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Northern Icelandic marine microalgae as health promoting multi-functional feed protein and lipid source for Atlantic salmon (*Salmo salar*) – A whole algae concept

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Report Summary

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Title	Northern Icelandic marine microalgae as health promoting multi-functional feed protein and lipid source for Atlantic salmon (*Salmo salar*) – A whole algae concept	
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Summary:	An increase in fish consumption, combined with a decrease in wild fish biomass, is driving the aquaculture industry at a rapid pace. Today, farmed seafood accounts for about half of all global seafood demand for human consumption. As the aquaculture industry continues to grow, so does the market for aquafeed. Currently, some of the feed ingredients are coming from low-value forage fishes (fishmeal) and terrestrial plants. The production of fishmeal can't be increased as it would affect the sustainability and ecosystems of the ocean. Similarly, increasing the production of terrestrial plant-based feed leads to deforestation and increased freshwater use. Hence, alternative, and environmentally sustainable sources of feed ingredients need to be developed. Testing initially 13 Icelandic marine microalgae species regarding their potential to accumulate high protein or lipid in response to alterations of phosphate or nitrogen (standard or 5 % of standard) as well as salinities (20, 30 and 40 PSU) in the culture media in combination with different photoperiods (12:12, 16:08, 08:16 and 24 h). The results obtained showed that In most cases nitrogen starvation combined with high light (16:08 h) and high salinity stress (40 PSU) led to significant shifts in the relative protein and lipid contents of the tested species. Selecting eight species-cultivation constellations, further up-scales were conducted (5 L and 100 L) to gain biomass for further analysis and experiments (e.g., harvest and process optimizations, qualitative and quantitative amino and fatty acid compositions, compound screenings and LC-analysis, aquafeed development and feed analysis as well as effect screenings, including cytotoxical and anti-oomycete screenings as well as Atlantic salmon feeding trials using parr and post-smolts). Since Atlantic salmon usually does not feed on microalgae and the cell wall of these autotrophic microorganisms has been proven to reduce the digestibility of proteins and lipids, Chlorophyceae cells were disrupted prior to their integration into the feed. In the feeding trials, fish performances, feed efficiency, lipid metabolism and final product quality were assessed to investigate the potential of the four whole-cell-microalgae products in experimental diets specifically formulated with low fish meal content. The results revealed that farmed Atlantic salmon parr and post-smolt can grow successfully on an 73-78.9% microalgae diet when supplemented with fish protein hydrolysates (FPH), low percentages of fishmeal (10%) both originating from Nile Tibia combined with zooplankton meal the brine shrimp *Artemia salina* and the copepod *Acartia tonsa*.	
Keywords:	*Atlantic salmon, aquafeed, microalgae, inducement of high lipid and protein*	

Contents

1. Introduction

The FAO of the UN predicts that the world's population will reach 9.7 billion by 2050, and the demand for food is set to increase by 50%. Fish, particularly farmed salmonids (e.g., salmon, trout, charr), can offer one solution to meet this increased demand. Farming of salmonids uses feed inputs more efficiently than terrestrial animal protein production systems (e.g., beef, poultry, and pork). Typical feed conversion ratio (FCR) for salmonids is 1.2 g feed g gain^{-1} compared to 1.8–6.3 g feed g gain^{-1} for livestock. This is due to higher dietary protein and energy retention efficiency in salmonid fish (23–31%) compared to terrestrial farm animals (5–21%). Also, since fish are poikilothermic and expend less energy maintaining their position in the water column, edible yields of farmed salmonids are higher (68%) than terrestrial livestock (38–52%). Salmonid farming in integrated multitrophic Aquaculture (IMTA) occupies low carbon footprints and those farmed in Norway, Chile and Canada may, in fact, be the most ecologically sustainable products on the global food protein market. However, it's important to note that salmonids are highly piscivorous and the industry remains greatly dependent upon global ocean resources, albeit to a far lower degree than previous decades (Tibetts 2018).

Figure 1. Atlantic salmon Post-smolt.

Atlantic salmon (*Salmo salar* L.) have considerable commercial, conservation, recreation and subsistence value as farmed, hatchery-produced and wild populations (Hindar et al. 2011, Dixon 2017, Fig. 1). Wild Atlantic salmon are known to feed opportunistically and to use a wide array of invertebrate and fish prey items (Lear 1972, Jacobsen & Hansen 2001). In aquaculture, Atlantic salmon are cultured in freshwater tanks and raceways in flow-through systems prior to transfer to sea. In the marine environment, they are farmed in sea cages where they depend solely on formulated feeds (FAO 2017). Atlantic salmon feeds are formulated for various stages of fish development and production cycles in freshwater and seawater. While freshwater feeds contain 16-24% lipid and 45-54% protein, respectively, the protein content in the salmon dietary is reduced (around 8% lower) and lipid content increased (up to 16% higher) during their seawater grow-out phase to market-size (~ 4 kg, FAO 2017). A wide range of ingredients is used in the formulation of Atlantic salmon feeds,

comprising proteins, essential fatty acids, vitamins and minerals (cf. Fig. 2). Several protein supplements such as high-quality fishmeal, plant protein products (soybean meal, corn gluten meal, canola meal, pea meal), animal by-product meal (poultry by-product meal, meat meal, blood meal, hydrolyzed feather meal) and crustacean meal (krill, shrimp, crab) are used in salmon feed formulation depending upon economics and availability (FAO 2017). Previous studies have proven that the inclusion of small amounts (<10% of the diet) of algae in fish feed (aquafeed) resulted in positive effects in growth performance and feed utilisation efficiency. Marine algae have also been shown to possess functional activities, helping in the mediation of lipid metabolism, and therefore are increasingly studied in human and animal nutrition (Norabuena et al. 2015).

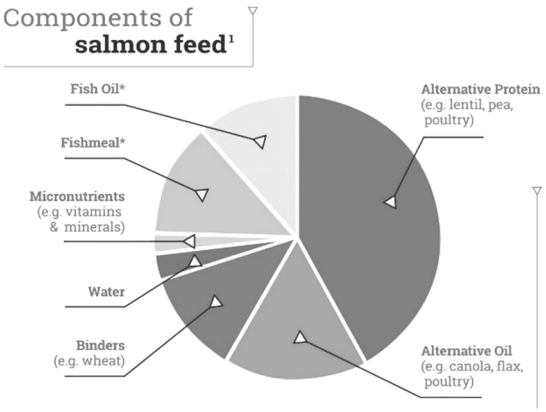

Figure 2. from http://bcsalmonfarmers.ca/growing-worlds-best-fish/

Fish meal and fish oil are abundantly used in fish feed (aquafeed) largely due to their content of high-quality proteins and beneficial vitamin composition, highly unsaturated fatty acids (HUFA) and omega-3 long chain polyunsaturated fatty acid (n-3 LC-PUFA) (FAO 1996). A global survey estimated aquaculture consumption of fish meal and fish oil at above 4,000 and 800 thousand tonnes/year, equating to 68.2% and 88.5% of the yearly global supply, respectively (Norabuena et al. 2015). It is anticipated that the supply of fish meal and fish oil will be exhausted by 2040 (e.g., Duarte et al. 2009). However, because of their current limited supply, raising prices and contamination with a range of pollutants, heavy metals and toxins, alternative raw materials are increasingly being used in aquafeed formulation (e.g., Sirakov et al. 2013).

With the wild fish supplies being unable to meet the demand from aquaculture, farmed fish are increasingly exposed to dietary plant materials, often without a comprehensive understanding of their impacts on fish health and ability to resist pathogens (Martin & Król 2017). Beyond that, fast development of aquaculture and increasing fish demand has led to intensification of fish culture, magnifying stressors for fish and thus heightening the risk of disease (Reverter et al. 2014).

Microalgae are a heterogeneous group of photosynthetic single-cell prokaryote (cyanobacteria) and eukaryote microorganisms, which are located in various environments, under a wide range of temperatures, pH and nutrient conditions (e.g., Meng et al. 2009). Microalgae play a key role in natural ecosystems by supplying organic matter and specific molecules, such as polyunsaturated fatty acids (PUFAs), to higher organisms such as fishes (e.g., Bellou et al. 2014). The biodiversity of microalgae is enormous, estimated at more than 100 000 up to 1 000 000 species of which only around 3000-5000 have been analysed and studied. In contrast, only around 30 species are currently used in biotechnological processes (e.g., Richmond 2004). Microalgae can biosynthesize, metabolize, accumulate and secrete a great diversity of primary and secondary metabolites, including high-value molecules, such as lipids, carbohydrates, proteins, vitamins and pigments (e.g., Pulz & Gross 2004). Many of these metabolites are valuable substances with potential applications in the food, pharmaceutical and cosmetics industries (e.g., Yamaguchi 1997, Gouveia et al. 2008).

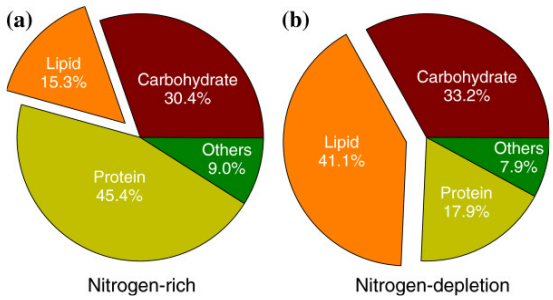

Figure 3. Example of the biochemical composition of a Chlorophyceae during a) standard and b) nutrient depleted culture conditions (Ho et al. 2014)

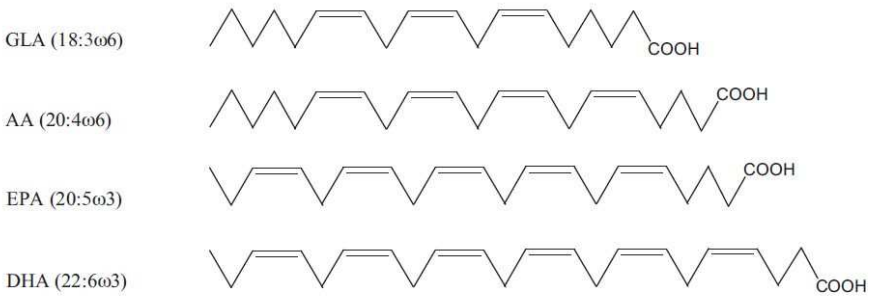

Figure 4. Chemical structure of polyunsaturated fatty acids of high pharmaceutical and nutritional value (Gouveia et al. 2008).

Proteins and lipids form the largest fractions of microalgae cells followed by carbohydrates (e.g., Templeton et al. 2012, Fig. 3). Lipids originate entirely or in part from two distinct types of biochemical subunits: ketoacyl and isoprene groups (Fahy et al. 2011) and are divided into eight categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids and prenol lipids (derived from condensation of isoprene subunits). Out of these categories fatty acids are of main biotechnological interest, particularly long chain polyunsaturated fatty acids (=LC-PUFAs, more than 18 carbons) which cannot be synthesized by higher plants and animals, only by microalgae (Pulz & Gross 2004), such as γ-linolenic acid (GLA, 18:3ω6), arachidonic acid (AA, 20:4ω6), eicosapentaenoic acid (EPA, 20:5ω3) and docosahexa-enoic acid (DHA, 22:6ω3) (Chini Zittelli et al. 1999, Bandarra et al. 2003, Donato et al. 2003, Molina Grima et al. 2003, Spolaore et al. 2006, Fig. 3). In species belonging to the genera *Porphyridium*, *Dunaliella*, *Isochrysis*, *Nannochloropsis*, *Tetraselmis*, *Phaeodactylum*, *Chlorella* and *Schizochytrium*, lipid content varies between 20 and 50% of dry biomass (=DW, Bellou et al. 2014). However, higher lipid accumulation (up to 80 % DW) can be reached by varying the culture conditions. Most microalgae accumulate lipids under specific environmental stress conditions, such as extreme temperatures, pH, salinities and nutrient limitations (e.g., Amaro et al. 2011, Bellou & Aggelis 2012, Hu et al. 2008, 2013, Msanne et al. 2012, Scholz & Liebezeit 2012a, b, 2013). Therefore, the management of the culture conditions is a common approach used for improving lipid accumulation in microalgal cells (Fig. 4).

Proteins are composed of different amino acids and hence the nutritional quality of a protein is determined basically by the content, proportion and availability of its amino acids (Becker 2007). Some species of microalgae are known to contain protein levels similar to those of traditional protein sources, such as meat, egg, soybean, and milk (Gouveia et al. 2008), but the quality of the proteins can vary dramatically, depending on digestibility and the availability of essential amino acids (Boisen & Eggum 1991). Basically, the amino acid composition of the protein of microalgae is very similar

between species (Brown 1991) and relatively unaffected by growth phases and light conditions (Brown et al. 1993a, b). In comparison to higher plants in which proteins are often considered as incomplete protein source as they commonly lack one or more of the essential amino acids (=EAA), algae are generally regarded as a viable protein source, with EAA composition meeting FAO requirements for aquaculture feeds (FAO 1991). In contrast to lipids, total proteins are the main biomass composite that decreases significantly when lipids or carbohydrates are accumulated due to stress conditions (e.g., Stehfest et al. 2005). Therefore, it is necessary to optimize in the present project the culture conditions for the production of lipid- and protein-rich microalgae separately and combine both products during the final aquafeed production in different concentrations.

The ability of fish to resist pathogens and cope with stress depends to a large extent on their nutritional status; the need for dietary interventions that would improve fish health has become widely recognised as central to sustainable aquaculture and the future of industry (Martin & Król 2017). Several studies have proven that the addition of algae in fish diets resulted in several positive effects such as the increase in growth performance, feed utilisation efficiency, carcass quality, physiological activity, intestinal micro biota (Mustafa & Nakagawa 1995, Valente et al. 2006), disease resistance (Satoh et al. 1987), improved stress response (Wassef et al. 2005), counteracting intestinal inflammation produced by soybean meal (Grammes et al. 2013), modulation of the lipid metabolism (Nakagawa 1997), and improved protein retention during the winter period of reduced feed intake (Nakagawa et al. 1993). Predominantly in the context of disease resistance and immune stimulation, studies have been shown that particularly marine microalgae contain an array of promising functional metabolites (e.g., Dias et al. 2012; Martin & Król 2017). For example, the microalgal carbohydrate beta-1,3-glucan is considered responsible to initiate host defense reactions in response to pathogen surface molecules (Yaakob et al. 2014). In addition, treating fish leucocytes with beta-1,3-glucan or adding this to fish food raises the non-specific and specific immunity level and the protection against infection (Belmonte et al. 2014, Martin & Król 2017).

Particularly pathogenic oomycetes have the ability to infect a wide range of plant and animal hosts and are responsible for a number of economically important diseases. Saprolegniosis, a disease affecting aquaculture broodfish, fish eggs and juvenile fish in hatcheries worldwide, is caused by the pathogenic oomycete *Saprolegnia parasitica* (Thoen et al. 2011, Earle & Hintz 2014). *S. parasitica* represents a serious problem in the aquaculture industry (e.g., van West 2006, Phillips et al. 2008). It is estimated that 10% of all hatched salmon succumb to saprolegniosis, causing major financial loss in an industry accounting for approximately 30% of the global fish production for consumption (e.g., van West 2006, Fregeneda-Grandes et al. 2007, Phillips et al. 2008). Until 2002, *S. parasitica* was kept under control through the use of Malachite green; however, due to its carcinogenic and toxicological effects, treatment with this chemical has been banned internationally (e.g., Robertson et al. 2009) and an adequate replacement has been still not developed. In this context, microalgae extracts have been reported to favor various activities like enhancement of immunostimulation, antifungal, antimicrobial and antipathogen properties in fish and shrimp aquaculture due to active principles such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics or steroids (e.g. Scholz & Liebezeit 2006, Chakraborty & Hancz 2011, Citarasu 2010, Scholz et al. 2017).

2. Objectives and Aims of the Study

Marine microalgae, as base of the aquatic food web, are rich sources of high-value molecules, such as proteins, carbohydrates and vitamins. Utilizing northern Icelandic microalgae strains maintained at BioPol ehf, optimizations of culture conditions will be combined with compound and selected activity screenings (cf. Fig. 5). Further up-scales combined with culture and processing optimizations finally led to the aimed multifunctional aquafeed, which includes, besides high-lipid and high-protein contents, also essential vitamins, carbohydrates and carotenoid pigments (e.g., β-carotene).

Specifically, we aimed to

- to induce the synthesis of high-lipid and high-protein contents in the microalgae cells by application of different culture conditions.
- Conduct compound screenings.
- use LC-MS systems to provide information regarding the quantitative and qualitative compositions of the lipids and proteins.
- develop suitable salmon-feed-formula based on the obtained information.
- conduct toxicity (LUMIStox, MTT assay) and anti-*Saprolegnia* screenings to find first hints on promising anti-pathogen activities of the products (extracts of lipid- and protein-rich whole microalgae powder)
- conduct feeding trials on juvenile and adult salmons.

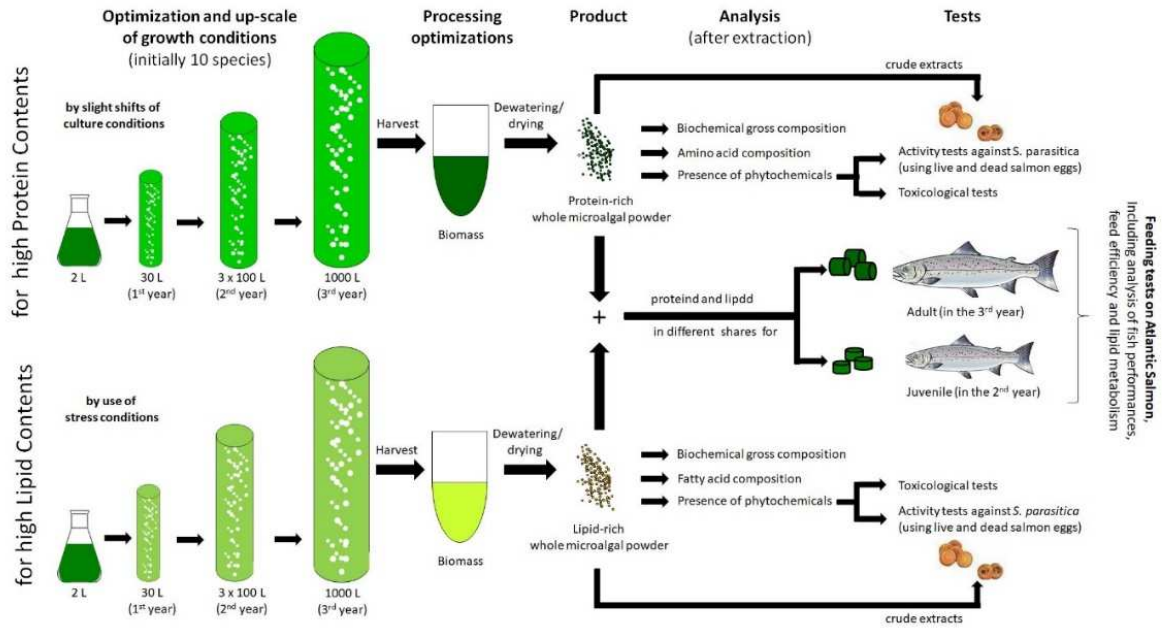

Figure 5. Schematic overview of the project.

3. Material and Methods

3.1 Algal Strains and Culture Conditions

3.1.1 Algal Species

Initially 13 microalgae species were selected for the experiments due to their reliable growth patterns.

Table 1. List of microalgae and cyanobacteria which were used in the experiments, including specifications of growth conditions (media, temperature, light conditions, and nitrogen (N) source). Furthermore, the laboratory code contains besides the name and taxonomic order initials information regarding year of isolation, habitat, and location.

No	Species	Laboratory Code (e.g., year and location of isolation, group designation)	Medium	Optimum growth by		
				T [°C]	PAR [µmol m^{-2} s^{-1}]	N-source
Cyanobacteria						
1	*Spirulina subsalsa*	14CY-P-SS-SKA	BG-11	16	30	$NaNo_3$
2	*Synechococcus*	14CY-P-SYN-SKA	BG-11	16	15	$NaNo_3$
3	*Synechocystis*	15CY-P-SYNC-SKA	BG-11	16	30	$NaNo_3$
Chlorophyceae						
4	*Dunaliella tertiolecta*	15CH-P-DT-WF	f/2	18	80	urea
5	*Chlorella salina*	16CH-P-CS-SKA	f/2	18	80	urea
6	*Nannochloropsis salina*					
Rhodophyceae						
7	*Porphyridium purpurerum*	13RHO-P-PP-SKA	f /2b	18	50	$NaNo_3$
Prymnesiophyceae						
8	*Isochrysis galbana*	13PR-P-IG-SKA	f/2	18	50	$NaNo_3$
Bacillariophyceae (diatoms)						
9	*Amphiprora paludosa*	14DIA-P-AP-ISA	f/2 Si	14	40	$NaNo_3$
10	*Amphora ovalis*	14DIA-P-AO-ISA	f/2 Si	14	40	urea
11	*Asterionella glacilialis*	14DIA-P-AG-HRU	f/2 Si	14	40	$NaNo_3$
12	*Coscinodiscus wailesii*	14DIA-P-CW-SKA	f/2 Si	14	40	urea
13	*Cylindrotheca closterium*	15DIA-P-CC-SKA	f/2 Si	14	40	$NaNo_3$

Abbreviations: PAR, photosynthetic active radiation measured in PFR, Photon fluence rates; P, phytoplankton species; SKA, Skagaströnd (Húnaflói); HRU, Hrútafjörður; ISA, Ísafjörður. Growth media: f/2b, f/2 Brackish Medium (f/2 Medium diluted 1:1 with sterilised freshwater, (Guillard 1975); f/2 + Si, f/2 Medium enriched with silicate; f/2b +Si, f/2 Brackish Medium enriched with silicate; f/2, f/2 Medium (Guillard 1975); BG-11 (Stanier et al. 1971).

