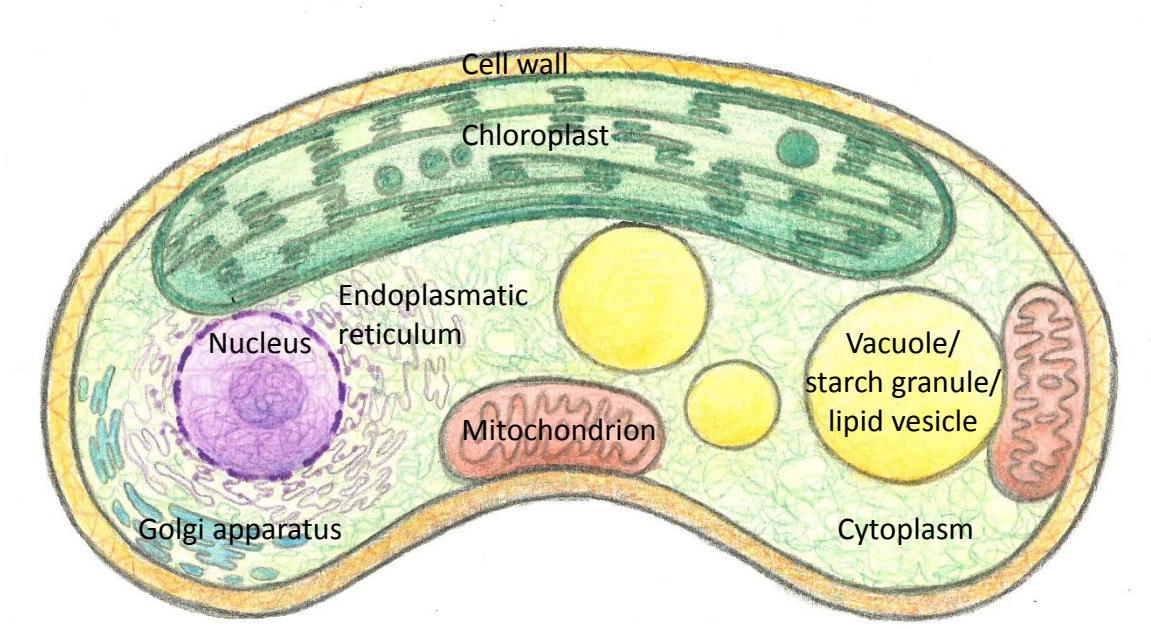

Figure 2 General cell structure of a eukaryotic microalga (based on [21-23])

Some of the global challenges facing humanity in which microalgae have been proposed as a solution follow below:

- Capture of carbon dioxide to reduce atmospheric greenhouse gases, *e.g.* in connection to coal-fired power plants and cement-production industry
- Wastewater remediation, *i.e.* removal of nitrogen and phosphorus from sewage[24]
- Biofuel production such as biodiesel[11, 18, 24, 25], bioethanol[26, 27] and hydrogen[28], thereby closing the carbon cycle instead of relying on fossil fuels
- Feed production, *e.g.* for aquaculture and land-based animal husbandry[29-32], reducing pressure on fish stocks and freeing up agricultural land used for feed production
- Food production[30, 33-35] and fortification[36, 37]
- Production of nutraceuticals such as LC n-3 PUFA, vitamins, pigments and antioxidants[38-47]
- Production of vaccines and cosmeceuticals[6, 48-52]
- … and many other applications[53-62]

From low-value, high volume applications (such as biofuel production), to high-value, low volume applications (such as pharmaceutical production), microalgae evoke sustainable solutions to a wide range of current issues.

3.2. Microalgae in human nutrition

Certain microalgal species are consumed by humans as food. Historically, the cyanobacteria of *Arthrospira* species have been eaten after being gathered from lakes in present-day Mexico and Chad; also, various species of *Nostoc* (*sphaeroides, flagelliforme*, and *commune*) found growing on land in Asian countries were – and still are – part of local diets[16, 63, 64]. Current large-scale cultivation of whole microalgal biomass for human consumption is dominated by *Arthrospira* and *Chlorella*[16, 57, 65]. Additionally, *Dunaliella* and *Haematococcus* are cultivated for β-carotene and astaxanthin, respectively[50, 65, 66]. Various species are also cultivated heterotrophically (*i.e.* they are cultured in dark fermenters) for docosahexaenoic acid (DHA), which is added to infant formula and other foods[67-70]. Generally, studies suggest that certain microalgae could be processed into food or food ingredients for human consumption based on i) the amino acid profile and ii) the presence of LC n-3 PUFAs. The amino acid profiles of microalgae are often found to contain higher amounts of essential amino acids than most land plants; in other words microalgal amino acid profiles can be comparable to those of soy or egg [7, 71]. As for the fatty acids, some microalgae are capable of producing the LC n-3 PUFAs, notably eicosapentaenoic acid (EPA) and DHA, which land plants cannot synthesize, and which are associated with certain health benefits. Apart from energy, well-balanced protein and LC n-3 PUFAs, microalgae can provide *e.g.* minerals and micronutrients[50].

Some microalgae are added to conventional foods in attempts to create functional foods. Functional foods lack a legal definition in Europe, but can be interpreted as a food (natural or processed) with clinically proven and documented health benefits beyond basic nutritional effects[72-74]. In Europe such foods for which a health claim is made, must conform to the European Food Safety Authority's (EFSA's) regulation. Several accepted health claims apply to nutrients found in microalgae, *e.g.* EPA and DHA[3]. However, studies on adding microalgae to foods are often focused on consumer acceptance and technical properties[36, 37, 75-79], while ignoring if the product provides true nutritional benefits. For example, one study added *Spirulina* to ice cream, replacing as much as half of the stabilizer (glycerol monostearate) and increasing the iron content of the ice cream[76]; however, since the amount of *Spirulina* added only comprised 0.15% of the ice cream in total, the ice cream was still not a good source of iron: roughly 10 liters of ice cream would be needed to reach the recommended daily intake of iron. Furthermore, the form of the iron and its bioaccessibility was not assessed. So, in spite of enthusiasm for microalgae, there is a major gap between adding microalgae to food and creating a functional food.

3.2.1. Lipids

Lipids are a heterogeneous group of molecules, defined by their extractability into non-polar solvents and their limited solubility in water[80, 81]. Lipids are often divided into three classes[82]: i) fatty acids, which are part of most neutral and polar lipids, ii) neutral lipids, in which the entire molecule is hydrophobic and iii) polar lipids, which have hydrophilic and hydrophobic regions within the same molecule. While all lipid classes can be found in microalgae, some species of microalgae are adept at storing excess energy in the form of neutral lipids[83].

3.2.1.1. Fatty acids

A fatty acid is a hydrocarbon chain with a –COOH group (carboxylic acid) at one end[80], as illustrated in **Figure 3**. As such, fatty acids are relatively uncommon in their free form in nature, but are an important component of most triacylglycerols and polar lipids (discussed below). Fatty acids exist at various lengths. Generally, the fatty acids present in plant and animal triacylglycerols, are 12-20 carbons long, with an even number of carbons[80, 82]. In this dissertation, fatty acids longer than 18 carbons are referred to as "long chain". Most fatty acids are linear (*i.e.* they do not branch), though kinks can be present in the chain as a result of double bonds[80]. Fatty acids with double bonds are referred to as unsaturated fatty acids; a single double bond results in a monounsaturated fatty acid, and further desaturation results in a polyunsaturated fatty acid[22, 84]. Contrary to the convention of organic chemists, who number the carbon atoms starting at the acidic head group, those who are interested in the physiological effects of fatty acids, start counting at the methyl terminus when describing the position of the double bond: a fatty acid with a double bond between the third and fourth carbon from the end is referred to as an n-3 fatty acid[85, 86]. Similarly, fatty acids in which the first double bond occurs between the sixth and seventh carbon from the end, are n-6 fatty acids, with distinct physiological properties from n-3 fatty acids[85]. Fatty acids are often referred to in shorthand, *e.g.* "C18:1 n-9" with the numbers indicating (from left to right) the number of carbon atoms (18), the number of double bonds (1) and the location of the first double bond from the methyl end (carbon 9).

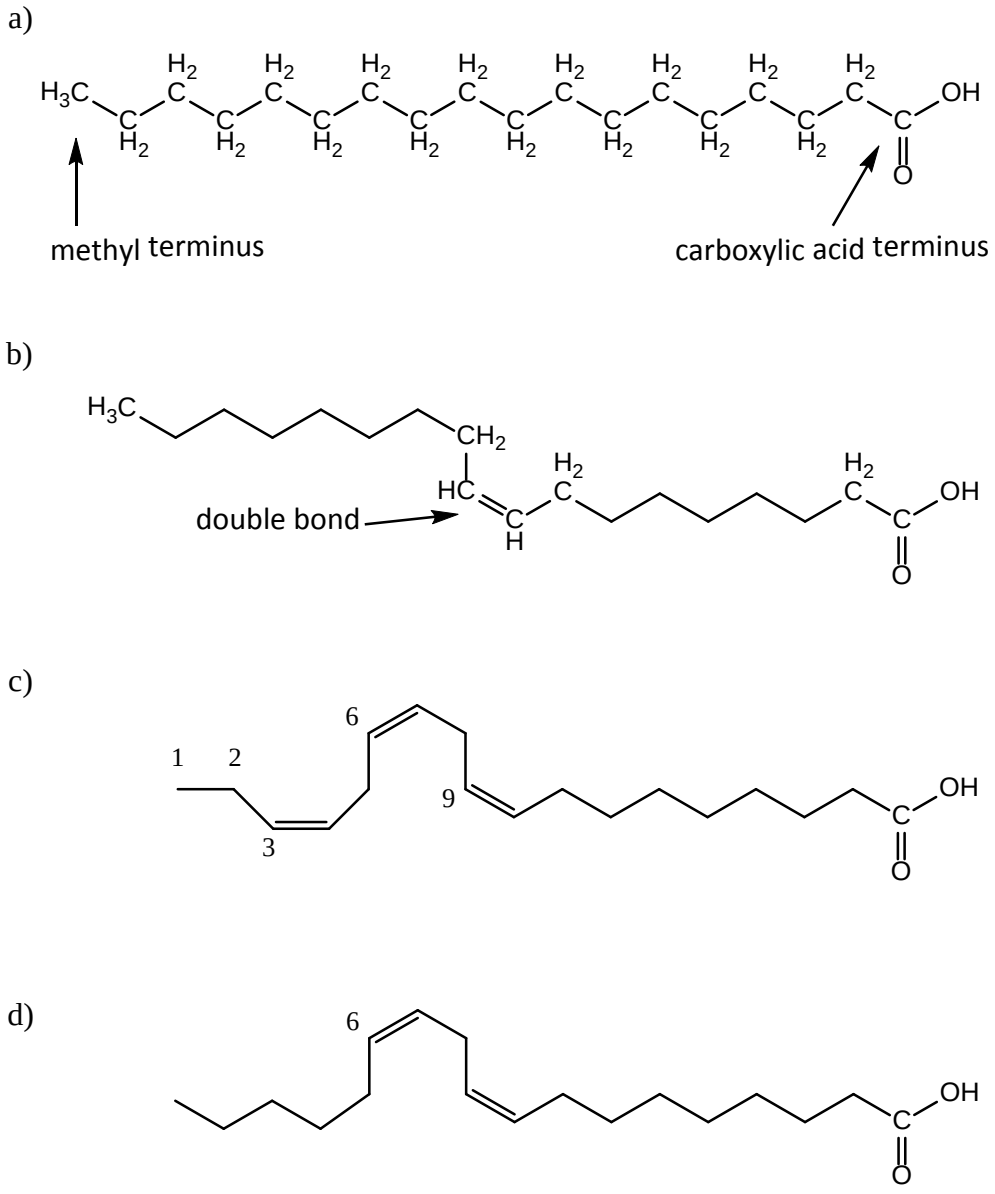

Figure 3 a) stearic acid, C18:0, a saturated fatty acid, with all carbon and hydrogen atoms shown; b) oleic acid, C18:1 n-9, a monounsaturated fatty acid, presented in a reduced structure with only key carbon and hydrogen atoms shown; c) α-linolenic acid, C18:3 n-3, a polyunsaturated n-3 fatty acid, shown in an attenuated structure; d) linoleic acid, C18:2 n-6, a polyunsaturated n-6 fatty acid.

3.2.1.2. Neutral lipids

The vast majority of lipids found in food are neutral lipids in the form of triacylglycerols[22, 80, 81]: three fatty acids esterified to glycerol form an excellent molecule in which to store energy, as these can be packed densely as an intracellular lipid droplet[87], see **Figure 4**. Triacylglycerols are found as constituents of many foods, such as meat, nuts and cheese or are present in nearly pure form *e.g.* in various oils and fats such as olive oil, butter and lard[81]. Though microalgae are not necessarily considered to be food, some species are known to contain 20-50% of the cells' dry weight as triacylglycerols[83].

Another type of lipid molecule for which algae are known are pigments, a small but visible fraction. Carotenoids are pigments synthesized mainly in plants and algae as part of their light-harvesting complexes and serve not only to broaden the spectrum which can be absorbed by the chloroplasts[12], but also have a photoprotective function, by scavenging free radical species[50, 81]. Carotenes can be considered neutral lipids since they contain only carbon and hydrogen atoms. Carotenes are one type of carotenoid, the other type of carotenoid is xanthophylls. Xanthophylls are structurally very similar to carotenes but contain oxygen atoms, making them slightly polar. Although they are not necessarily true neutral lipids, carotenoids and other pigments often partition with the bulk neutral lipid during lipid extraction.

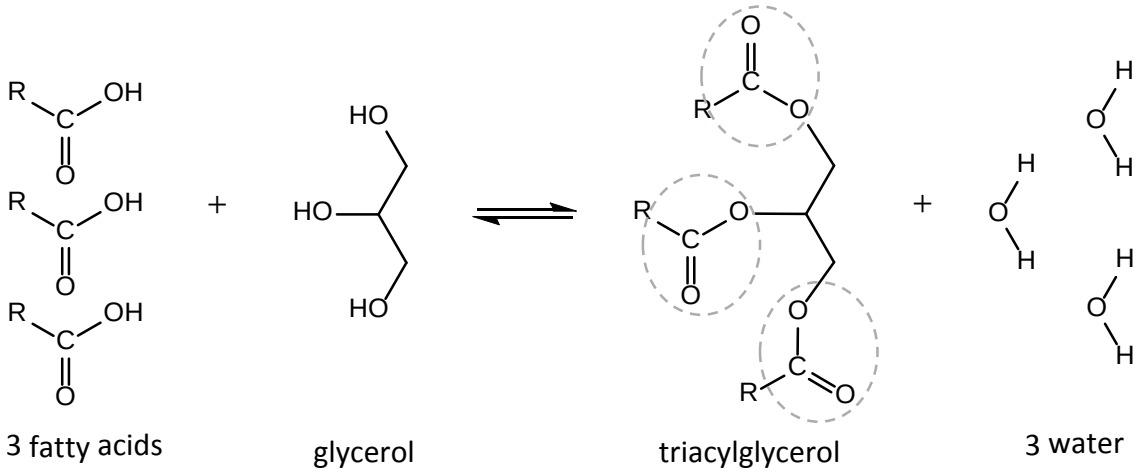

Figure 4 Three fatty acids condense with glycerol to form a triacylglycerol, the main constituent of fats and oils, with three molecules of water formed in the reaction. The ester bonds formed in the reaction are highlighted. R stands for the fatty acid acyl chain and can be different for all three fatty acids.

