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Cell wall disruption increases bioavailability of *Nannochloropsis gaditana* nutrients for juvenile Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

In this study the correlation between the accessibility of nutrients and *in vivo* nutrient digestibility was tested on the marine microalga *Nannochloropsis gaditana* in juvenile Nile tilapia (*Oreochromis niloticus*). It was hypothesized that disrupting the cell walls of microalgae increases the nutrient accessibility and digestibility. *N. gaditana* biomass was subjected to physical treatments (pasteurization, freezing, freeze drying) or mechanical treatments (bead milling) to influence its cell wall integrity. These treatments resulted in an up to 4 x increase in *in vitro* accessibility of *N. gaditana* nutrients, assessed from measurements of leaching and susceptibility to protein hydrolysis. Apparent digestibility coefficients of macronutrients, dry matter, energy, phosphorus and calcium of untreated and treated microalgae biomass were determined in triplicate, at a 30% diet inclusion level. Bead milling the algae led to the highest increase in *in vivo* digestibility of dry matter, energy, protein, fat, ash and calcium on ingredient level, compared to untreated algae biomass. This includes an increase in apparent digestibility coefficient (ADC) of protein and fat from 62 to 78% and from 50 to 82%, respectively. ADCs of total carbohydrates and of phosphorus were not affected by algal cell disruption. *In vivo* digestibilities of *N. gaditana* dry matter, energy, protein, and fat were positively correlated ($p < .001$; $r \geq 0.91$) with the nutrient accessibility of *N. gaditana*, as estimated with *in vitro* nutrient leaching analyses. This shows that the *in vitro* methods used 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 the microalga *Nannochloropsis gaditana* in Nile tilapia.

1. 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 among various microalgal species (Sarker et al., 2016; Teuling et al., 2017b). The assumption is that in some algae the nutrient digestion can be limited by the presence of the algae cell walls, limiting the *in vivo* accessibility of the intracellular nutrients. Consequently, it is expected that for those algae the *in vivo* digestibility can be improved by improving the nutrient accessibility after disruption of the cell wall structure. The aim of this study was 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 have a high variability between studies (67–86%) (Burr et al., 2011; Gong et al., 2017; Safari et al., 2016; Sarker et al., 2016; Teuling et al., 2017b; Tibbetts et al., 2017). In some of these studies, the protein digestibility was low. This low protein digestibility has been suggested to be related to differences in cell wall structure and associated nutrient accessibility of the algae and cyanobacteria. This cell wall hardness of microalgae and cyanobacteria has been quantified previously (Teuling et al., 2017b), but has not yet been quantitatively related to protein digestibility in fish or other animal studies. Studies have shown, however, that treating microalgae with (high pressure) homogenization (Janczyk et al., 2007; Komaki et al., 1998; Sommer et al., 1991; Tibbetts et al., 2017) can increase the *in vivo* accessibility and the digestibly of nutrients and natural colorants in rats and fish. Effect of cell wall disruption on nutrient digestibility in fish is limited to a single study by Tibbetts et al. (2017). They described how homogenization of the freshwater alga *Chlorella* sp., increased the ingredient protein ADC from 79.5% to 85.4% (at dietary inclusion levels of 30%)

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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[a]						
	UNT	PAS	FRD	FRO	L40	BEM	%CV[d]
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)	500.1	508.6	533.2	488.8	487.2	490.7	0.42
Crude protein (N * 4.84)	387.3	393.9	412.9	378.5	377.3	380.0	0.42
Crude fat	160.9	162.1	129.7	173.3	156.6	146.3	2.15
Total carbohydrates[b]	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	1.0	2.7	2.3	4.5	5.7	5.9	19.93
NSP[c]	159.4	165.4	133.2	153.8	159.7	118.0	1.91
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

[a] 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.
[b] Total carbohydrates comprise starch and NSP.
[c] NSP = total carbohydrates – starch.
[d] Coefficient of variation.

in juvenile Atlantic salmon (*Salmo salar* L.). Methods that can be used to disrupt algal cell walls can in general be divided into four categories: enzymatic, chemical, physical and mechanical methods (Lee et al., 2012). Examples of each are the use of cellulases (enzymatic), alkaline, acid and organic solvents (chemical), bead milling, high-pressure homogenization and microfluidics and ultrasonication (mechanical) and thermal treatments and freeze drying (physical) (Lee et al., 2012; Middelberg, 1995; Tibbetts et al., 2017). 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 (Doucha and Lívanský, 2008; Middelberg, 1995), while keeping the composition intact. Physical methods like freezing and freeze drying (Mazur, 1969) and thermal treatments (Mendes-Pinto et al., 2001; Ometto et al., 2014) are milder since they can be employed to damage cell wall structures without completely disrupting the walls. 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 (van Rooijen et al., 2013).

To clearly discern the effect of the 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 (Middelberg, 1995). 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 (Ometto et al., 2014; Tibbetts et al., 2017). Other treatments like bead milling can result in complete breakdown of algae cells. The 'broken cells' can be analyzed 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. These cell disruptive treatments aimed to increase the protein accessibility and subsequent protein digestibility. Nutrient accessibility of the treated and of untreated *N. gaditana* was measured *in vitro*, and nutrient digestibility was measured *in vivo* in triplicate in juvenile Nile tilapia (*Oreochromis niloticus*).

2. Materials and methods

2.1. Materials

All chemicals used for the *in vitro* study and for analyses of 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). The biomass was provided in 2 batches that were harvested in June and July of the same year. No stabilizers or other chemicals were added to the biomass. After harvesting, the *N. gaditana* biomass was centrifuged to a paste of 20% [*w*/w DM]. The biomass was washed by diluting the paste 1:1 with tap water, following by another centrifugation to a final DM of 20% [*w*/w]. Pancreatin used originated from porcine pancreas (Sigma product number P3292).

2.2. Nannochloropsis gaditana treatments

N. gaditana biomass was treated by 5 different methods, aimed at decreasing the cell wall integrity and increasing the accessibility of

intracellular nutrients. After the treatment (as described below), the algae products were dried to > 900 g dry matter (DM)/kg material by either drum drying or freeze drying (details are discussed below). Subsequently, the dried algae products (Table 1) were used as test ingredients of extruded fish feeds.

The (washed) algal paste (see the Materials section) received no further treatment (UNT), or received 1 out of 5 physical or mechanical treatments (hot pasteurization, PAS; freezing, FRO; freeze drying, FRD; cold pasteurization L40 and bead milling, 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 −18 °C. The FRO samples were thawed at 4 °C (after ~2 weeks frozen storage). Freezing and thawing was performed in small batches to ensure microbial safety. Serving as a positive control, the BEM sample was diluted with demineralized water 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 post-harvest physical treatment, similar to pasteurization, without applying elevated temperatures. No additives were used in the production of L40. Except 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 for 1 week. The freeze drying shelves used were not temperature controlled during the freeze drying process. The UNT ingredient was considered as the negative control and the BEM sample as a positive control.

