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Microalgae as feed ingredients for Atlantic salmon

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FACULTY OF BIOSCIENCES AND AQUACULTURE

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Preface

This dissertation is submitted in fulfillment of the requirements for the degree of Philosophiae Doctor (PhD) at The Faculty of Biosciences and Aquaculture (FBA), Nord University (Nord), Bodø, Norway. The original research presented in the thesis is part of the project "Large-scale production of fuels and feed from marine microalgae" funded by Department of Energy (DoE), USA (Project No.DE-EE0003371) and part of the COFASP ERA-NET project "MARINALGAE4aqua" funded by the Research Council of Norway (Project No. 260190, Alger4laks).

The project team consisted of the following members:

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Mette Sørensen, Professor, FBA, Nord University: Main supervisor

Kiron Viswanath, Professor, FBA, Nord University: Project leader and Co-supervisor

Yangyang Gong

Bodø, September 24, 2018

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List of abbreviations

ALA: α-linolenic acid

DE: Digestible energy

DHA: Docosahexaenoic acid

DL: Digestible lipid

DP: Digestible protein

EPA: Eicosapentaenoic acid

FAO: Food and Agriculture Organization of the United Nations

FBW: Final body weight

FCR: Feed conversion ratio

LA: Linoleic acid

IBW: Initial body weight

NSPs: Non-starch polysaccharides

n-3 LC-PUFAs: n-3 long-chain polyunsaturated fatty acids

n-6 LC-PUFAs: n-6 long-chain polyunsaturated fatty acids

PUFAs: Polyunsaturated fatty acids

OECD: Organization for Economic Co-operation and Development

PAP: processed animal protein

PER: Protein efficiency ratio

SFAs: Saturated fatty acids

SGR: Specific growth rate

T: Temperature

TAG: Triacylglycerol

TGC: Thermal growth coefficient

WG: Weight gain

Abstract

Fish meal and fish oil are limited resources and they are to a large extent replaced with land–based plant-derived ingredients in commercial salmon feeds. Plant ingredients cannot provide all the nutrients required by salmonids. Atlantic salmon fed plant-derived ingredients may not have ideal lipid composition and nutritional quality desired by the current and future population. There is thus a need for protein and oil sources with balanced nutritional profile and which are more reliable and sustainable. Photosynthetic microalgae can be considered as sustainable alternatives to fish meal and fish oil or plant-derived ingredients. The content of nutrients–such as protein and lipid as well as fatty acids–in different microalgae varies, and hence their potential as a feed ingredient for carnivorous fish has to be investigated thoroughly. The general objective of this PhD thesis was to investigate the potential of microalgae as feed ingredients for Atlantic salmon. The main response variables were nutrient digestibility, growth performance, feed utilization, chemical composition and intestinal health of the fish. The specific objectives addressed were: 1) Determine the apparent digestibility coefficients of microalgae when fed to Atlantic salmon. 2) Determine the effects of incorporating microalgae in extruded fish meal-based or commercial-like plant-based diets for Atlantic salmon on nutrient digestibility, growth performance, feed utilization, chemical composition and intestinal health of the fish. 3) Investigate the efficacy of different means such as thermo-mechanical treatment or feed additives to improve utilization of nutrients in microalgae.

The microalgae incorporated diets were readily accepted by the fish. The microalga *Nannochloropsis oceanica* was more digestible than *Desmodesmus* sp. Incorporation of microalgae at 10% in both fish meal-based and plant-protein based salmon feeds had no negative effect on growth, feed utilization, condition indices, health parameters and proximate composition of Atlantic salmon. Thermo-mechanical processing (extrusion) can be used as a cost-effective method to improve digestibility of nutrients from microalgae. Use of feed additives did not improve feed utilization. An increased content of PUFAs was noted in whole body composition of Atlantic salmon fed

Nannochloropsis oceanica combined with one of the feed additives or those fed *Scenedesmus* sp., which is considered favorable from a nutritional point of view.

Abstract in Norwegian – Samandrag på norsk

Fiskemel og fiskeolje er begrensede ressurser og har derfor i stor grad blitt erstattet med planteråvarer i kommersielt fôr til laks. Planteråvarer kan ikke alene oppfylle alle ernæringsmessige behov hos laksefisk, og dessuten bidrar planteoljer til en endret fettsyresammensetning i laksen. Dette har medført diskusjoner rundt laks som kilde for langkjedede flerumettede omega 3- fettsyrer, og om innholdet er høyt nok av de ønskede fettsyrene. Det er et stort ønske om å finne nye bærekraftige protein- og oljekilder med en balansert næringssammensetning som kan produseres i skalerbare volum ved økende etterspørsel. Fotosyntetiske mikroalger kan utgjøre et bærekraftige alternativ til fiskemel og fiskeolje eller plantebaserte råvarer i fiskefôr. Innholdet av næringsstoffer som protein og fett samt fettsyresammensetning varierer mellom ulike mikroalger, noe som tyder på at deres potensial som fôringrediens til karnivor fisk kan variere mellom ulike kilder. Det overordnede målet med denne doktorgradsavhandlingen var å undersøke potensialet for mikroalger som fôrbestanddeler til atlantisk laks. Utnyttelse av mikrolagebaserte dietter ble evaluert ved å måle tilsynelatende fordøyelighet, tilvekst, fôrutnyttelse, kjemisk sammensetning av fisken og fiskens tarmhelse. De spesifikke målene var følgende: 1) Å estimere tilsynelatende fordøyelighet av næringsstoffer til ulike mikroalger fôret til atlantisk laks. 2) Undersøke effekten av ulike innblandingsnivåer i fiskemelbaserte eller plantebaserte dietter på fordøyelighet, vekstprestasjon, fôrutnyttelse, kjemisk sammensetning og fiskens tarmhelse. 3) Evaluere bruk av en thermo-mekanisk behandling (ekstrudering) samt to ulike fôrtilsetningsstoffer for øke utnyttelsen av næringsstoffer av fôr med mikroalger.

Fisken viste god appetitt på fôrene med mikroalger. Mikroalgen *Nannochloropsis oceania* var mer fordøyelig enn *Desmodesmus* sp. Innblanding av mikroalger opp til 10% i enten fiskemelbaserte eller planteproteinbaserte fôr til laks hadde ingen negativ effekt på vekst, fôrutnyttelse, tilstandsindekser, helseparametere og kjemisk sammensetning av atlantisk laks. Resultatene viste også at ekstrudering kan brukes som en kostnadseffektiv metode for å forbedre fordøyelsen av næringsstoffer fra mikroalger. Bruk av tilsetningsstoffer forbedret ikke fôrutnyttelsen. Økt innhold av

PUFA ble observert i helkroppssammensetningen av atlantisk laks fôret *Nannochloropsis oceanica* kombinert med et av tilsetningsstoffene eller fisk som ble fôret *Scenedesmus* sp. Økt innhold av PUFA er gunstig og gjør laksen sunnere som menneskemat.

1 Introduction

1.1 Aquaculture has the potential to feed the growing world population

According to Food and Agriculture Organization of the United Nations (FAO), by 2050 the world's population will be nearly 10 billion. It is expected that the demand for food from animal origin will increase as the population grows and the expanding middle class acquires greater purchasing power. In 2016, about 88% of the 170.9 million tons of global fisheries and aquaculture production was utilized for direct human consumption (FAO, 2018). Although global capture fisheries have plateaued in recent decades (**Figure 1**), aquaculture has continued its growth, with an average annual growth rate of 5.8% between 2001 and 2016. Increasing demand for seafood and the growing awareness of the health benefits of fish will sustain the growth of the aquaculture sector (FAO, 2018).

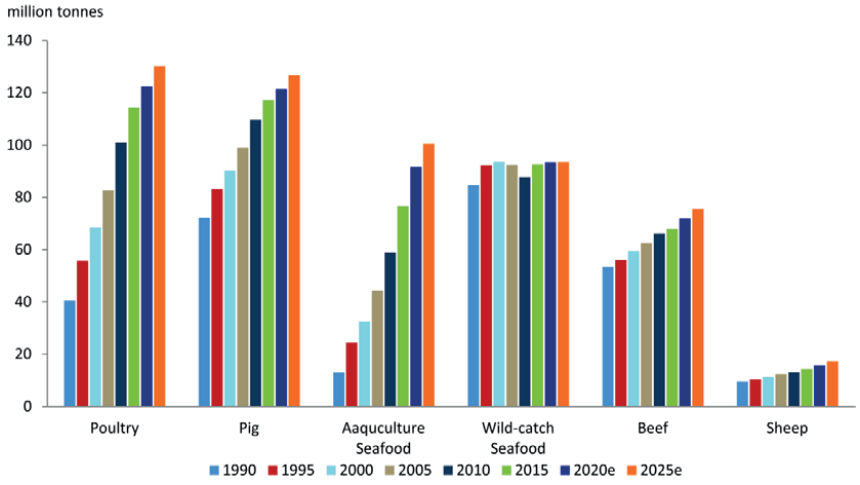


Figure 1. Trends in global production of food from animal origin, 1990-2025(e) (OECD, 2018).

1.2 Norwegian aquaculture

The Norwegian aquaculture industry has grown significantly during the last decades, both in terms of production volume and value. Aquaculture production has increased from approximately 150, 000 tons to more than 1.3 million tons since 1990s (**Figure 2**). Norwegian aquaculture is dominated by Atlantic salmon (*Salmo salar*) farming, accounting for approximately 95% of total volume produced in 2017. Currently, Norway is the largest producer of salmon–responsible for 50% of global production–followed by Chile, Scotland, Canada, and the Faroe Islands. Salmon production in Norway grew annually by 10% during the 20-year period from 1992 to 2012. Since 2012, salmon production has stagnated or even reduced about 5% (from 2015 to 2016) due to sea lice- and disease-related mortality. In 2017, salmon production amounted to 1.22 million tons. Salmon is the most important species for Norwegian seafood industry, with over 68% of the total export value (NOK 64. 7 billion) in 2017 (Norwegian Seafood Council). It is estimated that the production of salmonids in Norway would reach 5 million tons by 2050 (Olafsen et al., 2012).

