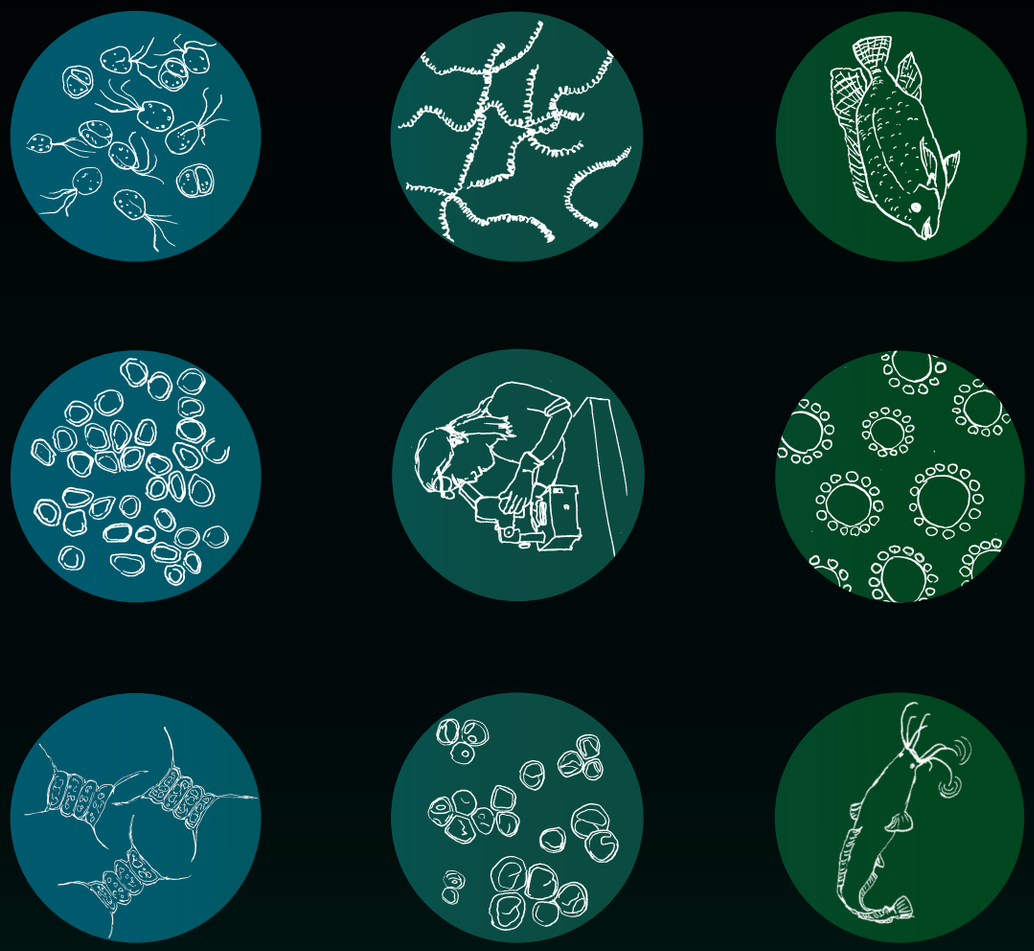
Unicellular protein

Isolation, techno-functionality and digestibility



Emma Teuling

2018 Unicellular protein: isolation, techno-functionality and digestibility Emma Teuling

Propositions

1. For microalgae, protein accessibility dominates protein digestibility.
   (*this thesis*)

2. The emulsifying ability of proteins as determined by the critical protein concentration is affected by the volume fraction of oil.
   (*this thesis*)

3. Substrate specificity of lytic polysaccharide monooxygenase subclasses cannot be predicted by their amino acid sequence similarities.

4. Promoting the home use of antiseptic hand soaps puts public health at risk.

5. The new norms (2014) of the Dutch mental healthcare association stimulate abuse of the DSM IV classification to benefit patients in their insurance coverage.

6. The difficulties in forming majority cabinets in The Netherlands are not related to the number of active political parties.

Propositions belonging to the doctoral thesis:

'**Unicellular protein: isolation, techno-functionality and digestibility**'

Emma Teuling
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Unicellular protein: isolation, techno-functionality and digestibility

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Unicellular protein: isolation, techno-functionality and digestibility

Emma Teuling

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ABSTRACT

Unicellular, photosynthetic microalgae and cyanobacteria are potential new protein sources for both food and feed. Variations between these sources are reflected in chemical variations (e.g. in the chemical composition) and in structural variation (e.g. in cell wall robustness). The aim of this thesis was to understand how chemical and structural variations between unicellular photosynthetic sources affect the application of unicellular protein as techno-functional ingredients and as a fish feed ingredient.

To study the applicability of unicellular protein as techno-functional ingredient in food, proteins were extracted and isolated from the cyanobacterium *Arthrospira (spirulina) maxima* and the microalgae *Nannochloropsis gaditana, Tetraselmis impellucida* and *Scenedesmus dimorphus*. Chemical variations observed between photosynthetic unicellular sources were reflected in variations in protein extractability. The isolates (62–77% w/w protein) varied in protein solubility as a function of pH and ionic strength, especially at pH < 4.0. Isolates from *N. gaditana*, *T. impellucida* and *A. maxima* were able to form emulsions ($d_{3,2}$ 0.2–0.3 μm) at pH 8.0. The amount of each isolate needed to form emulsions varied between the isolates (9–74 mg protein / mL oil), but was within the range of proteins from both similar (photosynthetic) sources (algae and sugar beet leaves) and other protein sources (dairy, legume and egg). Minor differences were observed in the pH dependence of flocculation amongst the isolate stabilized emulsions.

To study the applicability of microalgae and cyanobacteria as dietary protein sources for fish, *Chlorella vulgaris*, *S. dimorphus*, *N gaditana*, and *A. maxima* biomass was incorporated in fish feed (30% inclusion) and fed to Nile tilapia and African catfish. The cell walls of these unicellular sources used were quantified to vary in their robustness to mechanical degradation (structural variations). Although protein digestibility varied between the unicellular sources (ranging from 67–83%), the protein digestibility did not relate to the variations in unicellular cell wall robustness. There was no difference between both fish species regarding the nutrient digestibilities of the unicellular sources. Subjecting *N. gaditana* biomass to treatments that decrease its cell wall integrity increased *in vitro* accessibility of microalgae nutrients up to 4 times. The increased *in vitro* accessibility correlated with an increased *in vivo* digestibility of protein in Nile tilapia, confirming that nutrient accessibility plays an important role in the nutrient digestibility of microalgae in fish.

In conclusion, chemical variations observed between photosynthetic unicellular sources were reflected in variations in protein extractability. Structural variations between the sources were reflected in variations in *in vivo* protein accessibility and subsequent protein digestibility.

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1 General Introduction.

By 2050 the world population is expected to increase by 30% compared to 2017, reaching approximately 9.8 billion [1]. To feed this number of people, food and feed production need to be increased by 50 percent in this time period [2]. A significant issue in both the food and feed production is the availability of protein(rich) sources. Consequently, there is an interest in exploring alternative protein sources. These alternative protein sources should be produced in a sustainable way, not competing with current food and feed production and in addition have a reasonable protein digestibility relative to its price in comparison to current sources used in feed formulation. One class of alternative protein sources that has the potential to meet these requirements are the unicellular, photosynthetic microalgae and cyanobacteria.

For application in food products, the microalgal and cyanobacterial proteins need to be extracted and isolated to be free of colour and highly soluble. Such levels of purification are not necessarily required for the application in feed. The isolation procedure required for food yields a considerable side stream. This side stream contains many valuable nutrients, of which the focus will be on proteins in this thesis. When using these insoluble (or membrane-bound) protein fractions in feed, and the soluble isolates in food, both the feed and food markets can be supplied with algal or cyanobacterial protein. In this thesis, both fields of application of proteins from unicellular sources (various microalgae and one cyanobacterium) are explored. The first aim of this thesis is to provide a better understanding of the extent to which photosynthetic unicellular sources differ in their chemical and protein composition, and how these differences affect protein digestibility in fish, protein extractability and techno-functionality of the protein isolates obtained. For unicellular protein digestibility in fish, the effect of cell wall hardness, protein accessibility and fish species on unicellular protein digestibility in fish was elucidated.

MICROALGAE AND CYANOBACTERIA AS PROTEIN SOURCES

Protein contents

Microalgae and cyanobacteria encompass a wide variety of photosynthetic unicellular organisms, ranging from marine prokaryotes to freshwater eukaryotes and belonging to over eleven phyla [3]. The only microalgae and cyanobacteria currently holding a GRAS status as food ingredients are *Dunaliella bardawil*, *Chlorella vulgaris* and *Arthrospira* (*spirulina*) *platensis* [4]. These sources are also already applied industrially in feed: in aquaculture hatcheries (for molluscs, crustaceans and zooplankton) [5]. They are, however, not yet commercially applied as a protein source in formulated fish feeds. Microalgae that do not hold the GRAS status but are applied commercially in aquaculture hatcheries are from the genera *Nannochloris*, *Haematococcus*, *Tetraselmis*, *Pyramimonas*, *Nannochloropsis*,

Isochrysis, *Pavlova* (*Monochrysis*) and *Schizochytrium* [6]. In addition, microalgae from the genus *Scenedesmus* have been studied extensively in food and feed related research. The protein contents of these microalgae and cyanobacteria vary greatly, between 12 and 72% (w/w on DM) (Table 1). This variation is partially due to growing conditions, seasonal conditions and due to differences between species within each genus [7, 8] (Table 1). Although variation in protein content due to species and growing conditions also occurs in other protein sources, the variations observed in protein contents within a single genus of a microalga or cyanobacterium are larger than those of for example wheat (9–20% protein), lupin (24–48 %) and soy (31–43%) [9].

Table 1: Protein contents of commonly used microalgae and cyanobacteria [% w/w, on dry weight basis].

Genus	Protein content		References
	Mean ± SD	Range [a]	
Arthrospira	54 ± 17	21–72	*7, 10, 11*
Chlorella	46 ± 13	27–58	*11-14*
Dunaliella	28 ± 9	20–40	*12, 15, 16*
Haematococcus	25 ± 2	24–26	*17-19*
Isochrysis	30 ± 7	22–42	*15, 20-22*
Nannochloris	38 ± 7	30–52	*15, 23*
Nannochloropsis	42 ± 10	18–59	*11, 14, 22, 24, 25*
Pavlova (Monochrysis)	26 ± 3	20–29	*15, 22*
Scenedesmus	41 ± 15	12–54	*12-14*
Schizochytrium	14 ± 2	12–16	*26-28*
Tetraselmis	33 ± 6	26–47	*11, 15, 20, 21, 29*

[a] Range due to varying culture conditions and different species within the same genus.
N.b.: no protein contents were found for *Pyramimonas sp.*

Protein types

Although few studies report protein extraction and protein isolation from unicellular sources, plenty of data is available on the types of proteins that are present in these sources. These data can be used to steer protein extraction and isolation processes, and can help to predict which proteins will be retained during such an isolation process. The most well-known protein that is present in all unicellular sources discussed above is the carbon fixating enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco). In cyanobacteria and microalgae, Rubisco consists of 8 large and 8 small subunits (L_8S_8) [30], and does not have post-

translational modifications or prosthetic groups [31] [i,ii]. Typically, the molecular weight (Mw) of the large subunit of Rubisco is rather conserved over various organisms, and ranges from 52–54 kDa (depending on the organism) [31] [ii]. The Mw of the small subunit of Rubisco is more variable between organisms, and ranges from 10–17 kDa (also depending on the organism) [31] [i]. The L_8S_8 form of Rubisco has an Mw of 500–570 kDa. The interactions between the 16 subunits are typically noncovalent, and are a combination of hydrophobic interactions, hydrogen bonding and salt bridges [32]. There are indications of disulfide bridging between 2 large subunits in Rubisco molecules from spinach [33], although Rubisco from other sources do not contain any disulfide bridges (e.g. from *Galdieria* sp. [34]). Next to Rubisco, all photosynthetic organisms contain light harvesting proteins. Light harvesting proteins in microalgae have subunits of 21–44 kDa [31, 35, 36] [iii]. They are expected to be associated into multimeric complexes, similar to the light harvesting complex-II (LHC-II) proteins from spinach. Spinach LHC-II proteins are trimers, where each monomer consists of 10 polypeptide chains each (PDB ID 1RWT) [37]. These proteins can form super complexes with photosystem II via antenna proteins [38]. Cyanobacteria synthesize multimeric blue pigmented phycocyanins for light-harvesting [3], with subunits of 15–22 kDa [39].

A typical amino acid profile of Rubisco (taking the microalgae *Nannochloropsis gaditana* as example) is shown in Table 2. The amino acid composition of microalgae and cyanobacteria in relation to others sources, with respect to the nutritional quality (essential amino acids) is discussed in more detail in Chapter 2. Based on the amino acid composition, the LS_8SS_8 form of the *N. gaditana* Rubisco has a (computed) pI of 5.68. Isoelectric points of other Rubisco entries were computed to be in the range of 5.88–8.00 [40] [iv].

[i] Uniprot search terms: rbcS/cbbS genes in *Arthrospira* sp., *Nannochloropsis* sp., *Scenedesmus* sp. and *Tetraselmis* sp. Accession numbers used: D4ZVW5, W6SIC7, K1VV20, A0A023PJK0 and K9ZWI1

[ii] Uniprot search terms: rbcL/cbbL genes in *Arthrospira* sp., *Nannochloropsis* sp., *Scenedesmus* sp. and *Tetraselmis* sp. Accession numbers used: T1RH29, Q3S3D2, B5VXI0, D4ZVW7, Q1KVV0 and K9ZV74.

[iii] Uniprot search terms: LHC genes in *Tetraselmis* sp. (accession numbers used: A0A061RA39, A0A061RJR5, A0A061SK82, A0A061S745, A0A061SA24, A0A061R6B3, A0A061R2N8, A0A061S1P5, A0A061R213, A0A061S9W9 and O22496); LHC genes in *Scenedesmus* sp. (accession numbers used: A2SY33, A2SY34, A2SY35, A2SY32); and LHC genes in *Nannochloropsis gaditana*. (accession numbers used: K8YPQ7, W7TX20, W7UAI7, W7T6P5, W7TFG9, W7TZB5, W7TTD7, W7UBF0, W7U2H0 and W7TCK1)

[iv] Accession numbers used: 4MKV, 1WDD, 1RLC, 1RLD, 1RBL, 1RSC, 1BWV,1BXN, 1EJ7, 1IWA, 2YBV, 3AXM, 3AXK, 3ZXW, 4F0M, 4F0K, 4F0H, 1UPM, 1UPP, 1IR1, AA1, 1RCX, 1RXO, 1RBO, 1RCO, 8RUC, 1AUS, 2VDH, 2VDI, 2V67, 2V68, 2V63, 2V69, 2V6A, 1UW9, 1UWA, 1IR2 and1GK8.

Table 2: Amino acid profile [w/w %], of the large subunit [a] (LS), small subunit [b] (SS) and of the 16 subunit complex [c] (L_8S_8) of Rubisco from *Nannochloropsis gaditana*.

	ALA	ARG	ASN	ASP	CYS	GLN	GLU	GLY	HIS	ILE
LS	6.3	7.2	4.2	5.5	1.3	3.3	7.2	4.7	2.0	6.3
SS	3.9	8.5	4.1	4.2	2.5	4.7	9.4	2.4	2.5	6.8
L_8S_8	5.8	7.5	4.2	5.2	1.6	3.6	7.7	4.1	2.1	6.4
	LEU	**LYS**	**MET**	**PHE**	**PRO**	**SER**	**THR**	**TRP**	**TYR**	**VAL**
LS	8.4	6.2	3.9	5.2	2.9	3.9	5.6	2.8	7.0	6.1
SS	7.5	3.9	2.4	4.5	5.3	4.2	5.5	4.5	10.9	2.4
LS_8SS_8	8.2	5.6	3.5	5.0	3.4	4.0	5.6	3.2	7.9	5.2

[a,b] Data obtained from [40] with accession codes [a] K9ZV74 and [b] A0A023PJK0.
[c] Calculated from [a] and [b].

PROTEIN EXTRACTION

Unicellular cell disruption

Before the proteins (described above) can be obtained from unicellular sources, they need to be extracted from the biomass. In microalgae and cyanobacteria, proteins are present in the cytoplasm, bound to cell walls and membranes or enclosed in cell organelles (e.g. in starch sheath enclosed pyrenoids in *Tetraselmis* species [41]). To liberate the cytoplasmic proteins from the biomass (thereby increasing their bioavailability and extractability), the cell walls of the organism need to be disrupted. The energy and/or mechanism needed to disrupt the cell walls depends on amongst others the cell wall structure and cell size [42, 43].

Although some microalgae do not have a cell wall at all [44], the majority of microalgae have cell walls. Microalgal and cyanobacterial cell walls lack the lignin structures that are present in terrestrial plants [45, 46], and are often cellulose based (e.g. in *Nannochloropsis gaditana*, *Chlorella* sp. and *Scenedesmus* sp. [47-50]) or peptidoglycan based (in cyanobacteria like *Arthrospira* sp. [51]). Next to these basic cell wall structures, microalgal and cyanobacterial cell walls are sometimes coated with (amongst others) mucus layers [3] or algaenan layers [47, 49]. The total variety in cell wall structures in microalgae is therefore much wider than commonly found in plants (both terrestrial and aquatic). Microalgal cell walls can further be protected by external ornaments like sheaths or scales [3]. It is generally assumed (although without scientific proof) that peptidoglycan cell walls of cyanobacteria are less robust against mechanical disruption than the cellulose cell walls of microalgae, and that additional coatings and ornaments add to this robustness. Currently there are no standardized methods to determine cell wall strength. Examples of methods that are used in research are discussed in more detail in the paragraph "**quantifying cell wall strength**". Despite these efforts, no data sets are available at this time that support the assumed relation between cell wall type and cell wall strength.

Methods that disrupt cell walls (without quantifying cell wall strength) can be divided into four categories: enzymatic, chemical, physical and mechanical methods [52].

Examples of these include the use of carbohydrases (like cellulases), alkaline and organic solvents, thermal treatments, freeze drying, bead milling and ultrasonication [52, 53]. Using chemicals to hydrolyze the cell walls is likely to also hydrolyze the proteins present in the cells. Similarly, the use of carbohydrases poses a risk of protein hydrolysis by introducing proteases to the system, since often there are some proteases present in commercial carbohydrases. Mechanical and physical methods are therefore considered to be most suitable to extract high molecular weight, native-like proteins from microalgae and cyanobacteria.

Quantifying unicellular cell wall strength

Research that focussed on measuring the hardness of cell walls of unicellular sources often used gas decompression of cells [54] and indentation and disruption of cells [55, 56]. In a comparative study using gas decompression [54], the bacterium *Salmonella typhimurium* and the unicellular green alga *Chlamydomonas eugametos* had similar tensile strengths (100 and 95 atmospheres, respectively). Both organisms have cellulose-free cell walls, the walls are peptidoglycan and glycoprotein based [57, 58], which may explain the similarity in breaking strengths. Another reason for this similarity might be the limitations of this technique: the authors of the abovementioned study ([54]) describe how, next to cell wall thickness and cell wall microstructure, the breaking strength as quantified by gas disruption depends on the gas diffusion rate, cell size and cell shape. These factors limit the applicability of gas decompression as a tool to compare cell wall hardness of various microalgae and cyanobacteria. Another way of measuring cell wall strength described in literature is atomic force microscopy (AFM) (e.g. [55, 56]). AFM measures the force needed to indent and to break cell walls. Unlike gas decompression, the cell breaking force measured with AFM does not depend on cell size, as was shown for microbial cells [56]. Algal and microbial cell wall strengths measured with AFM differed greatly between studies. For example, the cell wall strength of *Chlorococcum* sp. cells was reported to be 619 kPa and 775 kPa [42] (nitrogen-depleted and nitrogen repleted cells respectively) whereas another study reported cell wall strengths of 58 MPa and 2 MPa for *Scenedesmus dimorphus* cells [59] (dehydrated and hydrated cells respectively). Cell wall strengths of up to 22.4 GPa were measured in diatoms containing silica cell walls [60]. None of these studies, however, have measured differences in cell wall strength between various species of microalgae with cellulose-based cell walls. Differences measured between the various studies mentioned above may therefore be representative of differences in cell wall strengths between the unicellular species, but also of differences in the experimental set-up used. Overall, these results indicate that the AFM technique could be applied to measure differences in cell wall strength between various microalgae and cyanobacteria, but that the cell wall strength measured may depend on the experimental setup used.

Unicellular protein extraction

Considering mild protein extractions from cyanobacteria and microalgae, published work primarily describes the use of bead milling to disintegrate cells. These studies (e.g. [29, 61, 62])

report protein extractabilities of 21–85% from *Arthrospira*, *Chlorella* and *Tetraselmis*. Some studies have used other means of (mild) cell disruption techniques, including high pressure cell disruption [63] and sonication [64] on *Chlorella* species, leading to protein extraction yields of 6–35%. It should be noted that these protein extraction yields never reach 100%. This is (in most cases) not due to a lack of cell disruption, but to the inability to extract proteins that are membrane (or cell wall) bound and proteins that are enclosed in cell organelles. It should be noted that the extractable protein fractions obtained from plants and microalgae include considerable fractions of low Mw proteinaceous material, i.e. amino acids and peptides, rather than high Mw proteins. For example, 36–56% of the total proteinaceous material extracted from microalgae [29] and sugar beet leaves [65] is of low Mw (< 14 kDa). This low Mw fraction is often not retained during further isolation of the large Mw proteins present, due to inclusion of dialysis and/or acid precipitation steps.

Protein deteriorating reactions during protein extraction

Proteolysis by endogenous proteases

During protein extraction from plants, the release of naturally present proteases can decrease the yield of high Mw proteins. For example, in wheat, pea and kale, proteolytic activity induced losses in (high Mw) protein extraction yields of up to 40% within 2 h after protein extraction [66]. Since proteolytic activity is also reported in microalgae [67], it is possible that this can influence the protein extraction yield in microalgae as well. At this moment, however, no data is available on protease related protein yield losses in microalgae or cyanobacteria.

Polyphenol – protein interactions

Protein extraction from plant sources can be influenced by the presence of phenolic compounds and the (natural) occurrence of enzymatic reactions. An important enzymatic reaction that can take place upon protein extraction from plant sources is the oxidation of *o*-diphenols to *o*-quinones, and the subsequent covalent binding of *o*-quinones to the proteins present [68]. This reaction is catalyzed by enzymes categorized as polyphenol oxidases (PPOs). PPO-mediated binding between phenolic compounds and proteins is known to decrease protein solubility [69] and digestibility [70]. Therefore, the reaction presents an essential challenge that needs to be overcome for successful extraction and application of plant-derived proteins. The presence of phenolic compounds and PPO activity is for example a main issue in the production of sunflower protein products, as was described by González-Pérez [71]. The PPOs involved in the reaction are widespread in terrestrial plants, including mosses [72], ferns [73] and flowering plants [74]. In contrast, both multicellular and unicellular green algae (chlorophytes) lack the genes that encode PPOs [72, 73]. This means that protein extraction from macro- or microalgae is not susceptible to protein-deteriorating reactions due to intrinsic PPO activity. Protein extraction from these aquatic sources thus does not require (in contrast to extraction from terrestrial plant sources) the use of chemicals like sulfite or costly processing conditions like low temperatures or oxygen deprivation to yield high quality (both techno-functional and nutritional) protein extracts.

Next to enzyme-mediated binding, polyphenols can also bind non-covalently to proteins [75]. Polyphenols (including tannins) are part of the defensive mechanism in plants [76] and seaweeds [77]. The polyphenols and or the complexes formed have been shown to inhibit proteolytic enzymes and subsequent digestion of the proteins present [78]. In addition, several studies have shown a negative correlation between the total phenolic content of various seaweeds and their *in vitro* protein hydrolysis [79, 80]. In general, brown seaweeds appear to have higher phenolic contents (expressed as gallic acid equivalents) than red and green seaweeds (Table 3). The phenolic contents of microalgae and cyanobacteria are in the same range as some brown seaweeds (Table 3). No information is available, however, on the effect of phenolic compounds in microalgae on protein digestion.

Table 3: Phenolic contents of various microalgae, cyanobacteria and seaweeds, in gallic acid equivalents (GAE) on dry matter.

	mg/g DM (GAE)	**References**
Microalgae		
Chlorella sp.	0.75–7.7	*11, 81*
Nannochloropsis sp.	1.39–8.0	*11, 81*
Tetraselmis sp.	1.71–20.0	*11, 81*
Scenedesmus obliquus	1.94	*81*
Cyanobacteria		
Arthrospira (spirulina) platensis	10.7	*11*
Arthrospira (spirulina) maxima	3.2–12.9	*82*
Brown seaweeds		
Laminaria digitata	0.9–8.44	*79, 83*
Laminaria hyperborea	1.0–2.3	*83*
Saccharina latissima	2.3–6.8	*83*
Alaria esculenta	3.1–14.9	*83*
Fucus vesiculosus	46.7	*80*
Ascophyllum nodosum	58.7	*80*
Red seaweeds		
Hypnea charoides	8.44	*79*
Hypnea japonica	8.48	*79*
Palmaria palmata	4.8–5.5	*80*
Chondrus crispus	4.0–4.3	*80*
Green seaweed		
Ulva lactata	8.99	*79*

APPLICATION OF MICROALGAE AND CYANOBACTERIA IN FOOD AND FEED

Techno-functional properties in foods

Solubility

If new protein ingredients are intended as an alternative to currently used protein sources in food (e.g. dairy and egg), the solubility of the novel proteins is an important techno-functional property to study. Protein solubility is dependent on the type of protein (e.g. the amino acid composition and amino acid sequence) and the conditions in which they are solubilized (e.g. the pH and ionic strength of the solvent used).

At the pH where the net charge of a protein is zero (the isoelectric point or pI), the protein molecules will (typically) aggregate, leading to decreased solubility. Thus at a pH further away from the pI, solubility will be increased. The pI of a protein depends on its amino acid composition, and therefore varies between proteins. There are, however, exceptions to the solubility behavior as a function of pH, e.g. in whey proteins. Native whey proteins are typically soluble over the full pH range [84]. Caseins [85], as well as legume proteins (e.g. protein isolated from soy bean, pea and faba bean [86]), show the typical U-curve as a function of pH. In other words, these proteins are well soluble in alkaline conditions, are least soluble near the pI, and at lower pH (below the pI) their solubility is restored again. In recent years, a few solubility curves as a function of pH of algae and cyanobacterial proteins were published [87 29, 88, 89], of which examples are shown in Figure 1. These data show that the majority of proteins from these sources have very similar solubility curves as a function of pH. Microalgal and cyanobacterial proteins are more soluble in higher pH ranges, and have a minimum solubility at pH ≤ 4. Different than for the above mentioned caseins and leguminous proteins, microalgal and cyanobacterial protein solubility was not restored at pH < 4.0 in any of the reported solubility curves. It should be noted that for soy there are also examples where the solubility is not restored at low pH (Figure 1). As is discussed later, this might be due to differences in ionic strength. The point of minimum solubility of the unicellular proteins (pH 4) cannot directly be related to the pI of Rubisco, which is calculated to be at a higher pH range (5.88–8.00). The point of minimum solubility is thus considered to be due to the presence of other proteins next to Rubisco. Interactions between the protein molecules and (naturally present) charged carbohydrates like uronic acids [90] are expected to affect the pH dependent solubility of the proteins, including the lack of the U-shaped curve and the point of minimum solubility.

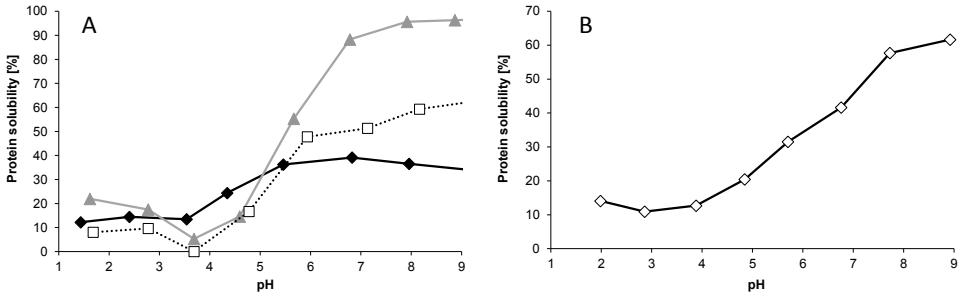

Figure 1: Protein solubility as a function of pH of (A) *Porphyridium cruentum* (◆), *Phaeodactylum tricornutum* (□) and soy bean (▲), adapted from [87] and (B) *Arthrospira (spirulina) platensis* (◇), adapted from [88].

Protein solubility also depends on the ionic strength of the solvent. Data on solubility behaviour of microalgal and cyanobacterial protein under different solvent conditions is very limited, and only available for *Tetraselmis impellucida* proteins. Protein solubility (as a function of pH) of proteins extracted from *T. impellucida* [29] were reported to be independent of ionic strength; the solubility curves were similar at ionic strengths of 30, 200 and 500 mM (Figure 2 A). In comparison, soy glycinin [91] and proteins from sugar beet leaves [92] (Figure 2 B) exhibit a strong dependence on ionic strength at lower pH ranges. For sugar beet leaf protein concentrate for example, solubility at pH ≥ 6 was 100% at ionic strengths of 10 mM and 500 mM (Figure 2 B, [92]). At lower pH values, however, the protein solubility was restored to 100% under low ionic strength conditions (10 mM), but was not restored under high ionic strength conditions (500 mM), at which the protein solubility was 20%.

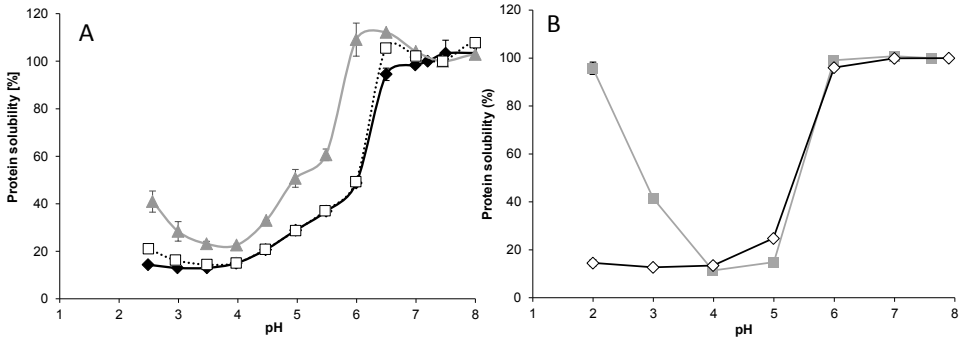

Figure 2: Protein solubility as a function of ionic strength of (A) *Tetraselmis impellucida* at ionic strengths of 0.03 M (▲), 0.2 M (□) and 0.5 M (◆), 100% = 5 mg protein / mL; adapted from [29] and (B) sugar beet leaf protein at ionic strengths of 10 mM (■) and 500 mM (◇), 100% = 10 mg protein / mL; adapted from [92].

Emulsification

Due to their amphiphilic character, proteins can act as emulsifying agents. Upon droplet formation in an oil-in-water system, proteins can adsorb at the oil-water interface and thereby reduce the interfacial tension. This prevents droplet coalescence and subsequent phase separation. The majority of the (few) publications on algae protein emulsion properties uses the emulsifying capacity to quantify the emulsifying ability of microalgal protein (e.g. [17, 93] and [63]). The emulsifying capacity is then defined as the oil volume [mL] that could be emulsified per g of protein. Emulsifying capacities reported of microalgal protein vary in the range of 534 [17] to 3740 [93] mL oil / g protein. The disadvantage of using emulsifying capacity, however, is that in some cases the volume of the oil fraction added is to the point of phase inversion (i.e. at oil fractions of ≥ 0.64 or ≥ 0.74; depending on the packing type). In consequence, in such studies the point of phase inversion is dominated by the oil fraction instead of the protein characteristics. This also means that the emulsifying capacity depends on the protein concentration in the original aqueous phase.

Another way to characterize the emulsion behavior of protein is by the droplet size ($d_{3,2}$) of the emulsions obtained after homogenization, and the effect of protein concentration on this droplet size. The $d_{3,2}$ decreases with increasing protein concentration, until a minimum droplet size ($d_{3,2,min}$) is reached. The protein concentration at which the $d_{3,2} = d_{3,2,min}$ is known as the critical protein concentration (C_{cr}). The C_{cr} can be used to quantify the emulsion forming ability of proteins [94]. At protein concentrations below the C_{cr}, proteins can adsorb to the oil-water interface formed during droplet formation, but cannot cover the complete surface at $d_{3,2} = d_{3,2,min}$. At protein concentrations at or above the C_{cr}, there is enough protein to cover the interface formed during droplet formation, at $d_{3,2} = d_{3,2,min}$ [94, 95]. In contrast to the emulsifying capacity, the C_{cr} is dependent on both interfacial and molecular protein properties (e.g. radius, net charge, exposed hydrophobicity). Additionally the C_{cr} depends on system characteristics like the volume oil fraction and ionic strength. Data on C_{cr}, of algae and cyanobacterial protein is limited to only one publication on *T. impellucida* [96], where a C_{cr} of 3.9 mg protein /mL was determined for a 30% [w/w] oil-in-water emulsion at pH 7 and I = 10 mM (Figure 3). This is for instance higher than the C_{cr} of whey protein isolate (at 2.6 mg / mL) determined under similar conditions. Another Rubisco-rich protein, isolated from sugar beet leaves was shown to have a C_{cr} of 2.1 (at pH 8 and I = 10 mM) and 5.4 mg protein / mL oil (at I = 500 mM) [92]. This sugar beet leaf protein C_{cr} value was determined in a 10% [w/w] oil-in-water emulsion, however, which is 3x less oil than what was used in the previously mentioned emulsion research on algae protein and whey protein (using 30% [w/w] oil). The C_{cr} value obtained of the sugar beet leaf protein should therefore be multiplied by factor 3 for fair comparison to the C_{cr} values for algae protein and whey protein.

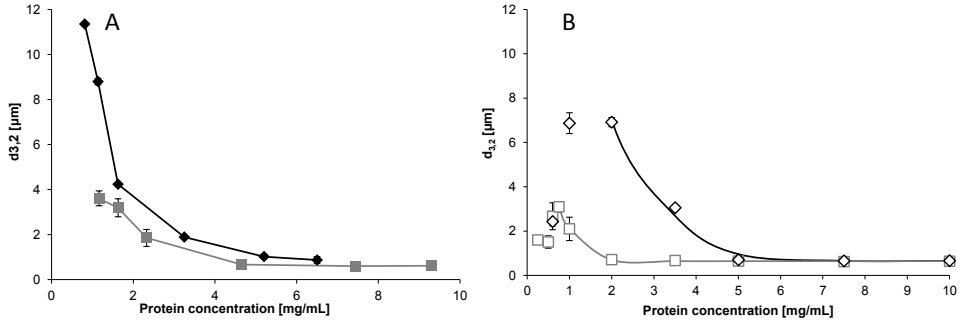

Figure 3: Droplet size ($d_{3,2}$) as a function of protein concentration of emulsions stabilized with (A) *Tetraselmis impellucida* protein isolate (◆) and whey protein isolate (■), both at pH 7 and an ionic strength of 10 mM (adapted from [92]) and with (B) sugar beet leaf protein concentrate at pH 8 and an ionic strength of 10 mM (□) and at 500 mM (◇) (adapted from [97]).

After emulsification, the emulsion droplets formed can flocculate as a function of pH and ionic strength. Droplet flocculation occurs when the attractive interactions (e.g. exposed hydrophobicity) between adsorbed protein layers on emulsion droplets are greater than the repulsive interactions (e.g. electrostatic repulsion and steric repulsion) between them [97]. Very few studies are published in which the effect of ionic strength and/or pH is studied on microalgal or cyanobacterial protein stabilized emulsions. The available data, from Schwenzfeier et al. [96] and Benelhadj et al. [88], show that the pH dependence of microalgal and cyanobacterial protein stabilized emulsions is very diverse (Figure 4). Unlike *A. platensis* protein and more common proteins like whey protein, *T. impellucida* protein is able to stabilize emulsions at low pH ranges (pH ≥ 4). This distinctive characteristic of *T. impellucida* protein was later attributed to the presence of co-extracted charged polysaccharides in the protein isolate [90].

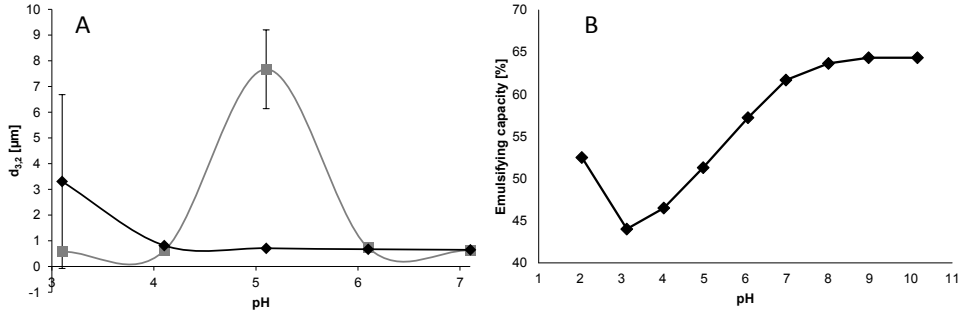

Figure 4: The effect of pH on the droplet size ($d_{3,2}$) (A) of emulsions stabilized with *Tetraselmis impellucida* protein isolate (◆) and whey protein isolate (■) (adapted from [96]) and on the emulsifying capacity (B) of *Arthrospira platensis* protein (adapted from [88]).

Nutritional quality of unicellular protein for humans

Next to the techno-functional properties discussed above, the nutritional quality of novel protein ingredients is also of importance for human nutrition. The nutritional quality of proteins for fish are discussed below. For humans, the quality of a protein as dietary ingredient relies on the content of essential amino acids and the digestibility of these amino acids [98]. More specifically, it relies on the content and digestibility of the limiting essential amino acid(s) of a protein ingredient. To quantify this protein quality, different scoring systems have been used. The most recent scoring system is the digestible indispensable amino acid score; DIAAS. This value is calculated from the amino acid composition and the (preferably ileal) digestibility of each amino acid [98]. Since the DIAAS is not known of microalgal or cyanobacterial protein, the amino acid score (AAS) is used to quantify the nutritional quality of the proteins. The AAS is calculated for each individual amino acid (AAS_{AA}) as shown in equation 1 [98].

$$AAS_{AA}[\%] = 100 \times \frac{mg\ of\ dietary\ indispensable\ amino\ acid\ in\ 1\ g\ of\ the\ dietary\ protein}{mg\ of\ dietary\ indispensable\ amino\ acid\ in\ 1\ g\ of\ the\ reference\ protein} \quad (1)$$

In which the dietary indispensable amino acids for humans are histidine, isoleucine, leucine, lysine, threonine, tryptophan, valine, the sum of cysteine + methionine and the sum of phenylalanine + tyrosine. The reference protein is the human amino acid requirement. The requirements selected were those for adults (> 18 years old) and for children (3–10 years old) was selected, estimated by the FAO [98]. The dietary protein is the protein of which the AAS is calculated, using the amino acid composition on protein basis. The final AAS of that protein is the lowest value (i.e. corresponding to the first limiting amino acid) of all AAS_{AA} values obtained. For completeness, the AAS_{AA} values for both the first and second limiting amino acid are shown (when applicable), referred to as AAS_{AA1} (i.e. the final AAS) and AAS_{AA2}, respectively. Proteins having a final AAS of ≥ 100% do not have any limiting indispensable amino acids and can therefore be regarded as proteins with a potentially high nutritional quality.

Considering the main protein of algae, Rubisco, the AAS for adults is 125%, and for children 118% (Table 4). These Rubisco AAS values are in range of the AAS values of the total AA composition of microalgae (85–136%). The majority of the microalgae shown in this overview have AAS values of > 100% and are thus expected to fulfil the (amino acid) nutritional needs of both adult humans and growing children. The microalgae that had AAS values < 100% (ranging between 81–92%) were limited by the combination methionine and cysteine (for both adults and children). With regard to their AAS values, the majority of the microalgae shown in Table 4 can be compared to the more commonly used protein sources soybeans and milk, which have AAS values of 122–125% and 141–148%, respectively. The true nutritional quality of microalgal protein still relies on the digestibility of the amino acids, however, as explained above. Digestibility of microalgal protein is expected to be limited when proteins are enclosed by cell walls. Therefore, to reach the full potential of microalgal that is reflected by their AAS values, the proteins present in microalgae need to be made accessible to digestion using cell disruption techniques, as described above.

Table 4: Amino acid score (AAS) of various microalgae, Rubisco, soybean, milk and fish meal, for humans [a].

Source		Adult (> 18 years)			Child (3–10 years)		
		AAS_{AA1} [f]		# $AAs_{limiting}$ [g]	AAS_{AA1} [f]		# $AAs_{limiting}$ [g]
Microalgae[b]	Class						
Dunaliella tertiolecta	Chlorophyceae	85	MET + CYS	1	81	MET + CYS	1
Nannochloris atomus	Chlorophyceae	113	-	0	108	-	0
Chroomonas salina	Cryptophyceae	122	-	0	114	-	0
Nannochloropsis oculata	Eustigmatophyceae	93	MET + CYS	1	88	MET + CYS	1
Tetraselmis chui	Prasinophyceae	114	-	0	109	-	0
Tetraselmis suecica	Prasinophyceae	115	-	0	114	-	0
Isochrysis galbana	Prymnesiophyceae	122	-	0	117	-	0
Isochrysis galbana (T-iso)	Prymnesiophyceae	136	-	0	128	-	0
Pavlova lutheri	Prymnesiophyceae	126	-	0	118	-	0
Pavlova salina	Prymnesiophyceae	92	MET + CYS	1	88	MET + CYS	2[f]
Rubisco (*Nannochloropsis gaditana*)		125	-	0	118	-	0
Soybean (*Glycine max*)[c]		125	-	0	122	-	0
Milk (*Bos taurus*)[d]		148	-	0	141	-	0
Fish meal (anchovies)[e]		130	-	0	125	-	0

[a] Based on the amino acid requirements estimated by the FAO [98], using amino acid compositions of [b] Brown et al [15], [c] Cervantes-Pahm et al. [99] and Panthee et al. [100], [c] Belitz et al. [101] and Williams et al. [102], [d] the Uniprot database [31] (accession codes K9ZV74 and A0A023PJK0), and [e] NRC [103] (using fish meal with international feed number 5-01-985). [f] AAS_{AA1} is the AAS of the first limiting amino acid (and therefore the total AAS) and [g] # $AAs_{limiting}$ is the total number of amino acids that have a AAS value < 100%, [h] The AAS_{AA2} value was 96%, with the second limiting amino acid being HIS.

Microalgae and cyanobacteria in fish feed

To meet the increasing demands in aquaculture feed, it has been attempted to (partially) replace the traditionally used fish meal protein by other feed ingredients. This replacement mostly focused on terrestrial plant protein sources, often from soy, lupine and canola origin, and animal byproducts, including meals made from rendered meat, blood and poultry [104]. Although unicellular sources are already applied in aquaculture hatcheries and in fish as feed supplements (e.g. as colorants and antioxidants [105]), they are not yet commercially applied as protein sources in fish feed for the grow-out phase of fish.

In order to evaluate the quality of novel (fish) feed ingredients [104], data is needed on various aspects of the ingredient. More specifically, Glencross et al. have defined a number of key parameters that are needed for this ingredient evaluation. These key parameters are (1) chemical characterization, (2) ingredient digestibility, (3) ingredient palatability, (4) nutrient utilization and (5) processing functionality. For unicellular sources, only the first key parameter is well described, since both their chemical composition and

the effect of seasonal variation and growing conditions of microalgae and cyanobacteria is well documented, e.g. [7, 8]. The amino acid composition is described in more detail in the next paragraph. The other key parameters have not yet been extensively studied (in feed applications for the grow-out phase of fish). The latter is possibly a result of the (current) generally low availability and high price of microalgae and cyanobacteria (the availability is for example recently described by Vigani et al. [106]), with exception of *Arthrospira*/*Spirulina* biomass [107]. Nutrient digestibility (key parameter 2) can be considered as one of the most important parameters for ingredient evaluation. As described above, digestibility data (on ingredient level) of microalgae and cyanobacteria in fish in their grow-out phase is very limited. The data that is available on this topic is discussed in more detail below. Since relatively more experiments are performed on the growth aspects of fish upon dietary inclusion of unicellular sources, these results are also discussed below.

AAS of unicellular protein for fish

Although not commonly used as a quality indicator for fish feed ingredients, the AAS previously calculated for human nutrition (using equation 1) can also be applied to fish nutrition. Different to the AAS calculations for humans, the amino acid arginine is additionally included as an indispensable amino acid for AAS calculations for fish. As a reference protein, the amino acid requirements of tilapia (*Oreochromis* spp.) and of Atlantic salmon (*Salmo salar*) were used, as reported by the NRC [103] (and re-calculated on protein basis). Different than for both growing children and adult humans, the microalgae studied were shown to fall below the requirements of both tilapia and salmon (AAS values < 92%; Table 4). According to the NRC [103], tilapia requires high amounts of histidine in its diet; 1% histidine in the diet, which is 4% on protein requirement basis. This is reflected in the AAS values of the microalgae for tilapia (45–62%), with histidine being the major limiting amino acid for tilapia. This is similar, however, to the AAS values of fish meal protein (69%, also with histidine as the first limiting AA). Next to histidine, the majority of microalgae is limited in the combination of methionine and cysteine, for tilapia. For salmon, the histidine requirements are lower (0.8% on diet, or 2% of the required protein [103]), which is both observed in overall AAS values of microalgae (61–92%, which are higher than for tilapia) and in the limiting amino acids. For salmon, the combination of methionine and cysteine, and lysine are more limiting in the microalgae than histidine. Fish meal has all the amino acids required at sufficient levels (AAS value of 107%) for salmon. The microalgae AAS values compare well to those of soybean (AAS of 81%). Based on their amino acid patterns and the nutrient requirements of salmon and tilapia, microalgae cannot be used as single protein source in fish, and/or as complete replacement of fishmeal protein in fish.

Table 5: Amino acid score (AAS) of various microalgae, Rubisco, soybean, milk and fish meal, for tilapia and salmon [a].

Source		Nile tilapia (*Oreochromis* spp.)			Atlantic salmon (*Salmo salar*)		
		AAS_{AA1} [f]	AAS_{AA2} [g]	# $AAs_{limiting}$ [h]	AAS_{AA1} [f]	AAS_{AA2} [g]	# $AAs_{limiting}$ [h]
Microalgae[b]	Class						
Dunaliella tertiolecta	Chlorophyceae	54 MET + CYS	54 HIS	3	61 MET + CYS	73 MET	4
Nannochloris atomus	Chlorophyceae	53 HIS	72 MET + CYS	5	79 LYS	81 MET + CYS	4
Chroomonas salina	Cryptophyceae	53 HIS	89 MET + CYS	2	82 HIS	93 LYS	2
Nannochloropsis oculata	Eustigmatophyceae	59 MET + CYS	62 HIS	3	67 MET + CYS	83 MET	4
Tetraselmis chui	Prasinophyceae	53 HIS	73 MET + CYS	4	82 MET + CYS	82 HIS	4
Tetraselmis suecica	Prasinophyceae	53 HIS	87 MET + CYS	3	82 HIS	91 LYS	3
Isochrysis galbana	Prymnesiophyceae	62 HIS	78 MET + CYS	3	88 MET + CYS	94 LYS	3
Isochrysis galbana (T-iso)	Prymnesiophyceae	59 HIS	91 MET + CYS	2	92 LYS	92 HIS	2
Pavlova lutheri	Prymnesiophyceae	59 HIS	-	1	85 LYS	91 HIS	2
Pavlova salina	Prymnesiophyceae	45 HIS	59 MET + CYS	4	66 MET + CYS	69 HIS	4
Rubisco (*Nannochloropsis gaditana*)		62 HIS	-	1	85 LYS	96 HIS	2
Soybean (*Glycine max*)[c]		65 MET	80 HIS	5	81 MET	98 LYS	2
Milk (*Bos taurus*)[d]		73 HIS	79 ARG	3	65 ARG	-	1
Fishmeal (anchovies)[e]		69 HIS	-	1	107 -	-	0

[a] Based on the amino acid requirements estimated by the NRC [103] (on protein basis), using amino acid compositions of [b] Brown et al [15], [c] Cervantes-Pahm et al. [99] and Panthee et al. [100], [c] Belitz et al. [101] and Williams et al. [102], [d] the Uniprot database [31] (accession codes K9ZV74 and A0A023PJK0), and [e] NRC [103] (using fish meal with international feed number 5-01-985). [f] AAS_{AA1} is the AAS of the first limiting amino acid (and therefore the total AAS), [g] AAS_{AA2} is the AAS of the second limiting amino acid, and [h] # $AAs_{limiting}$ is the total number of amino acids that have a AAS value < 100%.

Growth aspects upon using unicellular sources as fish meal replacements

Various studies have investigated the effect of fish meal replacement in feed by microalgal or cyanobacterial biomass on fish growth (Table 6). In these types of research, diets with varying levels of unicellular sources as fish meal replacements were fed to fish. In these studies, the highest percentage of fish meal that could be replaced in the diets without negatively affecting fish growth and survival was taken as the optimal replacement level. In Table 6, an overview of the optimal levels of replacement recorded in each study is given. It should be noted that some of these optimal levels reported were the maximum levels of fish meal that was replaced in the experiment. The true optimal replacement levels may thus have been higher than those shown in Table 6. Based on fish growth and survival rates, fish meal could be replaced between 7–100% by unicellular sources [108-113]. These data show large variations in fish performance and growth responses upon replacement of fish meal by unicellular sources, both between the fish species and between the unicellular species. This variation may be caused by the various species used, but also by the variation in experimental design that was applied in the studies (including the feeding method and fish age). For example, in many of these studies, fish meal was not the only protein source in the diet, which may increase the fish meal replacement levels possible. However, this does not explain the differences between the studies where 7% and 100% fish meal could be replaced. In the study where 7% of the fish meal could be replaced, other sources of protein were also available in the diets (including 6% wheat gluten and 6% soy protein concentrate) [109]. In contrast, in the study where 100% of the fish meal could be replaced, fish meal was the major protein source in the reference diet, making the unicellular source the major protein source in the diet in which 100% of the fish meal was replaced [114]. In the AAS discussion above, it was shown that microalgae are limited in their amino acid compositions for both Nile tilapia and Atlantic salmon. These limitations may be the reason why not all fish meal could be replaced by unicellular sources in the studies using tilapia [108] and salmon [109, 112]. In those studies where higher levels of fish meal replacement were tested than the optimum reported [108, 109], fish growth decreased at higher levels of fish meal replacement. Specifically, in salmon [109] the specific growth rate (SGR) measured for fish fed a diet with 3% fish meal replacement was equal to the control group (no fish meal replacement; SGR of 1.03 %/day), but decreased to 0.96 %/day and 0.82 %/day at fish meal replacements of 6 and 12%, respectively. In tilapia [108], the SGR was equal for fish fed diets in which 0–40% of fish meal was replaced (4.59–4.72 %/day), but decreased to 1.78–3.65 %/day at increasing levels of fish meal replacement (60–100%). In those studies, it is unclear whether this decrease in SGR can be attributed to the decreased feed intake in fish fed diets with increasing levels of unicellular sources. The decreased growth is sometimes attributed to a lower digestibility of the unicellular source used (e.g. in [109]). The nutrient digestibility was, however, only determined on a diet level in these studies [109-111, 113, 114], not on ingredient level. Nutrient digestibility of the unicellular sources on ingredient level can elucidate the results discussed and shown above. The (limited) data that are available on ingredient digestibility of microalgae and cyanobacteria are described below.

Table 6: Optimal [a] replacements of fish meal with microalgae and cyanobacteria in fish diets.

Unicellular species used as fish meal replacement	Fish species	Initial fish weight [g] [b]	Feeding method [c]	Levels of replacement tested [%]	"optimal" [a] replacement level [%]	Inclusion level in diet [d] [%]	Reference
Phaeodactylum tricornutum	Atlantic salmon (*Salmo salar*)	325	S	0/3/7/14	7	6	109
Navicula sp.	red drum (*Sciaenops ocellatus*)	222	R	0/5/10	10 [e]	21	113
Tetraselmis suecica	European sea bass (*Dicentrarchus labrax*)	69.5	S	0/10/20	20 [f]	16	111
Isochrysis .sp (T-ISO)	European sea bass (*Dicentrarchus labrax*)	142	S	0/17/33	33	14	110
Desmodesmus sp. [g]	Atlantic salmon (*Salmo salar*)	168	S	0/13/26	26	20	112
Spirulina maxima	Mozambique tilapia (*Oreochromis mossambicus* P.)	0.28	R	0/20/40/60/80/100	40	21	108
Arthrospira platensis	Catla carp (*Catla catla*)	0.53	R	0/25/50/75/100	100	31	114
Arthrospira platensis	Rohu carp (*Labeo rohu*)	1.27	R	0/25/50/75/100	100	31	114

[a] The "optimal" replacement level is the percentage of fish meal that could be replaced without causing negative effects on fish growth and survival. N.B.: this optimal level was sometimes equal to the maximum replacement level tested; [b] Average weight of the fish at the start of each trial. [c] S = fed to apparent satiation, R = fed restrictively. [d] Inclusion level of the unicellular source in the diet [w/w %] at the "optimum" level of fish meal replacement. [e] Next to fish meal, also soy protein concentrate was replaced. [f] Next to fish meal, also wheat meal was replaced. [g] the *Desmodesmus* sp. used was defatted biomass.