3.2.1.3. Polar lipids

Polar lipids are a diverse group of molecules, but share the characteristic that there are hydrophilic and hydrophobic regions within the same molecule, *i.e.* they are amphiphilic[88], see **Figure 5**. Separating the aqueous environment of each cell from the surrounding environment is a lipid bilayer, composed of polar lipids arranged so that polar head groups are in contact with water while the hydrophobic tails associate to form a water-repelling barrier[88]. Many polar lipids contain a glycerol backbone, with up to two fatty acids esterified to the backbone, forming a hydrophobic tail[81]. Esterified to the third position of the glycerol is a hydrophilic head group. The hydrophilic head group may be charged, as is the case in phospholipids, or highly water-soluble, such as a sugar

moiety in the cases of glyceroglycolipids[88]. Glyceroglycolipids, such as monogalactosyl-diacylglycerol and digalactosyldiacylglycerol, are of special interest in this work, since they are lipids found in plants and microalgae as part of their photosynthetic membranes[89-91].

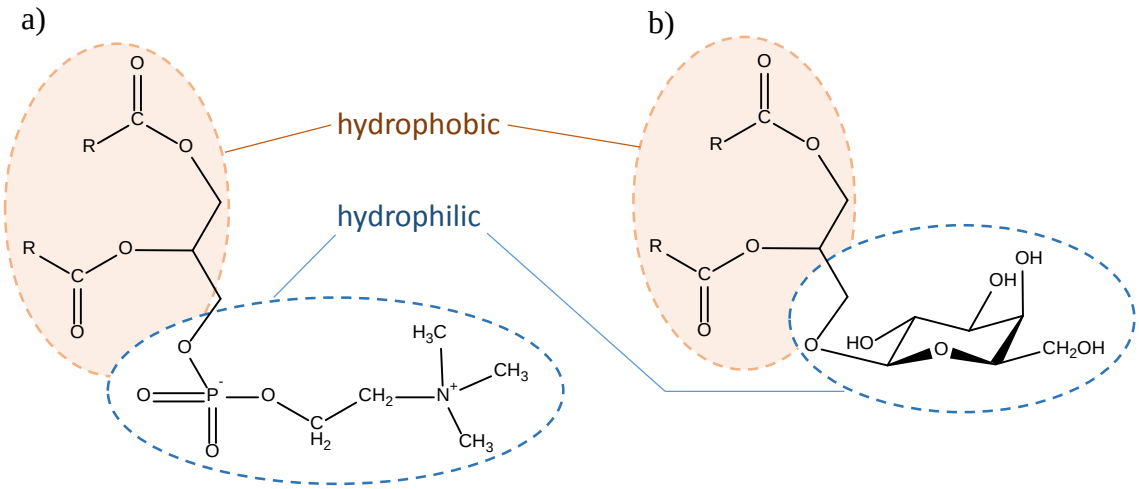

Figure 5 a) Phosphatidylcholine, an example of a phospholipid. Note that both the phosphorus and the nitrogen atom carry a charge, making that part of the molecule hydrophilic, while the two fatty acid residues are hydrophobic. b) monogalactosyldiacylglyerol, an example of a glyceroglycolipid which, analogous to the phospholipids, has a two hydrophobic fatty acid residues and a hydrophilic sugar moiety.

3.2.1.4. LC n-3 PUFA-production by microalgae

Various genera of microalgae have been investigated for their production of LC n-3 PUFAs, see **Table 1**. Most of the listed genera grow photoautotrophically, *i.e.* they use sunlight as their energy source and carbon dioxide as their carbon source[17]. Since fixing carbon in itself requires energy and the microalgae tend to shade each other, there have been attempts to boost the LC n-3 PUFA yield and/or productivity by culturing some species heterotrophically[92], *i.e.* in dark fermenters to which an organic carbon source such as sugar is supplied[93-96]. Although this approach has proven successful for *Crypthecodinium cohnii*[39, 70], it has not been successful in all cases, possibly because the LC n-3 PUFAs are part of the photosynthetic membranes of some species[90, 97], a fraction which can be expected to be reduced under heterotrophic fermentation. Other strategies for increasing the LC n-3 PUFA yield or productivity include optimization of any or several of the following: light intensity and length of the light cycle[98-101]; temperature[100-105]; rate of carbon dioxide supply[98, 105]; source and concentration of various nutrients[104] (including nitrogen[100, 102, 105, 106], phosphorous[100, 106, 107], sulfur[106] and silica[106]); pH of the medium[108]; salt concentration of the medium[105, 108]; and dilution rate in continuous cultures[98, 109, 110]. Currently, no single strategy is known to increase the yield or productivity of LC n-3 PUFA in all microalgae; instead culture conditions need to be tailored to each species.

Table 1 Some microalgae investigated for production of EPA and/or DHA. The yield is given in fatty acids per liter of culture; n.d. stands for no data; note that culture conditions vary between references.

Genus	**EPA %** (of dry biomass)	**DHA %** (of dry biomass)	**EPA yield** (mg/L)	**DHA yield** (mg/L)	**Reference(s)**
Amphidinium sp.	0.4-2.2	0.3-2.4	4.0-8.3	2.0-4.4	[95, 107, 111]
Aplanochytrium sp.	n.d.	n.d.	0	1	[96]
Asterionella sp. (tentative)	0-0.5	0-0.2	n.d.	n.d.	[111]
Aurantiochytrium spp.	n.d.	n.d.	1-2	14-18	[96]
Biddulphia sinensis	1.8	0	3.0	0	[107]
Chaetoceros spp.	0-2.4	0-0.4	0-4.2	0	[112-115]
Chlorella spp.	0-4.4	0	0-37	0	[42, 100, 107, 112]
Chroococcus sp.	0	0	n.d.	n.d.	[116]
Chroomonas salina	<0.1	0.3	<1	0.7	[69, 73]
Coccolithus huxleyi	1.5	0	3.8	0	[107]
Criccosphaera carteri	2.0-2.2	0	3.7-4.4	0	[107]
Crypthecodinium cohnii	0	0.8-1.3	0	15-20	[112]
Cryptomonas sp.	0.2-1.6	<0.1-0.6	<1-3.8	<1-2.0	[69, 70, 73]
Dunaliella spp.	0-1	0	0-14	0	[100, 107, 111, 115]
Emiliania huxleyi	2.4	0	5.1	0	[70, 76]
Fragilaria familica	1.3	<0.1	n.d.	n.d.	[113]
Heteromastrix rotunda	1.8	<0.1	3.1	<1	[107]
Isochrysis sp.	0-0.9	0-4.7	<1-8.0	0-40	[73, 75-79, 115, 117, 118]
Monochrysis lutheri	2.1	0	4.7	0	[107]
Monodus subterranaeus	0-3.8	0	0-96	0	[112]
Nannochloropsis spp.	0-8.2	0-0.7	0-14	0-2.6	[42, 107, 112, 116, 118-120]
Nitzschia spp.	1.1-1.5	n.d.	n.d.	n.d.	[92, 100]
Oblongichytrium	n.d.	n.d.	0-3	1-4	[96]
Oocystis sp.	0.1	0	n.d.	n.d.	[116]
Pavlova sp.	<0.1-3.2	0-1.3	<1-4.0	0-1.2	[42, 98, 107, 112-116, 119]
Phaeodactylum tricornutum	0.2-5.5	0-0.4	1.3-130	0-11	[42, 107, 112, 115, 116, 119]
Porphyridium spp.	<0.1-3.8	0	<1-69	0	[42, 100, 107, 112, 116]
Prorocentrum spp.	<0.1-0.6	0.2-0.8	<1-2.3	<1-1.5	[71, 73]
Prymnesium parvum	<0.1	<0.1-0.2	n.d.	n.d.	[111]
Pseudokirchneriella sp.	0	<0.1	n.d.	n.d.	[116]
Pseudopedionella sp.	2.5	<0.1	4.9	<1	[107]
Rhodomonas sp.	0.4-1.4	0-0.6	1.8-7.8	<1-4.0	[116, 118]
Schizochytrium aggregatum	1.0-1.2	0-0.3	6.1-9.3	0-<1	[112]
Synechococcus sp.	0	0	n.d.	n.d.	[100, 116]
Tetraselmis spp.	0.2-0.5	<0.1	<1-5.1	0	[113, 116, 118]
Thalassiosira spp.	0.3-1.7	0.1-0.5	<1-1.2	<1	[112, 113, 115, 119, 121]
Thraustrochytrium spp.	0.3	0.5-1.0	0-3	1-16	[72, 73, 95]
Tribonema sp.	0.3	0	n.d.	n.d.	[116]

3.2.1.5. Health effects of LC n-3 PUFAs

Although humans are capable of *de novo* fatty acid-synthesis (therefore, excess energy from sugary foods is stored as fat)[84], we cannot form n-6 and n-3 fatty acids. Human enzymes cannot insert a double bond at the n-6 and n-3 positions and are also inefficient at elongating 18-carbon fatty acids[85, 122-124]. The efficiency of conversion of n-3 PUFA (α-linolenic acid, shown in **Figure 3**) to LC n-3 PUFAs (such as EPA and DHA shown **Figure 6**) is further reduced by the presence of n-6 fatty acid (linoleic acid, **Figure 3**), which competes for the same enzymes[125, 126]. The ratio of n-6:n-3 PUFAs in a standard Western-style diet is considered to be high (about 16:1) and is implicated in many common diseases; ratios between 1:1 to 1:4 have been suggested to be beneficial in the prevention of the implicated diseases[47, 127-132]. There are several suggested mechanisms for the physiological effects of LC n-3 PUFAs, including alteration of membrane fluidity, alteration of gene transcription and the formation of signaling molecules, eicosanoids[47, 85, 122, 123, 125, 127, 129, 133, 134]. The eicosanoids are involved in the processes of inflammation and its resolution, vasodilation, platelet aggregation, pain and fever[85, 122, 123, 129, 135]. Since the listed processes are part of many human disorders, LC n-3 PUFAs – or lack thereof – may be involved in a plethora of diseases[134]. Amongst the abundance of health effects attributed to LC n-3 PUFAs, the European Food Safety Authority, with its stringent demand for documentation, recognizes only a few health claims for LC n-3 PUFAs[3]:

- EPA and DHA contribute to the normal functioning of the heart (at 250 mg/day) and normal blood triacylglycerol levels (2 g/day) and maintenance of normal blood pressure (3 g/day)[3]
- DHA contributes to the maintenance of normal brain and eye function in adults (250 mg/day)[3]
- DHA contributes to the normal development of brain and eye in fetuses (maternal intake 350 mg/day) and infants (100 mg/day)[3]

Given the health effects of LC n-3 PUFAs and the inefficiency with which they are formed in humans, many consumers are eager to get 250 mg dietary LC n-3 PUFAs daily, as recommended by EFSA[136].

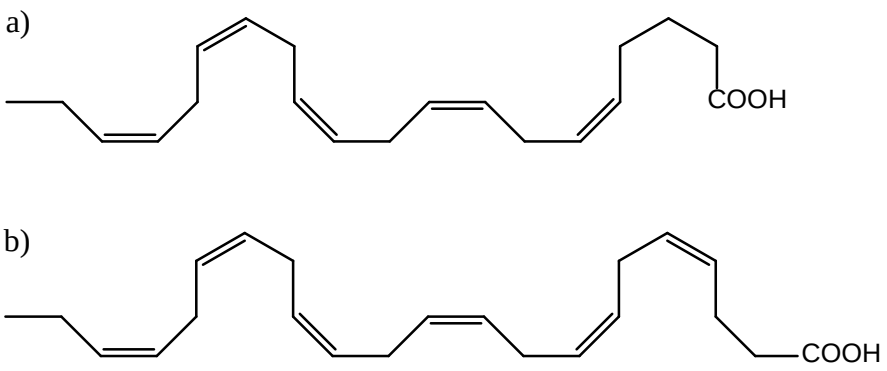

Figure 6 The structure of LC n-3 PUFAs: a) EPA b) DHA

3.2.2. Proteins

Apart from lipids, proteins are the other major nutrient which make microalgae an interesting source of human nutrition: some species of microalgae contain all essential amino acids in proportions as they are required by humans, unlike most crop plants which tend to be deficient in at least one amino acid[137].

3.2.2.1. Amino acids and peptides

Amino acids are small molecules with a central carbon atom which in most cases connects i) a hydrogen atom, ii) a carboxylic acid group, -COOH, iii) an amino group, -NH_2 and iv) a side chain –R [88], see **Figure 7**. The side chain gives each amino acid its unique physiochemical properties. For example, the branched hydrocarbon side chain of leucine is hydrophobic, while the alcohol group of serine is hydrophilic[88]. Humans can synthesize roughly half of the amino acids, while the so called essential amino acids must be provided in the diet[88]. The carboxylic acid and amino group of two amino acids can form a covalent bond, known as a peptide or amide bond, forming a dipeptide; adding a third amino acid results in a tripeptide, *etc.*[88]. In summary, individual amino acids can form links to other amino acids, with the side chains conferring unique properties on the polypeptide.

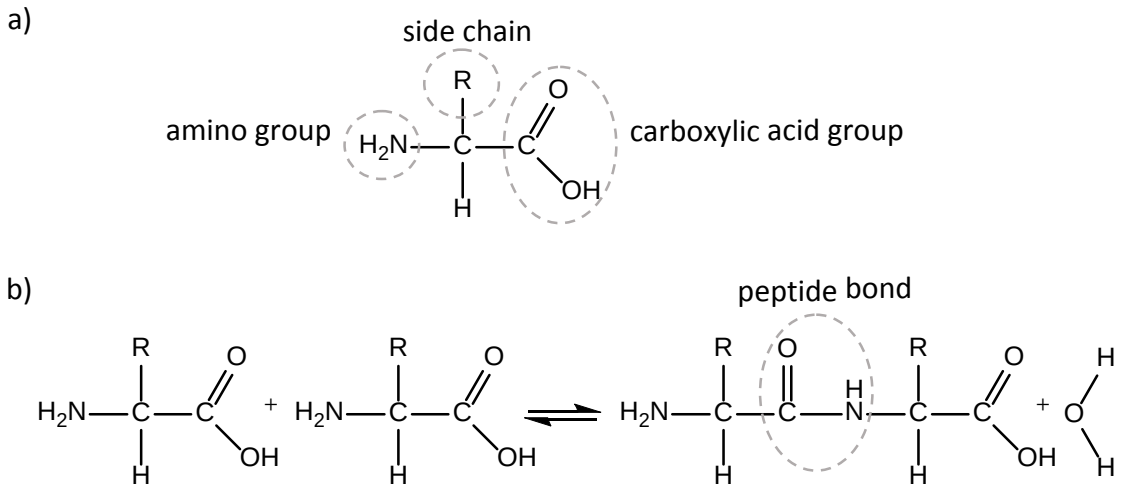

Figure 7 a) the general structure of an amino acid, where R can be any of *ca.* 20 side-chains; b) two amino acids condense to form a dipeptide; the peptide bond is also known as the amide bond.

3.2.2.2. Proteins

Proteins are long polymers of amino acids. The different properties of the amino acids' side chains give proteins an amphiphilic character. Interactions of side chains of similar character (in the case hydrophobic interactions) or complimentary character (in the case of electrostatic interaction) along the length of the polypeptide cause proteins to fold into complex three-dimensional structures[88]. A protein's tertiary structure is key to its biological function, *e.g.* enzymatic, structural, signaling,

etc.[88, 138]. Change to the native conformation of a protein is known as denaturation and can be induced by heat, pressure and shear, extremes in pH, and the binding of detergents or small molecules such as urea[88]. When denaturation is irreversible, such as when an egg is boiled, it results in permanent loss of biological function[88]. However, in the context of food, denaturation can be desirable, *e.g*. by inactivating protease inhibitors (a group of anti-nutrients found in legumes) or increasing the emulsification properties of proteins[138].