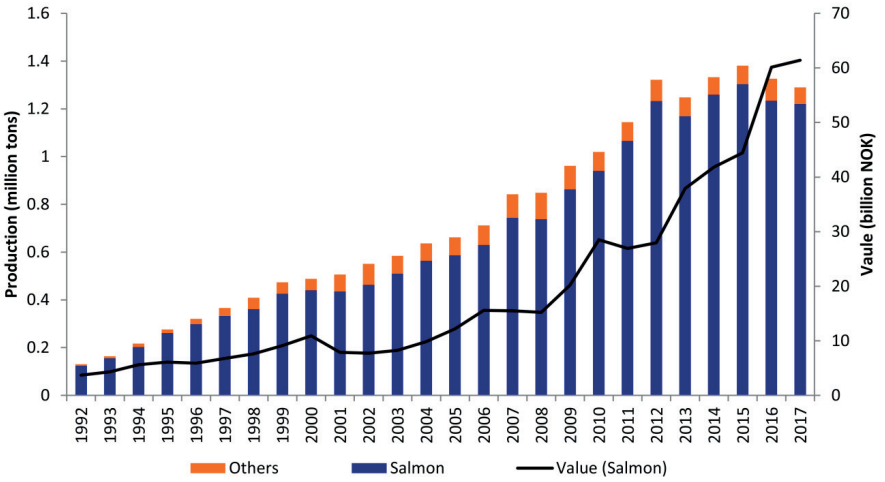


Figure 2. Norwegian salmon production (blue bar) in relation to the production value (black line) (Statistics Norway)

1.3 Demand for feeds for the growing aquaculture sector

The need for compound feed is increasing with the growth of the aquaculture sector and production intensification. In 2012, an estimated 24.3 million tons i.e. 47.6% of total global aquaculture production was based on the direct use of commercially manufactured feeds (Tacon and Metian, 2015). Total industrial compound aquafeed production increased almost six-fold during the course of the last two decades (Tacon and Metian, 2015). According to the 2017 Alltech Global Feed Survey, aquaculture industry used 39.9 million tons of feed in 2016, a 12% increase compared to 2015. In 2017, around 1.66 million tons of feeds were used to produce Norwegian Atlantic salmon (Norwegian-Seafood-Federation, 2018). By 2050, Norwegian salmon aquaculture sector requires 6 million tons of feed (Olafsen et al., 2012). This feed has to be produced using high quality feed ingredients that are sustainable. Furthermore, the feed should provide the required nutrients, at recommended levels, to ensure good growth and health of salmon, the fillet of which should meet the high-quality product expectations of future consumers.

1.4 Fish meal and fish oil in Norwegian salmon feed

Fish meal is recognized as a high-quality, highly digestible and palatable feed ingredient for farmed fish and shrimp. It is a complex product containing essential nutrients as well as many compounds that are biologically active (Hardy, 2010). Fish meal that is currently used in Norwegian salmon feed is mainly produced from forage fishes such as anchoveta, capelin, sprat, blue whiting, and sand eel. Trimmings (e.g. herring, capelin, mackerel) from human food fish are also used in Norwegian salmon feeds (Ytrestøyl et al., 2015). Norse-LT 94 fish meal is a high quality fish meal that is produced from fresh material and dried at low temperature. The typical Norse-LT 94 fish meal contains 6-10% moisture, >68% crude protein (of which 18-32% is water soluble), 6-10% crude fat and 13-16% ash (**Table 1**) (Storebakken et al., 2015, De Santis et al., 2016). Fish meal also supply significant amount of long chain omega-3

polyunsaturated fatty acids (n-3 LC-PUFA), cholesterol (6% of fish meal lipid) and phospholipids (17-27% of fish meal lipid) and 2-5% phosphorus (Scolari et al., 2000, Tocher et al., 2008, ARRAINA, 2015, Storebakken et al., 2015). Fish meal has high levels of B-vitamins (e.g., riboflavin, vitamin B_{12}, niacin, pantothenic acid and vitamin B_6), bioavailability of which are higher than those of plant protein ingredients. Fish meal also contains several low molecular weight nitrogen substances that may affect feed intake, fish growth and health; taurine, hydroxyproline, creatinine, histidine-related peptides (such as anserine and carnosine), nucleotides, and free amino acids (Aksnes et al., 2006, Aksnes et al., 2008, Kousoulaki et al., 2009, Wu and Bechtel, 2012). Fish meal positively affects feed pellet quality; improves binding, starch gelatinization and pellet durability (Samuelsen et al., 2014).

Fish oil is the main source of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), that exert a range of health benefits. The fish oil used in Norwegian salmon feed is mainly produced from the same fatty fish species and trimmings that are used for producing fish meal (Ytrestøyl et al., 2015). Around 22% of fish oil that was used in 2012 was derived from silage and trimmings from fisheries and processing industry (Ytrestøyl et al., 2015).

Table 1. Chemical composition of a typical fish meal

	Fish meal
Composition, kg^{-1}	
Dry matter, g	915
Crude protein, g	685
Crude fat, g	82
Ash, g	152
Gross Energy MJ kg^{-1}	20.0
Essential amino acids, g 16 g^{-1} N	
Arginine	5.27
Histidine	1.87
Isoleucine	3.69
Leucine	6.26
Lysine	6.92
Methionine	2.42
Phenylalanine	3.37
Threonine	3.65
Tryptophan	0.73
Valine	4.03
Minerals	
Phosphorus, g kg^{-1}	23
Calcium, g kg^{-1}	26
Zinc, mg kg^{-1}	125
Other valuable nutrients	
EPA+DHA, g kg^{-1}	20
Phospholipids, g kg^{-1}	26
Cholesterol, g kg^{-1}	6
Taurine, g kg^{-1}	3
Choline, mg kg^{-1}	5.3
Riboflavin, mg kg^{-1}	9.7
Vitamin B12, mg kg^{-1}	430
Niacin, mg kg^{-1}	85
Pantothenic acid, mg kg^{-1}	17.3
Vitamin B6, mg kg^{-1}	4.8

Sources: Sugiura *et al.*, 1998; Scolari *et al.*, 2000; Hua *et al.*, 2005; Tocher *et al.*, 2008; Krogdahl *et al.*, 2015a; SPAROS, 2015; Storebakken *et al.*, 2015; De Santis *et al.*, 2016

Since 1997, production of fish meal and fish oil has declined by 2 million tons due to depletion of wild stocks as a result of overharvesting and climate change-related challenges (e.g., El Niño). The production of the finite resources is not expected to grow significantly, because of quota restrictions. The global production of fish meal and fish oil has remained fairly stable for the last two decades. In 2015, aquaculture industry used 70 and 73% of the world's production of fish meal (4.73 million tons) and fish oil (0.85 million tons), respectively (**Figure 3**). However, to meet the future challenges such as population growth and the ensuing aquatic food demands, the aquaculture industry should shift its dependence from fish meal and fish oil to alternative feed ingredients; to support a rapid, but sustainable development. For the last decades, identification of alternative raw materials has been the main task of the aquafeed sector; to increase flexibility and reduce vulnerability to fluctuating fish meal and fish oil prices.

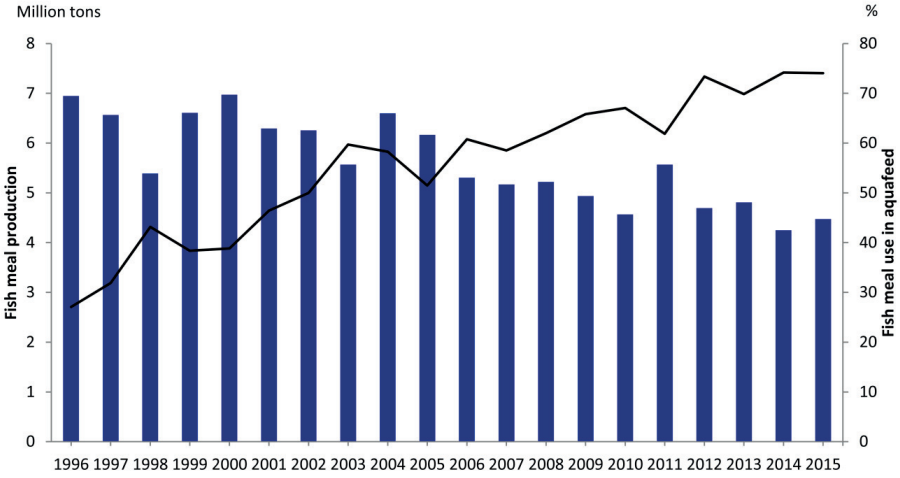


Figure 3. Global fish meal production (blue bar) and percentage of fish meal used in aquaculture (black line) from 1996-2015

1.5 Plant ingredients for Norwegian salmon feed

Use of plant ingredients in diets for carnivorous fishes like Atlantic salmon is the established practice in Europe (Sheperd et al., 2017; Ytrestøyl et al., 2015) (**Table 2**). In 2014, the average inclusion level of fish meal in Norwegian salmon feed was only 17% and that of plant ingredients was 74% (Ytrestøyl et al., 2015, Marine-Harvest, 2018). Fish oil inclusion level in Norwegian salmon feeds was reduced from 24% in 1990 to 9% in 2014 (Ytrestøyl et al., 2015, Marine-Harvest, 2018). Replacement of fish meal and fish oil with plant-derived ingredients and oils has enabled the aquaculture sector to grow without both overexploitation of fish stocks and negatively affecting the marine ecosystem. The main plant protein sources in Norwegian salmon feed are soy protein concentrate, followed by sunflower expeller, and wheat gluten (Ytrestøyl et al., 2015, Sørensen et al., 2011). A small amount of faba beans, pea protein concentrate, corn gluten and horse beans are also used in salmon feed (Ytrestøyl et al., 2015). Rapeseed oil is the main plant oil that is used in Norwegian salmon diet. Wheat is the main starch, but pea and tapioca are also used as binders (Ytrestøyl et al., 2015).