3.1.2 Cultivation

Prior to cultivation in liquid culture, the microalgae were purified from bacterial contaminants by spreading cells on 1.5% medium agar plates with 5 µg mL^{-1} tetracycline and 5 µg mL^{-1} kanamycin (Guillard 1975). The absence of bacteria was verified by epifluorescence microscopy (BX51, Olympus

Corporation, Tokyo, Japan/Axiophot, Carl Zeiss AG, Oberkochen, Germany) using the dye 4',6-diamidino-2-phenylindol (DAPI). Subsequently each isolate was maintained in culture in 500 mL flasks (250 mL culture volume) under sterile conditions at 16±2°C, 12:12 h light: dark (L:D) regime and at an irradiance of 30-80 µmol photons m^{-2} s^{-1} using Master TL-D 18W/840 light (Phillips, Germany), corresponding to species specific optima (cf. Table 1, Fig. 6). Cultures were adapted to the standard culturing conditions described above at a saturating nitrogen concentration of 800 µM, ca. 90% of original medium, for two weeks. The nitrogen to phosphorous ratio of the medium during all experiments was 17:1. Furthermore, sodium nitrate ($NaNO_3$) was used as main nitrogen source besides urea for Chlorophyceae and most diatoms. Artificial seawater salt (Tropic Marin Classic®, GmbH Aquarientechnik, Wartenberg) dissolved in de-ionised water was used with a salinity of 30 and pH of 8.3, corresponding to environmental factors recorded at the sampling sites from which the species originated in northern Iceland. Salinity, pH and conductivity were measured using handheld probes (YK-31SA, YK-2001PH SI Model 33**,** Engineered Systems and Designs-Model 600, Philips W9424). Photon fluence rates (PFR, 400–700 nm) were measured with an underwater spherical quantum sensor LI-193SA connected to a Licor Data Logger LI-250A (LI-COR Lincoln, NE, USA).

Figure 6. Cultivation of microalgae and cyanobacteria in different locations. A) 2016: in the climate chamber at BioPol ehf. B) 2018: at the BioPol laboratory. The maximum temperature change per hour was ±5.5"C (depending on season); C), D) since 2019 in an external facility which provides the optimal temperature gradient from the highest to lowest shelf (difference 3°C). The overall temperature change per hour is ±0.2"C (measured on the highest shelf).

3.2 Microalgae Experiments

3.2.1 First Screening for high Lipid and Protein inducing Culture Conditions and Media Compositions

The small-scale experiments were conducted in 100 mL Erlenmeyer flasks (50 mL culture volume) in batches of 13 species and 36 different culture media per run in 2018 (cf. Fig. 7). Besides the different culture media also four different photoperiods were tested (12:12 h, 16:8 h, 8:16 h and 72:0 h light:dark cycles with an irradiance of 50 µmol photons m^{-2} s^{-1} using Master TL-D 18W/840 light (Phillips, Germany), and three salinities (20, 30, 40 PSU = Practical salinity units), resulting overall in 470 test assays. The trials started by filling the Erlenmeyer flasks each with 25 mL fresh culture medium, add 25 mL dense algal culture (8.8 x 10^6 – 8.3 x 10^9 cells per millilitre) and incubating them at a temperature of 16±2°C for 192 hours. As culture media BG-11, f/2, f/2b, and f/2Si (cf. Table 1 for the individual species/culture medium pairing) were applied, reducing in each run either phosphate or nitrate in the media (95% reduction). As control, the nutrient compositions of the standard culture media were used. All media receipts are attached in the appendix (9.2). The flasks were careful shaken every day and the position of replicate flasks was randomly changed every second day to eliminate any location effect due to minor changes in external conditions. After 192 hours incubation all test and control assays were harvested and processed as described in 3.4. The biochemical gross composition analysed as described in 3.5.1.

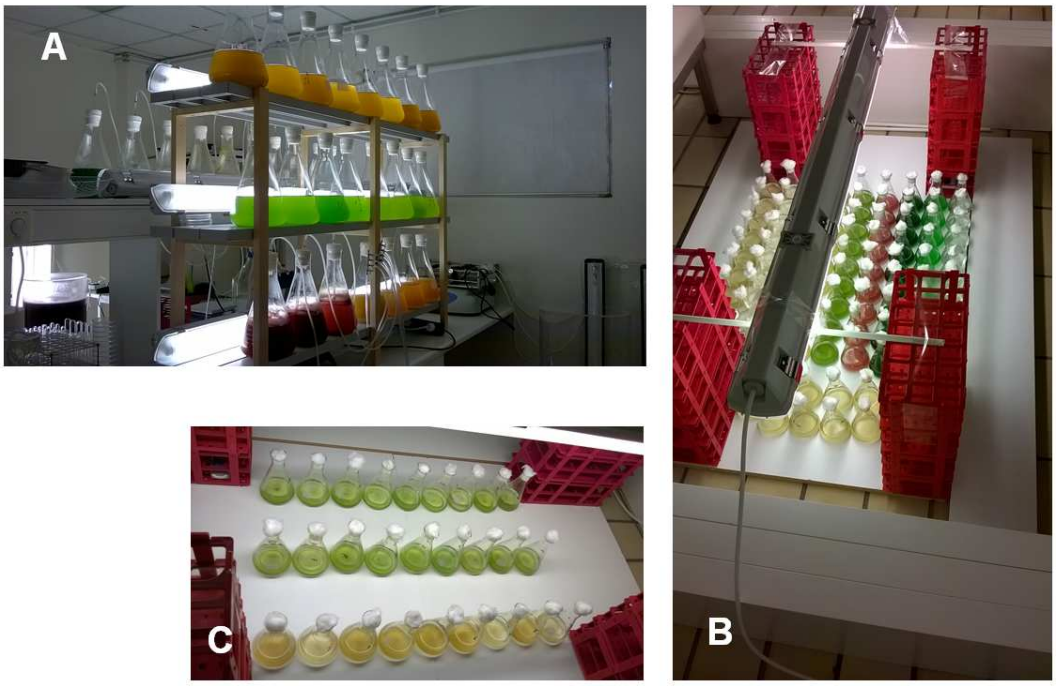

Figure 7. Conditions prior and during the small-scale experiments, showing the up-scale cultures in 2 L Erlenmeyer flasks (1600 mL culture volume, A), the design of the experiment at the beginning of the 12: 12 h run using 10 species in 36 media (B), and some of the assays after 8 days (C).

3.2.2 First up-scale and Investigation regarding the Influence of Harvest and Processing Methods on the Recovery Yield of Proteins and Lipids

To gain more biomass for further biochemical analysis (e.g., qualitative and quantitative lipid and protein compositions) cultivations were conducted in 5 L flasks (e.g., Fig. 8D), utilizing eight out of the 470 test assays from the former screening experiments (3.2.1) for which the highest lipid and protein yields were recorded (Table 2) and for which interesting features in the compound pre-screening were recorded (data not shown). An additional aim was to test the effect of different harvest methods on the lipid and protein yield. For this purpose, five biomass samples were selected (Table 2, No 1, 2, 3, 5, and 7). As different harvest methods centrifugation (control), autoflocculation by alkaline pH shifts (NaOH, KOH, $Ca(OH)_2$; pH 12) and biological flocculants (Modified Larch-Tannin and chitosan) were tested, whereas as alternative processing methods lyophilization (control) and hot air drying (30, 40, 50 and 60°C) were compared. The flocculation procedure was carried out according to previously published sources with a focus on comparing the flocculants (Castrillo et al. 2013). For each test, 150 mL of harvested algae culture were pH adjusted in a beaker and the respective flocculant was added. Algae suspensions were stirred at 300 rpm for 3 min and then left for settling. Flocculation efficiency (FE, η) was determined after 30 min by sampling and determining OD_{680} 2 cm below suspension surface, using the formula

$$\eta = 1 - (OD_f/OD_i)$$

with OD_f being OD_{680} after flocculation and OD_i as initial OD_{680}.

Chitosan was obtained from BioLog Heppe GmbH (Biolog Heppe GmbH 2017) and was prepared as 10 g/L stock solution in 1% v/v acetic acid. Modified Larch-Tannin (Polysepar CFL-PT), supplied as quaternary, cationic ammonium-tannate, was obtained from Separ Chemie (Separ-Chemie 2017). CFL-PT is marketed as emulsion breaker and fixation agent for the treatment of wastewater or process water. According to REACH specification noted in the respective data sheet (Separ-Chemie 2015), it exhibits low toxicity (LD50 oral: 2260 mg/kg for *Rattus rattus* and LC50 73.9 mg/L for *Labeo rohita*) and is "easily biodegradable." For removal of AOM, samples were centrifuged at 6,000 × g for 15 min and resuspended in fresh medium (Henderson et al. 2010). For the drying process a horizontal dryer with hot air flow (Tray dryer, model no. FDTHQQZ) was used.

Table 2. List of the selected assays for the up-scale cultivations in 3.2.2 and 3.2.3.

No	Species	Medium	Cultivation Parameter	Outcome
1	*S. subsalsa*	BG11	standard nutrients, 08:16 h light:dark, 30 PSU	high protein
2	*D. tertiolecta*	f72	nitrogen depletion, 16:08 h light:dark, 40 PSU	high protein
3	*C. salina* I	f72	phosphate depletion, 12:12 h light:dark, 40 PSU	high protein
4	*C. salina* II	f72	nitrogen depletion, 16:08 h light:dark, 40 PSU	high protein
5	*N. salina* I	f72	nitrogen depletion, 12:12 h light:dark, 20 PSU	high protein
6	*N. salina* II	f72	nitrogen depletion, 16:08 h light:dark, 20 PSU	high protein
7	*C. wailesii* I	f/2Si	nitrogen depletion, 12:12 h light:dark, 40 PSU	high lipid
8	*C. wailesii* II	f/2Si	nitrogen depletion, 16:8 h light: dark, 40 PSU	high lipid

As culture media BG-11, f/2, f/2b, and f/2Si were applied (cf. Table 2 for the individual species/medium pairing). The culture conditions, except for the media compositions, salinities and photoperiods (Table 2), were as described in 3.1.2. The experiments started by filling 3000 mL fresh prepared media to

2000 mL dense grown microalgae cultures in the 5 L bottles, under continuously aeration via pumps. All species were in their stationary growth phase when the fresh medium was added. After 504 h the 5 L were harvested using the standard method (3.4). For the five selected assays of the harvest and processing trial 1 L from each was used. The biomasses were subdivided into eight equal portions and analysed subsequently after the procedures as described in 3.5.1.1 and 3.5.1.2. In addition, the biomasses from the control assays were employed for the quantitative and qualitative analysis of amino and fatty acid compositions (cf. 3.5.2) as well as the compound screenings described in 3.5.3. The latter samples were used afterwards in the toxicological as well as anti-oomycete screening (3.8.1). Finally, all data obtained during the analysis were utilized to develop overall four whole-cells-microalgae aquafeeds (cf. 3.6).

Figure 8. Examples of up scales, comprising different volumes of 2 L and 5 L flasks and 5 L bubble columns (B, D, E) as well as experiments in the two 100 L PBRs (A, C).

3.2.3 Cultivations in 100 L Photobioreactors (PBRs)

To gain biomass for the aquafeed development and feeding trials, cultivations in photobioreactors (PBRs) were conducted, utilizing bubble column PBRs, made from transparent ACRYL (KUS-Kunststofftechnik, Recklinghausen, Germany). The cultivations were conducted in two 100 L bubble column PBRs in parallel (ø 400/390 mm, length 1000 mm, Fig. 8C), using in each case species/culture conditions constellations described in Table 2. The up scales were identical for all five test-species and included the following capacity steps: 4 x 2 L (Fig. 30E), 4 x 5 L (Fig. 30B, D), 40 and 80 L. The latter two steps took already place in the PBRs (Fig. 30A), using a temperature of 17±0.5°C and a light to dark cycle of 12:12 hours during all steps of the upscales. The cultivations started by filling 20 L fresh culture medium into each PBR which was adapted to the desired composition (Table 2). The runs were repeated several times to gain the necessary amount of biomass (up to 5 times per species). The use of 100 L PBRs replaced cultivations in 1000 L, avoiding thereby problems related to process timely such high volumes. The biomasses for the aquafeed development and feeding trials were harvested by addition of Modified Larch-Tannin and processed via drying at 40 °C.

3.3 Determination of Microalgae Growth

Growth was monitored by conducting cell counts and by measuring dry matter. Counting of cells during the experiments were performed under a light microscope (BX51, Olympus Corporation, Tokyo, Japan / Axiophot, Carl Zeiss AG, Oberkochen, Germany), using a Neubauer improved counting chamber with 0.1 mm depth (LO Laboroptik GmbH, Germany). During the long-term experiments, cell counts were conducted every second day. Only cells were counted, which exhibited red fluorescence under ultraviolet light. At least 500 cells were counted in each sample at a 400× magnification. The specific growth rate (μ) was calculated with the following equation:

$$\mu = \frac{\ln_{c1} - \ln_{c0}}{t_1 - t_0}$$

where c_1 and c_0 are the number of cells at time t_1 and t_0.

In addition, dry cell weight (DCW) or simply dry weight (DW) was measured in triplicates by using 0.45 µm cellulose acetate filters (WHA10404006 Whatman®). Filters were pre-dried for 10 min at 105°C to remove any moisture. Subsequently 40 mL of the algal culture was filtered and dried for 24 h at 70°C and then weighed to measure the dry weight, then expressed as grams per litre.

3.4 Harvest and Processing

Biomasses were harvested by centrifugation at 8000× g for 10 min at 4°C (Heraeus Biofuge Primo R, Thermo Fisher Scientific GmbH, Dreieich Germany). Harvested cells were processed by lyophilization (24 h; Modulyo desk top lyophilisator 3981, Edwards High Vacuum Int, Sussex, England).

3.5 Biochemical Analysis

If not otherwise mentioned, all chemicals used in this study were of the highest purity from Sigma/Aldrich.

3.5.1 Gross Compositions

3.5.1.1 Crude Protein

For pre-treatment, stock solutions were prepared with approximately 500 mL of ultrapure water and some drops of 2 N NaOH to adjust to pH 12. A sample of 1 g of freeze-dried biomass was added to 50 mL of the stock solution and the mixture heated at 40 °C with stirring for 1 h. Separation of the solid–liquid mixture was conducted by centrifugation at 5000 g for 10 min. Samples of the supernatant were taken for protein and for amino acid analysis (cf. 3.5.2.2). Protein was measured using the Lowry method as described by Herbert et al. (1971) with bovine serum bumin as a standard. In brief, 5 mL 1.0 N NaOH was added to the biomass aliquot and incubated for 5 min in a boiling water bath (95°C,

Typ 1083, GFL mbH, Burgwedel, Germany). After cooling, 2.5 mL of the reactive mixture (5% Na_2CO_3 + 0.5% $CuSO_4$ x 5 H_2O in 1.0% Na-K-Tartarate; ratio 25:1 v/v) were added and incubated for 10 min at room temperature. This was followed by the addition of 0.5 mL Folin-phenol reagent (1.0 N) and incubation for another 15 min. After centrifugation (Omnifuge 2.0 RS, Heraeus Sepatch, Osterode, Germany), the intensity of the resulting blue colour was determined at 650 nm.

3.5.1.2 Crude Lipids

The total lipid determination was based on the method of Ryckebosch et al. (2014), using a chloroform–methanol solvent mixture (1:1). Briefly, the solvent mixture (6 mL) was added to 100 mg lyophilized microalgae powder and the tube was vortex mixed for 30 s. The mixture (2 mL) and water (2 mL) were then added, and the tube was vortex mixed again and subsequently centrifuged at 2,000 rpm for 10 min. The aqueous layer was removed, and the solvent layer was transferred into a clear tube. The remaining solids were re-extracted with 4 mL solvent or mixture. The combined solvent layers were passed through a layer of anhydrous sodium sulfate using Whatman No. 1 filter paper in a funnel. The solvent was removed by rotary evaporation at 40 °C after which the lipid content was determined gravimetrically. The extraction was performed in quadruplicate. The resulting percentage of extracted lipids is the sum of three extractions performed in series.

3.5.1.3 Carbohydrates

Total soluble carbohydrates were determined using the phenol sulphuric acid method of Kochert (1978) and Ben-Amotz et al. (1985) incorporating the modifications of Mercz (1994) and Buttery (2000). The samples were homogenised in 1 M H2SO4, and after heating at 100 °C for 60 min, 0.3 mL of the supernatant was transferred into a fresh vial and made up to 2 mL with deionized water. Sets of glucose standards were prepared and 1 mL of 5 % (w/v) phenol solution was added to all samples, after which 5 mL concentrated H2SO4 was added. Absorbance was read at 485 nm. In the feed analysis, the carbohydrate content was measured after a two-step H2SO4 hydrolysis and HPAEC-PAD analysis, as described by Verspreet et al. (2020). The total carbohydrate level was estimated by summing monosaccharides and uronic acids, each corrected for water uptake during hydrolysis.

3.5.1.4 Dry Matter and Ash Contents

Dry matter content was evaluated by weighing samples before and after overnight drying at 105 °C, while ash content was determined after subsequent drying at 550 °C (4 h) (Atkinson et al. 1984).

3.5.2 Qualitative and Quantitative Lipid and Protein Analysis

3.5.2.1 Fatty Acids

The Lyophilized microalgae biomass (100 mg) was dissolved in 50 mL ethanol and vortexed for 5 min. The solution was then extracted via sonication for 30 min using ultrasonic water bath at room temperature. The solvent extract was then filtered through Whatman No. 1 filter paper and the procedure is repeated with another 50 mL of ethanol for a second and third round of extraction. The filtered extracts were pooled and evaporated to dryness using a rotary evaporator (Heidolph Instruments GmbH and Co.KG, Schwabach, Germany) at 30 °C, and stored at -20 °C until further

analysis. The ethanol fraction was then used for LC-MS analysis. Fatty acids in the crude samples (~3 mg) were extracted using chloroform and methanol (1:2 v/v) by an ultrasonic device for 10 min. To separate the residue, the mixture was separated by centrifugation at 3000 rpm for 10 min, and the liquid phase was transferred into a glass tube. These extraction steps were repeated three times to obtain maximum extract. After the extraction, several ml of Milli Q water was added, the glass tube was shaken using vortex. Then, to separate impurities (which were dissolved in the water) and the organic solvent phase containing fatty acids, the glass tube was centrifuged at 3000 rpm for 10 min. The water was removed, and the organic solvent phase was dried once. Derivatization into FAMEs were performed with acetyl chloride and methanol (5:100 v/v) (West & West 2009). A 10ml acetyl chloride and methanol was added into the dried glass tube, and the derivatization was achieved by reacting under heat at 100 °C for 60 min using a heat block. After cooling at room temperate, the glass tube was added 2 ml of hexane and shake well, and then an aliquot of the upper phase was transferred to a new glass vial. This extraction step was repeated two more times. The final removal of hexane was performed on a 40 °C hot plate with nitrogen stream. Then, the sample was resolved with an accurate amount of hexane (300 µL). Finally, 1 µL of the sample was injected into a GC–MS for fatty acid analysis. Using this method, non-esterified fatty acids were obtained (Bouzabata et al. 2014). The identification of FAMEs was assisted by National Institute of Standards and Technology Library (NIST 17 version 2.3) and was confirmed manually. The mono-unsaturated fatty acids were manually detected by the presence of intense peaks of m/z 55 and m/z 69 along with the molecular weight peak. Meanwhile, the di-unsaturated fatty acids were detected by the presence of m/z 55 and m/z 67 intense peaks and m/z 74 and m/z 87 intense peaks were features of saturated fatty acids.

3.5.2.2 Amino Acids

The Amino acids analysis was performed using 100 mg of sample, which was hydrolysed in 10 mL of 6 N HCl under vacuum at 110 °C for 24 h. After hydrolyzation, the sample was filtered (using filter with pores of 0.45 µm), washed with 3 x 1 mL of 0.1 N HCl, evaporated to dryness under nitrogen at 40 °C, and the dry residue was redissolved in 2 mL of distilled water. The method given by Graser et al. (1985) was used for amino acid determination. The HPLC consisted of a Perkin Elmer Series 200 pump, equipped with a Perkin Elmer Series 200 Autosampler and a Perkin Elmer Altus A-10 FL Detector. The gradient elution was used for the separation. Mobile phase A consisted of a methanol/acetonitrile solution in a 12:1 (v/v) ratio, mobile phase B was 23 mM sodium acetate pH 5.95. After applying a linear gradient for 75 min at a flow rate of 1 mL min 1 from 0% to 53.5% B, an equilibration step was performed with 100% A for 20 min.