3.2.2.3. Proteins in microalgae

Relatively little is known about microalgal proteins, contrary to the comparatively advanced knowledge on plant, human and microbial proteins[139]. As for other organisms, the synthesis of specific proteins depends on the physiological state of the cell, *e.g.* the amount of available nitrogen[140]. According to Schwenzfeier *et al*. (2011), microalgae do not accumulate distinct storage proteins, but rather contain many different types of protein[141]. Since microalgae are phylogenetically diverse, it is difficult to generalize about their proteins. However, studies of various microalgae mention proteins which probably apply to most cells, namely protein involved in catalysis, signal transduction, structure, movement and electron transfer as well as transporters and chaperones[142, 143]. As for RuBisCO, an important enzyme of carbon-fixation which can dominate the protein profile of some plants, investigated microalgae have not been found to contain an overwhelming amount of RuBisCO, *i.e.* less than 6% of the total protein[144]. In the thermophilic microalga *Galdieria sulphuraria* the proteins were reported to be strictly associated with polysaccharides, hindering enzymatic protein extraction[34]. Unlike muscle protein, which is dominated by myofibrillar proteins which form structures of the sarcomere filaments[145], microalgae are likely to be dominated by globular and membrane-associated proteins.

3.2.2.4. Protein nutritional quality

Different sources of protein have different nutritional quality. For protein to be considered high-quality, it must contain the essential amino acids at or above concentrations as determined by the World Health Organization[146] and a digestibility equal or greater to that of milk protein or egg whites[138]. While protein from animal sources generally contains essential amino acids in concentrations corresponding to the reference values, many plants lack at least one amino acid, *e.g.* cereals have low amounts of lysine[137, 138]. Digestibility describes how much of the food's nitrogen is absorbed relative to the total nitrogen[138]. Digestibility of animal protein tends to be high (> 90%), while the digestibility of protein from plant sources ranges from high (96% for wheat flour) to low (70% for corn cereal). Factors which decrease the digestibility are i) processing (which can either be detrimental, as in the case of the Maillard reaction, or beneficial, as when denatured protein becomes more accessible to proteases) and ii) the presence of various compounds which either hinder the action of enzymes (anti-nutrients such as trypsin inhibitors, tannins, or dietary fibers) or

hinder the absorption of nutrients (anti-nutrients such as lectins)[138]. Microalgae may have long been proposed as a protein source based on the general composition of the dry biomass, in which as much as 60% may be crude protein[30]. The amino acid profiles of microalgal proteins are often known to correspond to or exceed the reference values[30, 147-153]. Fewer studies have addressed microalgal protein digestibility, though these generally indicate that microalgae proteins are not as digestible as a reference protein such as casein[30]. In other words, while microalgae appear to be a promising source of protein, based on the amount of total protein and the protein's amino acid composition, the proteins' digestibility must also be studied to determine if the protein is of high nutritional quality.

3.3. Sustainability aspects of microalgae as a source of nutrients

3.3.1. Microalgal cultivation

Many non-dietary advantages for using microalgae as a food source have been suggested. Microalgae are touted as having a higher photosynthetic efficiency than land-based plants, *i.e.* they are capable of converting more of the incoming sunlight into biomass[32, 154, 155]. As for cultivation, microalgae may be grown with less consumption of freshwater, especially the marine species[154, 156]. Furthermore, culture of microalgae does not require arable land, thus competition with food production may be avoided[11, 156, 157]: microalgae may be cultured in photobioreactors or open ponds placed on marginal land or even in the ocean[32, 158]. Furthermore, depending on the prevalent climate at the culture location, it may be possible to culture algae all year around[156, 157], thereby improving food security[11]. In other words, microalgal culture has been proposed as alternative or supplement to traditional agriculture, requiring less resources while potentially producing more biomass *per* hectare.

In spite of the advantages mentioned above, some resources will still be needed to culture microalgae. Depending on the culture system, freshwater may be needed to compensate for evaporative losses[154] or as cooling water[159]. Other operations associated with algal culture may also require freshwater, such as harvest, downstream-processing and cleaning of equipment. Furthermore, nutrients including nitrogen, phosphorous and carbon will need to be supplied. While these elements are present in abundance in municipal wastewater[11], it is highly unlikely that such wastewater would be used in the production of food[160]. Possibly, food grade process waters, *e.g.* from food industry could be used as a source of nitrogen, carbon and phosphorous in algae cultivation. Otherwise, nitrogen and phosphorous – with energetically demanding production or mining methods – will need to be supplied in purified form. Depending on the source (*e.g.* glucose

or purified, compressed carbon dioxide), even carbon represents a significant energy investment[32]. Finally, energy will also be required for such operations as mixing, aerating, auxiliary lighting[161], temperature control, harvesting, cell disruption, *etc*.; this energy is only as sustainable as its source. Thus, there are resources beyond light and carbon dioxide which must be supplied to microalgae in order for them to produce molecules useful to humans.

3.3.2. Downstream processing of microalgae to recover lipids and proteins

3.3.2.1. Solvent extraction

When microalgal lipids are sold in purified form, it follows that the biomass must have been fractionated. Exactly how the biomass is fractionated industrially is not clear, since processes are proprietary, but extraction with organic solvents is a likely method[39]. Although chloroform-methanol methods are popular for analytical purposes[95, 99, 107, 111, 117, 120, 162-171], the presence of residual chlorinated solvent cannot be tolerated in food applications[6, 172, 173]. It is likely that industrial extraction of microalgae is carried out with solvents such as isopropanol, ethanol, hexane or mixtures thereof[6, 25, 39, 165, 172-177]. Supercritical fluid extraction has been suggested as an alternative extraction method[39, 174, 178, 179], but requires costly equipment which is difficult to scale up[180]. The application of any organic solvent is likely to denature the protein fraction[181]. Furthermore, whatever the exact method of the solvent extraction, the fact remains that organic solvents are hazardous for workers and environment, since they tend to be flammable and toxic. Solvent-free methods, with less impact on both the environment and denaturation-prone proteins in the residual biomass would thus be preferable to traditional solvent extraction. The solvent used in the pH-shift process is water, which is non-toxic and safer than organic solvents and easier to handle than supercritical carbon dioxide. Since freshwater is a limited resource in many parts of the world, it may be possible to further reduce the amounts of water utilized when processing microalgae by using culture medium in the pH-shift process.

3.3.2.2. The pH-shift process

Although the pH-shift process (explained below in section 4.1) was primarily developed to recover proteins with good techno-functional properties, it has also been suggested as a lipid fractionation method[9, 182]. In a related procedure, comprising a single acidic pH-adjustment to roughly 5.5 on a fish muscle-water homogenate, the oil yield was reported to be 60-74%, depending on the type of acid added (the highest yield was achieved with tartaric acid)[182]. It has been pointed out that the pH-shift process does not achieve pure fractions[183], as is common after solvent extraction and refinement of the oil. However, it is not always necessary to spend the energy to achieve pure fractions since foods tend to be complex mixtures. Furthermore, a partial fractionation could be an advantage if it leads to more of the biomass being utilized directly as human food. In keeping with

the principle of the European waste hierarchy[184], it would be preferable to use as much of the biomass as possible for human food[48]. Thus, although the pH-shift process does not achieve pure fractions, it does not use toxic solvents, and might lead to a larger fraction of the biomass being used by humans directly.

3.3.3. Fish, the current major source for LC n-3 PUFA

3.3.3.1. The state of the world's fisheries and aquaculture

Like humans, fish are very inefficient at producing LC n-3 PUFAs: instead, fish bioaccumulate the LC n-3 PUFAs originating from microalgae[185]. It follows that although aquacultured fish in 2014 accounted for half of the fish consumed on the planet[5], the aquacultured fish must be fed LC n-3 PUFAs. Currently, the main source of LC n-3 PUFAs is fish oil, with 75% of the produced fish oil going into aquaculture[4, 5]. The fish used to make fish oil are mainly (roughly 90% in 2011) captured marine species[5]. Of the marine fish stocks, 29% were overfished in 2014, while 61% were being harvested at the maximum biologically sustainable yield[5]. The world's marine fisheries have been in a state of slow decline since 1996, though the price of fish oil has increased as aquaculture has expanded[5]. As the world's population is projected to grow in coming years, the *per capita* fish consumption is also predicted to increase[5]. Concurrently, with growing affluence, the demand for LC n-3 PUFA-containing nutraceuticals and functional food can also be expected to grow. It will be necessary to utilize the available fish resources more efficiently[5], but to surpass the upper limit set by the currently available fish resources, alternative biomasses, such as microalgae, will need to be developed to cover the demand for LC n-3 PUFA.

3.3.3.2. Something is fishy about the fish

Although fish is an excellent source of protein, vitamins, minerals and LC n-3 PUFAs, fish may also contain various harmful substances including heavy metals and dioxins[2]. Experts agree that in general the benefits of eating fish overweigh the risks[2, 186], but consumers may be confused by local recommendations to avoid fish (*e.g.* fish from the Baltic Sea[187]). Additionally, consumers may choose not to eat fish for moral reasons or personal preferences. In this context, microalgae have been suggested as a vegetarian alternative to fish oil[69], and possibly even to fish[79]. Furthermore, by tightly controlling the culture medium and carefully selecting strains which do not produce toxins, it should be possible to culture microalgae free from toxicants. Thus, microalgae might be able to augment and replace fish as a source of LC n-3 PUFAs, without the environmental pollutants associated with fish.

4. Development of a pH-shift process for *Nannochloropsis*

The pH-shift process is an established method for fractionating animal or plant biomass and is applied in industrial scale both to recover muscle protein and to refine defatted soy meal. The general process is explained in section 4.1. Section 4.2 explains how the processes was developed, with section 4.2.3 detailing the process for *Nannochloropsis* in seawater, as published in Paper I. Finally, in section 4.3, the product of the process is characterized.

4.1. General introduction to the pH-shift process for protein separation

The pH-shift process is applied to various proteinaceous raw materials to fractionate soluble proteins from non-soluble components[9, 10, 188-194]; another name for the process is "isoelectric solubilization/precipitation"[195]. A schematic representation of the process is given in **Figure 8**. Generally, the process starts by adding water to the biomass and homogenizing the slurry, allowing the intracellular content to come into contact with the water. Solubilization of the proteins is then carried out, usually at high pH by adding alkali, *e.g.* sodium hydroxide, but it is also possible – depending on the initial raw material – to solubilize some proteins at low pH by adding acid, *e.g.* hydrochloric acid[9, 10]. Centrifugation encourages non-soluble material to sediment into a pellet, while the majority of proteins remain in solution. After separating the supernatant from the pellet, the proteins in the supernatant are precipitated at their isoelectric point. The majority of the proteins are recovered after a second centrifugation which yields a watery supernatant, and the proteinaceous pellet, which can then be processed further.

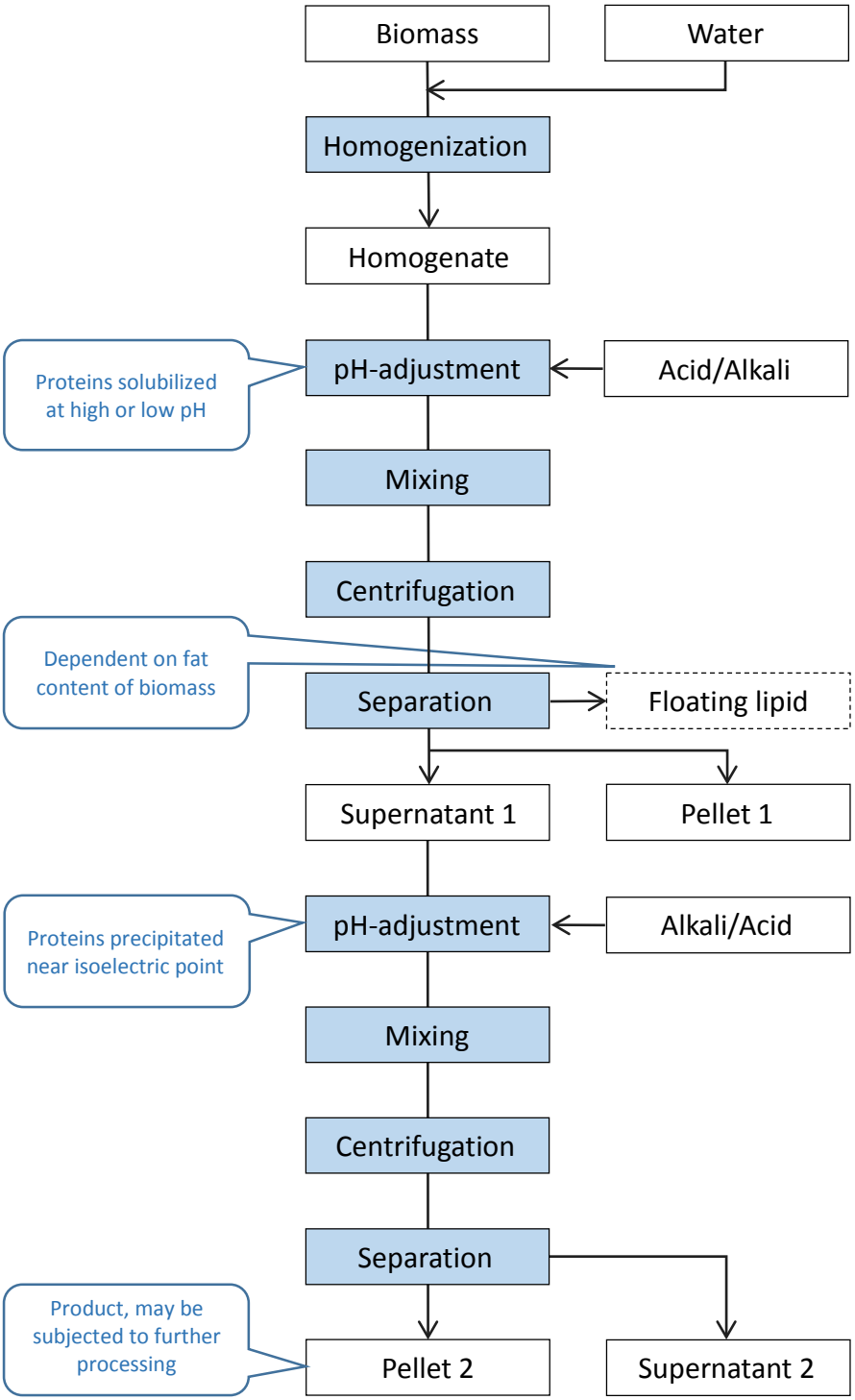

Figure 8 The general pH-shift process. Raw materials and products of the process are shown in white; process operations are shaded.

4.1.1. Common raw materials used in the pH-shift process

Both plant and animal raw materials are subjected to the pH-shift process at industrial scale. The common aim for both plant and animal raw material is to recover proteins with good sensory quality (*i.e.* a product which looks and smells appetizing) and good techno-functional properties (*e.g.* the capability to retain water, or to form a gel)[196, 197]. For plant material, *e.g.* for soy[188] and wheat germ[189], it is common that the raw material is defatted before subjecting it to the pH-shift process. Fats and oils are commonly removed from food raw materials by extraction with hexane, a process which may cause (at least part) of the proteins to denature irreversibly[197]. Conversely, the whole animal raw material is usually processed without prior extraction of the oils, to recover muscle protein[9] from *e.g.* fish frames[10, 192-194, 198]. When subjecting fish with a high lipid content to the pH-shift process, it is under some circumstances possible to recover a third fraction, beyond the insoluble material and aqueous proteins: a floating fat layer has been observed in several studies[182, 199]. The details of the pH-shift process differ for plant and animal raw materials. The pH-shift process on animal material is generally carried out refrigerated (1-15 °C), to repress the growth of food spoilage organisms, reduce lipid oxidation as well as protein denaturation[9]. In contrast, pH-shift processing on dry, defatted plant material does not have the same concerns and is therefore often carried out at room temperature or is even heated to increase protein recovery[190, 191, 197, 200]. Thus, any proteinaceous raw material can be treated with the pH-shift process, with adaptions made to the process to suit the raw material.