Table 2. Protein and lipid content (% as is) of commercially available feed ingredients for Atlantic salmon

Feed ingredients	Moisture	Protein	Lipid	References
Fish meal LT	7-10	69-72	10-12	ARRAINA, 2015
Krill meal	7-9	58-62	16-20	ARRAINA, 2015
Soy protein concentrate	4-6	60-63	<0.5	ARRAINA, 2015
Pea protein concentrate	4-6	76-78	1-2	ARRAINA, 2015
Wheat gluten	3-5	79-82	4-6	ARRAINA, 2015
Sunflower meal	6-8	26-29	2-3	ARRAINA, 2015
Corn gluten meal	8-10	60-62	2-4	ARRAINA, 2015
Soybean meal	7-9	45-47	2-4	ARRAINA, 2015
Wheat meal	9-12	11	1.5	ARRAINA, 2015
Faba bean meal	9.6	27.5	5.2	Ouraji et al. (2013)
Faba bean protein concentrate	10.7	55.3	2.8	De Santis et al. (2016)

1.6 Challenges concerning the use of plant ingredients in salmon feed

The shift from marine to plant-based ingredients has affected the nutrient retention and health of salmon. The fatty acid profiles of plant oils differ remarkably from those of fish oil. A change in the fatty acid composition of feeds is reflected in the fatty acid composition of flesh. The levels of oleic acid (18:1n-9), linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) were increased, but those of EPA and DHA were decreased in salmon fed rapeseed oil compared to fish fed fish oil (Sprague et al., 2016, Shepherd et al., 2017). The content of EPA and DHA in Norwegian farmed salmon fillets has decreased from 2.74 g/100 g to 1.05 g/100 g (a 62% reduction from 2005) (NIFES, 2018). The health benefits of salmon is hence questionable (Sprague et al., 2016). Salmon fed plant oil have altered lipid metabolism (Torstensen et al., 2000, Todorčević et al., 2008), intestinal morphology (Moldal et al., 2014, Caballero et al., 2003, Bou et al., 2017a). In addition, the fish becomes susceptible to stress and diseases (Martinez-Rubio et al., 2014, Montero et al., 2015, Bou et al., 2017b, Holen et al., 2018). Other challenges concerning some plant ingredients include reduced nutrient digestibility and bioavailability, lower feed intake, presence of anti-nutrients and undesirable substances, and their interferences with digestion, absorption and metabolism of nutrients (Ringø et al., 2009, Hemre et al., 2016, Krogdahl et al., 2010). Nutrient deficiencies, nutrient imbalances or antinutritional factors may reduce the feed intake, growth performance and disease resistance, resulting in increased mortality and economic losses (Olsvik et al., 2011, Shepherd et al., 2017, Asche and Sikveland, 2015). Changes in the feed formula also influence the feed production process parameters, processability and the technical properties of salmon feed (Draganovic et al., 2011, Samuelsen and Oterhals, 2016). For example, harder feed pellets (with higher breaking strength) were observed with high levels of wheat gluten and/or soy protein concentrate in the diets (Draganovic et al., 2011).

Plant ingredients are included in feeds as a sustainable solution to avoid overexploitation of wild fish sources (Naylor et al., 2009). However, sustainability of using plant-based feeds for aquaculture is debated in view of their environmental

impact; the use of fresh water resources, arable land, fertilizers and pesticides for cultivating the plants (Fry et al., 2016). Besides, the extensive use of plant ingredients make the salmon industry, at least in Europe, highly dependent on imported feed ingredients, such as soy protein concentrate. The nutrient limitations and sustainability issues, associated with the use of plant ingredients, have motivated researchers to develop feed ingredients that are more reliable, scalable and sustainable and that have an optimized nutritional profile. Identification and evaluation of new feed ingredients will increase formulation flexibility; helping to vary feed composition depending on market prices without impacting the physical or nutritional quality.

1.7 Novel ingredients-a path to sustainability

Novel sources of feed ingredients should ideally have a nutritional profile that meet the nutrient requirements of farmed fish, contribute to sustainability and should have the potential for production scale up to meet the future ingredient needs. Certain ingredients that are not used today will become important in the future. Examples of ingredients are processed animal protein (PAP) such as blood products and PAP from poultry (Sørensen et al., 2011), zooplanktons (Colombo-Hixson et al., 2013, Hatlen et al., 2016), microbial ingredients (Overland et al., 2010), and insect meal (Henry et al., 2015). However, ingredients from the marine environment are preferred due to their EPA and DHA contents. Norway has the potential for industrial production of microalgae, seaweed, blue mussel, tunicates and polychaete worms. Cultivation of such species may give opportunities for the production of biomass that could be used as feed ingredients/additives in salmon feed and make Norway more self-sufficient in the production of feed ingredients. Sustainable marine feed ingredients can also come from sources that are not directly consumed by humans, especially from lower trophic level in the marine ecosystem in Norwegian Sea (copepod). Thus, marine ingredients produced from microalgae, copepod, seaweed, blue mussel, tunicates and polychaete worms may be alternative resources to fill the gap (Taelman et al., 2013, Julián and Mariana, 2018).

1.8 Microalgae in diets for Atlantic salmon

Microalgae are a diverse group of relatively simple, unicellular aquatic organisms. There are approximately 200,000 species, most of which are capable of performing photosynthesis. The most abundant microalgae divisions are Bacillariophyta (diatoms), Chlorophyta (green algae) and Chrysophyta (golden algae). The phylum Cyanophyta includes photosynthetic bacteria, but is also referred to as 'microalgae' (blue-green algae). Photosynthetic microalgae utilize carbon dioxide (CO_2), light energy and inorganic nutrients to produce organic biomass and oxygen. A few microalgae species are able to grow heterotrophically in the absence of light, using organic carbon sources instead of CO_2 (Smetana et al., 2017). Microalga lies at the base of the food web, and is food for aquatic animals in the marine environment. They are essential for commercial rearing of various marine species; they are food for all growth stages of bivalve molluscs, larval stages of some crustacean species, and very early growth stages of some fish species (Brown et al., 1997, Conceição et al., 2010). Furthermore, algae are used to produce large quantities of zooplankton (rotifers, copepods, brine shrimp) which in turn serve as food for larvae and early-juvenile stages of crustaceans and fish (Reitan et al., 1997). Microalgae are also used directly in the larval tanks, where they are believed to play a key role in maintaining the water quality, providing nutrients to the larvae, and controlling the microbes (Spolaore et al., 2006). Commercial-scale culturing of some microalgae, *Chlorella* sp. (*Chlorella vulgaris; Chlorella pyrenoidosa*), *Haematococcus pluvialis*, *Dunaliella salina* is now well-established (Shah et al., 2018, Shields and Lupatsch, 2012).

The nutritional profiles of microalgae vary considerably; depends on algal species, strains and environmental factors, growth conditions and nutrient availability (Roy and Pal, 2015, Suzuki et al., 2018). Chemical composition of many microalgae strains grown under different growth conditions is already published (Becker, 2007, Batista et al., 2013, Shah et al., 2018). The crude protein and/or lipid contents of microalgae vary among species, but are comparable to or even higher than some of the currently used feed ingredients (**Table 2 & 3**). However, microalgae may also accumulate high

concentrations of non-protein nitrogen such as nucleic acids, inorganic nitrogen (nitrate, nitrite, and ammonia), pigments (e.g. chlorophyll, phycoerythrin), glucosamides and cell-wall materials, which are different from those in fish meal and might be poorly utilized by fish (Li et al., 2011, Safi et al., 2013, Templeton and Laurens, 2015). Some microalgae have the ability to accumulate large amounts of lipids (Araujo et al., 2011). The cultivation conditions of photoautotrophic microalgae such as *Nannochloropsis* sp. grown in outdoor ponds or photobioreactors can be manipulated to produce high levels of EPA (Borowitzka, 2013). Although Thraustochytrids *Schizochytrium* sp. is not an alga, it is referred to as heterotrophic 'microalgae' and the products are commonly marketed as being derived from microalgae. These heterotrophic, fungus-like Stramenopiles may contain 55-75% lipid in the dry matter and up to 50% of total fatty acids in this organism is DHA (Leyland et al., 2017). Microalgae also contain vitamins (e.g. a-tocopherol and ascorbic acid), minerals, sterols and other biomolecules such as carotenoids (e.g. astaxanthin, lutein, beta-carotene, fucoxanthin), minerals, phycobiliprotein, peptides, phenolic compounds, beta-1,3-glucan and sulfated polysaccharide (Fabregas and Herrero, 1986, Buono et al., 2014, Yaakob et al., 2014, Liu, 2017).