3.5.3 Compound Screenings

3.5.3.1 Flavonoids

Total flavonoid content was determined by a calorimetric method (Zishen et al. 1999). Approximately 0.5 mL of each extract with the concentration of 100 µg/mL was added with 1 mL 100 % methanol to make up to 3 mL. The mixture was left for 5 min after the addition of 0.4 mL distilled water and 0.3 mL of 5% sodium nitrite ($NaNO_2$). Approximately 2 mL of 1 M sodium hydroxide (NaOH) and distilled water was added to make up to 10 mL after 0.3 mL of 10% aluminium chloride ($AlCl_3$) was added and left at room temperature for 1 min. The mixture was left for 15 min after being shaken. The

absorbance was measured at 510 nm and the concentrations of total flavonoids were determined as quercetin equivalents per mg of extracts, QE/mg.

3.5.3.2 Phenolic Compounds

Polyphenols were extracted from the microalgal culture using 80% ethanol. Total phenol estimation was carried out using the Folin–Ciocalteau reagent. Phenols react with phosphomolybdic acid in Folin–Ciocalteau reagent in alkaline medium and produce a blue coloured complex (molybdenum blue) and the colour intensity was measured at 650 nm (Malik 1980). Extracts which showed interesting activities in the effect screening (3.7) were subjected to analysis via GC-MS. Evaporated extracts were diluted with *n*-hexane, and 1 µL of this was sampled for the gas chromatography. Analysis thereof was performed in a HP 6890 chromatograph equipped with a programmable vaporization temperature inlet (PTV). This machine was coupled to an HP 5973 mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA) in scan mode in order to determine the fractional composition of the extracts. The injector temperature was maintained at 280 °C in a pulsed, splitless mode. A GC program temperature ramp was set at 70 °C for 3 min, and then increased at a rate of 10 °C/min. up to 300 °C, to afford the best separation through a capillary HP-5 MS column. The transfer line was maintained at 300 °C. Mass spectra was obtained at 70 eV and compared through direct matching by using a NIST Mass Spectral Search Program and a NIST/EPA/NIH Mass Spectral Library.

3.6 Feed Formulation

Table 3. List of the selected components (%) in the developed whole-cell-microalgae-aquafeeds (WCMAF) for salmons.

Components	**Developed Aquafeeds**			
	WCMAF I	**WCMAF II**	**WCMAF III**	**WCMAF IV**
S. subsalsa	12.5	5.3	19.0	10.0
D. tertiolecta	30.5	35.3	31.2	20.1
C. salina I	2.3	8.5	5.4	9.5
C. salina II	3.7	-	7.4	10.0
N. salina I	5.2	8.2	0.5	12.0
N. salina II	3.5	4.4	2.6	2.2
C. wailesii I	10.0	10.2	5.5	10.2
C. wailesii II	5.3	7.0	5.1	0.8
Fishmeal (Talipia)	15.0	10.0	10.0	5.0
PHP	10.0		-	-
SPH	-	10.0		-
ASAT			11.3	18.4
others	2.0	2.0	2.0	1.8
Proximate composition (%)				
Moisture	21.5	19.4	20.1	18.3
Protein	34.1	36.2	50.4	56.3
Lipid	5.2	5.5	4.9	5.4
Fiber	1.1	1.0	1.2	1.3
Ash	5.0	5.1	5.0	4.8
Carbohydrates	31.4	32.3	34.4	32.0
Energy (kJ g^{-1})	11.3	11.5	11.7	11.9

Abbreviations: PHP, insoluble partly hydrolysed protein fraction; SPH, water-soluble protein hydrolysate; ASAT, *Artemia salina* and *Acartia tonsa* meal. As others were pregelatinized starch and wheat flour designated.

Based on the results from 3.2.1 and 3.2.2 overall four whole-cell-microalgae-aquafeeds (WCMAF) were developed. Formulation and proximate composition of the experimental diets are shown in Table 3. The individual qualitative and quantitative amino and fatty acid as well as vitamin and mineral compositions of the feeds are given in Tables A1–A4 (9.1 appendix). The Chlorophyceae biomasses (*Dunaliella, Chlorella, Nannochloropsis*) were additionally disrupted by bead milling (DYNO®-MILL-Mulitlab, Spring Lake, MI, USA). Milling was performed in a 0.514 L milling chamber filled to 64.2% (volume-based) with 0.5 mm beads (YTZ® grinding media, Tosoh, Tokyo, Japan). The pump and mill settings were set so that the average residence time was 15 min. Besides the high protein and lipid containing whole-cell-microalgae powder, commercially available feed ingredients were used (designated as "others" in Table 3), and diets were formulated to meet the dietary requirements of salmon, including vitamin and mineral requirements (NRC 2011). Specifically, small and balanced amounts of pregelatinized starch and wheat flour were used to compensate for the resulting modification of the overall nutritional value of the experimental diets due to algal addition (cf. Table A5 for suppliers). The control diet (Skretting UK, Cheshire, UK) was composed of fish meal (35%), soy protein concentrate (14%), wheat gluten (10%), wheat (4%), sunflower meal (3%), faba beans (5%), soybean oil (20%), fish oil (5%, South American and Northern hemisphere fish oil 70:30) and premixes (4%) including crystalline DL-methionine, lysine, vitamins and minerals. The four experimental diets were high in algae protein (>70%) and low in fish-derived protein:

- Diet WCMAF I contained 73% disrupted microalgae biomass, 15% fishmeal (from *Oreochromis niloticus*) and 10% partly hydrolysed fish protein (PHP),
- Diet WCMAF II included 78.9% disrupted microalgae biomass, 10% fishmeal and 10% soluble protein hydrolysate (SPH),
- Diet WCMAF III consisted of 76.7% disrupted microalgae biomass, 10% fishmeal and 11.3% mix of *Artemia salina* and *Acartia tonsa* (ASAT) and
- Diet WCMAF IV contained 74.8% disrupted microalgae biomass, 5% fishmeal and 18.4% ASAT.

All diets were iso-nitrogenous and iso-lipidic in content (Table 3). The molecular weight profile of the PHP and SPH supplement are outlined in Table A6 (9.1 appendix). Celite was included in all experimental diets to allow for apparent digestibility analyses. All ingredients were combined in a commercial baker's mixer and mixed thoroughly before addition of 2–3 L of water (at 80 °C) per 20 kg of diet and further mixed. The experimental diets were extruded (1-2- and 5-mm pellets) in a single screw extruder (PM-80, Bottene, Vicenza, Italy), dried at 40 °C in a dehumidifying oven over a 24-h period and stored at -20° C in airtight bags until use. There were no differences in physical quality or sinking properties of the four diets. The tested protein hydrolysates were produced from whole aquacultured Nile tilapia (*Oreochromis niloticus*) frozen directly after harvest. Following thawing, lipids and bone were removed from the blue whiting and the remaining raw material was enzymatically hydrolysed. The water-soluble protein hydrolysate fraction (SPH) and the insoluble partly hydrolysed protein fraction (PHP) were separated and spray-dried to prevent thermal damage to the protein. The SPH hydrolysate was composed of lower molecular weight peptides and single amino acids and contained 91% protein, of which 96% was soluble. The PHP hydrolysate was composed of low and medium molecular weight peptides and contained 68% protein, of which 18% was soluble (appendix Table A6). As final feed components hatched and reared *Artemia salina* and *Acartia tonsa* were harvested, freeze dried and milled (ASAT). The brine shrimp *A. salina* was cultured according to the methods given by Tiro (1980) and fed with Nannochloropsis, Isochrysis and Pavlova, whereas the copepod *A. tonsa* was reared as described by Støttrup et al. (1986) and were fed with *Rhodomonas*, *Dunaliella* and *Tetraselmis*.

3.7 Feed Analysis

3.7.1 Determination of Vitamins

Overall, 30 g of feed samples were weighted, grinded and transferred to a 250-mL Erlenmeyer flask, and 3 g of ascorbic acid and 65 mL of KOH–ethanol solution (prepared dissolving 50 mL of ethanol in 15 mL of KOH 60% (w/v)) were added. The samples were shaken continuously overnight at room temperature. Subsequently, the samples were transferred to a separating funnel where liquid extraction with 25 mL of hexane during shaking the funnel for 5 min was carried out. This procedure was repeated two more times. The organic phases were joined and washed two times with 25 mL of MilliQ water. Then, the organic phase was collected and evaporated to dryness in a vacuum rotary evaporator at 40° C and the residue was re-dissolved in 5 mL of methanol (Perales et al. 2005) After the extraction processes, the samples were filtered through a 0.45 µm membrane filter and were injected into the chromatographic system for analysis.

Chromatographic separation was carried out using a Varian ProStar System with Star Chromatography Workstation and LC control software (Varian Analytical Instruments) comprising a ProStar 240 solvent delivery modules, a Model ProStar 410 AutoSampler with a sample loop of 50 µL, a Model ProStar 330 photodiode array detector with the Polyview 2000™ program and a microsorb C18 250 mm × 4.6 mm column with particles of 5 µm equipped with a microsorb C18 10 mm × 4 mm guard column. All the experiments were conducted at 30 °C. Before analysis, the column was conditioned making the mobile phase flows through the system for 20 min at 2.0 mL min^{-1}. During the chromatographic separation the mobile phase was kept isocratic, at 2.0 mL min^{-1} and the acquisition of the data were done at 230 nm (absorption of K_1, D_3 and E vitamins), 280 nm (absorption of A, E, D_3 and K_1 vitamins) and 300 nm (absorption of K_1, D_3 and E vitamins).

For estimation of ascorbic acid, 1 g of the microalgae sample was ground and homogenized in 5 mL of 4% trichloroacetic acid (TCA); the volume was made up to 10 mL with double-distilled water and centrifuged at 2000× g for 10 min. The supernatant was treated with activated charcoal or bromine. Dehydroascorbic acid reacts with 2, 4-dinitro phenyl hydrazine to form osazones, which dissolve in sulphuric acid to give an orange-coloured solution whose absorbance was measured spectro-photometrically at 540 nm (Roe & Kuether 1943).

3.7.2 Trace Elements and Mineral Analysis

For mineral analysis, algae (0.4 g) were mixed with AgNO3 (1 M, 1 mL) and HNO3 (5 mL, 67–69%), incubated for 2 h at 105 °C and transferred to a new tube. The sample was diluted with ultrapure water to a volume of 15 mL, centrifuged (20 min, 4000 rpm) and the supernatant was transferred to another tube. The washing step was executed two more times by adding 15 mL of water, shaking, centrifugation, and collecting the supernatant. The volume of the combined supernatant phases was adjusted to 50 mL with water, after which, the mixture was analysed by inductively coupled plasma atomic emission spectroscopy (Thermo iCAP analyzer, Thermo Scientific) for P, K, Ca, Na, and Fe content. The trace elements determined were Zn, Se, Mn and Cu. This was achieved by digesting the sample using 5 mL of nitric acid and perchloric acid (Almoaruf et al. 2003). The digested samples were centrifuged, and the digested solutions were analysed via atomic absorption spectrophotometry (Buck Model 205, East Norwalk, USA).

3.7.3 Gross Energy, Fibre and Moisture Content

The gross energy content of feed samples was determined using adiabatic bomb calorimeter using the Gallenkamp Auto bomb system. A 1 g of dried sample was placed into a crucible. Nickel firing wire was fixed between the electrodes and then a cotton string was wound from the pellet around the firing wire and the shorter electrode. The electrode assembly was then put into the calorimeter bomb. The water jacket of the bomb was filled with tap water and the calorimeter vessel was filled with water at 21-23 °C and weighed to exactly 3 kg. The calorimeter vessel was placed into the water jacket. Before firing the calorimetry bomb the thermometer reading was recorded as the initial temperature. The bomb was then fired and when the temperature stabilised it was again recorded (final temperature). The energy content (KJ/g) of the sample was calculated as:

$$\% \text{ Gross Energy} = - \frac{\left[\left(\text{Final Temperature} - \text{Initial Temperature}\right) \text{x } 10.82\right] - 0.0896}{\text{Sample weight (g)}}$$

where 10.82 is the factor of heat capacity of the system and 0.0896 represents the combined energy value (expressed in KJ) for the wire and the cotton thread used on the analysis (Karalazos 2007).

Moisture content was determined by subjecting the sample with known weight to drying in an oven at 100-102°C for 16 h. The loss in weight is reported as moisture content. The percentage dry matter content = 100 - % moisture content.

Crude fibre was determined as loss of ignition of dried lipid free residues after digestion with 1.25% or 0.255 N H_2SO_4 and 1.25% or 0.313 NaOH 10 mL of acetone was added to dissolve any organic constituent. The percentage fibre was obtained by the formula

$$\% \text{ Crude fibre} = \frac{\text{Weight of residue - weight of ash x 100}}{\text{Sample weight}}$$

Nitrogen-free extract (% carbohydrate) was determined by subtracting sum of (moisture % + % crude fat + % crude protein + % ash) from 100 (Dawodu et al. 2012).

3.7.4 *In Vitro* Digestibility

The standardized in vitro digestion protocol proposed by Minekus et al. (2014) was used to evaluate the nutrient digestibility of the aquafeeds. Blanks containing digestive fluids, enzymes, and bile but no algae, were included in each analysis to account for nutrients not coming from algae digestion. Digestion tests were preceded by pH adjustment experiments to accurately determine the amount of NaOH and HCl needed to reach the pH values prescribed in the consensus protocol (Minekus et al. 2014). As recommended by Brodkorb et al. (2019), the substrate:fluid ratio for oral phase simulation was adapted to obtain a swallowable bolus with a paste-like consistency. Water was added to reach the standard mass at the start of digestion.

For the evaluation of protein digestibility, the digestion was stopped by adding a protease inhibitor (1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride). Subsequently, the digesta was centrifuged (5 min, 5000 x g) and supernatant aliquots were examined. Firstly, the total amount of nitrogen in the supernatant was measured with an automated Dumas protein analysis system (Vario EL Cube, Elementar, Langenselbold, Germany) and compared with the amount of nitrogen in the initial algae biomass. Secondly, the degree of protein hydrolysis was estimated by the quantification of the

primary amino groups and comparison with the total nitrogen in the sample. Samples were incubated overnight in 2% sodium dodecyl sulfate solution at 4 °C and added to a freshly prepared mixture of o-phthalaldehyde (OPA) and N-acetylcysteine (NAC) in borate buffer, as described before (Spellman et al. 2003). By using an L-isoleucine calibration series and absorbance measurement at 340 nm (Tecan Infinite 200 PRO, Tecan, Männedorf, Switzerland), the concentration of primary amino groups can be determined (Leni et al. 2020). The degree of protein hydrolysis was calculated as the ratio of primary amino groups to the total algae nitrogen. A correction was made for primary amino groups and total nitrogen in the blank. For the assessment of the lipid digestibility, digestion was ended by adding chloroform: methanol, as proposed by Brodkorb et al. (2019), immediately followed by lipid extraction, as described above. In case lipid extraction could not be executed instantly after digestion, a lipase inhibitor was added (4-bromophenylboronic acid in methanol, 5 mM inhibitor/mL digesta), the digesta was snap frozen, stored at -20 °C and extracted with chloroform:methanol on another day. After lipid extraction, the amount of free fatty acids (FFAs) was determined after their derivatization to fatty acid diethylamides and subsequent GC-FID analysis (Kangani et al. 2008). A famewax column (30 m length, 0.32 mm internal diameter, 0.25 µm film thickness, Restek, Bellefonte, PA, USA) was used for GC separation, pentadecanoic acid (C15:0) as the internal standard, and a lauric acid (C12:0) dilution series for detector calibration. Carbohydrate digestibility was tested by executing the standard digestion protocol followed by centrifugation (5 min, 5000 x g) and incubation with *Aspergillus niger* amyloglucosidase (A1602), which is needed to simulate the activity of intestinal brush border enzymes (Minekus et al. 2014). To this end, 1 mL of supernatant was transferred to a new recipient, the pH adapted to 4.5 and *A. niger* amyloglucosidase (12 U) was added. Next, the mixture was incubated for 60 min at 37 °C, heated (10 min at 95 °C) to inactivate enzymes, and the hydrolysate diluted and analysed by HPAEC-PAD, as described above. Finally, the concentration of dissolved orthophosphate in the digesta was measured. After the heat inactivation of enzymes (10 min at 95 °C), the digesta was cooled to room temperature and diluted so that dissolved orthophosphate could be measured with either the LCK348 or LCK 350 total/ortho phosphate kit (Hach Lange, Düsseldorf, Germany) according to the manufacturer's instructions.

3.8 Effect Screenings

3.8.1 Cytotoxicity and Anti-Parasite Screenings

In all screenings described in the following, aqueous pre-extracted solutions of the four whole-microalgae aquafeeds (WMAFI-IV) were used. In addition, extracts from the compound screening were also employed due to their interesting compound profile (8 samples; cf. Table 2, page 12).

3.8.1.1 Cytotoxicity Studies

The bacterial (*Vibrio fischeri*) luminescence inhibition (Lumistox, Dr. Bruno Lange, Düsseldorf, Germany) test was conducted according to ISO guidelines (1998) at 15 ± 1 °C on water samples with salinity adjustment to 35‰ at pH 7. The exposure time was 30 min. The lyophilized bacterial reagent was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM N# 7151, Braunschweig, Germany). Each sample dilution (or control) was performed in triplicate. The EC_{50}

values for the Lumistox test were calculated by graphical interpolation, according to the ISO guidelines (1998).

An RTgill-W1 fish cell line was used to evaluate the cytotoxic effects associated with the different experimental aquafeeds. This cell line was cultured in 75 cm^2 flasks at 20 °C, under a humidified atmosphere. Cells were grown in Leibovitz's L-15 medium which contained L-glutamine and L-amino acids and which was supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were split twice each week, using PBS/EDTA to wash and trypsin/EDTA mixture to detach the cells. To expose the cells to the mycotoxins, cells were seeded into transparent, flat-bottomed 96-well plates by adding 100 µL of cell suspension (2.5 x 104 cells/mL) per well. These were left overnight for cell attachment. Cells were then exposed for 24 h to serial dilutions of aquafeeds at the following concentration ranges: 0.012–50 µg/mL (0.019–78 µM). Cells were also exposed to serial dilutions of SDS as a positive control, and to the highest concentrations of solvent, either methanol or DMSO (0.08–10% v/v), to eliminate cytotoxic effects exerted by the vehicle. In addition, cells receiving only the medium were used as negative controls. Every aquafeed concentration and the negative and solvent controls were tested in triplicate in each plate, and at least three independent experiments were carried out for each experimental aquafeed.

A triple assay consisting of the alamarBlue™ (Invitrogen™), CFDA-AM (5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester) (both Thermo Fisher Scientific) and the neutral red uptake (NRU) assays was performed on the RTgill-W1 following the method described in Lammel et al. (2013). After exposure, the medium was removed and the cells were washed with PBS, before adding MEM containing 1% NEAA, 1.25 % (v/v) alamarBlue™ and 4 µM CFDA-AM to each well. Cells were incubated in this solution for 30 min in darkness under the culture conditions for the cell line. Afterwards, fluorescence intensity was measured at excitation/emission wavelengths of 532/590 nm and 485/535 nm for the alamarBlue™ and the CFDA-AM assay respectively. Cells were then washed with PBS and incubated in NR solution (0.03 mg/mL) for 1h. After a wash with PBS to remove excess of NR, the dye retained in viable cells was extracted with a solution of absolute ethanol:glacial acetic acid 1:1 (v/v). NR fluorescence was measured at excitation/emission wavelengths of 532/680 nm. Fluorescence readouts were corrected by subtracting the fluorescence intensity measured in cell-free wells and normalized to the vehicle control and/or the cells receiving only the medium.

3.8.1.2 Anti-Oomycete Screenings

The method used for the determination of minimum inhibitory concentration (MIC) in this study for the anti-oomycete activity assays was performed with some modifications introduced by Madrid et al. (2015). In this assay, 80 µL of Griffin's sporulation medium (Griffin 1978) were added to replicate wells of a flat-bottomed, 96-well polystyrene plate. Each extract (dissolved in ethanol (EtOH)) was serially diluted in 1% aqueous solution of EtOH in water at 10 times the desired test concentrations (200, 150, 125, 100, 75, 50, 25, 12.5, 6.25, and 3.125 µg/mL) to find a preliminary minimum inhibitory concentration (MIC) interval. Ten microliters of each dilution were added to triplicate wells, followed by addition of 10 µL of zoospore suspension. For *Saprolegnia parasitica*, 100 µL of Gypsum (G-Y) medium was added to all wells 24 h after exposure to the test extracts. All wells were scored for the presence or absence of *Saprolegnia* growth 48 h after the start of the experiment. All experiments were conducted three times with triplicates at each test concentration. A 1% aqueous solution of EtOH in water used as negative control, while bronopol was the positive control.