4.1.2. Microalgae in the pH-shift process and related processes

4.1.2.1. Microalgae in classic pH-shift processes

The pH-shift process and variations thereof, has been applied to various microalgae, as is briefly detailed below. An early report[201] from 1978 used sodium hydroxide (pH 11.5) and the reducing agent β-mercaptoethanol (0.5%) to extract proteins from *Scenedesmus acutus* with subsequent precipitation at the isoelectric point (pH 3.5)[201]. A few years later (1981), the same group investigated defatted *Spirulina platensis* flour, determining that the proteins had high solubility (> 80%) above pH 6 and an isoelectric point of pH 3[202]; furthermore, a rudimentary *in vitro* digestion model indicated the produced isolate had high (> 70%) protein digestibility[202]. The same authors later studied techno-functional properties of defatted *Spirulina* subjected to pH-shift processing, namely water and fat sorption capacity, emulsification capacity, foam capacity and foam stability[203]. In a further development of this early work, whole *Spirulina* biomass (*i.e.* non-defatted) was subjected to the pH-shift process and the product's techno-functional properties studied in detail[204-207]. More recently, proteins were recovered from defatted *Nannochloropsis* spp. with solubilization at pH 11 at 60 °C for 5 h and precipitation at pH 3.2 and 5 °C[176]: when the defatted *Nannochloropsis* raw material was compared to non-defatted *Nannochloropsis*, more

proteins were extracted from the non-defatted biomass[176]. The authors hypothesized that polar lipids acted as surfactants, aiding the proteins' extraction of the non-defatted *Nannochloropsis*; an alternative explanation could be that during the lipid extraction process, proteins denatured in such a way that they became permanently insoluble. In another recent study, *Nannochloropsis* proteins were recovered by solubilization at pH 12, and precipitation at pH 4, followed by removal of salts (by dialysis) and lipids (by hexane-extraction)[208]. Although the pH-shift process has been applied to microalgae of different taxonomic groups, with and without defatting, the listed studies share similarities in the protein-solubility curves, with high solubility above neutral pH and low solubility in the acidic range.

4.1.2.2. Microalgae in pH-shift related processes

While the studies listed above can be considered "classic" pH-shift processes, other studies report on processes reminiscent of the pH-shift process. In one such study, the total nitrogen of whole, defatted cells of *Porphyridium cruentum*, *Nannochloropsis oculata* and *Phaeodactylum tricornutum* were extracted at wide range of pH values for analytical purposes; as above, a high nitrogen solubility was noted above pH 6[209]. Schwenzfeier *et al.*, 2011 reported on a sophisticated protein purification of *Tetraselmis* sp.: the process included bead-milling of the biomass, centrifugation of mildly alkalized algae homogenate at 40 000×g, dialysis of the supernatant, subjection of the dialysate to ion-chromatography, dialysis of the eluate, de-colorization of the eluate by lowering the pH, recovery of the precipitated proteins by centrifugation and subsequent washing and re-solubilization of the proteins[141]. The same authors went on to study various techno-functional properties of the isolated *Tetraselmis* proteins in detail[210-212]. Meanwhile, Safi *et al.*, 2014, used a variation of the pH-shift method to extract proteins from whole microalgae (*Arthrospira platensis*, *Chlorella vulgaris*, *Haematococcus pluvialis*, *Porphyridium cruentum* and *Nannochloropsis oculata*) at pH 12 and 40 °C for 2 h[213]. The authors concluded that the warm alkaline extraction was more successful in recovering proteins than ultrasonication and manual grinding, but less successful than high-pressure homogenization of the microalgae in distilled water[213]. In summary, the pH-shift process and similar processes have been applied to various microalgae, for various purposes, though recovery of proteins has been the overall goal.

4.1.2.3. Motivations for applying the pH-shift process to microalgae

The greater context of the pH-shift process, of course, has a major impact on both the process conditions and the product. Generally, the motivation for developing a particular process is not explicitly stated. For example, Gerde *et al.*, 2013, reports on treating defatted *Nannochloropsis* with a pH-shift process[176]; presumably, defatting was done to simulate a process in which a pure lipid fraction (oil) was first removed from the algae and the remaining biomass required upgrading. Meanwhile, other studies[141, 210-212] aim for the recovery of highly purified proteins with good techno-

functional properties, possibly as replacement for soy protein, with the intention of recovering biodiesel from the remaining biomass. In both examples, authors strive to utilize as much of the biomass as possible with different applications, an approach which aims to add value to the biomass[18, 181]. Although side-streams from algal processing may still find a use *e.g.* as animal feed, our motivation was to apply as few processing steps as possible, to minimize dependence on fresh water and to utilize as much of the algal material as possible directly as human food.

4.2. pH-shift processing of wet *Nannochloropsis* in seawater

4.2.1. Raw materials

4.2.1.1. Microalgae used in Papers I & II

For the overall project, microalgae were chosen which were i) known to produce the LC n-3 PUFAs EPA and/or DHA (*cf.* **Table 1**), ii) not known to produce toxins, iii) were commercially available and iv) well-studied. Three species of microalgae fitted the requirements: *Isochrysis galbana*[104, 114, 116, 118, 165, 167, 177, 214-218], *Nannochloropsis oculata*[99, 104, 116, 118-120, 167, 177, 218-222] and *Phaeodactylum tricornutum*[42, 103, 105-107, 109, 110, 112, 116, 119, 172, 177, 214, 218, 223-226]. For Papers I & II, wet microalgae were desirable, since drying may have an effect on the proteins and thus also the pH-shift process[227]. At the onset of the study, *Nannochloropsis* was the only of the three aforementioned microalgae which were found commercially available as a wet paste. Thus, for Papers I & II, the same lot of wet *Nannochloropsis oculata* biomass at 33% dry weight of aquaculture grade was purchased in frozen form from PhytoBloom[228], Portugal, and stored at -80 °C.

4.2.1.2. Composition of *Nannochloropsis* used in Papers I & II

On a dry weight basis, the *Nannochloropsis* used in Papers I & II consisted of 11% total fatty acids, 19% protein, about 37% carbohydrates and 34% ash, see **Figure 9** and Paper I. The fatty acid profile was dominated by palmitic acid (C16:0, accounting for 28% of all fatty acids), palmitoleic acid (C16:1 n-7; 31%) and EPA (C20:5 n-3; 18%). Other fatty acids present at 1-8% of the total fatty acids were myristic acid (C14:0), oleic acid (C18:0), linoleic acid (C18:2 n-6) and arachidonic acid (C20:4 n-6); this fatty acid profile falls within the range of values reported previously for *Nannochloropsis*[112, 116, 119]. Of the fatty acids, 66% were found in the neutral lipid fraction, 20% in the glycolipid fraction, and 14% in the phospholipid fraction, comparable to values previously reported for nitrogen-depleted *Nannochloropsis*[229]. The amino acid profile of the *Nannochloropsis* suggested that the microalga is a good source of protein since all the essential amino acids were present at a percentage higher than as recommended by the World Health Organization[146]; the

amino acid profile agreed reasonably well with a previously published profile[148]. Thus, the presence of significant amount of LC n-3 PUFA and protein in *Nannochloropsis* was confirmed.

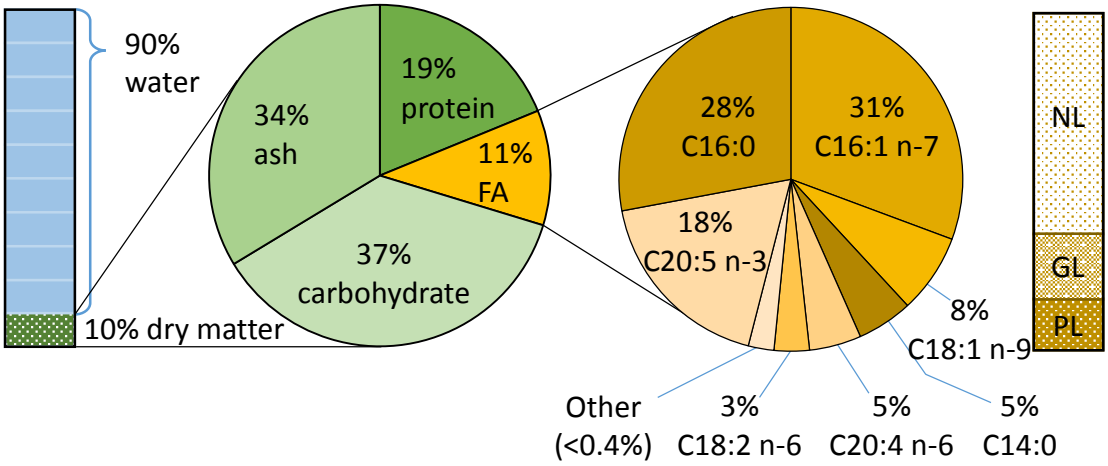

Figure 9 Composition of *Nannochloropsis* biomass mixed with seawater, used in the pH-shift experiments (Papers I & II). Of the 10% dry weight, roughly a third each was ash and carbohydrate, with 19% protein and 11% FA, fatty acids. Palmitic acid, palmitoleic acid and EPA dominated the fatty acids, with smaller amounts of myristic, oleic, linoleic and arachidonic acid. Preliminary data indicates that the fatty acids partitioned into NL, neutral lipids (about 66%), GL, glycolipids (about 20%) and PL, phospholipids (about 14%).

4.2.1.3. Seawater

The water used in the pH-shift process was seawater (filtered and autoclaved, with a conductivity of 44 mS/cm) collected at the Sven Lovén Centre for Marine Sciences at Tjärnö, Sweden. The rational for using seawater was to reduce the amount of freshwater and energy used in the pH-shift processing of the marine microalgae *Nannochloropsis*: the dewatering step of harvesting a microalgae culture is energetically costly and it seemed counterproductive to remove water in one step of the process, just to add it back in the next step. Thus, seawater was added to the wet *Nannochloropsis* biomass to simulate a culture, which had been dewatered to 10% dry weight (instead of 33% as in the purchased *Nannochloropsis* biomass, or 100% as in dried biomass). However, autoclaving of the seawater was done for reasons of storage stability alone and was not intended to be part of the process. The seawater's salt can be expected to have an impact on the protein solubility[141, 209]: anions, such as chloride, can interact with proteins' positively charged side groups, thereby shifting the isoelectric point towards a lower pH-value[230].

4.2.2. Processing steps considered in the developed *Nannochloropsis* pH-shift process

4.2.2.1. Cell disruption

Homogenization is the first step of the pH-shift process. Homogenization of *Nannochloropsis* cells is complicated by the cells' small diameter (2-4 μm[231]), and the cell wall containing cellulose and algaenan[232]. See **Table 2** for some previously investigated methods for breaking microalgal cell walls. The choice of disruption method depends in part on the desired outcome[82, 180, 233]. For pH-shift processing, the intracellular content needed to come into contact with the surrounding solution, demanding a method which broke open the majority of the cells. The development of heat was unwanted during the process, since heat can denature proteins and increase lipid oxidation; thus methods such as microwaving and autoclaving were excluded. Of the disruption methods tested in the presented work, the following gave no visible cell disruption, as assessed by phase-contrast microscopy: sonication with a probe (Branson sonifier 250, 50% duty cycles, up to 30 min), mechanical blending (Ultra Turrax, speed 6 for 5 min), Potter-Elverhjem homogenizer (piston worked by hand for 5 min), and grinding cells in liquid nitrogen in a mortar (7 min). The only method, which visibly disrupted the cells, was bead-beating. Beat-beating was developed for faster, more complete disruption by firstly decreasing the bead size and secondly increasing the tube's headspace to roughly 50%[*]. Heat which evolved in the vigorous shaking was removed by periodically cooling the sample on ice. One disadvantage of bead-beating yet to be addressed is that suspension adheres to the glass beads, resulting in significant loss. As an alternative to bead-beating, high-pressure homogenization might allow cell disruption in a continuous setup, with less loss. However, in spite of some biomass loss, bead-beating was chosen since it was the only available in-house method which resulted in cell disruption.

[*] The design of the MP Bio FastPrep bead-beater results in frequent breakdowns and expensive repairs; the Retsch MM400 bead-beater has performed more reliably and allows larger sample volumes to be processed.

Table 2 Some cell disruption methods applied to microalgae at the lab-scale. See [234-236] for disruption specifically on *Nannochloropsis*.

Method	**Reference(s)**
Sonication	6, 162, 168, 213, 234-239
Microwave heating	236-238
Pulsed electric field	235
High voltage electric discharge	235
High-pressure homogenization	213, 235
Autoclaving	237, 240
Hot water bath treatment	213, 236
Bead-milling and bead-beating	6, 178, 237
Osmotic shock	237
Laser treatment	236
Mechanical blending	236
French press	201
Spray-drying	240
Enzymatic treatment	205, 206
Chemical disruption *e.g.* with hydrochloric acid or sodium hydroxide	213, 240
Combination methods, *e.g.* warm water incubation followed by high-pressure homogenization	241

4.2.2.2. Protein solubilization- and precipitation-pH

Nannochloropsis proteins were found to be highly soluble in seawater already at the native pH of the lysate, pH 7. More than 80% of the initial protein was detected in the supernatant after the first centrifugation at pH 6-10, with a gradual decline in solubility as the pH was raised towards pH 12, as is shown in **Figure 10**. Below pH 5, the protein solubility was < 15% and below pH 4 the solubility was < 8%. The obtained solubility curve's shape and scale is comparable to that reported previously for *Tetraselmis*[141], and *Spirulina*[202], suggesting that considerable similarities exist between various microalgal proteins, even though they belong to distinct taxonomic groups[231, 242, 243]. The solubility curves were used to develop a process (see section 4.2.3) with solubilization at pH 7.

The pH at which protein solubilization is carried out may have an impact on the outcome of the pH-shift process: at more extreme pH-values the proteins become more unfolded, influencing the refolding pattern and subsequently down-stream processing[244]. Thus, to investigate the impact of the solubilization pH, another process version was carried out with solubilization at pH 10 by adding sodium hydroxide. At pH 10, the proteins were still highly soluble (about 90%, as shown in **Figure 10**), however, the pellet from the first centrifugation was larger: about 20% of the initial volume, compared to 10% at pH 7. Since the larger pellet trapped more of the aqueous phase with the soluble proteins, less supernatant was recovered in the first separation, resulting in a lower yield

for both proteins (70% *vs*. 80%) and lipids (81% *vs*. 90%). The solubilization pH did not appear to affect the precipitation pH: For both versions, the protein solubility was < 8% at pH 4 and below (**Figure 10**). Thus, the same precipitation pH (3) was used to compare the solubilization at pH 7 to solubilization at pH 10. The pellet of the second centrifugation was slightly more compact for the pH 10 process, making it slightly easier to decant the watery supernatant and thereby remove more water (about 60% of the pre-centrifuge volume, instead of 50% as in the pH 7 process). The product's overall composition with respect to protein, amino acid profile, total fatty acids and fatty acid profile was nearly the same for both processes, irrespective of the solubilization pH (see section 4.3 for the macronutrient profile of the pH 7-process). In summary, although the second centrifugation of the pH 10-process allowed more water to be removed, there was a trade-off in the form of a yield reduction for both lipids and proteins in the first centrifugation. Thus, pH 7 was chosen as the solubilization pH to reduce unnecessary loss of yield and addition of sodium hydroxide.

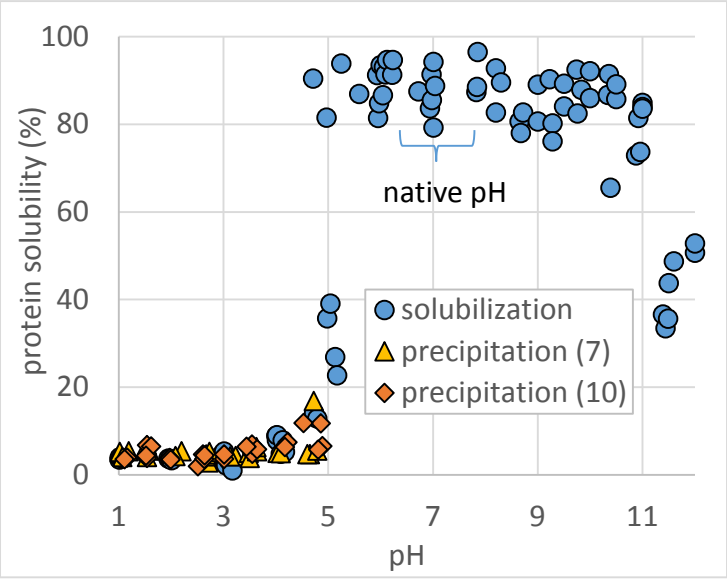

Figure 10 Protein solubility of *Nannochloropsis* in seawater in the solubilization step (blue circles) and precipitation step (yellow triangles for pH 7 solubilization, orange diamonds for pH 10 solubilization). Solubility is expressed as the protein concentration of supernatant (sup.) 1 divided by the protein concentration of the lysate for the solubilization step. The native pH of *Nannochloropsis* in seawater was pH 7 from where the pH was adjusted from 1-12 to determine the solubility. In the second part of the experiment, the proteins were solubilized at pH 7 or 10 and then precipitated at pH 1-5. Solubility is expressed as protein concentration of sup. 2, divided by protein concentration of sup. 1.