Table 3. Protein and lipid content (% as is) of different microalgae species and other plant ingredients commonly used in aquafeeds

Microalgae	Moisture	Protein	Lipid	References
Nannochloropsis oceanica	5.1	45.3	8	Skrede et al. (2011)
Nannochloropsis	9.1	29	51.3	Ju et al. (2009)
Nannochloropsis gaditana	3	52.5	15.5	Teuling et al. (2017)
Phaeodactylum tricornutum	3	47.5	7.2	Skrede et al. (2011)
Isochrysis galbana	11.2	17.8	14.4	Skrede et al. (2011)
Nannochloropsis + Isochrysis	9.2	42.1	18.2	Walker and Berlinsky (2011)
Scenedesmus dimorphus	5.1	40.7	8.1	Teuling et al. (2017)
Nanofrustulum sp. (defatted)	3.15	11.9	3.1	Kiron et al. (2012)
Thalassiosira weissflogii	15.2	18.3	12.9	Ju et al. (2009)
Tetraselmis sp.	10.8	27.9	3.8	Kiron et al. (2012)
Tetraselmis suecica	5.9	45.8	7.5	Cardinaletti et al. (2018)
Tisochrysis lutea	10	41.7	23.4	Cardinaletti et al. (2018)
Spirulina sp.*	10	44	-	Burr et al. (2011)
Spirulina sp.*	17.8	61.3	5.5	Sarker et al. (2016)
Spirulina sp.*	9.9	53.5	2.6	Safari et al. (2016)
*Arthrospira maxima**	9.6	72	5.6	Teuling et al. (2017)
Chlorella vulgaris	5.9	63.5	10.3	Teuling et al. (2017)
Chlorella sp.	5	54.5	9.4	Sarker et al. (2016)
Chlorella sp.	7.4	47.4	7	Shi et al. (2017)
Schizochytrium sp.*	3.5	11.9	54.1	Sarker et al. (2016)
Schizochytrium sp.*	1.6	13.2	61.4	Kousoulaki et al. (2016)
Haematococcus pluvialis	-	10	42	Barros et al. (2012)
Haematococcus pluvialis (defatted)	5.5	40.3	0.9	Ju et al. (2012)
Algae protein concentrate	4.4	78.3	4.1	Waghmare et al. (2016)
Rapeseed meal	7-9	34-36	2-4	ARRAINA, 2015
Cottonseed meal	7.8	42	9.4	Sauvant et al. (2004)
Peanut meal	8	44.7	9.3	Sauvant et al. (2004)

* Asterisks indicates that the listed species is not microalgae, but are referred to as 'microalgae' in the literature

Based on chemical composition, some microalgae have great potential as feed ingredients for Atlantic salmon (**Table 4**). However, only a few them are used in

commercial salmon feeds. The heterotrophic 'microalgae' *Schizochytrium*, is used as a source of DHA and the photoautotrophic microalgae *Haematococcus* is used instead of synthetic astaxanthin (Griffiths et al., 2016, Kousoulaki et al., 2016, Sprague et al., 2017). However, replacement of fish meal and plant ingredients currently used in salmon feeds with protein-rich microalgae remains a challenge. Microalgae are diverse and need to be thoroughly tested to ensure their safety as well as to understand their effects on growth, feed utilization, nutrient digestibility, animal health and product quality as well as feed quality (Glencross et al., 2007).

Rigid cell walls and complex chemical composition of the cell walls hinder intracellular nutrient accessibility, leading to a decreased nutrient digestibility and feed utilization (Teuling et al., 2017, Tibbetts et al., 2017). Cost-effective processing technologies are needed to disrupt cell walls and improve nutrient availability of microalgae to achieve widespread acceptance in commercial salmon feeds (Teuling et al., 2017, Tibbetts et al., 2017). Extrusion has been found effective in cell disruption of *Nannochloropsis oceanica* i.e. for the extraction of intracellular valuables (Wang et al., 2018). Nutritional value of microalgae may be further enhanced by using feed additives. More research is needed to evaluate the nutritional value and efficiency of microalgae as well as the potential of pre-processing and feed additives to improve the nutrient utilization by the targeted species.

Table 4. Recent studies on application of microalgae biomass as feed ingredients for Atlantic salmon

Microalgae species	% level of fish meal/fish oil in control feed	% level of fish meal/fish oil/microalgae in experimental feeds	Initial weight, feeding period, water temperature	Effects of microalgae	Authors
Nanofrustulum sp.	28/8.7	26.6/8.7.8.7, 25.2/8.7/17.4	173 g 12 weeks 8 °C	Growth, feed utilization and whole body composition revealed no significant differences	Kiron et al. (2012)
Tetraselmis sp.	28/8.7	26.6/8.7/3.7, 25.2/8.7/7.4	173 g 12 weeks 8 °C	Growth, feed utilization and whole body composition revealed no significant differences	Kiron et al., 2012)
Schizochytrium sp.*	24.8/15.3	24.8/14.8/1, 0/12.2/6, 24.8/0/15	213 g 12 weeks 10.2 °C	No signs of toxicity, stress, inflammation or other negative effects	Kousoulaki et al. (2015)
Schizochytrium sp.*	27/27	26/0/5.5 26/0/11	1534 g 19 weeks 6.5-13.8 °C	No effect on overall weight gain, but lower growth rate and higher FCR (11%)	Sprague et al. (2015)
Desmodesmus sp.	69/13.5	60/12.5/10, 51/11.5/20	167 g 10 weeks 7.6 °C	No negative effects on nutrient digestibility (protein, lipid), feed utilization, growth, whole body composition and gut health	Kiron et al. (2016)
Schizochytrium sp.*	15/6.6	15/3.8/2.5, 15/10.1/5 15/17.2/5	400 g 12 weeks 8.8 °C	Growth, FCR, protein digestibility did differ, but lipid digestibility reduced; retention efficiency (EPA+DHA) improved	Kousoulaki et al. (2016)
Phaeodactylum tricornutum	53.6/20	50.6/20/3, 47.6/20/6	325 g 82 days 7.9 °C	No negative effects on nutrient digestibility, feed utilization, growth and whole body composition (protein, lipid and ash)	Sørensen et al. (2016)

*Asterisks indicates that the listed species is not microalgae, but are referred to as 'microalgae' in the literature

2 Objectives

The general objective of this PhD thesis was to investigate the potential of microalgae as feed ingredients for Atlantic salmon. The main response variables were nutrient digestibility, growth performance, feed utilization, and chemical body composition and intestinal health of the fish (**Figure 4**). The specific objectives addressed were:

1. To determine the apparent digestibility coefficients of microalgae when fed to Atlantic salmon (**Paper I**).

2. To determine the effects of microalgae in extruded fish-meal-based diets or commercial-like plant-based diets for Atlantic salmon on nutrient digestibility, growth performance, feed utilization, chemical composition and intestinal health of the fish (**Paper II, III** and **IV**).

3. To investigate the efficacy of feed additives and/or thermo-mechanical treatment of microalgae (extrusion, double extrusion or. cold pelleting); whether the process or/and the additive is/are efficient in improving nutrient utilization of microalgae incorporated diets for Atlantic salmon (**Paper I, IV**).

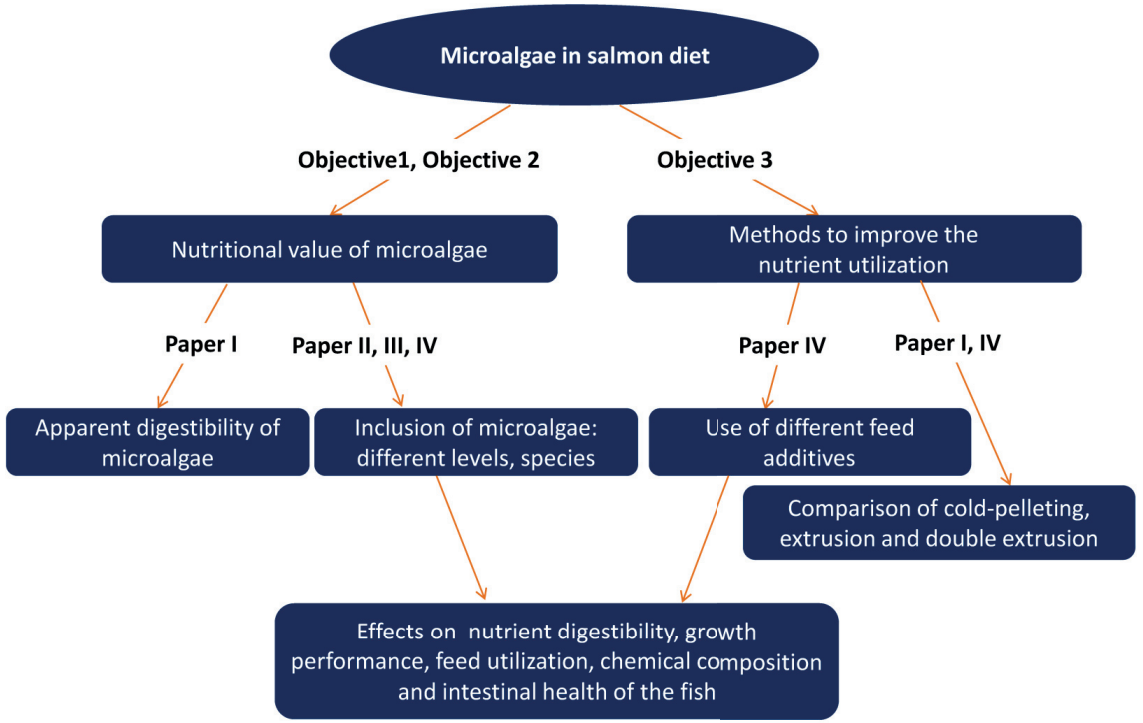


Figure 4. The main structure and topics discussed in this thesis

3 Results and discussion

3.1 Nutritional value and efficacy of microalgae

3.1.1 Microalgae varieties

Several varieties of microalgae (e.g. *Chlorella*, *Spirulina*, *Nannochloropsis*, *Tisochrysis*, *Tetraselmis*, *Scenedesmus*, *Schizochytrium, Haematococcus pluvialis, Phaeodactylum tricornutum*) have been explored for aquafeed applications (Shah et al., 2018). The studies presented in **Papers I-IV** have investigated nutrient digestibility and utilization of the microalgae *Nannochloropsis*, *Desmodesmus* and *Scenedesmus*, at different inclusion levels in salmon diets (**Table 5**). Microalgae can be produced phototrophically, heterotrophically or even mixotrophically (Huntley et al., 2015). In this thesis the main focus has been on photosynthetic microalgae which make use of light energy, CO_2 and dissolved ions in the water to synthesize complex molecules that constitute their biomass. *Nannochloropsis oceanica* and *Desmodesmus* sp. described in **Papers I** and **II** were cultivated at the facilities of Cellana (Kona Pilot Facility, Kailua-Kona, Hawaii, USA) using two-stage cultivation system (i.e. combination of photobioreactors and ponds). They were harvested, spray-dried and made available as defatted products after lipid extraction using solvents. The microalgae *Scenedesmus* sp. studied in **Paper III** and *Nannochloropsis oceanica* in **Paper IV** were produced in closed photobioreactor and obtained as spray-dried powder from Allmicroalgae Natural Products (Lisbon, Portugal).