2.3. In vitro assessment of treatments on algal cell wall integrity

2.3.1. Microscope analysis

The algae products were qualitatively 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.

2.3.2. 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. The hydrolysis parameters were chosen based on the known optimal conditions for porcine pancreatin (Minekus et al., 2014). The aim of the *in vitro* analyses was to quantify the changes in *in vitro* accessibility induced by the processing conditions applied on the algae, not to mimic *in vivo* hydrolysis conditions. 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 for 2 h 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 Eq. 1.

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

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) (Butré et al., 2014); 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) (Berman et al., 2000). 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. Although similar to previously published methods (*e.g.* by Tibbetts et al., 2016), the method used was developed specifically for the biomass used. 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. This method was not based on a published method and was developed specifically for this study and the biomass used. 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 Jasaitis et al. (1987). The algal products (100 mg each) were dispersed in 10 mL Milli-Q water. The samples were stirred for 1 h at room temperature and were then brought to 37 °C. The pH of the suspensions was adjusted to pH 3 using 1 M HCl. The initial pH of the suspensions was recorded. Subsequently, the pH of the samples was increased from pH 3 to pH 8 using 1 M NaOH. The alkaline buffering capacity ($BC_{alkaline}$, mmol NaOH/g sample) was calculated using eq. 2

$$BC_{alkaline} = \frac{C_{NaOH} \times V_{NaOH}}{m_{sampledryweight}} \times \frac{1}{pH_{final} - pH_{initial}} \qquad (2)$$

in which C_{NaOH} and V_{NaOH} are the concentration and volume of NaOH used to bring the samples from the initial pH ($pH_{initial}$; 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.

2.4. Digestibility trial

In the current experiment, fish 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 approved this experiment (conducted in 2016) and evaluated it as not being an animal experiment according to Dutch legislation (Act on Animal Experiments), as the procedures applied to the animals in this experiment were judged to be below the threshold.

2.4.1. 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

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[a]	1.00	0.70
Yttrium oxide	0.02	0.02
Test ingredients (w/w %)		
Nannochloropsis gaditana[b]	–	30.00

[a] Mineral premix composition (mg/kg reference diet): 50 iron (as $FeSO_4·7H_2O$); 30 zinc (as $ZnSO_4·7H_2O$); 0.1 cobalt (as $CoSO_4·7H_2O$); 10 copper (as $CuSO_4·5H_2O$); 0.5 selenium (as Na_2SeO_3); 20 manganese (as $MnSO_4·4H_2O$); 500 magnesium (as $MgSO_4·7H_2O$); 1 chromium (as $CrCl_3·6H_2O$); 2 iodine (as $CaIO_3·6H_2O$). 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.

[b] 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.

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 (Aqua Optima, Aqua Optima AS, Trondheim, Norway). More details about the settling units are provided by Amirkolaie et al. (2006). 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 µS/cm - 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.

2.4.2. 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 (as described in § 2.2), excluding the marker. Yttrium oxide (Y_2O_3) was added to all diets (0.20 g/kg, which is equal to 0.17 g/kg yttrium) as an inert marker to calculate apparent digestibility coefficients of the treated and untreated *N. gaditana*. The content of inert marker was kept equal in all diets to ensure equal accuracy in measuring the ADCs. All diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands). The dried algae products were mixed with the other dietary ingredients (including the marker), hammer milled (Condux LHM20/16, Hanau, Germany), and subsequently extruded using a Clextral BC45 laboratory scale twin-screw extruder (Clextral, Firminy, France) through a 2 mm die into sinking pellets. Oils were added prior to the extrusion process. After extrusion, the pellets were dried in a tray drier (70 °C for 3 h) and subsequently cooled to ambient temperature. Prior to feeding, the diets were sieved (2 mm) to remove fine particles. The compositions of the diets were formulated to meet the nutrient requirement for Nile tilapia (National Research Council, 2011), to ensure the fish were not exposed to nutrient deficiencies.

2.4.3. 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 h. The amount of feed given was registered and was restricted at a 90% satiation level of 17.1 g DM/kg$^{0.8}$/d. The satiation level is based on experience with satiation feeding in the Wageningen University experimental facilities and is 19 g DM/kg$^{0.8}$/d for Nile tilapia of the weight range used. From the start until 15 min after each feeding, sediment was collected using settling units. Uneaten feed pellets were counted in the sediment to determine true feed intake. The amount of uneaten feed in a tank was compensated by giving additional feed to that tank during the next feeding. The amount (g DM) of feed given per tank was also 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. During week 6, feces were collected from each tank separately overnight, starting 30 min after the afternoon feeding until the morning feeding, for 5 days. 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. On the last day of the trial, the fish were not fed and were batch-weighed again to determine their growth performance.

2.4.4. Chemical analyses on ingredients, feed and feces

Prior to analysis, the fecal samples were freeze dried. Algae and fecal samples were homogenized 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 (1999b)). *Ash content* was determined on the dried samples by incineration for 1 h at 550 °C (ISO 5984 (2002)). 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 (2005)). The 6.25 factor used is known to overestimate the protein content of microalgae. Therefore, the protein contents of the algae ingredients were additionally expressed as N * 4.84, based on previous amino acid analyses performed on *N. gaditana* (Teuling et al., 2017a).

Total starch was determined in duplicate using the total starch assay (AOAC Method 996.11 (AOAC, 2012)) 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 (Teuling et al., 2017b). 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 (Teuling et al., 2017b).

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

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

	Diets[a]							
	REF	UNT	PAS	FRD	FRO	L40	BEM	%CV[e]
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)	371	410	418	415	404	403	406	0.52
Crude protein (N * 6.25; N * 4.84)[b]	371	383	390	387	377	376	379	0.52
Crude fat	93.0	108.8	111.3	103.7	113.8	111.6	122.7	0.70
Total carbohydrates[c]	408.8	328.0	333.1	326.9	330.5	326.8	324.3	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	22.22	15.43	16.30	18.30	17.17	17.24	17.23	5.01
Xylose	21.02	14.77	15.49	15.44	14.77	14.27	14.51	5.02
Mannose	3.89	10.36	10.26	7.41	13.11	10.22	12.00	3.78
Galactose	18.13	19.92	20.28	20.41	18.62	21.02	18.76	2.73
Glucose	322.6	246.8	250.4	244.3	245.9	243.5	240.5	1.62
Ribose	0.20	1.53	1.22	1.28	1.20	1.26	1.04	23.95
Uronic acids	17.33	14.42	14.52	15.05	15.09	14.40	15.28	2.54
Starch	240.6	181.4	176.8	192.5	181.4	149.7	178.8	4.16
NSP[d]	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

[a] 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.
[b] Crude protein of the basal diet was calculated as N * 6.25. Crude protein of the test diets was calculated as (0.7 * N * 6.25) + (0.3 * N * 4.84).
[c] Total carbohydrates comprise starch and NSP.
[d] NSP = total carbohydrates – starch.
[e] Coefficient of variation.

standard (ISO 9831 (1998)).