4.2.2.3. Solubilization time

Some of the previous studies, in which the pH-shift process was applied to microalgae, included extraction times of 1 – 5 h[176, 203, 213], presumably to allow dissolved protein to reach equilibrium with the aqueous phase, maximizing the protein solubilization. Other studies either did not purposely consider time as a factor in the extraction process[141, 204] or failed to specify it[201, 202]. In a pilot experiment (no replicates), *Nannochloropsis* was disrupted by bead beating and the pH adjusted to 11.4. With one-hour intervals, aliquots were withdrawn which were centrifuged at 4 000×g and the protein concentration of the supernatant was quantified. Over an interval from 0 to 5 h, no change in protein concentration was observed. Since added time appeared to have no effect on the outcome, the process as presented in Paper I does not purposely include a time factor. However, for the pH to remain stable, it was necessary to wait a few (5-10) minutes after adding acid or alkali (alkali in particular) since the algae biomass buffered strongly around pH 9. It is possible that we did not observe a time effect protein solubilization of *Nannochloropsis* biomass in contrast to a previous report[176], because the study used defatted and dried *Nannochloropsis*[176]; presumably, the proteins denatured irreversibly under the influence of organic solvents and drying. In summary, the developed process does not contain extended extraction times, though the pH adjustments can be expected to take a few minutes due to buffering.

4.2.2.4. Centrifugal force during separation

Centrifugation is applied during the pH-shift process to first separate non-soluble matter from soluble proteins and then again to separate insoluble proteins from the surrounding water. The speed with which the insoluble material sinks depends on its density and mass, the soluble phase's density and its frictional coefficient and the centrifugal force applied to the system[245]. In practical terms, the factors which are easy to adjust are the magnitude of the centrifugal force and the time it is applied. Envisioning that the pH-shift process might be applied in large scale, we aimed to keep centrifugation time below 30 min and centrifugal force below 10 000×g. In pilot experiments, the effect of centrifugal force from 800 to 18 600×g for 10 min was investigated during the first separation step of the pH-shift process. Disrupted *Nannochloropsis* separated into two distinct layers at 4 000×g, with the proteins remaining in suspension at alkaline pH (pH 7-12), forming a dark green, opaque supernatant. Since 4 000×g for 10 min i) was sufficient to create two phases in both separation steps of the pH-shift process, ii) is achievable in an industrial decanter[246] and iii) is the upper limit of "low-speed" centrifugation as defined by the original patent[9], it was the condition chosen for the pH-shift process as presented in section 4.2.3 and Papers I & II.

4.2.3. A pH-shift process suitable for *Nannochloropsis*

Following documentation of maximum and minimum solubility points for wet *Nannochloropsis* biomass in seawater (**Figure 10**), the final version of the pH-shift process on *Nannochloropsis* was developed (**Figure 11**): *Nannochloropsis* microalgae, 10% dry weight in seawater (20% on wet weight basis), were disrupted by bead beating. The pH-adjustments were carried out on ice. At the native pH (7) of the resulting lysate, the proteins showed good (> 80%) solubility, see **Figure 10**. Centrifugation was carried out at 4 000×g, 4 °C, and 10 min, resulting in a small pellet, about 10% of the initial volume. The supernatant, containing the majority of the solubilized proteins was recovered by pouring it off. Next, the supernatant's proteins were precipitated by adding hydrochloric acid. Precipitation at pH 3 was chosen since it was well into the low solubility range, and since it represented a compromise between volume of acid added and the pellet volume: at pH 3, roughly 50% of the water could be removed in the supernatant by decantation after centrifugation at 4 000×g, 4 °C, for 10 min. Further addition of acid resulted in a more compact pellet, but below pH 3, large additions of 1.0 M hydrochloric acid resulted in only small changes in pellet volume. In summary, the key process operations for the developed pH-shift process developed on *Nannochloropsis* in seawater were: solubilization of the proteins at pH 7, removal of insoluble material by centrifugation at 4 000×g, and recovery of the proteins by precipitation at pH 3 and centrifugation at 4 000×g.

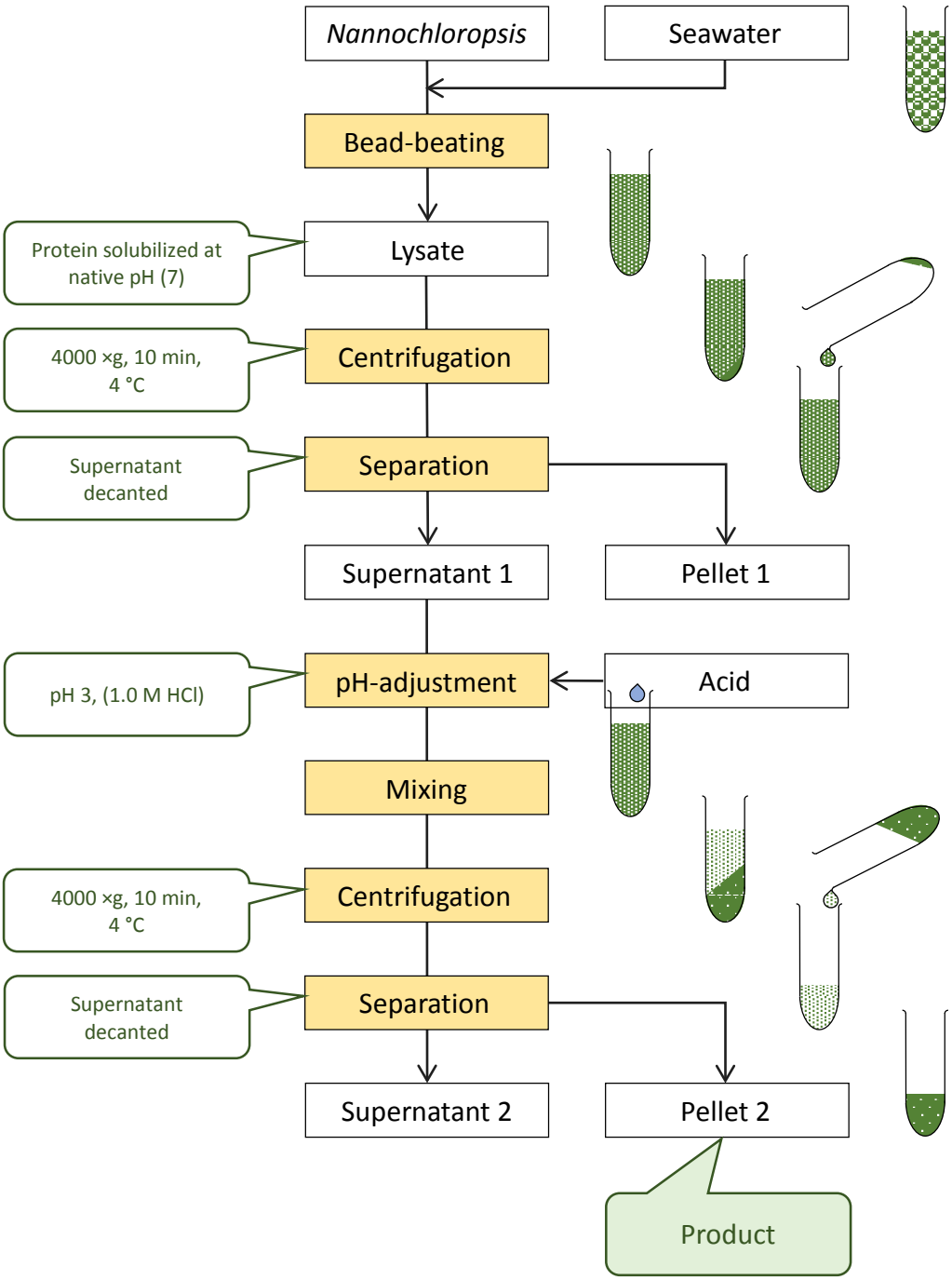

Figure 11 The pH-shift process developed for *Nannochloropsis* in seawater.

4.3. Macronutrient partitioning during the pH-shift process

The pH-shift process effected a slight concentration of lipids and protein to 12% and 23%, respectively, on a dry weight basis. In total, the process recovered 86% lipids and 85% of the protein. Thus, the developed pH-shift process could represent a method for converting *Nannochloropsis* biomass to a functional food containing both high-value LC n-3 PUFAs and proteins with fewer processing steps than conventional processing of fractions after solvents extraction. An overview of the lysate composition and the pH-shift process's product is given in **Figure 12**. However, the initial hypothesis, namely that the pH-shift process could be used to recover separate lipid and protein fractions, was not confirmed. Instead of forming a distinct floating lipid layer, the lipids partitioned into the same fractions as the proteins. Details of the distribution of individual macronutrients are given in the following sections.

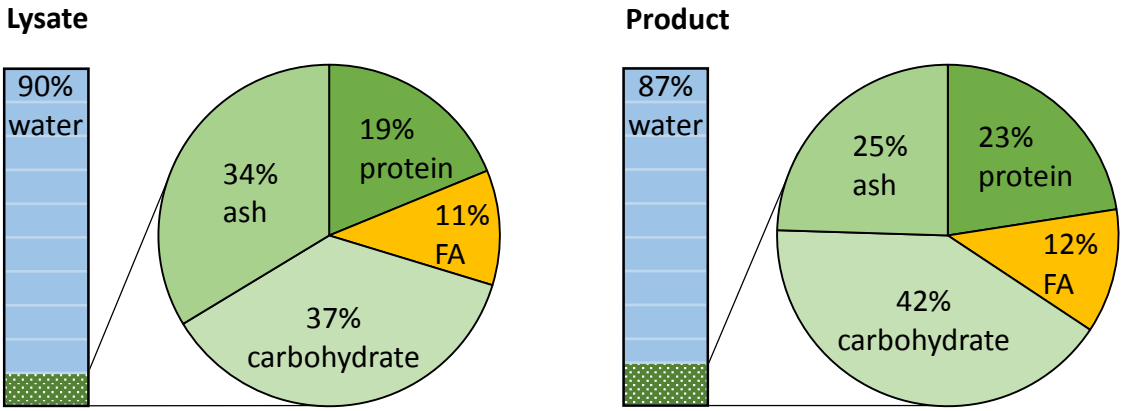

Figure 12 Basic composition of the lysate and product of the pH-shift process (with solubilization at pH 7).

4.3.1. Lipids in the pH-shift process

Lipids did not form a separate oil layer in the first centrifugation, but were instead found to be present in the same fractions as the proteins. Although the fact that the lipids and proteins were found in the same fraction does not necessarily imply chemical interaction between the two, it does remain plausible. Preliminary data on lipid class composition (**Figure 9**) indicates that one-third of the fatty acids were present in glycolipids and phospholipids, molecules which are inherently amphiphilic. Amphiphilic lipids may stabilize emulsions by surrounding droplets of neutral lipids. This system further contained protein. Cytoskeletal protein has been proposed to interact with membrane phospholipids, thus partially remaining in solution with the proteins in the first centrifugation of the pH-shift process[247]. Beyond cytoskeletal proteins, this system contained chloroplast proteins. Proteins of the light-harvesting complexes are embedded in the chloroplast

membrane[248] and thus, in their natural state, associate with phospholipids and glycolipids present in the chloroplast membrane[90]. In the presence of both amphiphilic proteins (light-harvesting, cytoskeletal and other) and amphiphilic lipids (glycolipids and phospholipids), neutral lipids may be kept in solution, which could explain why no separate lipid layer was obtained in the first centrifugation step of the pH-shift process, and likewise why the lipids were recovered with the precipitated protein in the second separation.

4.3.1.1. Total fatty acids: partitioning and yield

The pH-shift process increased the concentration of total fatty acids in the product marginally. The material entering the process contained 11% (range = 0.6%, n = 3) fatty acids by dry weight, while the pH-shift process product contained 12% (range = 1.5%, n = 2) fatty acids. In the first separation, no clear fractionation of the fatty acids was observed: both the pellet and the supernatant contained 10-11% fatty acids by dry weight. However, the pellet volume was small in comparison to the supernatant, thus 90% of the total fatty acids remained in the supernatant and continued on to the next processing step. In the second separation, little lipid remained in the supernatant, with the majority of the total fatty acids being present in the pellet, *i.e.* the product. Over the entire process as a whole, 85% of the ingoing fatty acids were recovered in the pellet.

4.3.1.2. Fatty acid profile

The pH-shift process did not observably change the fatty acid profile of the raw material. In the raw material as well as the product, the major fatty acids C16:1 n-7, C16:0 and C20:5 n-3 represented 31%, 28% and 18% of the total fatty acids; neither was any change noted in the distribution of minor fatty acids. However, on a dry matter basis, the amount of most fatty acids increased slightly, with the change for C20:5 n-3 being 1.9% to 2.3%. These results indicate that i) the pH-shift process did not selectively fractionate the fatty acids and ii) that the product is a source of LC n-3 PUFA comparable to the content found in cod fillet[249]. To achieve the daily intake of 250 mg of LC n-3 PUFAs, as recommended by EFSA[136], 84 g of wet product (**Figure 12**) would be needed.

4.3.2. Proteins in the pH-shift process

4.3.2.1. Protein solubility

The material entering the pH-shift process was 19% (range 0.7%, n = 4) protein on a dry weight basis. In the first separation step, both the pellet and the supernatant were about 17% protein (range = 2.0% and 1.9%, respectively, n = 2), indicating that proteins were evenly distributed through the pellet and supernatant. After precipitation at pH 3 and the second separation step, the supernatant was only about 1% protein (range = 0.1%, n = 2), while the pellet was 23% protein (range = 1.0%, n = 2), indicating that the process concentrated protein relative to the initial lysate.

4.3.2.2. Protein yield

The protein yield in the first separation of the pH-shift process, in which the supernatant is recovered and further processed, was 80% of the initial protein. Possibly, the yield could be increased by diluting the raw material further, *e.g.* by running the process at 5% dry weight instead of 10% dry weight: less protein might then be entrapped in a more diluted pellet, thereby reducing the loss[183]. Alternatively, the pellet could be washed and re-centrifuged to recover more of the proteins[204, 207]. However, in the processes as described in section 4.2.3, the remaining 20% of protein were not detectable, instead about 15% of the protein remained unaccounted for. Given that most of the fatty acids were accounted for (see section 4.3.1), the unaccounted protein suggests that the discrepancy was due to the protein quantification method (see section 6.2.1, below) rather than sampling errors and general losses during the process. This hypothesis is further supported by a measured increase in overall protein yield for the pH-shift process compared to the yield over step 1: the overall yield increased from 80% to 86%. In spite of an incomplete mass balance, the overall recovery of protein in the pH-shift process was good.

4.3.2.3. Amino acid and polypeptide profile

The amino acid profile (% of individual amino acids per total amino acids) of the raw material and the pH-shift processed *Nannochloropsis* was found to be nearly identical. The amino acid profile also agreed with *Nannochloropsis* profiles previously published[120, 148]. When comparing the initial material to the pH-shift process product the only difference noted was that the relative amount of proline dropped from 10% to 8.5%. It is unclear why proline would be selectively removed by the pH-shift process. However, since proline is not an essential amino acid, its removal by the pH-shift process is not crucial from a nutritional point of view. Although the digestibility needs to be assessed to determine the protein quality, the relative amount of essential amino acids per total amino acids in *Nannochloropsis* exceeded the values recommended by the WHO[146], thus suggesting that *Nannochloropsis* is a source of balanced protein.