Table 5. Inclusion levels (%) of microalgae and fish meal in the study diets in **Paper I-IV**

	Paper I	**Paper II**	**Paper III**	**Paper IV**
	6 Diets	3 Diets	3 Diets	4 Diets
Fish meal	75, 53, 53; 70, 49, 49	69, 59, 49	10, 5, 2.5	15, 7.5, 7.5, 7.5
Plant protein ingredients	0, 0, 0; 5, 3.5, 3.5	5, 5, 5	51, 50, 47	44, 46, 46, 46
Nannochloropsis oceanica (defatted)	30	10, 20		
Desmodesmus sp. (defatted)	30			
Scenedesmus sp. (whole algae)			10, 20	
Nannochloropsis oceanica (whole algae)				10

The chemical composition of microalgae used in **Paper I-IV** is shown in **Table 6**. The nutritional profiles of microalgae vary considerably; the values depend on microalgae species, strains, growth conditions and processing. The protein content of the algal biomass was calculated using nitrogen-to-protein conversion factors of 6.25 and 4.78, as employed by Tibbetts et al. (2015). The conversion factor 6.25 may overestimate the protein content because the total nitrogen in algae includes non-protein sources such as nucleic acids, inorganic nitrogen (nitrate, nitrite, and ammonia), pigments (chlorophyll, phycoerythrin), glucosamides and N-containing components in the cell walls (Safi et al., 2013, Templeton and Laurens, 2015, Li et al., 2011). The chosen factor must be based on the algal species, growth stage and lipid extraction methods (Safi et al., 2013, Tibbetts et al., 2015). Lourenço et al. (2004) studied 12 marine microalgae species and suggested the N conversion factor of 4.78. The crude protein content of microalgae described in this thesis ranged from 26.9-45.7% (N × 6.25) or 20.6-35.0% (N × 4.78). Protein content (N × 6.25) of the microalgae is comparable to that of the currently employed plant ingredients (e.g. corn gluten meal, soybean meal). However, the crude protein contents of the microalgae were lower compared to fish meal (**Table 2**). One main reason for the lower protein content in microalgae tested could be the higher ash and/or carbohydrate (e.g. fiber) content. The ash contents in fish meal (9-12%) and freshwater green algae *Scenedesmus* sp. (8.3%) were the lowest and that

in marine algae *Nannochloropsis oceanica* (23.3%) was the highest. Marine algae usually have higher content of salt adsorbed to the cell surface and salt is also present in the intercellular water compared to freshwater microalgae (Zhu and Lee, 1997). The amino acid profile of the microalgae (used in **Paper I-IV)** is comparable to that of fish meal, the exception being those of lysine and methionine (**Table 6**). Crude lipid content was in the range 1.0–14.2%. The microalgae *Nannochloropsis* sp. and *Desmodesmus* sp. are able to accumulate large amount of lipids and are thus chosen for biofuel production (Scott et al., 2010, Mata et al., 2010). The *Nannochloropsis* sp. and *Desmodesmus* sp. biomass were obtained after lipid extraction, and this explains the low lipid content of the microalgae in **Paper I** and **II**. The microalgae used in **Paper III** (*Scenedesmus* sp.) and **Paper IV** (*Nannochloropsis oceanica*) were incorporated as whole algae, without lipid extraction. The lipid content of microalgae *Scenedesmus* sp. (9.1%) and *Nannochloropsis oceanica* (14.2%) was comparable to fish meal (10-12%) (**Table 6**).

Table 6. Chemical composition of microalgae studied in **Paper I-IV**

	Nannochloropsis oceanica (defatted)	*Desmodesmus* sp. (defatted)	*Scenedesmus* sp. (Whole algae)	*Nannochloropsis oceanica* (Whole algae)	Fish meal LT 70[1]
Moisture	2.2	11.4	5.6	3.3	7 - 10
CP × 6.25	42.9	26.9	45.7	36.4	69 - 72
CP × 4.78	32.8	20.6	35.0	27.8	
Lipid	4.2	1.0	9.1	14.2	10 - 12
Ash	23.3	16.0	8.3	22.6	9 - 12
Fiber			15.8	9.3	
Energy	18.8	16.6	15.0	17.4	20.0 - 20.5
Amino acids g 16 g N^{-1}					
Lysine	4.7	5.5		5.7	7.5
Methionine	1.5	1.9		2.4	2.6
Arginine	4.9	5.3		5.8	5.6
Histidine	1.6	1.7		1.7	1.8
Isoleucine	3.8	3.7		4.5	3.8
Leucine	7.5	7.7		8.1	6.7
Phenylalanine	4.5	4.7		4.0	3.6
Threonine	4.5	4.6		3.6	3.9
Tryptophan	1.5	1.7		-	1.0
Valine	5.3	5.3		5.4	4.2

1. ARRAINA, 2015

3.1.2 Experimental feed production

The experimental feeds used in the studies described in this thesis were cold-pelleted (**Paper I**) or extruded (**Papers I, II, III** and **IV**) (**Table 7**). The cold-pelleted feeds in **Paper I** were produced at the feed laboratory of Nord University, Bodø, Norway. The extruded feeds in **Paper I** and **Paper II** were produced at the Center for Feed Technology (ForTek), Norwegian University of Life Sciences, Ås, Norway. The feeds were processed using a twin-screw cooking extruder (BCTG 62/20 D, Bühler, Uzwil, Switzerland). The feeds in **Paper III** and **Paper IV** were produced at SPAROS, Lda using a pilot-scale twin-screw extruder (Clextral BC45, Clextral, France). Feed manufacturing technology can affect the utilization of feeds by farmed fish (Sørensen, 2012, Glencross

et al., 2011). Feeding gilthead sea bream (*Sparus aurata*) with extruded feeds compared to pelleted feeds, improved growth and digestibility of energy and starch (Venou et al., 2009). Extrusion significantly improved the energy digestibility of the diets fed to rainbow trout (*Oncorhynchus mykiss*) compared to diets prepared using screw-press pelleting technology (Glencross et al., 2011). **Paper I** examined the digestibility of diets and microalgae; when the diets were manufactured using either cold-pelleting process or extrusion technology. The experimental feeds were formulated for estimating the nutrient digestibilities, and the values were determined following the principle of Cho and Slinger (1979). The main ingredients used for the reference feed were LT fish meal (Norsildmel AS, Bergen, Norway) and fish oil. Two test feeds were formulated by blending (% w/w basis) either *Nannochloropsis* sp. or *Desmodesmus* sp. meal with the basal control feed at a ratio of 70:30. The experimental feeds presented in **Paper II** were prepared to investigate the effect of incorporation level of *Nannochloropsis oceanica* in a fish meal–fish oil based diet on nutrients digestibility, growth, feed utilization, body composition and intestinal health of salmon. The control feed (1C) was based on LT fish meal (Norsildmel AS, Bergen, Norway), while in the test feeds algal biomass *Nannochloropsis oceanica* replaced 10% (1L) and 20% (1H) of fish meal. In **Paper III**, the experimental feeds were designed to test the potential of microalgae to replace fish meal in low fish meal diets. The main protein sources of the experimental diets were soy protein concentrate, pea protein concentrate and potato concentrate. The experimental diets were formulated to contain 1) 10% fish meal and no microalgae (CT), 2) 5% fish meal and 10% microalgae (*Scenedesmus* sp.) (SCE10) and 3) 2.5% fish meal and 20% microalgae (SCE20). The aim of **Paper IV** was to investigate effects of thermo-mechanical pre-processing of microalgae (extrusion) without or with feed additives; to understand their ability to improve nutrient utilization of microalgae by Atlantic salmon. Four plant-based experimental feeds (mixture of soy protein concentrate, pea protein concentrate, wheat gluten and faba beans) were formulated; a diet with 15% LT fish meal and no microalgae (CO), another diet containing 7.5% LT fish meal and 10% of the microalga *Nannochloropsis oceanica* (NC), and two diets containing 7.5% LT fish meal, 10% of the

microalgae *Nannochloropsis oceanica* and 2 commercial feed additives (0.06% Digestarom PEP MGE150 (Biomin GmbH, Getzersdorf, Austria) (ND), or 1% ZEOFeed (ZEOCEM AS, Bystré, Slovakia) (NZ)).

Table 7. Codes of experimental feeds used in **Paper I-IV**

Paper	Feed codes
I	Cold-pelleted: Fish meal based control diet (PC), *Nannochloropsis* sp. 30% (PN), *Desmodesmus* sp. 30% (PD); Extruded: Fish meal based control diet (EC), *Nannochloropsis* sp. 30% (EN), *Desmodesmus* sp. 30% (ED)
II	Fish meal based control diet (1C), *Nannochloropsis oceanica* 10% (1L) and 20% diet (1H)
III	Plant based control diet (CT), *Scenedesmus* sp. 10% (SCE10) and 20% diet (SCE20)
IV	Plant based control diet (CO), *Nannochloropsis oceanica* 10% (NC), *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% (ND), and *Nannochloropsis oceanica* 10% + ZEOFeed 1% (NZ)