Crude fat content was determined in triplicate using the Berntrop method with acid pre-hydrolysis (ISO 6492 (1999a)).

Maillard reaction products and cross-linked amino acids were used as indicators of protein damage during ingredient and feed processing. The Maillard reaction products (MRPs) (furosine, carboxyethyllysine (CEL) and carboxymethyllysine (CML)), cross-linked amino acids (lanthionine (LAN) and lysinoalanine (LAL)) and lysine were quantified in feeds and ingredients using UPLC-MS 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 (Krause et al., 2003). 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". 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 room temperature. The samples were re-suspended in 1 mL Milli-Q water, sonicated for 5 min and centrifuged (5 min, 20 °C, 19,000 g). The supernatants were diluted 100× 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 g). All compounds were determined using an Accela UHPLC System (Thermo Scientific, San Jose, CA, USA) using an Acquity UPLC BEH Amide column (2.1 × 150 mm, 1.7 µm particle size) with an Acquity BEH Amide Vanguard precolumn (2.1 × 50 mm, 1.7 µm particle size). The compounds were analyzed using selected reaction monitoring (SRM) method. Eluent A was Millipore water, containing 0.1% (v/v) formic acid and eluent B was acetonitrile containing 0.1% (v/v) formic acid. The diluted samples (1 µL) were injected onto the column which was maintained at 35 °C. The elution profile used was as follows: 0–2 min isocratic on 90% B; 2–17 min linear gradient from 90% to 40% B; 17–19 min isocratic 40% B; 19–21 min linear gradient from 40 to 90% B; 21–35 min isocratic on 90% B. The flow rate was 350 µL/min. Mass spectrometric data were obtained by analyzing samples on a LTQ-VelosPro (Thermo Scientific) equipped with a heated ESI probe coupled to the UHPLC system. The capillary voltage was set to 3 kV with the source operation in positive ion mode for CML, furosine, lysine, CEL and $^{13}C_6$,$^{15}N_2$-lysine and in negative ion mode for LAL and LAN. The normalized collision energy was set at 35 for labelled lysine, CML and CEL and at 30 for furosine, LAL, LAN and lysine. The *m/z* width on the fragment was set to 5. Compounds were quantified using an external standard calibration curve by plotting MS peak area of the SRM fragment divided by the MS peak area of the SRM fragment of $^{13}C_6$,$^{15}N_2$-lysine, used as internal standard. 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 eq. 3.

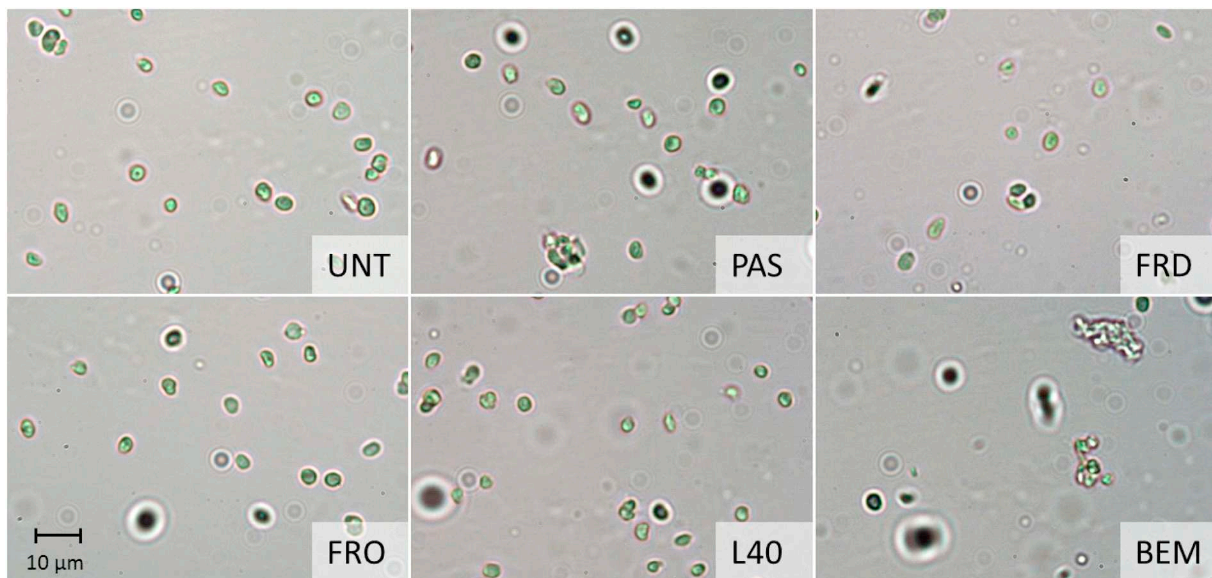

Fig. 1. Microscope images of *Nannochloropsis gaditana* biomass at a magnification of 40×. UNT, PAS, FRD, FRO, L40 and BEM: untreated, pasteurized, freeze dried, frozen-thawed, commercially processed (NutriSpring® Liquid 40, AlgaSpring, NL) and bead milled biomass, respectively. With exception of FRD,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

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

Peak $area_{sample}$, Peak $area_{spiked\ sample}$ and Peak $area_{standard}$ 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). Data were acquired and analyzed using Xcalibur 2.2 (Thermo Scientific).

2.4.5. Growth parameters

The feed conversion ratio (FCR) was calculated on dry matter intake using eq. 4.

$$FCR\ [-] = \frac{Total\ feed\ intake}{Total\ body\ weight\ gain} \quad (4)$$

Total feed intake per tank (g) was calculated on dry matter basis, total body weight gain per tank (g) was used as is.

The specific growth rate (SGR) was calculated using eq. 5.

$$SGR\ [\%] = \frac{(\ln(average\ final\ fish\ weight) - (\ln(average\ initial\ fish\ weight))}{Duration\ of\ trial} \times 100 \quad (5)$$

Average fish weight (g/fish) was calculated as the total weight of all fish in a tank, divided by the number of fish in that tank. The duration of the trial was 42 days.