The spore germination inhibition test was carried out with the agar dilution method described by Hu et al. (2013). In brief, the chosen *Saprolegnia* isolate (ATCC 42062 *S. parasitica*) was grown on potato dextrose agar (PDA) plates for 7–14 days, after which time spores were harvested from sporulating colonies and suspended in sterile distilled water (SDW). The concentration of spores in suspension were determined using a haemocytometer (Neubauer Improved counting chamber, Marienfeld, Germany) and adjusted to 1×10^4 CFU/mL approximately. The agar plates were prepared with the required concentration of the active samples, added to 10 mL of molten PDA (about 65 °C). Ten microliters of the spore suspension were spotted in triplicate on these plates which were then incubated at 25 °C for 72 h. The minimum oomyceticidal concentration (MOC) was defined as the lowest concentration of the aquafeed solutions that prevented visible growth or germination of spores.

In vitro anti-oomycete activities of the samples were assessed on the basis of mycelia growth inhibition rate (MOC; Madrid et al. 2015). Each extract was added to an assay flask containing hot sterilized PDA (about 65 °C) and final concentrations were adjusted to corresponding concentrations based on the initial tests. After mixing with a vortex, aliquots (10 mL) of treated medium were poured into 7 cm diameter Petri dishes. The *Saprolegnia*-colonized rapeseeds were inoculated in the centre of the prepared media. The mycelial growth diameter was measured after inoculation at 25 °C for 48 h. The growth inhibition rate was calculated from mean values as:

$$\%IR = 100\ (x - y)/(x - z)$$

where IR is the growth inhibition rate; x, the mycelial growth in control; y, the mycelia growth in sample; and z, the average diameter of the rapeseeds.

Saprolegniosis in Atlantic salmon aquaculture is mainly a problem in incubating eggs and newly hatched fry (Langvad 1994). Atlantic salmon eyed eggs of the AquaGen strain were used for the experiments, utilizing the protocol given by Thoen et al. (2011). The eggs had been incubated for 22 days at 5 °C and were disinfected with iodine upon arrival in the laboratory and were gradually over 24 h adapted to the laboratory water temperature used during the experiments (9 °C). Two different challenge experiments were performed with *S. parasitica*, employing only extracts which showed activities in the spore germination inhibition as well as mycelia growth inhibition test. Groups of dead eggs were exposed to spores, including motile zoospores, sporocysts and possibly germinating sporocysts, and groups of live eggs were exposed to Saprolegnia by co-incubation with infected dead eggs. *Saprolegnia* spores were produced according to the method described by Stuelandet al. (2005), and the concentration in the challenge suspensions was adjusted to 1.0×10^4 spores L^{-1}. The spore suspensions were confirmed to contain both motile zoospores and sporocysts.

To test the ability of extracts to inhibit the infection of dead eggs by *S. parasitica*, groups of 20 eggs were killed by immersion for 1 min in water at a temperature of 60°C. One group of 20 eggs was thereafter exposed to *Saprolegnia* spores at 111°C, overnight in 1000-mL glass bottles. The eggs were then rinsed and transferred to autoclave sterilized aquarium water for further incubation. All of the exposed eggs were examined by microscopy after rinsing and after 3 days of incubation at 111°C. Eggs for the co-incubation experiment were prepared as described above and inspected microscopically before distribution to the test units.

Live eggs were assigned to duplicate groups of 100 eggs each per Saprolegnia isolate and distributed in two separate compartments in small-scale hatching trays (one tray with two compartments of 100

eggs per isolate) equipped with a closed flow-through system. The eggs were spread to form an even layer covering the floor of the trays. In each compartment, groups of four eggs pre-infected with the respective isolates were placed upon the layer of live eggs and the extracts (200 µg/mL) were added. The four infected eggs per compartment were carefully placed in the corners of an imagined square on the layer of live eggs. In addition to the groups with infected eggs, two groups of four dead eggs that were not exposed to *Saprolegnia* spores were introduced in compartments with 100 live eggs to serve as non-infected control groups. In a third experimental unit the eggs were also not infected but the extract was added. The experimental units were maintained and inspected daily for 10 days. At termination of the experiment, the number of live eggs newly infected by hyphae from each of the introduced dead eggs was counted. The live eggs were considered as infected when they were entangled in hyphae and did not unfasten when the dead eggs were moved (Thoen et al. 2011). As indicator for the inhibition of infection, the number of eggs infected per chamber was recorded and calculated versus the uninfected ones.

3.8.2 Feeding Experiments with Atlantic Salmon

The methods for the trials were chosen in the present project due to their high potential of comparability of the results since in both plant (algae)-based feeds were tested on parr and smolt.

3.8.2.1 Experiment I (Parr)

The first experiment was exactly conducted as described by Egerton et al. (2020). Overall, 450 Atlantic salmon (body weight ~33.7 g) were anaesthetised, weighed and then randomly distributed into 15 tanks (30 fish per tank) of 1000 L freshwater capacity, within a fully controlled multi-tank recirculation system (RAS), three tanks were used as control (commercial feed, Skretting UK, Cheshire, UK) and three tanks were randomly assigned to each of the four dietary treatments. Dissolved oxygen levels were recorded throughout (9.1 ± 0.71 mg L^{-1}). Fish were held at 12°C, under 12:12 light: dark cycle, and total ammonia and nitrite levels were regularly monitored using Aquamerck test kits (Merck, Germany) and were maintained within optimal levels. Fish were fed the respective experimental diet twice daily to apparent satiation for 84 days. Then, after a 24 h gut evacuation period, all fish were anaesthetised and weighed.

3.8.2.2 Experiment II (Post-Smolt)

The second experiment was conducted according to the method given by Kiron et al. (2012). The rearing unit consisted of 520-L fiber glass tanks (approx. 450-L water volume), each having a flow-through system supplying seawater at a rate of 0.5 L sec^{-1} from a depth of 250 m. The water temperature in the tanks was 8 °C and the dissolved oxygen saturation was maintained above 90% during the rearing period. The fish husbandry followed the practices approved by the State Authorities who periodically examined the setup. Lighting conditions were arranged so as to gradually illuminate the rearing hall from 06:00 to 22:00 (16:8 day:night). Atlantic salmon post-smolts (62 g; 0-year) were maintained for a period of five months on commercial feeds until they were used for the trial. At the start of the feeding trial the fish had an average weight of 173.1 g. Fish were sorted and allotted randomly to each feed group (4 groups + control). Twenty-five fish were introduced into each of the 15 tanks (triplicate tanks per treatment), achieving an average density of 9.7 kg m^{-3}. Atlantic salmon

were fed manually six times weekly with the experimental feeds: once daily from 07:30 to 11:00 (in three cycles to satiety). The hand feeding regime was chosen to observe the appetite of the fish. Apparent feed intake was recorded daily during the entire 12-week period.

3.8.2.3 Sampling and Performance Parameters

An initial as well as a final sample of 6 fish per tank (15 per treatment) were randomly collected and euthanised, and samples of fillet and liver (3 fish per tank) and the whole body (3 fish per tank) were collected and stored at -20°C, until proximate analysis. The chemical composition of the faeces and fish samples was determined via proximate composition analysis according to standard methods (AOAC 2005). Lipid was determined by dichloromethane:methanol extraction (2:1) technique (Folch et al. 2005), with the substitution of chloroform with dichloromethane for safety reasons and the addition of butylated hydroxytoluene (BHT) (50 mg L^{-1}) to reduce lipid oxidation during processing. Standard formulae were used to assess growth and feed utilisation parameters over the experimental period, and were recorded according to the methods described by Francis et al. (2007); these included initial and final average weight (g), average feed consumption (g $fish^{-1}$), gain in weight (g and %), dress-out percentage (DP), food conversion ratio (FCR), specific growth rate (SGR, % day^{-1}), feed ratio (% of body weight), fillet yield percentage (FY%), and condition factor (K). In addition, Apparent digestibility coefficient of dry matter (ADC DM), protein production value (PPV), protein efficiency ratio (PER) and hepatosomatic Index (HSI) were calculated as the following:

$$ADC\,DM\,(\%) = (1 - (\frac{marker\,in\,feed}{marker\,in\,faeces})) \times 100$$

$$SGR\,(\%) = \frac{lnW_2 - lnW_1}{Feeding\,days} \times 100$$

$$FCR = \frac{Feed\,consumed/fish}{W_2 - W_1}$$

$$PPV = \frac{(W_2 \times CP_2 - W_1 \times CP_1)}{Protein\,consumed}$$

$$PER = \frac{W_2 - W_1}{Protein\,consumed/fish}$$

$$HSI(\%) = \frac{W_l}{W_f} \times 100$$

Where, W_1 and W_2 are average initial and final fish weights, respectively; CP_1 and CP_2 are the crude protein of the fish at the beginning and end of the feeding trial, respectively; W_l is the weight of liver and W_f is the weight of fish at the time of sampling and ln is the natural logarithm.

3.9 Statistical Analysis

Measures were carried out in triplicate (*n* = 3), and the results are given as mean values and standard deviations. Statistical analyses were performed using Microsoft Excel® and the SPSS® computer programs (SPSS Statistical Software, Inc., Chicago, IL.). All data were subjected to Analysis of Variance (ANOVA), or non-parametric alternative where appropriate, and pairwise comparisons were conducted by Tukey's test. Spearman's correlation was carried out on weight gain in relation to the alpha diversity indices; Shannon and Chao1.

4. Results

4.1 The Effects of Culture Conditions and Media Compositions on the Accumulation of Lipids, Proteins and Carbohydrates

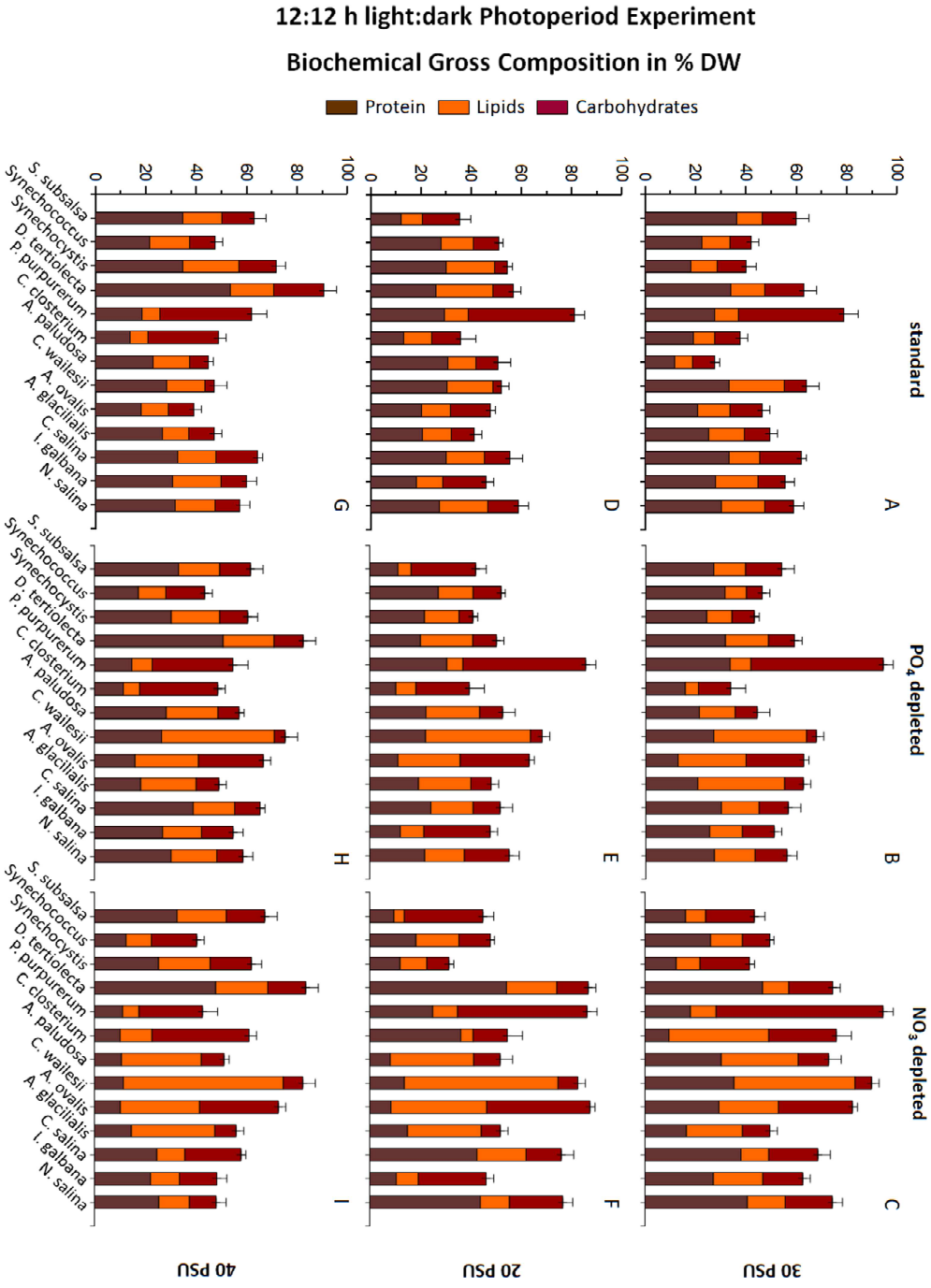

Figure 9. Results of the 12:12 h light:dark photoperiod experiment, utilizing different nutrient compositions (standard, phosphate or nitrogen depleted) as well as salinities: 30 PSU (A, B, C), 20 PSU (D, E, F) and 40 PSU (G, H, I). Measured were protein, lipid and carbohydrate gross compositions. Data are means of replicate measurements ± SD.

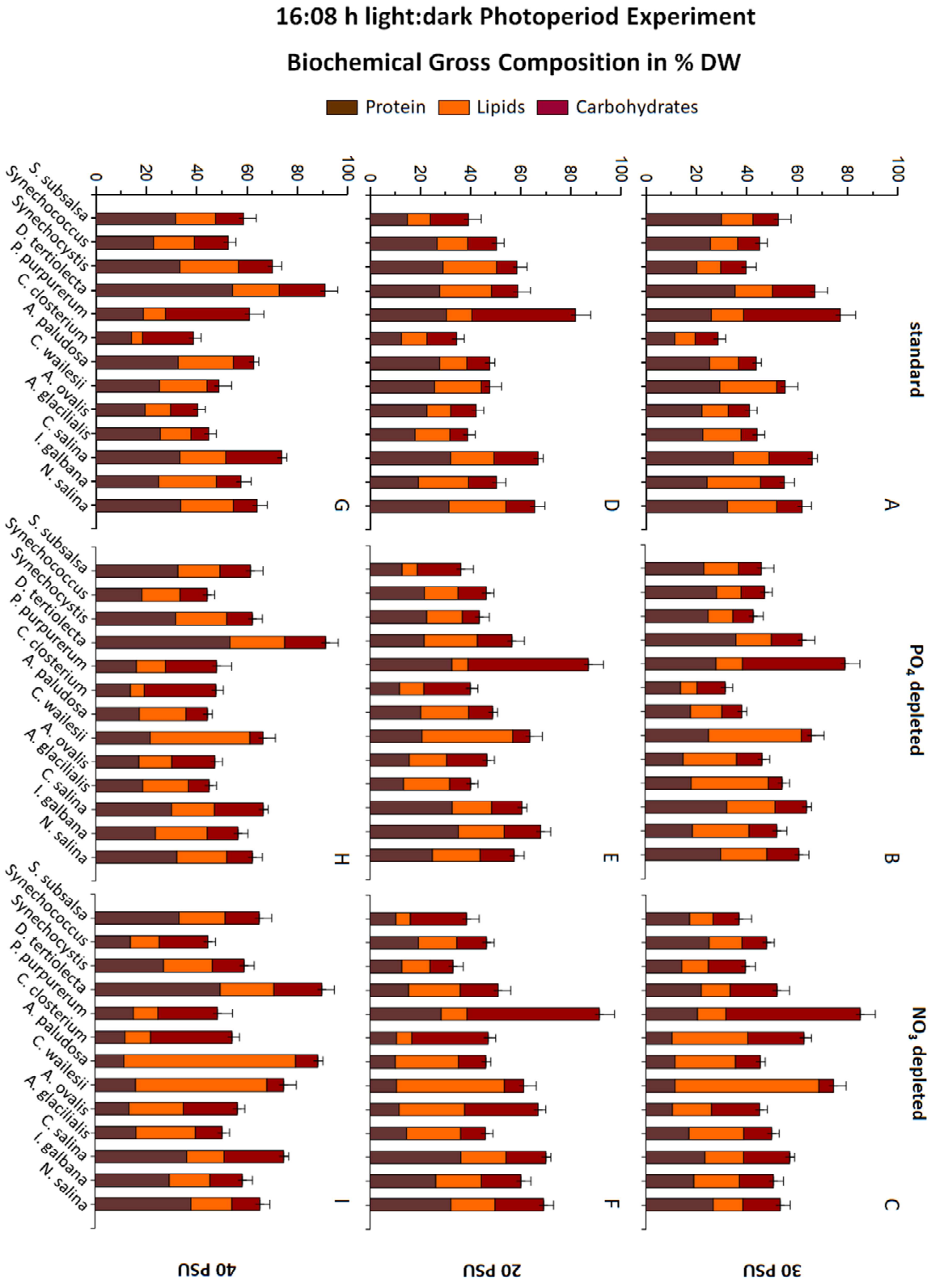

Figure 10. Results of the 16:08 h light:dark photoperiod experiment, utilizing different nutrient compositions (standard, phosphate or nitrogen depleted) as well as salinities: 30 PSU (A, B, C), 20 PSU (D, E, F) and 40 PSU (G, H, I). Measured were protein, lipid and carbohydrate gross compositions. Data are means of replicate measurements ± SD.

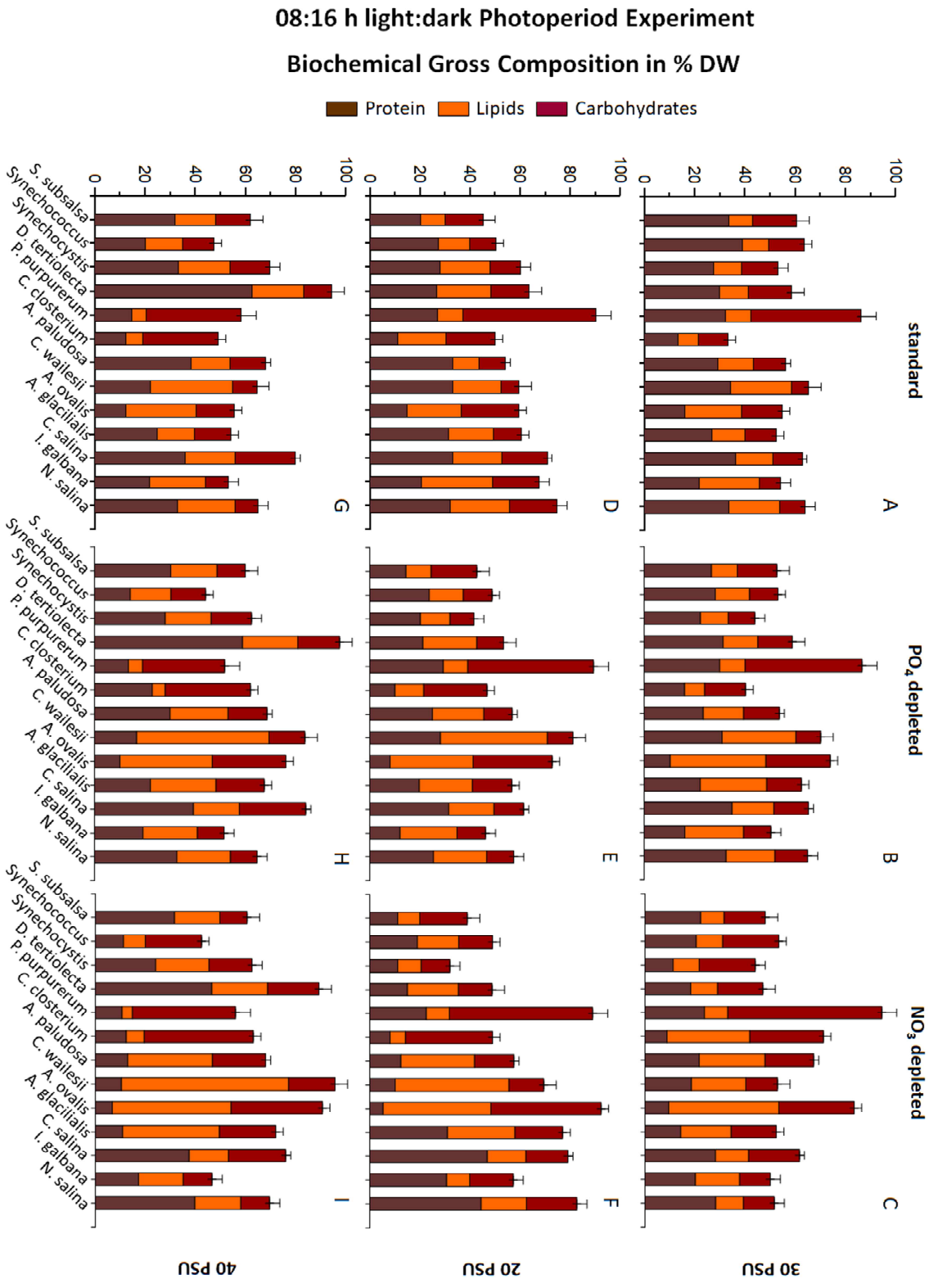

Figure 11. Results of the 08:16 h light:dark photoperiod experiment, utilizing different nutrient compositions (standard, phosphate or nitrogen depleted) as well as salinities: 30 PSU (A, B, C), 20 PSU (D, E, F) and 40 PSU (G, H, I). Measured were protein, lipid and carbohydrate gross compositions. Data are means of replicate measurements ± SD.