3.1.3 Nutrient digestibility of experimental feeds

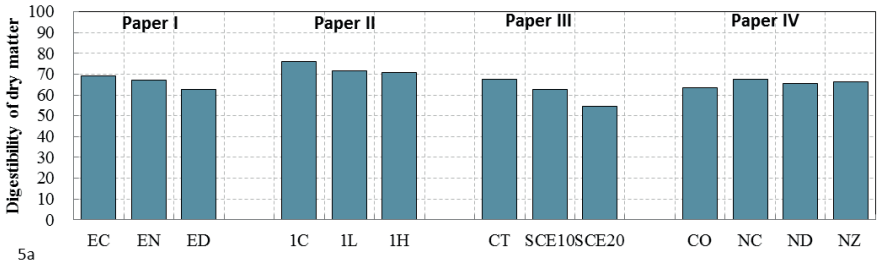
Digestibility of dry matter (70-77%), protein (86-88%), lipid (93%) and energy (83-86%) in the fish meal-based reference feed in **Paper I** and **II** is in line with those reported in other studies of similar-sized Atlantic salmon (Hatlen et al., 2012, Albrektsen et al., 2018). The digestibility values of dry matter (63-68%), protein (82-88%), lipid (91-94%) in plant-based control feed in **Paper III** and **IV** are generally lower or similar compared to fish meal-based feed in **Paper I** and **II** (**Figure 5a-d**). The inclusion of microalgae caused a decrease in digestibility of dry matter (55-72%) (**Paper I, II, III**) and protein (69-85%) (**Paper I, II, III**), and lipid digestibility (79-92%) (**Paper II, III, IV**) compared to the control feeds. These results agree with findings in Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) fed diets containing either 30% *Nannochloropsis gaditana* or 30% *Scenedesmus dimorphus*, and studies of mink (*Mustela vison*) fed diets containing 6-24% *Nannochloropsis oceanica* (Teuling et al., 2017, Skrede et al., 2011). Digestibilty of ash was either reduced (**Paper III**) or

improved (**Paper I, II, IV**) with incorporation of mciroalgae compared to control feed (**Figure 5**). The increased digestibility of ash was also observed in Nile tilapia and African catfish fed *Nannochloropsis gaditana* incorporated feeds (Teuling et al., 2017). Overall, the digestibility of protein was more severely reduced in **Paper III** with incorporation of 10/20% *Scenedesmus* sp. compared to results reported for 10%/20% incorporation of *Desmodesmus* sp. (Kiron et al., 2016), 10%/20% *Nannochloropsis oceanica* (**Paper II, IV**) or 30% *Nannochloropsis oceanica* (**Paper I**) (**Figure 5b**). Lipid digestibility was also slightly lower in **Paper III** compared to **Paper II** and **Paper IV**, as well as values reported by Kiron et al. (2016), but not to the same extent as that of protein. These findings suggest that nutrient digestibility of the three microalgae sources, in particular digestibility of protein, differs. Such variations in digestibility of protein, lipid and energy among different microalgae species were reported by other studies (Teuling et al., 2017, Skrede et al., 2011).

The reduction in dry matter, protein and lipid digestibility of the microalgae feeds compared to the control feeds is most likely explained by chemical composition of microalgae, the rigid cell wall and increased concentration of indigestible cell wall components in the biomass (Glencross et al., 2012). Microalgae have complex carbohydrate such as cellulose, pectins and hemicelluloses (Scholz et al., 2014, Baudelet et al., 2017). Carnivorous fishes do not have the capacity to digest non-starch polysaccharides (NSPs) and thus they act as non-nutritive filler in the feed (Krogdahl et al., 2005, Irvin et al., 2016). Besides, studies have shown that NSPs have negative effects on lipid and energy digestibility (Espinal-Ruiz et al., 2014, Irvin et al., 2016, Leenhouwers et al., 2006, Refstie et al., 1999, Aslaksen et al., 2007). Aslaksen et al. (2007) and Lekva et al. (2010) found a linear reduction in lipid digestibility with increased cellulose level (0-18%) in the feeds of Atlantic salmon and Atlantic cod (*Gadus morhua* L.). The non-starch polysaccharides from cereals and legumes disturb fat micelle formation and increase viscosity of gut contents leading to a reduced gastric emptying rate, which may affect fat digestion in farmed fish (Espinal-Ruiz et al., 2014, Refstie et al., 1999, Leenhouwers et al., 2006, Overland et al., 2009, Sinha et al., 2011). However, Kraugerud et al. (2007) found that dietary inclusion of cellulose or soy-NSP

(10%) did not have any negative effect on digestibility of lipid. Cellulose inclusion up to 15% did not influence the digestibility of lipid in freshwater rainbow trout as well (Hansen 2007). The low lipid digestibility observed in the present thesis could be attributed to the lower digestibility of the dietary saturated fatty acids (SFAs), as already reported in other studies employing Atlantic salmon (Kousoulaki et al., 2016, Kousoulaki et al., 2015). At low temperature, Atlantic salmon has only limited capacity to efficiently digest SFAs in the diet (Ng et al., 2004, Menoyo et al., 2003, Menoyo et al., 2007). The lower lipid digestibility observed in **Paper III** could be linked to increased levels of dietary SFAs in SCE10 (10% *Scenedesmus*) and SCE20 (20% *Scenedesmus*) diets. Such an explanation does not hold good for the lower lipid digestibility observed in **Paper IV** since the SFAs in the different diets were similar. Lipid digestibility is also dependent on the position of the fatty acids on the triacylglycerol (TAG) (Nielsen et al., 2005, Mu and Høy, 2004). However, the position of the SFAs in the tested microalgal TAG are unknown, and the effect of positioning on lipid digestibility should be investigated in future studies.

The effect of NSPs on protein digestibility was reported to be marginal (Hansen and Storebakken, 2007, Glencross et al., 2012, Irvin et al., 2016). The results found in the thesis are in accordance with these previous studies. More likely the lower digestibility of protein in microalgae diets in the current studies is explained by non-protein nitrogen in microalgae. Microalgae may accumulate high concentrations of non-protein nitrogen, which is different from fish meal and might be poorly used by fish (Safi et al., 2013, Templeton and Laurens, 2015, Li et al., 2011). If the protein content in feed is reported as N x 6.25, the indigestible non-protein nitrogen from microalgae is included in the calculations, resulting in underestimation of digestible protein of the feeds. The underlying reason for the variations of nutrient digestibility in microalgae and microalgae feeds remains unknown and warrants further investigation.



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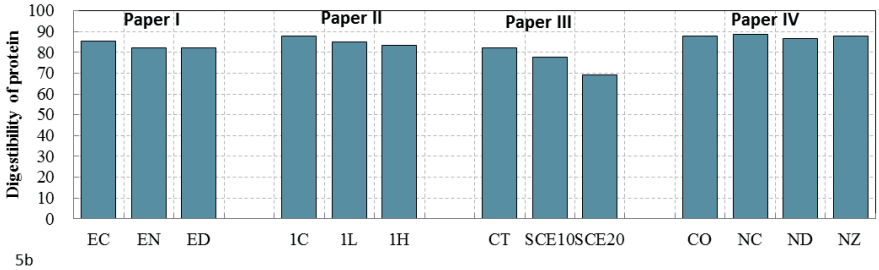

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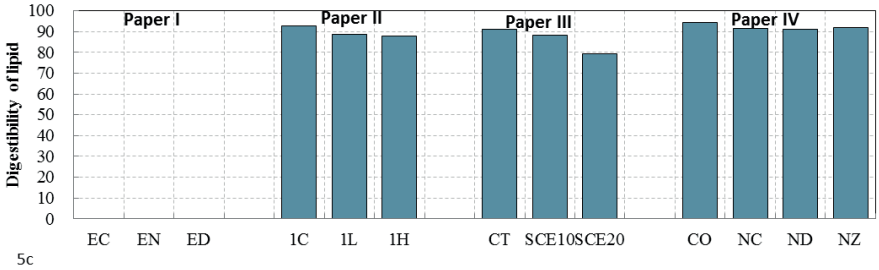

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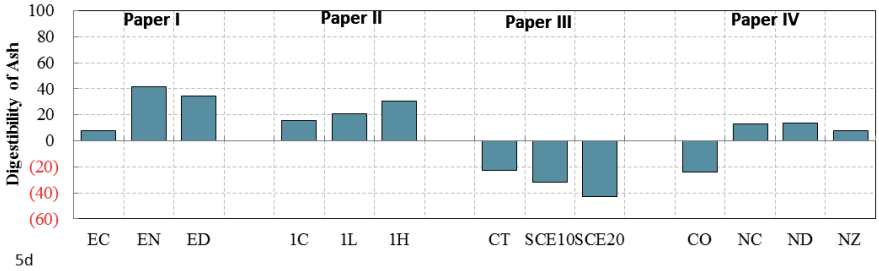


5d

Figure 5a, 5b, 5c, 5d. Apparent digestibility coefficients (%) of dry matter, lipid, protein and ash in Atlantic salmon fed the experimental diets in **Paper I-IV**

Note:

Paper I: Fish meal based control diet (EC), *Nannochloropsis* sp. 30% (EN), *Desmodesmus* sp. 30% (ED);
Paper II: Fish meal based control diet (1C), *Nannochloropsis oceanica* 10% (1L) and 20% diet (1H);
Paper III: Plant based control diet (CT), *Scenedesmus* sp. 10% (SCE10) and 20% diet (SCE20);
Paper IV: Plant based control diet (CO), *Nannochloropsis oceanica* 10% (NC), *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% (ND), and *Nannochloropsis oceanica* 10% + ZEOFeed 1% (NZ)

3.1.4 Effect of microalgae on feed intake, growth and feed utilization

Growth and feed intake were recorded in the **Papers** except for **Paper I** as the main purpose in the latter was to evaluate the digestibility of different microalgae species in the fish. Atlantic salmon in **Paper II-IV** had good growth performance during the course of the experimental period. Mortality was not recorded and the final weights of fish were approximately twice that of their initial weights (**Paper II-IV**, **Table 8**). The growth in **Paper II-IV**, however, varied depending on the microalgae and inclusion level. AquaGen strain employed in the studies had almost similar initial weight in **Paper II-IV** (**Table 8**). The fish were kept in same facilities and the feeding experiments were conducted in the same flow-through system at the Research Station, Nord University, Bodø, Norway.