2.4.6. Apparent digestibility coefficients

Apparent digestibility coefficients (ADCs) of the dietary components (ADC_{diet}) in the diets were calculated using eq. 6 (Cho and Slinger, 1979).

$$ADC_{diet}\ [\%] = \left(1 - \left[\frac{Y_{diet}}{Yfeces}\right] \times \left[\frac{N_{feces}}{N_{diet}}\right]\right) \times 100\% \quad (6)$$

Where Y_{diet} is the content of the inert marker yttrium in the diet and Y_{feces} is the content of inert marker (yttrium) in the feces. N_{feces} is the content of the dietary component in the feces and N_{diet} is the content of the dietary component in the diet. All contents of marker and nutrients were expressed in g/kg DM. ADCs of the dietary components in the test ingredients ($ADC_{ingredient}$) were calculated using eq. 7 (Bureau and Hua, 2006).

$$ADC_{ingredient}\ [\%] = ADC_{test\ diet} + (ADC_{test\ diet} - ADC_{reference\ diet}) \times \left(\frac{0.7 \times N_{reference\ diet}}{N_{test\ ingredient}}\right) \quad (7)$$

Where $ADC_{test\ diet}$ is the ADC of the dietary component in the test diet and $ADC_{reference\ diet}$ is the ADC of the dietary component in the reference diet. $N_{reference\ diet}$ is the content of the dietary component in the reference diet and $N_{test\ ingredient}$ is the content of the dietary component in test ingredient. All nutrient contents were expressed in g/kg DM. The protein ADCs of both the diets and nutrients were calculated based on N*6.25 protein contents.

2.5. 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 ($p < .05$) between means were detected using Tukey's test. Correlation coefficients between the *in vitro* and *in vivo* data were examined using Pearson's correlation coefficient (PROC CORR). *In vitro* data comprise the degree of hydrolysis, nitrogen solubility, ion leaching and buffering capacity and the *in vivo* data comprise the ADC's of macronutrients, dry matter, energy, phosphorus and calcium on ingredient level. Correlation coefficients between various *in vitro* measurements (ion leaching method than in the DH and nitrogen solubility) were also were examined using Pearson's correlation coefficient (PROC CORR). A selection of the nutrient ADCs were further tested for linear and quadratic relations with *in vitro* data using PROC GLM. Data selected for PROC GLM analyses were *in vivo* protein and fat ADCs on ingredient level and *in vitro* nitrogen solubility, degree of hydrolysis and ion leaching. Probability levels of < 0.05 were considered to be statistically significant, and levels between 0.05 and 0.1 were considered a trend.

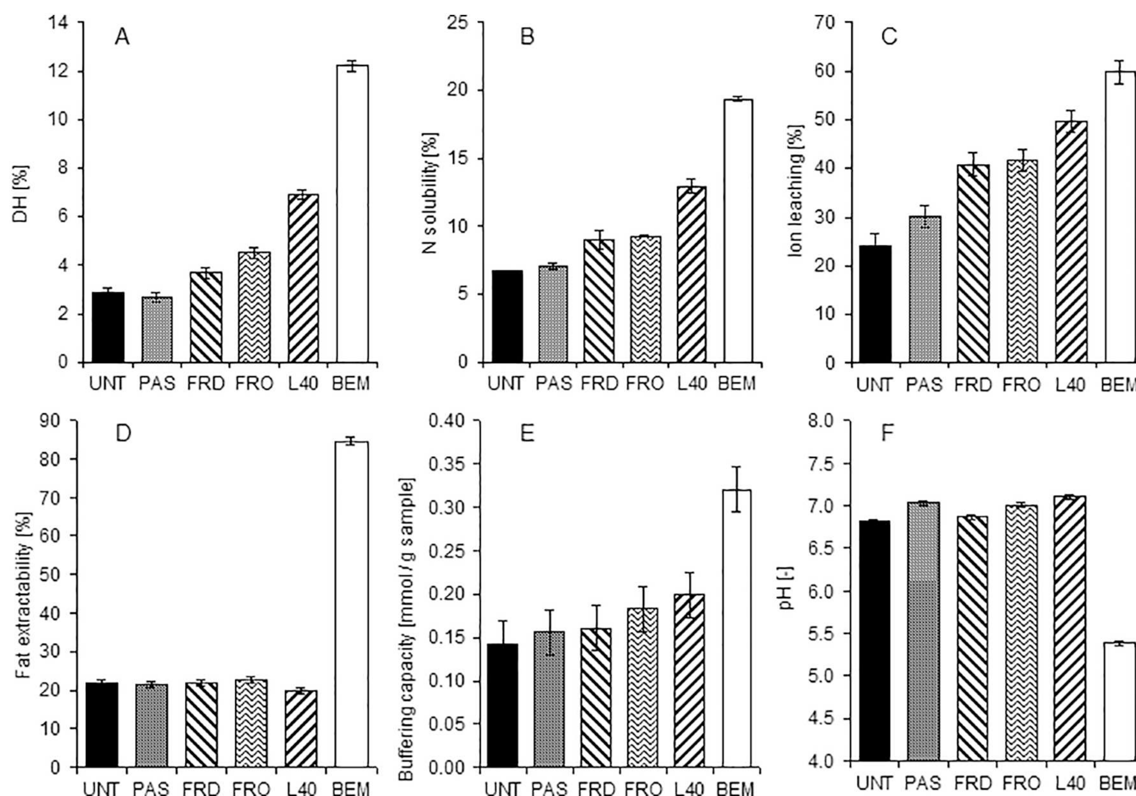

Fig. 2. Degree of protein hydrolysis (DH; A), nitrogen solubility (B), ion leaching (C), fat extractability (D), (alkaline) buffering capacity (E) and pH (F) of *Nannochloropsis gaditana* biomass that was untreated (■; UNT), pasteurized (▩; PAS), freeze dried (▧; FRD), frozen-thawed (▩; FRO), commercially 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.

3. Results & discussion

3.1. Cell wall integrity and nutrient accessibility

Qualitative light microcopy analyses (Fig. 1) showed that the untreated (UNT) and physically treated algae (PAS, FRD, FRO and L40) contained only intact cells. 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).

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 (Fig. 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 2.3–4.3 times increase in nutrient accessibility compared to UNT. The pH of BEM (pH 5.4) was lower than the pH of the other algae products (pH 6.8–7.1) (Fig. 2F). 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. The increase measured in nutrient leaching by cell disruption (BEM) is lower than that published by Tibbetts et al. (2017). They reported an increase in protein solubility by factor ≈ 7 after treating *Chlorella* sp. with high pressure homogenization. Differences measured in protein release between these studies are expected to be caused by the protein solubility analysis (using a pH 8 buffer or 0.2 M KOH) and by differences in algal species. As shown previously (Teuling et al., 2017a), the fraction of soluble protein present in algal biomass differs greatly between microalgal species.