The polypeptide profile was not changed significantly by the pH-shift process, as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) see **Figure 13**. Major bands present in the initial lysate were also present in later fractions of the pH-shift process, such as supernatant 1 (S1) and the product (P2). When comparing the lysate and S1 to the product and supernatant 2 (S2) two differences emerged: i) P2 and S2 gave bands which were slightly more diffuse than the initial material and ii) P2 and S2 gave darker bands at shorter polypeptide length. In combination, these two observations suggest that mild proteolysis took place during pH-shift process, though this was not detectable when the protein degree of hydrolysis was addressed specifically (see **Figure 17**). Nonetheless, there was no major change in band pattern, confirming that the pH-shift process did not selectively remove distinct protein fractions.

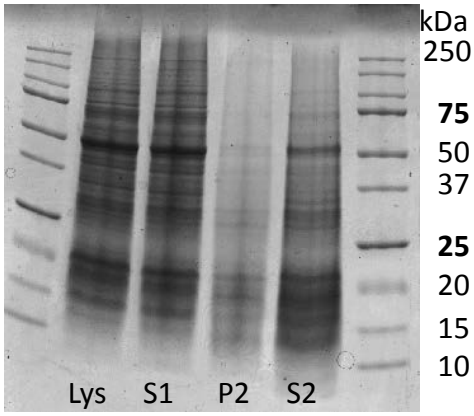

Figure 13 The SDS-PAGE polypeptide profile of *Nannochloropsis* proteins at the beginning of the pH-shift process (Lys), the supernatant of the first centrifugation (S1) and both pellet and supernatant after the second centrifugation (P2 and S2, respectively). Fragment size is indicated in kDa to the right of the protein ladder. Note that different amounts of protein were loaded in the various wells.

4.3.3. Other nutrients and aspects of the pH-shift process

4.3.3.1. Carbohydrates

The initial *Nannochloropsis* biomass was determined to be about 37% (range = 3.3%, n = 2) total carbohydrates, on a dry weight basis. Although the method for carbohydrate quantification[250] showed considerable variation, the product was found to be about 42% carbohydrates (range = 4.5%, n = 2). These results suggest that the pH-shift process increased the concentration of total carbohydrates. The types of carbohydrates were not analyzed, but previous research determined that cells walls of *Nannochloropsis* contain algenan and cellulose[232], which is not degradable by human digestive enzymes[251]. Thus, *Nannochloropsis* could provide dietary fibers to a functional food.

4.3.3.2. Ash

The initial *Nannochloropsis* biomass was determined to be about 36% (range = 4.8%, n = 3) ash, on a dry weight basis. The product of the pH-shift process was 25% (range = 2.4%, n = 3) ash, indicating that inorganic matter was removed in the pH-shift process.

4.3.3.3. Color

Most fractions of the pH-shift process were intensely green and opaque. The green color of the product diminished over time, turning beige. The color change is thought to be caused by Mg^{2+} leaving the porphyrin ring of chlorophyll, a reaction which takes place at low pH and is known as pheophytinization[252, 253]. If the product is left at pH 3, the green color slowly degrades, even at frozen storage. Although further study is needed to confirm that the green color can be stabilized by neutralizing the product, it may be possible to choose if the color of the product should be beige, by keeping the product at low pH, or green, by raising the pH of the product.

Judging by previous work, green color is not always desired in algae protein isolate[34, 141], presumably because green color limits the potential application of the protein isolate. Green color is a disadvantage, if the algae protein isolate is intended for a wide range of applications analogous to soy protein isolate. Soy protein is wide-spread in processed foods, including products such as cereals, meat products, dairy products, dessert cakes and baked goods[254], none of which are usually associated with a green color. However, other studies have added whole microalgae to food without attempting to remove the green color[36, 37, 75, 76, 78]. In these cases, the microalgae are added to enhance the nutritive value of the product. Indeed, some studies have added algae specifically to give the product color[77, 79]. Thus, the pH-shift process product's color may be chosen to be either green or beige, depending on the application.

5. *In vitro* digestion of Nannochloropsis

While the lipid and protein content of *Nannochloropsis* holds potential as human nutrition, it is not enough for nutrients to be present: the nutrients in any food also need to be accessible for uptake. Accessibility is defined as "the fraction of the component that is released from the food matrix into the juices of the gastrointestinal tract"[255]. Accessibility is distinct from bioaccessibility, a measure of how much of a component is absorbed, measured for example in cell-models or as concentration in the bloodstream. Paper II investigates the accessibility of lipids and proteins of *Nannochloropsis* both before and after pH-shift processing.

5.1. General introduction to the digestive tract and the process of digestion

The function of the gastrointestinal tract is to digest macromolecules in food into small molecules which can be absorbed[84]. In simplified terms, the gastrointestinal tract is a tube running through the body from mouth to anus[84]. An overview is given in **Figure 14**. Although the tube is within the body, the tube's content is technically part of the outside environment[84]. The major compartments of the gastrointestinal tract are the mouth, stomach, small intestine and large intestine[84]. Secretory organs, namely the salivary glands, pancreas, gallbladder and liver excrete solutions including enzymes and bile acid, to facilitate the degradation and absorption of nutrients[84].

Food tends to be a complex mixture of lipids, proteins, digestible carbohydrates and indigestible fibers[255]. Dietary fibers have been proposed to affect lipid digestion, *e.g.* by increasing the viscosity, adsorbing lipids and bile acid, and interacting with the lipases[255]. Insoluble dietary fibers, such as cellulose, are present in many microalgae[30, 44], but are resistant to human digestive enzymes[251]. Since *Nannochloropsis* itself is surrounded by a cellulosic cell wall[232], it is unlikely that any of the intracellular lipids or proteins are available for digestion or absorption in the human digestive tract, unless the cell walls are disrupted, warranting a study of *Nannochloropsis* in an *in vitro* digestion model.

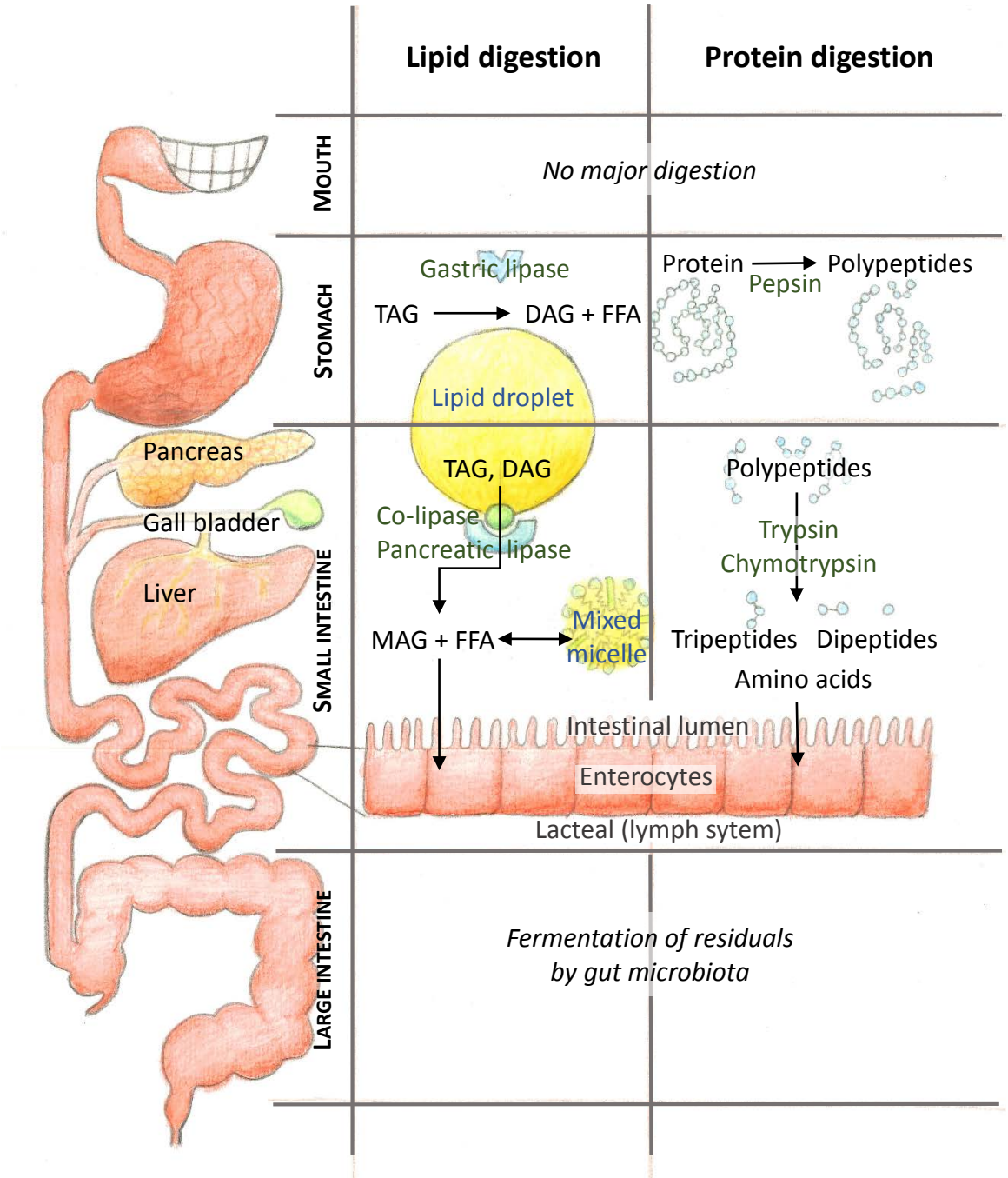

Figure 14 The simplified digestive tract: major compartments and digestion of lipids and proteins; note that structures are not to scale. **Left panel**: the major compartments and organs of the digestive tract. **Center panel**: Lipid digestion, which in adults starts in the stomach when triacylglycerol (TAG), present in lipid droplets, is converted by gastric lipase to diacylglycerol (DAG) and free fatty acids (FFA); in the small intestine, hydrolysis of TAG and DAG to monoacylglycerol (MAG) and FFAs is catalyzed *e.g.* by pancreatic lipase, anchored to the surface of lipid droplets by co-lipase; a pool of MAGs and FFAs diffuses in and out of mixed micelles (stabilized by bile acids from the liver) before being absorbed by enterocytes. **Right panel**: Protein digestion, which starts in the stomach by the action of pepsin, which hydrolyzes the long chains of proteins into polypeptides at acidic pH; hydrolysis continues in the small intestine, where polypeptides are cleaved into tripeptides, dipeptides and amino acids, which in turn are absorbed by enterocytes.

5.1.1. General lipid digestion

Lipid digestion takes place by the action of gastric lipase in the stomach and various lipases secreted by the pancreas in the small intestine. The main dietary lipid being triacylglycerols[84, 256], it is the most studied lipid molecule in terms of its digestion. When two of a triacylglycerol's three fatty acids have been hydrolyzed into one monoacylglycerol and two free fatty acids, these remaining molecules can be absorbed[257, 258]. In other words, triacylglycerols are usually hydrolyzed to a total of 67%. Of this 67% hydrolysis, about 10-25% takes place in the stomach, with the remaining hydrolysis occurring in the small intestine[259, 260]. Human pancreatic lipase is the main hydrolyzer of triacylglycerols and diacyglycerols in the small intestine[259], although gastric lipase also makes a continued contribution[261]. Pancreatic lipase is a water-soluble enzyme, therefore it can only act on the surface of lipid globules[84]. The larger lipid globules are emulsified by mechanical disruption and they are stabilized by bile acids and other amphiphilic molecules[84, 255]. However, emulsified lipid droplets are inaccessible to pancreatic lipase, therefore amphiphilic co-lipase is required to anchor pancreatic lipase to the lipid droplet[84, 255, 259, 261]. As fatty acids are hydrolyzed from their parent molecule, they tend to associate with other hydrophobic molecules (including monoglycerides) into micelles, from where they diffuse into intestinal epithelial cells (a.k.a. enterocytes)[84]. Other enzymes, notably pancreatic-related lipase protein 2 and carboxyl ester hydrolase, are thought to have broad substrate specificity, removing one fatty acid from galactosyldiacylglycerols and phospholipids[89, 259, 262]. Acting together, the various lipases remove fatty acids from dietary lipids, allowing the fatty acids and monoacylglycerols to be absorbed by enterocytes[84].

5.1.2. General protein digestion

Physiological protein digestion is effected by pepsin in the stomach as well as a suite of enzymes in the small intestine[84]. An empty stomach is a highly acidic environment, at pH 2 or even lower[263]. As food from the mouth enters the stomach, the pH of the stomach rises, which causes cells lining the stomach to secrete hydrochloric acid and pepsin in its inactive form[84]. At low pH, pepsin is activated and hydrolyzes peptide bonds of proteins, forming shorter polypeptides and thus providing about 20% of the protein digestion[84]. In the small intestine, the partially digested protein is joined by excretions of the pancreas: bicarbonate which raises the pH and the proteolytic enzymes trypsin and chymotrypsin[84, 264]. These enzymes further hydrolyze the protein into peptides, and the peptides in turn are hydrolyzed by exopeptidases, forming amino acids and short oligopeptides[84]. Amino acids, dipeptides and tripeptides are absorbed into enterocytes by active transport[84].

5.1.3. Microalgae digested in various models

Microalgae have been subjected to various digestions and digestion models, in order to study the availability of a range of nutrients in a number of different models. Static models are popular since they are reproducible, ethically uncomplicated and cheaper than the alternatives such as dynamic models, animal studies, and clinical trials[265, 266]. Static *in vitro* digestion models consist of a test tube containing the studied material, to which digestive enzymes and fluids are added, simulating various parts of the gastrointestinal tract. However, there is a wide variety of modeled digestive compartments, the amounts and more importantly the activities of the enzymes added, volumes and composition of the digestive fluids, residence time, *etc.* Since a plethora of *in vitro* digestion models exist, comparison of the studies' results is difficult.

5.1.3.1. Previously published studies on microalgal lipids

Relatively few studies have investigated the digestion of lipids and lipid-soluble compounds from microalgae specifically. In one *in vitro* study, *Nannochloropsis oculata* or carotenoid- and tocopherol-extracts thereof were subjected to stomach and small intestine simulations of 30 min each[267]. The authors concluded that although the digestion model was not physiologically accurate, results suggested that extracts had higher accessibility, possibly a result of matrix disruption during extraction[267]. The authors raise an important point: although microalgal lipids are often touted as beneficial for human health, some proposed applications[75, 77, 78] of microalgae (*e.g.* delivery of carotenoids or LC n-3 PUFAs) would benefit from demonstrating that the lipids are accessible for uptake: nutrients might otherwise remain inaccessible within the capsule of the cell wall. Indications that the cell wall may hinder the uptake of both intracellular and other dietary nutrients comes from animal studies in which addition of *Nannochloropsis* to the feed resulted in lower apparent lipid digestibility[150] and intestinal damage[268], possibly a result of the microalgae acting as dietary fiber. While no human studies have been conducted on whole *Nannochloropsis*, one clinical study assessed the bioaccessibility of LC n-3 PUFAs: *Nannochloropsis* oil, rich in glycolipids, was compared to krill oil, containing predominantly phospholipids[269]; over ten hours, the appearance of LC n-3 PUFAs in blood plasma was monitored, with both sources resulting in a significant increase of EPA and demonstrating that the polar lipid-rich *Nannochloropsis* oil can be an effective source of EPA[269]. In summary, although fatty acids from microalgal oils have good bioavailability, there are indications that the oil may remain inaccessible inside the cell walls of some microalgae, such as *Nannochloropsis*, warranting closer investigation.