Microalgae may affect palatibility of the feeds. Higher, equal or low feed intake has been reported in investigations with fish species such as Atlantic cod, European sea bass (*Dicentrarchus labrax*) and gilthead sea bream, when fed diets containing microalgae at different inclusion levels (Palmegiano et al., 2009, Walker and Berlinsky, 2011, Tibaldi et al., 2015, Vizcaíno et al., 2014). Atlantic salmon offered feeds containing 12% dried whole cells microalgae *Phaeodactylum tricornutum* had reduced feed intake (Sørensen et al., 2016). On the other hand, salmon in the studies described in this thesis readily accepted the microalgae incorporated feeds and we did not observe any negative effects on feed intake. As indicated in **Paper II**, partial substitution of LT fish meal with *Nannochloropsis oceanica* increased the feed intake. The higher feed intake of 1H group could be considered as compensation for the slightly lower digestible lipid and energy content in the feeds (**Table 9**). In experiments with Atlantic salmon and rainbow trout it has been shown that fishes compensate for

the lower feed energy by increasing their feed intake (Bendiksen et al., 2002, Boujard et al., 2004). When Atlantic salmon were fed 10 and 20% *Scenedesmus* sp. (**Paper III**) or 10% pre-extruded *Nannochloropsis oceanica* (**Paper IV**), no differences in feed intake were observed among dietary groups. Taken together, we suggest that microalgae incorporated diets are highly palatable. These findings are in line with the results of Kiron et al. (2012) and Sprague et al. (2015) who reported no effect on feed intake when Atlantic salmon were fed *Nanofrustulum* sp. or *Tetraselmis* sp. at 10% inclusion rate, or *Schizochytrium* sp. at 11% inclusion level.

Despite the relatively low levels of fish meal in diets (2.5-15%) employed in the studies in **Paper III** and **IV**, the observed growth was within the normal range of similar-sized salmon. The growth results (SGR, 0.74-1.12 % day^{-1}) of our studies were comparable to growth estimates (0.7-1.0 % day^{-1}) from tables given by Austreng et al. (1987), when considering both fish size (100-600 g) and water temperature (6-8 °C). The SGR values obtained in the present thesis were slightly higher than those reported earlier for fish of similar size and water temperature (Kiron et al., 2012, Hatlen et al., 2012). The growth rate was lower compared to the results of Albrektsen et al. (2018) (IBW 213 g, SGR 1.4 % day^{-1}, TGC 3.7) and Kousoulaki et al. (2015) (IBW 213 g, SGR 1.6 % day^{-1}, TGC 4.0) grown in higher water temperature (9.1-10 °C). Feeding Atlantic salmon with 10 or 20% *Nannochloropsis oceanica* did not affect the final body weight, weight gain, specific growth rate, and thermal growth coefficient negatively (**Paper II**). The growth rate in salmon was sustained by higher feed intake. However, we noted an impaired feed utilization, i.e. a higher feed conversion ratio, and lower retention of lipid and energy with increasing *Nannochloropsis oceanica* in the feed (**Paper II**). The growth performance (SGR) in salmon fed control (CT) and SCE10 diet (10% *Scenedesmus* sp.) was slightly higher (**Paper III)** compared to salmon of comparable size fed fish meal-based diets in **Paper II** and **Paper IV** (**Figure 6**). The optimal DP/DE ratio for Atlantic salmon to achieve its maximal growth i.e. up to 2.5 kg appears to be 19-21 g MJ^{-1} (Einen and Roem, 1997, Refstie et al., 2001). The DP/DE ratio in CT and SCE10 diet (21 g MJ^{-1}) were closer to this optimal ratio compared to the ratios of diets in **Paper II** and **Paper IV**, which may partly explain the higher SGR found in **Paper III**

(**Table 9**). The higher DP/DE ratio (23-24 g MJ^{-1}), found in **Paper II,** may indicate inadequate energy and the fish may have to catabolize protein for maintenance and growth (Hung et al., 2017, Einen and Roem, 1997). The findings in **Paper III** also suggest that fish meal incorporation can be reduced to 5% when diets are balanced for amino acids and other essential nutrients. However, inclusion of *Scenedesmus* sp. up to 20% (Diet SCE 20) could not sustain the growth of the fish at lowest fish meal inclusion (2.5%). The fish fed SCE20 had significantly lower final body weight, weight gain, specific growth rate, thermal growth coefficient and higher feed conversion ratio compared to the fish fed control diet in **Paper III**. It is assumed that some water soluble compounds present in fish meal that are important for feed intake and growth were insufficient in high plant-low fish meal diets (Kousoulaki et al., 2013, Kousoulaki et al., 2018). No differences in final body weight, weight gain, specific growth rate, thermal growth coefficient and feed conversion ratio were observed between the low microalgae group (7.5% fish meal/10% *Nannochloropsis* sp.) and control group (15% fish meal) in **Paper IV**. These findings were generally in accordance with the results obtained by Kiron et al. (2012). They reported no effect on growth and feed conversion ratio when Atlantic salmon were fed *Nanofrustulum* sp. or *Tetraselmis* sp. at 10% inclusion rate. But several studies have also observed negative effects on growth and/or feed conversion ratio when Atlantic salmon were fed diets with *Desmodesmus* sp. (10/20% inclusion level), *Schyzochrytrium* sp. (11% inclusion level), or *Phaedactylum tricornutum* (12%) (Sprague et al., 2015, Kiron et al., 2016, Sørensen et al., 2016). Based on available literature, we cannot readily explain the different results on feed intake and growth of fish fed diets containing different microalgae. The responses depend on the fish species, fish size, microalgae species and feed formulation, digestibility as well as nutritional composition of diets (Glencross et al., 2007, Jobling, 2016).

Table 8. Growth and feed utilization of Atlantic salmon studied in **Paper II-IV**

	Paper II			**Paper III**			**Paper IV**			
	1C	1L	1H	CT	SCE10	SCE20	CO	NC	ND	NZ
IBW	214.5	213.8	218.0	228.4	230.8	228.1	227.9	228.5	225.3	227.3
FBW	429.0	420.2	407.8	473.6	451.0	416.7	422.8	415.1	417.3	423.3
WG	100.2	96.3	86.9	107.1	95.4	82.6	85.4	81.6	86.2	85.2
TGC	2.6	2.5	2.4	3.5	3.2	2.8	2.7	2.6	2.7	2.8
FCR	0.81	0.86	1.00	0.76	0.88	0.97	0.90	0.95	0.89	0.89
PER	2.2	2.2	2.0	2.7	2.4	2.1	2.5	2.4	2.5	2.5
REP.	6	6	6	6	6	6	5	5	5	5
T		7.1			7.4				7.5	
D		84			65				68	

IBW, Initial body weight, g; FBW, Final body weight, g; WG, Weight gain; TGC, Thermal growth coefficient; FCR, Feed conversion ratio; PER, Protein efficiency ratio; REP., Replications for each dietary group; T, Temperature, °C; D, Feeding days

Note:

Paper II: Fish meal based control diet (1C), *Nannochloropsis oceanica* 10% (1L) and 20% diet (1H);

Paper III: Plant based control diet (CT), *Scenedesmus* sp. 10% (SCE10) and 20% diet (SCE20);

Paper IV: Plant based control diet (CO), *Nannochloropsis oceanica* 10% (NC), *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% (ND), and *Nannochloropsis oceanica* 10% + ZEOFeed 1% (NZ)

Table 9. Proximate composition of the experimental diets on a dry matter basis (%) used in **Paper II-IV**

	Paper II			**Paper III**			**Paper IV***			
	1C	1L	1H	CT	SCE10	SCE20	CO	NC	ND	NZ
Protein	56.4	53.5	50.1	49.2	49.3	48.9	44.4	43.1	42.3	42.9
Lipid	21.1	20.6	20.0	21.1	22.5	21.0	29.5	28.2	30.2	29.5
Ash	11.4	12.3	13.2	5.8	5.6	5.9	8.9	8.9	9.0	9.6
Energy	23.6	23.6	23.1	24.5	24.8	24.9	23.8	23.0	23.5	23.3
DP	49.6	45.5	41.8	40.5	38.3	33.8	39.0	38.1	36.6	37.7
DL	19.5	18.3	17.6	19.2	19.8	16.7	27.8	25.7	27.5	27.1
DE	20.3	19.2	18.3	19.0	18.0	15.9	21.0	20.0	20.3	20.4
DP/DE	24.5	23.6	22.8	21.3	21.2	21.3	18.5	19.1	18.0	18.5

DP, Digestible protein; DL, Digestible lipid; DE, Digestible energy (MJ kg^{-1}); DP/DE, g MJ^{-1}

* The gross energy content of feeds in **Paper IV** was not analyzed but calculated based on 23.7, 39.5 and 17.2 MJ kg^{-1} for protein, lipids and starch, respectively. The digestible energy was calculated using the digestibility of protein and lipid found in **Paper IV**, while the digestibility of starch was set to 50% (Einen and Roem, 1997, Aslaksen et al., 2007).

Note:

Paper II: Fish meal based control diet (1C), *Nannochloropsis oceanica* 10% (1L) and 20% diet (1H);

Paper III: Plant based control diet (CT), *Scenedesmus* sp. 10% (SCE10) and 20% diet (SCE20);

Paper IV: Plant based control diet (CO), *Nannochloropsis oceanica* 10% (NC), *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% (ND), and *Nannochloropsis oceanica* 10% + ZEOFeed 1% (NZ)

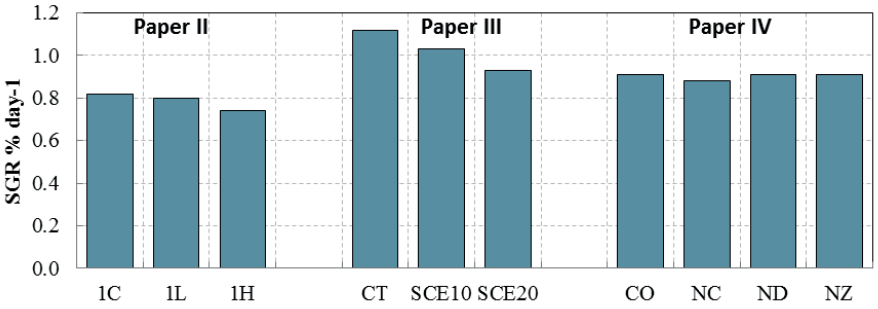


Figure 6. Specific growth rate (SGR, % day^{-1}) of Atlantic salmon fed experimental diets in **Paper II-IV**

Note:

Paper II: Fish meal based control diet (1C), *Nannochloropsis oceanica* 10% (1L) and 20% diet (1H);