The milder physical treatments performed on the algae (PAS, FRD, FRO and L40) did not affect the fat extractability. In contrast, a 4× increase in fat extractability was measured in BEM compared to UNT (Fig. 2 D). The physical treatments did increase the other nutrient accessibility parameters measured. This increase was up to factor 2.4 compared to UNT (Fig. 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% (Fig. 3). The normalized data were used to describe the nutrient accessibility. The data indicate that a maximum fat extractability was only reached after bead milling the algae (BEM). This underpins the importance of including an acid hydrolysis step in fat content analyses of intact microalgae, and 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. Possibly, the duration or temperature used in the pasteurization treatment was not sufficient to damage the cells to such an extent that nutrients could leach out. It is expected that a more severe pasteurization treatment could increase nutrient leaching and associated nutrient accessibility to a higher extent. The ranking of normalized accessibility parameters

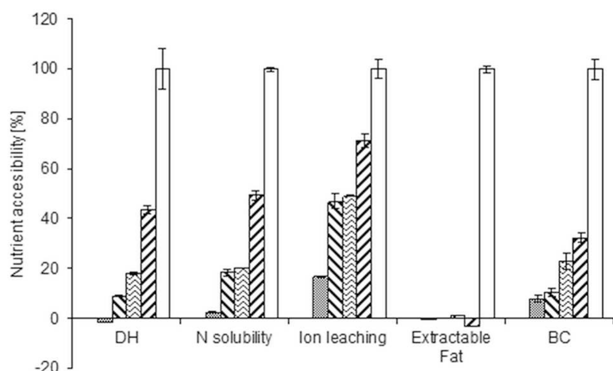

Fig. 3. Degree of protein hydrolysis (DH), nitrogen solubility, ion leaching, extractable fat and buffering capacity (BC) of *Nannochloropsis gaditana* biomass that was untreated (■), pasteurized (▩), freeze dried (▧), frozen-thawed (▨), commercially processed (NutriSpring® Liquid 40, AlgaSpring, NL; ▨) 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.

(DH, nitrogen solubility, ion leaching and BC) for each treatment was the same. Differences were found, however, in the relative effects of the treatments between the *in vitro* methods used.

For PAS, FRD and FRO, the determined accessibility was higher when using the ion leaching method than when using the DH and nitrogen solubility methods. This shows that ions leached out more rapidly than nitrogen or protein and/or than proteolytic enzymes could enter the cells. Proteins, including proteolytic enzymes, are larger molecules than ions. Proteins can thus only enter 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, an indication for protein solubility, and the DH were similar. This similarity was also reflected in a high Pearson correlation coefficient between these parameters ($r > 0.986$, $p < .001$). During pasteurization, freezing and/or freeze drying, the cells were apparently damaged to such an extent that ions could leach out, but larger molecules such as proteins could not.

There is a discrepancy between the microscopic analysis and the measurements of nutrient leaching, DH and BC. This 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. At a higher degree of damage, the cell wall permeability for larger molecules like algae proteins and digestive enzymes was increased. This is visualized in Fig. 4, illustrating the DH over nitrogen solubility (showing a linear correlation) and DH over ion leaching (showing a quadratic correlation).

Table 4
Contents of Maillard reaction products[a], cross-linked amino acids[b] 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[c])						
	Analyzed					Corrected[d]	
	Furosine	CEL	CML	LAL	Total lysine[c]	FL	(True) lysine[c]
Ingredient[e]							
UNT	2.32	0.05[g]	0.18	n.d.	46.48	7.24	42.42
PAS	2.71	0.08[g]	0.25	0.20	49.37	8.45	44.64
FRD	1.31	0.06[g]	0.22	0.14[g]	43.48	4.09	41.19
FRO	11.38	0.07[g]	0.39	0.19	41.20	35.57	21.28
L40	0.79	0.07[g]	0.18	0.13[g]	47.00	2.46	45.62
BEM	4.64	0.07[g]	0.24	0.56	40.90	14.49	32.79
Diet[f]							
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

n.d.: Not detected.
[a] Furosine, carboxyethyllysine (CEL), carboxymethyllysine (CML) and fructosylysine (FL).
[b] Lanthionine (LAN) was not detected in the ingredients and diets.
[c] Protein contents used were calculated as N$*$6.25.
[d] “Total lysine” is the uncorrected lysine content, “true lysine” is the lysine content corrected for the regenerated lysine. Lysine was corrected for regenerated lysine content originating from fructosyllysine by true lysine – (furosine $*$ 1.75). Fructosyllysine was calculated as 3.125 $*$ furosine.
[e] 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.
[f] 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.
[g] Below the detection limit of 0.17–0.18 g CEL or LAL/kg protein.

3.2. Effect of algae treatments on protein quality

3.2.1. 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 (van Rooijen et al., 2013). The *N. gaditana* biomass, reference diet and test diets did not contain the cross-linked amino acid product LAN, and either no or only trace amounts of LAL (Table 4). Of the algae products, FRO had the highest content of MRPs

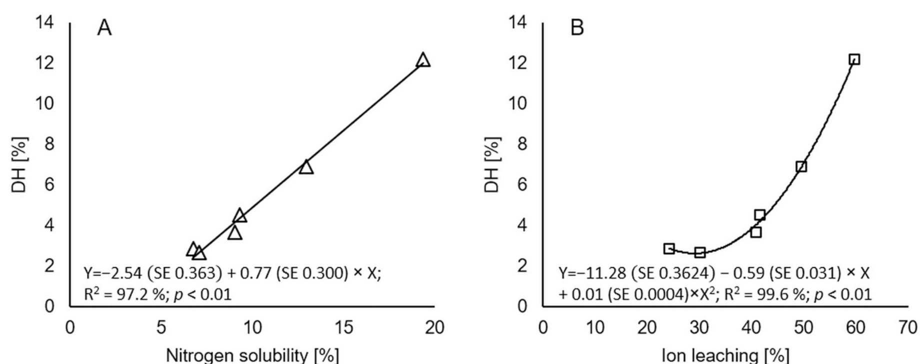

Fig. 4. Relationships between the degree of protein hydrolysis (%; DH) and the nitrogen solubility (A) and ion leaching (B) of *Nannochloropsis gaditana* biomass. Solid lines indicate significant relationships.