Unicellular protein digestibility in fish

At the start of this PhD project (2013), only 1 article was published reporting the apparent digestibility coefficients (ADCs) of microalgal / cyanobacterial protein on ingredient level [115]. Only recently (since 2016), more studies were published reporting these ingredient ADCs. The data are still limited, however, to a total of 5 studies, studying 5 unicellular species and 4 fish species; of which the protein ADCs reported are shown in Table 7. The protein ADCs of these sources (*Arthrospira* sp., *Chlorella* sp., *Schizochytrium* sp., *Nannochloropsis* sp. and *Desmodesmus* sp.) ranged between 67–90%. These unicellular protein ADCs were shown to be within the same range as protein ADCs of various plant protein concentrates and meals, when compared within the same digestibility trials [115, 116]. Additionally, the microalgal and cyanobacterial ADC data show that microalgal and cyanobacterial protein digestibility in fish was different between fish species [115], unicellular species [117, 118] and is dependent on the inclusion level of the unicellular source in the diet [119]. These types of variation are also known for other protein sources that are similar to each other, including for example differences in protein ADCs of various legumes within a single fish species [120] and variation in protein ADCs of a single leguminous protein ingredient (e.g. soy protein concentrate [121]) between fish species. The differences in protein ADC between the unicellular sources are expected to depend on the intrinsic properties of the proteins and differences in protein accessibility caused by different cell wall matrices (which was e.g. postulated in [117]). The term protein accessibility is a measure for the extent to which (unicellular) protein is accessible to enzymes, both *in vitro* and *in vivo*. Data on the role of cell walls and related accessibility of nutrients is very limited. In this PhD thesis, the effect of cell wall robustness and of nutrient accessibility on nutrient digestibility in fish is therefore studied (**chapter 3** and **4**). Just before finishing this thesis, an article was published by Tibbetts et al. whose results emphasise the role of cell walls in microalgae nutrient digestibility in fish [119]. More specifically, they showed that by disrupting the tough cell walls of *Chlorella* sp., protein digestibility was increased from 79.5 to 85.4% ADC (at dietary inclusion levels of 30%).

Table 7: Apparent digestibility coefficients (ADCs) of unicellular protein in various fish species.

Unicellular species	Fish species	Inclusion level [%]	ADC protein [a] [%]	Reference
Arthrospira sp.	Nile tilapia (*Oreochromis niloticus*)	30	86.1	[117]
	Caspian great sturgeon (*Huso huso*)	30	75.6	[116]
	Atlantic salmon (*Salmo salar*)	30	84.7	[115]
	Arctic charr (Salvelinus alpinus)	30	82.2	[115]
Chlorella sp.	Nile tilapia (*Oreochromis niloticus*)	30	80.0	[117]
	Atlantic salmon (*Salmo salar*)	6–30	70.2–89.7 [b]	[119]
Schizochytrium sp.	Nile tilapia (*Oreochromis niloticus*)	30	81.7	[117]
Nannochloropsis sp. [c]	Atlantic salmon (*Salmo salar*)	30	72.4	[118]
Desmodesmus sp. [c]	Atlantic salmon (*Salmo salar*)	30	67.1	[118]

[a] Protein contents were calculated as N * 6.25; [b] range due to various inclusion levels and the use of whole algal cells and disrupted algal cells; [c] the algae used were defatted prior to inclusion in the diet.

Differences in nutrient digestibility between fish species

For fish to be able to digest the inner-cell nutrients of microalgae (like protein), the algal cell walls need to be disrupted. This disruption can take place either prior to feeding (e.g. in ingredient preparation or during feed production) or by the digestive system and related anatomy of the fish. Since digestive systems vary greatly between fish species, microalgal or cyanobacterial nutrient digestibility will vary accordingly between fish species (as was also shown in [115]). It should be noted that in the text below, only species of fish that have a stomach are taken into account.

To estimate differences in nutrient digestion between fish species, the fish' trophic level can be used. Trophic levels are the position of a species in a food chain; for fish, these levels range from 2 (herbivores) to 5 (carnivorous predators). For example, trophic levels of the herbivorous Nile tilapia, the omnivorous African catfish, and the carnivorous Atlantic salmon are 2.00, 3.76 and 4.50, respectively [122]. In general, fish that feed on a lower trophic level are expected to be adapted to eating plant material (e.g., these fish have long digestive tracts) and fish that feed on a higher trophic level are expected to be adapted to diets of high nutrient density (e.g., these fish have shorter digestive tracts). Additionally, fish from a lower trophic level are in general more efficient in degrading carbohydrates than fish from a higher trophic level [123]. Some fish species have specific anatomical adjustments to feed on plant materials. For example, tilapia species have pharyngeal (i.e. throat) teeth to physically grind plant tissue [124] which benefits the enzymatic degradation in the subsequent digestive processes.

For microalgae in particular, carbohydrate degradation by fish may play an important role in microalgal nutrient digestibility. The increased carbohydrase activity in digestive systems of fish that feed on a lower trophic level [123], could help to make nutrients within algal cells (like proteins) more accessible to the fish' digestive enzymes. A potential challenge, however, is that microalgae and cyanobacteria are known to contain various carbohydrates that are not part of the natural diet of fish. As explained previously, the majority of microalgae have cellulose based cell walls, whereas cyanobacteria have peptidoglycan cell walls. Although all fish possess starch/glycogen degrading enzymes, most fish lack the enzymes needed for cellulose degradation [125]. Presence of cellulases is reported in herbivorous fish [126, 127], but since these cellulases are secreted by the fish' gut microflora the released glucose may not be available for the fish themselves [126-128].

Another difference between fish' digestive systems that is thought to be relevant for microalgal and cyanobacterial digestibility is the stomach pH. Nile tilapia, for example, is known for its acidic stomach conditions (which can reach pH 2 at 7 h after feeding) [129]. In comparison, the stomach conditions of African catfish are less acidic (it can reach pH 3.5 at 8 h after feeding [130]). The stomach conditions of Nile tilapia may be more suitable to chemically disrupt or weaken the cell walls of microalgae and cyanobacteria than those of African catfish. To conclude, it is expected that a low trophic level fish like Nile tilapia, as well as its acidic stomach conditions and pharyngeal teeth, would be more capable of accessing and subsequently digesting microalgal and cyanobacterial nutrients than a trophic level fish like African catfish or Atlantic salmon.

AIM AND OUTLINE OF THESIS

The overall aim of this thesis is to understand how the chemical and structural variation between unicellular green sources affects the application of unicellular protein in food and feed.

Firstly, to test the variability in protein extractability of various unicellular sources, we analyzed the chemical composition of four different unicellular sources: *Arthrospira (spirulina) maxima*, *Nannochloropsis gaditana*, *Tetraselmis impellucida* and *Scenedesmus dimorphus* (**chapter 2**). Using a mild process, proteins were isolated from each of these sources. It is described how the sources vary in both protein extractability and in the composition and solubility of the final protein isolates obtained. The protein isolates obtained in **chapter 2** are used in **chapter 3**, to study their emulsion behavior as a function of protein concentration and pH. The emulsion behavior of the protein isolates is linked to the molecular and interfacial properties of the proteins in the isolates.

For optimum use of microalgae and cyanobacteria in both food and fish feed, it is valuable to study the nutrient digestibility in the side streams produced in **chapter 2**. Since the soluble and insoluble fractions of the unicellular sources were shown to contain the same protein composition (**chapter 2**), and for feasibility reasons, it was decided to study the nutrient digestibility of the complete algal biomass, as presented in **chapter 4**. In this study we also include the effect of fish species by feed the unicellular sources to fish from 2 different trophic levels. In this study, four unicellular sources (*A. maxima*, *Chlorella vulgaris*, *N. gaditana* and *S. dimorphus*) are fed (at a 30% inclusion in the diets) to herbivorous fish (Nile tilapia) and omnivorous fish (African catfish). We describe the effect of cell wall hardness of the unicellular sources on nutrient digestibility. From the results obtained, it was hypothesized that for these types of sources, nutrient accessibility dominates nutrient digestibility. This hypothesis is tested in **chapter 5**, by subjecting *N. gaditana* biomass to five different treatments that influence its cell wall integrity. The algae samples are fed to Nile tilapia to study the nutrient digestibly. The nutrient accessibility is tested *in vitro* and is subsequently linked to the *in vivo* nutrient digestibility. In the final chapter (**chapter 6**), using results obtained in the previous chapters and from external work, the applicability of microalgae and cyanobacteria as protein sources for food and feed is discussed.

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2 Comparison of protein extracts from various unicellular green sources.

Photosynthetic unicellular organisms are considered as promising alternative protein sources. The aim of this study is to understand the extent to which these green sources differ with respect to their gross composition and how these differences affect the final protein isolate. Using mild isolation techniques, proteins were extracted and isolated from four different unicellular sources (*Arthrospira (spirulina) maxima*, *Nannochloropsis gaditana, Tetraselmis impellucida* and *Scenedesmus dimorphus*). Despite differences in protein contents of the sources (27–62% w/w) and in protein extractability (17–74% w/w), final protein isolates were obtained that had similar protein contents (62–77% w/w) and protein yields (3–9% w/w). Protein solubility as a function of pH was different between the sources and in ionic strength dependency, especially at pH < 4.0. Overall, the characterization and extraction protocol used allows a relatively fast and well described isolation of purified proteins from novel protein sources.

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INTRODUCTION

Photosynthetic single cell organisms (microalgae and cyanobacteria) have received interest as potential alternative protein sources for the food and feed industry. These organisms belong to over eleven phyla and are biologically very diverse, ranging from marine prokaryotes to freshwater eukaryotes [1]. Despite the interest, there is a lack of studies describing the detailed chemical composition of these organisms and of protein isolates that can be derived from them. The aim of this study is to understand the extent to which these green sources differ with respect to their gross composition, which is relevant for the feed industry. Secondly we aim to understand how these differences affect the final protein isolates, which could be later applied in the food industry. The protein isolates obtained were studied with respect to their chemical composition and techno-functional properties. Four different unicellular sources (*Arthrospira (spirulina) maxima*, *Nannochloropsis gaditana, Tetraselmis impellucida* and *Scenedesmus dimorphus*), encompassing in total 3 different phyla, were used to extract and further isolate proteins.

The research approach for proteins from these unicellular sources can be expected to develop the same way as the approach that has been developed in the past 50 year for proteins from seeds from leguminous plants, like soy, pea and lupines. These legumes are biologically related and studies showed that they contain similar types, or classes of proteins. Leguminous proteins include the well-known multimeric vicilin (7S) and legumin (11S) globulin fractions that, in soy, account for > 80% of the total proteins [2, 3]. It is known, however, that differences in non-protein compounds present in legume seeds, like high contents of starch (e.g. pea) and oil (e.g. soy), necessitate changes in protein isolation procedures [4]. In addition, significant differences have been found between the techno-functional properties and thermo-stability of protein isolates obtained from various legumes [4]. These differences are in part due to impurities caused by differences in the legumes' biomass composition, but are also partly due to differences in the intrinsic molecular properties of the proteins. For example, the multimeric state of leguminous proteins, make them quite distinctly different from for instance the monomeric whey proteins. For the study of proteins from unicellular sources, an example should be taken from these past studies on leguminous proteins. Similar to leguminous sources, the gross composition of unicellular green sources like microalgae and cyanobacteria varies greatly (Table 1). Extreme differences in composition between species have been reported, with values for protein and carbohydrate contents ranging from 6–72% (w/w dry matter) and 8–64% (w/w dry matter), respectively [5, 6]. It is important to note that the reported differences within one species, due to differences in growing or harvesting conditions can be at least as large as the differences between species [6, 7]. The variation within the composition of the cyanobacterium *Arthrospira* sp. and the microalgae *Nannochloropsis* sp., *Scenedesmus* sp. and *Tetraselmis* sp. is shown in Table 1. It should be noted that part of this variation may be caused by the different methods used in literature to measure protein, carbohydrate and lipid contents.

Table 1: Gross chemical composition of microalgae and cyanobacteria [% w/w] on dry weight basis.

Species	Phylum	Protein		Carbohydrate		Lipid		Ash		Total	Reference
		Mean ± SD	range*	Mean ± SD	range*	Mean ± SD	range*	Mean ± SD	range*		
Arthrospira	Cyanobacteria										
A. platensis		35[a] ± 10	2–43	36[f] ± 22	11-66	7[i] ± 2	4–8	n.d.	-	63–92	8
		35[a] ± 7	20–43	33[f] ± 18	9–66	7[i] ± 2	4–13	n.d.	-	59–99	8
		65[b] ± 5	59–72	15[f] ± 4	11–20	7[j] ± 0.4	6–7	n.d.	-	85–89	6
A. maxima		67[b] ± 3	63–70	14[f] ± 4	10–20	6[j] ± 1	6–7	n.d.	-	86–90	6
Arthrospira sp.		47[a]	-	14[f]	-	11[k]	-	8	-	80	9
Nannochloropsis	Ochrophyta										
N. gaditana		44[a] ± 5	32–51	n.d.	n.d.	27[l] ± 4	20–30	8± 2	5-14	66–89	10
		52[a,c] ± 9	37–59	21[f,c] ± 5	16–27	27[l,n] ± 6	21–36	n.d.	--	100[c]	11
		41[d]	-	25[g]	-	26[m]	-	n.d.		92	12
Tetraselmis	Chlorophyta										
*T. impellucida***		36[e]	-	24[h]	-	19[j]	-	15	-	94	13
T. chuii		31[e]	-	12[f]	-	17[l]	-	n.d.	-	60	14
Tetraselmis sp.		26[b]	-	9[f]	-	14[l]	-	14	-	63	15
		30[b]		8[f]		13[l]		17		68	15
Scenedesmus	Chlorophyta										
S. *dimorphus*		50[a]	-	6[f]	-	23[l]	-	2	-	83	16
S. *obliquus*		31[a] ± 13	11–43	28[f] ± 10	16–44	19[k] ± 3	16–24	10 ± 5	5–18	79–98	9
S. *almeriensis*		44[d]	-	25[g]	-	25[m]	-	n.d.	-	94	12

Protein content determined by [a] Lowry after alkaline treatment, [b] N*6.25, [d] combustion with TGA-MS, [e] amino acid composition. **Carbohydrate content** determined by [f] Dubois, [g] combustion with TGA-MS, [h] acid hydrolysis with HPAEC. **Lipid content** determined by [i] sulfo-phospho-vanillin, [j] Soxhlet, [k] Folch, [l] Bligh & Dyer, [m] combustion with TGA-MS.

[c] expressed as percentage of the organic fraction.

* Range due to varying culture conditions.

** *Tetraselmis* sp. used by Schwenzfeier et al. was later confirmed by the supplier to be *T. impellucida*.

n.d. Not determined.

Similar to leguminous seeds, various proteins in microalgae and cyanobacteria are from similar classes and types. This means that they will share certain intrinsic molecular properties (e.g. multimeric state), which are important for their techno-functional properties. For example, all photosynthetic organisms contain a form of the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) which catalyzes carbon fixation. In microalgae and cyanobacteria, it is present in the so-called form I, which consists of 8 large and 8 small subunits [17]. No post-translational modifications and prosthetic groups of Rubisco have been reported in online databases [11, 18, i,ii]. The Mw of Rubisco's large subunit over most reported species seems to be quite constant, and in *N. gaditana*, *T. impellucida*, *S. dimorphus* and *A. maxima* the Mw is found to be between 52–54 kDa [18,ii]. The small subunit is more variable in size and structure between species than the large subunit [19], and is known to have a Mw range of 10–17 kDa in these genera [18,i]. Additionally, photosynthetic organisms contain various proteins that are active in light harvesting. In microalgae, these proteins are associated to the light harvesting complexes (LHC). The major LHC protein in *N. gaditana* is the violaxanthin–chlorophyll a binding protein (VCP), with a Mw of 22 kDa [20, 21]. Other LHC proteins in *N. gaditana* also have molecular weights in the 21–32 kDa range [18,iii]. LHC proteins of *Tetraselmis* sp. and of Scenedesmus have molecular weights of 24–44 kDa and 26–27 kDa, respectively [18iv,v]. The LHC proteins of these sources are expected to be multimeric, similar to the LHC-II proteins from spinach. Spinach LHC-II proteins are trimers, where each monomer consists of 10 polypeptide chains each (PDB ID 1RWT) [22]. These proteins can form super complexes with photosystem II via antenna proteins [23]. Cyanobacteria do not contain LHCs but synthesize blue pigmented phycocyanins for light-harvesting [1]. These multimeric phycocyanins have subunits with molecular masses between 15–22 kDa [24]. Overall, Rubisco and the light harvesting proteins / phycocyanins in the four unicellular sources are all multimeric and have monomeric units in the same size range (15–54 kDa). It is therefore expected that these proteins will behave the same during protein extraction and isolation as a function of ionic strength (association / dissociation of the multimers) and dialysis.

Few studies have been performed on mild protein extraction from microalgae and cyanobacteria. Devi et al. reported an aqueous protein extraction from defatted *Arthrospira* (*Spirulina*) *platensis,* with a yield up to 85% [25]. Postma et al. also performed a mild extraction

[i] Uniprot search terms: rbcS/cbbS genes in *Arthrospira* sp., *Nannochloropsis* sp., *Scenedesmus* sp. and *Tetraselmis* sp. Accession numbers used: D4ZVW5, W6SIC7, K1VV20, A0A023PJK0 and K9ZWI1

[ii] Uniprot search terms: rbcL/cbbL genes in *Arthrospira* sp., *Nannochloropsis* sp., *Scenedesmus* sp. and *Tetraselmis* sp. Accession numbers used: T1RH29, Q3S3D2, B5VXI0, D4ZVW7, Q1KVV0 and K9ZV74.

[iii] Uniprot search terms: LHC genes in *Nannochloropsis gaditana*. Accession numbers used: K8YPQ7, W7TX20, W7UAI7, W7T6P5, W7TFG9, W7TZB5, W7TTD7, W7UBF0, W7U2H0 and W7TCK1.

[iv] Uniprot search terms: LHC genes in *Tetraselmis* sp. Accession numbers used: A0A061RA39, A0A061RJR5, A0A061SK82, A0A061S745, A0A061SA24, A0A061R6B3, A0A061R2N8, A0A061S1P5, A0A061R213, A0A061S9W9 and O22496.

[v] Uniprot search terms: LHC genes in *Scenedesmus* sp. Accession numbers used: A2SY33, A2SY34, A2SY35, A2SY32.

of protein and reported a *Chlorella vulgaris* protein extractability of 32–42% [26]. Ursu et al. reported a soluble protein yield of 35% [w/w] from *Chlorella vulgaris*, using high pressure cell disruption (2700 bar) at pH 7 [27]. Schwenzfeier et al. reported a *T. impellucida* protein extractability of 21% [w/w] under mild conditions, with a final protein isolate yield of 7% ([w/w] and protein isolate purity of 64% [w/w] [13]. Most studies published on protein extraction from microalgae and cyanobacteria however, involve harsh chemical or physical treatments to disintegrate the cells, which affect the quality of the proteins. By using harsh chemicals (e.g. organic solvents) or physical treatments (e.g. high temperatures), proteins can lose their native tertiary structure or can be hydrolyzed to peptides or amino acids. This will affect the application possibilities in foods, for which techno-functional properties like good solubility, emulsification and gelling behavior are desired. For example, heating has been shown to reduce protein solubility in alfalfa leaves, whereas acid precipitation can retain protein solubility [28]. In this study, the aim was to isolate the proteins in a structure as close to the native structure as possible, to provide a base-line observation of the intrinsic properties of the proteins.

For this study, protein sources were selected from three different unicellular photosynthetic phyla: one cyanobacterium (*Arthrospira maxima*), one heterokontophyta (*Nannochloropsis gaditana*) and two chlorophyta (*Tetraselmis impellucida* and *Scenedesmus dimorphus*). A mild isolation technique was used to avoid possible negative effects to the structure and conformational state of the proteins.

MATERIALS & METHODS

Materials

Nonviable samples of *Nannochloropsis gaditana* (NAN), *Scenedesmus dimorphus* (SCE) and *Arthrospira (spirulina) maxima* (ART) were kindly provided by AlgaSpring (Almere, The Netherlands) as a frozen paste (microalgae) or a dried powder (cyanobacteria). Nonviable *Tetraselmis impellucida* (TET, Instant Algae, strain CCMP892) was purchased from Reed Mariculture (Campbell, CA, USA) as a frozen paste. The TET material was the same product that was used in the work by Schwenzfeier et al. [13]. The growing conditions of the biomass samples were not provided by the suppliers. All samples were stored frozen (-20 °C) prior to use. All chemicals used were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma Aldrich (St. Louis, MO, USA), unless stated otherwise. All water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA), unless stated otherwise.

Protein isolation

Protein was isolated from the microalgae and cyanobacteria using the isolation method described before [13]. Algae paste or cyanobacteria powder was diluted or dispersed to 12% w/w dry matter in a potassium phosphate buffer with a final pH of 8.0 and a final concentration of 50 mM. The cells were disrupted using an agitation bead mill DYNO®-Mill type MULTI LAB (Willy A. Bachofen Maschinenfabrik, Muttenz, Switzerland). The bead

milling (recirculation) time was adjusted for each source. The ART, TET, NAN and SCE samples were recirculated for 20, 30, 45 or 60 min respectively, per 1 L sample, using a set pump speed of 1.5 L/min and a tube inner diameter of 0.8 cm. These times were used to reach complete cell disruption for each source, as confirmed by microscopic analyses. The 0.3 L grinding chamber was filled with 190 mL (approximately 65% [v/v]) yttria-stabilized zirconia SiLiBeads grinding beads, type ZY Premium, of 0.4–0.6 mm (Sigmund Lindner, Warmensteinach, Germany). Water cooled to 2 °C was recirculated through the cooling jacket of the grinding chamber, and the samples were kept on ice to ensure that the sample temperature at the bead mill outlet never exceeded 21 °C. The bead milled biomass was centrifuged (70,000 x g, 30 min, 4 °C), with exception of NAN. The NAN sample was first centrifuged at 16,000 x g (30 min, 4 °C), then filtered using a Whatmann paper filter and subsequently the filtrate was centrifuged at 70,000 x g (30 min, 4 °C). The protein extractability was defined as the amount of protein in the supernatant (algae juice; AJ) divided by the amount of protein in the corresponding biomass * 100% (i.e. g protein in AJ / 100 g protein in the biomass). The AJ of all samples was dialyzed (MWCO 12,000–14,000) against demineralized water and subsequently against a potassium phosphate buffer ("buffer A", pH 7.6, 35 mM) at 4 °C, to remove low Mw peptides and non-proteinaceous nitrogen. Each dialyzed algae juice (AJD) was applied on a glass filter (pore size 2) containing the anion exchange adsorbent Streamline DEAE (GE Healthcare, Uppsala, Sweden) in a volumetric ratio of 2:1. The DEAE was previously washed with an excess of demineralized water and then equilibrated with buffer A in a DEAE:buffer volumetric ratio of 1:2. The eluent was applied three times to ensure maximum protein binding (elution under gravity took 30–60 minutes). The DEAE was washed with buffer A in a DEAE:buffer volumetric ratio of 1:2. Bound protein was eluted by applying buffer A containing 2 M NaCl, in a DEAE:buffer volumetric ratio of 1:2. The eluate was dialyzed (MWCO 12,000–14,000) against demineralized water and subsequently against buffer A at 4 °C, yielding the crude algae soluble protein isolate (CASPI). The CASPI was acidified to pH 3.5 with 1 M HCl and then kept at 4 °C for 1 hour. The acidified CASPI was centrifuged at 4700 x g for 30 min at 4 °C. The pellet was redissolved in water by adjusting the pH to 7.6 with 1 M NaOH and the algae soluble protein isolate (ASPI) obtained was freeze dried, or stored frozen with 0.5 M sucrose. The protein isolation yield was defined as the amount of protein in each ASPI divided by the amount of protein in the corresponding biomass * 100% (i.e. g protein in ASPI / 100 g protein in the biomass). At all isolation steps, aliquots of samples were freeze dried as such, and additional aliquots were stored frozen with 0.8 M sucrose for further analyses. ASPIs derived from ART, TET, NAN and SCE will be further referred to as ASPI-A, ASPI-T, ASPI-N, and ASPI-S, respectively.

Compositional analyses

All samples were freeze dried prior to analysis except for the aliquots needed for moisture content determination. All analysis results of the freeze dried samples were expressed on a dry weight basis, assuming a residual moisture content of 10% after freeze drying (which was the typical moisture content measured in the freeze-dried biomass).

Dry matter content. Dry matter content of liquid samples was determined gravimetrically in triplicate by drying the samples overnight at 80 °C followed by 3 h at 105 °C.

Ash content. Ash content was determined gravimetrically in triplicate by burning freeze dried samples overnight at 550 °C. Ash content was additionally determined on washed biomass. For this, freeze dried biomass was dispersed in water (6% w/w dry matter), stirred on a magnetic stirrer for 1 h, and subsequently centrifuged (10 min, 4,500 g, 20 °C). The supernatant was discarded and the pellet was re-suspended and centrifuged in the same manner, two times. The washed biomass was oven dried (overnight at 80 °C followed by 3 h at 105 °C). Ash content was determined of the dried washed biomass.

Amino acid composition. Amino acid composition was determined in duplicate according to ISO method 13903:2005, with exception of tryptophan. Analysis of tryptophan content was only performed for the biomass, and not for the derived fractions. Tryptophan was determined in duplicate by a commercial laboratory (NutriControl, Veghel, The Netherlands). Standard deviations were found to be on average < 0.5% of the mean. In the worst case the standard deviation was 11.8%.

Total protein content and nitrogen – to protein conversion factors. Total nitrogen content was determined in triplicate with the Dumas method using a Flash EA 1112 N analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and D-methionine for calibration. Nitrogen-to-protein (N-Prot) conversion factors k_p and k_a were calculated as described previously [13]. The first N-Prot factor, k_p, was calculated as the ratio between the sum of amino acid residues (total protein content) and total nitrogen content (including non-proteinaceous nitrogen). The second N-Prot factor, k_a, was calculated as the ratio of the sum of amino acid residues (total protein content) to nitrogen from recovered amino acids (proteinaceous nitrogen only). Due to acid hydrolysis during amino acid quantification, asparagine (ASN) and glutamine (GLN) cannot be distinguished from (ASP) and glutamic acid (GLU). Therefore, the nitrogen recovered from amino acids was calculated assuming either 100% ASN/GLN or 100% ASP/GLU. Presented protein contents of samples are based on the total nitrogen contents and using the calculated N-Prot factors.

Lipid content and fatty acid composition. Lipid content was determined gravimetrically in duplicate according to Folch et al. [29]. Bead milled biomass (1.5 g) was mixed with dichloromethane:methanol (2:1; 100 mL). The mixture was homogenized by sonication (20 s) and shaken for 2 h (200 rpm, 20 °C). Water (25 mL) was added to reach a methanol:dichloromethane:water ratio of 8:4:3, and the mixture was centrifuged (20 min, 4000 g, 20 °C). The upper layer was removed and the dichloromethane /pellet mixture was stored for 12 h at 4 °C. The mixture was paper filtered and flushed with dichloromethane. The dichloromethane was evaporated in a rotatory evaporator. Fatty acid composition was analyzed in duplicate on bead milled biomass according to Breuer et al [30]. In short, lipids were extracted with chloroform:methanol (ratio 4:5 v/v), followed by transesterification of the fatty acids to fatty acid methyl esters (FAMEs). FAMEs were quantified by GC-FID using a Nukol column, as described by Breuer et al. A triglyceride (C15:0) was used as an internal standard. The GC was calibrated using TraceCERT FAME standards purchased from Supelco

(#CRM18918, #18913-1AMP and #CRM18920, Supelco, Bellefonte, PA, USA). Annotation % of the fatty acids was calculated by assuming all unidentified GC peaks were unidentified FAMEs (< 8% of total peak area). To quantify the unidentified FAMEs, molecular weights were used of FAMEs with similar retention times (< 30 s difference).

Sugar composition and total uronic acid content. Neutral carbohydrate composition was determined in triplicate according to the procedure by Englyst and Cummings, using inositol as internal standard and a pre-hydrolysis with H_2SO_4 (72% w/w) [31]. Alditol acetates formed were analyzed by gas chromatography (Focus-GC, Thermo Scientific, Waltham, MA, USA), using arabinose, galactose, glucose, fucose, mannose, rhamnose, ribose and xylose as standards. Total uronic acid content was determined in triplicate according to an automated colorimetric m-hydroxydiphenyl assay based on Ahmed et al. [32], using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Samples were pre-hydrolyzed as described in the neutral carbohydrate composition method. Adaptations to this method were the concentrations used of sodium tetraborate (23.7 mM) and m-hydroxydiphenyl (0.04% in 0.5% NaOH). Galacturonic acid (0–100 µg / mL) was used for calibration.

SDS-PAGE and immunoblotting. SDS-PAGE was performed in duplicate under reducing conditions (10 mM β-mercapthoethanol) on a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used as a molecular weight marker. Gels (Mini-Protean TGX) were either stained with Instant Blue coomassie stain (Expedeon, San Diego, CA, USA) or transferred to a 0.2 µm pore-size nitrocellulose membrane (Bio-Rad Laboratories) for immunoblotting. Immunoblot assays were carried out with standard reagents according to the protocol. Rabbit polyclonal antibodies against the large subunit of Rubisco (#MBS715138, MyBioSource, San Diego, CA, USA) were detected with polyclonal goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase (#P0448, Dako, Carpinteria, CA, USA), using Clarity Western ECL (Bio-Rad Laboratories,) as a substrate. To reduce the influence of co-precipitated soluble proteins in the insoluble fractions of the biomass, freeze dried aliquots of the pellet fractions were washed with a potassium phosphate buffer (50 mM, pH 8.0) prior to analyzing them with SDS-PAGE.

Protein solubility

ASPI of each alga or cyanobacterium was dispersed in Milli-Q water, and the pH was adjusted to 8.0. Samples that were not completely soluble were stirred overnight (4 °C). All samples were subsequently centrifuged (10 min, 10,000 g, 20 °C), and the supernatant was used for further analyses. In all cases, > 80% of the protein was soluble, and the amount of ASPI dispersed was adapted per source to yield a final concentration of soluble proteins of 5 mg/mL in each supernatant. A buffer of 3.65 mM potassium phosphate, pH 7.6 was adjusted to ionic strengths of I = 0.01, 0.20 or 0.50 M with NaCl. The NaCl concentrations of the buffers were 0, 0.19 and 0.49 M, respectively. The ASPI supernatants were diafiltered with the potassium phosphate buffers of various ionic strengths. Subsequently, the protein

solutions were adjusted to pH 2.0 using 1 M HCl, resulting in ionic strengths of I = 0.01 (SD = 0.002), 0.21 (SD = 0.006) and 0.49 (SD < 0.001) M. Using a pH-stat, the pH of the protein solutions was adjusted up to pH 8.5 with unit intervals of 0.5 using 0.2 M NaOH. At each pH, an aliquot of each protein solution was taken for further analyses. Actual pH and NaOH additions were recorded during the pH adjustments. The aliquots were kept at 4 °C for 1 hour and subsequently centrifuged (10 min, 10,000 g, 4 °C). The protein concentration of the supernatants was determined using the BCA protein assay (Pierce ThermoScientific, Waltham, MA USA). Protein concentrations calculated were corrected for the dilutions by NaOH titration and aliquots taken during the pH adjustments. Due to the pH adjustments, the final ionic strengths were calculated to be I = 0.01 (SD = 0.003), 0.19 (SD = 0.006) and 0.48 (SD = 0.010) M for samples with initial ionic strengths of I = 0.01, 0.20 and 0.50 M respectively. At each ionic strength the protein solubility at pH 8.0 was set at 100%.

RESULTS AND DISCUSSION

Before describing the protein isolation and the composition of the isolates obtained, it is important to consider the chemical composition of the biomass. This information is relevant for the extraction of the proteins for food applications, but may also provide relevant information about the non-protein compounds, which can be used for other applications such as the aquaculture industry.

Biomass characterization

Chemical composition

For all samples 92–99% [w/w] of the total dry matter of the starting material was accounted for (annotated) in the gross compositional analysis (Table 2). Protein contents differed greatly between the four materials, with values of 61.7, 45.0, 35.8 and 26.6% [w/w] measured for respectively ART, NAN, TET and SCE. The total carbohydrate content was found to be quite similar for all sources, ranging between 15.1–21.5% [w/w], including 0.7–2.2% [w/w] uronic acids. Total lipid contents ranged between 12.1–29.3% [w/w]. These gross composition analysis results fall within the ranges reported in literature [6, 9, 13-15]. It should be noted that growing and harvesting conditions can greatly influence the chemical composition of algae and cyanobacterial biomass [6, 7].

Fatty acid composition

The fatty acid composition of unicellular organisms is relevant for the nutritional quality of the sources, especially in the aquaculture industry [33]. Specifically, the essential fatty acids and other omega-3 and -6 fatty acids are of relevance for assessing the nutritional quality. The two essential fatty acids were identified in the unicellular sources: linoleic acid (4–32 mol% of FA_{tot}) and α-linolenic acid (13 and 32 mol% of FA_{tot} in TET and SCE respectively). Other omega-3 and -6 fatty acids present were eicosapentaenoic acid (EPA) and γ-linolenic acid. NAN and TET contained EPA (31 and 3 mol% of FA_{tot}, respectively). ART and TET contained γ-linolenic acid (23 and 4 mol% of FA_{tot}, respectively). No docosahexaenoic acid

(DHA) was detected in the samples. Out of the four sources, NAN and TET can be considered interesting sources for aquaculture, because they contain EPA. Overall, all four samples contained high amounts of palmitic acid, i.e. palmitic acid accounts for 21–30 mol% of the total amount of fatty acids (FA_{tot}) (Table 3). In addition, The contents and type of fatty acids that formed the majority of the FA_{tot} in these sources, as indicated with an asterisk in Table 3, were similar to literature findings for NAN [34, 35], TET [36, 37], SCE [16, 36] and ART [38, 39].

Table 2: Gross chemical composition of the starting materials [% w/w] on dry weight basis.

Component	***A. maxima***		***N. gaditana***		***T. impellucida***		***S. dimorphus***	
Proteins[a]	61.7	± 0.5	45.0	± 0.6	34.7	± 0.1	26.6	± 2.6
Carbohydrates	15.1	± 0.2	16.5	± 0.2	17.9	± 0.2	21.5	± 0.2
Neutral	13.7	± 0.2	15.8	± 0.3	15.7	± 0.2	20.8	± 0.3
Charged	1.2	± <0.1	0.6	± <0.1	2.2	± <0.1	0.7	± <0.1
Lipids[b]	12.1	± 0.2	29.3	± 0.2	23.1	± 0.7	25.2	± 2.1
Ash[c]	6.3	± <0.1	8.4	± 0.2	17.3	± <0.1	18.2	± <0.1
Total annotated	95.2		99.1		93.0		91.5	

[a] Based on total amino acid analysis, i.e. including peptides and free amino acids.

[b] Determined as MeOH/CH_2Cl_2 soluble material.

[c] All measurements were performed on the biomass as such. Ash contents of washed biomass, thus excluding contribution of extracellular material, were 2.9 ± 0.1, 3.9 ± 0.0, 11.8 ± 0.1 and 16.7 ± 0.01 % w/w for *A. maxima*, *N. gaditana*, *T. impellucida* and *S. dimorphus* respectively.

Carbohydrate composition

The carbohydrate composition is indicative of the types of oligo- and polysaccharides present in the unicellular sources. Oligo- and polysaccharides can act as fibers in food or feed, but can also be co-passengers during the isolation of proteins. Glucose and galactose were the major carbohydrate constituents (28–66 mol% and 8–19 mol% of total carbohydrates, respectively) of all four starting materials (Table 4). Charged sugars (uronic acids) accounted for 3–11 mol% of the total carbohydrates in all starting materials. These uronic acids may form complexes with proteins during isolation, as was for instance shown in the emulsion properties of an algae protein isolate [40]. In the microalgae (NAN, TET and SCE) mannose was also a major carbohydrate (14–32 mol%), while it was only a minor part of the carbohydrates in ART (2 mol%). The high glucose content was expected, since in all four sources the storage carbohydrates are glucose-based polymers [41-44]. In addition, the cell walls of *Nannochloropsis gaditana* [44, 45] and *Scenedesmus dimorphus* consist primarily of cellulose [46, 47]. Another difference between the samples was the rhamnose and ribose content. The rhamnose content was higher in ART and NAN than in TET and SCE (5–6 mol% and < 1 mol%, respectively). The highest ribose amount was found in ART (10 mol%), compared to 6, 5 and 2 mol% in NAN, TET and ART, respectively.

Table 3: Fatty acid composition of biomass (%mol of total fatty acids); ±SD.

Shorthand	**C14:0**	**C14:1 cis9**	**C16:0**	**C16:1**	**C16:2**	**C16:3**	**C16:4**	**C18:0**	**C18:1 n9**	**C18:2 n6**
Trivial name	Myristic acid	Myristoleic Acid	Palmitic acid	Palmitoleic acid	Hexadeca-dienoic acid	Hexadeca-trienoic acid	Hexadeca-tetraenoic acid	Stearic acid	Oleic acid	Linoleic acid
A. maxima	0.8 ± 0.2	0.8 ± 0.4	*29.9 ± 4.6	3.1 ± 1.0	n.d. -	0.9 ± 0.3	n.d. -	1.1 ± 0.3	5.9 ± 0.5	*32.2 ± 3.2
N. gaditana	7.2 ± <0.1	1.0 ± <0.1	*23.5 ± 0.1	*21.8 ± 0.2	0.6 ± <0.1	1.3 ± <0.1	n.d. -	n.d. -	4.4 ± <0.1	3.9 ± <0.1
T. impellucida	2.2 ± <0.1	1.5 ± <0.1	*30.1 ± 0.5	5.0 ± 0.1	2.0 ± <0.1	3.2 ± <0.1	*11.0 ± 0.1	0.9 ± <0.1	*11.2 ± 0.1	*7.5 ± 0.2
S. dimorphus	2.0 ± 0.3	1.8 ± 0.6	*20.7 ± 0.3	11.1 ± 0.2	n.d. -	3.4 ± 0.1	*16.4 ± 0.3	n.d. -	7.4 ± 0.4	5.2 ± 0.3

Shorthand	**C18:3 n3**	**C18:3 n6**	**C18:4**	**C20:1**	**C20:2 n6**	**C20:3 n3**	**C20:5 n3**	**C22:1**	**Total annotated**
Trivial name	α-Linolenic acid	γ-Linolenic acid	Stearidonic acid	Gondoic acid	Eicosa-dienoic acid	Eicosa-trienoic acid	Eicosa-pentaenoic acid (EPA)	Erucic acid	(% w/w)
A. maxima	n.d. -	*23.2 ± 1.7	n.d. -	n.d. -	2.2 ± 0.8	n.d. -	n.d. -	n.d. -	98.1
N. gaditana	n.d. -	n.d. -	n.d. -	n.d. -	n.d. -	5.3 ± <0.1	*31.0 ± 0.2	n.d. -	96.9
T. impellucida	*12.6 ± 0.3	3.5 ± 0.1	3.4 ± 0.1	1.6 ± 0.4	n.d. -	1.2 ± 0.1	2.8 ± 0.7	0.5 ± 0.2	92.1
S. dimorphus	*32.1 ± 0.5	n.d. -	n.d. -	n.d. -	n.d. -	n.d. ±	n.d. -	n.d. -	100.0

* Major fatty acids (sum up to > 70 mol% of total fatty acids of the species).
n.d.: Not detected (< 0.5 w/w% of total fatty acids).

Table 4: Monocarbohydrate composition of total carbohydrates in biomass [mol%]; ±SD.

	Rha	Fuc	Ara	Xyl	Man
A. maxima	5.81 ± 0.05	0.83 ± 0.11	1.24 ± 0.29	3.04 ± 0.12	1.85 ± 0.16
N. gaditana	4.63 ± 0.16	0.88 ± 0.12	1.47 ± 0.21	1.85 ± 0.09	14.29 ± 0.15
T. impellucida	0.78 ± 0.02	0.20 ± 0.06	2.60 ± 0.15	1.15 ± 0.10	32.41 ± 0.19
S. dimorphus	0.84 ± 0.02	0.61 ± 0.02	0.85 ± 0.10	1.54 ± 0.10	16.18 ± 0.27
	Gal	**Glc**	**Rib**	**UA**	
A. maxima	11.40 ± 0.13	59.06 ± 0.27	9.65 ± 0.27	7.12 ± 0.18	
N. gaditana	18.59 ± 0.15	48.66 ± 0.10	6.10 ± 0.14	3.53 ± 0.19	
T. impellucida	18.39 ± 0.05	28.42 ± 0.17	4.94 ± 0.16	11.11 ± 0.03	
S. dimorphus	7.79 ± 0.02	66.54 ± 0.27	2.46 ± 0.02	3.19 ± 0.08	

Overall, the carbohydrate composition measured in the starting materials was similar to what has been described in literature, with high glucose, galactose and mannose contents for *Tetraselmis* impellucida (30, 38, 7 mol%, respectively) [13], *Nannochloropsis* sp. (46, 17 and 34 mol%, respectively) [48] and *Scenedesmus* sp. (38–70, 11–31 and 1–7 mol%, respectively) [49]. In *Arthrospira* sp., the major carbohydrate constituents are similar to the present findings and are reported to be glucose and galactose (59–74 and 10–20 mol%, respectively) [50]. The differences in carbohydrate composition between the sources can affect both the isolation process, as well as the techno-functional properties of the proteins isolated.

Protein composition

SDS-PAGE analysis showed major bands at ~50 kDa and at > 250 kDa in all sources (Figure 1 A). The ~50 kDa band corresponds to the large subunit of Rubisco, as shown by immunoblotting (Figure 1, B). The > 250 kDa proteins are expected to be protein aggregates. Bands between 10–17 kDa detected in all sources are expected to represent the small subunit of Rubisco [18][vi]. Other major proteins detected varied between sources and were 25–27 kDa (TET), 15–18 kDa (ART), 26 and 38 kDa (SCE) and 15–20, 23, 30 and 39 kDa (NAN). Additionally, these sources contained various proteins that are part of the photosynthetic complex. The TET bands of 25–27 kDa and ~40 kDa are in the range of light harvesting complex (LHC) proteins reported for this genus (24–44 kDa) [18][vii]. The intense 15–18 kDa bands in ART match the molecular mass of phycocyanin sub-units (15–22 kDa) [24]. The ~26 kDa band found in SCE matches the

[vi] Uniprot search terms: rbcS/cbbS genes in *Arthrospira* sp., *Nannochloropsis* sp., *Scenedesmus* sp. and *Tetraselmis* sp. Accession numbers used: D4ZVW5, W6SIC7, K1VV20, A0A023PJK0 and K9ZWI1

[vii] Uniprot search terms: LHC genes in *Tetraselmis* sp. Accession numbers used: A0A061RA39, A0A061RJR5, A0A061SK82, A0A061S745, A0A061SA24, A0A061R6B3, A0A061R2N8, A0A061S1P5, A0A061R213, A0A061S9W9 and O22496.

reported presence of a 27 kDa LHC in *Scenedesmus* sp. [18viii, 51]. The ~23 kDa band detected in NAN is expected to be the 22 kDa violaxanthin–chlorophyll a binding protein (VCP) [20, 21].

Amino acid composition

Despite differences in the protein composition of the four sources, the overall amino acid profiles of the starting materials were very similar to each other (Figure 2). The standard deviations of the mean of 14 out of 18 analyzed amino acids were 4–14% amongst the various sources. The amino acids that showed the highest deviations between the sources were CYS, PRO, ARG and TRP. The compositions measured are similar to what has been reported earlier [14]. When comparing to common food protein sources, like soy and bovine milk [52-55], the microalgae and cyanobacterium amino acid compositions are more similar to soy proteins than to bovine milk (Figure 2). This is illustrated by the high linear regressions between the determined amino acid compositions and the literature values of microalgae [14], soy [52, 53] and bovine milk [54, 55] with determination coefficients of $R^2 = 0.89$, 0.82 and 0.66, respectively. Compared to bovine milk, the unicellular sources have proportionally half the amounts (in w/w% total amino acids) of GLX and proline and more than twice the amounts of GLY and ALA. Compared to soy proteins, the unicellular sources have over 60% more MET, ALA and TRP and approximately 50% less HIS..

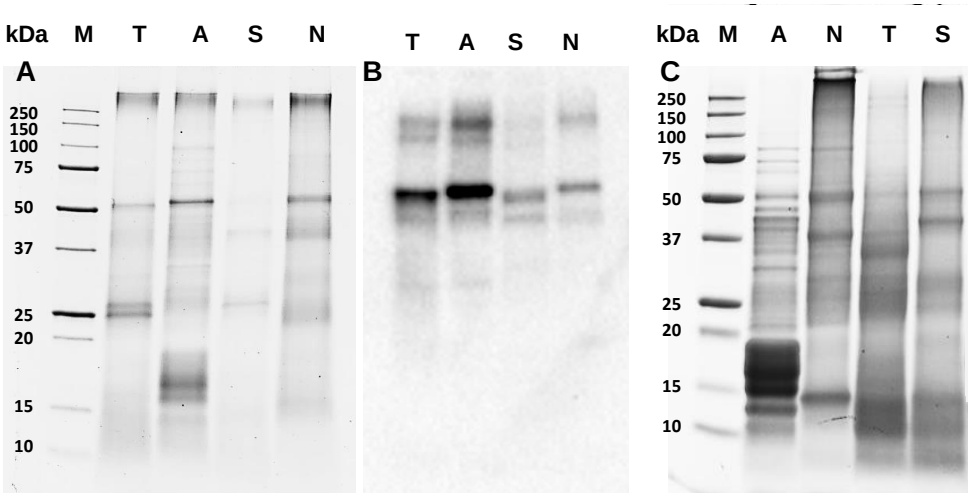

Figure 1: (A) SDS-PAGE gels stained with coomassie of bead milled biomass, under reducing conditions and corresponding Western Blot, detecting rabbit polyclonal antibodies against the large subunit of Rubisco with polyclonal goat anti-rabbit immunoglobulins (B). (C) SDS-PAGE gels stained with coomassie of ASPIs. M = molecular weight marker, T = *T. impellucida*, A = *A. maxima*, S = *S. dimorphus* and N = *N. gaditana*.

[viii] Uniprot search terms: LHC(x) genes in *Scenedesmus* sp. Accession numbers used: A2SY33, A2SY34, A2SY35, A2SY32.

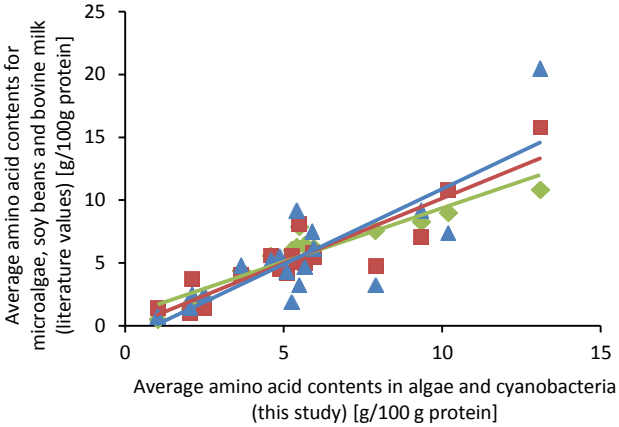

Figure 2: Comparison of average amino acid contents of various microalgae (green) [14], soy beans (red) [52, 53] and bovine milk (blue) [8, 54, 55] with *A. maxima*, *N. gaditana*, *T. impellucida* and *S. dimorphus* (this study). The lines depict linear regressions with determination coefficients of R^2 = 0.89, 0.82 and 0.66 for microalgae, soy beans and bovine milk, respectively.

Nitrogen-to-protein conversion factors

The nitrogen-to-protein conversion k_p factors of the four sources ranged from 3.88 to 5.88 (Table 6), indicating that the use of the standard k_p factor 6.25 would overestimate the protein contents of these samples up to 1.6 times. The k_a factors obtained were much more similar amongst the four sources than the k_p factors, with a lower limit (ASX/GLX = 100% ASP/GLU) ranging between 5.37–5.49 and an upper limit (ASX/GLX = 100% ASN/GLN) ranging between 6.30–6.37. This shows that the difference in N content between these sources is determined by variations in non-proteinaceous-nitrogen rather than by variations in the amino acid composition (and thus the k_a factors). These differences are also reflected in the proteinaceous nitrogen to total nitrogen ratios (N_{AA}/N_T). ART was analyzed to have the highest N_{AA}/N_T of 80–98% in the biomass and SCE had the lowest N_{AA}/N_T of 62–72%.

Protein extraction

Large differences were found in the protein extractability between the four sources. After bead milling and centrifugation, 17, 41, 58 and 74% [w/w] of the total protein in the biomass was extracted for SCE, TET, NAN and ART, respectively (Table 7). The unicellular sources have different types of cell walls, which affected the duration of bead milling needed to disrupt the cells. *Scenedesmus* sp. and *N. gaditana* cell walls are mainly composed of cellulose, with an outer hydrophobic algaenan layer [45-47]. Cell walls of *Tetraselmis* sp. consist of various carbohydrate acids and neutral carbohydrates [42, 56]. The cell walls of cyanobacteria (including *Arthrospira* sp.) are mainly composed of peptidoglycans [57]. It is generally assumed that peptidoglycan cell walls of cyanobacteria are less robust than the cellulose cell walls of microalgae. Indeed, more time was needed to break the cells of SCE and NAN than of ART (60 and 45 minutes, compared to 20 min, respectively, per L sample (12% w/w dry matter)). Cell walls of TET were less recalcitrant than the cellulose based cell

walls of SCE and NAN, but more recalcitrant than ART cell walls (30 min bead milling / L at 12% dry matter). All samples were bead milled until most cells were disrupted (as verified by light microscopy). No relation could be found between protein extractability of the bead milled samples and the cell wall recalcitrance to disintegration by bead milling. Part of the proteinaceous material extracted was found to be low molecular weight (LMW; < 12–14 kDa) peptides or free amino acids, based on the lower protein yield after dialysis of the algae juice (AJD): 12, 27, 48 and 36% [w/w] in SCE, TET, NAN and ART respectively. This means that 21–51% of the soluble proteinaceous material was of LMW, with ART having the highest LMW fraction. The high molecular weight protein fraction contains the (intact) proteins of interest and was therefore used for further isolation. Previous publications on algal protein extractability showed a similar protein extractability for *T. impellucida* (21% [w/w]) [13] and a 30% [w/w] protein extractability for *Nannochloropsis* sp. [58]. No data was found on protein extractability from *Arthrospira* and *Scenedesmus* species. The proportion of LMW proteinaceous material in the extracts (12–36%) was similar to what was reported by Schwenzfeier et al., who showed that 38% [w/w] of the extracted proteins of *T. impellucida* is of low Mw [13]. The final protein isolates, obtained after AEC and acid precipitation, had protein contents of 62–77% [w/w], corresponding to protein yields of 3–9% [w/w]. The differences in protein content between the starting materials were thus reduced during the isolation process. The yields are not high, but the aim of the method was to obtain representative fractions of the soluble part of the proteins. It should, however, be noted that the soluble proteins may represent only part of all proteins in the algae, and that the proteins represent only part of the total nitrogen. The processing step in which most of the solubilized protein was lost for all sources is the AEC step, where 68–78% [w/w] of the protein in AJD was not bound to the DEAE. The combination of the AEC step and the acid precipitation step increased the protein purity the most (with 26–250%). The TET results were very similar to the work by Schwenzfeier et al., in which a *T. impellucida* protein isolate was obtained with a protein content of 64% [w/w] and a protein yield of 7% [w/w] [13].