5.1.3.2. Previously published studies on microalgal proteins

Microalgae have long been proposed as a source of protein[30, 270]. As stated above, both the amino acid profile as well as the digestibility determine the protein quality[138, 146]. Since studies of protein balance (with feces collection) require expensive animal or clinical studies, a common starting point

is to evaluate the protein accessibility by *in vitro* digestion studies. However, comparison of results from *in vitro* studies is often difficult, since nearly every study uses its own method and often fails to note the activity of the proteolytic enzymes used[63, 149, 152, 202, 271, 272]. Nonetheless, *in vitro* studies suggest that drying *Spirulina* reduces the amount of protein available for digestion[202]. Furthermore, extracting the lipids with ethanol from dry, disrupted *Nannochloropsis granulata* was found to increase the amount of digestible protein compared to the disrupted, non-extracted microalgae[152]. There is reason to believe that the protein of whole microalgae remain encapsulated by the cell walls of some species[30, 273]: when *Nannochloropsis oceanica*, *Isochrysis galbana* and *Phaeodactylum tricornutum* replaced 24% of mink feed, the crude protein digestibility dropped from 90% to 70% for *Nannochloropsis*, but only marginally for *Phaeodactylum*[150]. Thus previous studies show that many microalgae species have amino acid profiles indicative of high nutritional value, though the accessibility of the protein may be dependent on microalgal species and the degree of cell disruption.

5.2. The *in vitro* digestion model of Paper II

In Paper II, the accessibility of pH-shift processed *Nannochloropsis* lipids and proteins was studied in a static *in vitro* digestion model. The digestion model was based on that of the Infogest consensus model, but with some modifications. The Infogest digestion model is a recent (2014) attempt by an international consortium of scientists to standardize static *in vitro* digestion models and thereby permit results from different studies to be compared[264]. The standardized method allows for some variation: *e.g.* if only lipids or proteins are being digested, there is no need for an oral step. However, in Paper II, an oral amylase digestion was included since the algal cell wall contains carbohydrates, and the possibility to digest these could affect the subsequent digestion of lipids and proteins of *Nannochloropsis*. Also for reasons of physiological relevance, the standard method was altered to include gastric lipase, purchased from Germe, France. Although the standard Infogest method recognizes the importance of gastric lipase, the final protocol does not contain gastric lipase since it was not commercially available at the time of print[264]. With gastric lipase available, Paper II presents a gastric step as a hybrid between two established protocols[264, 274], with details of the digestion given in **Figure 15**. Although lipolysis has not been compared specifically with and without gastric lipase, there is preliminary data suggesting that when gastric lipase is used, more acid needs to be added in the simulated intestinal step in order to keep the pH at 7. According to experts on gastric lipase, the enzyme can remain active also in the small intestine[261]. Thus, gastric lipase was included in the digestion model, to make the method and results more physiologically accurate.

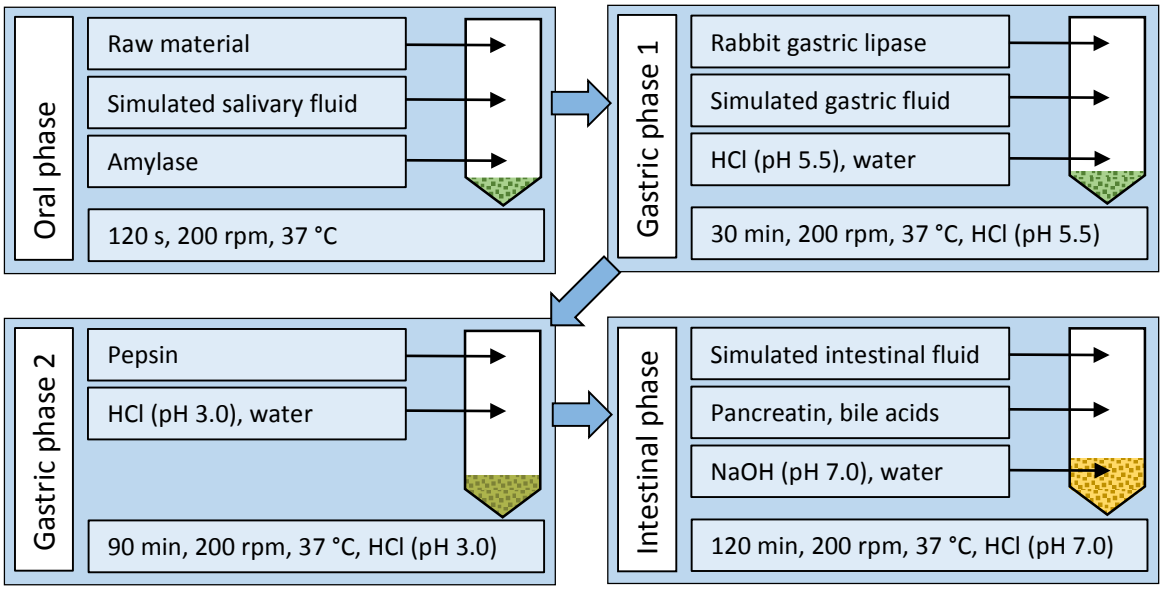

Figure 15 The *in vitro* digestion model used to assess the accessibility of fatty acids and proteins. The simulation of the mouth and small intestine are according to the Infogest protocol, while the gastric step is a hybrid between Capolino *et al*.[274] (phase 1) and the Infogest protocol[264] (phase 2).

5.3. Accessibility of *Nannochloropsis* macronutrients before and after pH-shift processing

5.3.1. Accessibility of *Nannochloropsis* fatty acids

The accessibility of *Nannochloropsis* lipids was assessed by measuring free fatty acids after *in vitro* digestion of various products of the pH-shift process. Whole algae (before pH-shift processing) were compared to i) lysate (after cell disruption), ii) the products of the pH-shift process, and iii) the product after a heat-treatment at 72 °C for 15 s, simulating a down-stream pasteurization of the product. The fatty acids present in intact *Nannochloropsis* were not accessible: less fatty acids were detected in digested whole algae than in the digested negative control sample (only seawater), see **Figure 16**, suggesting that *Nannochloropsis* acted as dietary fiber, adsorbing other nutrients in the digestion mix. Breaking the cells open by bead-beating greatly increased the accessibility: 36% (standard deviation = 2.5%, n = 3) of total fatty acids were hydrolyzed in the digestion of broken cells. However, up to 46% (± 2.8%) of the fatty acids were hydrolyzed in the digested product of the pH-shift process, suggesting that further processing of the disrupted cells improves the fatty acid accessibility. The heat-treatment did not cause a statistically significant change in the amount of free fatty acids. The lipid hydrolysis of the pH-shift process product can be interpreted as being good, considering that at most, 67% hydrolysis (representing cleavage of two out of three fatty acids from a molecule of triacylglycerol) was expected.

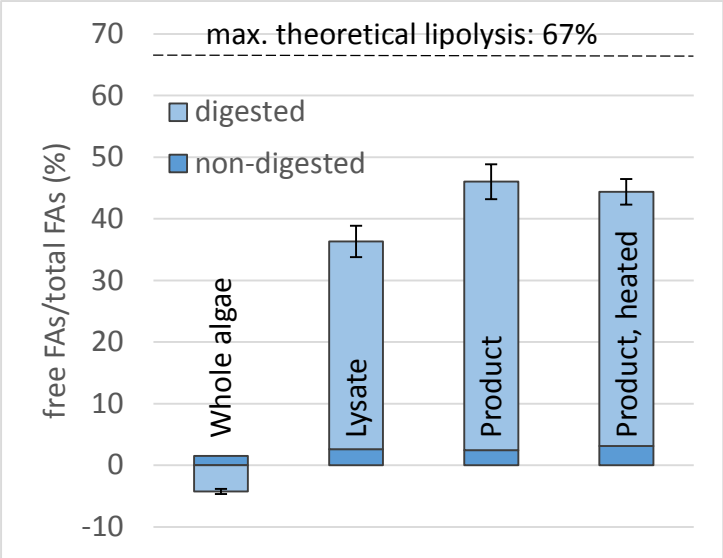

Figure 16 Lipolysis of fatty acids of *Nannochloropsis* in various stages of the pH-shift process: *Nannochloropsis* before pH-shift processing (whole algae), after cell disruption (lysate), after the entire process (product) and the product after heat-treatment. Error bars show standard deviation, with n = 3 for all treatment types, except the product, where n = 6. In all samples, 2-3% of the total fatty acids were found to be non-esterified before digestion (non-digested). For all of the digested values, the negative control (digested seawater) has been subtracted, leading to a negative value of digested whole algae.

The fatty acid profiles *pre*- and *post*-digestion were compared. Chemical modification of lipids during digestion was observed previously[255], as well as in Paper II: while C9:0 was not detected in the raw material, it was present in the digested samples. The digestive enzymes and bile acid were checked for the presence of C9:0, but none was found, thus, C9:0 must have been formed during digestion. The presence of C9:0 suggests that fatty acids were not only hydrolyzed from their parent molecule but they were also cleaved by oxidation. Conversely, less EPA was present in the free fatty acid fraction of the digested samples than the raw material, though this is not necessarily an indication that EPA was being oxidized: this could also be an indication that lipolysis of EPA was low. Low lipolysis might occur if the bond of the esterified EPA is inaccessible to the lipase, which might occur if EPA is predominantly present in the *sn*-2 position of triacylglycerols, phosopholipids and glycolipids. Gastric and pancreatic lipase are known to preferentially attack the *sn*-1 and *sn*-3 positions of triacylglycerols, leaving a monoacylglycerol with a fatty acid attached at the *sn*-2 position[259]. However, *in vivo* the monoacylglycerols are absorbed into micelles and from there by enterocytes[84]; in other words, the presence of free fatty acids in the digested samples is an incomplete measure of accessibility, since it misses the monoacylglycerols. An alternative method for determining accessibility could be to recover the micellar fraction of the aqueous phase by ultra-centrifugation[267]. Conversely, the micellar fraction may contain molecules which are not absorbed by enterocytes: for example, galactosylmonoacylglycerol is thought not to be absorbed by enterocytes, though it may be present in mixed micelles[89, 275]. Thus, while C9:0 is formed during the digestion, the fate of other fatty acids such as EPA requires further investigation.

5.3.2. Accessibility of *Nannochloropsis* proteins

The accessibility of *Nannochloropsis* protein was assessed by measuring degree of hydrolysis, *i.e.* the amount of broken peptide bonds, after *in vitro* digestion of various products of the pH-shift process. Casein, a protein known to have good digestibility, was compared to i) whole algae (before pH-shift processing), ii) lysate (after cell disruption), iii) the product of the pH-shift process, and iv) the product after a heat-treatment at 72 °C for 15 s. Proteins in intact *Nannochloropsis* were not accessible to the digestion enzymes used in the *in vitro* model, see **Figure 17**. Only 3.3% (standard deviation = 5.2%, n = 3) of peptide bonds were hydrolyzed in the whole *Nannochloropsis* cells subjected to the mammalian enzymes in the *in vitro* digestion. Cell disruption increased the hydrolysis to 36% (± 21%) of the peptide bonds, with further processing allowing up to 49% (± 9.4%) hydrolysis, comparable to the casein control (40% ± 12%). Given the large variance in the determination of the degree of protein hydrolysis, the only statistically significant difference is between whole *Nannochloropsis* and pH-shift processed *Nannochloropsis*, demonstrating the necessity to break open the cells for the intracellular content to become accessible to digestive enzymes and for uptake. These results are in line with a previous study[271] on *Scenedesmus* spp., another microalgae with a recalcitrant cell wall. The effect of different cell disruption methods on *in vitro* protein accessibility was assessed, and the study concluded that the more thoroughly the microalgae were disintegrated by bead-milling, the higher the protein digestibility was[271].

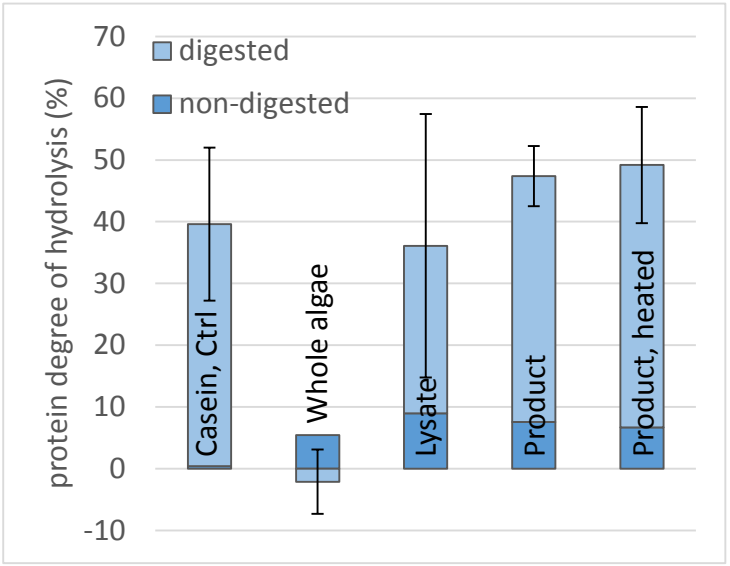

Figure 17 Protein degree of hydrolysis of *Nannochloropsis* in various stages of the pH-shift process: *Nannochloropsis* before pH-shift processing (whole algae), after cell disruption (lysate), after the entire process (product) and the product after heat-treatment. Casein, a protein with good digestibility was included as a positive control. Error bars show standard deviation, with n = 3 for all treatment types, except the product, where n = 6. Before digestion, 5-9% of the microalgal amino acids were not associated with a peptide bond. All digested values are presented with the negative control (digested seawater) subtracted, leading to a negative value for digested whole algae.

6. Analytical considerations

The main substances of interest in Papers I & II, were lipids and proteins. Sections 6.1 and 6.2 detail the rationale for choosing certain methods of lipid and protein analysis over other alternatives, and various experiences gained from working with the detailed methods, including advantages and disadvantages.

6.1. Lipid quantification and separation methods

Early work (Paper III) was used to develop a method for quantification of individual and total fatty acids, which was then used throughout Papers I, II & IV and is detailed in section 6.1.1. However, the developed direct transesterification method does not distinguish between esterified and free fatty acids, therefore a different method was needed for quantification of fatty acids released during digestion in paper II and is detailed in section 6.1.2.

6.1.1. Total fatty acids and fatty acid profile

6.1.1.1. Previously published methods

There is no shortage of methods for analyzing lipids in microalgae[276]: NMR[277], staining with lipophilic dyes[43, 278, 279], IR-spectroscopy[280, 281], Raman spectroscopy[282], non-linear microscopy[283, 284] are techniques which have been reported, but require either sophisticated instruments or only give information about the total lipid content and insufficient information about the fatty acid profile. Extraction of lipids by various methods using chloroform-methanol as solvents are widely used for total lipid determination [6, 95, 99, 101, 105, 111, 117, 118, 120, 162-170, 215, 234, 238, 285-287], but these methods suffer two major draw-backs: i) gravimetric determination is imprecise when sample sizes are small and other compounds are co-extracted with the lipids[173, 288, 289] and ii) the fatty acid profile requires a separate analysis[290, 291].

To determine the fatty acid profile of a lipid extract, transesterification is applied, which transfers fatty acids from their parent ester to methanol[80, 292]. The resulting fatty acid methyl esters (FAMEs) are relatively volatile compounds and thus separable by gas chromatography[293-296]. By omitting the extraction step and applying the transesterification directly to dry biomass, time can be saved[219, 297]. The resulting data can be used to identify and quantify individual fatty acids and by summing the individual fatty acids, the total fatty acids can be quantified.