(4.9 x more fructosyllysine and 2.2 x more CML than UNT). After FRO, BEM had the highest content of MRPs (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. The high MRP and low corrected lysine contents in FRO are expected to be due to variations in the applied drum drying. In the other treated algae products the degree of lysine modification (*i.e.* loss of lysine) was similar to UNT, having an within sample SE variation of 8.8%. These differences in MRP contents between the algae products could not be attributed to their nutrient accessibility. The results do indicate that the combination of algae treatments and processing (drying) can lead to significant (up to 50%) modification of the lysines.

3.2.2. Formation of Maillard reaction products during feed manufacturing

The MRP and cross-linked amino acid contents in the reference diet and test diets (Table 4) are slightly higher in content than reported values for pig feeds (Salazar Villanea, 2017), but lower than those in pet feeds (van Rooijen et al., 2014). There is no published data yet available on formation of Maillard reaction products or cross-linked amino acid products during algal processing. In fact, also no analytical measurements have been performed on the content of MRP products in extruded fish feeds. However, various studies have assessed the formation of MRP products by looking at indicators like the loss of lysine (*e.g.*Singh, 2016) or color of the kibbles (*e.g.*Ayadi et al., 2011). These studies indicate that the loss of lysine and color changes during extrusion are dependent on process conditions and ingredient blends. Therefore, to test whether the algae treatments affected MRP formation during further processing by extrusion of the ingredient blends into kibbles, MRP and lysine contents in the kibbles of the diets and in the test ingredients were compared. If no Maillard reaction took place during the feed manufacturing process, the MRP contents in the kibbles of the test diets should be equal to a 30/70% mixture of the MRP contents of the ingredient (30%) and the kibble of reference diet (70%). The majority of these measured MRP contents in the kibbles were higher (up to 87% increase) than these theoretical (30/70% mixture) contents. This effect was most pronounced in the diets containing FRD and FRO algae, in which fructosyllysine increased with 50–52%. In FRO, this corresponded to a 25% loss of lysine during feed production. No clear relation was be found, however, between the various algae treatments and the susceptibility of the algae products to MRP formation and lysine modification during feed production by extrusion. Overall, the data indicated that both drum drying and feed manufacturing by extrusion can induce MRP formation and related amino acid modification in algae. When microalgae are applied in extruded or pelleted feed, the algal products first have to be dried. As seen in this study, even using relatively mild conditions this drying step may have implications on the nutritional quality of microalgae. Furthermore, the current data imply that an increased nutrient accessibility may increase the reactivity of proteins during drying and feed manufacturing by extrusion.

3.3. Fish growth, feed utilization and survival

The design of the experiment was aimed at studying the impact of processing (cell disruption) on nutrient digestion of the algae used. Nevertheless, growth and performance data are shown (Table 5) to enable judgement of the quality of the experiment. Fish survival was over 97% for all dietary treatments during the experimental period. Absolute feed intake was the same for all tanks. Despite this, 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 = .694 and p = .966, respectively). For fish fed all other diets SGR was lower ($p < .05$). Out of all test diets, only BEM had a lower FCR than the reference The FCR of the other test diets was equal to or higher than that of 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 (Appler and Jauncey, 1983; Hussein et al., 2013, 2014). FCRs of reference diets in those studies were 1.1–1.91.

3.4. Apparent digestibility coefficients of nutrients, dry matter and energy

On ingredient level, treatments of *N. gaditana* affected the ADCs for DM, energy, protein, fat, ash and calcium ($p < .05$) (Table 6). For completeness, ADCs of nutrients on diet level (Table 6) and the ADCs of the total carbohydrate constituent monosaccharides and of all minerals analyzed on both diet and ingredient level (Table 7) are also provided. Starch and NSP ADCs at ingredient level were not reported in Table 6 since the algae biomass contained < 0.6% *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. Protein and fat digestibility of UNT were low with ADC values of 61.5 and 50.4%, respectively. These low ADCs are postulated to be caused by the presence of resistant cellulosic cell wall (Scholz et al., 2014), that hinder enzymatic hydrolysis and subsequent digestion by the tilapia. The protein and fat ADCs of the untreated algae are also lower than previously reported values (74.7% and 74.5% respectively) for *N. gaditana* in Nile tilapia and in Atlantic salmon (*Salmo salar*) (72%) (Gong et al., 2017; Teuling et al., 2017b). In a previous tilapia trial using the same diet composition, the reference diet was digested to the same extent in in the present trial (Teuling et al., 2017b). 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. Although BEM was produced from a different batch than the other microalgal products used in this study, no large batch-to-batch or seasonal effects were expected, since the batches were harvested in the same season of the same year. Additionally, the chemical composition of BEM is the same as that of the other algae products (Table 1).

Other (untreated) unicellular sources were reported to have protein ADCs of 80–85% for *Arthrospira* (*Spirulina*) sp. (Burr et al., 2011; Safari et al., 2016; Sarker et al., 2016; Teuling et al., 2017b), 82% for *Schizochytrium* sp. (Sarker et al., 2016), 73–81% for *Chlorella* sp. (Sarker et al., 2016; Teuling et al., 2017b; Tibbetts et al., 2017), 67–68% for *Scenedesmus dimorphus* (Teuling et al., 2017b) and 67% for *Desmodesmus* sp. (Gong et al., 2017), in various fish species. In these studies, fat ADCs were reported between 60 and 98% (Safari et al., 2016; Sarker et al., 2016; Teuling et al., 2017b; Tibbetts et al., 2017) and energy ADCs between 45 and 87% (Burr et al., 2011; Safari et al., 2016; Sarker et al., 2016; Teuling et al., 2017b; Tibbetts et al., 2017). Compared to these data on other microalgae and cyanobacteria, protein and fat ADCs of UNT were lower, and protein and fat ADCs of BEM were comparable. Energy ADCs of both UNT and BEM were in range with the literature data.

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, ranging from 27 to 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. Nevertheless, the increases measured were higher than those observed by Tibbetts et al. using *Chlorella* sp. and juvenile Atlantic salmon (Tibbetts et al., 2017). Tibbetts et al. (2017) report a 12–28% increase in energy, protein and fat ADCs at 30% inclusion level of the microalga. Both *Chlorella* sp. and *N. gaditana* have cellulosic cell walls (Allard and Templier, 2000; Scholz et al., 2014). The combined data from both Tibbetts' and this study suggest that cell disruption may increase the nutrient availability of more

Table 5
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 biomass[2] (g)	1019	1051	1027	1020	1034	1030	1047	11.0	0.303
Initial body weight (g/fish)	29.1	30.0	29.3	29.1	29.6	29.4	29.9	0.31	0.302
Final biomass[2] (g)	3175[ab]	3045[ab]	2923[b]	3095[ab]	2924[b]	3146[ab]	3268[a]	67.5	0.022
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.

[2] The biomass represents the total weight of fish in each tank.