Protein and amino acid distribution upon extraction

The soluble and insoluble fractions obtained after extraction of proteins from SCE, TET, NAN and ART had identical protein compositions (Figure 3). The identical protein composition was not caused by co-precipitation of soluble proteins in the pellets: pellets washed with a potassium phosphate buffer still had the same protein compositions. The similarity between insoluble and soluble protein upon extraction also shows in the amino acid compositions (Table 5), which are the same for the pellet and algae juice fractions (the average SD of the mean is 1.3%). Based on the SDS-PAGE and AA results, it was concluded that the majority of proteins in the insoluble fraction are essentially the same proteins as in the soluble fractions. This indicates that the insoluble fraction that is often referred to as hydrophobic proteins or cell wall bound proteins [59] does not necessarily have to consist of different proteins than those that are obtained in the extract.

Table 5: Amino acid profiles of samples obtained during the different isolation steps of the various unicellular sources [g / 100 g protein], with according standard deviations.

	CYS	MET	ASX	THR	SER	GLX	GLY	ALA	PRO
A. maxima									
Biomass	0.81 ± 0.01	2.51 ± 0.01	10.17 ± 0.04	5.11 ± 0.03	5.08 ± 0.04	14.63 ± 0.06	4.49 ± 0.01	7.61 ± 0.02	4.11 ± 0.09
Pellet	0.77 ± <0.01	2.42 ± <0.01	10.63 ± 0.01	5.21 ± <0.01	5.12 ± <0.01	13.37 ± 0.04	4.68 ± <0.01	7.59 ± 0.01	4.29 ± 0.04
AJ	0.72 ± <0.01	2.69 ± 0.01	10.45 ± 0.01	5.39 ± 0.01	5.30 ± 0.02	13.73 ± 0.04	4.56 ± <0.01	7.52 ± <0.01	3.98 ± 0.03
AJD	0.87 ± <0.01	2.68 ± <0.01	10.20 ± <0.01	5.33 ± <0.01	5.21 ± <0.01	14.69 ± 0.01	4.65 ± <0.01	7.72 ± 0.01	3.92 ± 0.04
CASPI	1.04 ± 0.01	2.91 ± 0.02	11.23 ± 0.01	5.42 ± 0.01	5.14 ± <0.01	14.98 ± 0.01	4.50 ± <0.01	7.84 ± 0.03	3.62 ± 0.11
ASPI	1.01 ± <0.01	3.03 ± 0.01	10.93 ± 0.02	5.54 ± <0.01	5.27 ± 0.01	12.56 ± 0.09	4.72 ± <0.01	7.95 ± 0.02	3.66 ± 0.06
N. gaditana									
Biomass	0.81 ± 0.01	2.37 ± 0.01	9.75 ± 0.04	5.10 ± 0.02	4.39 ± 0.01	13.21 ± 0.08	5.12 ± 0.01	7.45 ± 0.01	5.57 ± 0.13
Pellet	0.71 ± 0.01	2.61 ± 0.02	9.96 ± 0.05	5.36 ± 0.01	4.59 ± 0.01	11.77 ± <0.01	5.61 ± <0.01	7.53 ± 0.01	5.38 ± <0.01
AJ	0.79 ± <0.01	2.63 ± <0.01	9.66 ± <0.01	5.35 ± 0.01	4.46 ± 0.01	11.81 ± 0.01	5.60 ± 0.01	7.62 ± 0.01	5.40 ± 0.04
AJD	0.72 ± <0.01	2.66 ± <0.01	10.00 ± 0.02	5.41 ± <0.01	4.55 ± <0.01	11.86 ± <0.01	5.57 ± <0.01	7.13 ± 0.01	4.82 ± 0.01
CASPI	1.22 ± 0.01	2.56 ± 0.01	11.33 ± 0.03	5.52 ± <0.01	4.58 ± 0.01	13.15 ± 0.01	5.02 ± <0.01	6.64 ± <0.01	4.48 ± 0.04
ASPI	0.97 ± 0.03	2.68 ± 0.01	10.83 ± 0.02	5.50 ± 0.01	4.54 ± 0.01	12.35 ± 0.01	5.43 ± 0.01	6.85 ± 0.01	4.49 ± 0.05
T. impellucida									
Biomass	1.30 ± 0.01	2.71 ± <0.01	9.71 ± 0.02	4.95 ± 0.02	4.23 ± 0.03	12.01 ± 0.03	5.53 ± <0.01	8.78 ± <0.01	6.58 ± 0.08
Pellet	1.17 ± 0.01	3.01 ± 0.01	10.00 ± 0.05	5.14 ± <0.01	4.66 ± 0.01	11.56 ± 0.04	5.70 ± <0.01	7.75 ± <0.01	5.41 ± 0.03
AJ	1.61 ± 0.01	2.53 ± 0.02	9.84 ± 0.01	4.87 ± <0.01	3.93 ± 0.01	13.10 ± 0.04	5.74 ± 0.01	11.0 ± 0.03	7.91 ± 0.01
AJD	1.24 ± <0.01	3.07 ± 0.01	10.80 ± 0.02	5.64 ± 0.01	4.83 ± <0.01	12.16 ± <0.01	5.58 ± 0.01	6.84 ± <0.01	5.05 ± 0.06
CASPI	1.79 ± 0.01	2.70 ± <0.01	12.41 ± <0.01	6.14 ± 0.02	5.10 ± 0.01	14.09 ± <0.01	5.19 ± 0.01	7.05 ± 0.01	4.84 ± 0.07
ASPI	1.69 ± <0.01	3.21 ± 0.01	11.37 ± 0.03	5.78 ± 0.01	4.84 ± <0.01	12.15 ± 0.03	4.95 ± <0.01	6.70 ± <0.01	4.63 ± 0.02
S. dimorphus									
Biomass	1.14 ± 0.02	2.39 ± 0.01	11.10 ± 0.02	5.17 ± 0.03	4.66 ± 0.03	12.51 ± 0.02	5.81 ± 0.01	7.72 ± 0.03	5.41 ± 0.07
Pellet	1.20 ± 0.01	2.31 ± 0.01	12.75 ± 0.07	5.55 ± <0.01	4.96 ± 0.02	11.83 ± 0.08	6.56 ± 0.01	7.62 ± 0.02	4.98 ± 0.12
AJ	0.96 ± <0.01	2.51 ± <0.01	10.89 ± 0.03	5.53 ± 0.01	4.42 ± <0.01	13.59 ± 0.04	5.96 ± 0.01	9.12 ± <0.01	5.15 ± 0.14
AJD	1.79 ± <0.01	2.69 ± 0.01	12.07 ± 0.02	5.92 ± <0.01	4.56 ± <0.01	12.61 ± 0.03	5.61 ± 0.01	7.24 ± 0.01	5.11 ± 0.03
CASPI	2.29 ± 0.02	2.53 ± 0.01	12.69 ± 0.06	5.86 ± <0.01	4.48 ± 0.02	13.45 ± 0.04	5.44 ± <0.01	7.11 ± <0.01	5.05 ± 0.01
ASPI	1.98 ± 0.01	2.83 ± 0.01	11.52 ± 0.02	5.73 ± 0.03	4.25 ± 0.02	11.86 ± 0.28	5.32 ± <0.01	6.87 ± 0.01	4.69 ± 0.05
Average biomass[a]	1.02 ± 0.25	2.49 ± 0.16	10.18 ± 0.65	5.08 ± 0.09	4.59 ± 0.37	13.09 ± 1.14	5.24 ± 0.58	7.89 ± 0.60	5.41 ± 1.01
Literature[b]									
Algae[c]	0.51 ± 0.08	2.15 ± 0.48	9.02 ± 0.68	5.00 ± 0.61	5.57 ± 0.81	10.84 ± 0.74	6.00 ± 0.32	7.62 ± 0.51	6.28 ± 2.17
Soy beans[d]	1.48 ± 0.14	1.49 ± 0.09	10.83 ± 1.15	4.21 ± 0.41	5.63 ± 0.82	15.82 ± 3.40	5.60 ± 1.81	4.76 ± 0.51	5.13 ± 0.42
Bovine milk[e]	0.73 ± 0.02	2.51 ± 0.12	7.37 ± 0.03	4.30 ± <0.01	5.22 ± 0.03	20.45 ± 0.05	1.92 ± 0.07	3.25 ± 0.09	9.20 ± 0.09

[a] Average of amino acid compositions of *A. maxima, N. gaditana, T. impellucida* and *S. dimorphus* biomass, as analyzed in this study.

[b] Literature values adapted from [c 14], [d 52] and [53], [e 54] and [55].

n.d. Not determined.

Table 5 (continued**):** Amino acid profiles of samples obtained during the different isolation steps of the various unicellular sources [g / 100 g protein], with according standard deviations.

	VAL	ILE	LEU	TYR	PHE	HIS	LYS	ARG	TRP
A. maxima									
Biomass	6.31 ± 0.04	5.85 ± 0.02	9.29 ± 0.01	4.28 ± 0.01	4.93 ± 0.02	1.82 ± 0.05	4.93 ± 0.02	6.58 ± 0.05	1.50 ± 0.04
Pellet	6.37 ± 0.01	5.94 ± <0.01	9.70 ± <0.01	4.34 ± <0.01	5.73 ± 0.02	2.10 ± 0.01	4.69 ± <0.01	7.08 ± 0.01	n.d. -
AJ	6.53 ± 0.02	6.32 ± 0.02	9.53 ± 0.01	4.82 ± <0.01	4.63 ± <0.01	1.68 ± 0.01	5.41 ± <0.01	6.72 ± <0.01	n.d. -
AJD	6.36 ± 0.01	6.11 ± <0.01	9.32 ± <0.01	4.63 ± 0.01	4.60 ± <0.01	1.67 ± 0.02	5.38 ± <0.01	6.65 ± 0.01	n.d. -
CASPI	5.56 ± 0.01	6.41 ± 0.01	8.84 ± 0.02	5.64 ± 0.03	4.08 ± 0.01	1.40 ± 0.01	4.90 ± 0.01	6.50 ± 0.02	n.d. -
ASPI	5.68 ± 0.02	6.53 ± 0.01	9.23 ± 0.01	5.79 ± 0.04	4.35 ± <0.01	1.45 ± <0.01	5.12 ± 0.01	7.19 ± 0.01	n.d. -
N. gaditana									
Biomass	5.78 ± <0.01	4.90 ± 0.01	9.79 ± 0.01	3.45 ± 0.01	5.67 ± 0.02	2.14 ± 0.02	6.61 ± 0.02	5.60 ± 0.03	2.30 ± 0.05
Pellet	6.34 ± 0.01	5.03 ± <0.01	10.55 ± <0.01	3.76 ± <0.01	5.94 ± <0.01	2.40 ± 0.01	6.98 ± <0.01	5.48 ± 0.01	n.d. -
AJ	6.28 ± 0.01	4.99 ± <0.01	9.95 ± <0.01	3.98 ± <0.01	5.56 ± 0.01	2.37 ± 0.01	7.62 ± 0.02	5.92 ± 0.02	n.d. -
AJD	6.34 ± <0.01	5.18 ± <0.01	10.36 ± 0.01	4.03 ± 0.01	5.79 ± 0.01	2.41 ± 0.01	7.22 ± 0.01	5.96 ± 0.01	n.d. -
CASPI	6.19 ± <0.01	5.30 ± 0.01	9.13 ± 0.01	4.67 ± 0.02	5.09 ± 0.02	2.45 ± 0.03	6.74 ± 0.01	5.91 ± 0.03	n.d. -
ASPI	6.26 ± 0.01	5.37 ± 0.01	9.51 ± 0.05	4.87 ± 0.14	5.31 ± 0.02	2.04 ± 0.02	6.85 ± 0.02	6.15 ± 0.06	n.d. -
T. impellucida									
Biomass	5.93 ± 0.02	4.51 ± 0.02	9.11 ± 0.01	3.54 ± 0.02	6.19 ± 0.10	2.28 ± 0.02	6.04 ± <0.01	4.40 ± 0.01	2.21 ± 0.05
Pellet	6.14 ± 0.01	4.91 ± 0.01	10.03 ± 0.02	4.00 ± 0.05	6.94 ± <0.01	2.46 ± 0.02	5.97 ± 0.04	5.14 ± 0.01	n.d. -
AJ	5.93 ± 0.02	4.29 ± 0.01	8.51 ± 0.01	2.98 ± 0.03	5.45 ± 0.04	2.15 ± <0.01	6.65 ± 0.01	3.51 ± 0.03	n.d. -
AJD	6.21 ± 0.01	4.89 ± <0.01	9.72 ± 0.01	4.22 ± 0.01	6.73 ± <0.01	2.41 ± <0.01	5.88 ± <0.01	4.72 ± 0.01	n.d. -
CASPI	6.65 ± 0.01	5.17 ± 0.11	9.17 ± 0.01	0.23 ± 0.03	5.58 ± 0.04	2.26 ± 0.02	6.54 ± 0.01	5.10 ± 0.01	n.d. -
ASPI	6.32 ± <0.01	5.18 ± <0.01	9.17 ± 0.01	4.25 ± 0.02	5.68 ± <0.01	2.23 ± <0.01	6.46 ± <0.01	5.39 ± 0.02	n.d. -
S. dimorphus									
Biomass	5.80 ± 0.02	4.21 ± 0.01	9.13 ± 0.02	3.34 ± 0.01	5.86 ± 0.01	2.19 ± 0.02	6.03 ± 0.02	5.38 ± 0.03	2.14 ± 0.03
Pellet	6.02 ± <0.01	4.52 ± <0.01	9.51 ± 0.01	2.20 ± 0.01	6.20 ± 0.03	1.45 ± 0.01	6.75 ± 0.01	5.58 ± 0.03	n.d. -
AJ	6.62 ± 0.01	4.72 ± <0.01	9.03 ± 0.02	3.25 ± 0.03	5.23 ± 0.01	1.80 ± <0.01	6.43 ± 0.02	4.78 ± <0.01	n.d. -
AJD	6.23 ± 0.01	4.67 ± <0.01	8.77 ± 0.01	3.65 ± <0.01	5.67 ± 0.01	2.34 ± <0.01	5.64 ± <0.01	5.41 ± 0.02	n.d. -
CASPI	6.24 ± 0.01	4.78 ± <0.01	8.49 ± 0.01	3.72 ± <0.01	5.13 ± <0.01	2.10 ± 0.08	5.46 ± 0.01	5.19 ± 0.01	n.d. -
ASPI	6.27 ± 0.01	4.95 ± 0.02	9.44 ± 0.03	4.04 ± 0.01	5.79 ± 0.04	2.11 ± 0.02	5.69 ± 0.02	6.65 ± 0.02	n.d. -
Average biomass[a]	5.95 ± 0.24	4.87 ± 0.71	9.33 ± 0.32	3.65 ± 0.42	5.66 ± 0.53	2.11 ± 0.20	5.90 ± 0.70	5.49 ± 0.90	2.04 ± 0.37
Literature[b]									
Algae[c]	6.22 ± 0.26	4.74 ± 0.74	8.28 ± 0.55	4.40 ± 0.62	6.40 ± 0.54	1.88 ± 0.26	5.93 ± 0.35	7.88 ± 2.32	1.34 ± 0.26
Soy beans[d]	5.52 ± 0.88	4.51 ± 0.08	7.06 ± 1.08	4.12 ± 0.59	5.04 ± 0.20	3.78 ± 1.45	5.85 ± 0.94	8.11 ± 0.21	1.07 ± 0.01
Bovine milk[e]	6.13 ± 0.06	5.53 ± 0.02	9.19 ± 0.17	4.80 ± 0.04	4.66 ± 0.11	2.52 ± 0.01	7.50 ± 0.10	3.25 ± 0.04	1.47 ± 0.17

[a] Average of amino acid compositions of *A. maxima, N. gaditana, T. impellucida* and *S. dimorphus* biomass, as analyzed in this study.

[b] Literature values adapted from [c 14], [d 52] and [53], [e 54] and [55].

n.d. Not determined.

Table 6: Proteinaceous nitrogen and nitrogen-to-protein conversion factors k_a and k_p at each step of the isolation procedure of each of the unicellular sources.

Processing step	N_{AA}/N_T [%][a,b]			N-Prot factor k_p[c]	N-Prot factor k_a[d]		
A. maxima							
Biomass[e]	80	< x <	95	5.08	5.37	< y <	6.35
Biomass[f]	79	< x <	94	5.01	5.36	< y <	6.35
Pellet	78	< x <	92	4.91	5.36	< y <	6.30
Algae juice	78	< x <	92	4.97	5.38	< y <	6.34
Dialyzed algae juice	70	< x <	83	4.44	5.36	< y <	6.34
CASPI	71	< x <	85	4.55	5.37	< y <	6.42
ASPI	79	< x <	93	5.01	5.39	< y <	6.32
N. gaditana							
Biomass[e]	77	< x <	90	4.84	5.40	< y <	6.31
Biomass[f]	75	< x <	88	4.73	5.38	< y <	6.30
Pellet	71	< x <	82	4.45	5.42	< y <	6.28
Algae juice	75	< x <	86	4.64	5.38	< y <	6.22
Dialyzed algae juice	73	< x <	85	4.59	5.39	< y <	6.25
CASPI	73	< x <	87	4.64	5.35	< y <	6.32
ASPI	80	< x <	93	5.03	5.38	< y <	6.30
T. impellucida							
Biomass[e]	70	< x <	82	4.48	5.49	< y <	6.37
Biomass[f]	69	< x <	80	4.39	5.47	< y <	6.36
Pellet	71	< x <	83	4.52	5.48	< y <	6.35
Algae juice	65	< x <	76	4.13	5.45	< y <	6.37
Dialyzed algae juice	79	< x <	93	5.09	5.49	< y <	6.44
CASPI	82	< x <	98	5.16	5.29	< y <	6.32
ASPI	83	< x <	98	5.32	5.43	< y <	6.39
S. dimorphus							
Biomass[e]	62	< x <	72	3.88	5.37	< y <	6.30
Biomass[f]	60	< x <	71	3.78	5.35	< y <	6.29
Pellet	60	< x <	71	3.77	5.32	< y <	6.28
Algae juice	71	< x <	84	4.51	5.37	< y <	6.34
Dialyzed algae juice	75	< x <	88	4.74	5.37	< y <	6.35
CASPI	77	< x <	92	4.93	5.36	< y <	6.41
ASPI	80	< x <	94	5.03	5.36	< y <	6.28

[a] Proteinaceous nitrogen (NAA) as proportion of total nitrogen (NT).

[b] Lower limit represents theoretical value calculated with ASX/GLX = 100% ASP/GLU, upper limit calculated with ASX/GLX = 100% ASN/GLN.

[c] k_p values are the average of k_p calculated with ASX/GLX = 100% ASN/GLN and k_p calculated with ASX/GLX = 100% ASP/GLU. The standard deviations between the values were ≤ 0.001.

[d] Lower limit represents theoretical value calculated with ASX/GLX = 100% ASN/GLN, upper limit calculated with ASX/GLX = 100% ASP/GLU.

[e] Values include tryptophan. Since tryptophan was only analyzed in the biomass, the values in [f] are calculated without tryptophan to allow comparison with the other processing steps.

Table 7: Protein yield and protein content of samples at each processing step, on a dry matter basis; ±SD.

	A. maxima		*N. gaditana*		*T. impellucida*		*S. dimorphus*	
	Proteinaceous material [w/w%]	Proteinaceous yield [%]	Proteinaceous material [w/w%]	Proteinaceous yield [%]	Proteinaceous material [w/w%]	Proteinaceous yield [%]	Proteinaceous material [w/w%]	Proteinaceous yield [%]
Biomass	61.74 ± 0.51	100.00 -	44.99 ± 0.61	100.00 -	35.75 ± 1.90	100.00 -	29.08 ± 4.99	100.00 -
Pellet	59.54 ± 0.51	26.31 ± 2.24	40.86 ± 0.15	41.83 ± 15.24	34.85 ± 2.48	60.09 ± 6.49	31.16 ± 2.55	82.75 ± 10.19
AJ	63.90 ± 0.89	73.69 ± 11.85	43.08 ± 0.42	58.17 ± 19.90	34.68 ± 1.27	41.06 ± 5.99	18.79 ± 14.75	17.29 ± 10.07
AJD	60.77 ± 1.00	35.91 ± 9.16	50.39 ± 0.89	46.24 ± 13.40	52.09 ± 4.79	26.85 ± 5.35	17.85 ± 9.09	12.38 ± 2.08
CASPI	66.57 ± 2.60	7.92 ± 0.23	67.67 ± 10.18	10.79 ± 2.18	61.92 ± 3.54	8.60 ± 2.56	18.62 ± 4.07	3.89 ± 2.75
ASPI	76.68 ± 0.07	6.16 ± 1.14	76.79 ± 2.56	8.79 ± 1.60	66.37 ± 6.60	6.25 ± 2.38	62.47 ± 5.31	3.24 ± 1.31

Table 8: Monocarbohydrate composition of total carbohydrates in protein isolates [mol%]; ±SD .

	Rha	**Fuc**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**Rib**	**UA**
A. maxima	14.60 ± 0.01	2.64 ± 0.03	6.22 ± 0.01	4.95 ± 0.07	1.04 ± 0.03	2.90 ± 0.03	1.78 ± 0.25	67.30 ± 0.02	9.68 ± 0.03
N. gaditana	15.56 ± 0.06	8.47 ± 0.09	10.00 ± 0.01	10.51 ± 0.01	26.94 ± 0.13	14.82 ± 0.03	10.11 ± 0.06	8.21 ± 0.01	6.50 ± 0.02
T. impellucida	9.30 ± 0.30	n.d. -	18.21 ± 0.10	7.47 ± 0.33	5.04 ± 0.30	35.52 ± 0.16	4.25 ± 1.20	24.75 ± 0.11	6.58 ± 0.11
S. dimorphus	2.07 ± 0.07	1.12 ± 0.01	3.44 ± 0.07	2.60 ± 0.07	5.87 ± 0.08	5.23 ± 0.12	53.47 ± 0.76	32.46 ± 0.22	4.85 ± 0.07

n.d.: Not detected.

Table 9: Gross chemical composition of protein isolates [% w/w] on dry weight basis.

Component	*A. maxima*		*N. gaditana*		*T. impellucida*		*S. dimorphus*	
Proteins	76.7	± 0.1	76.8	± 2.6	66.4	± 6.6	62.5	± 5.3
Carbohydrates	9.2	± 0.4	8.9	± 0.3	24.4	± 0.4	19.8	± 1.1
Neutral	8.1	± 0.3	8.3	± 0.4	18.7	± 0.5	18.8	± 1.2
Charged	1.0	± <0.1	0.6	± <0.1	5.7	± 0.1	1.0	± 0.1
Total annotated	85.8		85.7		90.8		82.2	

Protein isolate characterization

Chemical composition

The final ASPIs contained 62–77% [w/w] protein and 9–24% [w/w] carbohydrates (Table 9). These components formed the majority of the ASPIs, representing 82–87% of the total dry weight. The isolates thus had higher protein contents than the biomass they were isolated from. Compared to the biomass, the total carbohydrate content is lower in ASPI-N and -A, and is increased in ASPI-T and -S. The ratio of uronic acids (charged carbohydrates) to protein (UA:P) of the isolates are 0.01, 0.01, 0.09, and 0.02 for ASPI -A, -N, -T, and -S. This means that for SCE, NAN and ART, the UA:P in the ASPI was about a factor 2 lower than in the biomass, while for TET it was a factor 1.4 higher. Previously, the presence of the charged carbohydrates in ASPI from *T. impellucida* was linked to higher stability of emulsions against flocculation around the pI [40]. The monocarbohydrate constituents of the total carbohydrates in the ASPIs are different than to those of the corresponding biomass (Table 8). Overall, carbohydrates containing rhamnose, arabinose and xylose represent a larger fraction (%mol) of the total carbohydrates in all ASPIs than in their initial biomass. Ribose is co-isolated in the protein isolation process of ART, TET and SCE and is the major carbohydrate constituent in the associated ASPIs (25–67 %mol of total carbohydrates). In contrast, the ribose fraction of ASPI-N carbohydrates is similar to that of the NAN biomass (6 vs 8 %mol of total carbohydrates). Likewise, mannose and galactose appear to be more co-isolated in NAN and TET respectively than in the other samples. The glucose fraction of the total carbohydrates decreases during the protein isolation of ART, NAN and TET (from 49–67 %mol to 2–10 %mol) but remains constant in SCE.

Protein and amino acid composition

The protein composition of the cyanobacterial ASPI-A was different from those of the three microalgal ASPIs (-N, -T and -S); the latter three are quite similar to each other (Figure 3). The microalgal ASPIs had a diverse protein composition (Figure 1 C), whereas ASPI-A contained one dominant group of proteins (15–18 kDa). Based on the intense blue color of the isolate and the dominance of the 15–18 kDa bands, this shows that the phycocyanins present in the biomass were predominantly retained during the isolation process. The ~50 kDa protein that is considered to be Rubisco's large subunit was much less pronounced in ASPI-A compared to the other ASPIs. The protein composition of ASPI-A was more homogenous than that of the ART biomass. The most intense bands detected in ASPI-N represent proteins of 15, 37 and 50 kDa and proteins of large Mw (> 250 kDa). Compared

to the NAN biomass, the 15 kDa band (attributed to the small subunit of Rubisco) was more pronounced in the isolate. ASPI-T mostly contained 10–15, 25–30, 35–37 and 50 kDa proteins. Compared to the TET biomass, the bands of 10–15 kDa were more pronounced and the large subunit Rubisco is less pronounced. The protein composition of ASPI-S was comparable to that of ASPI-N and ASPI-T, with major bands at < 15 kDa, 25–30, 37 and 50 kDa. Like in ASPI-N, large Mw proteins (> 250 kDa) were present in the isolate. Additionally, glycoprotein analysis with PAS staining revealed the presence of glycoproteins of ≥ 250 kDa in all ASPIs (data not shown). Overall, a shared property of the ASPIs is the presence of proteins that are subunits of multimeric proteins (i.e. Rubisco and phycocyanins), which may lead to similar techno-functionalities. Additionally, ASPI-N, -T and -S are more similar to each other than ASPI-A, based on their more diverse protein composition and presence of Rubisco.

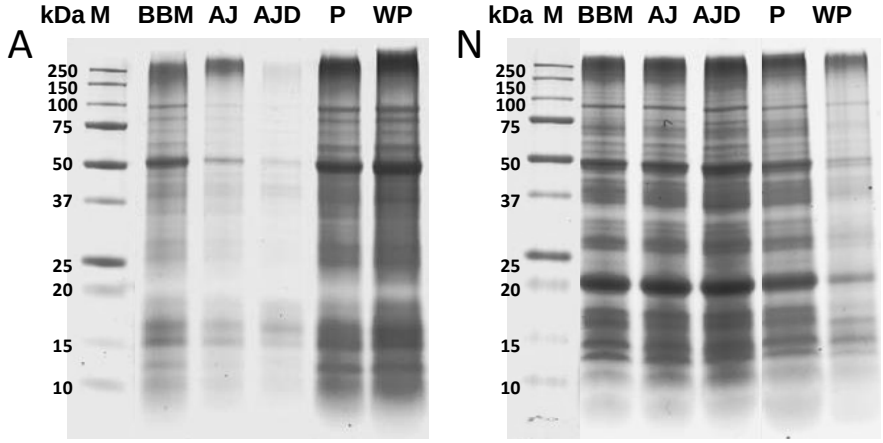

Figure 3: Coomassie stained SDS-PAGE gels with various fractions of *A. maxima* (A) and *N. gaditana* (N), under reducing conditions. BBM = bead milled biomass, AJ = algae juice (non-dialyzed), AJD = algae juice (dialyzed), P = insoluble fraction of the biomass, WP = washed pellet and M = protein molecular weight marker.

Solubility

Despite the differences in protein composition, the protein isolates of ART, NAN, TET and SCE displayed similar pH dependent solubility (Figure 4). At low ionic strength (I = 0.01), the proteins were completely soluble at pH > 6.5 (ASPI-T and ASPI-S) or at pH > 7.0 (ASPI-A and ASPI-N), and least soluble at pH 4.0–4.5. This point of lowest solubility is close to the computed pI based on amino acid compositions, which were calculated to be 4.98, 5.16, 4.94, and 4.98 for ASPI-A, -N, -T and -S respectively. For these calculations, GLU/GLN and ASP/ASN ratios of Rubisco from *Nannochloropsis gaditana* and *Tetraselmis suecica* (1.0:2.2 and 1.0:1.4 respectively) were used (based on [18][ix]). These pI values are lower than the

[ix] Accession numbers used: A0A023PJK0, Q3S3D2 and K9ZV74.

theoretical pI of Rubisco (5.88–8.00) [60x]. These differences are expected to be partially due to the presence of other proteins apart from Rubisco, as shown in the SDS-PAGE profiles. Additionally, the presence of protein bound uronic acids contribute to the overall charge and solubility of the ASPIs. Using a pKa value of 3.3 for uronic acids [61], the uronic acids present in the ASPIs were calculated to decrease the pI by 0.24–0.52 pH units.

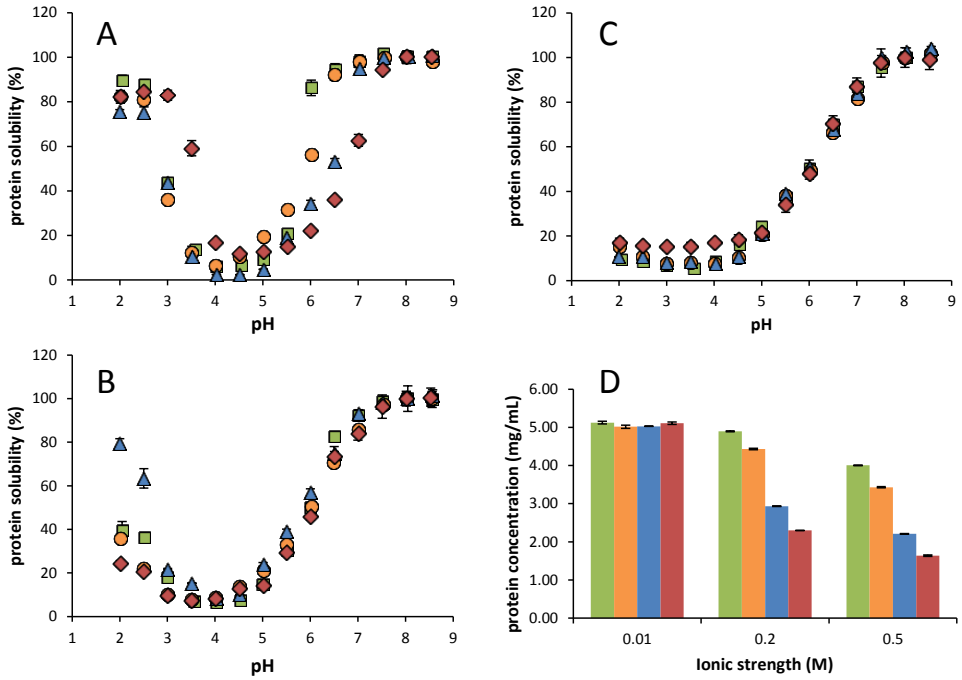

Figure 4: Protein solubility (starting concentration 5 mg protein /mL) as a function of pH of ASPI-T (green), ASPI-S (orange), ASPI-A (blue) and ASPI-N (red) at different ionic strengths (I = 0.05 M (A), 0.2 M (B) and 0.5 M (C). Solubility is expressed relative to pH 8.0 (=set as 100% soluble) (A, B, C) and the amount of solubilized protein at pH 8.0 as affected by ionic strength (D). Error bars indicate standard deviations.

Solubility increased again at pH values below the theoretical pI; below pH 3.0, > 80% of all ASPIs was soluble. It should be noted that all isolates were obtained using a similar isolation procedure, which would select proteins with similar solubility at the pH used for extraction and precipitation (pH 8.0 and 3.5). The point of minimum solubility of the ASPIs is lower than some conventional vegetal protein sources, including soy glycinin (pH 4.7–6.2) [62] and more comparable to that of sunflower helianthinin (pH 4.0–5.5) [63]. Values reported for unicellular proteins (from *Arthrospira platensis* [25], *Nannochloropsis* sp. [58] and *T. impellucida* [13] are very similar and are in the range of pH 3.0–4.0. At pH 8, protein solubility

[x] Accession numbers used: 4MKV, 1WDD, 1RLC, 1RLD, 1RBL, 1RSC, 1BWV,1BXN, 1EJ7, 1IWA, 2YBV, 3AXM, 3AXK, 3ZXW, 4F0M, 4F0K, 4F0H, 1UPM, 1UPP, 1IR1, AA1, 1RCX, 1RXO, 1RBO, 1RCO, 8RUC, 1AUS, 2VDH, 2VDI, 2V67, 2V68, 2V63, 2V69, 2V6A, 1UW9, 1UWA, 1IR2 and1GK8.

of all ASPIs was found to be dependent of ionic strength (Figure 4D). When increasing the ionic strength to I = 0.2 and I = 0.5 M, the protein solubility decreased. At low pH (< pH 4.5), this decrease was more apparent (Figure 4 A - C). Protein solubility of ASPI-T and ASPI-S was least dependent on ionic strength, since at pH 7.6, I = 0.2 M 85% [w/w] protein was in solution, whereas 38–39% [w/w] protein of the other ASPIs was in solution under these conditions. At I = 0.5 M, the solubility at pH 7.6 was lower than that at I = 0.2 M for all ASPIs (38–69% w/w), apart from ASPI-T (85% w/w). At low pH (≤ 4.0) and high ionic strength (I = 0.5 M), protein solubility was considerably decreased (4–10% [w/w]) for all ASPIs. The ionic strength dependence of the ASPIs was different from previously reported solubility profiles of a *T. impellucida* ASPI [13], which show a low ionic strength dependency at ionic strengths of 0.03–0.5 M [13] This difference in solubility between the two *T. impellucida* isolates is thought to be due to batch-to-batch variations in the microalgae. The behavior of the ASPIs was similar to that of sunflower helianthinin, of which the protein solubility at lower pH range decreases drastically at I = 0.25 M as compared to I = 0.03 M [63].

CONCLUSIONS

The aim of this study was to make a first step in the description of the differences in gross composition of various types of unicellular biomass and to understand how these differences affect the final protein isolate. A single isolation method was used in this study as a tool to isolate proteins from four different unicellular photosynthetic sources. The current protocol was not aimed at optimizing protein isolation yield. It aimed at enabling a relatively fast isolation of purified proteins from various novel protein sources, in order to compare the proteins' characteristics. The key findings of this study were that in spite of the different chemical compositions of the unicellular sources used, protein isolates were obtained with comparable purity (62–77% [w/w] protein) and proteinaceous yield (3–9% [w/w]). Additionally, protein solubility as a function of pH of the ASPIs was similar at low ionic strength (I = 10 mM). At higher ionic strengths (I = 0.2–0.5 M) differences in protein solubility between the sources were observed, especially at pH < 4.0. Overall, this study showed that the isolation method applied can yield protein isolates that have similar protein purity and solubility, regardless of the chemical composition and protein composition of the starting algal or cyanobacterial biomass.

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3 Characterizing emulsion properties of microalgal and cyanobacterial protein isolates.

Photosynthetic unicellular sources contain a large variety of proteins. The types of proteins vary between various microalgae/cyanobacteria. The aim was to study the effect of the variation in proteins and in non-proteinaceous components present in unicellular protein isolates on their emulsion behavior. Algae soluble protein isolates (ASPIs, 66–77% w/w protein) of *Nannochloropsis gaditana*, *Tetraselmis impellucida* and *Arthrospira* (*Spirulina*) *maxima* were studied, using commercially available WPI as a reference (93% w/w protein). All protein isolates were able to form emulsions stable against creaming ($d_{3,2}$ 0.2–0.3 µm) at pH 8.0. The amount of each ASPI needed (C_{cr}; on protein basis) to form these stable emulsions varied between the isolates, but was within the range of proteins from both similar (photosynthetic) sources (algae and sugar beet leaves) and other protein sources (dairy, legume and egg). Minor differences were observed in the pH dependence of flocculation amongst the ASPI stabilized emulsions. For the ASPIs, the expected correlation between interfacial and molecular properties (adsorption rate constant and ζ-potential) and the emulsion behavior (C_{cr} and droplet size as a function of pH) was absent.

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INTRODUCTION

In the past decades, most research on emulsion behavior of novel proteins has focused on proteins from leguminous proteins (e.g. soy, lupin and pea). More recently, interests have included Rubisco-containing protein sources such as leaves, microalgae and cyanobacteria. Leaf proteins have been shown to consist largely of Rubisco e.g. in sugar beet leaves [1]), whereas cyanobacteria and microalgae are known to contain a large variety of proteins, e.g. [2, 3]. This study aims to analyze the effects of this variation in proteins present in microalgae and cyanobacteria protein isolates on their emulsion behavior. In addition, the effect of non-proteinaceous components in these isolates on their emulsion behavior is analyzed.

In recent years, studies have been performed on the emulsifying properties of protein isolates from various microalgae and cyanobacteria. Examples of these are studies on *Haemotococcus pluvialis* [4], *Nannochloropsis gaditana* [5], *Chlorella vulgaris* [6], *Tetraselmis impellucida* [7, 8] and *Arthrospira platensis* [9]. In these studies the emulsifying capacity of the protein isolates was measured as the amount of oil stabilized by 1 g of protein, or by the minimum protein concentration needed to produce stable emulsions. The algae proteins used were shown to have similar or higher emulsifying capacities than isolates of whey protein [7], soy protein [6] and sodium caseinates [4, 6]. For better understanding, more information is, however, needed on the differences and similarities in the emulsion properties and the relation to composition of protein isolates from different algal and cyanobacterial sources.

Algal and cyanobacterial protein isolates so far described in literature have a lower purity (on protein content) than protein isolates from dairy and leguminous proteins. Algae and cyanobacteria isolates had reported protein contents of up to 64–77% [w/w] [2, 7, 9], whereas protein isolates containing > 90% w/w protein have been isolated decades ago from e.g. milk [10], soy beans [11], peas [12] and sunflower seeds [13]. In the algae and cyanobacterial protein isolates, the presence of other components is expected to affect the emulsifying properties. It has already been shown for example that charged carbohydrates (which may naturally occur in algae protein isolates) can play a role in stabilizing emulsions against flocculation [8]. Another difference with traditional sources (egg, milk) is that the major protein fractions of microalgae and cyanobacteria are multimeric and can thus associate or dissociate as a function of pH or ionic strength [14], affecting their emulsifying behavior. More specifically, protein isolates obtained from *N. gaditana* and *T. impellucida* were reported to be rich in Rubisco [2 7], a multimeric (8 small and 8 large subunits) photosynthetic enzyme of 534 kDa [15][i]. In contrast, isolates obtained from *A. maxima* and *A. platensis* had a lower Rubisco content, but were found to be rich in biliproteins like C-phycocyanin [2, 9]. C-phycocyanins are multimeric light-harvesting proteins that are thought to occur as trimers (3 α- and 3 β-subunits) in *Arthrospira* sp., forming a 112 kDa molecule [16].

To understand the emulsion behavior of algae protein isolates, a recently developed approach was used that enables the prediction of the emulsion behavior of proteins based

[i] Uniprot search terms: rbcL/cbbL and rbcS/cbbS and genes in *Nannochloropsis* sp. and *Tetraselmis* sp. Accession numbers used: T1RH29, Q3S3D2, K9ZV74, A0A023PJK0 and K9ZWI1

on the proteins' molecular and interfacial properties. This model was successfully applied to estimate the emulsion behavior of lysozyme, β-lactoglobulin and ovalbumin [17] and of less purified soy protein and sugar beet leaf protein concentrates [18]. The model estimates the minimal or critical protein concentration (C_{cr}), which is the protein concentration above which emulsions are formed in which the minimum droplet size is reached ($d_{3,2,min}$). Above this protein concentration (referred to as the protein-rich-regime), there is enough protein to cover the interface formed during droplet formation, at $d_{3,2} = d_{3,2,min}$ [17, 19]. The C_{cr} is dependent on both the interfacial and molecular protein properties, including the adsorption rate constant (k_{adsorb}), the adsorbed amount of protein on the interface (Γ_{max}) and the protein radius (R_p), and on system characteristics like the volume oil fraction (Φ_{oil}) and ionic strength.

In this work, the emulsion behavior of protein isolates from various unicellular sources was compared. In addition, the relation between emulsion behavior and protein isolate characteristics was studied. For this, algae protein isolates were derived from unicellular sources belonging to three different phyla: *Arthrospira maxima* (a cyanobacterium), *Nannochloropsis gaditana* (a heterokontophyta) and *Tetraselmis impellucida* (a chlorophyta).

MATERIALS & METHODS

Materials

Food grade, non-purified sunflower oil was purchased at a local supermarket and stored at 4 °C prior to use. Whey protein isolate (WPI; 93.4% [w/w] protein; N x 6.32 [20] was purchased from Davisco Foods International (BiPro®, Le Sueur, MN, USA). The WPI contained, by weight, 74.0% β-lactoglobulin, 12.5% α-lactalbumin, 5.5% bovine serum albumin and 5.5% immunoglobulin, according to specifications of the supplier. Isolation of algae soluble protein isolates (ASPIs) from *Tetraselmis impellucida* (ASPI-T, 66.4% w/w protein, N x 5.32), *Nannochloropsis gaditana* (ASPI-N, 76.8% w/w protein, N x 5.03) and *Arthrospira maxima* (ASPI-A, 76.7% w/w protein, N x 5.01) and nitrogen-to-protein conversion factors were determined as described previously [2].

The ASPI concentrations used in this study were expressed as mg protein / mL. The chemical composition and protein composition of the ASPIs are shown in Table 1 and Figure 1. Based on the SDS-PAGE pattern (Figure 1), ASPI-A was thought to contain mostly C-phycocyanin. ASPI-N and -T were rich in Rubisco (estimated to represent 20–40% of the proteins present). ASPI-A was stored frozen (- 20 °C) as a lyophilized powder. ASPI-N and ASPI-T were stored frozen in solution (- 20 °C) with 0.5 M sucrose to prevent protein insolubility by freeze-concentration. Spinach D-ribulose 1,5-diphosphate carboxylase (Rubisco) was purchased from Sigma-Aldrich (product # R8000). All other chemicals used were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. All water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

Table 1: Gross chemical composition of protein isolates [% w/w] ± SD on dry weight (sucrose-free) basis. Reprinted from **chapter 2**.

Component	*A. maxima*	*N. gaditana*	*T. impellucida*
Proteins	76.7 ± 0.1	76.8 ± 2.6	66.4 ± 6.6
Carbohydrates	9.2 ± 0.4	8.9 ± 0.3	24.4 ± 0.4
Neutral	8.1 ± 0.3	8.3 ± 0.4	18.7 ± 0.5
Charged	1.0 ± <0.1	0.6 ± <0.1	5.7 ± 0.1
Total annotated	85.8	85.7	90.8

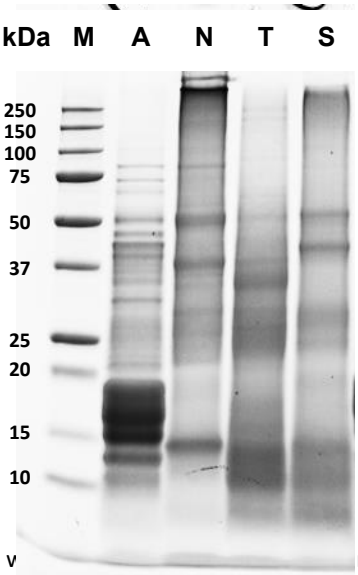


Figure 1: SDS-PAGE gels stained with [illegible] reducing conditions. M = molecular weight marker, A: ASPI-A, [illegible] ed from **chapter 2**.

Size exclusion chromatography (SEC)

The apparent molecular weight distribution of the ASPIs and of commercial Rubisco (included as a reference) was determined using SEC on an Äkta Micro equipped with a Superdex 200 Increase 10/300 GL. (GE Healthcare, Uppsala, Sweden). The proteins were solubilized in a filtered (0.45 µm pore size) 10 mM potassium phosphate buffer pH 8, at a protein concentration of 2 mg/mL, and centrifuged (10 min, 16,000 g, 10 °C). Supernatants were injected (50 µL) and eluted with the same buffer, at a flow rate of 0.75 mL/min. Absorbance was measured at 214 nm and 280 nm. Calibration was performed with ovalbumin (45 kDa), conalbumine (75 kDa), aldolase (158 kDa) and ferritin (440 kDa).

The percentage (w/w) of proteins present in multimeric state in each sample was calculated using literature data on Rubisco (for ASPI-N, ASPI–T and Rubisco) and literature data on C-phycocyanin for ASPI-A: C-phycocyanin has a preferred multimeric state of a trimer consisting of 3 α-subunits (19.6 kDa) and 3 β-subunits (17.7 kDa) [16]. Therefore, the

M_w of C-phycocyanin was assumed to be 112 kDa. Rubisco is typically present in a hexadecameric structure, consisting of 8 large (52–54 kDa) and 8 small (10–17 kDa) subunits [15][ii], and the average M_w of Rubisco was calculated to be 534 kDa. Based on these data, proteins of >560 kDa were considered multimeric (but could also contain soluble aggregates), proteins of 60–560 kDa were considered to be of intermediate size and proteins of < 60 kDa were considered monomeric in ASPI-N, ASPI–T and Rubisco. In ASPI-A, the range in which monomeric proteins are expected is at a lower MW, since the subunits are known to be of a smaller size than Rubisco (19.6 and 17.7 kDa), and form trimers of 112 kDa. Therefore, in ASPI-A proteins of > 112 kDa were considered multimeric (but this size range could also contain soluble aggregates), proteins of 36–112 kDa were considered intermediate sized and proteins of <36 kDa were considered monomeric.

Surface dilatational elastic modulus and adsorption kinetics

WPI and ASPIs were solubilized at 0.05 mg protein / mL in a 2 mM phosphate + 1 mM citrate buffer (pH 8) containing 4.43 mM NaCl, yielding an ionic strength of 10 mM. The surface elastic modulus and surface tension were measured in duplicate as a function of time, using an automated drop tensiometer at 20 °C (ADT, Teclis IT Concept, Longessaigne, France), according to the method described by Delahaije *et al.* [21].

The surface dilatational elastic modulus (E_d [mN/m]) was measured by inducing sinusoidal changes in the interfacial area with an amplitude of 5% and a frequency of 0.1 Hz. The surface elastic modulus was calculated from the measured changes in surface tension and surface area. The change in surface tension (γ_t [mN/m]) compared to that of the pure air-water interface (γ_0=71 mN/m) using the buffer only was expressed as the surface pressure. In the range (mN/m) where the various E_d/ Π curves superimpose, the initial slopes of the Π/t curves (dΠ/dt) were used to calculate the adsorption rate constant (k_{adsorb}), relative to that of WPI (k_{adsorb} = 1).

Emulsion preparation

Stock solutions of WPI and ASPI-A, -N and -T containing 1 mg/mL soluble protein were prepared in duplicate by solubilizing the respective isolate in 10 mM NaCl solution overnight at 4 °C, based on a method described previously [22]. To remove sucrose from the protein isolates, the solutions were diafiltered with 5 volumes 10 mM NaCl over 10 kDa NMWL regenerated cellulose membranes (Amicon Ultra-15 centrifugal filter units, Sigma-Aldrich, Saint Louis, MO, USA) for 10 min at 4100 x g at room temperature. After filtration, the concentration of the protein solutions was re-adjusted to 1 mg/mL protein when needed. The pH of the protein solutions (7.2–7.8) was adjusted to pH 8.0 with 0.2 M NaOH. The protein solutions were diluted to 0.025, 0.05, 0.08, 0.1, 0.3, 0.5, 0.65, 0.85 and 1.0 mg/mL protein with 10 mM NaCl. Due to a limited amount of protein isolate available for the experiments, emulsions were made containing 1% [w/w] sunflower oil (Φ_{oil} = 0.01). This is a 10–30 times lower Φ_{oil} than what is used in the literature. Using WPI, it was confirmed

[ii] Uniprot search terms: rbcL/cbbL and rbcS/cbbS and genes in *Nannochloropsis* sp. and *Tetraselmis* sp. Accession numbers used: T1RH29, Q3S3D2, K9ZV74, A0A023PJK0 and K9ZWI1.

that the C_{cr} of emulsions with Φ_{oil} = 0.01 was 10 times lower than the emulsions prepared with Φ_{oil} = 0.1, meaning that the diluted system can be compared to previously reported data on the more concentrated emulsions. The dispersions were pre-emulsified with an Ultra-Turrax (Type T-25B, IKA, Staufen, Germany) at 9500 rpm for 1 min. The pre-emulsions were passed 30 times through a laboratory scale homogenizer (Labhoscope HU-3.0, Delta Instruments, Drachten, The Netherlands) at a pressure of 150 bar. During homogenization the samples were cooled in ice water.

The influence of pH on droplet flocculation (from here on referred to as emulsion stability) was tested in duplicate on emulsions with 1% (w/v) sunflower oil, stabilized by WPI, ASPI-A, -N or -T in the protein-rich regimes. Since the C_{cr} was different between the protein samples, it was decided not to work with the same protein concentration for all samples, but to use protein concentrations that equal a similar surplus of protein, i.e. C ~ 1.5 x C_{cr}. Emulsions were prepared at pH 8 as described above using protein concentrations of 0.25, 0.60, 1.10 and 0.13 mg/mL for WPI, ASPI-A, -T and -N, respectively. Directly after homogenization, the pH of each emulsion was adjusted to pH 2–8 with 0.5 unit intervals using 0.2 M HCl and a pH-stat device. At each pH value, an aliquot was taken for physico-chemical analyses.

Droplet size determination

Droplet size distributions and volume surface average diameters ($d_{3,2}$) of the emulsions were determined by static light scattering (Mastersizer Hydro 2000SM, Malvern Instruments Ltd, Malvern, UK) at room temperature. Refractive indices of 1.46 and 1.33 were used for sunflower oil and water, respectively. Obscuration during the analyses was between 10% and 15% for all samples. The volume-surface average diameter ($d_{3,2}$) was reported as the average over three analyses on the same sample. For samples in which the influence of pH was studied, additional droplet size distribution measurements were performed after diluting the emulsions 1:1 in 0.5% (w/v) SDS, to see whether changes in $d_{3,2}$ were caused by droplet coalescence or flocculation. The data obtained in the concentration dependency experiment ($d_{3,2}$ over protein concentration) were fitted using the solver function of Microsoft Excel (version 14.0.7166.5000, Microsoft Corporation, Albuquerque, NM, United States) and equations 1 and 2, as described previously [18].

For C < C_{cr} (protein-poor regime): $$d_{3,2} = \alpha \times \left(\frac{1}{C} - \frac{1}{C_{cr}}\right) + d_{3,2,min} \quad (1)$$

For C ≥ C_{cr} (protein-rich regime): $$d_{3,2} = d_{3,2,min} \quad (2)$$

In which the C_{cr} is the minimal or critical protein concentration (mg/mL) needed to form emulsions with $d_{3,2} = d_{3,2,min}$, C is the protein concentration (g/L), $d_{3,2min}$ is the minimum average particle diameter (μm) and α (L/g) and C_{cr} are the fitting parameters. Protein concentrations higher than the C_{cr} are considered to be in the protein-rich-regime, protein concentrations lower than the C_{cr} are considered to be in the protein-poor-regime.

ζ-potential measurements

The ζ-potential of WPI solutions and of WPI or ASPI stabilized emulsion droplets as function of pH was determined using a particle electrophoresis instrument (Zetasizer, Nano series – ZSP, Malvern, United Kingdom), based on a previously described method [23]. WPI (5 mg/mL) was dissolved in 2 mM phosphate + 1 mM citrate buffers (pH 2–8) containing 4.43–9.76 mM NaCl, yielding an ionic strength of *I* = 10 mM for each buffer. The emulsions were diluted 500 times in the same buffers of the corresponding pH. The pH and conductivity of the diluted emulsions in the phosphate/citrate buffers were recorded; no adjustment was needed. ζ-Potential measurements were performed at room temperature at 150 V for protein solutions and at 40 V for emulsions. Samples were equilibrated inside the instrument for 2 min at 25 °C before starting a measurement. Data were collected and averaged over at least 5 sequential readings. The zeta potentials were calculated using the Smoluchowski model, as described previously [23]. The minimum absolute ζ-potential (the apparent isoelectric point) was calculated using a linear interpolation between pH 3.5 and 6.0 (R^2> 0.98 for all).