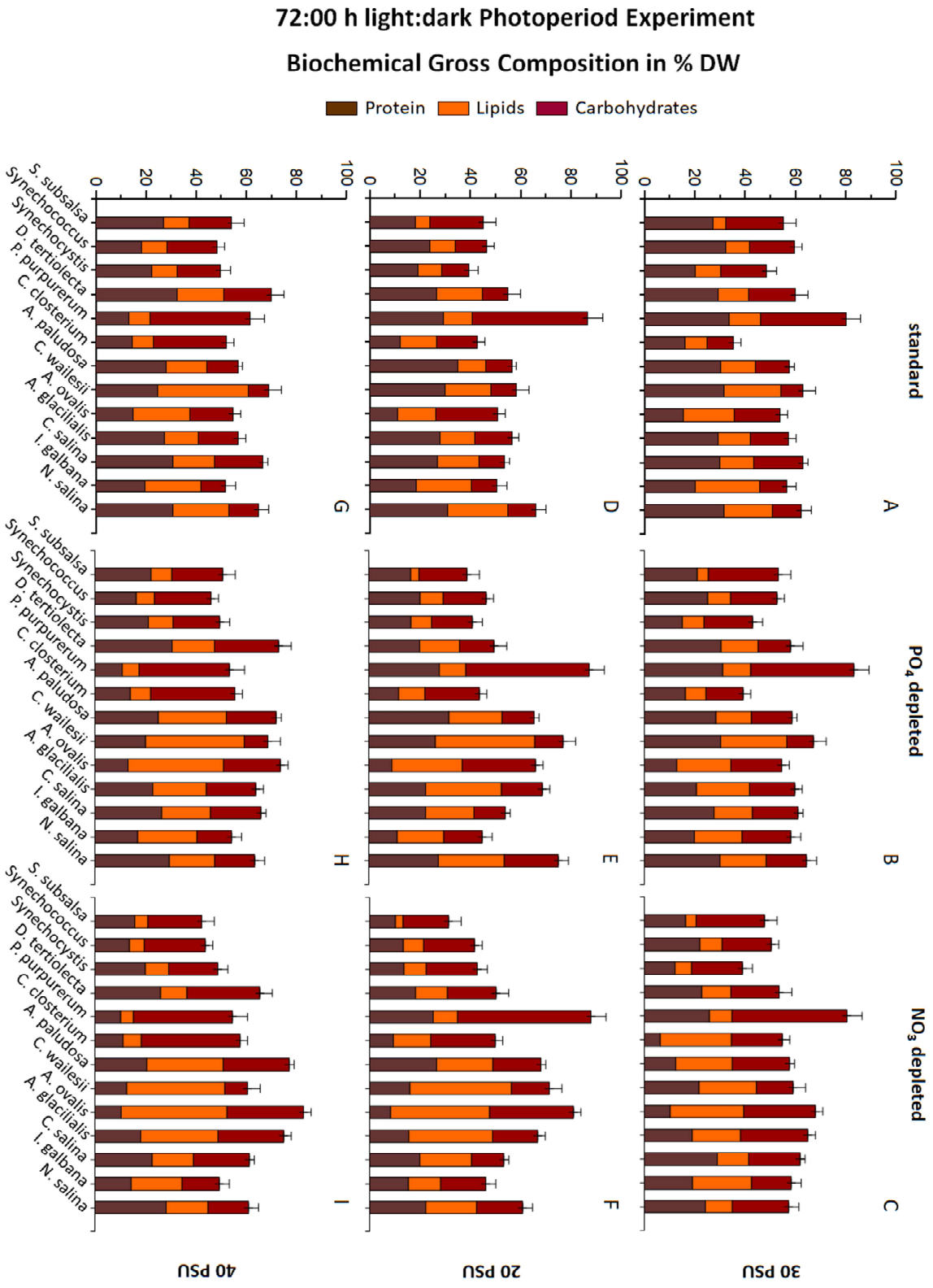

Figure 12. Results of the 72:0 h light:dark photoperiod experiment, utilizing different nutrient compositions (standard, phosphate or nitrogen depleted) as well as salinities: 30 PSU (A, B, C), 20 PSU (D, E, F) and 40 PSU (G, H, I). Measured were protein, lipid and carbohydrate gross compositions. Data are means of replicate measurements ± SD.

Testing initially 13 microalgae species (cf. Table 1) regarding their potential to accumulate high protein or lipid in response to alterations of phosphate or nitrogen (standard or 5 % of standard) as well as salinities (20, 30 and 40 PSU) in the culture media in combination with different photoperiods (12:12, 16:08, 08:16 and 24 h), the results obtained showed a highly heterogenic species-response picture of the northern Icelandic isolates (Figs. 9-12). In most cases nitrogen starvation combined with high light (16:08 h) and high salinity stress (40 PSU) led to significant shifts in the relative protein and lipid contents of the tested species. The highest relative protein value was found for the Chlorophyceae *Dunaliella tertiolecta* with 61.5% under nitrogen depletion combined with a photoperiod of 16 h and a salinity of 40 PSU (Fig. 9I). With lower but still significant protein contents were the Icelandic Chlorophyceae *Nannochloropsis salina* (I: nitrogen depletion, 12:12 h, 30 PSU and II: 16:08 h light:dark, 20 PSU; Figs. 9C and 10F) and *Chlorella salina* (I: nitrogen depletion, 12:12 h light:dark, 40 PSU and II: nitrogen depletion, 16:08 h light:dark, 40 PSU; Figs. 9I and 10I) recorded, with relative protein contents ranging between 47.9 and 54.2%. In contrast, the Icelandic *Spirulina subsalsa* showed its highest relative protein contents under standard nutrition combined with a reduced photoperiod of 08 h and a salinity of 30 PSU (32.2 %, Fig. 11A). Furthermore, the highest relative lipid contents were detected in the Icelandic isolate *Coscinodiscus wailesii* (diatom) with 60.5 and 61.3% under nitrogen depletion and a salinity of 40 PSU in the 12:12 h and 16:08 h light:dark assays, respectively (Figs. 9I and 10I). All values obtained during the screening are within the range of protein, lipid and carbohydrate amounts given in the literature for microalgae (e.g., Brown & Jeffrey 1992, 1995). These species-culture condition constellations mentioned above were chosen for the scale up cultivations and further investigations, leading to the development of the algae based whole-cell aquafeed for Atlantic salmon.

4.2 The Influence of Harvest and Processing Methods on the Protein and Lipid Recovery Yield

Table 4. Effect of different drying temperatures on the protein and lipid yield [%] obtained from the biomasses of the tested microalgae species.

	S. subsalsa	***D. tertiolecta***	***C. salina***	***N. salina***	***C. wailesii***
Protein					
30°C	30.2 ± 1.0^{c}	34.3 ± 1.6^{b}	39.3 ± 1.0^{a}	31.8 ± 1.2^{c}	15.5 ± 1.5^{d}
40°C	51.7 ± 1.8^{b}	62.4 ± 0.9^{a}	61.1 ± 1.1^{a}	52.1 ± 1.8^{c}	33.5 ± 0.5^{d}
50°C	53.2 ± 1.4^{a}	66.9 ± 1.1^{a}	65.7 ± 0.9^{a}	57.3 ± 1.9^{a}	35.7 ± 1.1^{a}
60°C	48.8 ± 0.8^{d}	55.5 ± 2.0^{b}	60.7 ± 1.1^{b}	50.2 ± 2.3^{c}	29.9 ± 1.5^{e}
Control*	**53.9 ± 1.0^{a}**	**68.5 ± 1.1^{a}**	**66.2 ± 2.0^{a}**	**58.7 ± 0.7^{a}**	**36.4 ± 0.5^{a}**
Lipid					
30°C	22.9 ± 1.3^{b}	18.3 ± 1.1^{c}	19.7 ± 1.0^{c}	11.3 ± 1.2^{d}	39.4 ± 2.1^{b}
40°C	29.4 ± 1.5^{b}	24.4 ± 0.4^{d}	27.3 ± 1.1^{c}	22.4 ± 1.5^{e}	58.2 ± 1.9^{a}
50°C	30.5 ± 1.0^{a}	28.9 ± 2.1^{a}	35.3 ± 0.7^{a}	25.9 ± 1.2^{a}	60.1 ± 0.5^{a}
60°C	29.1 ± 0.8^{c}	28.1 ± 1.3^{c}	33.3 ± 1.4^{b}	25.1 ± 0.3^{d}	59.8 ± 0.8^{a}
Control*	**31.8 ± 1.3^{a}**	**29.9 ± 1.5^{a}**	**34.7 ± 2.3^{a}**	**26.9 ± 0.9^{a}**	**61.4 ± 1.0^{a}**

* Samples were harvested by centrifugation and subsequently lyophilized.
Different letters indicate significant difference among treatments.

Table 5. Recovery rate [%] of protein and lipid yields after harvest using auto flocculation and biological flocculants *versus* the control.

	Control*[1]	Auto flocculation			Biological flocculants	
		NaOH	KOH	$Ca(OH)_2$	MLT	chitosan
Protein						
S. subsalsa	**54.1 ± 2.1**[a]	42.5 ± 1.1[d]	47.5 ± 0.9[b]	44.1 ± 0.6[c]	54.0 ± 0.5[a]	53.2 ± 2.3[a]
D. tertiolecta	**67.2 ± 2.2**[a]	56.5 ± 0.5[b]	56.9 ± 1.1[b]	53.30 ± 1.7[c]	66.9 ± 1.0[a]	66.3 ± 0.3[a]
C. salina	**66.9 ± 1.3**[a]	54.1 ± 0.6[c]	55.3 ± 1.3[b]	55.8 ± 1.4[b]	66.5 ± 0.7[a]	66.1 ± 1.1[a]
N. salina	**57.9 ± 1.0**[a]	49.2 ± 1.2[c]	49.0 ± 0.2[c]	52.2 ± 1.0[b]	57.3 ± 0.5[a]	57.1 ± 0.7[a]
C. wailesii	**36.1 ± 1.2**[a]	20.4 ± 2.0[e]	21.3 ± 1.5[d]	24.9 ± 1.1[c]	35.9 ± 0.5[a]	33.6 ± 1.1[b]
Lipid						
S. subsalsa	**33.7 ± 2.1**[a]	18.5 ± 1.4[d]	27.5 ± 0.8[c]	24.1 ± 0.3[c]	33.0 ± 2.0[a]	30.9 ± 1.3[b]
D. tertiolecta	**30.1 ± 2.3**[a]	16.5 ± 0.5[e]	16.9 ± 1.1[d]	25.3 ± 1.9[c]	30.2 ± 0.5[a]	29.2 ± 0.6[b]
C. salina	**34.3 ± 1.0**[a]	20.9 ± 0.9[d]	19.4 ± 1.7[e]	25.8 ± 1.7[c]	33.9 ± 0.3[a]	30.1 ± 1.1[b]
N. salina	**25.8 ± 2.0**[a]	19.1 ± 0.3[c]	19.0 ± 0.4[b]	20.2 ± 1.0[b]	25.3 ± 0.6[a]	23.3 ± 0.6[a]
C. wailesii	**60.9 ± 1.0**[a]	52.2 ± 1.8[d]	49.7 ± 2.5[e]	55.3 ± 0.7[c]	60.2 ± 0.5[a]	58.2 ± 1.1[b]

*[1] Samples were harvested by centrifugation and subsequently lyophilized.

Abbreviations: MLT = Modified Larch-Tannin

Different letters indicate significant difference among treatments.

NaOH, KOH, $Ca(OH)_2$; pH 12) and biological flocculants (modified Larch-Tannin and chitosan) were tested to simplify the harvest procedure for high microalgae biomasses, whereas as alternative processing methods lyophilization (control) and hot air drying (30, 40, 50 and 60°C) were utilized in the present study (Tables 4 and 5). The results showed for a drying temperature of 50 °C the best results regarding the relative protein and lipid contents of the tested microalgae (Table 4), whereas for the harvest modified Larch-Tannin was most effective.

4.3 Qualitative and Quantitative Lipid and Protein Compositions of the selected Microalgae under the chosen Culture Conditions

To assess the nutritional value of the protein fractions of the selected species grown under the culture conditions pointed out in 4.1, the amino acid (AA) compositions were first evaluated undisrupted samples (Fig. 13, Table 6). The AA compositions were highly heterogenous and varied from species to species. According to Wilson (1986) qualitative AA requirements for normal growth and metabolism of salmonids the presence of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are essential. All these essential AAs were present in the analysed species-condition constellations, although in varying concentrations. In particular, the biomass of the Icelandic isolate of *Dunaliella tertiolecta*, grown under nitrogen depletion with a salinity of 40 PSU and a light:dark cycle of 16:08 h, showed the highest concentrations of all essential AAs (>100 ng · mg DW^{-1}) when compared to the other species-culture condition constellations. For instance, tryptophan and arginine were detected in *D. salina* with a concentration of 49.789 and 73.185 ng · mg DW^{-1}, whereas lysine and histidine were found with concentrations of 24.215 and 24.723 ng · mg DW^{-1}, respectively.

Compared to the other species, extraordinary concentrations were found in *D. salina* for isoleucine, leucine, phenylalanine, threonine (all >150 ng · mg DW^{-1}) and valine (236.665 ng · mg DW^{-1}). Only methionine was the highest in *Spirulina subsalsa* with 8.025 ng · mg DW^{-1}, which also showed the highest alanine concentration (424.683 ng · mg DW^{-1}) when compared to the other species-culture condition constellations (Table 6).

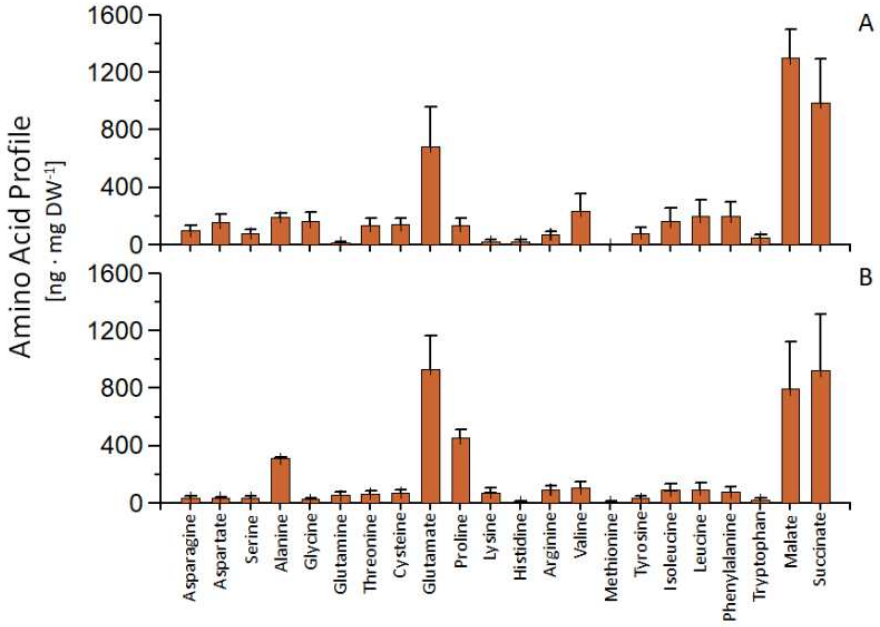

Figure 13. Amino acid profiles of *Chlorella salina* I (A) and *Nannochloropsis salina* I (B) (cf. Table 2 for the culture conditions and 4.1). Data are expressed as triplicated measurements ± SD.

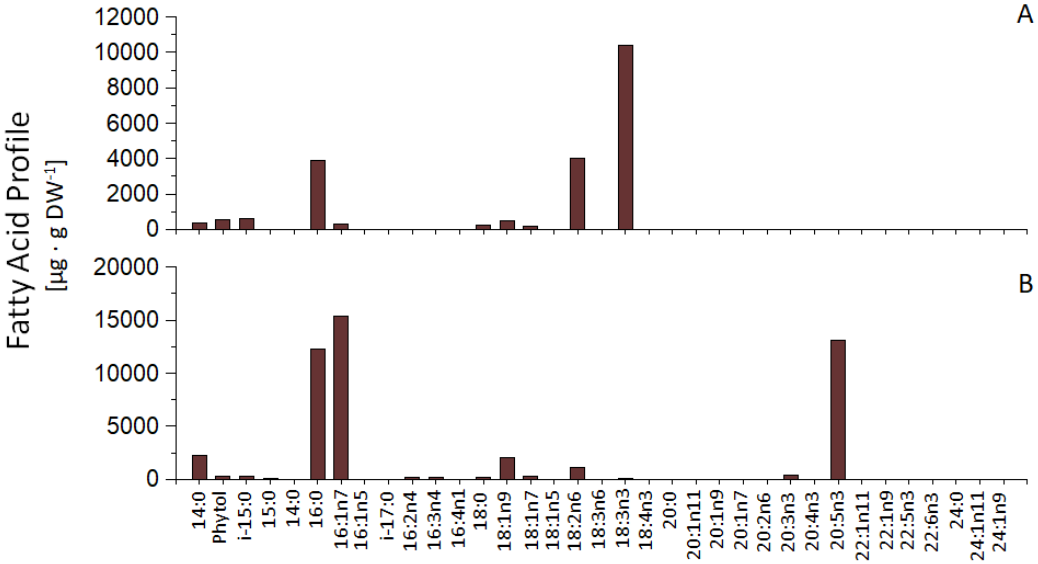

Figure 14. Fatty acid profile of *Chlorella salina* I (A) and *Nannochloropsis salina* I (B) (cf. Table 2 for the individual culture conditions).

Table 6. Amino acid composition of six of the tested species/culture condition constellations (cf. Table 2 for the culture conditions). (n.d. = not detected)

Amino acid (ng · mg DW^{-1})	***S. subsalsa***	***D. tertiolecta***	***C. salina* II**	***N. salina* II**	***C. wailesii* I**	***C. wailesii* I**
Asparagine	3.599	96.779	0.088	0.028	1.176	0.284
Aspartate	42.264	155.371	0.577	0.474	0.830	0.619
Serine	15.402	80.046	0.055	0.019	0.263	0.073
Alanine	424.683	195.559	152.9	311.105	72.757	95.408
Glycine	9.736	161.791	n.d.	n.d.	0.096	0.004
Glutamine	7.368	17.448	0.196	0.121	1.258	0.382
Threonine	19.529	153.440	0.114	0.056	0.209	0.081
Cysteine	19.664	140.470	n.d.	n.d.	n.d.	n.d.
Glutamate	58.471	85.203	0.231	0.192	1.170	0.362
Proline	13.672	134.806	9.777	5.724	9.910	1.389
Lysine	9.322	24.215	0.208	0.174	1.540	0.470
Histidine	1.399	24.723	0.116	0.126	0.112	0.164
Arginine	13.349	73.185	0.434	0.261	1.150	0.396
Valine	16.584	236.665	1.451	0.792	2.128	1.489
Methionine	8.025	0.148	n.d.	0.014	n.d.	n.d.
Tyrosine	10.199	81.946	2.255	0.899	2.549	1.730
Isoleucine	14.300	161.224	2.487	1.505	4.673	1.765
Leucine	15.462	198.071	3.909	2.000	8.516	4.586
Phenylalanine	15.582	199.000	3.339	1.958	5.876	4.133
Tryptophan	2.973	49.789	0.563	0.260	0.546	0.956
Malate	248.958	399.174	n.d.	n.d.	n.d.	n.d.
Succinate	151.441	98.858	n.d.	n.d.	n.d.	n.d.
Total	**2216.982**	**2767.911**	**178.671**	**325.708**	**114.759**	**114.291**

Dietary lipids are important in fish nutrition as energy sources, as carriers of lipid-soluble vitamins and minerals, and as sources of essential fatty acids (EFAs). To assess the nutritional value of the lipid fractions of the selected species grown under the culture conditions pointed out in 4.1, the fatty acid (FA) compositions were also evaluated on undisrupted samples (Fig. 14, Table 7). The total n-3 PUFA dietary requirement of salmonids, including ALA, EPA and DHA, has been reported to range from 1 to 2.5 % of the diet, depending on the species and experimental condition (Bou et al. 2017). In the analysed biomass, linoleic acid (18:2n6) was present in all microalgae fractions, with the highest levels in *Spirulina subsalsa* (1968.989 µg · g DW^{-1}), *Coscinodiscus wailesii* I (1013.086 µg · g DW^{-1}) and II (1259.345 µg · g DW^{-1}). Apart from *S. subsalsa*, α-linolenic acid (ALA, 18:3n3) and eicosapentaenoic acid (EPA, 20:5n3) were detected in all tested species-culture condition constellations, being the highest in *C. wailesii* II (ALA = 15.368 µg · g DW^{-1}, EPA = 258.574 µg · g DW). In contrast, docosahexaenoic acid (DHA, 22:6n3) was only present in *Nannochloropsis salina* II in low levels (0.133 µg · g DW^{-1}) (Table 7) which may lead in future to the inclusion of DHA obtained from microorganisms such as Thraustochytrids in the Atlantic Salmon aquafeed.