Table 6
Apparent digestibility coefficients (%; ADC[1]) of nutrients of the reference diet and of test diets with 30% inclusion of treated and untreated *Nannochloropsis gaditana*, and of nutrients of treated or untreated *Nannochloropsis gaditana* (on ingredient level, at 30% diet inclusion level) in juvenile Nile tilapia.

	In vivo trial								
ADC (%)	Diet[2]/*Nannochloropsis gaditana*[3]							p-values	
	CON	UNT	PAS	FRD	FRO	L40	BEM	SEM	Treatment
Diets									
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[4]	73.2	67.7	67.9	68.9	68.5	69.3	71.3	1.30	0.087
Starch[5]	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
N. gaditana									
Dry matter		48.4[c]	50.2[c]	50.6[bc]	55.2[bc]	61.2[ab]	66.3[a]	2.23	< 0.001
Gross energy		51.0[c]	50.1[c]	53.0[bc]	57.0[bc]	60.6[b]	69.2[a]	1.73	< 0.001
Crude protein		61.5[cd]	60.7[e]	60.6[de]	66.2[c]	72.9[b]	78.0[a]	1.00	< 0.001
Crude fat		50.4[c]	56.1[c]	57.8[c]	53.0[c]	66.4[b]	82.0[a]	1.62	< 0.001
Total carbohydrates		34.9	38.0	38.5	40.5	46.6	56.7	9.81	0.662
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]	55.6[ab]	72.8[a]	5.16	< 0.001

[1] Values presented are means. Mean values in a row with different superscript are significantly different ($p < .05$).

[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] 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.

[4] Total carbohydrates comprise starch and NSP.

[5] NSP = total carbohydrates – starch.

microalgae containing rigid cell walls when fed to monogastric fish.

Compared to BEM, the milder physical treatments performed on *N. gaditana* (PAS, FRO, FRD and L40) had less effect on nutrient digestibility. Pasteurization (PAS) had no effect on digestibility of any of the nutrients. L40 had higher ADCs of DM, energy, protein and fat than UNT. 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 (Köprücü and Özdemir, 2005; Leenhouwers et al., 2007). 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 where a phosphorus ADC of 58% was measured (Teuling et al., 2017b). The divergent phosphorus ADCs cannot be explained at this moment.

3.4.1. 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 *N. gaditana* biomass. The treated algae products and the corresponding diets had higher amounts of MRPs than the

Table 7
Apparent digestibility coefficients (%; ADC[1]) of the total carbohydrate constituent monosaccharides and of minerals of the reference diet and of test diets with 30% inclusion of treated and untreated *Nannochloropsis gaditana* and of the treated and untreated *N. gaditana* at ingredient level in juvenile Nile tilapia. Values are presented are means, in g/kg DM.

ADC (%)	Diet[2]/*Nannochloropsis gaditana*[3]							*p*-values	
	REF	UNT	PAS	FRD	FRO	L40	BEM	SEM	Diet
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
Cupper	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
N. gaditana									
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
Cupper		−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 ($P < .05$).
[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] 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.

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. 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 by the higher protein ADC of FRO (66%) compared to that of FRD (61%). The MRP contents in FRO were higher and the lysine contents were lower than in FRD. Despite this higher ADC, SGR and FCR were similar ($p = .22$ 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 the growth performance of the tilapia fed the FRO diet. 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.

3.4.2. Correlations between in vitro nutrient accessibility and 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 ($p < .001$ and $r \geq 0.91$ for all *in vitro* methods) (as presented in Table 9). This demonstrates that nutrient accessibility is an important factor in nutrient digestibility of microalgae.

In Nile tilapia, protein and fat are assumed to be predominantly hydrolyzed and absorbed in the proximal and mid part of the intestine (Tengjaroenkul et al., 2000). The increase in ADCs of these nutrients upon increasing the nutrient accessibility was in line with expectations. More specifically, a higher *in vitro* accessibility could indicate a higher accessibility to proteolytic enzymes in the stomach and mid part of the intestine. This correlation was not significant for non-starch carbohydrates ($p = .06$–0.07; $r = 0.66$–0.67). The ADC of non-starch carbohydrates did correlate with the ADCs of protein, DM and energy ($p < .03$ for all and $r = 0.73$–0.84). These correlations support the conclusion that there is a trend between nutrient accessibility and carbohydrate digestion. In this study the error on the measurement (SEM = 11%) decreased the significance of the correlations.

The correlations between the digestibility of protein and fat and the various methods used to assess nutrient accessibility were studied in more detail. It was assessed whether the correlations were linear or quadratic (Fig. 5 and Table 8). Protein ADC was linearly correlated to