Experimental and theoretical maximum adsorbed amount (Γ_{max})

The maximum amount of protein adsorbed at the oil-water interface was estimated from $d_{3,2,min}$ and from the lowest protein concentration at which $d_{3,2,min}$ was reached (C_{cr}), using equation 3 [24].

$$\Gamma_{max} = \frac{d_{3,2,min} \times (1-\Phi_{oil}) \times k_{adsorb} \times C_{cr}}{6 \times \Phi_{oil}} \quad (3)$$

In which Φ_{oil} is the volume fraction oil. The theoretical maximum amount of protein adsorbed at the oil-water interface ($\Gamma_{max,theory}$ in mg/m^2) was calculated using equation 4 [24].

$$\Gamma_{max,theory} = \frac{M_w \times 10^3}{\pi \times {R_{eff}}^2 \times N_a} \times \theta_\infty \quad (4)$$

In which M_w is the protein molecular mass (g/mol). θ_∞ (-) is the maximum protein packing density at the interface (0.547) [25], N_a is Avogadro's number and R_{eff} is the effective hard-sphere radius of the adsorbed protein (m). For these calculations literature data on Rubisco [15][iii] were used for ASPI-N and -T, on C-phycocyanin [16] for ASPI-A, and on β-lactoglobulin [15][iv] for WPI.. In these calculations a monolayer of proteins on the oil/water interface was assumed. The R_{eff} of the particle is larger than its actual hard-sphere radius (R_p), since the electrostatic interactions are taken into account. The R_{eff} can be approximated by equation 5 [24].

[iii] Uniprot search terms: rbcL/cbbL and rbcS/cbbS and genes in *Nannochloropsis* sp. and *Tetraselmis* sp. Accession numbers used: T1RH29, Q3S3D2, K9ZV74, A0A023PJK0 and K9ZWI1

[iv] Uniprot accession number P02754.

$$R_{eff} = R_p - \frac{1}{2} \times ln\left(\frac{x}{R_p}\right) \times k^{-1} \quad (5)$$

In which R_p is the radius of the protein (m) and κ is the inverse Debye screening length (m). The constant x (-) describes the net contribution of electrostatic repulsion, kinetic energy and hydrophobic interactions. The value of x was assumed to be similar to that of β-lactoglobulin ($1.77 \cdot 10^{-9}$, at pH 7.0) [24]. The R_p was calculated using equation 6.

$$R_p = \left(\frac{3 \times v \times M_w}{4 \times \pi \times N_a}\right)^{\frac{1}{3}} \quad (6)$$

In which ν is the partial specific volume of the protein ($0.73 \cdot 10^{-6}$ m^3/g) [26]. The κ was calculated using equation 7.

$$\kappa = \sqrt{\frac{2 \times N_a \times e^2 \times I}{\varepsilon_0 \times \varepsilon_r \times \varepsilon_B \times T}} \quad (7)$$

In which N_a is the Avogadro constant ($6.022 \cdot 10^{23}$ mol^{-1}), e is the elementary charge ($1.602 \cdot 10^{-19}$ C), I is the ionic strength (mol/m^3), k_B is the Boltzmann constant ($1.38 \cdot 10^{-23}$ J/K) and T is the temperature (K).

Microscopy

The state of droplet flocculation was analyzed by light microscopy using an Axioscope A01 (Carl Zeiss, Sliedrecht, The Netherlands) at 40x magnification. The emulsions were diluted 50 times in phosphate / citrate buffers (2 mM / 1 mM, pH 8) containing 4.43 mM NaCl (I = 10 mM), of the corresponding pH. To further confirm flocculation, the emulsion samples were diluted 1:1 in SDS (0.5% w/v), followed by a 25 times dilution in the previously mentioned citrate/phosphate buffers and re-analyzed by microscopy. A presence of separate droplets after inclusion of SDS was used as a confirmation of droplet flocculation in the initial emulsion.

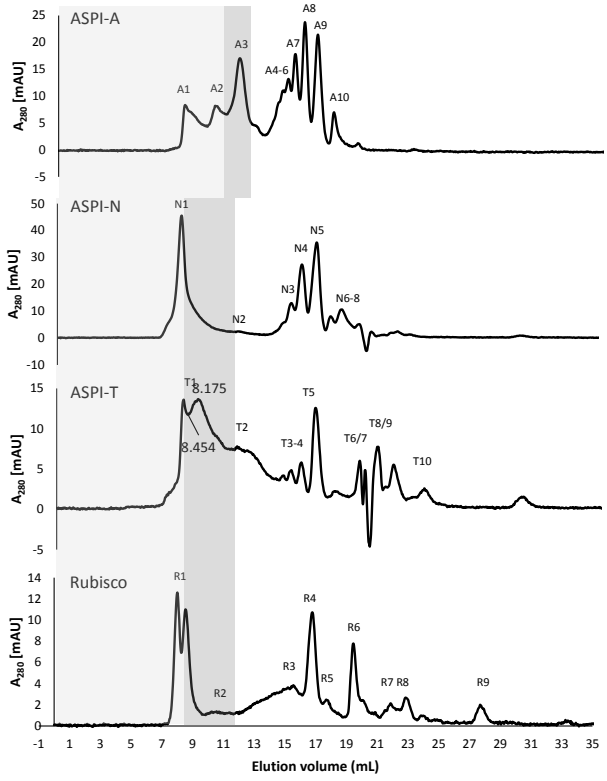
RESULTS AND DISCUSSION

Physico-chemical properties of the protein isolates

Molecular mass distribution

Based on their SDS-PAGE patterns, ASPI-N and APSI-T were considered to be rich in Rubisco (estimated to represent 20–40% of the proteins present) and ASPI-A was thought to contain mostly C-phycocyanin (Figure 1). Using SEC, the multimerity of the proteins was estimated. In the commercial Rubisco isolate, only 25% of proteins were in multimeric state (hexadecameric; > 560 kDa; Figure 2). The other proteins present were in monomeric state (69%; < 60 kDa) or were of intermediate size (6%; 60–560 kDa). The proteins in the Rubisco-rich ASPI-N and ASPI-T had a higher percentage of multimeric proteins (39 and 42%, respectively) than the proteins in the commercial Rubisco. The other proteins present in

ASPI-N and -T were monomeric. ASPI-A had a lower percentage of multimeric proteins (12%; > 112 kDa) and contained 23% proteins of intermediate size (36–112 kDa). The percentage of monomeric proteins in ASPI-A (65%; < 36 kDa) was similar to that of the other ASPIs (58 and 61%). Although similar to the commercial Rubisco, the high percentage of monomeric proteins found in the ASPIs was more than expected since literature data mostly comments on the associated state of Rubisco. Additionally, the method applied to obtain the isolates did not include the use of heat or denaturing/dissociating chemicals, as described previously [2]. The method was therefore expected to yield Rubisco in its 534 kDa (L8S8) associated form [15v].Rubisco is known to dissociate under influence of chemicals (urea, detergents and citraconylation), at pH 5.0–5.6 (turning the large subunits insoluble), and above pH 11 [14, 27]. It is not known however, how sensitive Rubisco is to processing and milder system conditions concerning dissociation and association of its subunits.



Peak #	ASPI-A	ASPI-N	ASPI-T	Rubisco
1	590	623	523	532
2	155	70	37	110
3	51	<10	<10	<10
4 - 11	<10	<10	<10	<10

	Multimeric	Intermediate	Monomeric
ASPI-A	12	23	65
ASPI-N	39	-	61
ASPI-T	42	-	58
Rubisco	25	6	69

Figure 2: Size exclusion chromatography elution patterns of ASPI-A, -N, and -T at 2 mg protein / mL, pH 8.0, I = 10 mM. Inserts: estimated molecular weights of the peaks (in kDa) and % of proteins present as multimeric, intermediate and monomeric proteins. Elution ranges of multimeric proteins are shaded in light gray, ranges of intermediate proteins are shaded in dark gray, and ranges of monomeric protein are not shaded in the chromatograph.

[v] Uniprot search terms: rbcL/cbbL and rbcS/cbbS and genes in *Nannochloropsis* sp. and *Tetraselmis* sp. Accession numbers used: T1RH29, Q3S3D2, K9ZV74, A0A023PJK0 and K9ZWI1

Interfacial rheology and adsorption kinetics

The surface dilatational elastic modulus (E_d) was plotted against the surface pressure (Π), as an indication of the interactions between the proteins adsorbed at the interface (Figure 3 A). Up to a surface pressure of ~ 6 mN/m, all E_d/ Π curves superimposed, thus the slope of the Π/t curves (dΠ/dt, Figure 3 B) between 0 and 6 mN/m could be used as an indication of the adsorption rate. The initial adsorption rate [mN / (m * s)] of ASPI-A (0.082 ± 0.011) was two times higher than that of the other proteins (0.034–0.037 ± ≤ 0.004) (Table 2). The relative adsorption rate constant (k_{adsorb} [-] calculated as dΠ/dt) relative to that of WPI of ASPI-A was 2.41 (± 0.32), compared to 1.09 (± 0.10) and 1.01 (± 0.08) for ASPI-N and -T. The time needed for proteins to reach the oil-water interface affects the proteins' ability to stabilize the emulsion droplets against coalescence during homogenization. Proteins with a higher k_{adsorb} (like ASPI-A) are thus expected to stabilize emulsions against coalescence during homogenization at lower protein concentrations than proteins with a lower k_{adsorb} (ASPI-N and -T).

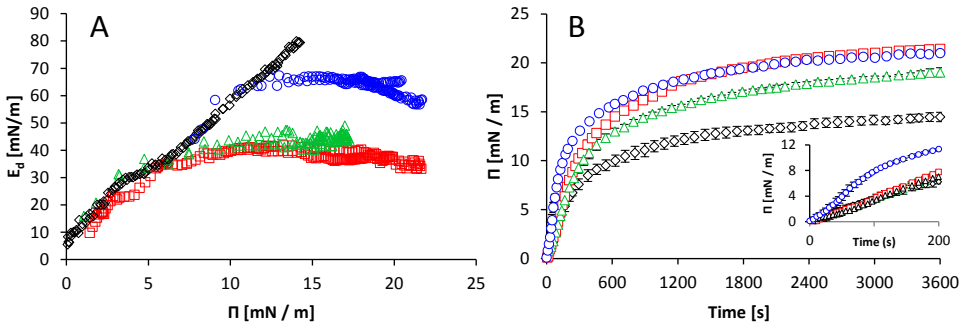


Figure 3: Elastic modulus (E_d) as a function of surface pressure (A) and surface pressure (Π) as function of time (B) for ASPI-A, (◯), ASPI-N (□), ASPI-T (△) and WPI (◇) at 0.05 mg protein / mL, pH 8.0 and I = 0.01 M. The inset shows the surface pressure at 0–200 s. Duplicate measurements are shown individually in A, whereas the means are shown in B with error bars indicating standard deviations.

Emulsifying ability

In the protein-rich regime (protein concentration ≥ C_{cr}; Figure 4), ASPI and WPI stabilized emulsions formed at pH 8 had droplet sizes of 0.2–0.3 µm (Table 2). In that respect all protein isolates were comparable. However, there were large differences in the efficiency of the proteins to stabilize the droplets formed during homogenization, as reflected in C_{cr}. The C_{cr} of ASPI-N (0.09 ± 0.02 mg/mL) was 2 times lower than that of WPI (0.17 ± 0.02 mg/mL) and 5–8 times lower than ASPI-A (0.41 ± 0.03 mg/mL) and ASPI-T (0.74 ± 0.01 mg/mL). In literature, C_{cr} values were reported for WPI stabilized emulsions (containing 10% or 30% w/w oil) [7, 22]. The C_{cr} is dependent on the Φ_{oil} used: a higher oil fraction requires a higher protein concentration to form an emulsion. To compare the literature C_{cr} values, made at higher Φ_{oil} amongst each other and to our C_{cr} values at lower the Φ_{oil}, the C_{cr} should be corrected for Φ_{oil}. (by C_{cr} / Φ_{oil}). Using this correction, the present findings for WPI (18

mg protein/mL oil) were in the same range as the (wide-spread) data in literature on e.g. WPI (12 mg/mL [7]), β-lactoglobulin (20 and 50 mg/mL [17, 22]) and whey protein concentrate (7 mg/mL [28]). Using the same approach, the C_{cr}/Φ_{oil} of ASPI-T (74 mg/mL) was found to be 6 times higher than that of a previously described protein isolate of another batch of *Tetraselmis impellucida* (13 mg/mL [7]). The C_{cr}/Φ_{oil} of ASPI-N (9 mg/mL) was similar to what was described for soy protein (10 mg/mL [18]), and lower than that of e.g. sugar beet leaf protein (21 mg/mL [18]) and whey protein (12–50 mg/mL [7, 29]). The C_{cr}/Φ_{oil} of the ASPI-A and -T (41 and 74 mg/mL) were thus 4–7 times higher than that of the soy protein, 2–4 times higher than the sugar beet leaf protein and 1–7 times higher than WPI. From the C_{cr}, the adsorbed amount of protein (Γ_{max} [mg protein/m^2]) was calculated (based on [24]), using equation 3 (Table 2). The Γ_{max} was lowest for ASPI-N (0.43 mg/m^2) and WPI (0.78 mg/m^2). The Γ_{max} of ASPI-A was 2–4 times higher (1.67 mg/m^2), and that of ASPI—T was 4–8 times higher (3.25 mg/m^2).

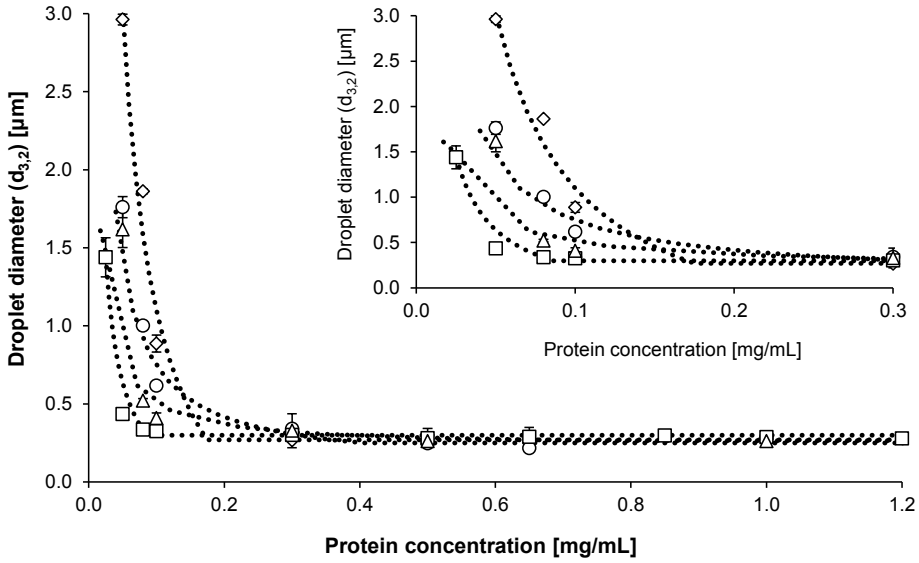


Figure 4: Average droplet diameter ($d_{3,2}$) as function of protein concentration of emulsions stabilized with ASPI-A, (◯), ASPI-N (□), ASPI-T (△) and WPI (◇) at I = 0.01 M and pH 8.0. All data points are average values of duplicate samples, each measured in triplicate. The error bars (smaller than the marker size) indicate the standard deviation between the duplicates. The dashed lines represent a fit of the data calculated with equations 1 and 2 and the fitting parameters shown in Table 3. The insert shows a close-up of the same data, using only protein concentrations of 0–0.3 mg/mL.

Table 2: Protein adsorbed amount at the interface derived from $d_{3,2}$ (Γ_{max}) and based on theoretical calculations ($\Gamma_{max,theory}$) at pH 8.0.

		Parameters	ASPI-A	ASPI-N	ASPI-T	WPI
From emulsions	Experimental values	Φ_{oil} [-]	0.01	0.01	0.01	0.01
		$d_{3,2,min}$ [µm]	0.25 ± 0.01	0.30 ± 0.02	0.27 ± 0.02	0.27 ± 0.01
		dΠ/dt [mN /(m*s)]	0.082 ± 0.011	0.037 ± 0.004	0.034 ± 0.003	0.034 ± 0.004
		k_{adsorb} [-]	2.41 ± 0.32	1.09 ± 0.10	1.01 ± 0.08	1.00 -
		C_{cr} [mg/mL]	0.41 ± 0.03	0.09 ± 0.02	0.74 ± 0.01	0.17 ± 0.02
	Calculated data	Γ_{max} [mg/m²][ab]	1.67	0.43	3.25	0.78
		R_{eff} [nm][bc]	4.40	18.7	6.8	3.7
Constants	Literature values	M_w [g/mol][cd]	111900*	534134 **	534134 **	36600 ***
From adsorbed layer	Calculated data[x]	K^{-1} [nm][de]	2.96	2.96	2.96	2.96
		R_p [nm][ef]	3.19	5.25	5.25	2.20
		R_{eff} [nm][fg]	4.06	6.85	6.85	2.52
		$\Gamma_{max,theory}$ [mg/m²][h]	1.96	3.15	3.15	1.67

[a] ± Indicates the SD of duplicate measurements; [b] calculated data using equation 3; [c] calculated data using equation 4; [d] Literature values from * [16], assuming a trimer; ** [15] using accession numbers T1RH29, Q3S3D2, K9ZV74, A0A023PJK0 and K9ZWI, assuming a L8S8 conformation; *** [15] using accession number P02754, assuming a dimer; [e] calculated data from equation 7; [f] calculated data from equation 6; [g] calculated using equation 5; and [h] calculated data using equation 4, using 4 °C and I = 10 mM.

Table 3: Fitting parameters (α, $d_{3,2\ min}$ and C_{cr}) and the coefficient of determination for fitting equations 1 and 2 using data shown in Figure 4.

$$d_{3,2}(C)fit = \begin{cases} \alpha \cdot \left(\frac{1}{C} - \frac{1}{C_{cr}} \right) + d_{3,2,min}, C < C_{cr} \\ d_{3,2,min}, C \geq C_{cr} \end{cases}$$

	ASPI-A	ASPI-N	ASPI-T	WPI
α	0.066	0.040	0.028	0.193
$d_{3,2,min}$ [μm]	0.249	0.299	0.265	0.270
C_{cr} [g/L]	0.406	0.086	0.742	0.175
R^2 [%]	88.2	88.7	86.1	83.0

The experimental values for Γ_{max} were compared to the theoretical Γ_{max} ($\Gamma_{max,theory}$) calculated using equations 4, 5, 6 and 7 (Table 2). For ASPI-A and -T the experimental Γ_{max} values were similar to the calculated $\Gamma_{max,theory}$. This shows that for these ASPIs from two types of microalgae an indication of the C_{cr} could be obtained from the known properties of the dominant proteins. However, for ASPI-N and WPI, the Γ_{max} was lower (2–7 x) than the $\Gamma_{max,theory}$. This is slightly surprising, since it suggests that the emulsion droplets were stable against coalescence (during homogenization) and flocculation (after homogenization) at a surface coverage lower than the maximum (0.547). In contrast, in work by Schwenzfeier et al. the experimental Γ_{max} was only slightly lower 1.56 mg/m^2 (based on a C_{cr} of 4 mg/mL) than the $\Gamma_{max,theory}$ (1.67 mg/m^2, with I = 10 mM) in WPI stabilized emulsions with 30% oil [7]. Moreover, Delahaije et al. report a higher experimental Γ_{max} (2.48 mg/m^2, using a C_{cr} of 5 mg/mL) than the minimum theoretical coverage (1.99 mg/m^2 at I = 86 mM) in WPI stabilized emulsions of 10% oil [29]. Summarizing, the experimental Γ_{max} values are different from the theoretical values in all these studies and also deviate between the different studies. It appears that the estimation of $\Gamma_{max,theory}$ values is very sensitive to the C_{cr} determination (e.g. by computation or by using lowest measured value). The major differences observed between the low experimental Γ_{max} in the present work and that of Schwenzfeier et al. and Delahaije et al. [7, 29] is postulated to be due to the lower oil fraction (1% compared to 10% and 30%). The lower Φ_{oil} may have generated a longer time between collision of emulsion droplets upon homogenization, giving the proteins more time to adsorb to the interface, and finally yielding a lower C_{cr}/Φ_{oil}.

The effect of pH on emulsion droplet size and ζ-potential

Emulsion stability (stability against pH induced flocculation) was studied using emulsions made in the protein-rich regime, at C = 1.5 x C_{cr}. After adjusting the pH from 8.0 to pH 6.0, no changes were observed in the $d_{3,2}$ ($d_{3,2} = d_{3,2,min}$) for all ASPI and WPI stabilized emulsions (Figure 5). At lower pH, increases in $d_{3,2}$ occurred for several samples. This increase in $d_{3,2}$ was due to flocculation as confirmed by microscopy (microscopy examples of WPI and ASPI-N emulsions are shown in Figure 6). For WPI stabilized emulsions, the emulsion droplets flocculated in the range from pH 3.5–6.0, but did not flocculate when the pH was further decreased to pH < 3.5. These results for WPI are very similar to what was published before on 10% w/w oil WPI stabilized emulsions [29]. The ASPI samples had a broader pH range at which the emulsions flocculated than WPI. Overall, the emulsions stabilized with various ASPIs showed similar pH dependence of flocculation. A small difference was that ASPI-N

stabilized emulsions flocculated in a broader pH range (pH < 5.8) than the other ASPIs (pH 2.0–5.4). The flocculation of the ASPI-T emulsions was a bit surprising, since in previous work 30% w/w oil emulsions stabilized with a protein isolate from *T. impellucida* showed no flocculation at pH < 4 [7]. This could be due to the presence of non-protein compounds as discussed below. At least it is a further indication (next to the previously discussed differences in C_{cr}) that indeed there were batch-to-batch differences between the *T. impellucida* biomass or the ASPIs derived from that. Of the ASPI stabilized emulsions, only ASPI-T emulsions showed complete dissociation of the flocculates formed at pH 2.0.

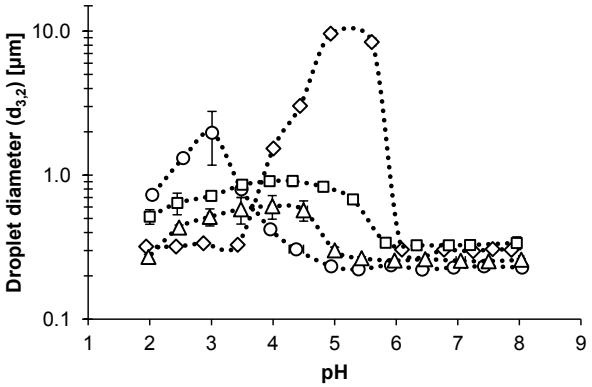


Figure 5: Average droplet diameter (d3,2) for ASPI- and WPI-stabilized emulsions as function of pH at protein concentrations of 1.10 mg/mL for ASPI-A (○), 0.13 mg/mL for ASPI-N (□), 0.60 mg/mL for ASPI-T (△) and 0.25 mg/mL for WPI (◇), respectively. All protein concentrations were 1.5 x Ccr. Error bars depict standard deviations between duplicate samples.

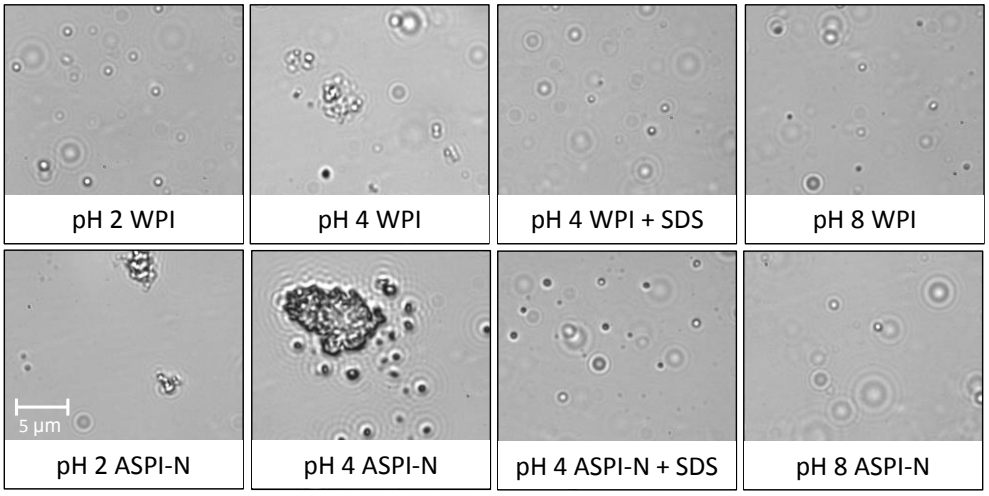


Figure 6: Light microscopy pictures of emulsions stabilized with 0.25 mg protein / mL WPI or 0.13 mg/mL ASPI-N at pH 8.0, after adjustment to pH 2.0 and 4.0, and after addition of SDS of the flocculated emulsions at pH 4.0.

In previous studies, the ζ-potential of proteins in solution was measured in order to explain or help predict their emulsion stability (e.g. [18]). Using WPI, it was confirmed that the maximum amount of flocculation occurred around the point (pH ~ 5; Figure 7) where the ζ-potential was close to zero. For WPI stabilized emulsions the minimum absolute ζ-potential was at the same pH as for the proteins (pH 5.0 ± 0.2, Figure 7). This pI of WPI stabilized emulsions is similar to that of that of the theoretical pI of β-lactoglobulin (pI = 4.83). Qualitatively, the trend (but not the absolute value) of the ζ-potential as function of pH was similar for the WPI solution and WPI stabilized emulsion droplets (Figure 7).

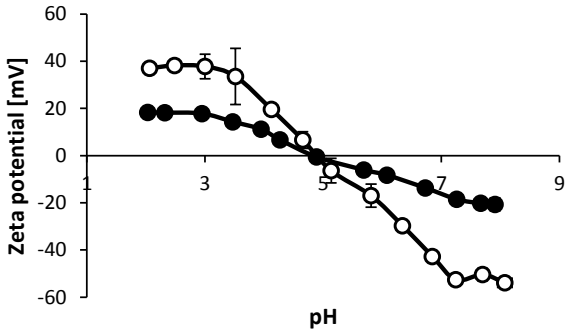


Figure 7: ζ-potential as function of pH of WPI (5 mg protein / mL at I = 10 mM; filled symbols); and of an emulsion stabilized with WPI (I = 10 mM and 0.25 mg protein / mL; open symbols). Error bars depict standard deviations between duplicate samples.

For ASPIs, protein ζ-potential measurements could not be performed, since the ASPIs absorbed the light of the laser (λ = 633 nm). For the ASPI stabilized emulsions the measurements could be performed, since the system could be more diluted due to higher scattering intensity of the emulsion droplets compared to the proteins in solution. Based on the results obtained for WPI, the ζ-potential as a function of pH of ASPI emulsions can be taken as an estimate of the ζ-potential of ASPI solutions. The ζ-potential curves measured of the 3 ASPI stabilized emulsions (Figure 8) superimposed to a single curve. The ζ-potential curves of the ASPI stabilized emulsions are similar to that of emulsions stabilized by sugar beet leaf proteins [18] and soy protein isolate [18]. However, the results with ASPI-T were quite different from earlier results with an ASPI from another batch of *T. impellucida* [7]. The minimum absolute ζ-potential (the apparent isoelectric point) of all ASPI stabilized emulsions was calculated to be at pH 4.0 (± 0.1). The similarity in ζ-potential of the droplets, as well as the apparent pI is not reflected in the flocculation behavior.

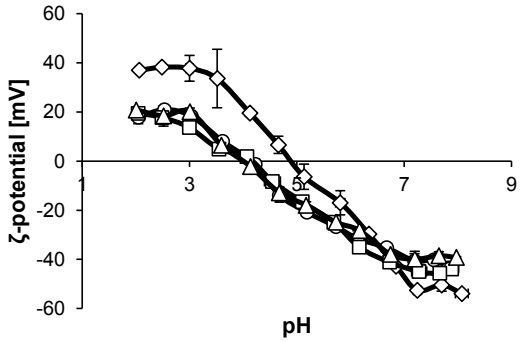


Figure 8: ζ-potential as function of pH of emulsions stabilized with ASPI-A, (○), ASPI-N (□), ASPI-T (△) and WPI (◇) at I = 0.01 M and 1.10, 0.13, 0.60 and 0.25 mg protein / mL, respectively. All markers are average values of triplicate measurements of duplicate samples, with the error bars indicating the standard deviation between the duplicates, for most samples the error bars are smaller than marker size.

A plot of $d_{3,2}$ versus the ζ-potential (Figure 9) shows that emulsions stabilized by the ASPIs and WPI have a small droplet diameter when the absolute ζ-potential is > 20–30 mV. This ζ-potential range is in the range of previously reported findings on emulsions stabilized by WPI (≥ 20 mV [7, 30]) and *T. impellucida* protein isolates (33 mV [8]). However, ASPI-A stabilized emulsions, and to a lesser extent also for WPI stabilized emulsions, behaved differently after being acidified through the iso-electric point. This is indicated by a different relation between $d_{3,2}$ and ζ-potential in the range where pH > pI and the range where pH < pI while for the other samples the two ranges overlap. For ASPI-A, the $d_{3,2}$ of the emulsion droplets is larger at a pH range where pH > pI than at pH < pI. This indicates that in ASPI-A stabilized emulsions (in contrast to emulsions stabilized by ASPI-N and -T) the flocculates formed at the pI do not dissociate for when further acidifying the pH to pH < pI. In WPI stabilized emulsions flocculation also occurs at low ζ-potential (< 20–30 mV), but the size of the flocculates differ for pH > pI and pH < pI. This behavior was not expected, since the absolute ζ-potentials and thus the net repulsive forces between the droplets were similar between the samples. This phenomenon is possibly an artefact caused by the low emulsion concentration (both oil and protein) used.

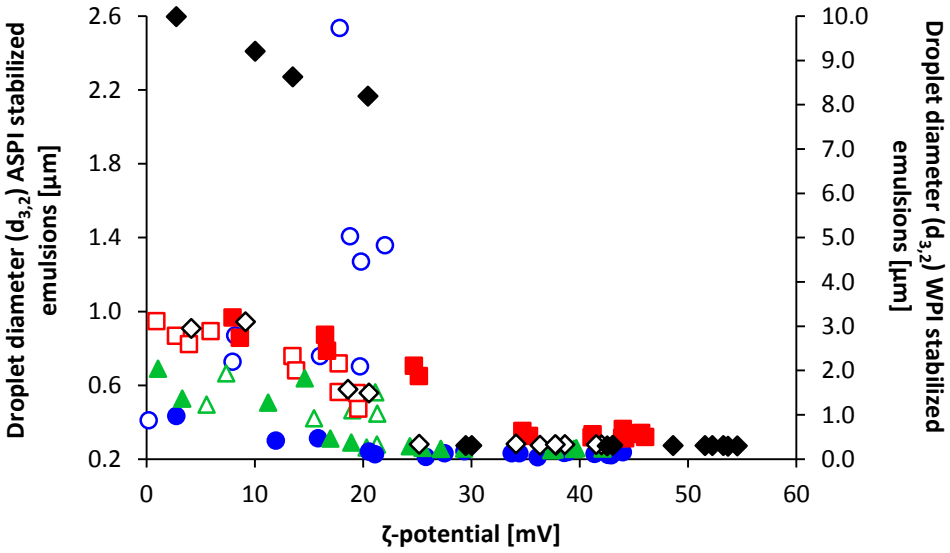


Figure 9: Average droplet diameter ($d_{3,2}$) as function of absolute ζ-potential of emulsions stabilized with ASPI-A (●), ASPI-N (■), ASPI-T (▲) and WPI (◆). Emulsions were prepared at pH 8.0 and acidified to pH 8.0–2.0. All unique data points of the duplicate measurements are shown as such. Open markers are data points at which the actual ζ-potential was negative; filled markers are data points at which the actual ζ-potential was positive.

Role of charged carbohydrates in emulsion behavior

In pure protein systems, the emulsion behavior of protein-stabilized emulsions can be predicted by the interfacial (k_{adsorb}) and molecular (ζ-potential) properties of the proteins. As discussed above, the emulsion stability of the ASPIs (as determined by $d_{3,2}$ as a function of pH) did not correlate to these protein properties. For example, based on the high k_{adsorb} of ASPI-A compared to the other ASPIs, a lower C_{cr} was expected for ASPI-A. However, the C_{cr} of ASPI-A was found to be the same range as the C_{cr} of the other ASPI's. Additionally, the pI of the ASPIs as determined by the ζ-potential as a function of pH did not correspond to the pH where maximum droplet flocculation ($d_{3,2}$) occurred in the ASPI-stabilized emulsions. These findings indicate that charged components present in the isolates, other than proteins, affected both the emulsifying behavior and emulsion stability of the ASPIs.

The protein contents of the ASPIs were 66–76%. The remainder mostly consisted of carbohydrates (9–24% w/w) of which a part are charged carbohydrates (7–23% of total carbohydrates; measured as uronic acids; UAs). Previous work showed that emulsions stabilized with another *T. impellucida* protein isolate (ASPI-T_{ref}) were stable against flocculation over a broad pH range [7]. This increased pH dependent stability was confirmed to be caused by the natural presence of UAs in the ASPI-T_{ref}. There were striking differences between the $d_{3,2}$ and ζ-potential as a function of pH of emulsions stabilized with ASPI-T_{ref} and that of emulsions stabilized with the present ASPI-T. Unexpectedly, the ratio of UA to protein between the two ASPI-Ts were similar (both 0.09; Table 1 and [8]). A possible

explanation for these differences is that the polysaccharides containing the UAs, the type of UAs present or their degree of methylation is different between the ASPIs, leading to different interactions with the proteins and/or emulsion droplets.

Another indication of the role of charged carbohydrates in the ASPIs was found in the ζ-potential as a function of pH and the apparent isoelectric points measured (Figure 8). These charges were different from the charges that were theoretically expected (Figure 10). These theoretical charges were calculated using the ASPIs' amino acid composition and the UAs (pKa 3.3) present. Contrary to the ASPI results, the change in ζ-potential of WPI as function of pH was similar to that expected based on the theoretical charge, using the amino acid composition of β-lactoglobulin (Figure 8 and Figure 10). This suggests that the ASPIs contained other charged components, not analyzed in both the present study and that of Schwenzfeier et al. that could influence the charge and emulsion behavior of the algae protein isolates. For example, a wide range of microalgal species (including *Tetraselmis* sp.) produce exopolysaccharides that contain (charged) sulfated polysaccharides [31]. These sulfated polysaccharides might be co-passengers in the protein isolation process applied, affecting the techno-functional properties of the isolates obtained.

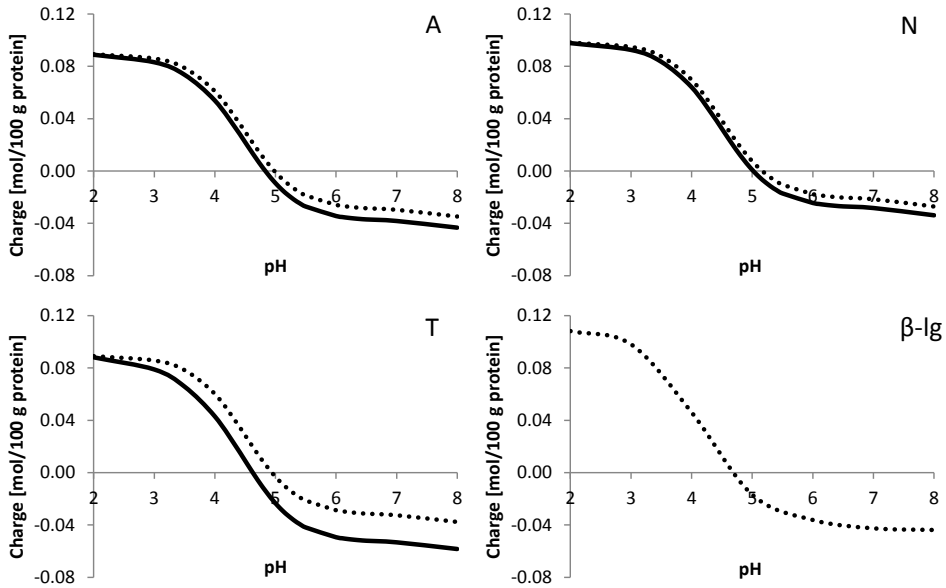


Figure 10: Theoretical charge (in eV) as a function of pH of ASPI-A (A): ASPI-N (N), ASPI-T (T) and β-lactoglobulin (β-lg). The charge was calculated on the amino acid composition of the ASPIs (data published in [2]) and literature data on β-lactoglobulin [15][vi]. Dotted lines depict the theoretical charge of the proteins only, continuous lines depict the theoretical charge of proteins with uronic acids present. Since β-lg does not contain uronic acids, only the dotted line is depicted in that panel.

[vi] Uniprot accession number P02754.

Table 4: Interfacial and emulsion properties of proteins from various animal and plant sources.

	Dairy (bovine)						Seeds/legumes		Potato		
	β-lg			WPI			Hel	SPI	Pat		PPIs
Reference	1	2	3	4	5	6	7	8	9	2	9
C_{cr}/Φ_{oil} [mg protein/mL oil]	20 [a]	50 [a]	n.d.	50 [b]	12 [a]	18 [c]	15 [b]	10 [c]	30 [e]		n.d.
$d_{3,2,min}$ [um]	0.26 [a]	0.18 [a]	n.d.	0.33 [b]	1.0 [a]	0.27 [c]	1.05 [b]	0.6 [c]	0.31 [f]	3.3 [a]	0.35 [f]
$d_{3,2,flocc}$ [um]	n.d.	14 [a]	n.d.	10.5 [b]	7.5 [a]	10 [c]	n.f	16 [c]	n.d.		n.d.
Q_H or k_{adsorb} [-]	1.0	1.0	1.0	1.0	1.0	1.0	n.d.	0.9	n.d.	0.73	n.d.
Γ_{max} [mg/m^2]	n.d.	n.d.	1.69 [a]	2.19 [d]	0.97 [a]	0.78 [c]	3.5 [b]	1.8 [c]	2.5 [f]		1.8 [f]

	Egg (chicken)				Gum	Leaves	Algae/cyanobacteria			
	Ova		Lys		GA	LSPC	ASPI-A	ASPI-N	ASPI-T	
Reference	1	3	1	3	5	8	6	6	6	5
C_{cr}/Φ_{oil} [mg protein/mL oil]	100 [a]	n.d.	250 [a]	n.d.	6 [a,*]	21 [c]	41 [c]	9 [c]	74 [c]	13 [a]
$d_{3,2,min}$ [um]	~0.3 [a]	n.d.	~0.5 [a]	n.d.	2 [a]	0.6 [c]	0.25 [c]	0.30 [c]	0.27 [c]	1 [a]
$d_{3,2,flocc}$ [um]	n.d.	n.d.	n.d.	n.d.	n.f.	8 [c]	2.0 [c]	0.9 [c]	0.6 [c]	3.5 [a]
Q_H or k_{adsorb} [-]	n.d.	0.19	n.d.	0.06	n.d.	1.1	2.41 [c]	1.09 [c]	1.01 [c]	
Γ_{max} [mg/m^2]	n.d.	1.80 [a]	n.d.	1.95 [a]	1.70	1.9 [c]	1.67 [c]	0.43 [c]	3.25 [c]	0.82 [a]

β-lg: β-lactoglobulin, WPI: whey protein isolate, Hel: helianthinin, SPI: soy protein isolate, Pat: patatin, PPIs, potato protease inhibitors, Ova: ovalbumin, Lys: lysozyme, GA: gum arabic, LSPC: sugar beet leaf protein concentrate, ASPI: algae soluble protein isolate from (-A): *Arthrospira maxima*, (-N): *Nannochloropsis gaditana* and (-T) *Tetraselmis impellucida*.

C_{cr}/Φ_{oil}: critical protein concentration corrected for the oil fraction; $d_{3,2,min}$: minimum droplet size (at $C > C_{cr}$), $d_{3,2,flocc}$: droplet size at pH at which flocculation occurred; Q_H: protein surface hydrophobicity, relative to WPI or β-lg; k_{adsorb}: protein adsorption rate constant, relative to WPI/ β-lg; and Γ_{max}: adsorbed amount of protein at the interface.

System conditions: [a] pH 7, I = 10 mM; [b] pH 7, I = 20 mM; [c] pH 8, I = 10 mM; [d] pH 7, I = 86 mM; [e] pH 7, I = 50 mM; [f] pH 7, I = 15 mM.

* Expressed on protein basis. On weight basis the C_{cr}/Φ_{oil} was 267 mg protein / mL oil.

n.f.: no flocculation detected.

n.d.: not determined.

Data retrieved from: 1 [17], 2 [22], 3 [24], 4 [29], 5 [7], 6 this study, 7 [32], 8 [18], 9 [33].

Algae protein isolates compared to other emulsifying agents

Literature data on interfacial properties and emulsion behavior of proteins from dairy and plant origin show a wide range of emulsion behavior and molecular and interfacial properties, like C_{cr} and Q_H/k_{adsorb} (Table 4). To allow a fair comparison, data was only used from studies that used similar system conditions as the present study (pH 7–8; I = 10–86 mM). Additionally, the C_{cr} was corrected with the Φ_{oil} used (C_{cr}/Φ_{oil}). Large variations were reported in C_{cr}/Φ_{oil} between studies using the same protein source (e.g. 20–50 mg protein/mL oil for β-lactoglobulin and 12–50 mg protein/mL oil for WPI). Between different protein sources however, these C_{cr}/Φ_{oil} values varied to a greater extent. More specifically, the C_{cr}/Φ_{oil} values were relatively high (100–250 mg protein/mL oil) for egg proteins (ovalbumin and lysozyme), compared to 12–50 mg protein/mL oil for dairy proteins and 10–15 mg protein/mL oil for leguminous proteins (helianthinin and soy proteins). The C_{cr}/Φ_{oil} of the algae proteins (9–74 mg protein/mL oil) were within the range observed for proteins from the other protein sources. Additionally, the C_{cr}/Φ_{oil} of the protein isolate derived from *N. gaditana* was also similar to that of gum arabic, a natural glycoprotein / polysaccharide mixture. This is postulated to be due to the presence of charged and neutral carbohydrates in the ASPI.

Usually, differences in C_{cr} are explained by differences in protein properties like Q_H [17, 18]. Similar to the variation in C_{cr}, a wide range is reported in Q_H or k_{adsorb} (relative to WPI or β-lactoglobulin) for proteins from various sources. Egg proteins are reported to have a far lower Q_H or k_{adsorb} compared to WPI or β-lactoglobulin (0.19 and 0.06 for ovalbumin and lysozyme, respectively). The Q_H or k_{adsorb} reported for leaf, leguminous and potato proteins were higher than this, but similar to each other (1.1, 0.9 and 0.7 for sugar beet leaf protein, patatin and soy protein isolate, respectively). The largest variety in k_{adsorb} is shown in algae proteins, as presented in this study, with values ranging from 1.01–2.41. Especially, the k_{adsorb} of ASPI-A is strikingly higher (2.41) than that of the other algae proteins (1.01–1.09) and protein from other sources (0.06–1.1). In contrast to expectations based on the model on prediction of emulsion behavior (developed by [17] and confirmed for leaf proteins [18], these increased adsorption kinetics of ASPI-A compared to ASPI-N and -T were not reflected in their emulsifying ability (C_{cr}/Φ_{oil}).

CONCLUSIONS

Variations in C_{cr}/Φ_{oil}, indicative over the emulsifying behavior, of the algae proteins were within the range of proteins from both similar (Rubisco rich; i.e. other algae, sugar beet leaf) and other protein sources (dairy, legume and egg). However, for the algae proteins, the expected correlation between interfacial and molecular properties and the emulsion behavior was absent. For instance, stability of algae protein stabilized emulsions against pH-induced flocculation was not reflected by differences in the emulsions' ζ-potential curves.

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4 Effect of cell wall characteristics on algae nutrient digestibility in Nile tilapia and African catfish.

This study aimed to assess the effect of cell wall hardness and fish species on digestibility of unicellular sources. The gross composition, and the composition and cell wall hardness were determined for four sources. These were 3 microalgae species (*Chlorella vulgaris*, *Scenedesmus dimorphus* and *Nannochloropsis gaditana*) and a cyanobacterium (*Arthrospira maxima*). Apparent digestibility coefficients (ADCs) of their nutrients were determined in Nile tilapia and African catfish, at a 30% diet inclusion level. It was hypothesized that herbivores can access and thus digest unicellular proteins better than omnivores, and that the differences in protein digestion between the fish species increase with the robustness of the cell walls. Differences in cell wall hardness were quantified as the cells' resistance to mechanical shear. *A. maxima* was least resistant to shear: the time needed to disrupt 50% of the cells was 2 min compared to 24–33 min for the other sources. Differences were also measured in nutrient digestibility between the sources in both fish species. Contrary to the basal diet, which was digested differently between the fish species, there was no fish species effect on nutrient ADCs of the unicellular sources. *A. maxima* had the highest protein ADCs in both fish species (81.4–82.5%), followed by *C. vulgaris* (80.7–80.9%), *N. gaditana* (72.4–74.7%) and *S. dimorphus* (67.0- 68.3%). Ingredient fat ADCs ranged between 65.1–89.1%. Unicellular non-starch polysaccharides (NSP), comprising the unicellular cell wall fraction, was not inert in both fish species (ADC >46.0%), which was attributed to fermentation. The digestibility data suggest that the differences in nutrient accessibility of unicellular sources are dominant over the differences in digestive systems between herbivorous and omnivorous fish. Nevertheless, nutrient digestibility of the unicellular sources did not relate to the mechanical cell wall hardness.

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INTRODUCTION

Single cell protein sources like microalgae and cyanobacteria have recently gained interest as protein sources for fish feed. In digestion trials with fish, microalgae and cyanobacteria are mostly fed in intact form, e.g. [1-3]. These unicellular sources vary in gross composition, and the composition and hardness of their cell walls. Both these ingredient properties and the digestive characteristics of the fish species are thought to affect the nutrient apparent digestibility coefficient (ADC) of unicellular sources.

Microalgae and cyanobacteria (from here on referred to as unicellular sources) are already applied industrially in aquaculture hatcheries (for mollusks, crustaceans and zooplankton) [4], but are not yet commercially applied as source of macro nutrients in formulated fish feeds. Still, the inclusion of intact unicellular sources in fish diets has been subject of several scientific studies (e.g. [5-9]). These studies mainly reported the effects on health and growth parameters, with dissimilar results of inclusion of unicellular sources in fish feed on these parameters. Health and growth parameters are next to nutrient digestibility also influenced by other factors. Consequently, these parameters are not a direct indication of the actual digestibility of the nutrients in these unicellular sources. To estimate the efficiency of unicellular sources as protein alternatives in fish feed, there is a need for data on their nutrient digestibility. Compared to growth studies, relatively few studies reported the digestibility of diets containing unicellular sources. Of these studies, even fewer reported the nutrient digestibility of the unicellular sources, as opposed to the digestibility of nutrients in the total diet. The available data are limited to 5 unicellular species and 4 fish species. Protein apparent digestibility coefficients (ADCs) of *Arthrospira* sp. were reported to be 75-86% in Caspian great sturgeon [10], Nile tilapia [11], Arctic charr [12] and Atlantic salmon [12]. ADCs of protein from *Chlorella* sp. and *Schizochytrium* sp. were reported to be 80 and 82%, respectively, in Nile tilapia [11]. ADCs of protein from *Nannochloropsis* sp. and *Desmodesmus* sp. were reported to be 72 and 67%, respectively, in Atlantic salmon [9]. In two of those studies, digestibility of unicellular protein was compared to digestibility of various plant protein concentrates and meals [10, 12] and was found to be in a similar range. Unicellular protein ADC was reported to be lower than the ADC of fish meal protein [10].

Sarker et al. [11] and Gong et al. [9] have recently shown that nutrient digestibility is different between unicellular sources (within a single fish species). The differences in protein digestibility between the various unicellular sources can, amongst others, be due to differences in the intrinsic properties of the proteins, differences in protein accessibility caused by different cell wall matrices, or differences in fish species. In the studies mentioned previously, intact algae were used, which means that protein and other nutrients in the cells are not directly in contact with the digestive enzymes. It is hypothesized that the cell walls of unicellular sources thus hinder nutrient accessibility, leading to a decreased nutrient digestibility. There is a large diversity in the cell wall structures of microalgae and cyanobacteria: from peptidoglycan cell walls to cellulosic cell walls [13, 14]. It is commonly assumed, but without clear experimental evidence, that

peptidoglycan cell walls are softer than cellulose based cell walls. Additionally, most fish lack the enzymes needed for cellulose degradation [15]. Thus, algae containing cellulosic cell walls are expected to be digested to a lesser extent than e.g. cyanobacteria with a peptidoglycan cell wall. Apart from differences between unicellular sources, also the differences between (monogastric) fish species should be taken into account. Both the presence of specific carbohydrases and the activity of the carbohydrases present in fish is fish species dependent. As was shown in a review by Stone [16], based on carbohydrase activity assays, fish from a lower trophic level are in general more efficient in degrading carbohydrates than fish from a higher trophic level.

The aim of this study was to assess the effect of cell wall hardness and the effect of fish species on nutrient digestion of various unicellular sources. It was hypothesized that herbivores are able to access and thus digest unicellular proteins better than omnivores, and that the differences in protein digestion between the fish species increases with the robustness of the cell wall of the unicellular sources. To test these hypotheses, 3 different types of microalgae (2 freshwater species and 1 marine species) and 1 type of freshwater cyanobacterium were fed to Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarus gariepinus*), at a 30% inclusion level in the diet. Additionally, mechanical cell wall hardness of the unicellular sources was measured as the rate at which the cells were disrupted using a bead mill.

MATERIALS & METHODS

Digestibility experiment

This experiment was conducted in accordance with the current Dutch law on experimental animals. The experiment consisted of two trials: one with Nile tilapia and one with African catfish. Both trials were identical regarding the experimental set-up, but the housing conditions were specific for the fish species. Each trial had a duration of 5 weeks (33 days). The sampling, measurements and procedures were identical for both trials.

Fish and housing conditions

Tilapia:

Male Nile tilapia (*Oreochromis niloticus*, Til-Aqua Silver NMT strain) were obtained from a commercial breeder (Til-Aqua International, Someren, The Netherlands) 2 months prior to the start of the experiment and were reared at the Wageningen University experimental facilities (Carus Aquatic Research Facility, Wageningen, The Netherlands). At the start of the experiment, a group of 525 unfed juvenile tilapia (mean body weight 37.4 g ± 0.5) was batch-weighed, and randomly allocated to 15 tanks (35 fish / tank). The tanks were connected to a recirculating water system and equipped with air stones, pumps and swirl separators (settling units). Throughout the experiment the following housing and water quality parameters aimed at optimal conditions for Nile tilapia were monitored: photoperiod (12 h light : 12 h dark), water temperature (28 °C, SD = 0.2), water volume (60 L / tank), tank inlet flow (7.0 L / min, SD = 0.2), pH (7.2, SD = 0.5), ammonium (< 1.0 mg / L),

nitrite (< 0.3 mg / L), nitrate (< 500 mg / L), salinity (2510–7250 µS / cm), and dissolved oxygen concentration (6.7 mg / L, SD = 0.4). Water temperature, pH, conductivity and dissolved oxygen concentration were measured daily. Ammonium, nitrite and nitrate concentrations were measured 3 times in the first week of the trial, and subsequently on a weekly basis.

Catfish:
Mixed sex African catfish (*Clarus gariepinus*) were obtained from a commercial breeder (Fleuren-Nooijen BV, Someren, The Netherlands) and reared at the Wageningen University experimental facilities (Carus Aquatic Research Facility, Wageningen, The Netherlands). Weighing and allocating the juvenile catfish (mean weight 66.7 g, SD = 2.9) was performed in the same way as described for the tilapia. Housing conditions were similar to the tilapia trial with the following exceptions: no air stones were used, the water temperature was maintained at 25.7 °C (SD = 0.4) and the salinity levels were maintained at 2810–4940 µS / cm throughout the trial. Other housing and water quality parameters were similar to the tilapia trial: photoperiod (12 h light : 12 h dark), water volume (60 L / tank), inlet flow (7.0 L / min, SD = 0.2), pH (7.2 °C, SD = 0.2), ammonium (< 1.0 mg / L), nitrite (< 1.0 mg / L), nitrate (< 400 mg / L) and dissolved oxygen concentration (7.0 mg / L, SD = 0.5).

Diets
In order to measure the digestibility of the 4 unicellular sources, 5 diets were formulated which were used in both trials: one reference diet (REF) and 4 test diets (Table 1 and Table 2). The test diets consisted of 70% reference diet and 30% dry (non-bead milled) biomass of either *Nannochloropsis gaditana* (NAN), *Arthrospira (spirulina) maxima* (ART), *Chlorella vulgaris* (CHL) or *Scenedesmus dimorphus* (SCE) (Table 3). The compositions of the diets were formulated to meet the nutrient requirement for both Nile tilapia and African catfish [17], to ensure the fish were not exposed to nutrient deficiencies. A wide spectrum of protein sources was used in the reference diet to rule out the influence of specific ingredients on algal nutrient digestibility.