Table 7. Fatty acid composition of six of the tested species/culture condition constellations (cf. Table 2 for the culture conditions). (n.d. = not detected)

Fatty acid (µg · g DW^{-1})	***S. subsalsa***	***D. tertiolecta***	***C. salina* II**	***N. salina* II**	***C. wailesii* I**	***C. wailesii* I**
14:0	120.531	46.102	56.183	34.267	103.784	103.201
Phytol	24.944	0.000	0.589	n.d.	n.d.	n.d.
i-15:0	314.060	1.192	8.193	4.849	8.718	14.999
15:0	n.d.	2.239	3.951	2.471	5.175	5.199
14:0	155.885	1.433	3.889	2.032	2.591	3.158
16:0	499.989	79.215	264.842	130.063	253.183	217.755
16:1n7	642.936	115.408	253.741	129.580	510.725	354.317
16:1n5	n.d.	n.d.	0.985	0.571	1.417	n.d.
i-17:0	n.d.	2.164	1.406	0.899	3.063	7.203
16:2n4	48.891	8.345	6.617	2.565	20.293	26.388
16:3n4	57.535	20.622	3.583	2.529	64.463	76.679
16:4n1	42.942	1.835	1.373	0.785	1.666	2.032
18:0	257.925	5.028	5.888	3.546	9.991	14.341
18:1n9	443.622	8.376	30.634	14.097	46.334	38.737
18:1n7	108.823	7.377	6.466	4.247	16.212	36.143
18:1n5	n.d.	1.648	1.538	0.522	n.d.	1.555
18:2n6	1968.989	2.991	27.806	8.729	1013.086	1259.345
18:3n6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:3n3	n.d.	0.626	3.799	0.786	13.454	15.368
18:4n3	n.d.	n.d.	0.790	0.543	4.662	3.782
20:0	29.001	0.561	0.579	n.d.	30.588	28.587
20:1n11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:1n9	n.d.	n.d.	n.d.	n.d.	1.767	n.d.
20:1n7	n.d.	0.522	n.d.	n.d.	1.058	2.129
20:2n6	31.938	1.215	n.d.	n.d.	35.350	32.422
20:3n3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:4n3	n.d.	n.d.	1.071	0.580	2.734	2.518
20:5n3	n.d.	31.103	189.720	90.542	190.153	258.574
22:1n11	n.d.	2.332	n.d.	n.d.	n.d.	1.924
22:1n9	n.d.	4.301	0.472	0.530	10.741	1.622
22:6n3	n.d.	n.d.	n.d.	0.133	n.d.	n.d.
24:0	n.d.	14.443	0.708	0.903	29.465	37.374
24:1n11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24:1n9	n.d.	1.405	n.d.	n.d.	6.237	3.342
22:6n3	n.d.	2.688	n.d.	n.d.	n.d.	2.610
Total	**4748.011**	**363.170**	**874.823**	**435.637**	**2396.91**	**2551.304**

4.4 Presence of other Compound Classes in the investigated Microalgae Biomasses

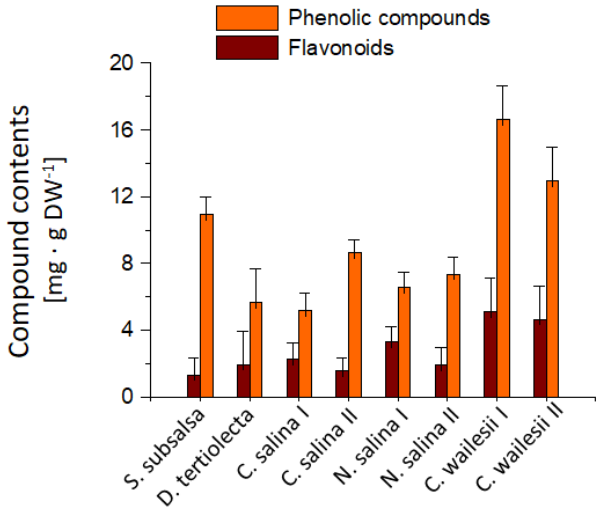

Figure 17. Results of the secondary metabolite screening considering phenolic compounds and flavonoids detected in biomass samples of the five selected species and eight specific culture conditions (Table 2). Data are expressed as triplicated measurements ± SD.

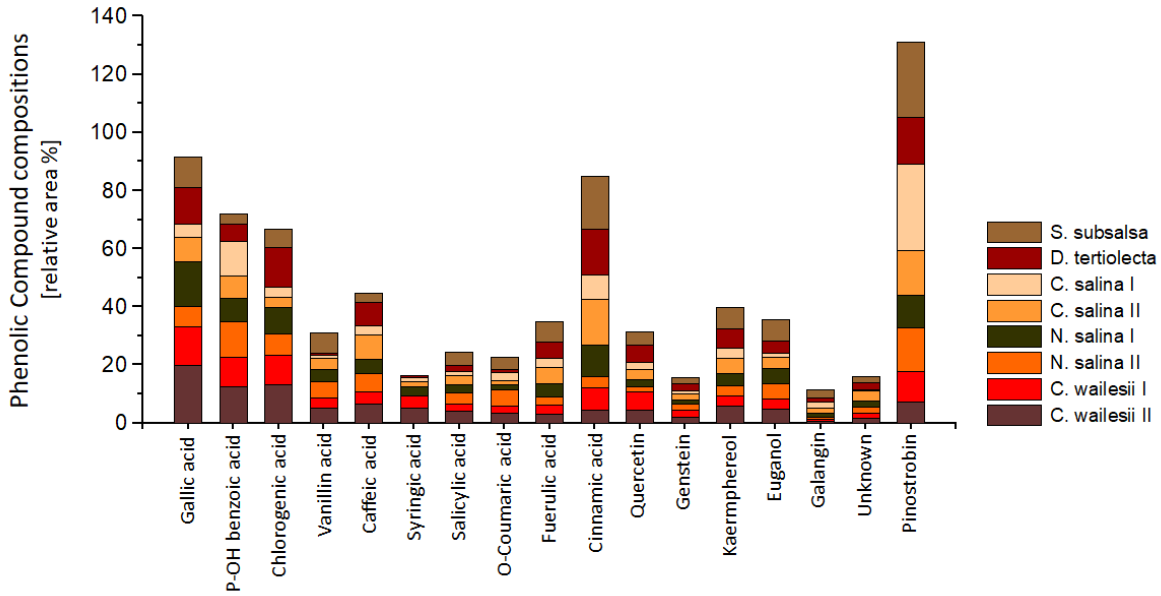

Figure 18. Phenolic compound compositions for the eight selected species/culture condition constellations obtained by GC-MS analysis.

The screening for the presence of phenolic compounds (including flavonoids) showed for both the highest values in the diatom *Coscinosdiscus wailesii* (up to 16.63 ± 2.1 mg · g DW^{-1}; Fig 17). Gallic, chlorogenic, cinnamic and p-OH benzoic acids were the most abundant constituents (>1 to <10 % of

the total chromatographic area) of phenolic acids (Fig. 18). From the flavonoids, pinostrobin was most abundant in all tested microalgae samples. The highest relative concentrations of pinostrobin were observed in *Spirulina subsalsa* (25.98%) and *Chlorella salina* I (29.73%). This compound has been detected in *Spirulina maxima* (Abd El-Baky et al. 2009) and is known to elicits intense apoptotic response from cultured leukaemia cells *in vitro*, strongly inhibits the Ca^{2+} signals involved in the control of G2/M phase cell cycle progression in *Saccharomyces cerevisiae* and shows potent antiviral effect against herpes simplex virus-1 (https://www.sigmaaldrich.com/IS/ en/product/sial/80614).

4.5. The Whole-Cell-Microalgae Aquafeed – *In Vitro* Digestibility, Toxicological Effects, Anti-Oomycete Activities and Atlantic Salmon Performance Parameter

4.5.1 *In Vitro* Digestibility

Initially, the four whole-cell-microalgae aquafeeds (WCMF I-IV), including the disrupted algae components (cf. Table 3), were digested *in vitro* and the amount of soluble organic matter was determined (Fig. 19A). All aquafeeds showed high relative values of the soluble organic matter, ranging between 77.3 and 84.7% for WCMFII and IV, respectively. Since carbohydrates, proteins, and lipids can only be absorbed in the intestine when they are degraded to smaller building blocks that are soluble in the liquid/micellar phase, a low organic matter solubility after digestion can be seen as an indication for a low combined digestibility of carbohydrates, proteins, and lipids.

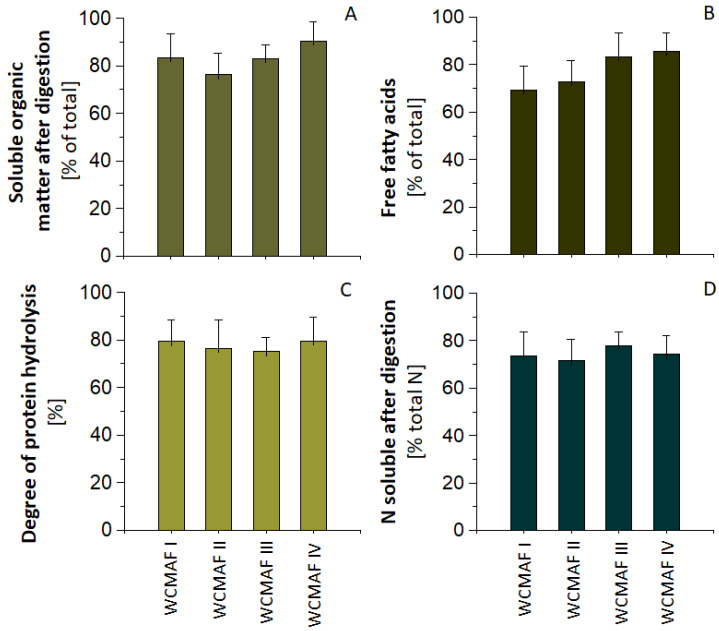

Figure 19. Results of the in vitro digestibility analysis of the four whole-cell-microalgae aquafeeds. (A) Organic matter soluble after digestion, expressed as a percentage of the initial organic matter level. (B) Free fatty acids (FFAs) released from algae biomass after digestion, expressed as % of the total fatty acid content measured as fatty acid methyl esters (%FA). N solubility after digestion (C), and degree of protein hydrolysis after digestion (D). The type of aquafeed had no significant impact on the degree of protein hydrolysis ($p = 0.114$, 1-way ANOVA). The degree of protein hydrolysis of the four aquafeeds ranged between 24.8 ± 2.6% and 30.8 ± 4.1%, with no statistical difference among samples ($p = 0.11$, one-way ANOVA).

Lipid digestibility was evaluated by comparing the concentration of free fatty acids (FFAs) after digestion with the total fatty acid content of the sample. It should be noted that this is not the same as the lipid bio-accessibility, i.e., the fraction available for uptake in the intestine. Indeed, not only FFAs but also other lipid degradation products such as monoacylglycerols participate in the formation of micelles that are taken up by enterocytes in the gut. On the other hand, the incorporation of entirely hydrolysed lipids in mixed micelles may be limited due to the presence of other cell compounds or due to the lipid localization in the cell. The aquafeeds WCMF III and IV displayed the highest lipid digestibility, with 83.2 ± 10.3% and 85.5 ± 8.1% of the fatty acids liberated as free fatty acids after digestion, respectively. Lower average values were observed for WCMF I and II (69.3 ± 9.9% and 72.8 ± 9.1%) (Fig. 19B).

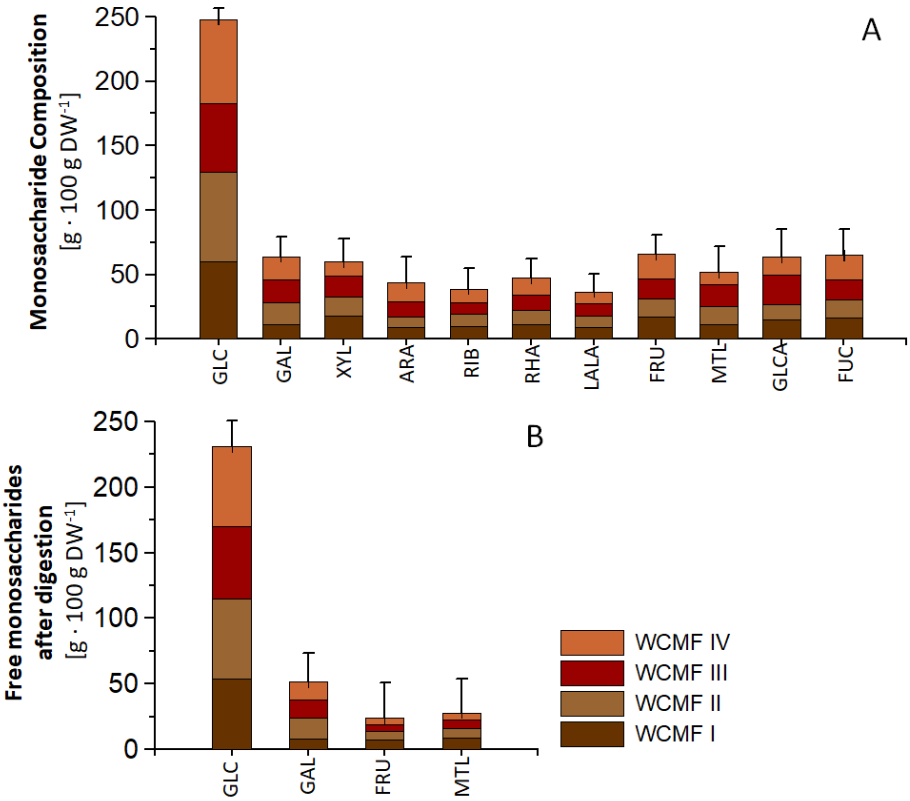

Figure 20. Total monosaccharide and uronic acid levels in the aquafeeds measured by anion exchange chromatography after acid hydrolysis (A). Free monosaccharides in the liquid digesta after digestion measured by HPAEC (B). Abbreviations: GLC = glucose, GAL = galactose, XYL = xylose, ARA = arabinose, RIB = ribose, RHA = rhamnose, GALA = galacturonic acid, FRU = fructose, MTL = mannitol, GLCA = glucuronic acid, FUC = fucose. Bars and error bars represent averages and standard deviations, respectively.

To examine protein digestibility, three different approaches were followed. First, the concentration of free amino acids after digestion was determined. Yet, the HPAEC profiles of digesta samples contained several interfering peaks, making reliable amino acid quantification impossible. Therefore, a second approach was followed, where the nitrogen fraction that is soluble after digestion was determined. This approach showed high but not significantly different variations in the nitrogen solubility of the four aquafeeds after digestion, ranging between 71.4 ± 8.1% (WCMF II) and 77.8 ± 6.3% (WCMF III) (Fig. 19D). According to the data, about 20-28.6% of all proteins were insoluble after digestion (Fig. 19C) and thus indigestible, while simultaneously over 70% of all nitrogen was present as free amino

groups (Fig. 19D). It can be speculated that at least part of the remaining soluble fraction is also bio-accessible, since brush-border peptidases are not included in the standard digestion protocol used in this study. Brush-border peptidases are required to complete protein and peptide degradation and play an important role in the activation of trypsinogen.

The monosaccharide composition analysis (Fig. 20A) shows that glucose was the main monosaccharide in all four aquafeeds, with concentrations ranging between 53.1% (WCMF III) and 69.8% (WCMF II) on a DW basis. Other monosaccharides, mainly galactose, xylose, and arabinose, were present in lower concentrations (Fig. 20A). When the aquafeed fractions underwent *in vitro* digestion, significant amounts of free glucose (61.5 g glucose/100 g DW) were detected in the case of the aquafeed WCMF IV (Fig. 20B). These data do not allow a precise prediction of the *in vivo* glucose release, since a detailed kinetic study of starch degradation was not performed and because of the limitations of *in vitro* digestion studies. Yet, these results do suggest that considerable glucose levels can become available for intestinal uptake during the digestion of the four aquafeeds and that glucose will contribute significantly to the energy content. It is noteworthy that algae biomass was not heated before digestion only physically disrupted. Therefore, the algal starch was not yet gelatinized and hence, partly resistant to hydrolysis by intestinal enzymes, as seen for other starch types such as cereal starches. The concentrations of the other free monosaccharides (<15%) were much lower than that of glucose (Fig. 20B). According to this, galactose, fructose, and mannitol will have a lower impact on the energy supply of Atlantic salmon. However, the latter two can still be relevant, as they belong to the so-called FODMAPs. FODMAPs are fermentable oligo-, di- and monosaccharides and polyols that are not or are incompletely absorbed in the small intestine. These compounds can cause gastro-intestinal problems with sensitive subjects upon consumption.

4.5.2 Toxicological Effects

Table 8. Assessment of the toxicological effects of the whole-cell-microalgae aquafeed and microalgae samples from the compound screening (4.4) using different assays. EC_{50} concentrations expressed in µg/mL (mean ± SEM, n = 3) and in µM at the three different endpoints tested in the triple assay in RTgill-W1 after 24 h of exposure.

Sample	**LUMIStox** luminescence inhibition [%]	**EC_{50}**					
		AM		**CFDA-AM**		**NRU**	
		µm/mL	µM	µm/mL	µM	µm/mL	µM
Aquafeeds							
WCMF I	12.5 ± 1.5	138.44 ± 1.15	342.38	187.52 ± 1.08	211.03	162.89 ± 0.59	234.25
WCMF II	15.4 ± 1.0	197.53 ± 0.59	341.33	119.19 ± 1.82	316.75	173.79 ± 0.33	275.70
WCMF III	18.5 ± 1.1	159.26 ± 0.93	320.58	108.26 ± 1.18	312.33	167.61 ± 2.43	280.36
WCMF IV	19.4 ± 1.9	137.25 ± 0.05	320.18	177.45 ± 1.09[c]	320.94	166.07 ± 2.23	243.56
Microalgae Extracts							
S. subsalsa	52.4 ± 1.9	113.05 ± 2.11	316.63	113.10 ± 2.09	216.71	193.09 ± 0.43	323.85
D. tertiolecta	15.4 ± 1.0	122.56 ± 3.10	334.53	150.42 ± 3.44	277.23	155.53 ± 0.69	338.48
C. salina I	58.5 ± 1.1	111.35 ± 2.50	336.34	119.23 ± 1.15	321.11	184.83 ± 14.01	269.33
C. salina II	29.4 ± 1.9	142.56 ± 4.19	306.12	103.28 ± 1.08	247.65	143.95 ± 10.61	308.84

N. salina I	27.3 ± 1.5	139.11 ± 3.27	338.53	169.05 ± 1.05	337.81	151.58 ± 5.08	375.07
N. salina II	27.9 ± 1.2	164.07 ± 5.43	329.14	115.36 ± 3.23	319.06	147.32 ± 3.05[c]	282.47
C. wailesii I	52 .5 ± 1.5	158.46 ± 0.51	312.37	157.54 ± 1.03	311.09	142.88 ± 0.64	344.33
C. wailesii II	57.4 ± 1.0	157.59 ± 1.53	311.33	151.08 ± 2.33	216.75	133.81 ± 1.26	365.72

Am, alamarBlue™; CFDA-AM, 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester, NRU, neutral red uptake

The effective concentrations that decreased mitochondrial activity or plasma or lysosomal membrane integrities in 50% of the cells (EC50) were calculated for each feed (WCMF I-IV, Table 8), utilizing a triple assay consisting of the alamarBlue™ (AM), CFDA-AM (5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester) and the neutral red uptake (NRU) assay. The values indicated that neither the developed aquafeeds nor the eight microalgae extracts from the compound screening were toxic for the fish cell line. In contrast, results from the LUMIStox assay indicated toxicity by the microalgae samples against the luminescent bacterium *Vibrio fischeri*, being the highest for *Spirulina subsalsa* and *Chlorella salina* I as well as *Coscinodiscus wailesii* I and II (values > 50% luminescent inhibition).

4.5.3 Anti-Oomycete Activities

Table 9. Assessment of the anti-oomycete activities of the whole-cell-microalgae aquafeed as well as microalgae extracts from the compound screening (4.4) using different assays.