6.1.1.2. Development of a direct transesterification method for microalgal fatty acids

Paper III compares several methods for fatty acid extraction and transesterification in three species of microalgae. The results provide indications that several common methods used in transesterification, yield essentially the same results. Furthermore, the results demonstrate that for species with recalcitrant cell walls such as *Nannochloropsis oculata*, the Bligh & Dyer method[298] extracts less fatty acids than the direct transesterification methods. The direct transesterification method developed in-house was based on that of Lepage & Roy (1986)[299] and used hydrochloric acid as a transmethylation catalyst in methanol. Transmethylation took place at 70 °C for 120 min. Compared to Christie's (1989) sulfuric acid method[294], the hydrochloric acid method requires less reaction time. Compared to boron trifluoride[297], hydrochloric acid is less toxic and cheaper. Drawbacks of the hydrochloric acid method are firstly that it is sensitive towards water (though acid catalysts are less sensitive than basic catalysts), requiring drying prior to analysis and secondly that no information about lipid classes can be gained unless these are extracted and separated before transmethylation. In spite of the drawbacks, the in-house method with hydrochloric acid was an improvement over extraction and gravimetric determination, which takes more time, requires more biomass, uses greater volumes of toxic solvents, and is imprecise since proteins are co-extracted with the lipids[289].

6.1.2. Free fatty acid quantification

For Paper II, lipid digestibility of crude and processed microalgae was determined as fatty acids liberated from their parent molecule, thus a method for quantifying the free fatty acids was needed.

6.1.2.1. Spectrophotometric method

Initially, a spectrophotometric method (Lowry & Tinsely, 1976, with minor modifications by Bernádez *et al*., 2005)[300, 301], was considered for the quantification of free fatty acids. The method requires the sample to be dissolved in chloroform, so microalgae and *in vitro* digested microalgae were extracted with chloroform-methanol, resulting in a distinctly green chloroform extract. Clearly, pigments were being extracted together with the lipids. *Nannochloropsis gaditana*, closely related to *Nannochloropsis oculata*, is known to contain chlorophyll a, violaxanthin and vaucheriaxanthin[302]. The spectrophotometric method by Lowry & Tinsely (1976) measures absorbance at 710 nm[300], the tail end of the chlorophyll a spectrum[302]. Options for how to proceed with the method were: i) to confirm that chlorophyll does not interfere with the measurements, *e.g.* by spiking oleic acid standard with varying amounts of chlorophyll a, which is expensive to buy in

its pure form and labor-intense to extract[303], or ii) to remove chlorophyll from the chloroform extracts, by a method which does not influence the amount of fatty acids present. Both options represented significant investments of time for method development, therefore free fatty acids were separated by solid phase extraction (SPE), as follows below.

6.1.2.2. Separation of lipid classes by solid phase extraction (SPE)

For recovering and quantifying free fatty acids in Paper II, the SPE method of Balasubramanian *et al.* (2013) was selected. The method separates lipid classes into i) neutral lipids, ii) free fatty acids and iii) polar lipids[304]. The method is based on that of Kaluzny *et al.* (1985)[305] and uses commercially available aminopropyl SPE cartridges, in which the solid phase is sandwiched between two frits placed inside a plastic barrel. Unknown to us at the time of the study, the plastic barrels leached palmitic acid and stearic acid, which interfered with the fatty acid analysis. Only after a tenacious literature search did I realize that the contaminants had been reported previously[306, 307]. The discovery of the contamination lead to the omission of palmitic and stearic acid from the free fatty acid profiles presented in Paper II while the contribution of contaminants to the *total* free fatty acids was < 3% and thus considered negligible and therefore included in Paper II.

Paper IV reports the presence of C16:0 and C18:0 contaminations from SPE-columns when eluting with 2% acetic acid in diethyl ether. Although the presence of said contaminants has been reported before[306, 307], the information is not searchable by databases such as Scopus and Web of Science. By dedicating an entire publication to the contaminations, hopefully a wider circle of readers will become aware of the potential pitfall of SPE-columns. In searching for the source of the contaminations, it was discovered that re-using (clean) columns somewhat reduced the amount of contaminations leached from the column but by no means removed them. Thus, it remains important to determine which fatty acids are present in the sample prior to SPE and if the presence of up to 60 µg C16:0 and C18:0 will change the fatty acid profile.

For preliminary determination of lipid class distribution of *Nannochloropsis* lipids (**Figure 9**), the method by Olmstead *et al.* (2013) was applied[229]. The method has been previously applied to separate lipid classes from *Nannochloropsis* extracts, with the authors reporting that fractions were well-separated[229]. The method uses other solvents than Balasubramanian *et al.* (2013), therefore it may be possible that less contaminants are leached from the SPE column; nonetheless, the presence of contaminants should be thoroughly investigated before the method is routinely applied.

6.2. Protein quantification and separation methods

6.2.1. Protein quantification

Protein quantification was key to mapping the protein solubility and protein yields in Paper I. Various methods considered for protein quantification are detailed below. Only the method by Slocombe *et al*. (2013) met the requirements of being i) specific for proteins (though there are compounds other than peptide bonds which react with the Folin reagent) ii) reproducible and iii) possible to carry out in the lab without purchasing expensive equipment.

6.2.1.1. Kjeldahl crude protein

The Kjeldahl method can be used to determine crude protein by assessing the total amount of nitrogen in a sample. However, results may be influenced by non-protein nitrogen-sources and the amino acid composition of the sample. While conversion factors exist for commonly analyzed matrices such as food, no such factors were established for the microalgae analyzed used in the presented studies at the time they were carried out[147].

6.2.1.2. Infrared spectroscopy

Infrared spectroscopy in the form of Millipore's Direct Detect system is performed by pipetting a very small sample volume (2 µl) onto a card which is inserted into the machine where it is dried and the infrared spectrum is recorded[308]. In a small pre-trial, the variance was unacceptably high. The small sample volume requires accurate pipetting and a homogenous sample, the latter which was not fulfilled by all sample types used in this project.

6.2.1.3. Lowry protein measurement

Two spectrophotometric methods were evaluated based on the Lowry method, in which protein first reacts with copper in alkali, followed by oxidation of Folin reagent[309]; specifically, the peptide bond is detected in the ensuing color change, though various phenolic compounds are known to also react with the reagents[310]. For any spectrophotometric quantification method, a standard is required, which ideally should be as similar as possible to the analyte. Bovine serum albumin might therefore appear to be an odd choice of standard; however, bovine serum albumin is readily available in pure form, distributed in accurate quantities and relatively inexpensive, in contrast to algal protein. Since the aromatic amino acids tryptophan and tyrosine are known to increase the absorbance in the Lowry assay[147], the amino acid profiles of bovine serum albumin[311] and *Nannochloropsis*[148] were compared. *Nannochloropsis* contains a comparable percentage of tryptophan and tyrosine to bovine serum albumin ($\Sigma_{TRP+TYR}$= 5.9% for *Nannochloropsis* and 5.3% for bovine serum albumin).

Bio-Rad kit

Trials were performed with a commercial kit, developed to be compatible with detergents such as SDS and sodium hydroxide, used in the sample preparation[312]. Three different freeze-dried microalgae were used in an attempt to find a preparation method applicable to microalgae with different cell walls: i) the thin-walled *Isochrysis galbana*[167], ii) the organic cell-walled *Phaeodactylum tricornutum*[313], and iii) the tough cellulosic-walled *Nannochloropsis oculata*[232]. Boiling in either 0.5 M NaOH, 1% SDS or both for 10 min was compared; boiling in 1% SDS resulting in the highest measured protein concentration for *Isochrysis* and *Phaeodactylum* but the lowest for *Nannochloropsis*. When the trial was repeated, the inter-day variability was found to be > 20% for some samples, an unacceptably large difference. On further examination, the method was found to be highly influenced by the dilution of the sample, with higher dilutions resulting in higher relative absorbance: when doubling the dilution, the measured protein concentration increased by 25% or more. Taking together the inconclusive results for sample preparation, the high variability and the non-linear response to dilution, the use of the Bio-Rad protein assay kit was discontinued.

Slocombe *et al*. (2013) method

The protein quantification method of Slocombe *et al*. (2013)[314] was used for protein measurements on Paper I. This method first extracts protein from dried algae by precipitating the protein with trichloroacetic acid and then quantifying it spectrophotometrically according to Lowry's method[314]. Disadvantages of the Slocombe *et al*. (2013) method are that it is time and labor-intense and there is some uncertainty in the weighing of the initial sample: according to the method, 5 mg of freeze-dried algae are to be weighed in, which demands high accuracy of the analytical balance being used. Advantages of the Slocombe *et al*. (2013) method include that it was developed specifically for microalgae, with i) the extraction developed to be effective for cells with recalcitrant cell walls, such as *Chlorella* and ii) it uses small sample sizes, which is an advantage when little microalgal biomass is available. Furthermore, variability was lower than in any other investigated method, with a coefficient of variation < 8% observed in initial trials.

6.2.2. Polypeptide profiling by SDS-PAGE

For Paper I, proteins from the fractions of the pH-shift process on *Nannochloropsis* were separated by SDS polyacrylamide gel electrophoresis (PAGE), a technique in which proteins are denatured and separated according to size in an electric field[245]. Depending on the purity of the proteins, the sample may be subjected to a purification step before denaturation in the loading buffer. A purification[315] was attempted for the *Nannochloropsis* pH-shift fractions; however, instead of distinct bands, lanes displayed one long smear, *i.e.* proteins did not separate. Eventually, suspecting

that the majority of the proteins were in fact hydrophobic membrane proteins[316], the purification step was omitted and the denaturation altered by adding SDS and urea[317] to the Lämmli loading buffer with β-mercaptoethanol. Furthermore, the denaturation was carried out for 60 min at room temperature instead of 95 °C for 5 min, to avoid the proteins from aggregating. To reduce streaking, presumably caused by aggregated proteins, the denatured sample was centrifuged at 15 000×g for 5 min at room temperature and the supernatant loaded onto the gel. Under the described conditions, it was possible to resolve the samples' proteins into distinct bands.

6.2.3. Degree of protein hydrolysis

For Paper II, protein digestibility was assessed by quantifying the broken peptide bonds and comparing to a theoretical value, calculated from the amino acids present in the original material. Two methods were considered, one based on Nielsen *et al*. (2001), in which the reaction between primary amines with o-phthaldialdehyde results in a compound which absorbs maximally at 340 nm[318], and the other on Adler-Nissen (1979), in the reaction between primary amines and trinitro-benzene-sulfonic acid results in a compound which absorbs maximally at 340 nm[319]. The Nielsen *et al*. (2001) method promises improvements over the Adler-Nissen (1979) method, including shorter analysis time, higher accuracy, less sensitivity to pH and less toxic reagents. However, it has been questioned if the Nielsen *et al.* (2001)-method indeed is less toxic, considering that it uses sodium tetraborate, a presumed human reproductive toxicant[320], therefore the method by Adler-Nissen (1979) was used in-house. Minor changes have been made firstly to scale the method down to microtiter scale: volumes were reduced by one-tenth and secondly the alternative wavelength (420 nm) described in the original method was used to avoid light absorption by the polystyrene 48-well plate[321]. In addition, due to the nature of the digested samples, light-scattering particles were removed by centrifugation at 2000×g for 5 min.

6.3. Total carbohydrate quantification

For quantifying total carbohydrates, the method by Herbert, Phipps and Strange (1971) was used, in which polysaccharides are hydrolyzed by sulfuric acid and react with phenol to form a colored compound which can be detected spectrophotometrically[250]. As the name suggests, the method does not distinguish between simple sugars (monomers, and oligosaccharides), simple polysaccharides (*e.g.* chrysolaminarin, which may be present in *Nannochloropsis*[51]), complex polysaccharides (*i.e.* combinations of different monomers) and complex macromolecules (including nucleic acids)[250]. The method was originally published for application to microbes and has since been both modified for microtiter scale and had its suitability for microalgae demonstrated[322].

7. Conclusion

The herein presented work has focused on application of the pH-shift process to *Nannochloropsis* to recover lipids and proteins without the use of organic solvents. Although the pH-shift process did not yield separate lipid and protein fractions, the product nevertheless was found to have potential as a food or feed. The main discoveries of the presented work are as follows:

- *Nannochloropsis* in seawater (10% on dry weight basis) has high protein solubility (> 80%) between pH 6 and 10 with low solubility (< 10%) at pH 4 and below.
- The protein solubility characteristics were used to develop a process in which *Nannochloropsis* was solubilized at pH 7 and then precipitated at pH 3.
- The main unit operations of the developed process were i) bead-beating for cell-disruption and ii) centrifugation at low g-force to separate soluble from non-soluble material. All unit operations are scalable.
- Protein yield was higher with the process version in which proteins were solubilized at the native pH (7) compared to solubilization at pH 10: 86% *vs.* 72%. The developed pH 7-process resulted in a product which related to the unprocessed material as follows: Water and ash content was reduced; total fatty acids and LC n-3 PUFAs were increased marginally; total carbohydrates and proteins were increased slightly.
- Although crude *Nannochloropsis* had potential as a source of LC n-3 PUFAs and nutritionally high-value protein, the *in vitro* digestion model demonstrated that the cell wall posed a formidable barrier with no digestion of *Nannochloropsis* lipids or proteins observed for the unprocessed material (intact microalgae). Cell disruption permitted roughly half of the theoretically accessible fatty acids to be hydrolyzed, and hydrolysis was further increased by pH-shift processing. pH-shift processing permitted roughly half of the peptide bonds to be hydrolyzed.
- Direct transmethylation on dry microalgal biomass with hydrochloric acid was developed as an analytical tool to determine total fatty acids and fatty acid pattern, with results comparable to direct transesterification methods with other catalysts. Compared to solvent extraction, the developed method saved time, reduced the use of toxic chemicals and recovered more fatty acids with less variability, especially for cells surrounded by a recalcitrant cell wall, such as *Nannochloropsis*.
- When using SPE to fractionate free fatty acids from other lipid classes, contaminations (palmitic and stearic acid) were discovered and these were traced to the plastic in the extraction columns. Although total free fatty acids could still be determined (contaminations contributed < 3% of the total mass), amounts of palmitic and stearic acid originating from the algae could not be accurately determined.

8. Future outlook

The presented work indicates that the LC n-3 PUFAs and protein in pH-shift processed *Nannochloropsis* might find application as a food ingredient. Various improvements on the pH-shift process might be possible. However, before *Nannochloropsis* can make the jump from aquaculture to human food, various knowledge gaps need to be filled:

- LC n-3 PUFAs are not only associated with health benefits: they are also more susceptible to oxidative deterioration than more saturated fatty acids[323]. Conversely, microalgae contain various compounds which protect against oxidation both during storage and *in vitro* digestion[324, 325]. Thus, investigation is warranted of the lipid stability of a pH-shift processed *Nannochloropsis*-product during both storage and *in vitro* digestion.
- Protein quality determination will require further experiments, to establish the digestibility of pH-shift processed *Nannochloropsis* in animal models[137, 146].
- The techno-functional properties of pH-shift processed *Nannochloropsis* have not been fully explored. Especially if the pH-shift process product is to be used with other food components, the ability of the product to form gels or stable emulsions requires further investigation. Factors of interest include the solubilization pH and the ionic strength of the surrounding medium.
- Given that other microalgae have protein solubility curves[141, 202, 207] similar to *Nannochloropsis*, the pH-shift process could be applied to other microalgal biomasses could be investigated and general principles for the pH-shift process on microalgae established.
- By adding carbohydrate depolymerizing enzymes such as cellulases, it may be possible to reduce the water-holding capacity of the product, thereby improving the dewatering of the process.
- *Nannochloropsis* is not known to produce any toxic secondary metabolites. However, tests are needed to confirm that *e.g.* the concentrations of nucleic acids are acceptable[30]. Analagours to other microalgae and microalgal products[326-328], acute and subchronic toxicity of *Nannochloropsis* and pH-shift process product should be assessed to compliment an already published study[268]. Furthermore, it may be necessary to demonstrate that the culture, harvest and processing of *Nannochloropsis* does not add heavy metals, persistent organic pollutants, or processing chemicals to the product, while harmful bacteria stay acceptably low[30].
- Once the safety of *Nannochloropsis* has been established, food products with the pH-shift process product can be developed and a trained test panel should determine various sensory

qualities of the food products, before the consumer acceptance is evaluated in an appropriate focus group.

- For the direct transesterification method, it may be possible to further reduce the incubation time and temperature[229].

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