Yttrium oxide (Y_2O_3) was added to all diets as an inert marker to calculate apparent digestibility coefficients of the unicellular sources. Commercially available roller dried biomass of marine NAN and freshwater ART, CHL and SCE was kindly provided by AlgaSpring B.V. (Almere, The Netherlands). All diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands). The dietary ingredients were mixed and hammer milled into meals and subsequently extruded (through a 3 mm die) into sinking pellets. Fat was included during the extrusion process, not as a coating. Prior to feeding, the diets were sieved to remove fine particles. The biomass, meals and diets were stored in the dark at -4 °C until use.

Table 1: Analyzed chemical composition of experimental diets fed to juvenile Nile tilapia and African catfish. Values are presented as mean ± SD, on dry matter, unless stated otherwise.

	Diets[a]				
	REF	**NAN**	**ART**	**CHL**	**SCE**
Dry matter (g/kg wet weight)	956.4 ± 0.3	913.1 ± 0.3	936.8 ± 0.2	938.6 ± 0.3	955.1 ± 0.3
Gross energy (kJ/g)	20.6 ± 0.1	21.9 ± 0.04	21.3 ± 0.04	21.6 ± 0.1	20.7 ± 0.1
Crude protein (N * 6.25) (g/kg)	376.0 ± 0.1	421.4 ± 2.5	481.0 ± 0.6	454.9 ± 1.0	388.9 ± 2.8
Crude fat (g/kg)	95.1 ± 1.1	115.1 ± 1.0	85.4 ± 1.1	94.3 ± 1.9	96.8 ± 0.8
Total carbohydrates[b] (g/kg)	400.3 ± 2.3	326.6 ± 2.0	324.3 ± 2.0	335.4 ± 3.4	357.4 ± 4.5
Rhamnose (g/kg)	2.3 ± 0.1	3.2 ± 0.2	3.7 ± 0.2	4.5 ± 0.3	2.0 ± 0.2
Fucose (g/kg)	0.7 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
Arabinose (g/kg)	27.5 ± 0.9	20.2 ± 1.0	20.6 ± 0.3	20.9 ± 0.7	20.8 ± 0.9
Xylose (g/kg)	28.2 ± 1.1	21.6 ± 0.8	22.4 ± 2.1	21.6 ± 0.9	23.5 ± 1.6
Mannose (g/kg)	4.9 ± 0.4	9.9 ± 0.3	3.6 ± 0.2	4.6 ± 0.4	13.1 ± 0.2
Galactose (g/kg)	19.1 ± 0.2	21.2 ± 0.2	17.7 ± 0.6	24.1 ± 0.3	17.9 ± 0.4
Glucose (g/kg)	299.1 ± 4.4	231.8 ± 0.9	235.9 ± 3.7	239.5 ± 3.6	262.5 ± 2.0
Ribose (g/kg)	1.8 ± 0.1	3.3 ± 0.01	4.0 ± 0.03	4.3 ± 0.03	2.4 ± 0.1
Uronic acids (g/kg)	16.8 ± 0.6	14.3 ± 0.9	15.5 ± 0.2	15.2 ± 0.6	14.4 ± 0.2
Starch (g/kg)	245.1 ± 4.3	161.7 ± 4.9	176.7 ± 3.9	162.5 ± 4.2	190.4 ± 3.3
NSP[c] (g/kg)	155.2 ± 4.8	164.9 ± 5.3	147.6 ± 4.4	172.9 ± 5.4	167.0 ± 5.6
Organic matter[d] (g/kg)	934.4 ± 0.1	927.9 ± 0.2	934.8 ± 0.5	940.0 ± 0.2	904.1 ± 0.6
Ash (g/kg)	65.6 ± 0.1	72.1 ± 0.2	65.2 ± 0.5	60.0 ± 0.2	95.9 ± 0.6
Yttrium (g/kg)	0.2 ± <0.01	0.2 ± <0.01	0.2 ± <0.01	0.2 ± <0.01	0.2 ± <0.01
Phosphorus (g/kg)	9.8 ± 0.02	9.2 ± 0.1	10.2 ± 0.02	10.4 ± 0.04	10.4 ± 0.01
Calcium (g/kg)	11.2 ± 0.05	9.3 ± 0.03	8.0 ± 0.01	8.1 ± 0.1	21.2 ± 0.1
Copper (mg/ kg)	20.0 ± 0.3	11.9 ± 0.4	16.9 ± 0.4	15.0 ± 1.0	14.4 ± 0.2
Iron (mg/ kg)	248.6 ± 3.8	583.0 ± 5.2	325.8 ± 4.9	483.5 ± 4.6	1089.1 ± 1.5
Magnesium (mg/ kg)	3.1 ± <0.01	3.2 ± <0.01	3.1 ± <0.01	3.0 ± 0.02	3.4 ± 0.04
Manganese (mg/ kg)	62.7 ± 0.2	89.7 ± 0.4	52.9 ± 0.1	58.0 ± 0.2	162.7 ± 0.4
Zinc (g/kg)	127.8 ± 5.6	97.0 ± 1.1	100.0 ± 1.9	104.7 ± 13.5	103.5 ± 4.3

[a] REF - reference diet; NAN, ART, CHL and SCE - 70% reference diet, 30% *Nannochloropsis gaditana*, *Arthrospira maxima*, *Chlorella vulgaris* and *Scenedesmus dimorphus*, respectively.

[b] Total carbohydrates comprise starch and NSP.

[c] Non-starch polysaccharides = total carbohydrates – starch.

[d] Organic matter = 1000 – ash.

Table 2: Feed formulation of experimental diets fed to juvenile Nile tilapia and African catfish.

	Diets				
	REF	**NAN**	**ART**	**CHL**	**SCE**
Basal ingredients (w/w%)					
Maize	13.4	9.4	9.4	9.4	9.4
Wheat	20.0	14.0	14.0	14.0	14.0
Wheat bran	8.0	5.6	5.6	5.6	5.6
Wheat gluten	12.5	8.7	8.7	8.7	8.7
Rape seed meal	12.5	8.7	8.7	8.7	8.7
Fish meal	12.5	8.7	8.7	8.7	8.7
Soybean meal	12.5	8.7	8.7	8.7	8.7
Fish oil	2.5	1.7	1.7	1.7	1.7
Soy oil	2.5	1.7	1.7	1.7	1.7
Calcium carbonate	0.8	0.6	0.6	0.6	0.6
Mono-calcium phosphate	1.2	0.8	0.8	0.8	0.8
L-Lysine HCl	0.2	0.1	0.1	0.1	0.1
DL-methionine	0.3	0.2	0.2	0.2	0.2
L-threonine	0.1	0.1	0.1	0.1	0.1
Vitamin-mineral premix[a]	1.0	0.7	0.7	0.7	0.7
Yttrium oxide	0.02	0.02	0.02	0.02	0.02
Test ingredients (w/w%)					
Nannochloropsis gaditana	-	30.0	-	-	-
Arthrospira maxima	-	-	30.0	-	-
Chlorella vulgaris	-	-	-	30.0	-
Scenedesmus dimorphus	-	-	-	-	30.0

[a] Mineral premix composition (mg / kg reference diet): 50 iron (as $FeSO_4 \cdot 7H_2O$); 30 zinc (as $ZnSO_4 \cdot 7H_2O$); 0.1 cobalt (as $CoSO_4 \cdot 7H_2O$); 10 copper (as $CuSO_4 \cdot 5H_2O$); 0.5 selenium (as Na_2SeO_3); 20 manganese (as $MnSO_4 \cdot 4H_2O$); 500 magnesium (as $MgSO_4 \cdot 7H_2O$); 1 chromium (as $CrCl_3 \cdot 6H_2O$); 2 iodine (as $CaIO_3 \cdot 6H_2O$). Vitamin premix composition (mg/kg reference diet): 10 thiamine; 10 riboflavin; 20 nicotinic acid; 40 pantothenic acid, 10 pyridoxine; 0.2 biotine; 2 folic acid; 0.015 cyanocobalamin; 100 ascorbic acid (as ascorbic acid 2-phosphate); 100 IU alpha-tocopheryl acetate; 3000 IU retinyl palmitate, 2400 IU cholecalciferol; 10 menadione sodium bisulphite (51%); 400 inositol; 1500 choline (as choline chloride); 100 butylated hydroxytoluene; 1000 calcium propionate.

Table 3: Analyzed chemical composition of unicellular sources included in diets that were fed to juvenile Nile tilapia and African catfish. Values are presented as mean ± SD, on dry matter, unless stated otherwise.

	Unicellular source[a]							
	NAN		ART		CHL		SCE	
Dry matter (g/kg wet weight)	970.1	± 0.3	904.1	± 0.5	940.6	± 0.3	949.3	± 0.3
Gross energy (kJ/g)	24.4	± 0.1	22.4	± 0.10	23.7	± 0.05	20.1	± 0.01
Crude protein (N * 6.25) (g/kg)	525.4	± 2.0	719.9	± 1.2	634.8	± 1.8	407.3	± 0.1
Crude fat (g/kg)	155.0	± 1.0	56.2	± 2.6	102.9	± 1.3	80.5	± 0.8
Total carbohydrates[b] (g/kg)	139.8	± 2.2	138.1	± 2.4	164.1	± 2.0	203.8	± 3.5
Rhamnose (g/kg)	6.0	± 0.3	7.4	± 0.04	10.8	± 0.2	1.5	± 0.3
Fucose (g/kg)	1.1	± 0.2	1.1	± 0.05	0.2	± 0.3	1.1	± 0.3
Arabinose (g/kg)	1.7	± 0.2	1.4	± 0.2	1.5	± 0.3	1.4	± 0.2
Xylose (g/kg)	2.2	± 0.1	3.5	± 0.2	4.4	± 0.1	2.6	± 0.2
Mannose (g/kg)	20.4	± 0.5	2.6	± 0.6	5.2	± 0.2	33.2	± 0.2
Galactose (g/kg)	26.5	± 0.6	16.2	± 0.2	38.3	± 0.4	16.0	± 0.8
Glucose (g/kg)	69.4	± 1.0	83.7	± 1.9	81.8	± 1.1	136.7	± 1.3
Ribose (g/kg)	7.1	± 0.2	11.2	± 0.04	12.0	± 0.5	4.1	± 0.4
Uronic acids (g/kg)	5.5	± 0.2	11.0	± 0.1	10.0	± 0.1	7.1	± 0.2
Starch (g/kg)	1.5	± 0.1	32.8	± 0.9	30.6	± 3.3	80.7	± 3.4
NSP[c] (g/kg)	138.4	± 2.2	105.3	± 2.6	133.5	± 3.8	123.1	± 4.8
Organic matter[d] (g/kg)	915.8	± 0.2	936.6	± 0.2	948.0	± 0.2	818.1	± 0.6
Ash (g/kg)	84.2	± 0.2	63.4	± 0.2	52.0	± 0.2	181.9	± 0.6
Phosphorus (g/kg)	7.8	± 0.1	10.5	± 0.02	11.2	± 0.2	10.7	± 0.1
Calcium (g/kg)	3.7	± 0.01	0.8	± <0.01	1.5	± 0.3	48.1	± 0.3
Copper (mg/kg DM)	3.3	± 1.8	6.6	± 1.2	2.9	± 0.9	5.7	± 1.1
Iron (g/kg)	1.2	± 0.01	0.5	± 0.01	1.0	± 0.04	2.9	± 0.02
Magnesium (g/kg)	3.8	± 0.01	2.8	± <0.01	2.6	± 0.1	4.2	± 0.01
Manganese (mg/kg DM)	14.0	± 0.05	2.9	± 0.02	4.5	± 0.1	39.1	± 0.1
Zinc (mg/kg DM)	4.0	± 0.1	9.2	± 5.4	4.2	± 2.6	5.7	± 0.7

[a] NAN - Nannochloropsis gaditana, ART - Arthrospira maxima, CHL - Chlorella vulgaris and SCE - Scenedesmus dimorphus.

[b] Total carbohydrates comprise starch and NSP.

[c] Non-starch polysaccharides = total carbohydrates – starch.

[d] Organic matter = 1000 – ash.

Experimental procedure

For both trials, the five experimental diets were randomly assigned to 15 tanks, resulting in three replicates per diet. The fish were hand-fed twice a day for 1 h at 9:00 and 15:30 hrs. The amount of feed given was registered and was restricted at a 90% satiation level of 17.1 g dry matter (DM) / $kg^{0.8}$ / d. From the start until 15 min after each feeding, sediment was collected using swirl separators. Uneaten feed pellets were collected in detachable bottles (250 mL) and counted in the sediment to determine true feed intake. The absolute amount (DM) of feed given per tank was identical within and between diets, and corrected when mortality occurred during the trials. In the tilapia trial, the feeding level was gradually increased from 0 to 100% (i.e. 90% satiation level) within the first two weeks of the trial. In the catfish trial, the feeding level was gradually increased from 0 to 100% in the first 3 days of the trial. From week 3 through week 5, feces were collected from each tank separately overnight (starting 30 min after the afternoon feeding until the morning feeding), 5 days a week. In the catfish trial, feces were additionally collected between the morning feeding (starting 30 min after the feeding) and the afternoon feeding in order to obtain sufficient material. Feces were collected using swirl separators attached to the outlet of each tank. The feces were collected in detachable bottles (250 mL) which were kept on ice during collection to minimize bacterial decomposition of the feces. A separate set of bottles was used for collection of uneaten feed pellets and the collection of feces. After collection, the feces and water collected in the bottles were separated by careful decantation, and the feces (the sediment) were transferred to aluminum trays and stored at -20 °C. The feces were pooled per tank, per week. Feces collected in week 5 in the tilapia trial were used for further analyses. In the catfish trial, feces from week 4 and 5 were pooled for further analyses to obtain sufficient fecal matter for analysis. Throughout the trials, fish behavior, morphology and mortality were monitored. On the last day of the trial, the fish were not fed and were batch-weighed again to determine their growth performance.

Chemical analyses on diets and feces

All chemicals used were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma Aldrich (St. Louis, MO, USA), unless stated otherwise. All water used was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). The feces were freeze-dried and finely ground prior to the analyses. Feed pellets were finely ground prior to the analyses.

Dry matter content was measured in triplicate by drying overnight at 70 °C followed by 3 h at 103 °C (ISO 6496). Subsequently, *ash content* was determined by incineration for 1 h at 550 °C (ISO 5984). In the ash sample, *yttrium oxide and minerals* (Ca, P, Mg, Mn, Cu, Fe and Zn) were determined by ICP-MS. The yttrium, phosphorus and calcium contents were determined in duplicate using inductively coupled plasma atomic emission spectroscopy (ICP-AES) by the Chemical Biological Soil Laboratory (Wageningen, The Netherlands).

Protein content was determined in triplicate by the Kjeldahl method (N * 6.25), using acetanilide as standard (ISO 5983).

Total starch content was determined in duplicate using the total starch assay (AOAC Method 996.11) from Megazyme (Megazyme International, Ltd, Wicklow, Ireland). D-glucose was used for calibration and standardized regular maize starch as a control.

Neutral carbohydrate composition was determined in duplicate based on the alditol acetates procedure by Englyst and Cummings, using a pre-hydrolysis with 72% H_2SO_4 followed by 1 M H_2SO_4 hydrolysis [18]. Inositol was used as an internal standard. The alditol acetates were analyzed by gas chromatography (Focus-GC, Thermo Scientific, Waltham, MA, USA), using arabinose, galactose, glucose, fucose, mannose, rhamnose, ribose and xylose as standards.

Total uronic acid content was determined in triplicate according to an automated colorimetric m-hydroxydiphenyl assay based on Ahmed et al. [19], using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Samples were pre-hydrolyzed with 72% H_2SO_4 followed by 1 M H_2SO_4 hydrolysis. Galacturonic acid (0–100 µg / mL) was used for calibration.

Gross energy was determined in triplicate with a bomb calorimeter (IKA-C-7000; IKA-Werke, Straufen, Germany) using benzoic acid as standard (ISO 9831).

Crude fat content was determined in triplicate using the Berntrop method with acid pre-hydrolysis (IS0 6492).

Apparent digestibility coefficients (ADCs) of the dietary components in the diets were calculated by equation 1.

$$\%\ ADC_{diet} = 100\% * \left(1 - \left[\frac{Y_{diet}}{Y_{faeces\ feces}}\right] * \left[\frac{N_{faeces}}{N_{diet}}\right]\right) \quad (1)$$

in which Y_{diet} and Y_{feces} are the contents of inert marker (yttrium) in diet and feces, respectively (g / kg DM) and N_{feces} and N_{diet} are the contents of the dietary component in feces and diet, respectively (g / kg DM). ADCs of the dietary components in the test ingredients were calculated by equation 2.

$$\%\ ADC_{ingredient} = ADC_{test\ diet} + \left(ADC_{test\ diet} - ADC_{ref\ diet}\right) * \left(\frac{0.7 * N_{ref\ diet}}{0.3 * N_{test\ ingredient}}\right) \quad (2)$$

in which $ADC_{test\ diet}$ and $ADC_{ref\ diet}$ are the ADCs of the dietary component in the test diet and the reference diet, respectively and $N_{reference\ diet}$ and $N_{test\ ingredient}$ are the contents of the dietary component in the reference diet and test ingredient, respectively (g / kg DM).

Cell wall hardness

Cell wall hardness of the selected microalgae and cyanobacterium was derived from the rate at which the cells were disrupted using a bead mill, as measured by protein release and decrease in cell count over time.

Cell disruption was performed in duplicate by bead milling. Dried algal and cyanobacterial biomass of NAN, ART, CHL and SCE were dispersed in Milli-Q water (9% DM) each. The cells were disrupted using a DYNO®-Mill type Research Lab (Willy A. Bachofen AG

Maschinenfabrik, Muttenz, Switzerland). Using an agitator speed of 2039 rpm, the samples were recirculated for 60 min under constant stirring at 120 rpm using an overhead stirrer. The 80 mL grinding chamber was filled with 52 mL (65% v/v) yttria-stabilized zirconia SiLiBeads grinding beads, type ZY Premium, of 0.3 mm (Sigmund Lindner GmbH, Warmensteinach, Germany). Cooling water recirculated through the cooling jacket of the grinding chamber and through a cooling coil in the sample. The sample temperatures never exceeded 25 °C. Aliquots of 1 mL were taken during bead milling. For the cell disintegration analysis, aliquots of these samples were diluted 1600x in Milli-Q water. For the protein release analysis, the rest of the samples were centrifuged (15,000 g, 10 min, 20 °C). The supernatants were stored at 4 °C and analyzed within 24 h.

Cell disruption was quantified using a flow cytometer (BD Accuri C6, BD Biosciences, San Jose, CA, USA), based on the method by Postma et al. [20]. A fixed volume of 15 µL was measured at a flow rate of 35 µL/min and a core size of 16 µm. Forward scattering FSC, static scattering SSC and total cell count were measured. All measurements had a minimum of 10.000 events. From the size distribution at t = 0, cell sizes were determined to be 4–5 µm (NAN), 4–6 µm (CHL and SCE) and 6–15 µm (ART). The total number of counts of particles in these size ranges was plotted as function of the bead milling time, as a percentage of the initial total count in these size ranges. To derive the time (min) needed for 50% cell breakdown, a normal distribution fit was made using the fitting function of Microsoft Excel (version 14.0.7166.5000, Microsoft Corporation, Albuquerque, NM, United States). This value (min) was used to estimate cell hardness.

Protein release was analyzed in triplicate as the soluble protein content of the supernatants, using a BCA protein assay (Thermo Scientific, Waltham, MA, USA) with BSA as a standard. Protein release at t = 60 min was set as 100%. The initial slope of protein release over bead milling time (% release / min) was used to estimate cell hardness.

Effect of processing on cell wall integrity

To test the effect of processing on cell integrity of the unicellular sources, protein solubility was measured of the ingredients (dried algal and cyanobacterial biomass), meals (30% dried biomass mixed with 70% basal diet ingredients) and diets (extruded meals). The diets were ground by mortar and pestle prior to analysis. Ingredients, meals and diets were dispersed (2.5% w/v) in a potassium phosphate buffer (pH 8.0, 50 mM) and mixed for 1 h on a head-over-tail mixer at room temperature. Subsequently, the dispersions were centrifuged for 10 min at 15,000 g, 20 °C. Nitrogen contents of the supernatants and of the ingredients, meals and diets were analyzed with the Dumas method using a Flash EA 1112 N analyzer (Thermo Fisher Scientific, Waltham, MA, USA), D-methionine for calibration and a nitrogen-to-protein conversion factor of 6.25. Protein solubility was expressed as the percentage of soluble protein out of the total protein present in each sample.

Statistical procedures

All statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA). Animal performance data, nutrient ADCs were tested for treatment effect, fish species effect and

their interactions using analysis of variance (ANOVA; PROC GLM). In the case of a significant interaction ($P < 0.05$), a pairwise comparison of the means was done using Tukey's test. Protein solubility data of the ingredients, meals and diets was tested for treatment effect using analysis of variance (ANOVA; PROC GLM). Significant differences ($p < 0.05$) between means were detected using Tukey's test.

RESULTS AND DISCUSSION

Fish growth, feed utilization and survival

The design of the tilapia and catfish trials was aimed at studying the impact of dietary inclusion of various unicellular sources on nutrient digestion. Nevertheless, growth and performance data are shown (Table 4) to enable judgement of the quality of the experiment. During both fish trials, fish survival was over 95% for all dietary treatments during the experimental period. The overall performance data of both tilapia and catfish were similar or superior to values reported in literature, for both the fish fed the reference diet as the test diets. Specifically, algae diet FCRs in tilapia are reported in the range of 1.01–1.91 (with 21–44% inclusion of algae), compared to FCRs of 1.03–1.91 of the reference diets in those studies [5, 21-23]. For catfish, an FCR of an algae diet (40% inclusion) was reported to be 1.3, compared to a reference diet FCR of 1.2 [24].

Apparent digestibility coefficient of nutrients, DM and energy

Dietary treatments affected the diet ADCs of DM, energy, protein, fat, total carbohydrates and NSP ($P < 0.02$) (Table 5). ADCs of the total carbohydrate constituent monosaccharides and of all minerals analyzed are provided in Table 6. ADCs of DM, energy, protein, fat and starch were also affected by fish species ($P < 0.001$). The difference between the fish species was present in lower ADCs (4–9% decrease) of diets fed to catfish compared to tilapia. This effect of fish species was expected based on reported differences between omnivorous and herbivorous fish [16]. Moreover, interactions between fish species and diet were present on the ADCs of protein ($P = 0.01$), energy ($P = 0.04$) and fat ($P = 0.03$). Strikingly, all these fish effects and the majority of the interactions detected at the diet level disappeared at ingredient level (Table 7). Thus the fish species effect was predominantly related to a different response to the basal diet and not to the unicellular sources. Burr et al. reported similar findings in a study in which *Arthrospira* sp. and various traditional plant ingredients were fed to Atlantic salmon and Arctic charr [12]. They showed that the protein ADCs of traditional plant ingredients were 11-21% lower in charr than in salmon, whereas this decrease was 3% for *Arthrospira* sp. In the present study, the majority of proteins in the basal diet were of a vegetal source (Table 2), and fish species dependency has been reported previously for plant protein digestibility [12, 25]. These literature results seem to be similar to our finding that the basal diet (containing traditional terrestrial plant based ingredients) showed a large difference in ADC between the two fish species, while the ADC for the unicellular sources did not. One could speculate that the differences in digestibility are related to intrinsic differences between ingredients from terrestrial sources (present in the

basal diets) and ingredients from aquatic sources (microalgae and cyanobacteria). The possible mechanisms behind this are however not clear.

On ingredient level, the ingredient ADC values for DM, energy, protein, fat, total carbohydrates and non-starch polysaccharides (NSP) were different between the unicellular sources ($P < 0.05$) for both Nile tilapia and African catfish (Table 7). No fish species effect was present on any of those ADCs ($P > 0.1$). An interaction effect between fish and unicellular sources was present however for the ADC of fat ($P = 0.002$). In both the tilapia and the catfish trial, the nutrients of ART and CHL were digested to the highest extent and the nutrients of NAN and SCE were digested to the lowest extent. In the tilapia trial, ADCs of protein ranged between 67.0% (SCE) and 82.5% (ART) and ADCs of fat ranged between 65.1 (SCE) and 84.3% (CHL). Total carbohydrate and NSP ADCs were of a broader range, between 21.6% (NAN) and 70.4% (CHL) for total carbohydrates, and 46.0% (NAN) and 97.8% (CHL) for NSP. Similar to the tilapia trial, ADCs of protein in the catfish trial ranged between 68.3% (SCE) and 81.4% (ART) and ADCs of fat ranged between 65.1% (NAN) and 89.1% (ART). ADCs of total carbohydrates ranged between 46.9% (NAN) and 84.6% (CHL) and NSP ADCs ranged between 66.9% (SCE) and 115% (CHL). ADCs of the total carbohydrate constituent monosaccharides and of all minerals analyzed are provided in Table 8.

Protein ADCs of CHL, ART and NAN were comparable to reported *Chlorella* sp., *Arthrospira* sp. and *Nannochloropsis* sp. ADCs in literature in Nile tilapia and other fish species. In Nile tilapia trials, a similar protein ADC was reported for *Arthrospira* sp. (80%) and a lower protein ADC for *Chlorella* sp. (73%) [11] compared to the present findings. Similar protein ADCs for *Arthrospira* sp. were reported in other fish species as well, with protein ADCs of 82% in Arctic charr [12], 85% in Atlantic salmon [12] and 79% in Caspian great sturgeon [10]. Also for *Nannochloropsis* sp. a similar protein ADC was reported (72%) in a trial using Atlantic salmon [9]. Literature values reported for energy ADCs of *Arthrospira* sp. and *Chlorella* sp. were higher than what was found in the current study. Energy ADCs of *Arthrospira* sp. were reported to be 80–86% in Arctic charr, Nile tilapia and Caspian great sturgeon [10-12] and ADCs of *Chlorella* sp. were reported to be 84% in Nile tilapia [11]. For *Nannochloropsis* sp. an energy ADC was reported in Atlantic salmon that was similar to the present findings for NAN (61%) [9]. Some previously reported lipid ADCs were in the same range as the present findings (79% for *Arthrospira* sp. in Caspian great sturgeon), but others were much higher (94-95% for *Arthrospira* sp. and *Chlorella* sp. in Nile tilapia) [10, 11]. For SCE, no literature data on ADC in fish were available.

Table 4: Growth performance, feed intake and survival rate of Nile tilapia (T) and African catfish (C) after 5 weeks of feeding diets with 30% inclusion of various unicellular sources.

	Species	Diets[a]						*P*-values		
		REF	NAN	ART	CHL	SCE	SEM	Diet	Fish	Diet*Fish
Initial body weight (g / fish)	T	37.7	36.8	37.6	37.3	37.5	1.20	0.618	<.0001	0.308
	C	66.1	69.5	66.7	65.4	65.7				
Final body weight (g / fish)	T	83.6	82.4	89.7	87.2	77.3	1.77	<0.001	<.0001	0.001
	C	162.7	163.3	178.5	173.4	149.3				
Feed intake (g DM/(fish · d))	T	1.32	1.32	1.32	1.34	1.32	0.001	0.007	<0.001	0.496
	C	2.54	2.52	2.54	2.52	2.52				
Growth (g / d)	T	1.39	1.38	1.58	1.51	1.21	0.036	<0.001	<0.001	<0.001
	C	2.92	2.84	3.39	3.27	2.53				
SGR (% / d)	T	2.41	2.44	2.64	2.57	2.19	0.036	<0.001	<0.001	0.044
	C	2.73	2.59	2.98	2.95	2.49				
Feed conversion ratio (g DM intake / g body gain)	T	0.95	0.96	0.84	0.89	1.10	0.01	<0.001	<0.001	0.234
	C	0.87	0.89	0.75	0.77	1.00				
Survival (%)	T	98	100	99	98	97	1.2	0.133	0.010	0.716
	C	95	99	95	95	96				

[a] REF - reference diet; NAN, ART, CHL and SCE - 70% reference diet, 30% *Nannochloropsis gaditana*, *Arthrospira maxima*, *Chlorella vulgaris* and *Scenedesmus dimorphus*, respectively.

Table 5: Apparent digestibility coefficient (%; ADC1) of diets with 30% inclusion of various unicellular sources in Nile tilapia (T) and African catfish (C).

ADC (%)	Species	Diet[2]						*P*-values		
		REF	NAN	ART	CHL	SCE	SEM	Diet	Fish	Diet*Fish
Dry matter	T	75.8^{A}	73.1^{B}	75.5^{A}	75.2^{A}	69.8^{A}	0.63	<0.001	<0.001	0.189
	C	68.0^{A}	65.9^{B}	69.4^{A}	68.8^{A}	65.0^{A}				
Gross energy	T	80.7^{a}	75.5^{b}	79.2^{a}	78.5^{a}	74.2^{bc}	0.54	<0.001	<0.001	0.037
	C	71.9^{c}	67.7^{d}	73.0^{bc}	71.8^{c}	68.8^{d}				
Crude protein	T	89.8^{a}	84.2^{c}	86.6^{b}	86.1^{b}	82.6^{c}	0.36	<0.001	<0.001	0.013
	C	83.7^{c}	79.5^{d}	82.7^{c}	82.4^{c}	78.8^{d}				
Crude fat	T	94.5^{a}	86.3^{c}	92.1^{a}	91.3^{ab}	86.7^{cd}	0.65	<0.001	<0.001	0.026
	C	88.2^{bcd}	78.7^{f}	88.4^{bc}	85.0^{de}	82.9^{e}				
Total carbohydrates[3]	T	62.4^{AB}	57.1^{B}	63.1^{AB}	63.6^{A}	61.4^{AB}	1.55	0.016	0.374	0.757
	C	62.0^{AB}	60.0^{B}	62.5^{AB}	65.4^{A}	62.1^{AB}				
Starch	T	98.1	98.1	97.6	98.7	97.7	0.27	0.157	<0.001	0.274
	C	99.1	99.2	99.5	99.4	99.1				
NSP[4]	T	5.7^{C}	16.8^{B}	21.9^{B}	30.5^{A}	19.5^{B}	3.24	<0.001	0.780	0.704
	C	3.7^{C}	22.0^{B}	18.6^{B}	33.4^{A}	19.7^{B}				
Organic matter[5]	T	78.5^{A}	74.7^{B}	77.8^{A}	77.2^{A}	73.0^{B}	0.60	<0.001	<0.001	0.175
	C	70.1^{A}	67.1^{B}	71.5^{A}	70.6^{A}	67.4^{B}				
Ash	T	36.3^{B}	52.7^{A}	41.9^{B}	43.7^{B}	40.1^{B}	2.84	<0.001	0.818	0.814
	C	37.5^{B}	50.6^{A}	40.4^{B}	39.5^{B}	43.1^{B}				
Phosphorus	T	57.9^{C}	63.1^{B}	68.7^{A}	67.1^{A}	51.9^{D}	0.78	<0.001	0.008	0.597
	C	59.5^{C}	64.6^{B}	69.6^{A}	67.5^{A}	54.8^{D}				
Calcium	T	16.7	36.1	6.1	28.2	31.1	7.92	0.200	<0.001	0.528
	C	49.3	54.4	46.9	50.9	48.2				

[1] Values presented are means. In the case of a significant interaction effect, means within the 2 rows per nutrient having a different lower case are significantly different ($P < 0.05$). In the case of a significant diet effect without an interaction effect, means within each row per nutrient having a different upper case are significantly different ($P < 0.05$).

[2] REF - reference diet; NAN, ART, CHL and SCE - 70% reference diet, 30% *Nannochloropsis gaditana*, *Arthrospira maxima*, *Chlorella vulgaris* and *Scenedesmus dimorphus*, respectively.

[3] Total carbohydrates comprise starch and NSP.

[4] Non-starch polysaccharides = total carbohydrates – starch.

[5] Organic matter = 1000 – ash.

Table 6: Apparent digestibility coefficient (%; ADC) of total carbohydrate constituent monosaccharides and of minerals of unicellular sources (at 30% inclusion levels) in Nile tilapia (T) and African catfish (C).

ADC (%)	Species	Unicellular source[2]					*P*-values		
		NAN	ART	CHL	SCE	SEM	Diet	Fish	Diet*Fish
Total carbohydrates	T	21.6^{C}	68.2^{AB}	70.4^{A}	56.9^{BC}	10.76	0.007	0.176	0.627
	C	46.9^{C}	66.3^{AB}	84.6^{A}	62.3^{BC}				
Rhamnose	T	-54.6^{BC}	49.8^{A}	-14.4^{AB}	-56.6^{C}	23.65	<0.001	0.203	0.110
	C	8.8^{BC}	56.2^{A}	48.2^{AB}	-100.3^{C}				
Fucose	T	73.9	42.4	292.1	34.5	37.19	0.271	0.553	0.466
	C	42.8	60.5	n.d.	17.8				
Arabinose	T	-177.7	-45.7	370.7	67.4	265.23	0.259	0.249	0.472
	C	-4.5	-215.4	154.1	-616.1				
Xylose	T	855.2	167.1	275.7	564.6	199.13	0.054	0.399	0.839
	C	639.9	198.3	251.3	284.8				
Mannose	T	74.9^{AB}	-4.6^{A}	-22.1^{A}	-8.1^{B}	28.83	0.004	0.955	0.278
	C	81.2^{AB}	-49.3^{A}	-45.5^{A}	58.4^{B}				
Galactose	T	9.2^{AB}	47.7^{A}	36.2^{A}	13.3^{B}	8.11	0.004	0.003	0.104
	C	53.7^{AB}	52.4^{A}	59.4^{A}	22.8^{B}				
Glucose	T	-8.4^{B}	79.5^{A}	84.6^{A}	70.0^{A}	6.24	<0.001	0.002	0.083
	C	27.6^{B}	81.0^{A}	101.2^{A}	82.2^{A}				
Ribose	T	76.4	100.0	96.4	91.8	7.37	0.107	0.750	0.731
	C	83.9	94.8	95.7	83.5				
Uronic acids	T	36.9	26.6	42.7	13.5	38.95	0.316	0.528	0.652
	C	4.1	34.6	64.9	-55.0				
Ash	T	82.6	55.3	65.3	43.3	9.61	0.271	0.553	0.466
	C	74.3	47.4	45.2	47.7				
Phosphorus	T	78.8^{B}	92.5^{A}	85.9^{A}	38.9^{C}	2.77	<0.001	0.594	0.553
	C	79.7^{B}	91.9^{A}	84.0^{AB}	44.7^{C}				
Calcium	T	172.2	-323.8	236.3	38.9	258.05	0.573	0.928	0.828
	C	90.5	-27.1	79.4	47.6				
Copper	T	-466.7^{B}	-79.3^{A}	-141.6^{A}	-182.8^{A}	37.34	<0.001	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.				
Iron	T	13.4^{A}	-61.1^{B}	-6.5^{AB}	11.5^{A}	13.92	0.017	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.				
Magnesium	T	84.3^{A}	87.0^{A}	85.4^{A}	57.7^{B}	1.42	<0.001	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.				
Manganese	T	4.1^{A}	-139.5^{B}	-44.4^{A}	7.2^{A}	15.31	<0.001	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.				
Zinc	T	20.2	-26.4	57.1	-5.2	18.65	0.061	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.				

[1] Values presented are means. In the case of a significant interaction effect, means within the 2 rows per nutrient having a different lower case are significantly different ($P < 0.05$). In the case of a significant diet effect without an interaction effect, means within each row per nutrient having a different upper case are significantly different ($P < 0.05$).
[2] NAN - *Nannochloropsis gaditana*, ART - *Arthrospira maxima*, CHL - *Chlorella vulgaris* and SCE - *Scenedesmus dimorphus*.
n.d.: not determined.
n/a : not applicable.

Table 7: Apparent digestibility coefficient (%; ADC[1]) of nutrients of unicellular sources (at 30% inclusion levels) in Nile tilapia (T) and African catfish (C).

ADC (%)	Species	Unicellular source[2]					*P*-values		
		NAN	ART	CHL	SCE	SEM	Diet	Fish	Diet*Fish
Dry matter	T	66.9^{B}	74.7^{A}	73.7^{A}	55.8^{C}	2.18	<0.001	0.217	0.331
	C	61.1^{B}	73.1^{A}	70.7^{A}	58.2^{C}				
Gross energy	T	65.1^{B}	75.8^{A}	73.9^{A}	58.5^{B}	1.66	<0.001	0.274	0.118
	C	59.5^{B}	75.3^{A}	71.6^{A}	61.4^{B}				
Crude protein	T	74.7^{C}	82.5^{A}	80.9^{B}	67.0^{C}	0.76	<0.001	0.328	0.161
	C	72.4^{C}	81.4^{A}	80.7^{B}	68.3^{C}				
Crude fat	T	74.5^{cd}	82.4^{abc}	84.3^{ab}	65.1^{d}	1.96	<0.001	0.321	0.002
	C	65.1^{d}	89.1^{a}	78.1^{bc}	68.3^{d}				
Total carbohydrates[3]	T	21.6^{C}	68.2^{AB}	70.4^{A}	56.9^{BC}	10.76	0.007	0.176	0.627
	C	46.9^{C}	66.3^{AB}	84.6^{A}	62.3^{BC}				
Starch	T	–*	85.3	108.2	96.9	4.99	0.102	0.203	0.108
	C	–*	103.5	103.7	99.7				
NSP[4]	T	46.0^{B}	77.5^{AB}	97.8^{A}	60.2^{B}	14.30	0.017	0.331	0.713
	C	70.2^{B}	70.1^{AB}	114.8^{A}	66.9^{B}				
Organic matter[5]	T	65.5^{B}	76.1^{A}	74.0^{A}	58.0^{B}	9.61	0.018	0.260	0.661
	C	59.8^{B}	74.7^{A}	71.9^{A}	60.1^{B}				
Ash	T	82.6^{ABC}	55.3^{C}	65.3^{B}	43.3^{AB}	2.17	0.018	0.260	0.661
	C	74.3^{ABC}	47.4^{C}	45.2^{B}	47.7^{AB}				
Phosphorus	T	78.8^{B}	92.5^{A}	85.9^{A}	38.9^{C}	2.77	<0.001	0.594	0.553
	C	79.7^{B}	91.9^{A}	84.0^{AB}	44.7^{C}				
Calcium	T	–**	–**	–**	38.9	–**	–**	–**	–**
	C	–**	–**	–**	47.6				

[1] Values presented are means. In the case of a significant interaction effect, means within the 2 rows per nutrient having a different lower case are significantly different ($P < 0.05$). In the case of a significant diet effect without an interaction effect, means within each row per nutrient having a different upper case are significantly different ($P < 0.05$).
[2] NAN - Nannochloropsis gaditana, ART - *Arthrospira maxima*, CHL - *Chlorella vulgaris* and SCE - *Scenedesmus dimorphus*.
[3] Total carbohydrates comprise starch and NSP.
[4] Non-starch polysaccharides = total carbohydrates – starch.
[5] Organic matter = 1000 – ash.
* NAN starch ADCs are omitted since NAN contains ≤0.1% (w/w% DM) starch. ADCs calculated were 32.8% and 92.8% for tilapia and catfish, respectively. When including the NAN data, no diet, fish or diet*fish effects were present ($P > 0.1$).
** NAN, ART and CHL calcium ADCs are omitted since these ingredients contain <0.4% (w/w% DM) calcium. ADCs calculated were 172.4%, -323.8%, and 236.3% for tilapia and 90.5%, -27.1% and 79.4% for catfish of NAN, ART and CHL, respectively. When including these data, the SEM was 9.76 and no diet, fish or diet*fish effects were present ($P > 0.1$).

Table 8: Apparent digestibility coefficient (%; ADC[1]) of total carbohydrate constituent monosaccharides and of minerals in diets with 30% inclusion of various unicellular sources in Nile tilapia (T) and African catfish (C).

ADC (%)	Species	Diet[2]					SEM	*P*-values		
		REF	NAN	ART	CHL	SCE		Diet	Fish	Diet*Fish
Total carbohydrates	T	75.8^{A}	73.1^{B}	75.5^{A}	75.2^{A}	69.8^{A}	0.63	<0.001	<0.001	0.189
	C	68.0^{A}	65.9^{B}	69.4^{A}	68.8^{A}	65.0^{A}				
Rhamnose	T	10.5^{bc}	-23.8^{d}	33.3^{ab}	-6.1^{cd}	-4.5^{cd}	4.79	<0.001	<0.001	0.027
	C	38.5^{ab}	22.9^{abc}	48.8^{a}	45.0^{a}	7.5^{bcd}				
Fucose	T	0.8^{B}	31.6^{A}	17.6^{A}	28.9^{AB}	15.0^{A}	5.00	0.005	<0.001	0.219
	C	-25.0^{B}	3.5^{A}	9.6^{A}	-14.1^{AB}	-7.0^{AB}				
Arabinose	T	-2.2	-6.8	-3.2	6.3	-0.7	4.06	0.340	0.817	0.550
	C	2.5	2.3	-2.2	5.9	-10.9				
Xylose	T	2.1	29.1	10.4	19.1	23.4	5.59	0.059	<0.001	0.926
	C	-45.1	-23.4	-32.8	-26.7	-32.7				
Mannose	T	-2.1^{B}	47.1^{A}	-2.6^{B}	-8.3^{B}	-6.6^{B}	5.87	<0.001	<0.001	0.108
	C	38.2^{B}	65.6^{A}	22.0^{B}	12.3^{B}	53.2^{B}				
Galactose	T	20.5^{ABC}	16.3^{BC}	27.7^{AB}	27.8^{A}	18.6^{C}	1.65	0.002	<0.001	0.107
	C	53.3^{ABC}	53.5^{BC}	53.0^{AB}	56.1^{A}	45.2^{C}				
Glucose	T	80.5^{A}	72.4^{B}	80.4^{A}	80.9^{A}	78.8^{A}	0.51	<0.001	<0.001	0.185
	C	82.0^{A}	77.1^{B}	81.9^{A}	84.0^{A}	82.0^{A}				
Ribose	T	100.0	85.1	100.0	97.4	95.9	3.25	0.070	<0.001	0.127
	C	71.3	79.2	88.4	89.5	77.4				
Uronic acids	T	18.9	21.1	20.6	23.7	18.1	4.17	0.259	<0.001	0.707
	C	4.9	4.8	11.4	17.1	-4.2				
Ash	T	36.3^{B}	52.7^{A}	41.9^{B}	43.7^{B}	40.1^{B}	2.84	<0.001	0.818	0.814
	C	37.5^{B}	50.6^{A}	40.4^{B}	39.5^{B}	43.1^{B}				
Phosphorus	T	57.9^{C}	63.1^{B}	68.7^{A}	67.1^{A}	51.9^{D}	0.78	<0.001	0.008	0.597
	C	59.5^{C}	64.6^{B}	69.6^{A}	67.5^{A}	54.8^{D}				
Calcium	T	16.7	36.1	6.1	28.2	31.1	7.92	0.200	<0.001	0.528
	C	49.3	54.4	46.9	50.9	48.2				
Copper	T	23.2^{a}	-9.2^{d}	10.6^{bc}	13.7^{ab}	0.8^{cd}	2.58	<0.001	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.	n.d.				
Iron	T	-28.5^{bc}	-0.4^{ac}	-43.0^{b}	-14.5^{abc}	4.9^{a}	6.40	0.002	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.	n.d.				
Magnesium	T	60.1^{b}	68.4^{a}	67.6^{a}	66.9^{a}	59.2^{b}	0.45	<0.001	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.	n.d.				
Manganese	T	-40.2^{c}	-18.5^{b}	-56.5^{d}	-41.2^{c}	-5.7^{a}	2.61	<0.001	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.	n.d.				
Zinc	T	8.3^{ac}	9.7^{ac}	0.2^{bc}	14.4^{a}	6.2^{ac}	2.32	0.017	n/a	n/a
	C	n.d.	n.d.	n.d.	n.d.	n.d.				

[1] Values presented are means. In the case of a significant interaction effect, means within the 2 rows per nutrient having a different lower case are significantly different ($P < 0.05$). In the case of a significant diet effect without an interaction effect, means within each row per nutrient having a different upper case are significantly different ($P < 0.05$).

[2] REF - reference diet; NAN, ART, CHL and SCE - 70% reference diet, 30% *Nannochloropsis gaditana*, *Arthrospira maxima*, *Chlorella vulgaris* and *Scenedesmus dimorphus*, respectively.

n.d.: not determined.

n/a: not applicable.

Effect of processing on cell wall integrity

In order to study the relation between cell wall hardness and nutrient digestibility, the unicellular sources used needed to be intact when fed to the fish. To estimate whether ingredient processing (e.g. harvesting and drying) and feed manufacturing (e.g. mixing and extruding) affected cell wall integrity, protein solubility of the (unicellular) ingredients, meals (30% dried biomass mixed with 70% basal diet ingredients) and diets (extruded meals) was measured. Protein solubility ranged between 8.1–14.9% for ingredients, 10.3–12.0% for meals and 6.6–8.0% for diets. There were no statistical differences in protein solubility between each meal and the derived diet ($P > 0.05$). Protein solubility of the ingredients were different ($P < 0.01$), with ART having a higher protein solubility (14.9%) than the other ingredients (8.1–8.6%). This could mean that the ART cells were more damaged during either the biomass harvesting or drying process compared to the other unicellular sources. The level of protein solubility in ART is still quite low, showing that the damage was only minor. Research has shown that upon completely breaking the cells of the similar cyanobacterium *Arthrospira platensis*, up to 85% of protein could be extracted [26]. Based on this, it is expected that no more than approximately 16% of the cells were damaged. With the ingredient and reference meal data, the expected protein solubility was calculated for each test meal. If the measured protein solubility exceeds the expected solubility, it would indicate damage of the unicellular cells due to feed processing. The calculated protein solubility values of the meals were slightly lower (2–11% decrease) than the measured values. This suggests that if the unicellular sources were damaged due to the mixing of diet ingredients prior to extrusion, it was only to a low extent. Upon extrusion, protein solubility of all the meals was decreased (8–36% decrease between meals and feeds). The differences in protein solubility between the diets are similar to those between the meals however, suggesting that the extrusion process did not affect cell wall integrity.

Cell wall hardness

Upon bead milling of the unicellular sources, a plateau for the released protein was reached within 50 minutes for all samples (Figure 1 A). Initial protein release, used as a measure for cell hardness, was similar for SCE, CHL and NAN (8-11% protein / min) but was considerably faster for ART (19% protein / min). Flow cytometry confirmed that ART cells disrupted faster than the other cells with 2 min bead milling needed to break 50% of all cells, compared to 24–33 min for the other samples (Figure 1 B). It must be noted that although flow cytometry was a suitable method to quantify cell disintegration, it was hindered by the presence of clusters of cell which caused the delayed onset in CHL, NAN and SCE. This cluster formation might be due to the drying step in the production of the biomass.

These data show that the unicellular sources were disintegrated at a lower rate than the rate at which protein was released. After 10 min bead milling for example, a fraction of >50% of the soluble protein was released from all samples tested, although only 11-39% of SCE, CHL and NAN cells were disintegrated at that time. This suggests that cells are first opened up by bead milling, allowing the release of soluble proteins, and are subsequently disintegrated further. There are no data on cell hardness in literature to

compare these results to, but information is available on the chemical composition of the cell walls of the unicellular sources. Like many cyanobacteria, ART is known to have a peptidoglycan cell wall [14]. Both NAN and SCE have an inner cellulose cell wall with an additional outer hydrophobic algaenan layer [13, 27, 28]. CHL also has a cellulose based cell wall, but lacks the additional algaenan layer (unlike other *Chlorella* species) [29]. It commonly assumed that peptidoglycan cell walls are softer than cellulose based cell walls. The presence of an algaenan layer in algal cell walls is known to increase the resistance of the cell wall to chemical degradation [30]. Therefore, it is expected that the algaenan-containing cell walls of NAN and SCE are harder than the CHL cell walls. Following this logic, the data on chemical composition match with the results on protein release and cell breakdown. More specifically, ART cell walls are known to be chemically softer than those of the microalgae, which correlated to a faster cell disruption upon bead milling. Furthermore, based on the chemical compositions, CHL cells are thought to be less tough than those of NAN and SCE, although no clear differences were observed between CHL, NAN and SCE in the bead milling experiment.

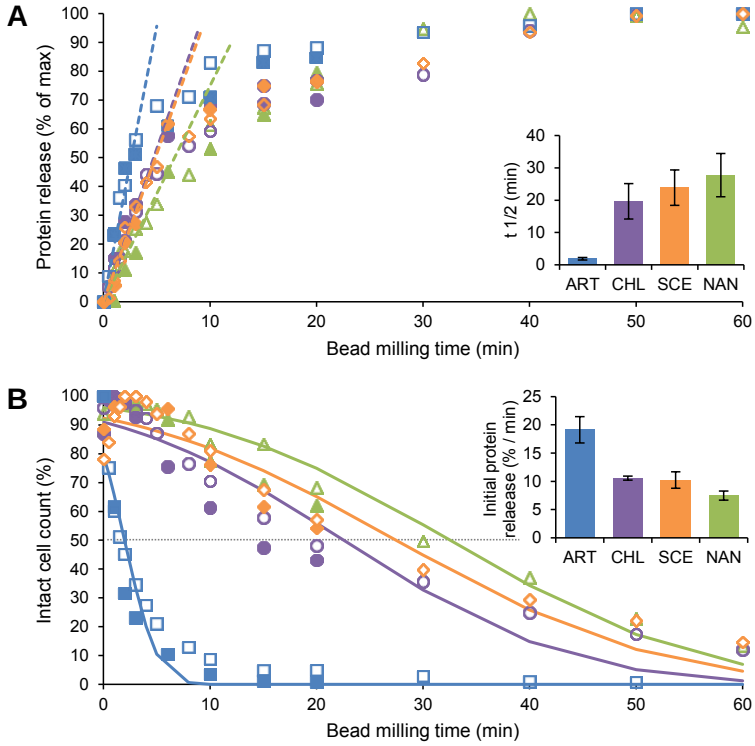

Figure 1: Protein release (**A**) (as % of total protein in the algae) and cell breakdown (**B**) (as % of total number of intact cells) as function of bead milling time of *N. gaditana* (NAN, ▲), *A. maxima*, (ART, ■), *C. vulgaris* (CHL, ●) and *S. dimorphus* (SCE, ◆). Open and closed symbols of the same type are duplicates of each other. In **A**, dotted lines represent initial protein release; the insert shows the slope of initial protein release (mean ± SD). In **B**, solid lines represent a normal distribution fit of cell breakdown; the dotted line represents an intact cell count of 50%. The corresponding bead milling time needed to reach this level for each sample is shown in the insert (mean ± SD).

Cell wall digestion and nutrient accessibility

Both protein and fat are mostly inside the algal and cyanobacterial cells, enclosed by cell walls. Therefore, protein and fat ADCs can be used as an indication for differences in *in vivo* nutrient accessibilities between the different unicellular sources. The ADCs of protein and fat of ART and CHL were higher than those of SCE and NAN in both fish species. In the catfish trial, the differences between the ADCs of the unicellular sources were more pronounced in fat digestion than in protein digestion. In the tilapia trial however, protein and fat ADCs show the same relative differences between the unicellular sources. This suggests that the differences in digestibility between the unicellular sources are not related to nutrient characteristics, such as amino acid sequence, but more likely to an external factor. Therefore, the limiting factor for nutrient digestibility is thought to be the accessibility of these nutrients.

In all diets, starch was almost fully digested in both Nile tilapia and African catfish (ADCs >97%). NSP from the basal diet was digested to a low extent in both fish species (ADCs of 3.7–5.7%). Conversely, NSP from unicellular sources was not inert in both fish species (ADCs of 46–115%). Other research has already shown that NSPs from terrestrial plants can indeed be utilized by both Nile tilapia [31, 32] and African catfish [33], but most ADC values for NSP are in lower ranges (2–24% in Nile tilapia [32, 34], and 4–56% in African catfish [33]. More in line with the present findings, Haidar and co-workers reported higher NSP digestibility in catfish (ADCs between 23–73%) and suggested that these variations can be attributed to the type and state of the NSP present [31]. In Nile tilapia, VFAs production and thus fermentation predominantly takes place in the distal part of the intestine [32, 35]. In CHL, SCE and NAN, cell walls are primarily composed of NSP [13, 27-29]. It is thus assumed that the cell walls of these unicellular sources are fermented in both fish species, presumably in the distal part of the intestine. Protein and fat are assumed to be for a large part hydrolyzed and absorbed in the proximal and mid part of the intestine in Nile tilapia and African catfish [36, 37]. Unicellular protein and fat were digested (65–89% ADC) in both fish species. Apparently, these nutrients were accessible to digestive enzymes in the proximal part of the intestine, before any cell wall (NSP) fermentation could take place. These results imply that the cells were already opened or damaged in or before they reached the proximal and mid part of the intestine. It is not clear what affected the cell wall integrity, but two possible options are the feed production process and *in vivo* digestive processes. The protein solubility data of ingredients, meals and diets (Figure 2) show that from the 4 ingredients only ART was slightly damaged prior to feed processing. This damage, however, cannot account for the high protein and fat ADCs measured in both fish for ART (> 80% for all). The feed manufacturing itself did not appear to have further damaged the cells of any of the sources. Thus, cell wall integrity was possibly affected in fish intestinal tracts. Typically, this would be assigned to the low pH stomach conditions in Nile tilapia (which can reach pH 2 at 7 h after feeding) [38]. However, both this acidity and expected stomach acidity of African catfish (pH 3.5 at 8 h after feeding) [39] are unlikely to hydrolyze the cellulose structures of algal cell walls to a large extent. For example, studies have shown that by pre-treating wheat straw (38–43% cellulose [40, 41]) in conditions that are considerably more severe than fish

stomach conditions (e.g. 20 min at 160 °C in 2.5% w/w H_2SO_4), ~90% of the cellulose was still retained [42]. A possible explanation for the ability of the digestive enzymes to reach the nutrients inside the cells may be the combination of processing and stomach conditions. Specifically, feed processing and/or the stomach conditions could loosen the cellulose structure, making the nutrients inside the cell more accessible to hydrolysis. From the bead milling experiment it was observed that protein was released faster than cells were broken down. Thus, cells do not have to be opened completely for the nutrients inside to be accessible.