Sample	**MIC** [µg · mL^{-1}]	**MOC** [µg · mL^{-1}]	**MIG** [%]	**Atlantic Salmon Egg Assays***	
				IR DE [%]	**IR LE** [%]
Aquafeeds					
WCMF I	>200	>200	0	n. d.	n. d.
WCMF II	150	125	25.0	78.3 ± 2.2	75.3 ± 1.3
WCMF III	>200	>200	0	n. d.	n. d.
WCMF IV	>200	>200	0	n. d.	n. d.
Microalgae Extracts					
S. subsalsa	100	100	38.0	27.3 ± 1.1	20.6 ± 2.2
D. tertiolecta	125	125	35.0	39.6 ± 2.3	35.4 ± 1.4
C. salina I	100	125	35.0	30.1 ± 2.3	27.1 ± 2.3
C. salina II	150	150	31.0	50.4 ± 1.0	48.9 ± 2.5
N. salina I	125	150	28.0	61.7 ± 5.9	59.2 ± 2.0
N. salina II	100	125	30.0	59.3 ± 1.1	57.8 ± 1.7
C. wailesii I	51	75	58.0	3.3 ± 0.2	2.1 ± 0.1
C. wailesii II	75	75	52.0	7.2 ± 0.3	5.5 ± 0.5

Abbreviations: MIC, minimum inhibitory concentration; MOC, minimum oomyceticidal concentration; MIG, mycelial growth inhibition; LE, live eggs; IR, infection rates; DE, dead eggs; n.d., not determined.
* using 200 mg/L from each extract.

The minimum inhibition concentration (MIC), can be defined as the minimum concentration of anti-oomycidal agent that is able to inhibit the visible oomycete growth after an incubation period of 48 h. This method has been used in the present study and is considered a fundamental instrument for the determination of susceptibility of microorganisms to anti-oomycidal agents. Table 9 presents the

values obtained for the MIC for the tested isolate (ATCC 42062 *Saprolegnia parasitica*), which, in the initial screening, presented some susceptibility to the microalgae extracts and only in one case minor inhibition of the mycelia growth was caused by the exposure to an aquafeed (WCMF II). In contrast, the aquafeeds WCMF I, III and IV were considered to have no inhibition effect against *Saprolegnia* with MIC values > 200.0 µg/mL, while the other nine samples showed anti-oomycete activities with MIC ≤ 150.0 µg/mL, in which extracts from *Coscinodiscus wailesii* I and II showed the most promising anti-*Saprolegnia* activities with MIC values of 51 and 75 µg/mL, respectively (Table 9).

The minimum oomyceticidal concentration (MOC) values of aquafeeds and ethanolic extracts against *Saprolegnia* spores after an exposure time of 72 h are also shown in Table 9. The results reveal that *Coscinodiscus wailesii* I and II extracts showed significant anti-oomycete activities with MOC values of 75 µg/mL, followed by *Spirulina subsalsa*, *Chlorella salina* I and *Nannochloropsis salina* II extracts with values of 100 µg/mL. The mycelial growth inhibition (MIG) percentage values of the tested samples against the *Saprolegnia* mycelium after 48 h exposure, revealed a reduction of the growth rate by extracts of *Coscinodiscus wailesii* I and II of 52% and 58%, respectively.

Utilizing dead (DE) and live (LE) Atlantic salmon eggs, the individual microalgae extracts as well as one aquafeed extract were tested (WCMF II) regarding the inhibition of infections caused by *Saprolegnia parasitica*. Although, the obtained infection rates (IR) showed only for both *C. wailesii* extracts (I and II) a significant reduction of the infections by *Saprolegnia*, all tested extracts showed some effect.

4.5.4 Atlantic Salmon Performance in the Feeding Trials

Table 10. Fish performances and feed efficiency in Atlantic Salmon Parr fed the four experimental diets during the first *in vivo* feeding trial.

	Control	WCMF I	WCMF II	WCMF III	WCMF IV
Survival (%)	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
Weight (g)					
Week 0	33.6 ± 0.4	34.5 ± 1.1	33.6 ± 0.5	33.6 ± 0.5	33.6 ± 0.2
Week 12	137.0 ± 2.5	136.4 ± 2.1	135.2 ± 1.8	136.7 ± 0.9	136.9 ± 1.5
SGR (% day^{-1})	1.71 ± 0.03	1.69 ± 0.02	1.67 ± 0.02	1.69 ± 0.03	1.69 ± 0.02
FCR	0.79 ± 0.01	0.81 ± 0.01	0.80 ± 0.01	0.79 ± 0.03	0.81 ± 0.02
PGR (% day^{-1})	1.87 ± 0.03	1.78 ± 0.02	1.85 ± 0.01	1.86 ± 0.02	1.83 ± 0.02
PER	2.57 ± 0.01	2.56 ± 0.02	2.58 ± 0.02	2.58 ± 0.01	2.58 ± 0.02
% NPU	51.1 ± 0.7	49.9 ± 0.5	52.3 ± 0.4	51.7 ± 1.2	51.3 ± 0.2
FDR (% day^{-1})	1.69 ± 0.02^{a}	1.61 ± 0.02	1.59 ± 0.01	1.59 ± 0.04	1.58 ± 0.03^{b}
DP %	90.6 ± 0.1	91.2 ± 0.1	90.5 ± 0.5	91.0 ± 0.1	91.3 ± 0.2
FY %	55.3 ± 0.8	55.1 ± 0.3	55.0 ± 1.0	54.9 ± 0.3	54.8 ± 0.7
HSI%	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
K	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0

Data are expressed as mean ± SD, n = 3; N = 18.
Abbreviations: FCR, food conversion ratio; SGR, specific growth rate; PER, protein efficiency ratio; PGR, protein growth rate; %NPU, net protein utilisation; FRD, fat deposition rate; DP%, dress-out percentage; FY%, Fillet yield percentage; HSI%, Hepato-Somatic index; K, Fulton's condition factor.
Values in the same row with different letters (a, b, c) are significantly different.

Table 11. The proximate composition (mg/g) of fillets and whole bodies of Atlantic Salmon Parr at commencement and the completion of the first *in vivo* feeding trial.

	Control	WCMF I	WCMF II	WCMF III	WCMF IV
Whole fish					
Moisture	700.0 ± 0.4	705.7 ± 1.1	702.8 ± 1.4	703.7 ± 1.1	706.2 ± 2.5
Lipid	90.9 ± 1.8	89.9 ± 2.2	87.4 ± 1.2	86.3 ± 2.4	86.0 ± 2.3
Protein	191.5 ± 1.0	197.3 ± 1.2	195.9 ± 0.9	193.4 ± 2.6	192.8 ± 1.3
Ash	17.3 ± 0.6	17.5 ± 0.4	17.2 ± 0.2	17.6 ± 0.6	17.6 ± 0.3
Fillet					
Moisture	729.3 ± 2.6	726.0 ± 2.1	729.1 ± 1.1	723.2 ± 3.2	730.3 ± 3.1
Lipid	41.7 ± 3.1	46.2 ± 1.7	43.9 ± 2.3	42.8 ± 2.3	42.5 ± 2.5
Protein	216.9 ± 1.7	219.6 ± 3.1	218.3 ± 1.4	218.1 ± 2.7	217.4 ± 1.9
Ash	13.7 ± 0.4	13.5 ± 0.6	13.7 ± 0.8	13.6 ± 0.5	13.7 ± 0.3

Data are expressed as mean ± SD, n = 3; no statistically significant differences between treatments for any parameter were observed by ANOVA.

Basically, growth performance, feed performance and body composition of salmon fed the algae-based whole-cell aquafeeds and those fed the control feed were not different in both 12-week trials with Atlantic salmon juveniles (parr and post-smolt) ($P > 0.05$) (Tables 10-13). Specifically, the inclusion of the algae did not reveal any statistically significant difference in the growth data of parr and smolt Tables 10 and 12. With respect to SGR, FCR and PER, all four algal products yielded similar performance values as that of the control feed. At the end of the trial, SGR values ranged from 1.67 to 1.69 for WCMF II and I, III, IV, respectively, in trial I and 0.54 zo 0.59 for WCMF IV and I, respectively, in trial II. The FCR values observed in the trials (0.80-0.81 and 1.12-1.16) are comparable to published ranges for salmon during the spring season (0.58 –1.18; Einen et al. 2007). Apart of WCMF IV in the second trial (Table 13), the protein efficiency ratio (PER) of the fish fed the algae-based aquafeeds (2.56-2.58 and 2.04-2.08) was close to that of the control groups in both trials (2.57 and 2.07), indicating that the replacement with algal protein may not have affected the rate of the protein utilization.

Table 12. Fish performances and feed efficiency in Atlantic Salmon post-Smolt fed the four experimental diets during the second *in vivo* feeding trial.

	Control	WCMF I	WCMF II	WCMF III	WCMF IV
Survival (%)	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
Weight (g)					
Week 0	174.5 ± 20.4	173.8 ± 19.7	173.6 ± 16.4	172.9 ± 18.0	175.5 ± 20.1
Week 6	226.4 ± 19.3	230.5 ± 20.6	230.3 ± 20.1	227.5 ± 22.7	226.8 ± 23.3
Week 12	273.9 ± 32.1	274.2 ± 33.3	271.6 ± 34.1	272.9 ± 35.0	267.1 ± 33.8
SGR (% day^{-1})	0.58 ± 0.04	0.59 ± 0.02	0.57 ± 0.03	0.57 ± 0.02	0.54 ± 0.01
FI (% BW day^{-1})	0.75 ± 0.05	0.76 ± 0.02	0.73 ± 0.05	0.72 ± 0.03	0.67 ± 0.03
FCR	1.15 ± 0.03	1.16 ± 0.03	1.15 ± 0.02	1.13 ± 0.01	1.12 ± 0.05
PER	2.07 ± 0.09[a]	2.08 ± 0.03	2.06 ± 0.05	2.04 ± 0.01	1.97 ± 0.09[b]

Values are given as mean ± SD; n = 3 replicate tanks. Values in the same row with different letters (a, b, c) are significantly different.

Abbreviations: SGR, Specific growth rate; FI, Apparent feed intake; BW, Body weight; FCR, Feed conversion ratio; PER, Protein efficiency ratio.

Table 13. Proximate composition of the whole fish and fillet from Atlantic salmon post-Smolt offered microalgae-based feeds for 12 weeks.

	Control	WCMF I	WCMF II	WCMF III	WCMF IV
Whole fish					
Moisture	726.1 ± 4.1	721.3 ± 5.7	721.4 ± 1.5	721.0 ± 3.1	721.2 ± 3.6
Lipid	273.2 ± 10.6	271.1 ± 10.2	283.5 ± 5.5	268.4 ± 5.3	267.2 ± 5.2
Protein	668.4 ± 12.3	677.1 ± 23.0	672.3 ± 11.2	660.4 ± 12.5	660.1 ± 10.6
Ash	86.4 ± 5.9	85.2 ± 5.5	83.9 ± 1.1	81.2 ± 4.6	80.5 ± 3.9
Fillet					
Moisture	753.9 ± 5.6	755.9 ± 2.2	753.1 ± 0.8	752.5 ± 4.0	750 ± 0.3
Lipid	129.4 ± 10.6^{a}	127.6 ± 5.3	122.4 ± 2.1	119.9 ± 11.1	90.9 ± 4.3^{c}
Protein	861.5 ± 12.2	869.5 ± 3.0	867.4 ± 5.3	864.1 ± 8.8	863.1 ± 10.3
Ash	61.7 ± 3.0	63.9 ± 5.8	62.5 ± 3.5	66.0 ± 2.3	62.8 ± 5.0

Values ($g \cdot kg^{-1}$ dry weight) are given as mean ± SD; n = 3 fish from each of the replicate tanks.
Different superscripts indicate statistically significant differences ($P < 0.05$), if any, between the groups in a particular row.

Apart from the reduced lipid content in WCMF IV fillets in trial II (Table 13), other biochemical components assayed in the whole fish and fillet did not show any significant difference. The lipid content of the fillet was highest for the control group, and this was significantly different ($P < 0.05$) from that recorded for the WCMF IV group. Although in the first trial a lipid reduction was not detected, a lipid lowering effect of algae diets has been observed previously. For instance, *Chlorella* extract was found to reduce lipid accumulation in the muscle of *Plecoglossus altivelis* (Nematipour et al. 1987). No significant differences in growth or feed performance were observed for algae-based feeds combined with protein hydrolysates from *Oreochromis niloticus* as well as milled *Artemia salina* and *Acartia tonsa* relative to the controls in both trials. Even though, adult Atlantic salmon, being carnivorous, may not be capable of tolerating high amounts of algal materials in their feed (Krogdahl et al. 2003, Torstensen et al. 2008), protein rich microalgae may be incorporated in salmon feeds for juveniles at higher amounts than those tested in the present study (>70%).

5. Discussion

In recent years, the aquaculture industry has succeeded in reducing the inclusion rates of fishmeal and fish oil in the feeds of farmed aquatic animals. However, due to the increase in production of farmed species there is still a growing demand for these ingredients (Naylor et al. 2009). Fishmeal is the principal source of protein in commercial aquatic feeds. As a result of the steep increase in price of fishmeal and the decline in fishery resources that go into the fishmeal production, there is an interest in developing alternatives to this finite component (Kiron et al. 2012). Feed for salmonids devoid of fish meal and fish oil, can only be developed if new feed resources become available that can supply the feed with protein and lipids (EPA and DHA) at a competitive price. Low content of EPA and DHA in the feed is a concern, because of the high correlation between content in feed and amount deposited in the flesh of Atlantic salmon (Berge et al. 2009, Østby et al. 2009). Use of diets with a high content of plant oils may also have adverse health effects in the fish and increase susceptibility for viral diseases (Martinez-Rubio et al. 2012, 2014) and parasites.

In the present study, fish protein hydrolysates (FPH) originating from Nile Tibia were tested as fishmeal replacement. FPH are products from either chemical (e.g., acid and alkaline) or enzymatic (e.g., protease) breakdown of fish proteins into single amino acids, peptides and oligopeptides (Kristinsson & Rasco 2000). High quality FPH can be produced from fish processing by-products, fishery by-catch, and low-value pelagic species not currently directly consumed by humans (Kristinsson et al. 2007, Egerton et al. 2018). They are considered a suitable source of protein for human and animal nutrition because of their balanced amino acid composition and their low molecular weight, allowing higher gut absorption rates (Benjakul et al. 2014, Egerton et al. 2018). Their addition at low concentrations (18-24%) has been found to significantly increase individual specific growth rates of adult Atlantic salmon (Hevrøy et al. 2005). More recently, Atlantic salmon, at the fast-growing seawater stage, were found to grow equally well on a diet consisting of plant proteins supplemented with 5% fishmeal, 5% fish soluble protein and 3% squid hydrolysate as on a fishmeal control diet (Espe et al. 2007). The supplemented fish-derived fractions of this diet increased palatability and provided sufficient bioavailable nutrients to compensate for the nutritional shortcomings of the plant protein ingredients (e.g., antinutrients and lower bioavailability of nutrients) (Espe et al. 2007). FPH added at appropriate levels, has been reported to increase survival and growth rates, decrease malformation rates, increase enzyme activity, modify nutrient transport patterns in the intestine, improve nutrient absorption and induce non-specific immune responses in larvae, fry and adult fish (Bøgwald et al. 1996, Cahu et al. 1999, Espe et al. 1999, Murray et al. 2003, Aksnes et al. 2006, Liang et al. 2006). FPH added to Atlantic salmon diets have resulted in positive immune modulation (enhanced levels of superoxide anion production in head kidney leucocytes) in adult fish (Bøgwald et al. 1996) and increased feed intake (12.5% greater than control) and growth (1.8% higher specific growth rate compared to control) in post-smolts (Hevrøy et al. 2005).

As microalgae protein is of good quality, with amino acid (AA) profiles comparable to that of other reference food proteins (Becker 2007), it could be a plausible alternative to fishmeal protein. For instance, leucine, arginine and lysine are generally predominant in microalgal protein (on average 7 g 100 g protein^{-1}), methionine, histidine and tryptophan are typically most limiting (on average 2 g 100 g protein^{-1}) and isoleucine, phenylalanine, threonine and valine are mid-range (on average 4 g 100 g

protein^{-1}) (Tibbetts 2017). An important factor when evaluating the protein quality of microalgae-based ingredients for nutrition is their concentrations of nucleic acids (RNA and DNA), which are sources of purines. Microalgae typically contain lower levels of nucleic acids and purines (4–6%) than other single-cell proteins like yeast and bacteria (8–20%). In addition, microalgae, which are the source of all photosynthetically fixed carbon in the food web of aquatic animals (Kwak & Zedler 1997), may be an ideal replacement for fishmeal in aquatic feeds. Meal from the brackish water cyanobacterium Spirulina has been incorporated into experimental fish feeds with some success (El-Sayed 1994, Olvera-Novoa et al. 1998, Nandeesha et al. 2001, Palmegiano et al. 2005). However, there have been few investigations of the use of marine microalgae in compound aquatic feeds (e.g., Jaime-Ceballos et al. 2006), especially not on microalgae produced in large-scale quantities. Microalgal co-products resulting from the production of third generation biofuels, which may be available in large amounts in the future are a source of human food and animal feed protein (Brennan & Owende 2010, Stephens et al. 2010). Mass produced microalgae are therefore a promising ingredient in aquafeeds.

While it is true that many essential dietary nutrients for wild salmonids originate from aquatic phytoplankton (microalgae) and other single-celled organisms, they are delivered through 'indirect' passage of nutrients up the aquatic food chain and rarely via 'direct' intake as salmonids do not actively seek to consume microalgae. Higher trophic predators like salmonids evolved to rely on a progression of intermediary organisms (e.g., grazing phytoplankton, zooplankton, forage fish, etc.) to extract nutrients from complex food matrices that make up 'base-of-the-food chain' organisms (e.g., phytoplankton). This upward passage and trophic accumulation of essential nutrients, referred to as food-chain amplification, transforms them into forms that the relatively simple monogastric digestive system of salmonids can assimilate and use for productive purposes like protein synthesis, growth, tissue repair, metabolic energy and reproduction. The practical implication is that, in the absence of food-chain amplification, reliance on transformative intermediary organisms represents a nutritional barrier for direct feeding of microalgae to most monogastric animals, especially cold water farmed salmonids. This is because their capacity to extract and utilize microalgal nutrients directly is limited by the highly recalcitrant cell walls of most microalgae, combined with the relatively short gastric (acidic) digestion phase in salmonid fishes. In the present study, the cell walls of the chlorophytes (*Dunaliella, Chlorella, Nannochloropsis*) used in the developed aquafeeds were disrupted prior to their integration, in order to increase the nutritional availability (Verspreet et al. 2020, 2021). Furthermore, studies into salmon feeding at high seas in the North-East Atlantic showed that shrimps accounted for 95% of the food in number, but only about 30% by weight (Jacobsen & Hansen 2001). Thus, selected zooplankton species, specifically the brine shrimp *Artemia salina* and the copepod *Acartia tonsa* were included into the developed whole-cell-microalgae aquafeeds in low concentrations after processing (WCMF III and IV).

Although different stated from several authors, under natural conditions, the composition of free AAs in microalgae can vary dramatically during the growing season. For example, a significant (up to 20 times) change in the intracellular concentration of free AAs was found in the diatom *Rhizosolenia delicatula* during its mass development (Martin-Jezequel 1992). In this case, the highest content of AA accounted for glutamic acid, glutamine, alanine, isoleucine, and lysine (the total content of which exceeded 65%). In periods when the development of *R. delicatula* did not occur, other AA were dominant such as serine, glycine, arginine, and aspartic acid (Kolmakova *&* Kolmakov 2019). A lot of attention is currently being paid to the dependence of AA composition in microalgae and cyanobacteria laboratory cultures on cultivation conditions (Ogbonda et al. 2007, Samek et al. 2013).

By the example of *Chlorella ellipsoidea* it was shown that the AA composition remained stable under outdoor and indoor illumination in a bioreactor (Wang et al. 2014). However, when *Chlorella* was cultivated in the dark (heterotrophic conditions) and artificially illuminated (autotrophic conditions), significant differences were found for all AAs except for alanine (Wu 2009). Also, the dependence of the AA composition on cultivation conditions was shown for the cyanobacterium *Spirulina platensis* and the chlrorphyceae *Scenedesmus quadricauda*. When they were cultivated, in addition to the photoperiod and light intensity, the composition of AAs were influenced by turbulence, pH, salinity, temperature, and the content of biogenic elements in the medium (Samek et al. 2013). Overall, there are contradicting data about the dependence of the AA composition in diatoms on growing conditions in culture. On the one hand, the study of Brown et al. (1996) indicates the absence of an obvious association between the AA composition, the photoperiod, and the light intensity in laboratory experiments with the *Thalassiosira pseudonana* Hasle & Heimdal diatom culture. On the other hand, the AA composition of diatoms depended on the addition of nitrogen to the medium (Dortch 1982). Moreover, even in the classic study of Hecky et al. (1973) using the example of six diatom species, it was shown that the composition of AAs in the cell wall and inside the cytoplasm can vary significantly, depending on environmental conditions (Kolmakova & Kolmakov 2019).