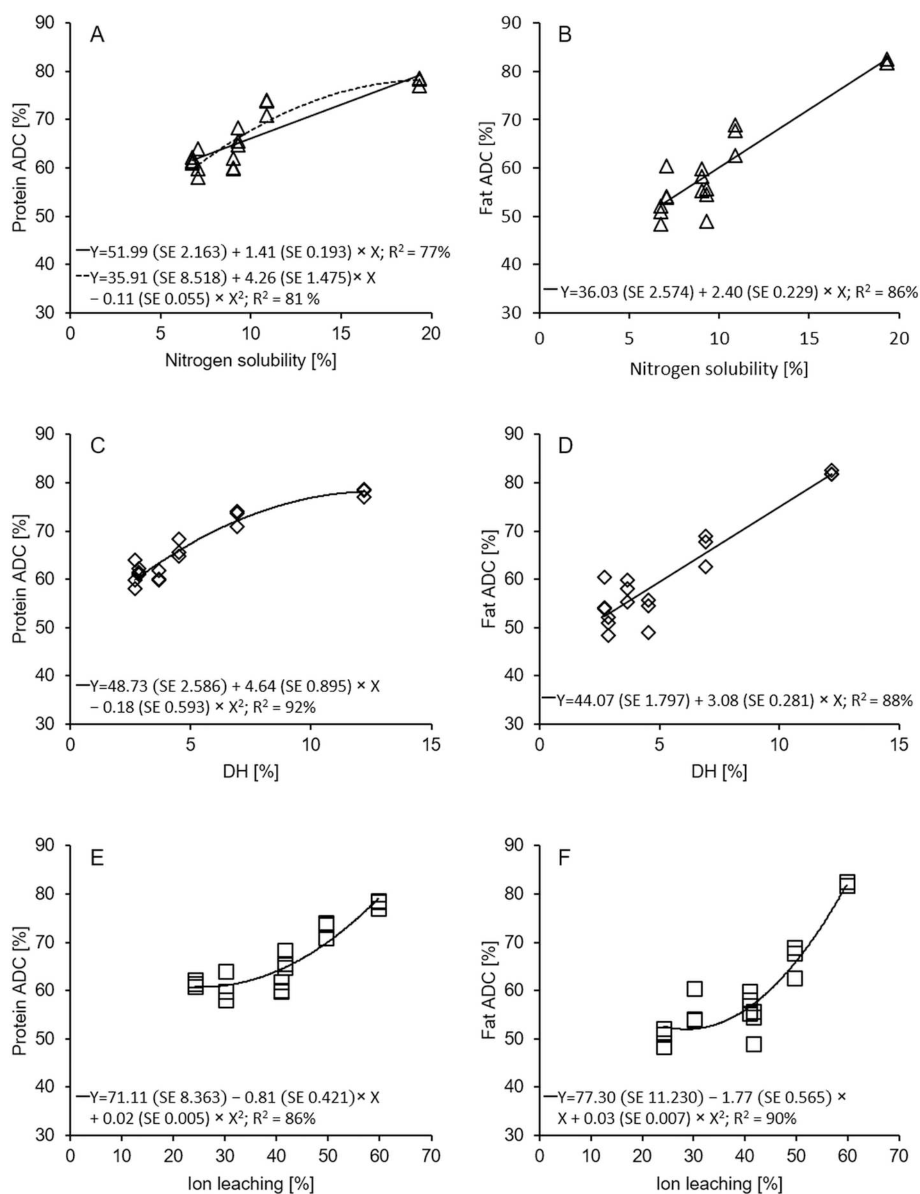

Fig. 5. Relationships between the apparent digestibility coefficients (%; ADC) of protein (A, C, E) and fat ADC (B, D, F) from *Nannochloropsis gaditana* in juvenile Nile tilapia and the nitrogen solubility (A, B), degree of protein hydrolysis (DH; C, D) and ion leaching (E, F) of *Nannochloropsis gaditana* biomass. Significant relationships ($p < .05$; either linear or quadratic) are indicated by solid lines. A tendency for a significant relationship ($p < .1$; only present in A) is indicated by the dotted line. Additional information on the equations are presented in Table 8.

the nitrogen solubility of the algae ($p < .001$; $r = 0.94$), although the quadratic function also tended to be significant ($p = .071$). Protein ADC was also correlated to DH ($r = 0.93$) and ion leaching ($r = 0.87$), but these relationships were curvilinear ($p = .008$ and $p = .007$, respectively). 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 appeared to level off at higher degrees of cell damage. Conversely, ion leaching occurred already at lower degree of cell disruption, as was also observed from Fig. 2. An increase in ion leaching therefore did not correlate to an increase in protein ADC at lower degrees of cell damage. Fat ADC was linearly correlated to the nitrogen solubility ($p < .001$, r = 0.93) and the DH of the algae products ($p < .001$, r = 0.94). In contrast with the DH and N solubility correlations with protein ADC, the increase in fat ADC did not yet reach a plateau at the maximum levels of cell disruption. Fat ADC was also correlated to ion leaching (r = 0.87), showing a curvilinear relationship ($p < .001$) similar 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 cell walls was found to be a limiting factor for *N. gaditana* nutrient digestibility in Nile tilapia.

4. Conclusions

This study showed that nutrient accessibility is a dominant limiting factor in nutrient digestibility of the microalga *Nannochloropsis gaditana* in Nile tilapia. Mechanical and physical processes increased *in vivo N. gaditana* 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.

Table 8
Linear and quadratic relationships fitted 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 [%]	p (linear component)[2]	p (quadratic component)[2]
Protein ADC (%)	N_{sol} [%]	Y = 51.99 (SE 2.163) + 1.41 (SE 0.193) × X	87.7	< 0.001	–
		Y = 35.91 (SE 8.518) + 4.26 (SE 1.475) × X − 0.11 (SE 0.055) × X^2	90.3	–	0.071
	DH [%]	Y = 56.14 (SE 1.173) + 1.91 (SE 0.183) × X	93.4	< 0.001	–
		Y = 48.73 (SE 2.586) + 4.64 (SE 0.895) × X − 0.18 (SE 0.593) × X^2	96.0	–	0.008
	Ion [%]	Y = 45.86 (SE 3.028) + 0.51 (SE 0.071) × X	87.2	< 0.001	–
		Y = 71.11 (SE 8.363) − 0.81 (SE 0.421) × X + 0.02 (SE 0.005) × X^2	92.5	–	0.007
Fat ADC (%)	N_{sol} [%]	Y = 36.03 (SE 2.574) + 2.40 (SE 0.229) × X	93.4	< 0.001	–
		Y = 32.39 (SE 11.299) + 3.04 (SE 1.956) × X − 0.02 (SE 0.073) × X^2	93.4	–	0.745
	DH [%]	Y = 44.07 (SE 1.797) + 3.08 (SE 0.281) × X	94.0	< 0.001	
		Y = 47.25 (SE 4.985) + 1.92 (SE 1.726) × X + 0.08 (SE 0.114) × X^2	94.2	–	0.503
	Ion [%]	Y = 27.86 (SE 4.894) + 0.81 (SE 0.115) × X	86.9	< 0.001	–
		Y = 77.30 (SE 11.230) − 1.77 (SE 0.565) × X + 0.03 (SE 0.007) × X^2	94.8	–	< 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] $p < .05$ relationships were considered significant, $p < .1$ relationships were considered a tendency for a significant relationship.

Table 9
Correlations between apparent digestibility coefficients of nutrients in juvenile Nile tilapia (at 30% diet inclusion level) to *in vitro* nutrient accessibility measurements[1] of treated or untreated *Nannochloropsis gaditana*.

	Correlation coefficients r [−][2]			
	DH	N_{sol}	Ion	BC
Dry matter	0.86***	0.82***	0.85***	0.83***
Gross energy	0.92***	0.90***	0.88***	0.90***
Crude protein	0.93***	0.88***	0.87***	0.89***
Crude fat	0.94***	0.93***	0.87***	0.93***
Total carbohydrates	0.46†	0.45†	0.43†	0.45†
Ash	0.42	0.35	0.55	0.35
Phosphorus	0.48*	0.47*	0.47*	0.51*
Calcium	0.84***	0.83***	0.92***	0.79***

[1] DH - degree of protein hydrolysis using pancreatin; N_{sol} - nitrogen solubility in a pH 8 buffer; Ion – conductivity in water; BC – alkaline buffering capacity.
[2] Pearson correlations (r) between the triplicate apparent digestibility coefficient values ($n = 18$) and the mean values of each *in vitro* measurement ($n = 6$). * = $p < .05$, ** = $p < .01$, *** = $p < .001$ and † = $p < .1$.

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