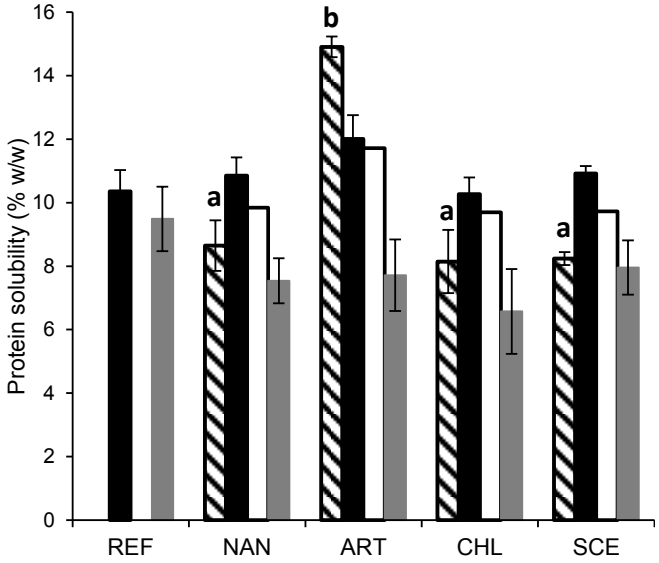

Figure 2: Protein solubility (in w/w% of total protein present) of ingredients (dried biomass; striped), meals (30% dried biomass mixed with 70% basal diet ingredients; black) and diets (extruded meals; grey). Reference meal and diet: REF, test ingredients, meals and diets: *Nannochloropsis gaditana* (NAN), *Arthrospira maxima* (ART), *Chlorella vulgaris* (CHL) and *Scenedesmus dimorphus* (SCE). Unfilled black bars present the expected meal protein solubility (30% ingredient, 70% meal). Error bars depict standard deviations between duplicates. Solubility values with different subscript letters are significantly different between the ingredients, meals or diets ($P < 0.05$).

Relation between cell wall hardness and nutrient digestibility

According to literature data on cell wall composition [13, 27-29] and the bead milling experiments performed in the present study, *Arthrospira* has softer cell walls than the other sources used. The literature data on the chemical cell wall composition suggest that CHL has harder cell walls than ART, and that SCE and NAN have the hardest cell walls. It is plausible that this cell wall hardness affects both the digestion kinetics in fish as well as the kinetics of mechanical rupture. These differences in cell walls between CHL and SCE/NAN however, could not be clearly detected in the bead milling experiment. Furthermore, these differences in cell wall hardness measured between the unicellular sources do not correlate

with the differences measured in protein and fat ADCs between the sources. This discrepancy between cell wall hardness and nutrient digestibility shows that cell wall hardness is not directly related to *in vivo* protein and fat digestion. The results do show, however, that algal/cyanobacterial nutrient accessibility is likely to be a limiting factor in nutrient digestibility in fish. Of course the experiments were limited to a relatively small number of different unicellular sources and fish species. However, the data suggest that limited nutrient accessibility of unicellular sources is indeed dominant over differences in digestive systems between herbivorous and omnivorous fish.

CONCLUSIONS

In this study, differences in mechanical cell wall hardness were demonstrated between various unicellular sources. Nutrient digestion in fish was also different between the unicellular sources. Furthermore, NSP from unicellular sources was not inert in both Nile tilapia and African catfish. Digestibility of the basal diets was different between the fish species. However, in contrast to the expectations, digestibility of nutrients from unicellular sources was not different between both fish species. The data suggest that differences in nutrient accessibility of unicellular sources are dominant over differences in digestive systems between herbivorous and omnivorous fish. These differences in nutrient accessibility were not related to the differences in mechanical cell wall hardness of algae and cyanobacteria.

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5 Increasing algae nutrient accessibility increases algae nutrient digestibility in Nile tilapia.

In this study the correlation between the accessibility of microalgae nutrients and their *in vivo* nutrient digestibility in fish was tested. It was hypothesized that increasing microalgae nutrient accessibility by disrupting their cell walls would increase microalgae nutrient digestibility in fish. To achieve the aim, *Nannochloropsis gaditana* biomass was subjected to five different treatments that influence its cell wall integrity. The treatments included physical treatments (pasteurization, freezing, freeze-drying) and mechanical treatments (bead-milling). These treatments resulted in increased *in vitro* accessibility of microalgae nutrients up to 4 times, as determined from nutrient leaching and susceptibility to protein hydrolysis. Apparent digestibility coefficients of the nutrients in untreated and treated microalgae biomass were determined in juvenile Nile tilapia (*Oreochromis niloticus*), at a 30% diet inclusion level. *In vivo* digestibility of protein and fat (both intracellular nutrients) was increased from 62 to 78% and from 50 to 82%, respectively. The *in vitro* accessibility data were positively correlated with the *in vivo* digestibility of protein ($p < 0.01$) and fat ($p < 0.01$). This shows that these methods are effective ways to assess the effect of mechanical and physical treatments on *in vivo* nutrient quality of a single ingredient. The results of this study confirm that nutrient accessibility plays a significant role in the nutrient digestibility of microalgae in fish.

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INTRODUCTION

In aquaculture, microalgae are currently predominantly used as live food for larvae. From the limited data available on the use of algae in compound feeds for the grow-out phase of fish, nutrient digestion from microalgae was found to vary greatly amongst various microalgal species [1, 2]. The assumption is that in some algae nutrient digestion can be limited by the presence of the algae cell walls, hindering *in vivo* accessibility of the intracellular nutrients. Consequently, it is expected that for those algae, the *in vivo* digestibility can be improved upon improving the nutrient accessibility, by disrupting the cell wall structure. The aim of this study is to correlate the accessibility of microalgae nutrients measured *in vitro* to the *in vivo* nutrient digestibility in fish.

Reported apparent digestibility coefficients (ADCs) of microalgal protein in fish range between 67–86% [1-5]. In these digestibility studies, the algae and cyanobacteria were incorporated into the fish feeds as intact cells. Two of these studies reported a lower protein digestibility in Nile tilapia for *Nannochloropsis gaditana* and *Scenedesmus dimorphus* than for *Arthrospira maxima* and *Chlorella vulgaris* [1, 2]. In both studies these differences in protein digestibility were suggested to be related to differences in cell wall structure and associated nutrient accessibility of the algae and cyanobacteria. In one of the studies, cell wall hardness of microalgae and cyanobacteria was studied [2], but this value was not correlated to the nutrient digestibility. For algae with low *in vivo* digestibility, disruption of the algae cell walls can be expected to increase nutrient accessibility and subsequent nutrient digestibly. Indeed, positive effects of increased accessibility of nutrients on *in vivo* parameters have been reported in multiple publications. For example, in rats the weight gain/total protein intake for *Scenedesmus acutus* was ~1.7 times higher after drum-drying the algae than after sun-drying or freeze-drying [6]. Ultrasonication and high pressure homogenization of *C. vulgaris* cells increased the protein digestibility in rats (an increase of up to ~ 20% of protein ADC) [7, 8]. For fish, one study was found that studied effect of altering algae nutrient accessibility on *in vivo* parameters [9].The flesh of trout fed homogenized astaxanthin containing algae showed higher levels of coloration than fish fed untreated algae [9].

The previously mentioned studies illustrated only a few methods for cell wall disruption. Such methods can in general be divided into four categories: enzymatic, chemical, physical and mechanical methods [10]. Examples of each are the use of cellulases (enzymatic), alkaline and organic solvents (chemical), bead milling and ultrasonication (mechanical) and thermal treatments and freeze-drying (physical) [10, 11]. To allow comparison of the effect of nutrient accessibility, the chemical composition and molecular structure of the nutrients should be the same between the treated and untreated algae. Enzymatic and chemical methods are believed to affect the integrity of inner-cell nutrients. For this reason, the use of physical and mechanical methods is preferred over the use of chemicals and enzymes. Mechanical methods like bead-milling can be used to completely disrupt cell walls [11, 12], while keeping the composition intact. Physical methods are milder, since they can be employed to damage cell wall structures without completely disrupting the walls. Freezing

and freeze-drying are for instance known to cause this type of cell wall damage due to ice crystal formation [13]. Thermal treatments have also been shown to damage algal cell walls, as detected by the release of intracellular organic matter (after heating for 30 min at 105–165 °C) [14], or increased extractability of lipids (after heating for 30 min at 121 °C) [15]. A disadvantage of thermal processing is the chance of Maillard reaction product formation. The Maillard reaction is a chemical reaction between amino acids and reducing sugars. The reaction is accelerated by heat and is known to reduce the nutritional quality of proteins and amino acids present [16].

To clearly discern the effect of the aforementioned disruption methods on nutrient digestibility it is important to quantify the extent of cell disruption. Cell disruption can be measured directly by microscopy or particle size analysis, or indirectly by measuring the release of intracellular products [11]. Disruption refers to treatments where the cell wall structure is compromised while the cells may still appear 'intact' in shape and size. Disruption can be quantified by the release of intracellular products [14]. Other, or longer treatments will result in complete breakdown of algae cells. The 'broken cells' can be analysed, or quantified using light microscopy and particle size analysis.

To test the effect of cell wall damage on microalgae nutrient digestibility, one type of microalgae (*Nannochloropsis gaditana*) was treated in 5 different ways, aiming to improve the protein accessibility and subsequent digestibility by damaging the algae cell walls. Nutrient accessibility of the treated and of untreated *N. gaditana* was measured *in vitro*, and nutrient digestibility was measured *in vivo* (in triplicate) in Nile tilapia (*Oreochromis niloticus*).

MATERIALS & METHODS

Materials

All chemicals used for the *in vitro* work and for analyses on ingredients, feeds and feces were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma Aldrich (St. Louis, MO, USA), unless stated otherwise. Commercially available biomass of marine *Nannochloropsis gaditana* (strain number AS1405) was kindly provided by AlgaSpring B.V. (Almere, The Netherlands). Pancreatin used originated from porcine pancreas (Sigma product number P3292).

Nannochloropsis gaditana treatments

N. gaditana biomass was treated by 5 different methods to create contrasts in cell wall integrity, aiming to create contrasts in the accessibility of intracellular nutrients. The biomass was provided in 2 batches that were harvested in the same season (harvested in June and July of the same year). After receiving their treatments (as described below), the algae products were dried to > 900 g DM / kg material by either drum drying or freeze drying. Subsequently, the dried algae products (Table 1) were used as test ingredients of extruded fish feeds.

Table 1: Analyzed chemical composition of treated or untreated *Nannochloropsis gaditana* biomass included in diets (30% inclusion level) that were fed to juvenile Nile tilapia. Values are presented are means, in g/kg DM, unless stated otherwise.

	***Nannochloropsis gaditana*[1]**						
	UNT	**PAS**	**FRD**	**FRO**	**L40**	**BEM**	**%CV[4]**
Dry matter (g DM /kg wet weight)	964.2	972.1	931.0	949.7	976.0	919.1	0.05
Gross energy (kJ/g)	24.5	24.4	24.2	24.6	23.6	24.7	0.35
Crude protein (N * 6.25)	482.2	494.4	496.4	464.2	475.5	451.0	0.43
Crude fat	160.9	162.1	129.7	173.3	156.6	146.3	2.15
Total carbohydrates[2]	160.4	168.0	135.6	158.3	165.3	123.9	1.45
Rhamnose	7.77	7.36	7.96	7.75	8.2	8.50	3.99
Fucose	1.65	1.25	1.60	1.55	1.8	2.00	13.26
Arabinose	1.46	1.65	1.42	1.79	1.8	1.21	9.94
Xylose	1.84	1.62	1.87	1.80	1.8	1.80	7.34
Mannose	26.0	28.7	16.2	35.5	28.6	37.2	1.97
Galactose	22.1	23.6	21.5	18.0	23.7	19.4	3.69
Glucose	83.6	85.1	68.2	76.1	82.9	36.6	1.13
Ribose	5.7	5.7	6.4	6.0	5.6	4.7	3.11
Uronic acids	10.4	10.4	10.5	9.8	10.9	12.5	8.40
Starch	0.1	0.3	0.2	0.5	0.6	0.6	1.50
NSP[3]	160.3	167.7	135.3	157.9	164.8	123.3	19.93
Ash	72.2	70.3	81.6	77.7	95.8	90.9	0.20
Phosphorus	12.8	13.1	8.3	11.0	11.8	11.3	0.47
Calcium	4.65	4.69	4.60	3.28	5.40	5.95	0.54
Copper	<0.01	<0.01	<0.01	0.01	0.00	<0.01	35.24
Iron	1.14	1.14	1.00	0.75	0.99	0.60	0.64
Magnesium	3.39	3.27	4.02	3.65	4.35	4.29	0.44
Manganese	0.21	0.21	0.24	0.18	0.20	0.22	0.40
Zinc	0.03	0.03	0.03	0.04	0.03	0.03	15.32

[1]: UNT, PAS, FRD, FRO, L40 and BEM: untreated, pasteurized, freeze-dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass of *Nannochloropsis gaditana*, respectively. With exception of FRD, all *N. gaditana* biomass was drum dried.

[2]: Total carbohydrates comprise starch and NSP.

[3]: NSP = total carbohydrates – starch.

[4]: Coefficient of variation.

After harvesting, the *N. gaditana* biomass was washed and centrifuged to a paste of 20% [w/w DM]. The washed paste received no further treatment (UNT), or received 1 out of 5 physical or mechanical treatments (PAS, FRO, FRD, L40 and BEM) that affect algal cell integrity by rupturing or weakening the cell walls. UNT, PAS, FRO, FRD and L40 were produced from 1 batch, BEM from a second batch. The PAS sample was pasteurized using a heat exchanger at 80 °C for 20 s. The FRO and FRD samples were frozen at -8 °C. The FRO samples were thawed at 4 °C (after ~2 wks frozen storage). Freezing and thawing was performed in small batches to ensure microbial safety. Serving as a positive control, the BEM sample was diluted to 14% [w/w DM] and subsequently bead milled to disrupt the algal cells. The bead milling was performed on a DYNO-Mill type ECM-AP05 LAB (Willy A. Bachofen Maschinenfabrik, Muttenz, Switzerland), using 0.5 mm yttria-stabilized zirconia grinding beads, type ZY Premium (Sigmund Lindner, Warmensteinach, Germany). The pump speed was set at 20 L / h and milling speed at 14 m / s. During milling, the milling chamber was cooled with running water to prevent protein deteriorating reactions and to ensure microbial safety. The algae suspension was passed through the mill 3 times to break the majority of the algal cells. Cell disruption was monitored by microscopy. L40 is a commercially available product ("NutriSpring® Liquid 40") provided by AlgaSpring. L40 is *N. gaditana* biomass (grown and harvested by AlgaSpring) that has received a physical treatment, similar to pasteurisation. No additives were used in the production of L40. With exception of the FRD sample, all the treated and untreated samples were drum dried, during which the algae products were dried within ~ 7 s on drums heated to 130 °C. The frozen FRD sample was freeze-dried. The UNT ingredient was considered as the negative control and the BEM sample as a positive control.

In vitro assessment of treatments on algal cell wall integrity

Microscope analysis

The algae products were analyzed by light microscopy using an Axioscope A01 (Carl Zeiss, Sliedrecht, The Netherlands) at a magnification of 40×, to identify whether the cells were broken by the various physical and mechanical treatments.

Nutrient accessibility

The effect of the various treatments on nutrient accessibility of *N. gaditana* was measured by the *in vitro* protein hydrolysis, amount of nitrogen and ion leaching into solution, fat extractability and buffering capacity.

In vitro protein hydrolyses were performed in duplicate, using a pH stat system. Each algal ingredient was dispersed in Milli-Q water (150 mg in 15 mL) and mixed for 50 min at room temperature, followed by 10 min mixing at 37 °C using a magnetic stirrer. Subsequently, the pH of the suspensions was adjusted to 8.0 with 0.2 M NaOH. Protein hydrolysis was performed with pancreatin, using 100 µL freshly prepared pancreatin solution of 3 mg / mL. During hydrolysis, the pH was kept constant using 0.2 M NaOH. Substrate blanks, samples incubated without addition of enzyme, were also measured in duplicate. The degree of hydrolysis (DH) was calculated using equation 1.

$$DH\ [\%] = V_b \times N_b \times \frac{1}{\alpha} \times \frac{1}{m_p} \times \frac{1}{h_{tot}} \times 100\% \quad (1)$$

in which V_b is the volume of NaOH added in mL; N_b is the normality of NaOH; α the average degree of dissociation of the α-NH group (1/α = 1.3 at 37 °C and pH 8.0) [17]; m_p the mass of protein weighed in in g; h_{tot} the total number of peptide bonds per gram of protein substrate (7.8 mmol / g for Rubisco) [18]. The DH calculated was corrected for the DH of the substrate blanks.

Nitrogen leaching was tested in duplicate by dispersing 50 mg of each algal product in 1 mL potassium phosphate buffer (pH 8.0, 50 mM). The dispersions were mixed in a head-over-tail rotator for 1 h, and subsequently centrifuged at 15,000 g for 10 min at 20 °C. The total nitrogen content of the supernatants and of the starting materials was determined with the Dumas method using a Flash EA 1112 N analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and D-methionine for calibration.

Ion leaching was tested in duplicate by dispersing 1 g of each algal product in 20 mL Milli-Q water. The dispersions were stirred for 120 min using a magnetic stirrer, before measuring their conductivity. To estimate the maximum conductivity, it was assumed that all ash in the ingredients could be solubilized and was NaCl. The calculated concentrations of NaCl were 3.36–4.50 mg / mL. NaCl solutions with these concentrations were used as conductivity calibrants.

Fat extractability was tested in duplicate using the Berntrop method with and without acid pre-hydrolysis (ISO 6492). The fat extracted without the acid hydrolysis step was expressed as a percentage of the fat extracted using the acid hydrolysis step.

Buffering capacity was measured in duplicate according to the method described by Butré *et al* [19]. The initial pH of the suspensions was recorded. Since the starting materials had a different initial pH, only the alkaline buffering capacity ($BC_{alkaline}$, mmol NaOH / g sample) was used, which was calculated using equation 2

$$BC_{alkaline} = \frac{C_{NaOH} \times V_{NaOH}}{m_{sample\ dry\ weight}} \times \frac{1}{pH_{final} - pH_{initial}} \quad (2)$$

in which C_{NaOH} and V_{NaOH} are the concentration and volume of NaOH used to bring the samples from the inital pH (pH_{intial}; pH 3) to the final pH (pH_{final}; pH 8). The $m_{sample\ dry\ weight}$ is the mass of the algae products, corrected for their DM content.

Digestibility trial

In the current experiment, animals were not exposed to invasive techniques or discomfort related to the experimental treatments. Fish were not anesthetized or euthanized as part of the experimental procedures. This experiment was evaluated by the Animal Welfare Body of Wageningen University. The Animal Welfare Body judged the procedures applied to the animals in this experiment to be below the threshold. So the current experiment, conducted in 2016, was evaluated as not being an animal experiment according to Dutch legislation (Act on Animal Experiments).

Fish and housing conditions

Male Nile tilapia (*Oreochromis niloticus*, Til-Aqua Silver NMT strain) were obtained from a commercial breeder (Til-Aqua International, Someren, The Netherlands) 2 months prior to the start of the trial and reared at the Wageningen University experimental facilities (Carus Aquatic Research Facility, Wageningen, The Netherlands). At the start of the trial, a group of 735 unfed juvenile tilapia (mean body weight 29.5 g, SD = 0.6) was batch-weighed in groups of 5 or 10 fish, and randomly allocated to 21 tanks (35 fish / tank). The tanks were connected to a recirculating water system and equipped with air stones, pumps and settling units. Throughout the trial the following housing and water quality parameters aimed at optimal conditions for Nile tilapia were monitored: photoperiod (12 h light : 12 h dark), water temperature (27.1 °C, SD = 0.5), water volume (60 L / tank), inlet flow (7.0 L / min), pH (6.8, SD = 0.5), ammonium (1.3 mg / L, SD = 2.2), nitrite (0.6 mg / L, SD = 0.6), nitrate (< 500 mg / L), conductivity (2000–9890 µS/ cm), and dissolved oxygen concentration (6.0 mg / L, SD = 1.3). Water temperature, pH, conductivity and dissolved oxygen concentration were measured daily. Ammonium, nitrite and nitrate concentrations were measured at least once a week.

Diets

The 7 formulated diets were: 1 reference diet (REF) and 6 test diets (Table 2 and Table 3). The test diets consisted of 70% reference diet and 30% dry treated or untreated *N. gaditana* biomass. All diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands). The dried algae products were mixed with the other dietary ingredients, hammer milled, and subsequently extruded (through a 2 mm die) into sinking pellets. Prior to feeding, the diets were sieved (2 mm) to remove fine particles. Oils were added prior to the extrusion process. The compositions of the diets were formulated to meet the nutrient requirement for Nile tilapia [20], to ensure the fish were not exposed to nutrient deficiencies. A wide spectrum of protein sources was used in the reference diet to rule out the influence of specific ingredients on the algae nutrient digestibility. Yttrium oxide (Y_2O_3) was added to all diets as an inert marker to calculate apparent digestibility coefficients of the treated and untreated *N. gaditana*. Feed pellets were finely ground prior to chemical analyses.

Table 2: Formulation of experimental diets fed to juvenile Nile tilapia.

	Diets	
	Reference diet	**Test diets**
Basal ingredients (w/w%)		
Maize	13.40	9.38
Wheat	20.00	14.00
Wheat bran	8.00	5.60
Wheat gluten	12.50	8.75
Rape seed meal	12.50	8.75
Fish meal	12.50	8.75
Soybean meal	12.50	8.75
Fish oil	2.50	1.75
Soy oil	2.50	1.75
Calcium carbonate	0.80	0.56
Mono-calcium phosphate	1.20	0.84
L-lysine HCl	0.20	0.14
DL-methionine	0.30	0.21
L-threonine	0.10	0.07
Vitamin-mineral premix[1]	1.00	0.70
Yttrium oxide	0.02	0.02
Test ingredients (w/w%)		
Nannochloropsis gaditana[2]	-	30.00

[1] Mineral premix composition (mg / kg reference diet): 50 iron (as $FeSO_4 \cdot 7H_2O$); 30 zinc (as $ZnSO_4 \cdot 7H_2O$); 0.1 cobalt (as $CoSO_4 \cdot 7H_2O$); 10 copper (as $CuSO_4 \cdot 5H_2O$); 0.5 selenium (as Na_2SeO_3); 20 manganese (as $MnSO_4 \cdot 4H_2O$); 500 magnesium (as $MgSO_4 \cdot 7H_2O$); 1 chromium (as $CrCl_3 \cdot 6H_2O$); 2 iodine (as $CaIO_3 \cdot 6H_2O$). Vitamin premix composition (mg/kg reference diet): 10 thiamine; 10 riboflavin; 20 nicotinic acid; 40 pantothenic acid, 10 pyridoxine; 0.2 biotine; 2 folic acid; 0.015 cyanocobalamin; 100 ascorbic acid (as ascorbic acid 2-phosphate); 100 IU alpha-tocopheryl acetate; 3000 IU retinyl palmitate, 2400 IU cholecalciferol; 10 menadione sodium bisulphite (51%); 400 inositol; 1500 choline (as choline chloride); 100 butylated hydroxytoluene; 1000 calcium propionate.

[2] Untreated, pasteurized, frozen-thawed, freeze-dried, commercially processed (NutriSpring® Liquid 40) or bead milled biomass of *Nannochloropsis gaditana*. With exception of the freeze-dried sample, all biomass was drum dried.

Table 3: Analyzed chemical composition of experimental diets fed to juvenile Nile tilapia. Values are means, in g/kg DM, unless stated otherwise.

	Diets[1]							
	REF	**UNT**	**PAS**	**FRD**	**FRO**	**L40**	**BEM**	**%CV[4]**
Dry matter (g DM /kg wet weight)	971.4	956.3	948.0	951.9	955.0	962.3	942.3	0.05
Gross energy (kJ/g)	20.6	22.0	21.6	21.6	22.0	21.5	21.7	0.41
Crude protein (N * 6.25)	360.4	392.5	396.2	395.5	385.9	388.0	382.7	0.83
Crude fat	93.0	108.8	111.3	103.7	113.8	111.6	122.7	0.70
Total carbohydrates[2]	397.1	313.7	314.5	311.2	315.6	326.8	315.8	1.54
Rhamnose	2.66	4.04	3.88	3.78	3.87	3.99	4.01	3.23
Fucose	0.79	0.77	0.77	0.92	0.80	0.85	0.96	8.08
Arabinose	21.6	14.8	15.5	17.4	16.4	17.2	16.2	5.01
Xylose	20.4	14.1	14.7	14.7	14.1	14.3	13.7	5.02
Mannose	3.89	10.4	10.3	7.41	12.5	10.2	11.3	3.78
Galactose	17.6	19.1	19.2	19.4	17.8	21.0	17.7	2.73
Glucose	313.4	236.0	237.4	232.5	234.8	243.5	226.6	1.62
Ribose	0.20	1.53	1.22	1.28	1.20	1.26	1.04	23.95
Uronic acids	16.8	13.8	13.8	14.3	14.4	14.4	14.4	2.54
Starch	240.6	181.4	176.8	192.5	181.4	149.7	178.8	4.16
NSP[3]	168.2	146.6	156.4	134.3	149.1	177.1	145.5	0.50
Ash	67.1	68.3	68.2	71.1	69.9	76.5	73.1	0.26
Yttrium	0.17	0.17	0.17	0.17	0.16	0.16	0.17	0.31
Phosphorus	9.75	10.8	10.9	9.36	10.2	10.4	10.3	0.34
Calcium	12.7	10.1	10.1	10.0	9.46	10.4	10.4	0.54
Copper	0.02	0.01	0.01	0.01	0.01	0.01	0.01	3.05
Iron	0.39	0.51	0.50	0.48	0.38	0.47	0.35	0.37
Magnesium	2.17	2.57	2.51	2.70	2.61	2.85	2.75	0.36
Manganese	0.05	0.11	0.11	0.11	0.09	0.11	0.11	0.52
Zinc	0.08	0.07	0.06	0.06	0.07	0.07	0.07	2.59

[1]: REF: reference diet. UNT, PAS, FRD, FRO, L40 and BEM: 70% reference diet, 30% *Nannochloropsis gaditana* biomass – untreated, pasteurized, freeze-dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass, respectively. With exception of FRD, all *N. gaditana* biomass was drum dried.

[2]: Total carbohydrates comprise starch and NSP.

[3]: NSP = total carbohydrates – starch.

[4]: Coefficient of variation.

Experimental procedure

The experiment lasted 6 weeks (42 days). The 7 experimental diets were randomly assigned to 21 tanks, resulting in 3 replicates per diet. The fish were hand-fed twice a day for 1 h at 9:00 and 15:30 hours. The amount of feed given was registered and was restricted at a 90% satiation level of 17.1 g DM / $kg^{0.8}$ / d. From the start until 15 min after each feeding moment, sediment was collected using settling units. Uneaten feed pellets were counted in the sediment to determine true feed intake. The amount (g DM) of feed given per tank was corrected for mortality during the trial, so that an absolute equal amount of feed (g DM) per fish were given for each of the treatments. The feeding level was gradually increased from 0 to 100% (i.e. 90% satiation level) within the first 10 days of the trial. From week 3 through week 6, feces were collected from each tank separately overnight (starting 30 min after the afternoon feeding until the morning feeding), 5 days a week. Feces were collected in 250 mL bottles kept on ice using settling units, transferred to aluminum trays and stored at -20 °C. The feces were pooled per tank, per week. Feces collected in week 6 were used for further analyses. On the last day of the trial, the fish were not fed and were batch-weighed again to determine their growth performance.

Chemical analyses on ingredients, feed and feces

Prior to analysis, the fecal samples were freeze-dried and subsequently air-dried for 4 h. Algae and fecal samples were homogenised with mortar and pestle. Homogenized feces were subsequently sieved to remove any fish scales present. Feed pellets were ground in a centrifugal mill (Retsch ZM 200, Germany) at 1200 RPM, to pass through a 1 mm screen.

Dry matter content was measured in triplicate by drying overnight at 70 °C followed by 3 h at 103 °C (ISO 6496). *Ash content* was determined on the dried samples by incineration for 1 h at 550 °C (ISO 5984). From the ash samples, *yttrium oxide and minerals* (Ca, P, Mg, Mn, Cu, Fe and Zn) were determined by ICP-MS.

Protein content was determined in triplicate by the Kjeldahl method (N * 6.25), using acetanilide as standard (ISO 5983).

Total starch was determined in duplicate using the total starch assay (AOAC Method 996.11) from Megazyme (Megazyme International, Ltd, Wicklow, Ireland). Total starch was determined by total starch assay method "c" and includes resistant starch, digestible starch and free glucose and maltodextrins. D-glucose was used for calibration and standardized regular maize starch as a control.

Neutral carbohydrate composition was determined in duplicate based on the alditol acetates procedure, as described previously [2]. The monosugar constituents of the total carbohydrates were expressed in anhydrous form.

Total uronic acid content was determined in triplicate according to an automated colorimetric m-hydroxydiphenyl assay, which was described previously [2].

Gross energy was determined in triplicate with a bomb calorimeter (IKA-C-7000; IKA-Werke, Straufen, Germany) using benzoic acid as standard (ISO 9831).

Crude fat content was determined in triplicate using the Berntrop method with acid pre-hydrolysis (IS0 6492).

Maillard reaction products and cross-linked amino acids were used as indicators of protein damage during ingredient and feed processing. The maillard reaction products (furosine, carboxyethyllysine and carboxymethyllysine), cross-linked amino acids (lanthionine (LAN) and lysinoalanine (LAL)) and lysine were quantified in feeds and ingredients using UPLC-MS, as described by Butré *et al*. [19]. In addition to the method described by Butré *et al*, also lysine and carboxyethyllysine (CEL) were analyzed. Furosine was analyzed to serve as an indicator for the early MRP fructosyllysine. During the acid hydrolysis step of the sample preparation, fructosyllysine is assumed to be converted to 32% furosine, 16% pyrosidine and 56% regenerated lysine [21]. Fructosyllysine was thus calculated as 3.125 * furosine content and lysine was corrected for regenerated lysine content originating from fructosyllysine by lysine content – (furosine content * 1.75). This corrected lysine content was denoted "true lysine" and the uncorrected lysine content (i.e., the total lysine content measured) was denoted "total lysine". The method of sample preparation, use of standards and the test for matrix effects were optimized for the samples of this experiment. Feeds and ingredients (~10 mg) were exactly weighed and solubilized in 1 mL 6 M HCl. The samples were incubated at 110 °C for 23 h and subsequently dried under N_2 at RT. The samples were re-suspended in 1 mL Milli-Q water, sonicated for 5 min and centrifuged (5 min, 20 °C, 19,000 x g). The supernatants were diluted 100x in eluent A (Millipore water containing 0.1% (v/v) formic acid) containing 0.5 mg/L $^{13}C_6$,$^{15}N_2$-lysine as internal standard and centrifuged (5 min, 20 °C, 19,000 x g). All compounds were analyzed using selected reaction monitoring (SRM) method (details are shown in Table 4). The normalized collision energy was set at 35 for CEL and at 30 for lysine. The SE on the calibration was 7.2% and the SE for samples was 8.8%. The limits of quantification for the feeds and ingredients were 0.092, 0.090, 0.046, 0.49, 0.090 and 0.082 g / kg sample for furosine, CEL, CML, LAN, LAL, and lysine respectively. To verify the accuracy of the analysis, i.e. that the analysis was not affected by matrix effects, several samples were spiked using furosine, CEL, CML, LAN, LAL and lysine standards. The standards were added to the samples to a final concentration of 0.01, 0.01, 0.05, 0.05, 0.50 and 5.0 mg / L for CEL, CML, LAL, furosine, LAN and lysine, respectively. The absence of matrix effects was determined by calculating the recovery of standards by comparing the signal from the standards injected alone and the standards added to the samples, using equation 3.

$$\text{Recovery of standard } [\%] = \frac{\text{Peak area}_{\text{sample}} - \text{Peak area}_{\text{spiked sample}}}{\text{Peak area}_{\text{standard}}} \times 100\% \quad (3)$$

Peak area$_{\text{sample}}$, Peak area$_{\text{spiked sample}}$ and Peak area$_{\text{standard}}$ are the MS peak areas of the SRM fragment in a sample, spiked sample, or standard, respectively. All peak areas were corrected using the internal standard. The calculated recoveries over all analyzed compounds from 5 spiked samples were 104% (SD = 10).

Table 4: Selected reaction monitoring conditions of Maillard reactions products, cross-linked amino acid products and lysine.

Compound	Parent mass (Da)	Fragment (m/z)	Ionization mode
Lysine	146	130	-
$^{13}C_6$,$^{15}N_2$-lysine	154	137	-
Carboxymethyllysine	204	84, 130	-
Lanthionine	208	120	+
Carboxyethyllysine	218	84, 130	-
Lysinoalanine	233	128, 145	+
Furosine	254	84, 130	-

Apparent digestibility coefficients

Apparent digestibility coefficients (ADCs) of the dietary components in the diets were calculated using equation 4.

$$ADC_{diet}[\%] = \left(1 - \left[\frac{Y_{diet}}{Y_{feces}}\right] \times \left[\frac{N_{feces}}{N_{diet}}\right]\right) \times 100\% \quad (4)$$

Y_{diet} and Y_{feces} are the contents of inert marker (yttrium) in diet and feces, respectively (g / kg DM) and N_{feces} and N_{diet} are the contents of the dietary component in feces and diet, respectively (g / kg DM). ADCs of the dietary components in the test ingredients were calculated using equation 5.

$$ADC_{ingredient}[\%] = ADC_{test\ diet} + (ADC_{test\ diet} - ADC_{reference\ diet}) \times \frac{0.7 \times N_{reference\ diet}}{0.3 \times N_{test\ ingredient}} \quad (5)$$

$ADC_{test\ diet}$ and $ADC_{reference\ diet}$ are the ADCs of the dietary component in the test diet and the reference diet, respectively and $N_{reference\ diet}$ and $N_{test\ ingredient}$ are the contents of the dietary component in the reference diet and test ingredient, respectively (g / kg DM).

Statistics

All statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA). Animal performance data and nutrient ADCs were tested for treatment effect using analysis of variance (ANOVA; PROC GLM). Significant differences ($p < 0.05$) between means were detected using Tukey's test. Correlation coefficients between the *in vitro* and *in vivo* data and between the various *in vitro* measurements were examined using Pearson's correlation coefficient (PROC CORR Probability levels of less than 0.05 were considered to be statistically significant, and levels between 0.05–0.1 were considered a trend. The *in* vitro data were tested for linear and quadratic relations with protein and fat ADCs using PROC GLM.

RESULTS AND DISCUSSION

Cell wall integrity and nutrient accessibility

Light microcopy analyses showed that the untreated (UNT) and physically treated algae (pasteurized, PAS; freeze-dried, FRD; frozen, FRO and the commercial product L40) contained only intact cells (Figure 1). A small number of intact cells were also observed in BEM, but the majority of the cells (60–80%) were broken, showing that BEM can serve as a positive control. Nutrient accessibility of *N. gaditana* was quantitatively assessed by *in vitro* protein hydrolysis (DH), nitrogen solubility, ion leaching, fat extractability and buffering capacity (BC).

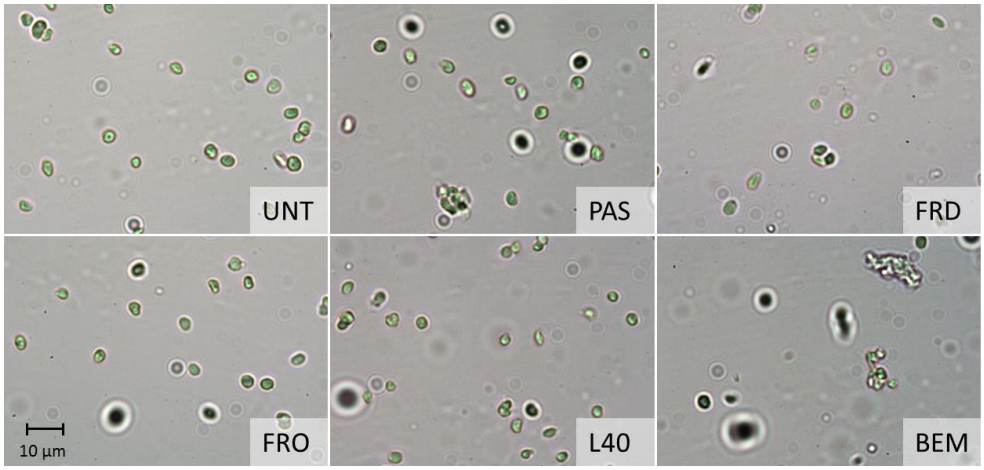

Figure 1: Microscope images of *Nannochloropsis gaditana* biomass at a magnification of 40×. UNT, PAS, FRD, FRO, L40 and BEM: untreated, pasteurized, freeze-dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass, respectively. With exception of FRD, all *N. gaditana* biomass was drum dried.

The untreated algae (UNT) reached a maximum DH (DH_{max}) of 2.4% after hydrolysis, and showed a nitrogen solubility of 6.8% and 24.2% ion leaching (Figure 2). The fat extractability was 22% and the BC was 0.14 mmol / g DM sample. Since UNT is considered to consist of intact cells, it is assumed that the nitrogen and ions measured in solution were mostly originating from the culture medium of the algae. The positive control (BEM) resulted in breakdown of cells as seen by microscopy. This breakdown resulted in a higher nutrient accessibility than UNT, with a DH_{max} of 12%, a nitrogen solubility of 19%, as well as 60% ion leaching, a fat extractability of 85% and a BC of 0.32 mmol / g DM sample. Overall, nutrient accessibility of BEM was a factor 2.3–4.3 higher than UNT. The pH of BEM (pH 5.4) was measured to be lower than the pH of the other algae products (pH 6.8–7.1) (Figure 2 F). Previous work on lab scale also showed that the pH of *N. gaditana* dispersions in water (~ pH 7) directly decreased to pH 5–6 upon bead milling. The drop in pH of BEM compared to the other treatments was therefore considered to be an additional indication of cell wall damage.

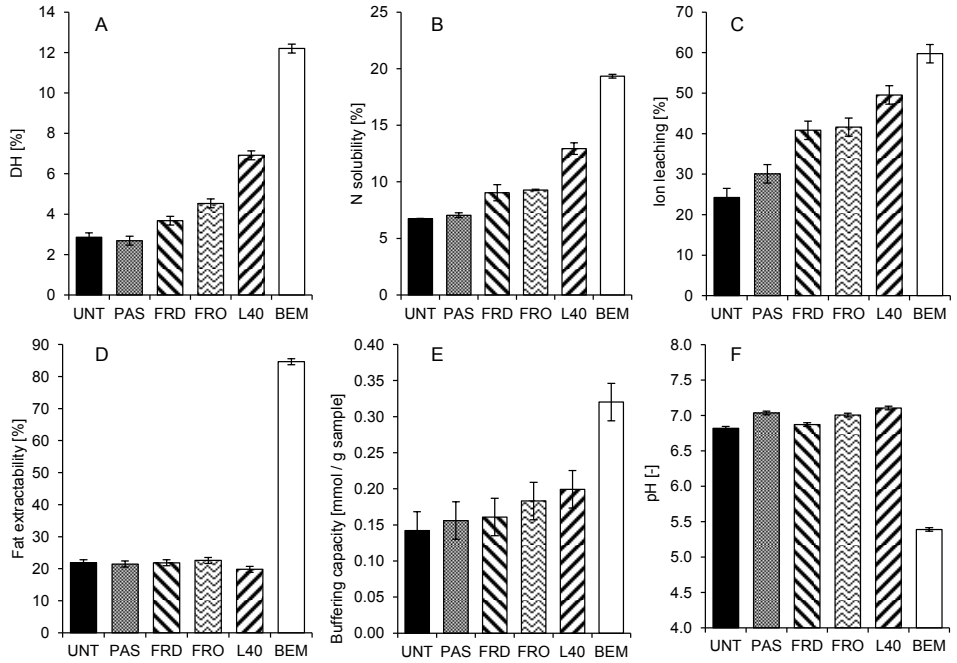

Figure 2: Degree of protein hydrolysis (DH; A), nitrogen solubility (B), ion leaching (C), fat extractability (D), (alkaline) buffering capacity (E) and pH (F) of *Nannochloropsis gaditana* biomass that was untreated (■; UNT), pasteurized (▩; PAS), freeze-dried (◪; FRD), frozen-thawed (▨; FRO), commercially processed (NutriSpring® Liquid 40, Algaspring, NL; ◩ ; L40) or bead milled (□; BEM). With exception of FRD, all *N. gaditana* biomass was drum dried. Error bars depict standard deviations.

The milder physical treatments performed on the algae (PAS, FRD, FRO and L40) did not affect the fat extractability (90–100% of the fat extracted in UNT was extracted in the physical treated algae). In contrast, a 4x increase in fat extractability was measured in BEM compared to UNT (Figure 2 D). The physical treatments did affect the DH, nitrogen solubility, ion leaching and BC, representing an increased nutrient accessibility of up to factor 2.4 compared to UNT (Figure 2). This shows that even though the cells were not broken (as seen in microscopy) the cell wall structure was compromised. To compare the effect of the four physical treatments over all the *in vitro* methods, the data of each method was normalized to UNT as 0% and BEM as 100% (Figure 3). The normalized data were used to describe the nutrient accessibility. The data indicate maximum fat extractability was only reached after complete disruption of the algae cell wall (BEM). This underpins the importance of including an acid hydrolysis step in fat content analyses of intact microalgae, as well as analyses of fecal samples of animals fed a microalgae-containing diet. The other accessibility data showed that among the physically treated algae, L40 had the highest nutrient accessibility values (up to 71% of the BEM nutrient accessibility, depending on the method of analysis). FRD and FRO had lower nutrient accessibilities than L40, but were

similar to each other, with up to 47% and 49% of the BEM nutrient accessibility, respectively. The similar results for FRO and FRD indicate that the drum drying process did not damage cells more or less than freeze-drying did. The least effective method to break the cells was pasteurization (under the conditions used); PAS had a slight increase in ion leaching (16% of BEM) compared to UNT, but no effect was found in any other *in vitro* tests. The ranking of normalized accessibility parameters (DH, nitrogen solubility, ion leaching and BC) for each treatment was the same, but there were differences in the relative effects of the treatments between the *in vitro* methods used.

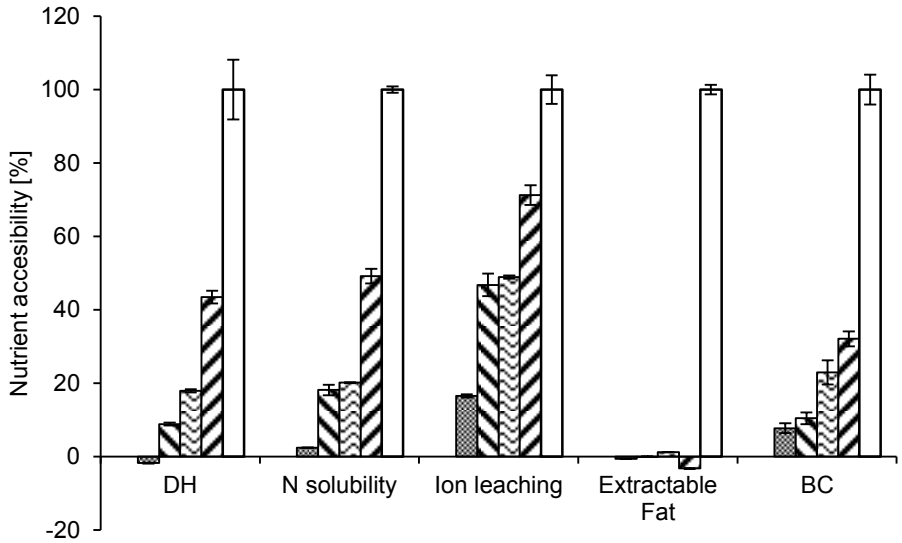

Figure 3: Degree of protein hydrolysis (DH), nitrogen solubility, ion leaching, extractable fat and buffering capacity (BC) of *Nannochloropsis gaditana* biomass that was untreated (■), pasteurized (▩), freeze-dried (▨), frozen-thawed (▧), commercially processed (NutriSpring® Liquid 40, Algaspring, NL; ◪) or bead milled (□). With exception of FRD, all *N. gaditana* biomass was drum dried. The presented values are relative to untreated biomass (set to 0%) and to bead milled biomass (set to 100%). Error bars depict standard deviations.

As described above, a higher accessibility was measured for PAS, FRD and FRO in the ion leaching method than in the DH and nitrogen solubility methods. This shows that ions leached out more rapidly than nitrogen or protein (as measured by nitrogen) and/or than proteolytic enzymes could enter the cells. Proteins (including proteolytic enzymes) are larger molecules than ions, and can thus only enter into, or leach out of the algae cells when the induced cell wall damage led to perforations large enough for the proteins to pass. At the same time, the results of the nitrogen solubility (as an indication for protein solubility) and the DH were similar; which was also shown in a high Pearson correlation coefficient between these parameters ($r > 0.986$, $p < 0.001$). During pasteurization, freezing and/or freeze-drying, the cells were apparently damaged to such an extent that ions could leach out, but larger molecules could not.

The discrepancy between the microscopic analysis and the leaching, DH and BC measurements indicates that the physical treatments applied (as opposed to the mechanical BEM treatment) did not completely disrupt the algae cell walls. Instead, these treatments only damaged the cell wall structure and thereby increased their permeability for ions, and at higher degree of damage, their permeability for larger molecules like algae proteins and digestive enzymes. This is visualized in Figure 4, illustrating the DH over nitrogen solubility (showing a linear correlation) and DH over ion leaching (showing a quadratic correlation).

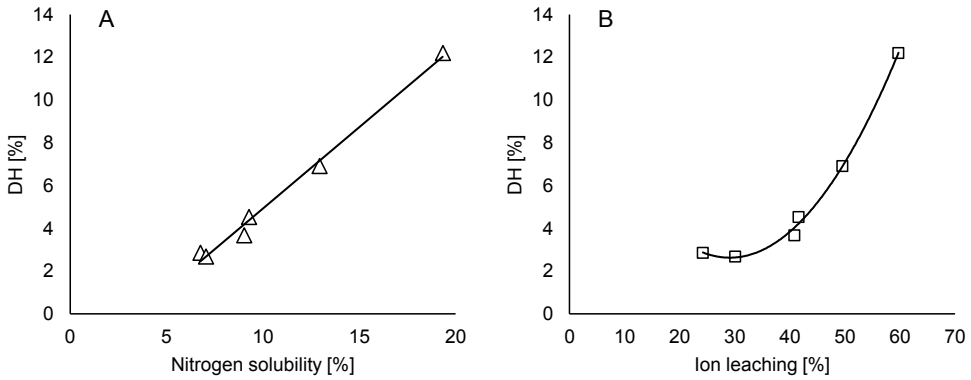

Figure 4: Relationships between the degree of protein hydrolysis (%; DH) and the nitrogen solubility (A) and ion leaching (B) of *Nannochloropsis gaditana* biomass. Solid lines indicate significant relationships ($p < 0.05$; either linear or quadratic). Equations used are (A) Y = -2.54 (SE0.363) + 0.77 (SE 0.03) · X; $R^2 = 97.3\%$; $p < 0.01$ and (B) Y = 11.28 (SE 0.624) – 0.59 (SE 0.031) · X + 0.010 (SE 0.0004) · X^2; $R^2 = 99.7\%$; $p < 0.01$.

Effect of algae treatments on protein quality

Maillard reaction products in algae

The formation of Maillard reaction products (MRPs) and amino acid crosslinking can be used as indications of protein damage during feed processing of ingredients [16]. The *N. gaditana* biomass, reference diet and test diets did not contain the cross-linked amino acid product LAN, and either no or trace amounts of LAL (Table 5). In the algae products, the advanced MRP CML contents ranged between 0.18–0.39 g / kg protein and CEL was present at 0.05–0.08 g / kg protein. In the algae products, fructosyllysine contents were calculated to range between 2.5–35.6 g / kg protein. FRO had the highest content of MRPs (4.9 x more fructosyllysine and 2.2 x more CML than UNT), followed by BEM (2.0 x more fructosyllysine and 1.4 x more CML than UNT). This was also reflected in a decrease in (corrected) lysine content of ~ 50% in FRO and ~ 23% in BEM, compared to UNT. In the other treated algae products the degree of lysine modification (i.e. loss of lysine) was similar to UNT (the variation was within the sample SE of 8.8%). These differences in MRP contents between

the algae products could not be attributed to their nutrient accessibility: FRO MRP content was much higher compared to the other algae products than its nutrient accessibility was. The results do indicate that the combination of algae treatments and processing (drying) can lead to significant (up to 50%) modification of the lysines.

Table 5: Contents of Maillard reaction products[1], cross-linked amino acids[2] and lysine in experimental diets fed to juvenile Nile tilapia, and of test ingredients included in those diets (30% inclusion level).

	Content (g/kg protein)						
	Analysed					Corrected[3]	
	Furosine	CEL	CML	LAL	Total lysine[3]	FL	(True) lysine[3]
Diet[4]							
REF	1.52	0.07	0.13	n.d.	45.72	4.74	43.07
UNT	1.62	0.10	0.17	n.d.	43.36	5.06	40.52
PAS	1.85	0.06	0.16	n.d.	44.93	5.79	41.69
FRD	2.18	0.07	0.20	n.d.	44.81	6.80	41.00
FRO	6.81	0.11	0.31	n.d.	39.30	21.29	27.37
L40	0.60	0.12	0.20	n.d.	45.17	1.88	44.11
BEM	2.76	0.07	0.15	n.d.	41.26	8.64	36.42
Ingredient[5]							
UNT	2.32	0.05*	0.18	n.d.	46.48	7.24	42.42
PAS	2.71	0.08*	0.25	0.20	49.37	8.45	44.64
FRD	1.31	0.06*	0.22	0.14*	43.48	4.09	41.19
FRO	11.38	0.07*	0.39	0.19	41.20	35.57	21.28
L40	0.79	0.07*	0.18	0.13*	47.00	2.46	45.62
BEM	4.64	0.07*	0.24	0.56	40.90	14.49	32.79

[1] Furosine, carboxyethyllysine (CEL), carboxymethyllysine (CML) and fructosylysine (FL).

[2] Lanthionine (LAN) and lysinoalanine (LAL) were not detected in the ingredients and diets.

[3] Fructosyllysine was calculated as 3.125 * furosine and lysine was corrected for regenerated lysine content originating from fructosyllysine by lysine – (furosine * 1.75). "Total lysine" is the uncorrected lysine content, "true lysine" is the lysine content corrected for the regenerated lysine.

[4] REF: reference diet. UNT, PAS, FRD, FRO, L40 and BEM: 70% reference diet, 30% *Nannochloropsis gaditana* biomass – untreated, pasteurized, frozen-thawed, freeze-dried, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass, respectively.

[5] UNT, PAS, FRD, FRO, L40 and BEM: untreated, pasteurized, freeze-dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass of *N. gaditana*, respectively. With exception of FRD, all *N. gaditana* biomass was drum dried.

* Below the detection limit of 0.17–0.18 g CEL or LAL / kg protein.

n.d.: Not detected.