The dietary essential n-3 LC-PUFAs, EPA and DHA, required by farmed salmonids have traditionally been supplied by fish oil, which is manufactured from wild-caught pelagic fish deemed unsuitable for direct human consumption, and this practice is no longer ecologically or economically sustainable (Tibbetts 2017). Most industrialized microalgae species do not accumulate high-value essential n-3 LC-PUFA. This essential lipid deficiency may relegate these species as poor nutritional value for use in salmonid feeds. Like other macronutrients, lipid content of microalgae varies widely, and fatty acid (FA) composition is also highly heterogeneous. The only discreet trend is that the lipid fraction of most species is dominated by the saturated FA (SFA) palmitic acid (16:0) and the monounsaturated FA (MUFA) oleic acid (18:1n-9); which combined generally account for about 40% of total FAs. Many marine and freshwater species, particularly *Scenedesmus* and *Tetraselmis*, produce significant levels (~10% of total FAs) of the n-3 PUFA α-linolenic acid (18:3n-3; ALA) which, once in the body, can be desaturated and elongated as a metabolic precursor for endogenous cellular biosynthesis of the essential n-3 LC-PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). This endogenous biosynthesis of n-3 LC-PUFA from ALA is rate-limiting in salmonids such as rainbow trout to a relatively low efficiency of 12–27% depending on various other dietary and farming conditions and thus essential n-3 LC-PUFA must still be added to salmonid diets. There are several, almost exclusively marine, photoautotrophic microalgae (reviewed by Colombo et al. (2006) that are good accumulators of EPA (up to 53% of total FAs), namely the marine genera *Chromophyte*, *Dunaliella*, *Isochrysis*, *Nannochloropsis*, *Pavlova*, *Phaeodactylum* and *Skeletonema*. However, the only ones evaluated in salmonid feeds are *Isochrysis* and *Nannochloropsis* (up to 28% of total FAs).

Nutrient digestibility vary considerable among various species of microalgae. A dose-response experiment was carried out with mink to study protein and lipid digestibility of *Nannochloropsis oceanica, Phaeodactylum tricornutum* and *Isochrysis galbana*, replacing fish meal in levels of 6, 12 and 24%, respectively (Skrede et al. 2011). The authors found significant linear reduction in digestibility of crude protein and lipid with increasing level of all three algaes. *N. oceanica* and *I. galbana* showed negative effects on protein digestibility already at 6% inclusion, *while P. tricornutum* showed negative effects at the highest inclusion level. Based on linear regression, apparent protein digestibility for *N. oceanica, P. tricornutum* and *I. galbana* was estimated to be 35.5%, 79.9% and 18.8%, respectively.

Digestibility of protein in Spirulina was estimated to be 85% for Atlantic salmon, when 30% of the algae was added to the test diet (Burr et al. 2011). Kousoulaki et al. (2015) reported an ADC of protein in the range of 87–88%, independent of inclusion level, 0–15%, of the thraustochytrid *Schizochytrium* sp. in the feed. The effect of *Schizochytrium* sp on retention of long chain polyunsaturated fatty acids was tested in Atlantic salmon parr during saltwater transfer (Miller et al. 2007), in post smolt Atlantic salmon growing from 213 g to approximately 800 g (Kousoulaki et al. 2015) and during growth from approximately 1534 g until doubling of weight (Sprague et al. 2015). These latter studies reported a significant effect on the amount of DHA in Atlantic salmon muscle when fed *Schizochytrium* biomass in the feed. In the experiment of Kousoulaki et al. (2015), however, highest content of DHA was reported at an inclusion level of 6% rather than 15% (Sørensen et al. 2016).

There has been considerable research on re-formulating aquafeeds using novel ingredients and nutritional supplements (e.g., exogenous enzymes, bioactive compounds and bioavailable trace metals) that complement plant proteins and help to meet the needs of aquaculture species (Sarker et al. 2007, Dalsgaard et al. 2012, Ringø & Song 2016, Liu et al. 2014). Creating sustainable feeds that promote fish welfare and maximise growth potential, while remaining cost-efficient, is a prominent challenge for the aquaculture industry. Furthermore, it is now recognised that the effects of dietary alterations on the gut microbiota of fish must be considered, as they play a key role in influencing fish health and growth (Merrifield et al. 2010, Kononova et al. 2019, Egerton et al. 2018). Some promising work is emerging to suggest that diets with very low or no fishmeal inclusion will be possible with careful formulation in the future (Espe et al. 2006, Kousoulaki et al. 2012). However, to date, it has been recommended that for optimal growth a minimum of 5% fishmeal is required to provide unidentified growth factors, thought to be naturally occurring trace and ultra-trace compounds such as amines and steroids (Hardy 2010, Espe et al. 2007). While a detailed understanding of the composition and structure of the intestinal microbiota in Atlantic salmon is still developing (Kononova et al. 2019), several studies have investigated the effects of alternative protein sources and high plant-protein/ low fishmeal diets (Green et al. 2013, Hartviksen et al. 2014, Gajardo et al. 2017, Schmidt et al. 2016). These studies were all carried out on adult Atlantic salmon. The results, however, have found significant differences in the gut microbiota composition from those reported for Atlantic salmon at sea (Llewellyn et al. 2016, Dehler et al. 2017), pointing to the necessity for conducting more detailed analysis of the developed aquafeeds in the present study.

6. Conclusions and Perspectives

In the present study, it was investigated whether Icelandic marine microalgae biomasses represent a potential feed source for Atlantic salmon. The results revealed that farmed Atlantic salmon parr and post-smolt can grow successfully on an 73-78.9% microalgae diet when supplemented with fish protein hydrolysates (FPH) originating from Nile Tibia combined with zooplankton meal from two selected species. FPH for instance can be produced from fish processing by-products, fishery by-catch, and low-value pelagic species not currently directly consumed by humans. When using sustainable processing practices, it can be one of multiple product streams derived from using the entire fish. Its high nutritional value allows it to be used in small quantities as a supplement to fortify diets. These characteristics suggest that its use could go some way towards sustainable food production and help reduce the volume of wild fish species used in aquafeeds, an important consideration in light of recent

biodiversity reports. Although the present study reports some significant results, not all growth performance indicators revealed statistically significant differences. Further research into the effects of the different diets over extended periods, and associated variations in gut microbiota using metabolomics and shotgun sequencing to ascribe digestive roles would be beneficial to gain a greater understanding of the interaction of dietary nutrients and gut microbiota and their effects on host health, development and growth. Regarding the effects of the developed aquafeeds on Saprolegniosis far more testing is necessary to either incorporate the extracts either directly into the aquafeed or develop it as a stand-alone agent.

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8. References

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9. Appendix

9.1 Qualitative and Quantitative Compositions of the whole-cell- microalgae aquafeeds

Table A1. Amino acid compositions of the four whole-cell-microalgae aquafeeds developed for Atlantic salmon.

Amino acid [ng · mg DW^{-1}]	**WCMAF I**	**WCMAF II**	**WCMAF III**	**WCMAF IV**	**ANOVA (one-way) p <**
Asparagine	1.611	48.620	1.884	129.158	0.001
Aspartate	27.171	55.770	23.144	232.627	0.01
Serine	18.427	23.324	16.948	140.591	ns
Alanine	1888.106	62.610	1613.930	112.696	0.001
Glycine	30.067	60.203	16.180	276.136	0.05
Glutamine	0.102	645.353	27.710	176.355	ns
Threonine	23.633	28.082	22.927	191.459	0.001
Cysteine	20.801	14.511	11.050	336.570	0.01
Glutamate	58.638	100.872	53.601	448.657	0.001
Proline	47.165	81.134	40.796	217.737	0.001
Lysine	34.675	6.019	37.461	47.706	0.01
Histidine	7.235	8.472	6.022	71.963	0.001
Arginine	57.559	60.382	58.892	277.641	0.001
Valine	130.202	70.994	142.862	328.366	0.001
Methionine	0.170	1.412	0.153	253.850	0.001
Tyrosine	57.345	33.869	67.545	190.685	0.05
Isoleucine	199.150	78.756	223.069	436.408	ns
Leucine	260.764	116.123	285.653	n.d.	0.001
Phenylalanine	189.117	118.143	210.247	611.898	ns
Tryptophan	22.619	33.954	27.513	93.801	0.001
Malate	266.290	1043.846	301.340	2094.594	0.05
Succinate	84.659	116.757	95.694	1012.087	0.01
Total	**3425.505**	**2809.206**	**3284.621**	**7680.985**	0.001

Table A2. Fatty acid composition of the four whole-cell-microalgae aquafeeds developed for Atlantic salmon

Fatty acid ($\mu g \cdot g\ DW^{-1}$)	**WCMAF I**	**WCMAF II**	**WCMAF III**	**WCMAF IV**	**ANOVA (one-way) p <**
14:0	2302.627	1159.948	1956.373	909.484	0.001
Phytol	n.d.	n.d.	n.d.	n.d.	0.01
i-15:0	78.480	40.681	52.612	n.d.	ns
15:0	135.348	95.191	117.493	92.110	0.001
14:0 Alc	n.d.	n.d.	n.d.	n.d.	0.05
16:0	6446.565	6233.190	5584.000	4821.383	ns
16:1n7	1762.465	1223.652	1609.866	1643.196	0.001
16:1n5	84.336	39.742	81.388	n.d.	0.01
i-17:0	n.d.	n.d.	n.d.	n.d.	0.001
16:2n4	267.899	54.501	67.582	n.d.	0.001
16:3n4	137.705	101.238	133.209	111.071	0.01
16:4n1	117.822	52.341	120.328	n.d.	0.001
18:0	661.263	1132.187	631.030	1036.892	0.001
18:1n9	3761.350	11372.449	3749.000	9533.036	0.001
18:1n7	723.066	1029.344	720.328	1213.799	0.001
18:1n5	101.233	48.005	94.567	80.852	0.05
18:2n6	1019.751	8357.124	864.776	3739.311	ns
18:3n6	n.d.	n.d.	n.d.	n.d.	0.001
18:3n3	312.029	1617.537	315.299	849.789	ns
18:4n3	626.211	260.261	646.418	328.449	0.001
20:0	51.466	169.310	41.791	95.961	0.05
20:1n11	294.503	182.391	283.313	289.875	0.01
20:1n9	2897.680	1611.594	2579.657	2211.782	0.001
20:1n7	77.469	70.177	72.612	144.882	0.01
20:2n6	78.421	214.569	75.866	163.806	ns
20:3n3	243.653	218.913	251.284	188.629	0.001
20:4n3	129.813	150.415	148.627	160.364	0.05
20:5n3	1750.898	1004.819	2040.284	1388.453	ns
22:1n11	5013.992	1932.653	4371.552	2043.733	0.001
22:1n9	358.063	260.879	309.075	321.602	0.01
22:5n3	207.693	205.310	216.388	323.332	0.001
24:0	35.774	22.174	n.d.	37.492	0.001
24:1n11	n.d.	43.227	n.d.	n.d.	0.01
24:1n9	8.393	5.481	n.d.	8.222	0.001
22:6n3	1759.274	1342.123	3199.015	2028.344	0.001
Total	**31445.242**	**40251.427**	**101911.046**	**33765.849**	0.001

Table A3. Vitamin composition of the four whole-cell-microalgae aquafeeds in mg · kg^{-1}. Vitamins in feeds were determined according to the method described by Kienen et al. (2008) (cf. 3.7.1).

Vitamin	WCMAF I	WCMAF II	WCMAF III	WCMAF IV	ANOVA (one-way) p <
Ascorbate	55.4	62.5	90.2	90.9	0.001
Thiamine (B_1)	6.2	8.3	11.2	13.6	0.001
Biotin (B_7)	0.40	0.57	0.70	1.14	0.001
Vitamin B_6	5.28	7.15	10.3	12.9	0.05
Folate	1.77	2.27	3.23	4.22	ns
Cobalamin	0.18	0.31	0.37	0.51	0.001
Vitamin E	64.2	63.5	66.1	63.4	ns
Pantothenic acid	9.9	16.7	25.3	21.7	0.001
Riboflavin	4.7	6.8	10.7	13.1	0.05
Niacin	43.9	50.1	64.2	76.5	0.01
Total	**191.93**	**218.2**	**282.3**	**297.97**	0.001

Table A3. Mineral and trace element composition of the four whole-cell-microalgae aquafeeds in mg · g DW^{-1}.

Element	WCMAF I	WCMAF II	WCMAF III	WCMAF IV	ANOVA (one-way) p <
Mineral contents					
P	18.6 ± 2.3	17.5 ± 2.0	17.9 ± 2.5	18.1 ± 2.5	ns
Mg	9.4 ± 1.0	9.8 ± 2.2	9.3 ± 2.0	9.1 ± 2.6	ns
Ca	3.6 ± 1.4	3.9 ± 1.3	3.8 ± 0.5	3.8 ± 0.6	ns
Na	8.8 ± 1.1	8.6 ± 2.0	8.6 ± 0.9	8.7 ± 0.7	ns
K	13.5 ± 2.7	13.9 ± 1.6	14.5 ± 2.3	14.1 ± 2.0	ns
Trace elements					
Fe	4.9 ± 0.3	4.5 ± 0.6	4.8 ± 2.2	4.9 ± 0.2	ns
Cu	0.8 ± 0.2	0.7 ± 0.3	0.8 ± 0.3	0.8 ± 0.4	ns
Zn	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	ns
Se	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	ns
Total	**59.68**	**59.0**	**59.8**	**59.6**	ns

Table A5. List of commercial ingredients used in the whole-cell-microalgae aquafeeds and their producer.

Commercial ingredient	Producer
Fishmeal	Petuna Seafoods, TAS
Pregelatinized starch	Starch Australasia

Table A6. Molecular weight profile of the soluble protein fractions of the partly hydrolysed protein hydrolysate powder (PHP) which contained 18% soluble protein and the soluble protein hydrolysate powder (SPH) which contained 96% soluble protein, used as experimental ingredients to supplement the developed whole-cell-microalgae aquafeeds for Atlantic salmon parr; kDa = kilo-Daltons.

	% of Soluble Protein		**% of Total Protein**	
	PHP	**SPH**	**PHP**	**SPH**
>20kDa	0.23	0.04	0.04	0.04
20-10kDa	0.49	0.26	0.09	0.25
10-5kDa	1.97	1.36	0.35	1.31
5-2kDa	6.94	6.09	1.25	5.85
2-1kDa	13.5	12.89	2.43	12.37
1-0.5kDa	27.7	27.35	4.99	26.26
<0.5kDa	49.18	52.01	8.85	49.93

9.2 Media Receipts

20. BG 11 Medium for Cyanobacteria (= BG 11)

	stock solution [g/100 ml]	nutrient solution [ml]
$NaNO_3$	15	10
$K_2HPO_4 . 3H_2O$	0.4	10
$MgSO_4 . 7H_2O$	0.75	10
$CaCl_2 . 2H_2O$	0.36	10
citric acid	0.06	10
ferric ammonium citrate	0.06	10
EDTA (dinatrium-salt)	0.01	10
Na_2CO_3	0.2	10
micronutrient solution *		1
de-ionized or distilled water		919

* Composition of the micronutrient solution (from Kuhl and Lorenzen 1964)

Add to 1000 ml of de-ionized or distilled water:

H_3BO_3	61.0 mg
$MnSO_4 . H_2O$	169.0 mg
$ZnSO_4 . 7H_2O$	287.0 mg
$CuSO_4 . 5 H_2O$	2.5 mg
$(NH_4)_6Mo_7O_{24} . 4H_2O$	12.5 mg

a) BG-11 Medium without Sodium Nitrate (=BG11-NaNO3)
Prepare BG-11 Medium (Medium 20) without Sodium Nitrate ($NaNO_3$) and add 929 ml instead of 919 ml of water.

Ref.: Stanier, R. Y., Kunisawa, R., Mandel, M. & Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol. Rev. 35: 171-205.

Rippka, R. & Herdman, M. (1993), modif.

f/2 Medium

Stocks		**per litre**
(1)	Trace elements (chelated)	
	Na_2 EDTA	4.16 g
	$FeCl_3.6H_2O$	3.15 g
	$CuSO_4.5H_2O$	0.01 g
	$ZnSO_4.7H_2O$	0.022 g
	$CoCl_2.6H_2O$	0.01 g
	$MnCl_2.4H_2O$	0.18 g
	$Na_2MoO_4.2H_2O$	0.006 g
(2)	Vitamin mix	
	Cyanocobalamin (Vitamin B_{12})	0.0005 g
	Thiamine HCl (Vitamin B_1)	0.1 g
	Biotin	0.0005 g

Medium	**per litre**
$NaNo_3$	0.075 g
$NaH_2PO_4.2H_2O$	0.00565 g
Trace elements stock solution (1)	1. 0 ml
Vitamin mix stock solution (2)	1. 0 ml

Make up to 1 litre with filtered natural seawater. Adjust pH to 8.0 with 1M NaOH or HCl. Sterilise by autoclaving for 15 minutes at 15 psi and use when cooled to room temperature.

Reference

Guillard RRL and Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervaceae* (Cleve) Gran. Can. J. Microbiol. **8**, 229-239.

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Note

For f/40 medium for calcifying *Emilianias*, simply dilute all stock solutions by 1/20th

f/2 Medium for growing diatoms: 2L Recipe

Modified (only quantities, not final concentrations, except in [2]) from Andersen, R. A., Berges, J. A., Harrison, P. J. and Watanabe, M. M. 2005. Recipes for freshwater and seawater media. In: Algal Culturing Techniques (R. A. Andersen, eds), pp. 429-538. Elsevier, Amsterdam.

This is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation, termed "f Medium" (Guillard, 1962) has been reduced by half (Guillard 1975). The original medium (Guillard, 1962) used ferric sequestrene; we have substituted $Na_2EDTA{\cdot}2H_2O$ and $FeCl_3{\cdot}6H_2O$.

Into 1950 mL of ASW (artificial sea water), add the following components (you can also add the Vitamins' stock after autoclaving via sterile filtering). Bring the final volume to **2 litres** with ASW. Autoclave. If silicate is not required, omit to reduce precipitation.

component	mass (g/mol)	stock (g/L dH_2O)	g in 100 mL	add	final conc. (M)	final conc. (g/L ASW)
$NaNO_3$	84.98	150 g/L	15 g	1 mL	8.82×10^{-4}	0.075 g/L
$NaH_2PO_4{\cdot}H_2O$	137.97	10 g/L	1 g	1 mL	3.62×10^{-5}	0.005 g/L
$Na_2SiO_3{\cdot}9H_2O$	284.04	60 g/L[1] 30 g/L[2]	6 g[1] 3 g[2]	1 mL	1.06×10^{-4} 0.58×10^{-4}	0.030 g/L[1] 0.015 g/L[2]
Trace metals stock	–	see recipe	–	1 mL	–	–
Vitamins stock	–	see recipe	–	0.5 mL	–	–

[1]Andersen & al., [2]Silicate-poor medium: can be used if precipitation is a problem. Preliminary observations showed no evident differences between both concentrations. Stocks will yield 100 × 2L f/2

f/2 Trace Metals Stock (will yield 100 × 2L f/2)

Into 80 mL of dH_2O, dissolve the EDTA **entirely**, then add the other components. Bring the final volume to **100 mL** with dH_2O. Primary stocks are prepared in 20 mL and stored at room temperature.

component	mass (g/mol)	1° stock (g/L dH_2O)	g in 10 mL	add	final conc. (M)	final conc. (g/L ASW)
$Na_2EDTA{\cdot}2H_2O$	374.24	–	–	0.88 g	1.17×10^{-5}	0.0044
$FeCl_3{\cdot}6H_2O$	270.30	–	–	0.63 g	1.17×10^{-5}	0.0032
$MnCl_2{\cdot}4H_2O$	197.01	179 g/L	1.79 g	0.2 mL	9.10×10^{-7}	1.79×10^{-4}
$ZnSO_4{\cdot}7H_2O$	286.00	21.9 g/L	0.219 g	0.2 mL	7.65×10^{-8}	2.19×10^{-5}
$CoCl_2{\cdot}6H_2O$	237.00	9.9 g/L	0.099 g	0.2 mL	4.20×10^{-8}	9.95×10^{-6}
$CuSO_4{\cdot}5H_2O$	249.00	9.8 g/L	0.098 g	0.2 mL	3.93×10^{-8}	9.79×10^{-6}
$Na_2MoO_4{\cdot}2H_2O$	237.88	6.2 g/L	0.062 g	0.2 mL	2.60×10^{-8}	6.18×10^{-6}

1° stocks will yield 50 × stocks

f/2 Vitamins Stock (will yield 200 × 2L f/2)

Into 80 mL of dH_2O, dissolve the thiamine · HCl, and add 1 mL of the primary stocks. Bring the final volume to **100 mL** with dH_2O. Primary stocks are prepared in a large volume and discarded. Filter-sterilize and store frozen. Thiamine is heat labile, so it will be largely destroyed after autoclaving, but also after a few days at room temperature.

component	mass (g/mol)	1° stock (g/L dH_2O)	g in x mL	add	final conc. (M)	final conc. (g/L ASW)
Thiamine · HCl (vitamin B_1)	333.27	–	–	0.04 g	2.96×10^{-7}	1.00×10^{-4}
Biotin (vitamin H)[a]	242.45	0.2 g/L	0.02 in 100	1 mL	2.05×10^{-9}	5.00×10^{-7}
Cyanocobalamin (vitamin B_{12})	1355.4	1.0 g/L	0.01 in 10	0.2 mL	3.69×10^{-10}	5.00×10^{-7}

1° stocks will yield 100 × stocks. [a]Biotin has a maximum solubility in H_2O of 0.2 g/L