Formation of Maillard reaction products during feed manufacturing

In the reference diet, CML, CEL and fructosyllysine contents were 0.13, 0.07 and 4.74 g / kg protein, respectively. In the test diets, CML, CEL and fructosyllysine contents ranged between 0.15–0.31, 0.06–0.12 and 1.88–21.3 g / kg protein, respectively. To put these values into perceptive, the MRP and cross-linked amino acid products in the feeds are slightly higher in content than in fish and pig feeds reported in literature, but lower than in pet feeds. In experimental fish feeds containing 30% feather hydrolysates, lower furosine contents (0.14–0.29 g / kg protein were reported [19] than in the present findings. In experimental pig feeds containing heavily processed rape seed meal, equivalents of CML contents of 0.2–0.4 g / kg protein, CEL contents of 0.2–0.4 g / kg protein and furosine contents of 0.7–1.5 g / kg protein were reported [22]. In commercial pet food, higher MRP contents were reported, with equivalents of 28–94 g / kg protein CML and 0.8–9.2 g / kg protein furosine [23].

To test whether the algae treatments affected MRP formation during further processing in the feed manufacturing process, MRP and lysine contents of the diets and the test ingredients were compared. If no Maillard reaction took place during the feed manufacturing process, the MRP contents of the test diets should be equal to a 30/70% mixture of the MRP contents of the ingredient (30%) and the reference diet (70%). The majority of these measured MRP contents were higher (up to 87% increase) than the theoretical (30/70% mixture) contents. This effect was most pronounced in the diets containing FRD and FRO algae (50–52% increase of fructosyllysine). In FRO, this corresponded to a 25% loss of lysine during feed production. No clear relation could be found, however, between the various algae treatments and the susceptibility of the algae products to MRP formation (and lysine modification) during feed production. Overall, the data indicated that both drum drying and the feed manufacturing process can induce MRP formation and related amino acid modification in algae. Furthermore, the data imply that an increased nutrient accessibility may increase the reactivity of proteins during drying and feed manufacturing.

Fish growth, feed utilization and survival

Fish survival was over 97% for all dietary treatments during the experimental period (Table 6). Absolute feed intake was the same for all tanks, with an average feed intake of 1.40 g DM / (fish * d) (SD = < 0.01). Despite equal feed intake, fish performance was different between fish fed the test diets compared to the reference diet. The fish fed the BEM diet and the L40 diet had similar specific growth rates (SGRs) as the fish fed the reference diet (p = 0.694 and p = 0.966, respectively). For fish fed all other diets SGR was lower (3–6% decrease; $p < 0.05$). The feed conversion ratio (FCR) of the reference diet was 0.96. Only the BEM diet had a lower FCR than the reference (0.90; i.e. a decrease of ~ 6%). In the other test diets, FCR was 2–11% lower than the reference diet. Fish performance was similar or superior to values reported in literature. FCRs of Nile tilapia reported for diets containing 19–39% algae, under restricted feeding, are 1.1–1.42 [24-26]. FCRs of reference diets in those studies were 1.1–1.91.

Table 6: Growth performance, feed intake and survival rate of Nile tilapia after 6 weeks of feeding a reference diet and diets with 30% inclusion of treated or untreated *Nannochloropsis gaditana*. Values are presented are means.

	Diets[1]								*p*-values
	REF	**UNT**	**PAS**	**FRD**	**FRO**	**L40**	**BEM**	**SEM**	**Diet**
Survival (%)	100	100	97	100	98	100	98	1.3	0.564
Feed intake (g DM / (fish · d))	1.40	1.40	1.40	1.40	1.40	1.40	1.40	0.002	0.678
Initial body weight (g / fish)	29.1	30.0	29.3	29.1	29.6	29.4	29.9	0.31	0.302
Final body weight (g / fish)	90.7^{ab}	87.0^{bc}	85.9^{bc}	86.8^{bc}	85.2^{c}	89.9^{bc}	95.2^{a}	1.07	<0.01
SGR (% / d)	2.71^{a}	2.53^{c}	2.56^{bc}	2.60^{bc}	2.52^{c}	2.66^{ab}	2.76^{a}	0.021	<0.01
Feed conversion ratio (g DM intake / g body gain)	0.96^{cd}	1.04^{ab}	1.04^{ab}	1.02^{ac}	1.06^{a}	0.97^{bc}	0.90^{d}	0.014	<0.01

[1]: REF: reference diet. UNT, PAS, FRD, FRO, L40 and BEM: 70% reference diet, 30% *Nannochloropsis gaditana* biomass – untreated, pasteurized, freeze-dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass, respectively. With exception of FRD, all *N. gaditana* biomass was drum dried.

Apparent digestibility coefficients of nutrients, DM and energy

The reference diet (REF) had DM, energy, protein and fat ADCs of 75.2, 82.0, 89.5 and 91.0%, respectively (Table 7). Dietary treatment affected the ADCs of these nutrients ($p < 0.001$ for all). No differences were present in ADCs for carbohydrates, starch, NSP and ash. Energy, protein and fat of all the test diets were digested to a lower extent (4–19% decrease) than the REF diet. ADCs of the total carbohydrate constituent monosaccharides and of all minerals analyzed are provided in Table 8.

On ingredient level, treatments of *N. gaditana* affected the ADCs for DM, energy, protein, fat, ash and calcium ($p < 0.05$) (Table 9). ADCs of the total carbohydrate constituent monosaccharides and of all minerals analyzed are provided in Table 10. Starch and NSP ADCs at ingredient level were not reported in Table 9 since the algae biomass contained less than 0.1% (w/w) starch, leading to large errors on both starch and NSP ADC values. The algae treatments had no effect on digestibility of total carbohydrates. Untreated (UNT) algae had a protein and fat digestibility of 61.5% and 50.4%, respectively. The protein and fat ADCs of the untreated algae are lower than previously reported values (74.7% and 74.5% respectively) for *N. gaditana* in Nile tilapia and in Atlantic salmon (72%) [2, 5]. In a previous tilapia trial using the same diet composition, the reference diet was digested to the same extent in in the present trial [2]. This indicates that the differences in digestibility observed should be attributed to the properties of the algae biomass. Although gross composition of

the algae used in the two studies was quite similar, differences in nitrogen solubility were observed between the two batches of biomass. The *N. gaditana* used in the present study was a different strain (AS1405) than the algae used in the previous study (AS1301). Therefore, the differences in nutrient digestibility might be caused by differences in nutrient accessibility due to strain differences, batch-to-batch differences or seasonal changes. Other (untreated) unicellular sources were reported to have protein ADCs of 80–85% for *Arthrospira* (S*pirulina*) sp., 82% for *Schizochytrium* sp. [1], 73–81% [1-4] for *Chlorella* sp. [1, 2], 67–68% for *Scenedesmus dimorphus* [2] and 67% for *Desmodesmus* sp. [5], in various fish species. In these studies, fat ADCs were reported between 65–98% [1-3] and energy ADCs between 61–87% [1-4]. Compared to this, fat and energy ADCs of UNT were lower, and fat and energy ADCs of BEM were comparable to them.

The DM, energy, protein and fat the digestibility in the positive control (BEM) was higher than in UNT, although the increase was not identical for each nutrient (27–63% increase in ADCs). It should be noted that these values are possibly an underestimation of the maximum nutrient digestibility of this microalga, since not all the cells were disrupted during bead milling.

Compared to BEM, the milder physical treatments performed on the algae (PAS, FRO, FRD and L40) had less effect on nutrient digestibility. More specifically, freezing the biomass (FRO) increased only the protein ADC by 8%, compared to UNT, and freeze-drying (FRD) increased only the fat ADC by 15%. Pasteurization (PAS) had no effect on digestibility of any nutrient. L40 had higher ADCs of DM, energy, protein and fat than UNT (19–32% increase, depending on the nutrient). It should also be noted that phosphorus ADCs on ingredient level were higher than what was reported for fish meals and plant based ingredients in Nile tilapia [27, 28]. Moreover, some phosphorus ADCs of the algae products were higher than 100%. These high values might be an artefact caused by the low phosphorus ADC of the reference diet (6% w/w). Feeds and feces of REF samples were re-analyzed for phosphorus content, yielding the same results. This low phosphorus digestibility in REF deviates from results obtained in a previous fish trial with a similar set-up and REF diet (phosphorus ADC of 58%) [2]. Although phytase activity in the microalgae might explain the higher phosphorus ADCs in the test diets compared to the REF diet, no records were found for genes encoding the production of phytase in the taxonomic division of chlorophyta [18]. Thus, the divergent phosphorus ADCs cannot be explained at this moment.

Table 7: Apparent digestibility coefficients (%; ADC[1]) of the reference diet and test diets with 30% inclusion of treated or untreated *Nannochloropsis gaditana* in juvenile Nile tilapia.

ADC (%)	Diet[2]								*p*-values
	REF	UNT	PAS	FRD	FRO	L40	BEM	SEM	Diet
Dry matter	75.2^{a}	67.2^{d}	67.7^{d}	68.0^{cd}	69.3^{cd}	67.7^{bc}	72.6^{ab}	0.62	<0.001
Gross energy	82.0^{a}	71.6^{d}	71.3^{d}	72.3^{d}	73.6^{cd}	75.0^{c}	77.7^{b}	0.54	<0.001
Crude protein	89.5^{a}	79.3^{e}	78.8^{e}	78.5^{e}	81.1^{d}	83.5^{c}	85.4^{b}	0.34	<0.001
Crude fat	91.0^{a}	73.8^{e}	76.1^{de}	78.6^{cd}	74.2^{e}	80.7^{c}	87.1^{b}	0.64	<0.001
Total carbohydrates[3]	73.2	67.7	67.9	68.9	68.5	69.3	71.3	1.30	0.087
Starch	97.5	97.4	97.6	97.1	97.6	97.7	96.7	0.31	0.314
NSP[4]	38.4	30.9	34.3	28.4	33.1	35.6	40.1	2.95	0.148
Ash	9.5^{b}	10.7^{b}	15.6^{ab}	16.4^{ab}	15.0^{ab}	26.8^{a}	20.0^{ab}	2.49	0.004
Phosphorus	5.7^{c}	36.8^{ab}	44.2^{ab}	29.6^{b}	39.3^{ab}	41.6^{ab}	44.0^{a}	2.61	<0.001
Calcium	66.7^{a}	57.1^{d}	57.9^{d}	62.9^{bc}	61.3^{c}	65.1^{ab}	67.7^{a}	2.69	<0.001

[1]: Values presented are means. Mean values in a row with different superscript are significantly different ($P < 0.05$).

[2]: REF: reference diet. UNT, PAS, FRD, FRO, L40 and BEM: 70% reference diet, 30% *Nannochloropsis gaditana* biomass – untreated, pasteurized, freeze-dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass, respectively. With exception of FRD, all *N. gaditana* biomass was drum dried.

[3]: Total carbohydrates comprise starch and NSP.

[4]: NSP = total carbohydrates – starch.

Table 8: Apparent digestibility coefficients (%; ADC[1]) of the carbohydrate constituent monosaccharides and of minerals of the reference diet and test diets with 30% inclusion of treated or untreated *Nannochloropsis gaditana* in juvenile Nile tilapia.

ADC (%)	Diet[2]								*p*-values
	REF	UNT	PAS	FRD	FRO	L40	BEM	SEM	Diet
Total carbohydrates	73.2	67.7	67.9	68.9	68.5	69.2	71.3	1.30	0.087
Rhamnose	70.2	71.5	66.8	69.2	69.8	65.0	69.5	3.33	0.846
Fucose	23.2	15.4	8.9	25.4	12.6	9.3	28.0	8.46	0.536
Arabinose	12.7	11.6	13.4	24.9	17.8	19.3	16.9	5.25	0.600
Xylose	-0.1	-10.5	-4.2	-3.4	-17.6	-8.3	-10.8	8.58	0.824
Mannose	34.6^{c}	70.1^{a}	67.9^{ab}	58.0^{b}	71.3^{a}	69.1^{ab}	75.8^{a}	2.47	<0.001
Galactose	42.4^{b}	51.1^{ab}	50.6^{ab}	52.7^{ab}	49.6^{ab}	57.0^{a}	54.5^{a}	2.26	0.014
Glucose	87.9^{a}	81.2^{c}	81.6^{c}	82.6^{bc}	82.5^{bc}	82.3^{c}	85.2^{ab}	0.59	<0.001
Ribose	-71.6^{c}	29.3^{ab}	-23.0^{bc}	7.6^{ab}	31.8^{ab}	46.3^{a}	50.4^{a}	12.92	<0.001
Uronic acids	12.9	6.2	8.9	11.2	11.2	13.6	15.6	2.26	0.156
Ash	9.5^{b}	10.7^{b}	15.6^{ab}	16.4^{ab}	15.0^{ab}	26.8^{a}	20.0^{ab}	2.49	0.004
Phosphorus	5.7^{c}	36.8^{ab}	44.2^{a}	29.6^{b}	39.3^{ab}	41.6^{ab}	44.0^{a}	2.61	<0.001
Calcium	66.7^{a}	57.1^{d}	57.9^{d}	62.9^{bc}	61.3^{c}	65.1^{ab}	67.7^{a}	0.69	<0.001
Copper	28.0^{a}	2.8^{cd}	-6.5^{d}	6.6^{bc}	14.3^{b}	-5.9^{d}	9.1^{bc}	2.20	<0.001
Iron	-9.1^{bc}	-1.9^{ab}	-4.7^{abc}	-1.3^{a}	-10.1^{c}	-4.7^{abc}	-8.1^{abc}	1.52	0.005
Magnesium	33.2^{c}	48.0^{b}	48.7^{b}	47.6^{b}	50.6^{ab}	54.8^{a}	52.9^{ab}	1.10	<0.001
Manganese	-20.0^{c}	5.0^{ab}	7.1^{a}	-5.1^{b}	-2.7^{ab}	-0.7^{ab}	0.6^{ab}	2.23	<0.001
Zinc	-7.1^{a}	-24.4^{ab}	-41.7^{bc}	-50.2^{c}	-50.3^{c}	-52.4^{c}	-39.0^{bc}	4.54	<0.001

[1]: Values presented are means. Mean values in a row with different superscript are significantly different ($P < 0.05$).

[2]: REF: reference diet. UNT, PAS, FRD, FRO, L40 and BEM: 70% reference diet, 30% *Nannochloropsis gaditana* biomass – untreated, pasteurized, freeze-dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass, respectively. With exception of FRD, all *N. gaditana* biomass was drum dried.

Table 9: Apparent digestibility coefficients (%; ADC[1]) of nutrients of treated or untreated *Nannochloropsis gaditana* (at 30% diet inclusion level) in juvenile Nile tilapia.

ADC (%)	*In vivo* trial								Correlations to *in vitro* measurements[3]			
	Nannochloropsis gaditana[2]						*p*-values		Correlation coefficients r[4]			
	UNT	**PAS**	**FRD**	**FRO**	**L40**	**BEM**	**SEM**	**Treatment**	**DH**	**N_{sol}**	**Ion**	**BC**
Dry matter	48.4^{c}	50.2^{c}	50.6^{bc}	55.2^{bc}	61.2^{ab}	66.3^{a}	2.23	<0.001	0.86***	0.82***	0.85***	0.83***
Gross energy	51.0^{c}	50.1^{c}	53.0^{bc}	57.0^{bc}	60.6^{b}	69.2^{a}	1.73	<0.001	0.92***	0.90***	0.88***	0.90***
Crude protein	61.5^{cd}	60.7^{e}	60.6^{de}	66.2^{c}	72.9^{b}	78.0^{a}	1.00	<0.001	0.93***	0.88***	0.87***	0.89***
Crude fat	50.4^{c}	56.1^{c}	57.8^{c}	53.0^{c}	66.4^{b}	82.0^{a}	1.62	<0.001	0.94***	0.93***	0.87***	0.93***
Total carbohydrates	34.9	38.0	38.5	40.5	46.6	56.7	9.81	0.662	0.46†	0.45†	0.43†	0.45†
Ash	13.2^{b}	29.1^{ab}	29.6^{ab}	26.1^{ab}	55.0^{a}	37.9^{ab}	7.88	0.050	0.42	0.35	0.55	0.35
Phosphorus	92.0	110.9	94.7	109.1	110.7	120.9	8.16	0.187	0.48*	0.47*	0.47*	0.51*
Calcium	-3.7^{c}	2.3^{c}	38.9^{b}	13.1^{c}	55.6^{ab}	72.8^{a}	5.16	<0.001	0.84***	0.83***	0.92***	0.79***

[1]: Values presented are means. Mean values in a row with different superscript are significantly different ($P < 0.05$).

[2]: UNT, PAS, FRD, FRO, L40 and BEM: untreated, pasteurized, freeze-dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass of *Nannochloropsis gaditana*, respectively. With exception of FRD, all *N. gaditana* biomass was drum dried.

[3]: DH - degree of protein hydrolysis using pancreatin; N_{sol} - nitrogen solubility in a pH 8 buffer; Ion – conductivity in water; BC – alkaline buffering capacity.

[4]: Pearson correlations between the triplicate ADC values (n = 18) and the mean values of each *in vitro* measurement (n = 6). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and † = $p < 0.1$.

Table 10: Apparent digestibility coefficients (%; ADC[1]) of the carbohydrate constituent monosaccharides and of minerals of treated or untreated *Nannochloropsis gaditana* (at 30% diet inclusion level) in juvenile Nile tilapia.

ADC (%)	*Nannochloropsis gaditana*[2]						*p*-values	
	UNT	PAS	FRD	FRO	L40	BEM	SEM	Treatment
Total carbohydrates	34.9	38.0	38.5	40.5	46.6	56.7	9.81	0.662
Rhamnose	72.5	64.0	68.5	69.4	61.2	69.1	5.71	0.754
Fucose	6.6	-12.4	27.9	-0.1	-4.9	32.5	17.23	0.406
Arabinose	-27.9	37.0	472.2	167.1	209.0	196.7	168.94	0.432
Xylose	-288.3	-128.4	-90.7	-494.0	-228.4	-302.9	259.21	0.897
Mannose	82.6	78.4	71.1	80.7	80.0	85.8	3.40	0.133
Galactose	67.7	65.1	72.8	66.3	83.0	80.9	7.52	0.435
Glucose	20.8	26.1	24.1	29.5	31.8	30.1	8.31	0.930
Ribose	37.7^{ab}	-18.9^{c}	13.5^{bc}	40.0^{ab}	56.4^{a}	62.9^{a}	7.98	<0.001
Uronic acids	-19.9	-6.4	4.8	4.6	16.3	24.3	11.82	0.192
Ash	13.2^{b}	29.1^{ab}	29.6^{ab}	26.1^{ab}	55.0^{a}	37.9^{ab}	7.88	0.050
Phosphorus	92.0	110.9	94.7	109.1	110.7	120.9	8.16	0.187
Calcium	-3.7^{c}	2.3^{c}	38.9^{b}	13.1^{c}	56.6^{ab}	72.8^{a}	5.16	<0.001
Copper	-275.4^{b}	-293.6^{bc}	-193.8^{b}	-38.4^{a}	-415.4^{c}	-234.2^{b}	27.27	<0.001
Iron	3.9^{ab}	-1.2^{ab}	5.8^{a}	-11.2^{b}	-0.6^{ab}	-6.5^{ab}	3.41	0.035
Magnesium	70.2^{ab}	72.6^{ab}	65.7^{b}	74.6^{ab}	79.8^{a}	76.1^{ab}	2.59	0.032
Manganese	19.8^{ab}	23.3^{a}	2.8^{b}	9.4^{ab}	11.2^{ab}	12.5^{ab}	3.73	0.025
Zinc	-147.5	-280.5	-302.7	-250.5	-322.8	-237.3	36.88	0.064

[1]: Values presented are means. Mean values in a row with different superscript are significantly different ($P < 0.05$).

[2]: UNT, PAS, FRD, FRO, L40 and BEM: untreated, pasteurized, freeze-dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, Algaspring, NL) and bead milled biomass of *Nannochloropsis gaditana*, respectively. With exception of FRD, all *N. gaditana* biomass was drum dried.

Correlations between algae processing and treatments on in vivo parameters

The results discussed above showed that there are significant effects of the algae treatments on the nutrient accessibility, nutrient digestibility and growth parameters of Nile tilapia. In this section, the relations between the processing effects, *in vitro* accessibility and *in vivo* measured digestibility are discussed.

Effect of processing on in vivo nutrient digestibility and growth parameters

The digestion data indicate that the MRPs formed did not affect protein digestion; i.e., the MRPs were digested to a similar extent as the unmodified protein in the algae. Specifically, the treated algae products and the corresponding diets had higher amounts of MRPs than the UNT algae and diet. Especially the FRO algae and FRO diet had increased MRP and modified lysine contents. However, protein ADC of FRO was not affected accordingly. This observation is supported by the findings of Butré *et al*., who reported furosine and LAN ADCs of 83–92% in trout [19]. There seems to be a relation between the increase in MRPs and loss of unmodified lysine in FRO and the growth parameters. This was shown for instance in the protein ADC of FRO (66%), which had higher MRP contents and a lower lysine content, being higher than the ADC of FRD (61%), which had lower MRP contents and a higher lysine content. Despite this higher ADC, SGR and FCR were similar (p = 0.22 and 0.61, respectively) between fish fed the FRO and FRD diets. The increased ADC, together with the similar fish performance indicated that lysine modification during algae treatments could have been a limiting factor in fish growth. However, the effects of algae treatments and feed processing on MRPs were relatively small compared to the positive effect of mechanical and physical treatment of the ingredients.

Effect of in vitro nutrient accessibility on in vivo nutrient digestibility

The *in vitro* data of nitrogen solubility, DH and BC were positively correlated with the *in vivo* digestibility of dry matter, energy, protein, and fat (p < 0.001 for all *in vitro* methods) (Table 9), demonstrating that nutrient accessibility is an important factor in nutrient digestibility of microalgae.

In Nile tilapia, protein and fat (both intercellular nutrients in microalgae) are assumed to be predominantly hydrolyzed and absorbed in the proximal and mid part of the intestine [29, 30]. The increase in ADCs of these nutrients upon increasing the nutrient accessibility was in line with expectations; a higher *in vitro* accessibility could indicate a higher accessibility to proteolytic enzymes in the stomach and mid part of the intestine. Although this correlation was not significant for non-starch carbohydrates (p = 0.06–0.07), the ADC of non-starch carbohydrates correlated with ADC of protein, DM and energy (p < 0.03 for all). This supports the conclusion that there is a trend between nutrient accessibility and carbohydrate digestion, but that the error on the measurement (SEM = 11%) decreased the significance of the correlation in this study.

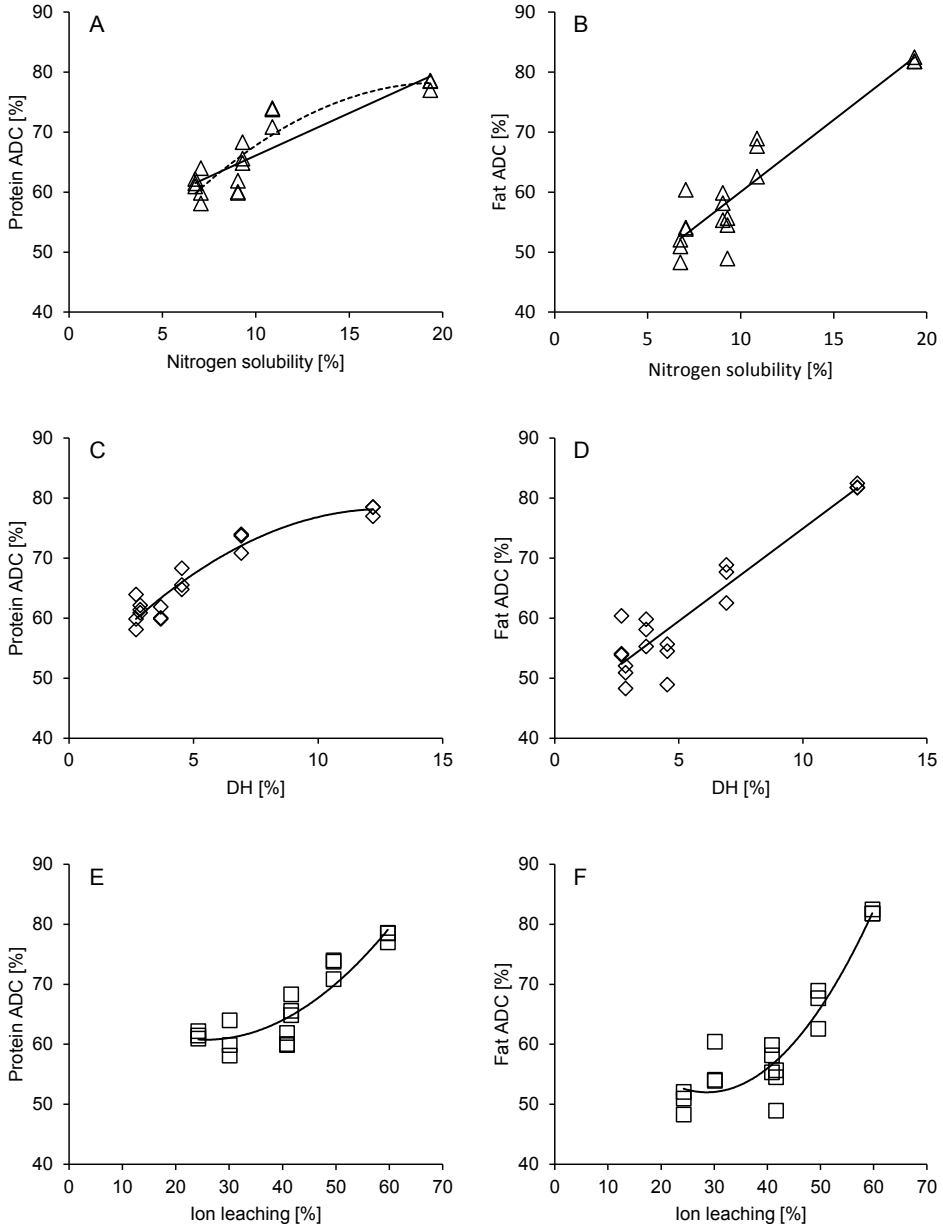

Figure 5: Relationships between the apparent digestibility coefficients (%; ADC) of protein (A, C, E) and fat ADC (B, D, F) from *Nannochloropsis gaditana* in juvenile Nile tilapia and the nitrogen solubility (A, B), degree of protein hydrolysis (DH; C, D) and ion leaching (E, F) of *Nannochloropsis gaditana* biomass. Significant relationships ($p < 0.05$; either linear or quadratic) are indicated by solid lines. A tendency for a significant relationship ($p < 0.1$; only present in A) is indicated by the dotted line. Equations are presented in Table 11.

Table 11: Linear and quadratic relationships estimated between protein and fat apparent digestibility coefficients (ADC) (Y) in juvenile Nile tilapia and *in vitro* nutrient accessibility data[1] (X) from *Nannochloropsis gaditana* biomass.

Y	X	Equation	R^2 [%]	*p* (linear component)[2]	*p* (quadratic component)[2]
Protein ADC (%)	N_{sol} [%]	$Y=51.99\ (SE\ 2.163) + 1.41\ (SE\ 0.193) \times X$	77.0	<0.001	-
		$Y=35.91\ (SE\ 8.518) + 4.26\ (SE\ 1.475) \times X - 0.11\ (SE\ 0.055) \times X^2$	81.6	-	0.071
	DH [%]	$Y=56.14\ (SE\ 1.173) + 1.91\ (SE\ 0.183) \times X$	87.3	<0.001	-
		$Y=48.73\ (SE\ 2.586) + 4.64\ (SE\ 0.895) \times X - 0.18\ (SE\ 0.593) \times X^2$	92.2	-	0.008
	Ion [%]	$Y=45.86\ (SE\ 3.028) + 0.51\ (SE\ 0.071) \times X$	76.1	<0.001	-
		$Y=71.11\ (SE\ 8.363) - 0.81\ (SE\ 0.421) \times X + 0.02\ (SE\ 0.005) \times X^2$	85.6	-	0.007
Fat ADC (%)	N_{sol} [%]	$Y=36.03\ (SE\ 2.574) + 2.40\ (SE\ 0.229) \times X$	87.3	<0.001	-
		$Y=32.39\ (SE\ 11.299) + 3.04\ (SE\ 1.956) \times X - 0.02\ (SE\ 0.073) \times X^2$	87.3	-	0.745
	DH [%]	$Y=44.07\ (SE\ 1.797) + 3.08\ (SE\ 0.281) \times X$	88.3	<0.001	
		$Y=47.25\ (SE\ 4.985) + 1.92\ (SE\ 1.726) \times X + 0.08\ (SE\ 0.114) \times X^2$	88.7	-	0.503
	Ion [%]	$Y=27.86\ (SE\ 4.894) + 0.81\ (SE\ 0.115) \times X$	75.6	<0.001	-
		$Y=77.30\ (SE\ 11.230) - 1.77\ (SE\ 0.565) \times X + 0.03\ (SE\ 0.007) \times X^2$	89.9	-	<0.001

[1]: N_{sol}: Nitrogen solubility - analyzed as the soluble percentage of total nitrogen present in the samples. DH: degree of protein hydrolysis - corrected for substrate blanks. Ion: ion leaching - analyzed as the percentage of the maximum conductivity using NaCl as a standard.

[2]: $p < 0.05$ relationships were considered significant, $p < 0.1$ relationships were considered a tendency for a significant relationship.

The correlations of the digestibility of protein and fat (both intracellular nutrients) with the various methods used to assess nutrient accessibility were studied in more detail, by assessing whether the correlations were linear or quadratic (Figure 5 and Table 11). Protein ADC was linearly correlated to the nitrogen solubility of the algae (p <0.001), although the quadratic function also tended to be significant (p = 0.071). Protein ADC was quadratically correlated to DH (p = 0.008) and ion leaching (p = 0.007). For both nitrogen solubility and DH, small damage to the cells (e.g. measured as < 10% nitrogen solubility and < 5% DH) correlated to significant increases in *in vivo* protein digestibility. However, for both correlations this effect appears to level off at higher degrees of cell damage. Conversely, ion leaching occurs already at lower degree of cell disruption, as was also observed from . An increase in ion leaching therefore did not correlate to an increase in protein ADC until the cells were damaged to such an extent that ~35% ion leaching was reached. Fat ADC was linearly correlated to the nitrogen solubility (p < 0.001) and the DH of the algae products (p < 0.001). In contrast with the DH and N solubility correlations with protein ADC, the increase in fat ADC did not reach a plateau yet, at the maximum levels of cell disruptions reached. Fat ADC was quadratically correlated to ion leaching (p < 0.001), with a similar correlation to that of protein ADC with ion leaching.

The data indicate that quantification of leaching nutrients and *in vitro* hydrolysis can be used as measures of nutrient accessibility. Furthermore, these *in vitro* parameters can be used to make a relative estimation of the effect of technical treatments on *in vivo* nutrient digestibility of a single ingredient source. Overall, the presence of intact algal cell walls was found to be a limiting factor for algal nutrient digestibility in Nile tilapia.

CONCLUSIONS

This study showed that nutrient accessibility is a dominant limiting factor in microalgal nutrient digestibility in fish. Mechanical and physical processes increased *in vivo* microalgal nutrient accessibility, leading to an increased protein and fat digestibility. The *in vitro* methods applied (protein hydrolysis, nitrogen solubility, ion leaching and buffering capacity) are useful tools to assess the effect of mechanical and physical treatments on *in vivo* nutrient quality of a single ingredient.

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6 General discussion

The aim of this thesis was to understand how chemical and structural variations between unicellular green sources affect the application of unicellular protein as techno-functional ingredients and as fish feed ingredient.

In this thesis the cell robustness of *Arthrospira maxima*, *Chlorella vulgaris*, *Nannochloropsis gaditana* and *Scenedesmus dimorphus* was quantified. This was done by determining the release of protein and the disruption of cells as function of bead milling time. These data showed a large variation in cell robustness between the various microalgae and cyanobacteria. The cell wall integrity was quantitatively linked to both *in vitro* and *in vivo* protein accessibility. Gross chemical composition of various microalgae and a cyanobacterium did not affect protein isolation yield and solubility profiles of the isolates obtained (**chapter 2**). The emulsion behavior of the protein isolates obtained differed between various sources, including the minimum protein concentration needed to form a stable emulsion and the pH dependency of the emulsion stability (**chapter 3**). Variation in cell wall robustness between unicellular sources was not correlated to variation in protein digestibility in fish (**chapter 4**). In contrast to expectations, unicellular protein digestibility was not affected by fish species (using a herbivorous species and an omnivorous species). The results obtained in **chapter 4** led to the hypothesis that *in vivo* protein accessibility of unicellular sources is correlated to *in* vivo protein digestibility in fish. This hypothesis was confirmed in **chapter 5** where a dominant (positive) effect of protein accessibility was demonstrated on unicellular protein digestibility in fish, by applying various cell-disrupting pretreatments to *N. gaditana* biomass prior to feed production.

Three major questions arose from the data obtained and discussed in this thesis. The first question is whether the unicellular protein extraction yields presented in **chapter 2** should be considered as 'low'. The second question arose from the variation in emulsion behavior observed between the different algae and cyanobacterial protein isolates. The question is whether the critical protein concentration (C_{cr}) is the right tool to quantitatively compare the emulsion abilities of various proteins. The last question is what is expected of the protein digestibility of the insoluble protein fraction (i.e. the side stream) of unicellular sources. In the *in* vivo digestibility experiments (**chapter 4** and **5**), whole unicellular biomass (either intact or cell disrupted) was fed to fish. For optimum use of microalgae and cyanobacteria in both food and fish feed, it is valuable to study the nutrient digestibility in the side streams produced in **chapter 2**.

VARIATIONS BETWEEN UNICELLULAR SOURCES - TERMINOLOGY

In this thesis, variations in cell wall robustness, protein extractability, protein solubility and protein (or nutrient) accessibility between the unicellular sources are studied.

Protein accessibility and *nutrient accessibility* are measures for the extent to which proteins and other nutrients present inside the cells (e.g. protein and fat) are accessible to enzymes, both *in vitro* and *in vivo*. In this thesis, it is assumed that proteins and other nutrients from the unicellular source (e.g. fat) will have a negligible accessibility to digestive enzymes. Only after cell walls are disrupted, either through pretreatment, processing, or the action of the digestive system, the nutrients are assumed to become accessible. In order for protein and fat digestion to take place in fish, it is hypothesized that these nutrients need to be accessible and that therefore the cells need to be disrupted, as will be discussed in more detail further in this chapter.

Cell wall robustness is the resistance of cells against cell wall disruption, e.g. by mechanical force (including bead milling) or by the digestive processes in fish (including stomach acidity and enzymes). Schematically, this is represented by the slope of the protein yield as function of the effort (mechanical force, digestive efforts) put into the cell disruption ("R" in Figure 1). Cells that are more robust (dotted line in Figure 1), for example due to their cell wall composition, will require more effort (mechanically or by digestive processes) to disrupt, or to make nutrients more accessible, than cells with less robust cell walls (solid and dashed lines).

Protein extractability represents the amount of protein that can be solubilized, as a percentage of the total protein present in the biomass, after complete disruption of the cells (illustrated by the plateau protein yield "E" in Figure 1). Two unicellular sources with a similar robustness may thus show a difference in extractability. It may also be that two sources with similar extractability have a difference in robustness (Figure 1).

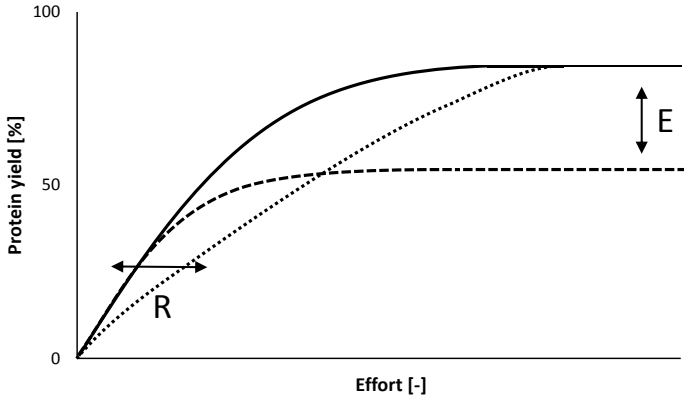

Figure 1: Graphical representation of how microalgae and cyanobacteria can differ in their protein extractability (the plateau height, E) and in their cell wall robustness (the slope, R). The lines represent theoretical unicellular sources that vary in their cell wall robustness and protein extractability.

The "s*oluble" protein fraction* represents fraction of protein that could be extracted after complete cell disruption (i.e. the protein extractability). This fraction containing the soluble proteins is termed *algae juice* in **chapter 2**. The "*insoluble" protein fraction* represents the fraction of protein that could not be extracted after complete cell disruption. The fraction containing the "insoluble" proteins is termed *pellet* in **chapter 2**. The quotation marks are used since the "insoluble" fraction represents both insoluble proteins and proteins that are intrinsically soluble but could not be liberated or extracted from the biomass. These extracted soluble proteins are thought to be bound to cell walls or membranes or locked inside cell organelles, as will be discussed in more detail further in this chapter.

FACTORS INFLUENCING UNICELLULAR PROTEIN EXTRACTABILITY

In **chapter 2** it is described how a major fraction of the proteinaceous material present in unicellular sources is 'lost' during the first steps in the protein isolation process, the protein extraction step and the dialysis step. The losses occur in the form of "insoluble" protein fractions and in low Mw protein fractions. Specifically, 26–83% of the total protein (on amino acid basis) present in various microalgae and a cyanobacterium were lost as "insoluble" fraction during bead milling and subsequent centrifugation at alkaline conditions (pH 8.0) (Figure 2). This corresponds to a protein extractability (Figure 1) of 17–74%. Of these extracted proteins (in the "algae juice" in **chapter 2**), 21–51% was lost during dialysis, indicating that this fraction consists of low Mw protein (<12–14 kDa). This resulted in a final extraction yield of high Mw proteins ("dialyzed algae juice" in **chapter 2**) of only 12–36% of the total amount of protein present in the algal and cyanobacterial biomass.

During discussions, and in comments of reviewers, the extraction yield of high Mw protein was often referred to as 'low'. The yields obtained are indeed lower than what is commonly found for leguminous protein isolation yields (64–88% [1]). They are, however, are similar to what was described previously for *Tetraselmis impellucida* (with a high Mw protein yield of 13%[2]), and for sugar beet leaves (12–33% [3]). The question that arises from these data is whether the (high Mw) unicellular protein extraction yields obtained in this study should be considered as 'low'. Instead, these yields may represent the total soluble amount of protein (i.e., the amount of cytoplasmic proteins) present in the unicellular sources, and should thus be considered as 100% yields of the soluble high Mw protein present. The question is therefore what the influence is of the extraction conditions, and if the high amount of low MW proteinaceous material could be the result of endogenous protease activity. Additionally, variations in biomass on the soluble fraction of microalgae are discussed below.

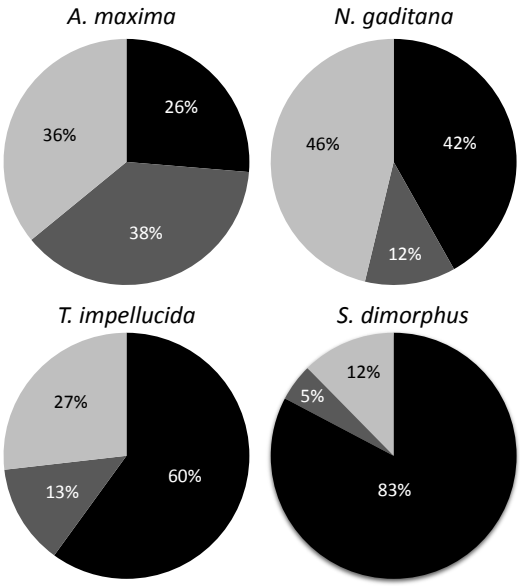

Figure 2: Fraction of protein that is not soluble (black) and fractions of protein that are soluble, of low Mw (< 12–14 kDa; dark grey) and high Mw (> 12–14 kDa; light grey), of various unicellular sources. The solubility was determined at pH 8, after complete disruption of the cells. Percentages shown are the percentage of each protein fraction from the total protein present in each biomass, based on amino acid quantification. For *N. gaditana*, strain AS1301 was used.

Effect of extraction conditions on protein yield

To test whether the (high Mw) protein extraction yield could be improved by altering the pH during extraction, *A. maxima* and *T. impellucida* biomass was dispersed in a potassium phosphate buffer (pH 8.0) to obtain a final biomass concentration of 6% (w/w) DM content and 50 mM buffer concentration. The biomass was bead milled until broken (method is shown in **chapter 2**) and subsequently brought to pH 2–8 (using 1 M HCl). The suspensions were stirred for 1 h at 4 °C and subsequently centrifuged (30 min, 60,000 g, 20 °C). The supernatant was dialyzed (12–14 kDa) against 10 mM NaCl at 4 °C for 48 h. The resulting proteinaceous extraction yield (as measured by N content in the dialyzed supernatant) as a function of pH is shown in Figure 3. These data show that the highest high Mw protein extraction yields were derived upon extraction at alkaline pH (pH 7 / 8). This is in line with what was observed previously in protein extraction of *Arthrospira platensis* [4] (which did not include a dialysis step), where a maximum extraction yield was reported at pH ≥ 7. It was therefore concluded that using a different pH in the extraction procedure of unicellular sources will not improve protein extraction yield considerably.

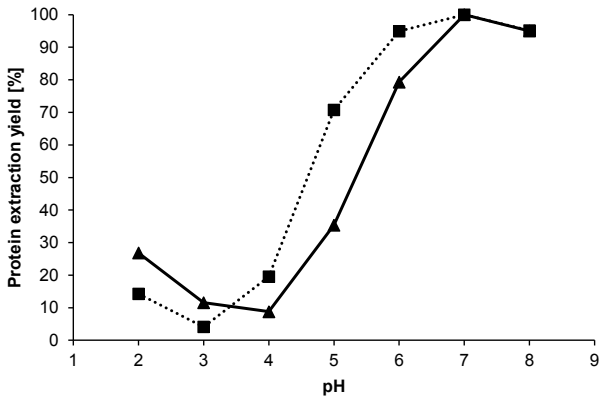

Figure 3: Protein extraction yield of *A. maxima* (solid line) and *T. impellucida* (dotted line) as a function of pH, relative to the maximum yield [i]. Extraction yield is shown after bead milling (at 6% w/w DM; 50 mM buffer), centrifugation and dialysis

In addition to the above, it was shown that the protein compositions of the extracted fractions and of the insoluble fractions were the same for each unicellular source tested (using reducing SDS-PAGE) (**chapter 2**). Furthermore, the amino acid compositions of the soluble and insoluble fractions were the same for each source (**chapter 2**). These observations are in line with SDS-PAGE results for soluble and insoluble fractions of *Tetraselmis impellucida* biomass [2] and of sugar beet leaves [5]. The solubilized proteins can therefore be seen as representative for all proteins present the cell, with the major difference being the state of the proteins (soluble versus insoluble). It is unclear why some of these proteins are in soluble state, whereas other proteins that appear to be similar (on SDS-PAGE) are not. It is hypothesized that (although for unknown reasons) a part of these proteins are cell-wall bound or membrane bound, causing the proteins to be unextractable and, at least appear, insoluble. Another possibility for the reduced protein extraction is that unicellular proteins may be locked inside cell organelles that are not disrupted by bead milling (under the used conditions). In some microalgae, for example, Rubisco can be stored in pyrenoids [6]. Both *Tetraselmis* species [7] and *S. dimorphus* [8] *can* contain pyrenoids, which are cell organelles often enclosed by starch sheaths [7]. The fact that Rubisco is enclosed by these structures may hinder its release during bead milling. It should be noted that the number and structure of pyrenoids present in an algal cell is influenced by culturing conditions (e.g. the CO_2 concentration [9] and dark/light regimes [10]), which may induce variability in the extractability of Rubisco within a single species of algae. For *Nannochloropsis* species, the pyrenoid is seen as a transient structure as it is only occasionally observed in this genus [11-13]. It is not certain whether the *N. gaditana* used in this study contained pyrenoids. Cyanobacteria (including *A. maxima*) do not contain

[i] Unpublished work. MSc thesis of Jingyang Li (under supervision of E. Teuling), "Effect of pH and ionic strength on protein yield and protein composition of soy and algal extracts". Laboratory of Food Chemistry, Wageningen University, 2017. *Performed within the STW "PROGRESS" project.*

pyrenoids [10], which may partially explain that the protein extractability for this species is higher than that of the others.

Overall, there is a significant amount of protein that is not liberated (extracted) after complete cell disruption (i.e. a low plateau height in Figure 1). These unextracted proteins are either insoluble, or they are bound to or entrapped by cell walls and cell organelles (including pyrenoids). Extraction yields could possibly be increased by applying harsh chemicals or enzymatic methods. These types of methods, however, may influence the quality and the applicability of the proteins extracted. In addition, the "insoluble" fractions (containing proteins that could not be liberated) should not be considered as a loss as they can be applied as fish feed ingredients.

Proteolysis by endogenous proteases

Of the proteinaceous material extracted after bead milling, 21–51% was of low Mw (< 12–14 kDa) (Figure 2, since it was removed during dialysis. This low Mw proteinaceous material (peptide or amino acids) can either be naturally present in the unicellular sources, or they can be formed during the extraction process due to hydrolysis by endogenous proteases. For example, from plants it is known that these types of 'losses' of low Mw proteinaceous material can be due to proteolysis by endogenous proteases [14], as was discussed in more detail in **chapter 1**. Proteolytic activity is also reported in microalgae [15], but no data are available on protease related protein yield losses in microalgae or cyanobacteria.

In the protein extraction work presented in this thesis, the temperature under which the protein extractions were performed ranged between 4 °C (e.g. during centrifugation, dialysis and pH precipitation) and 20 °C (during bead milling) (**chapter 2**). It was thus attempted to avoid any enzymatic processes present that could negatively affect protein quality and protein extraction yields. In addition, various unicellular sources were tested on the presence of proteolytic activity, to understand whether the large fraction of small Mw proteins (or peptides and free AAs) is a result of proteolysis. To do so, bead-milled biomass of various unicellular sources was incubated for 2 h at 37 °C [ii]. The protein composition before and after incubation was compared using SDS-PAGE (Figure 4). As a positive control, bead-milled biomass was also incubated under similar conditions in the presence of bovine trypsin (enzyme : substrate ratio of 1:100 w/w). In absence of trypsin, the incubated and non-incubated biomass showed similar SDS PAGE patterns (Figure 4), indicating a very limited, or even absence of proteolysis. When trypsin was present, most of the high and intermediate Mw molecular bands (> 10 kDa) disappeared, which shows that the unicellular proteins were susceptible to proteolysis by trypsin. Since no proteolysis was observed after a 2h incubation at 37 °C, it is a strong indication that proteolysis also does not occur during the protein extraction process (performed at much lower temperatures). Therefore, hydrolysis by endogenous proteases during protein extraction is

[ii] Unpublished work. MSc thesis of Kasper Brandt (under supervision of E. Teuling), "The influence of cell wall strength on *in vitro* hydrolysis and *in vivo* digestibility in Nile tilapia (*Oreochromis niloticus* L.)". Aquaculture and Fisheries group, Wageningen University, 2017. *Performed within the STW "PROGRESS" project.*

not expected to be the reason for the relatively high fractions of low Mw protein and subsequent "losses" of proteinaceous material (peptides and amino acids) during dialysis that are described in this thesis (**chapter 2**) and in literature (see **chapter 1** for examples). Instead, the low Mw fraction of proteinaceous material appear to be naturally present in these unicellular sources in the form of free amino acids or short peptides (<10 kDa).

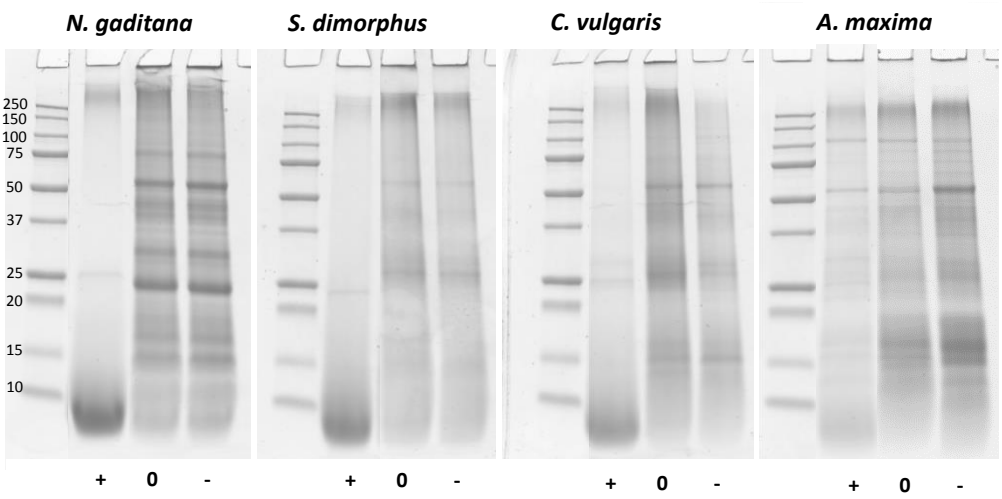

Figure 4: Coomassie stained SDS-PAGE gels of bead milled *N. gaditana* (strain AS1301), *S. dimorphus, C. vulgaris* and *A. maxima*, under reducing conditions [iii]. Samples were incubated with (+) or without (0) bovine trypsin (E:S = 1:100) for 2 h at 37 °C and pH 8.0. The negative control samples (-) were not incubated prior to SDS-PAGE sample preparation. The first lane of each gel contains a Mw marker (molecular weights in kDa shown on first lane).

Effect of strain-to-strain and seasonal variations on protein extractability

During the experimental work of this PhD thesis, a large variation in protein extractability (i.e. the plateau in Figure 1) was observed between algal strains and between batches. It is known that seasonal variations and harvesting conditions can considerably alter the gross chemical composition of microalgae and cyanobacteria (as is shown in [16, 17]). The effect of these altered compositions on protein extractability, however, is not yet studied and is therefore discussed below.

The results obtained with *Nannochloropsis gaditana* are based on multiple batches harvested in different years and various seasons and on 2 different strains (strain nos. AS1301 and AS1405). All *N. gaditana* biomass was grown at the same algae producer (AlgaSpring, Almere, The Netherlands), and harvested and processed (centrifuging to a

[iii] Unpublished work. MSc thesis of Kasper Brandt (under supervision of E. Teuling), "The influence of cell wall strength on *in vitro* hydrolysis and *in vivo* digestibility in Nile tilapia (*Oreochromis niloticus* L.)". Aquaculture and Fisheries group, Wageningen University, 2017. *Performed within the STW "PROGRESS" project.*

paste and subsequent freezing) in a similar way. Only small differences were measured in the gross composition of the biomass of the two different strains (Table 1); based on the compositional analyses presented in **chapter 4** (strain AS1301) and **chapter 5** (strain AS1405). The largest differences between the compositions are the protein versus carbohydrate contents, with 53% and 50% protein (N * 6.25), and 14% and 16% carbohydrates, in strains AS1301 and AS1405, respectively. Despite these minor differences in gross composition, considerable differences were observed in protein extractability (i.e. the % of protein that could be extracted from the total amount of protein present) between the strains. Of strain AS1301, 45–81% protein could be extracted (pH 8, without dialysis) (**chapter 2**); whereas only 6–13% protein could be extracted (using the same extraction method) from strain AS1405 [iv,v] (Figure 5). For strain AS1405, the protein extractability was determined using 9 batches of algae, harvested at different time-points during the year. The variation in protein extractability (6–13%) in this strain was shown to be due to seasonal (or batch-to-batch) variation (Figure 5, [v,iv]). In these data, the batch-to-batch variation is ± 21% (coefficient of variation). A similar variation (coefficient of variation of 32%) is observed in the extraction results of strain AS1301. The extractions performed (n = 3) on strain AS1301 were performed on 2 different batches. Therefore, it is expected that the variation in protein extractability of this strain is also due to batch-to-batch variation. The variation in protein extractability between the *N. gaditana* strains was larger than the difference in protein extractability between batches of the same strain.

Table 1: Strain-to-strain variation in the gross chemical composition of *Nannochloropsis gaditana* biomass [a] (in w/w % on DM; with exception of energy [kJ/kg DM]) and corresponding protein extraction yields (protein that was extracted as a percentage of the protein content of the biomass [w/w %]).

Strain	Energy	Proteins (N*6.25)	Lipids	Carbohydrates	Ash	Total annotated	Protein extraction yield
AS1301	2.4	52.5	15.5	14.0	8.4	93	45–81 [b]
AS1405	2.4	50.0	16.1	16.0	7.2	92	6–13 [c]

[a] The chemical composition was analyzed on a single batch of each strain.

[b] Range due to the use of 2 different batches and multiple extractions performed.

[c] Range due to seasonal, or batch-to-batch variation, using a total of 9 batches.

[iv] Unpublished work. MSc thesis of Jingyang Li (under supervision of E. Teuling), "Effect of pH and ionic strength on protein yield and protein composition of soy and algal extracts". Laboratory of Food Chemistry, Wageningen University, 2017. *Performed within the STW "PROGRESS" project.*

[v] Unpublished work. Research performed by René Kuijpers, "Seasonal variability in protein extractability of *Nannochloropsis gaditana*". Laboratory of Food Chemistry, Wageningen University, 2017. *Performed within the STW "PROGRESS" project.*

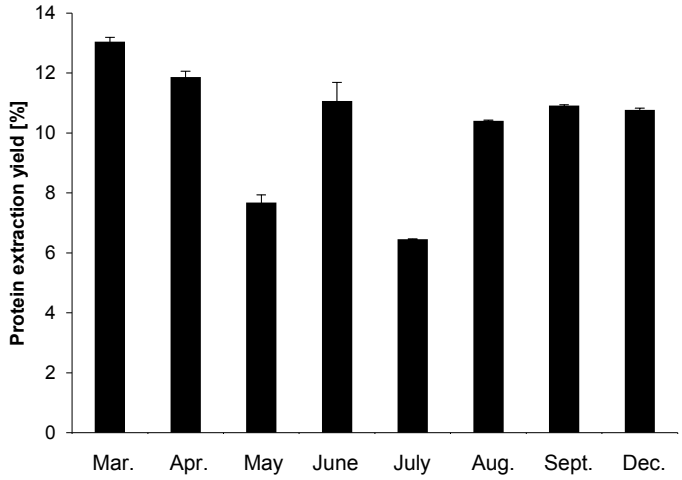

Figure 5: Seasonal variability in protein extractability (at pH 8) of *Nannochloropsis gaditana* biomass (strain AS1405) after bead milling [vi]. The yield is expressed as the protein that could be extracted from the biomass, as a percentage of the total protein content of the biomass (47 ± 4% [w/w on DM]). The biomass was harvested in various months of 2016 at AlgaSpring, The Netherlands. The error bars show the standard deviations between the dry matter and protein analyses.

Overall, the lack of variation in chemical composition between the samples does not explain the variation in protein extractability. It is believed that the variation in protein extractability between the strains is not due to experimental conditions that might be considered, like the sample production and the sample characteristics (dry matter content and viscosity) during bead milling and centrifugation. The sample production (including harvesting and freezing conditions) were similar for both strains. Variations in pyrenoids, if present at all (as explained above), are thus not a likely cause of the extractability variation between the strains. The dry matter content of the pastes obtained by centrifuging the biomass varied (18% and 30% for strains AS1301 and AS1405, respectively). Since these pastes were diluted to the same dry matter concentration prior to bead milling, this variation in dry matter content of the paste should not have affected cell disruption or sample behavior during bead milling. The differences in protein extractability could have been due to differences in viscosity [18] and in cell robustness (**chapter 4**), if the cell disruptive efforts applied (e.g. bead milling time or speed) were insufficient (Figure 1). It was observed that *N. gaditana* suspensions were more viscous than suspensions of the other algae/cyanobacteria at equal concentrations, but no differences in viscosity were recorded between the two *N. gaditana* strains. Moreover, final cell disruption was not likely to be affected by variations in viscosity or cell robustness, since bead milling was performed until the cells were visually disrupted (using light microcopy), not using a standardized duration

[vi] Unpublished work. Research performed by René Kuijpers, "Seasonal variability in protein extractability of *Nannochloropsis gaditana*". Laboratory of Food Chemistry, Wageningen University, 2017. *Performed within the STW "PROGRESS" project.*

of milling. Unfortunately, differences in bead milling time needed to disrupt the cells (i.e. the cell wall robustness) between the 2 strains were not recorded. Overall, it is therefore unknown what causes the observed in the ratio of unextracted (insoluble or bound) and extracted (cytoplasmic) proteins observed between the strains.

In conclusion, although the gross composition and protein composition of the two *N. gaditana* strains used were similar, the protein extractability was significantly different between the strains. These data indicate the significance of including the strain information in research performed on protein extraction (and on *in vivo* digestibility, as will be discussed below). Unfortunately, the majority of research published on extraction and techno-functionalities of unicellular protein only reports the genus and species of the source used in the experiments performed (e.g. in [4, 19-22]). Moreover, in some cases even the type of species is not mentioned (e.g. [2, 23]) and only the genus information is included. Only a small number of these publications report the strain or culture collection of the species used (e.g. [18, 23, 24]). The strain-dependent variation appears to be underestimated in this field of research. The lack of attention to this strain-to-strain variation may lead to seemingly contradictory information on protein extractability and protein techno-functionality. Essentially, strain selection of unicellular sources seems essential for predicting the applicability of unicellular species as novel protein sources in food and feed.

C_{CR} AS A TOOL TO COMPARE EMULSION BEHAVIOR OF PROTEIN INGREDIENTS

In **chapter 1** it is described how various methods are used in literature to quantify the emulsifying ability of proteins. One commonly used method is the emulsifying capacity, which describes the volume of oil that could be emulsified per g of protein. In this method, a specific volume or concentration of protein solution is used, to which oil is added (under homogenization) until phase inversion occurs. Due to this methodology, the emulsifying capacity parameter is often dominated by the volume fraction of oil (Φ_{oil}) at which phase inversion occurs, instead of by the characteristics of the protein ingredient. In the emulsion work presented in **chapter 3**, a method was used in which various emulsions are prepared with a fixed Φ_{oil} and a varying concentration of protein in the water phase. In these experiments, the Φ_{oil} used is below the point of phase inversion. The parameter measured in these experiments is the droplet size ($d_{3,2}$) of the emulsions prepared. In this work, the critical protein concentration (C_{cr}) was selected as a quantitative tool to characterize the emulsion ability of a protein. The C_{cr} is the protein concentration above which emulsions are formed which have a minimum droplet size ($d_{3,2,min}$). In contrast to the emulsifying capacity, the C_{cr} depends on both the interfacial and molecular protein properties, including the adsorption rate constant (k_{adsorb}), the adsorbed amount of protein on the interface (Γ_{max}) and the protein radius (R_p). Previous work on various dairy proteins [25] (including β-lactoglobulin and WPI), potato proteins (patatin [25]) and leaf proteins (sugar beet leaf [26]), have confirmed the relationship between C_{cr} and interfacial and molecular protein properties. This relation between protein properties and C_{cr} makes the use of C_{cr} a potentially useful tool to compare the emulsifying abilities of different proteins.

From the data obtained in this PhD thesis (**chapter 3**) and from literature, the C_{cr} values appear to be sensitive to variation between experiments. These variations give rise to the question how sensitive C_{cr} is to the way the emulsion experiments are performed and to the determination of the exact value of C_{cr}.

Table 2: Variations in the critical protein concentration observed for β-lactoglobulin and whey protein isolate.

	β-lactoglobulin		**Whey protein isolate**	
Reference	*25*	*27*	*28*	Chapter 3
C_{cr}/Φ_{oil} [mg protein/mL oil]	20 [a]	50 [a]	12 [a]	18 [c]
Φ_{oil} [-]	0.1	0.1	0.3	0.01

C_{cr}/Φ_{oil}: critical protein concentration corrected for the oil fraction;
Φ_{oil}: oil fraction of the emulsion [-];
System conditions: [a] pH 7, I = 10 mM; [b] pH 7, I = 20 mM; [c] pH 8, I = 10 mM.

The determination of C_{cr} appears to be sensitive to the way the C_{cr} value is obtained from the droplet size measurements performed on the emulsions. The C_{cr} can either be computed (or interpolated) from the data points measured (emulsion droplet size over protein concentration), as was done in **chapter 3** and in literature (e.g. *26*). Another way to define the C_{cr} is to use the lowest protein concentration that was tested, at which the droplet size equals $d_{3,2,min}$ (e.g. used in *25, 27*). The first approach was applied in **chapter 3**, yielding C_{cr} values of 0.17 mg protein / mL for WPI and 0.09, 0.41 and 0.74 mg protein / mL for isolates obtained from *N. gaditana*, *A. maxima* and *T. impellucida*, respectively. If the second approach would have been used, the C_{cr} values for WPI and the algae protein isolates would have been 0.3, 0.1, 0.5 and 1.0, respectively. These C_{cr} values determined would thus be 13–74% higher using the second approach than in the first approach. An example of variation caused by the second approach (using C_{cr} as minimum protein concentration tested at which $d_{3,2} = d_{3,2,min}$) is for example shown in the variation of C_{cr} of β-lactoglobulin measured by Delahaije et al. in two different studies in which the same system conditions and the same Φ_{oil} are used *25, 27*. Delahaije et al. reported C_{cr} values of 2 and 5 mg/mL, resulting in C_{cr}/Φ_{oil} values of 20 and 50 mg protein / mL oil (Table 2). This shows that especially in the second approach, the number of data points taken in the protein concentration range near the C_{cr} will greatly affect the final C_{cr} determined. It should be noted that also computation or interpolation of the C_{cr} from the surrounding data points (i.e. the fist approach) is likely to be prone to errors when insufficient data points in the protein concentration range near the C_{cr} are included.

Next to the above described sensitivity of the C_{cr} value to the method this value is obtained from the emulsion data, the C_{cr} also appears to be sensitive to the oil fraction (Φ_{oil}) used. To allow comparison between studies, the C_{cr} values obtained from literature and those obtained within this PhD were standardized on the Φ_{oil} used in the emulsions (using C_{cr}/Φ_{oil}) (as shown in **chapter 3**). The C_{cr} ought to be linearly correlated to the Φ_{oil}. The choice of Φ_{oil} should therefore not affect the C_{cr}/Φ_{oil} measured, as long as the Φ_{oil} chosen is

below the point of phase inversion. Based on the emulsion capacity data provided by Karaca et al. using protein isolates of various legumes [29], this point of phase inversion was calculated to be at a Φ_{oil} of 0.56–0.58. This is close to the theoretical maximum packing density of oil droplets in water (at Φ = 0.64 for hexagonal packing). In **chapter 3**, this linear correlation was also confirmed for WPI: the C_{cr} of emulsions with Φ_{oil} = 0.01 was measured to be 10 times lower than that of emulsions prepared with Φ_{oil} = 0.1. Upon comparing the C_{cr}/Φ_{oil} values to literature, however, it appears that there may be a minimum Φ_{oil} needed for reliable C_{cr} quantification. More specifically, it is expected that above a certain Φ_{oil}, the sensitivity of C_{cr} quantification to variations in homogenizing process as a function of the Φ_{oil} may become negligible. An example of the data this hypothesis is based on are the C_{cr}/Φ_{oil} values of WPI stabilized emulsions presented in **chapter 3** (18 mg protein/mL oil) and in a study by Schwenzfeier et al. (12 mg protein/mL oil [28]) (Table 2). In these studies, similar system conditions were used: pH 7 (**chapter 3**) and pH 8 (Schwenzfeier et al.), both at ionic strengths of 10 mM, and the experiments were performed at the same laboratory (using the same equipment). The main difference between the studies is the Φ_{oil}, which was 0.01 and 0.3 in this study and that of Schwenzfeier et al., respectively. A lower Φ_{oil} yields a lower number of emulsion droplets in the system, and therefore also yields an increased distance between those droplets. When plotting the approximate distance between emulsion droplets (using $d_{3,2,min}$ = 0.3 μm) as a function of $\Phi_{oil,}$ a logarithmic correlation is observed (Figure 6). This variation in distance between emulsion droplets as a function of Φ_{oil} has consequences for the emulsion formation during homogenization. It is hypothesized that in systems with a lower Φ_{oil} (e.g. the 0.01 used in **chapter 3**) there is a longer time between collision of oil droplets upon homogenization than in systems with a higher Φ_{oil}.(e.g. in the 0.3 used in [28]). This prolonged time is expected to give the proteins more time to adsorb to the interface, finally yielding a lower C_{cr}/Φ_{oil} than what would be determined for systems with a higher Φ_{oil}. There appears to be a typical distance between oil droplets above which the system (quantified by C_{cr}) is no longer sensitive to variations in Φ_{oil}. In other words, the expectation is that there is a minimum Φ_{oil} above which the C_{cr}/Φ_{oil} determined will be constant, regardless of the Φ_{oil} used. As mentioned above, for C_{cr} quantifications, the Φ_{oil} chosen should stay be the point of phase inversion (i.e. $\Phi_{oil} \leq$ 0.57–0.64; Figure 6). From Figure 6, it is estimated that the C_{cr}/Φ_{oil} will be constant when the droplet distance is below ~3E-7 μm, correlating to a Φ_{oil} of > ~0.125. Quantifying the exact value for the minimum Φ_{oil} would be a valuable next step in protein emulsifying research.

Overall, the C_{cr} can be considered as a useful tool to quantitatively compare the emulsifying abilities of various proteins, or protein ingredients. It is, however, sensitive to Φ_{oil}, and to the way the C_{cr} is retrieved from the data obtained. It is hypothesized that this variation can be decreased using a minimum Φ_{oil} (> ~ 0.125) and sufficient data points in the protein concentration range near the C_{cr}.

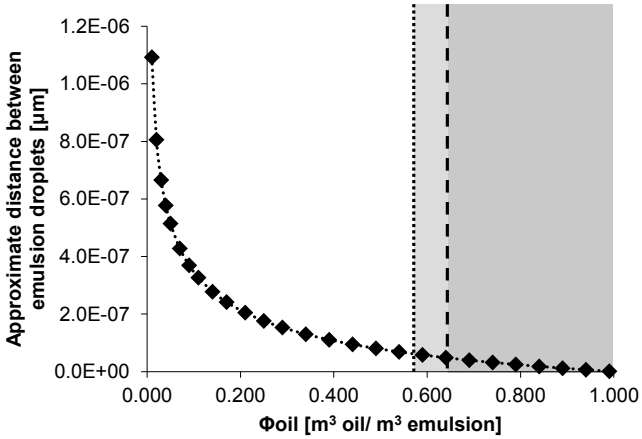

Figure 6: Approximate distance between emulsion droplets [µm] as a function of the volume fraction oil (Φ_{oil}) [-] used in an emulsion. A droplet size of 0.3 µm is assumed. The grey areas indicate the Φ_{oil} ranges at which phase inversion of the emulsion is expected. These ranges are based on experimental data (Φ_{oil} = 0.57, based on [29]; dotted line), and theory (Φ_{oil} = 0.64, assuming hexagonal packing; dashed line).

NUTRIENT DIGESTIBILITY OF UNICELLULAR PROTEIN FRACTIONS IN FISH

For optimum use of unicellular sources in both food and fish feed, it would be beneficial if the side streams produced during extraction of proteins for food applications (e.g. the "insoluble" fractions that are discussed above) could be used in the fish feed industry. In the work presented in this PhD thesis, the nutrient digestibility of the whole biomass of unicellular sources was studied, using either intact cells (**chapter 4**) or disrupted cells (**chapter 5**). The data obtained in **chapter 4** reveal that protein digestion took place in all unicellular sources tested (67–83% ADC) even though the cells were fed in an untreated ("intact") form. The protein ADC of a microalgae increased when the cells of the biomass were disrupted (**chapter 5**). This was attributed to an increased *in vivo* protein accessibility, i.e. an increased extent to which proteins of unicellular sources are accessible to enzymes. Using the data obtained in these chapters, the expected digestibility of the insoluble fraction created during the protein extraction process (**chapter 2**) was estimated, as is discussed below. The potential of this fraction (or side stream) is discussed excluding the effect of processing of both the ingredient (i.e. the algae and cyanobacteria) and the feed. Naturally, the potential value of any protein ingredient will be influenced by further (feed) processing of the fractions, as is for example shown in the formation of Maillard reaction products during processing (e.g. [30] and **chapter 5**).

To clarify the different algal and cyanobacterial fractions discussed in this section, a (schematic) fractionation overview is shown in Figure 7. In the first fish trial performed within this PhD research (**chapter 4**), the nutrient digestibility of whole algal and cyanobacterial cells (i.e. Figure 7, fraction A) was studied. In that study, 4 different

unicellular sources were used that varied in (amongst others) their cell wall composition and in the corresponding resistance of the cell walls to mechanical disruption. The protein digestibility of these unicellular sources was limited (67–83% apparent digestibility coefficient; ADC) in both African catfish and Nile tilapia, and varied between the unicellular sources.

Since protein and fat digestibility (both inner-cell nutrients) showed the same relative differences between the unicellular sources, it was hypothesized that digestibility of these nutrients is affected by their accessibility. The accessibility of proteins and fat to digestive enzymes is hypothesized to be negligible when these nutrients are present in intact cells (i.e. not disrupted; Figure 7, fraction A). Since digestion of protein and fat took place (≥ 65% ADC), these nutrients were, apparently, accessible to digestive enzymes. The differences in accessibility of protein and fat between the various unicellular sources is hypothesized to be related to differences in the cell walls of these sources (type, structure, etc.). Cell robustness (Figure 1), however, when quantified by the bead milling time needed to disrupt the cells, did not relate to these differences in protein and fat digestibility between the species. To confirm the role of nutrient accessibility to nutrient digestibility in unicellular sources, *Nannochloropsis gaditana* biomass was subjected to various treatments that influenced its cell wall integrity (**chapter 5**), including a treatment that completely disrupted the cell walls (i.e. Figure 7, fraction B). These treatments increased nutrient accessibility (measured *in vitro*) and, in Nile tilapia, also increased the protein digestibility (from 62 to 78% ADC) and the fat digestibility (50 – 82 % ADC). These results confirmed that cell walls of unicellular sources hinder the digestibility of inner-cell nutrients (proteins and fat) of these sources in fish. However, the protein digestibility of the disrupted cells (78%) is still not very high. Additionally, these results did not yet elucidate what protein ADC levels can be expected of the side streams produced during protein extraction processes (i.e. Figure 7, fraction D), and how this would compare to the nutrient ADCs of a soluble protein extract (Figure 7, fraction C).

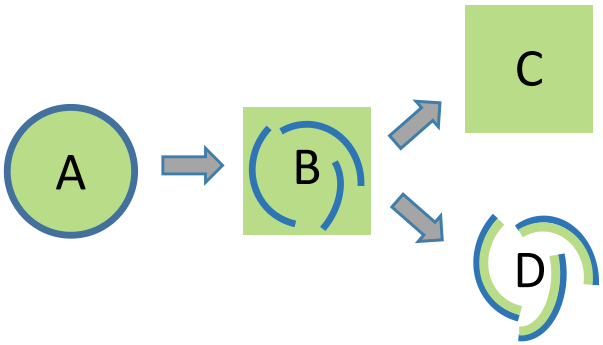

Figure 7: Schematic overview of microalgal and cyanobacterial fractions obtained during protein extraction processes. A = untreated biomass; B = cell disrupted biomass; C = soluble fraction of the biomass; D = insoluble fraction of the biomass.

Protein digestibility of intact cells

It is hypothesized that protein and fat digestibility from unicellular sources in fish is dominated by the presence or absence of an intact cell wall (i.e. the accessibility; **chapter 5**). However, as mentioned above, the protein digestibility from "intact" cells of *A. maxima, C. vulgaris*, *N. gaditana* and *S. dimorphus* was 67–83% ADC in both Nile tilapia and African catfish (**chapter 4**). Similar results were obtained for the fat digestibility (65–89% ADC). In this experiment, the unicellular sources were incorporated in the feed as untreated cells, which were therefore considered to be intact cells (i.e. Figure 7, fraction A). Both protein and fat are nutrients that are mostly present inside microalgal and cyanobacterial cells, and were thus enclosed by cell walls in these samples. Since digestion did take place, protein and fat were accessible to digestive enzymes in the proximal intestine. This is before any fermentation of cell wall carbohydrates can take place (which is usually in the distal intestine). The cells must have already been (at least partially) opened in or prior to reaching the proximal and mid intestines of both fish species. In **chapter 4** it is discussed how ingredient and feed processing most likely did not cause damages to the cells to such an extent that protein and fat could be accessible and subsequently digested to > 65% ADC. It is described how, subsequent to minor processing damages, the cell walls were probably further disintegrated by the acidic stomach conditions of both Nile tilapia and African catfish, enabling hydrolysis and digestion of protein and fat to take place.

From another perspective, it can be debated that the protein ADCs determined in Nile tilapia are quite low, considering the fact that both cyanobacteria [31-33] and microalgae [32, 33] are part of the natural diet of this fish species. Nile tilapia are filter-feeding fish known for their ability to feed on low-trophic level material. Their digestion efficiencies (a value based on the organic content of the food and feces) of the cyanobacterium *Microcystis aeruginosa* was reported to be 58.6 to 78.1% [34]. This is higher than that the filter-feeding carp species bighead and silver carp, which had a 25–30% digestion efficiency for the same cyanobacterium species [35]. According to Moriarty et al., the ability of Nile tilapia to feed on cyanobacteria is due to the low stomach pH of this fish species, which he showed was able to lyse the cell walls of cyanobacteria [31]. The difference in digestion efficiency between the fish species may thus be due to the presence of a stomach (and related low pH) in tilapia, compared to the absence of a stomach in carp species. Although many of the above mentioned publications use the terminology 'algae' or 'microalgae', and some researchers have indeed included true microalgae in their research (e.g. [36]), utilization and cell wall lysis of unicellular sources in Nile tilapia has only been shown for cyanobacteria [31, 34]. Possibly, Nile tilapia is only capable of lysing cyanobacterial cells due to the cell wall differences between cyanobacteria (peptidoglycan) and microalgae (often cellulose based), as discussed in **chapter 1** and **chapter 4**. This would explain why the protein and fat ADCs of the cyanobacteria *A. maxima* (82% and 83%) were higher than that of the microalgae *N. gaditana* (75% and 75%) and *S. dimorphus* (67% and 65%). It does not explain, however, the similarity in protein and fat ADCs between *A. maxima* and the microalgae *C. vulgaris* (81% and 84%) (**chapter 4**). This difference may be caused by differences the cellulosic cell walls of the microalgae: both *N. gaditana* and *S. dimorphus* have cellulose based cell walls with

an additional outer hydrophobic algaenan layer [37-39]. These types of cell walls may be more resistant to acid degradation (in the stomach) than the cellulose based cell wall of *C. vulgaris* that lack the algaenan layer [40]. It could also be hypothesized that freshwater species like Nile tilapia (and African catfish) would be more capable of digesting microalgae originating form freshwater environments. However, the results obtained in **chapter 4** oppose this hypothesis, since the protein and fat ADCs were highest for the freshwater *C. vulgaris*, followed by the marine species *N. gaditana* and least for the freshwater species *S. dimorphus.* Overall, due to the low stomach pH in Nile tilapia, and to a lesser extent in African catfish, all intact cyanobacteria are expected to be digested to a relatively high extent in these fish species. The combination of cellulose and algaenan in the cell walls of intact *N. gaditana* and *S. dimorphus*, are expected to be the reason for limited nutrient digestion of these sources in both fish species.

Protein digestibility of insoluble side streams

As stated above, it is hypothesized that protein digestibility from unicellular sources in fish is dominated by the presence of an intact cell wall (i.e. the accessibility; **chapter 5**). Hence, upon disrupting the cells and thereby increasing the protein accessibility, the cell wall is no longer a limiting factor in protein digestibility for fish. Upon fractionating the disrupted biomass, "soluble" and "insoluble" streams are created (Figure 7, fractions C and D). It is hypothesized that the protein digestibility from these fractions is dominated by the solubility of the proteins and by their accessibility. In this case accessibility is expected to differ between proteins that are entrapped in cell walls and organelles, bound to cell walls and soluble proteins. These variations between the fractions and the expected digestibility of these fractions are discussed below.

Unicellular protein fractions

The "insoluble" fraction (Figure 7, fraction D) has a few similarities to the cell disrupted biomass (Figure 7, fraction B). As is shown in **chapter 2**, the total biomass and the soluble and insoluble fractions of each microalgae and cyanobacterium used have near identical protein compositions (as analyzed with reducing SDS-PAGE). Additionally, with exception of *Scenedesmus dimorphus*, the protein contents of those fractions are also near identical (Table 3). Although other chemical components were not analyzed in these fractions, the total carbohydrate content of the various fractions is also expected to be similar, based on the carbohydrate contents analyzed by Schwenzfeier et al. on *Tetraselmis impellucida* biomass (24% carbohydrates) and on the insoluble fraction of this biomass (28% carbohydrates) [2]. It should be noted, however, that the type of carbohydrates does differ between the soluble and insoluble fractions [2]. It is expected that the carbohydrates of the insoluble fraction predominantly consist of cell wall carbohydrates, a type of non-starch polysaccharides (NSP). This cell wall fraction may affect nutrient digestion in fish. In a number of fish species (including Nile tilapia [41] and African catfish [42]), protein digestibility decreased with increasing content of soluble NSP in the feed. On the other hand, cell wall polysaccharides from microalgae are also suggested to be the functional ingredient that can

counteract soy bean meal induced intestinal inflammation (as was e.g. shown with *Chlorella vulgaris* in Atlantic salmon [43]). Apart from these polysaccharides no major differences are measured and/or expected between the biomass and the soluble and insoluble fractions of the unicellular sources used.

Table 3: Protein content of the total biomass and of soluble and insoluble fractions [a] of various unicellular sources, on a dry matter basis; ±SD. Adapted from **chapter 2**.

	Arthrospira maxima	***Nannochloropsis gaditana***	***Tetraselmis impellucida***	***Scenedesmus dimorphus***
Biomass (fraction A/B)	62 ± 0.5	45 ± 0.6	36 ± 1.9	29 ± 5.0
Algae juice (fraction C)	64 ± 0.9	43 ± 0.4	35 ± 1.3	19 ± 15
Pellet (Fraction D)	60 ± 0.5	41 ± 0.2	35 ± 2.5	32 ± 2.6

[a] fractions A, B, C and D refer to the fractions illustrated in Figure 7.

In vitro protein accessibility

As mentioned above, the differences between the "soluble" and "insoluble" fractions are expected to lie in the accessibility of the proteins present and in the different state the proteins are in (soluble versus insoluble). Concerning the accessibility, it is not known whether the interactions of protein with the cell walls/membranes and their possible entrapment in cell organelles hinder their accessibility to proteolytic enzymes. The tryptic hydrolysis performed on cell disrupted biomass of *A. maxima*, *C. vulgaris*, *N. gaditana* and *S. dimorphus* (Figure 4) showed that trypsin was able to hydrolyze the majority of the high Mw and intermediate Mw protein present in each sample. This indicates that also the "insoluble" protein fraction, representing 26–83% of the proteins present (Figure 2), could be and were hydrolyzed by trypsin, i.e. digestive enzymes, under the conditions used. Thus, insolubility and/or entrapment (cell wall, membranes, and organelles) of the proteins present did not pose a profound restriction on proteolysis. To gain more understanding on the accessibility of the proteins in the "insoluble" fraction to proteolysis, preliminary research [vii] was conducted on the *in vitro* protein hydrolysis (using trypsin) of "soluble" and "insoluble" fractions of *C. vulgaris* and *A*. *maxima*. The results obtained indicated that the proteins of the "insoluble" fraction (i.e. Figure 7, fraction D) could be hydrolyzed (thus the proteins were accessible to proteolytic enzymes), although the final degree of protein hydrolysis measured was somewhat lower than that of the proteins in the "soluble" fraction. More specifically, for *A. maxima* the degrees of hydrolysis obtained were 10.2, 9.1 and 7.8% for the cell disrupted, "soluble" and "insoluble" fractions, respectively, and for *C. vulgaris* the degrees of hydrolysis were 9.2, 9.5 and 6.9% for the cell disrupted, soluble and insoluble fractions, respectively (Table 4). The proteins in the "insoluble" fraction of the unicellular sources are thus expected to be essentially accessible to digestive enzymes in fish. Based on the decreased DH, the protein ADC of the "insoluble" fraction is hypothesized to be lower, however, than that of the "soluble" fractions.

Table 4: Apparent digestibility coefficients (%; ADC[a]) of protein in Nile tilapia and degrees of protein hydrolysis (%, DH[c]) of various fractions[b] of unicellular sources.

	***N. gaditana* (AS1301)**	***N. gaditana* (AS1405)**	***A. maxima***		***C. vulgaris***		***S. dimorphus***
	ADC%	ADC%	ADC%	DH%	ADC%	DH%	ADC%
Intact (**A**)	74.7	61.5	82.5	-	80.9	-	67.0
Disrupted (**B**)	-	78.0	-	9.1	-	9.2	-
Soluble fraction (**C**)	-	-	-	10.2	-	9.5	-
Insoluble fraction (**D**)	-	*75*[d]	-	7.8	-	6.9	-

[a] Data from chapters 4 and 5.

[b] Fractions A, B, C and D refer to the fractions illustrated in Figure 7.

[c] Tryptic hydrolysis, data from [vii].

[d] Calculated value, assuming a protein extractability of 13% (Figure 8) and a protein ADC of 100% for fraction C.

Role of protein solubility on in vivo proteolysis and digestion in fish

The location of protein and peptide degradation and subsequent digestion in the digestive tract of fish is expected to be different for soluble and insoluble fractions. It can be hypothesized that the kinetics in digestion differ between soluble and insoluble protein fractions. As will be explained below, protein solubility can either lead to a decreased digestibility (due to an increased passage rate in the gastrointestinal tract), or it may lead to an increased digestibility (due to increases accessibility to proteolysis). Data suggesting an increased passage rate of soluble protein fractions compared to insoluble protein fractions was proved by Harter et al [44]. Harter et al. estimated the cumulative protein ADC in the gastrointestinal tract of African catfish. They observed that diets that only varied in starch and fat ingredients had a significant difference in protein "ADC" in the stomach. Since no protein (or peptides / amino acids) is absorbed in the stomach, Harter et al. suggested that this observation may be due to differences in protein solubility and the related passage rate of the protein fractions. In other words, the data suggest that soluble protein fractions have a shorter passage time in the gastrointestinal tract than insoluble fractions. The shorter residence time of proteins in the stomach could lead to a lower degree of hydrolysis by pepsin, resulting in larger peptides (or larger protein fractions) entering the proximal intestine. In humans, it is known that proteins that are insoluble in the stomach (e.g. caseins) are hydrolyzed to a lesser extent than proteins that are soluble in the stomach (e.g. whey) [45]. This decreased protein hydrolysis is also shown to lead to a decreased amino acid uptake (lower amino acid concentrations in the blood) [45]. In fish, little is known about the fate of proteins that are hydrolyzed to a lesser extent in the stomach (i.e. larger peptides/proteins), in the rest of the digestive tract; i.e. whether these peptides can be further hydrolyzed and absorbed by the fish. Additionally, in aquaculture, very little is

[vii] Unpublished work. MSc thesis of Kasper Brandt (under supervision of E. Teuling), "The influence of cell wall strength on *in vitro* hydrolysis and *in vivo* digestibility in Nile tilapia (*Oreochromis niloticus* L.)". Aquaculture and Fisheries group, Wageningen University, 2017. *Performed within the STW "PROGRESS" project.*

known about the proteolysis kinetics of soluble and insoluble proteins in the gastrointestinal tracts of fish. *In vitro* protein hydrolysis experiments, however, have been performed on soluble and insoluble protein fractions. Data on pepsin hydrolysis is not available, but various studies have been performed on tryptic hydrolyses. These studies on trypsin hydrolysis of soluble and insoluble proteins show contradicting results. In a study conducted by Tonheim et al. [46], soluble protein fractions of various fish meals were hydrolyzed by a mixture of trypsin, chymotrypsin and a bacterial protease (at pH 8 and 22 °C). Digestibility was calculated as the fraction of nitrogen in the hydrolyzed sample that remained soluble after TCA precipitation. The researchers report a higher digestibility of the soluble fractions (up to 3x higher) of all 7 fish meals tested, after enzyme incubations of 1 and 12 h. In contrast, a study by Salazar-Villanea et al. [47] report a only minor differences in protein hydrolysis of soluble and insoluble rapeseed meal proteins. In this study, protein hydrolysis was performed using a mixture of trypsin, chymotrypsin and porcine intestinal peptidase at pH 8 and 39 °C. The degree of hydrolysis and the kinetics of the hydrolysis were determined with the pH-stat method. The data obtained indicate that the soluble proteins of rapeseed meal hydrolyzed to a slightly lower extent than the insoluble proteins (1.3–1.5 x lower). In contrast, the kinetics of the hydrolyses determined reveal that the soluble proteins were hydrolyzed faster than the insoluble proteins, but this difference was only marginal. *In vitro* data on differences in peptic and tryptic hydrolyses between soluble and insoluble proteins is very relevant to elucidate *in vivo* hydrolysis and digestion processes in fish, but these data cannot yet be obtained from the literature published thus far.

The data provided by the *in vivo* digestive study of Harter et al. [44] suggest an overall positive effect on protein ADC by protein solubility. Their data indicate that proteins that had a short residence time in the stomach (and were therefore thought to be soluble proteins) were, overall, better digestible to African catfish (increased ADC%) than the protein fractions that had a longer residence time in the stomach. Based on these data, it can be hypothesized that the hydrolysis rate of pepsin in the stomach is higher for soluble protein fractions than for insoluble proteins fractions. Subsequently, this increased hydrolysis rate could compensate for the shorter residence time of the soluble proteins in the stomach. Data that may support this hypothesis are provided by Sveier et al. [48]. They report that variations in the gastrointestinal residence time of fish meal protein in Atlantic salmon (generated by large particle size of the fish meal) did not affect overall nitrogen digestibility. Overall, the (*in vivo*) data imply that regardless of shortened stomach residence times, a higher protein solubility would, in the end, result in a higher protein digestibility in fish.

Role of protein solubility in digestion of unicellular sources

The discussion above dealt with the effect of protein solubility on protein hydrolysis and digestibility in fish. In unicellular sources, however, the “insoluble” fractions are expected to also contain soluble proteins that are entrapped by cell walls, cell membranes, and/or organelles (and that are thus less accessible). The *in vitro* hydrolysis experiments discussed above indicated that the reduced accessibility of these proteins reduced, but not inhibited,

protein hydrolysis. *In vivo*, protein digestion is both related to protein accessibility and protein digestibility. Using these factors, the overall expected digestibility of the various protein fractions (Figure 7) of unicellular sources in fish is discussed below.

When discussing the protein solubility unicellular sources in relation to digestive processes in fish, the stomach pH of fish should be taken into account. The pH of the stomach is affected by fish species [49] and by the feed [50]. For example, the stomach pH of tilapia fed a standard diet (of pH 5.8) was recorded to drop to pH 3.2 at 3 h after feeding and to pH 2 at 7 h after feeding [50]. It can be assumed that immediately after feed ingestion, the stomach pH was close to that of the feed, i.e. pH ~ 5.8. These pH variations will affect protein solubility of the feed in the stomach. Taking the diet with pH 5.8 as example, the (cytoplasmic) proteins present in *T. impellucida* and *A. maxima* are expected to be > 80% soluble at pH 5.8 (right after feeding), and < 30% soluble at pH 2–3 (a few hours after feeding) (Figure 3) in the stomach of Nile tilapia. Similarly, the (cytoplasmic) proteins of *Nannochloropsis oculata* are expected to be completely soluble at pH 5.8, and < 10% soluble at pH 2–3 [20]. Thus, proteins of microalgae that were initially soluble during the protein extraction process are likely to turn insoluble after a certain time in the stomach, depending on the initial stomach pH and the diet.

In the discussion above, it was discussed how protein solubility may affect protein digestibility in fish. The data obtained in this thesis, using the 2 *N. gaditana* strains, also suggest an effect of protein solubility on protein digestibility. As was discussed above and is shown in Table 1, the 2 strains of *N. gaditana* used varied greatly in the fraction of "soluble" versus "insoluble" protein (i.e. the fraction that could be extracted and the unextracted fraction). Next to these differences in protein solubility, the *in vivo* protein digestibility in fish was also shown to vary between the strains. The protein ADC in Nile tilapia of the *N. gaditana* strain AS1301 was 75% (using intact cells) (**chapter 4**), whereas it was 62% for the *N. gaditana* strain AS1405 (also using intact cells) (**chapter 5**); Table 4. The experimental set-up, diet formulation and husbandry conditions were near identical between the 2 fish trials conducted. Additional proof for the similarities between the trials was provided by the equal protein digestibility of the control diet in both trials (89.8% and 89.5% ADC in the trials conducted in **chapter 4** and **5**, respectively). Therefore, the variation in protein ADCs obtained between the strains can be attributed to differences in those strains. These ADCs are obtained from inclusion of "intact" cells (i.e. Figure 7, fraction A) in feed. Since variations in growing conditions of microalgae can affect cell wall thickness and cell wall strength (as was previously shown in *Nannochloropsis* sp. [51]), the variation in protein digestibility between the strains may be attributed to variations in *in vivo* protein accessibility (i.e. the "cell robustness" and "effort" shown in Figure 1) and subsequent digestibility. However, the extractability experiments (Table 1), which were performed on cells that were completely disrupted, indicate a large difference in the fraction of soluble protein between the strains. It thus appears that the decreased fraction of soluble protein (i.e. the extractability) of strain AS1405 compared to strain AS1301 is reflected in the decreased *in vivo* protein digestibility of this strain. In other words, the data imply that

soluble (or extractable) protein fractions are better digestible in fish than the “insoluble” (unextractable) protein fractions (i.e. Figure 7, fraction D).

However, the extractability and digestibility data obtained from both *N. gaditana* strains additionally indicate that the protein present in the insoluble fraction is (at least partially) digested by Nile tilapia. This observation is in line with the *in vitro* protein hydrolysis results discussed above). More specifically, taking strain AS1405 as example, the protein extractability or solubility was 6–13%. The protein ADC of this strain was 78% (for cell disrupted *N. gaditana*) (**chapter 5** and Table 4). Since maximally 13% of the protein in strain was soluble, and assuming a protein ADC of 100% for this soluble fraction (Figure 7, fraction C), the insoluble protein fraction (Figure 7, fraction D) is calculated to have a protein ADC of at least 75% (Table 4). It should be noted that the true overall protein ADC is likely to be higher, since not all biomass was disrupted in this experiment (it was estimated that 60–80% of all cells were disrupted) (**chapter 5**). Overall, it is hypothesized that the protein of the insoluble fraction is digested by Nile tilapia to a similar extent as the cell disrupted biomass, but to a lower extent than the soluble protein fraction. The difference in protein ADC of the 2 strains in intact form (not disrupted; Figure 7, fraction A) are attributed to differences in the cell robustness (e.g. due to variations in cell wall thickness or composition) and to the ratio soluble to insoluble protein.

Overall, it is hypothesized that protein digestibility in fish of the insoluble fraction, i.e. the side stream obtained by protein extraction processes for food (Figure 7, fraction D), will be higher than that of the intact cell fraction (i.e. Figure 7, fraction A) due to an increased protein accessibility. The protein digestibility of the insoluble fraction is expected to be lower than the protein ADC of a soluble protein fraction (i.e. Figure 7, fraction C). Regardless of the protein source, to progress knowledge of nutrient digestibility in fish, more insight needs to be gained in the fate of soluble and insoluble protein fractions during digestion within the gastrointestinal tract.

CONCLUDING REMARKS

In this thesis, it is described how various protein fractions of microalgae and cyanobacteria can be applied in both food and fish feed. For food applications, proteins were extracted from diverse microalgae and cyanobacteria. Based on these results, it was concluded that variation in protein extractability between unicellular sources used is not related to differences in the gross chemical composition or in protein composition of the sources. Techno-functional protein isolates can be obtained from unicellular sources, with solubility and emulsion behaviors that are in range of those of more conventional vegetable proteins (e.g. legumes).

Additionally, the results from this thesis indicate that when unicellular sources are to be applied in fish feed, cell disruption is necessary to reach the full potential of these sources as digestible protein ingredients for fish. When fed in intact form, protein and fat digestibility of unicellular sources is dominated by differences in the cell walls. The digestibility of these nutrients is not affected by fish species. As an alternative to the whole

biomass, the insoluble side streams created during the food protein extraction processes can also be applied in fish feed. These side streams have the same protein compositions and protein contents as the original biomass and that of the soluble, extracted, protein streams. It is expected that the insoluble side streams have protein digestibilities similar to that of disrupted algal biomass.

Furthermore, a large influence of strain-to-strain variation was observed. It is advisable to gain more understanding of this variation in unicellular sources on their protein extractability and digestibility. The results from this thesis indicate the genera, species and also the strain of a unicellular source will affect both the food and feed applicability of the source, by affecting the *in vitro* protein extractability and *in vivo* protein digestibility.

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Summary

The increase in world population has led to an interest in exploring alternative protein sources. One class of alternative protein sources are the unicellular, photosynthetic microalgae and cyanobacteria. These sources are biologically very diverse and range from marine prokaryotes to freshwater eukaryotes. Variations between these sources are reflected in chemical variations (e.g. in the chemical composition) and in structural variation (e.g. in cell wall robustness). The aim of this thesis was to understand how chemical and structural variations between photosynthetic unicellular sources affect the application of unicellular protein as techno-functional ingredients in food and as fish feed ingredient.

In **chapter 1**, the potential of photosynthetic unicellular organisms as sources of protein ingredients for food and feed was explored. The protein content of these sources varied greatly between species, but also within species due to due to growing conditions and seasonal conditions. While many studies refer to differences in robustness of unicellular cell walls it is found that there are few studies that quantity this variation in robustness. This cell wall robustness is relevant since cell walls need to be disrupted in order to extract proteins. It is hypothesized that this cell disruption is also necessary for *in vivo* digestion of unicellular protein. Cell disruption and subsequent protein extraction can be done by several mild processes. These processes, as well as the techno-functional properties of unicellular protein for food and its nutritional quality for both food and feed are discussed.

The extent to which various unicellular green sources differ with respect to their gross composition (chemical variation) was described **chapter 2**, as well as how these differences affect protein extraction, protein isolation and the final protein isolate obtained. Using mild isolation techniques, proteins were extracted and isolated from *Arthrospira (spirulina) maxima*, *Nannochloropsis gaditana, Tetraselmis impellucida* and *Scenedesmus dimorphus*. Despite differences in protein contents of the sources (27–62% w/w) and in protein extractability (17–74% w/w), final protein isolates were obtained that had similar protein contents (62–77% w/w) and protein yields (3–9% w/w). Protein solubility as a function of pH was different between the sources and in ionic strength dependency, especially at pH < 4.0.

The protein isolates obtained in **chapter 2** are used in **chapter 3**, to study their emulsion behavior as a function of protein concentration and pH. In this chapter, the emulsion behavior of these protein isolates (using those obtained from *N. gaditana*, *T. impellucida* and *A. maxima*) was compared, using commercially available WPI as a reference. In addition, the relation between emulsion behavior and protein isolate characteristics was studied. All protein isolates were able to form emulsions ($d_{3,2}$ 0.2–0.3 µm) at pH 8.0. The amount of each isolate needed (C_{cr} in mg protein/mL) to form these stable emulsions varied between the isolates, but was within the range of proteins from both similar (photosynthetic) sources (algae and sugar beet leaves) and other protein sources (dairy, legume and egg). Minor differences were observed in the pH dependence of flocculation amongst the ASPI stabilized emulsions. For the ASPIs, the expected correlation

between interfacial and molecular properties (adsorption rate constant and ζ-potential) and the emulsion behavior (C_{cr} and droplet size as a function of pH) was absent.

To assess the potential of photosynthetic unicellular sources as fish feed ingredients, the nutrient digestibility of various sources was studied in herbivorous and omnivorous fish, as presented in **chapter 4**. By using sources which varied in cell wall composition, the effect of cell wall robustness and fish species on digestibility of unicellular sources could be studied. Initially, the gross composition (chemical variation) and the cell wall robustness (structural variation) of the sources were determined for the microalgae *Chlorella vulgaris*, *S. dimorphus* and *N. gaditana*, and the cyanobacterium *A. maxima*. Apparent digestibility coefficients (ADCs) of their nutrients were determined in Nile tilapia and African catfish, at a 30% diet inclusion level. It was hypothesized that nutrients become more accessible in herbivores, and that herbivores can therefore digest unicellular proteins better than omnivores. Additionally, it was hypothesized that the differences in protein digestion between the fish species increase with the robustness of the cell walls. Differences in cell wall robustness were quantified as the cells' resistance to mechanical shear. *A. maxima* was least resistant to shear: the time needed to disrupt 50% of the cells was 2 min compared to 24–33 min for the other sources. Contrary to the basal diet, which was digested differently between the fish species, there was no fish species effect on nutrient ADCs of the unicellular sources. *A. maxima* had the highest protein ADCs in both fish species (81.4–82.5%), followed by *C. vulgaris* (80.7–80.9%), *N. gaditana* (72.4–74.7%) and *S. dimorphus* (67.0- 68.3%). Ingredient fat ADCs ranged between 65.1–89.1%. Unicellular non-starch polysaccharides (NSP), comprising the unicellular cell walls, was not inert in either fish species (ADC >46.0%) which was attributed to fermentation. The digestibility data suggest that the variation in nutrient digestibility is caused by variations in nutrient accessibility. Additionally, the differences in nutrient accessibility between unicellular sources are dominant over the differences in digestive systems between herbivorous and omnivorous fish. Nevertheless, nutrient digestibility of the unicellular sources did not relate to the measured mechanical cell wall robustness.

From the results obtained in **chapter 4**, it was hypothesized that for photosynthetic unicellular sources, nutrient accessibility dominates nutrient digestibility. This hypothesis was tested in **chapter 5**, by subjecting *N. gaditana* biomass to five different treatments that influence its cell wall integrity. The treatments included physical treatments (pasteurization, freezing, freeze-drying) and mechanical treatments (bead-milling). These treatments resulted in increased *in vitro* accessibility of microalgae nutrients up to 4 times, as determined from nutrient leaching and susceptibility to protein hydrolysis. Apparent digestibility coefficients of the nutrients in untreated and treated microalgae biomass were determined in Nile tilapia, at a 30% diet inclusion level. *In vivo* digestibility of protein and fat (both intracellular nutrients) was increased from 62 to 78% and from 50 to 82%, respectively, with the highest ADCs obtained for the bead-milled biomass. The *in vitro* accessibility data were positively correlated with the *in vivo* digestibility of and fat. This shows that these methods are effective ways to assess the effect of mechanical and physical treatments on *in vivo* nutrient quality of a single ingredient. The results of this study confirm

that nutrient accessibility plays a significant role in the nutrient digestibility of microalgae in fish.

From the data obtained in **chapters 2–5**, three major questions arose concerning (1) protein extraction yields, (2) emulsion analysis and (3) protein digestibility of insoluble unicellular protein fractions. These questions are discussed in **chapter 6**, based on (additional) data obtained during this PhD project and literature data. It is shown that (1) the relatively low protein extraction yields obtained from the unicellular sources tested are not caused by the pH conditions chosen during extraction or by proteolysis by endogenous enzymes. The unextracted proteins are considered to be either insoluble, or bound to or entrapped by cell walls and cell organelles. Concerning emulsion analyses (2), the C_{cr} can be considered as a useful tool to quantitatively compare the emulsifying abilities of various proteins, or protein ingredients. It is, however, sensitive to the volume fraction of oil (Φ_{oil}), and to the way the C_{cr} is retrieved from the data obtained. It is hypothesized that this variation can be decreased using a minimum Φ_{oil} (> ~ 0.125) and sufficient data points in the protein concentration range near the C_{cr}. Concerning the use of unicellular sources in both food and fish feed (3), it would be beneficial if the side streams produced during protein extraction processes (an insoluble fraction) aimed at food applications could be used in the feed industry. In **chapter 4** and **5**, the nutrient digestibility was only studied of the whole biomass. In **chapter 6**, it is hypothesized that protein digestibility in fish of the insoluble fraction will be higher than that of intact cells due to an increased protein accessibility. The protein digestibility of the insoluble fraction is expected to be lower than the protein ADC of a soluble protein fraction. The *in vivo* protein digestibility was greatly influenced by strain-to-strain variation of unicellular sources. Such a strong effect of this variation was also observed in the *in vitro* protein extractability. Based on this, it was concluded that strain selection of a unicellular source affects its potential as a protein source for both food and feed applications.

In conclusion, chemical variations observed between photosynthetic unicellular sources were reflected in variations in protein extractability. Structural variations between the sources were reflected in variations in *in vivo* protein accessibility and subsequent protein digestibility.

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Emma

About the author

Emma Teuling was born on September 17th 1987 in Amersfoort, The Netherlands. After graduating from secondary school (HAVO, De Amersfoortse Berg) in 2004, she spent a year abroad as an exchange student at Clay High School in Oregon, Ohio, USA. After returning to The Netherlands, she studied Food Innovation & Management at the university of applied sciences (HBO) Van Hall Larenstein in Velp and Wageningen, The Netherlands. As part of her BSc program she completed three internships, at Remia b.v., Campbell's Netherlands and NIZO food research. Following her final internship and graduation, she worked as a scientific assistant at NIZO food research (Ede, The Netherlands) in a "High Protein Foods" project of Top Institute Food and Nutrition (TIFN) for one year. Subsequently, she started her MSc studies in Food Technology at Wageningen University, with a specialization in ingredient functionality. Her MSc thesis (performed at the Laboratory of Food Chemistry) focused on the effect of structural modifications of food proteins on polyphenol-protein interactions. In 2013, she started her PhD research at the Laboratory of Food Chemistry and at the Aquaculture and Fisheries Group of Wageningen University. The PhD project was part of a protein innovation framework of STW (now known as the NWO domain TTW) and the Dutch Ministry of Economic Affairs. During her PhD, Emma was supervised by prof. dr. Harry Gruppen, dr. ir. Peter Wierenga and dr. ir. Johan Schrama. The results of her PhD are presented in this thesis. Currently, Emma is working as a protein scientist at Cosun R&D, Dinteloord, The Netherlands.

List of publications

- Teuling, E., Schrama, J. W., Gruppen, H., Wierenga, P. A. Characterizing emulsion properties of microalgal and cyanobacterial protein isolates. *To be submitted*.
- Teuling, E., Wierenga, P. A., Agboola, J.O., Schrama, J. W., Gruppen, H. Cell wall disruption increases bioavailability of microalgal nutrients for juvenile Nile tilapia (*Oreochromis niloticus*). *Submitted*.
- Teuling, E., Schrama, J. W., Gruppen, H., Wierenga, P. A. Effect of cell wall characteristics on algae nutrient digestibility in Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarus gariepinus*). *Aquaculture*, *479*, 490-500 (2017).
- Teuling, E., Wierenga, P. A., Schrama, J. W., Gruppen, H. Comparison of protein extracts from various unicellular green sources. *Journal of Agricultural and Food Chemistry*, 65(36), 7989-8002 (2017).

Overview of completed training activities

DISCIPLINE SPECIFIC ACTIVITIES

Conferences and meetings

- Minisymposium feeding the future, Wageningen, NL, 2013
- International conference on food digestion, (INRA / COST), Wageningen, NL, 2014
- European conference for marine natural products [†] (In Conference Ltd), Glasgow, Scotland, 2015
- Food colloids (WUR/TNO), Wageningen, NL, 2016

Courses

- Industrial food proteins (VLAG), Wageningen, NL, 2013
- Basic statistics (PE&RC), Wageningen, NL, 2014
- Advanced statistics: design of experiments (WIAS), Wageningen, NL, 2014
- Advanced food analysis (VLAG), Wageningen, NL, 2015
- Dynamic light scattering and zeta potential (Sysmex), Breda, NL, 2015
- Biorefinery for biomolecules [†] (VLAG), Wageningen, NL, 2015
- Advances in feed evaluation science [‡] (Wageningen Academy), Wageningen, NL, 2015

GENERAL COURSES

- PhD week 2014 (VLAG), Baarlo, NL, 2013
- Interpersonal communication (WGS) Wageningen, NL, 2013
- Teaching and supervising thesis students (ESD) Wageningen, NL, 2013
- Project & time management (WGS) Wageningen, The Netherlands, 2014
- Techniques for writing and presenting a scientific paper (WGS) Wageningen, NL, 2015
- Career assessment (WGS), Wageningen, NL, 2016
- Brain training (WGS), Wageningen, NL, 2017

OPTIONALS

- Laboratory of Food Chemistry PhD trip [†,‡], Germany, Denmark, Sweden and Finland, 2014
- Organizing the Laboratory of Food Chemistry PhD trip to Germany, Denmark, Sweden and Finland, 2013-2014
- Laboratory of Food Chemistry PhD trip [†,‡], Japan, 2016
- Laboratory of Food Chemistry PhD presentations, 2013 – 2017
- Laboratory of Food Chemistry BSc/MSc student presentations and colloquia, 2013 - 2017

USED SYMBOLS AND ABBREVIATIONS

† poster presentation, ‡ oral presentation

INRA: French National Institute for Agricultural Research

COST: European Cooperation in Science and Technology

TNO: Netherlands Organization for Applied Scientific Research

VLAG: graduate school for Food Technology, Agro-Biotechnology, Nutrition, and Health Sciences

PE&RC: Graduate School for Production Ecology & Resource Conservation

WGS: Wageningen Graduate Schools

ESD: Educational Staff Development of WUR

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