Paper III: Plant based control diet (CT), *Scenedesmus* sp. 10% (SCE10) and 20% diet (SCE20);

Paper IV: Plant based control diet (CO), *Nannochloropsis oceanica* 10% (NC), *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% (ND), and *Nannochloropsis oceanica* 10% + ZEOFeed 1% (NZ)

3.1.5 Whole body proximate composition

As presented in **Paper II** and **Paper IV**, the whole body proximate composition of Atlantic salmon was not affected when fish were fed microalgae feeds. These results are in line with other experiments with Atlantic salmon fed either *Desmodesmus* sp., *Nanofrustulum* sp. or *Tetraselmis* sp (Kiron et al., 2012, Kiron et al., 2016). In contrast, whole body lipid content was significantly lower and protein content higher in fish fed 20% *Scenedesmus* sp. (SCE20) compared with the other dietary groups **in Paper III**. The lower lipid content in fish fed SCE20 can be explained by lower availability of energy from *Scenedesmus* sp. compared to fish meal (Einen and Roem, 1997, Qiu et al., 2018). Whole body protein (42-44%) of fish in **Paper IV** was lower and the lipid content (28-30%) was higher than values (protein 55-57%, lipid 36-37%) found in **Paper II** and **III** (**Table 9**). The ash content of the fish in **Paper IV** was in line with the values found in **Paper II** and **III**. The proximate composition can vary with life stages of the fish and is also influenced by endogenous factors such as genetics, size and sex, as well as

exogenous factors such as feed composition, feeding frequency and environment (Shearer, 1994). The differences in the present study might be due to the variations in dietary protein (42-56%) and lipid values (20-30%) since fish, as mentioned earlier, had same genetic background, similar fish size (**Table 8**), same feeding regime and were reared in the same flow-through system (Einen and Roem, 1997, Dessen et al., 2017) (**Table 9 & 10**).

Table 10. Proximate composition of the whole fish on a dry matter basis (%) in **Paper II-IV**

	Paper II			**Paper III**			**Paper IV**			
	1C	1L	1H	CT	SCE10	SCE20	CO	NC	ND	NZ
Protein	55.5	55.6	56.3	55.6	54.6	56.6	50.3	50.7	50.7	50.7
Lipid	36.4	36.3	35.8	37.3	37.4	35.7	41.9	42.2	39.3	39.1
Ash	6.0	6.3	6.4	5.6	5.8	6.3	5.4	5.8	5.6	5.5

Note:

Paper II: Fish meal based control diet (1C), *Nannochloropsis oceanica* 10% (1L) and 20% diet (1H);

Paper III: Plant based control diet (CT), *Scenedesmus* sp. 10% (SCE10) and 20% diet (SCE20);

Paper IV: Plant based control diet (CO), *Nannochloropsis oceanica* 10% (NC), *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% (ND), and *Nannochloropsis oceanica* 10% + ZEOFeed 1% (NZ)

3.1.6 Fatty acid composition

Microalgae are primary producers of fatty acids in the marine environment. Retention of PUFAs in Atlantic salmon fed the DHA rich *Schizochytrium* sp. (Kousoulaki et al., 2016) or EPA rich *Phaeodactylum tricornutum* (Sørensen et al., 2016) were studied previously. The fatty acids in the microalga *Scenedesmus* sp. used in **Paper III** is dominated by C16:0, C16:1, linoleic acid (18:2n-6, LA) and α-linolenic acid (C18:3n-3, ALA) (Custódio et al., 2014), while *Nannochloropsis oceanica* in **Paper IV** contains mostly C16:0, C16:1, LA and EPA (Patil et al., 2007). Neither *Scenedesmus* sp. nor *Nannochloropsis oceanica* is known to contain DHA. Inclusion of the microalga has lowered the DHA content in algae-incorporated diets compared to the control diet (**Table 11**). However, DHA in whole fish fed algae-incorporated diets and the control

diet were not significantly different (**Paper III** and **Paper IV**). On the other hand, the alga fed fish even showed a higher content of Σn-3 PUFAs (**Paper III**), Σn-6 PUFAs (**Paper III** and **Paper IV**) and ΣPUFAs (**Paper III** and **Paper IV**). The increased content of Σn-6 PUFAs of fish fed algal diet can be attributed to the higher content of LA in the whole body and Σn-3 PUFAs can be attributed to ALA, EPA and DHA in whole fish. The slightly elevated levels of EPA and ΣPUFAs observed in fish fed the SCE10 (**Paper III**) or NZ diets (**Paper IV**) are also noteworthy. An earlier study has revealed the ability of 1-3% zeolite (clinoptilolite) to modulate fatty acid profiles in rainbow trout (Danabas, 2011). The increased ΣPUFAs contents in whole body of Atlantic salmon fed microalgae *Scenedesmus* sp. and *Nannochloropsis oceanica* with feed additive ZEOFeed (clinoptilolite) is an important observation from nutritional point of view.

Table 11. Fatty acid composition (% of total fatty acids) of the experimental diets and the whole fish in **Paper III-IV**

	Paper III			Paper IV			
	CT	SCE10	SCE20	CO	NC	ND	NZ
Diets							
C16:0	13.0	13.6	14.2	10.2	9.9	10	9.9
C18:1n-9	36.4	37.0	36.6	39.1	39.9	40.0	40.1
C18:2n-6	14.3	14.0	15.0	14.3	14.5	14.4	14.4
C18:3n-3	4.9	4.7	6.3	6.0	6.1	6.1	6.1
EPA	3.3	3.2	4.1	5.5	5.7	5.6	5.6
DHA	9.1	8.8	7.3	4.5	4.0	4.0	4.0
Fish							
C16:0	13.8	13.6	13.4	10.8	10.8	10.7	10.5
C18:1n-9	37.4	37.0	36.9	37.3	37.3	37.4	37.6
C18:2n-6	13.9	14.5	14.3	11.8	12.1	12.1	12.2
C18:3n-3	4.5	5.2	4.9	4.2	4.3	4.3	4.3
EPA	2.9	3.6	3.2	2.8	2.9	3.0	3.0
DHA	8.1	8.2	8.6	6.8	6.6	6.6	6.6
Σn-6 PUFAs	14.3	14.9	14.6	13.9	14.2	14.2	14.3
Σn-3 PUFAs	15.6	17.0	16.8	17.2	17.1	17.1	17.3
ΣPUFAs	29.8	31.8	31.4	31.1	31.3	31.3	31.6

Note:

Paper III: Plant based control diet (CT), *Scenedesmus* sp. 10% (SCE10) and 20% diet (SCE20);

Paper IV: Plant based control diet (CO), *Nannochloropsis oceanica* 10% (NC), *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% (ND), and *Nannochloropsis oceanica* 10% + ZEOFeed 1% (NZ)

3.1.7 Intestinal health

In order to confirm the suitability of the microalgae as a feed component, effect on health should be assessed using histological and/or molecular tools. The microalgal-feeds did not alter the histomorphology of distal intestine (**Paper II**). This observation was confirmed by examining the expression of selected marker genes related to inflammation and intestinal immune system. In conclusion, there were no

signs of distal intestinal inflammation in the microalga-fed groups in this study, in accordance with our previous study using *Desmodesmus* sp. (Kiron et al., 2016). These findings, taken together, suggest that the *Nannochloropsis* did not induce inflammatory reactions in the distal intestine of the fish.

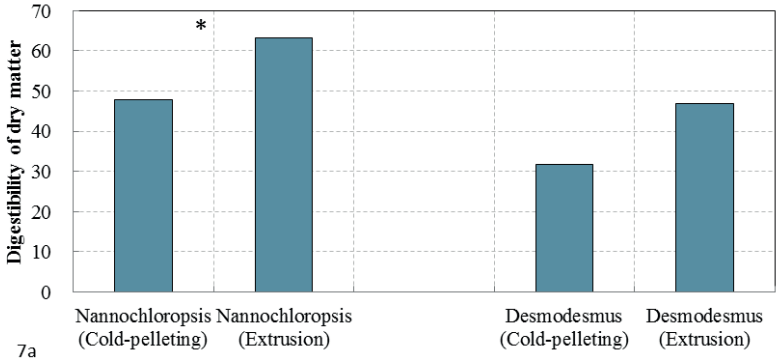
3.2 Means to improve utilization of nutrients in microalgae

3.2.1 Microalgae cell wall disruption by thermo mechanical treatment

We evaluated the digestibility of nutrients in the defatted microalgae biomass from *Nannochloropsis* sp. and *Desmodesmus* sp., employing cold-pelleted feeds and extruded feeds (**Paper I**). We observed differences in digestibility of nutrients in *Nannochloropsis* sp. and *Desmodesmus* sp. (**Figure 7a-b**). The digestibility of dry matter and protein in *Nannochloropsis* sp. were significantly higher than those of *Desmodesmus* sp. The digestibility of dry matter in *Nannochloropsis* sp. of the extruded feeds was significantly higher compared to that in the cold-pelleted feeds. We observed an increasing trend in digestibility of dry matter in *Desmodesmus* sp. after extrusion (p = 0.053). Extrusion did not improve digestibility of protein in *Nannochloropsis* sp., but protein digestibility in *Desmodesmus* sp. was significantly improved. Based on the results from **Paper I**, it was concluded that extrusion improved digestibility, especially for the low digestible microalgae. In **Paper IV**, extrusion was used for thermo-mechanical pre-processing of microalgae before they were mixed with other ingredients and extruded to produce pellets (double-extrusion). In **Paper III**, *Scenedesmus* sp. was used without any processing, and hence the cell wall was intact. The digestibility values of dry matter and protein in microalgae-included feeds were similar in **Paper II** and **IV**, while the values in **Paper III** were lower (**Figure 5**). In addition, we obtained higher lipid digestibility in **Paper IV** compared to results in **Paper II** and **III** (**Figure 5**). The difference in nutrient digestibility of different microalgae and different microalgae-incorporated diets can be attributed to feed ingredient composition, microalgae cell wall characteristics, cell wall components and different pre-treatment

of the microalgae per se. The cell wall characteristics of *Nannochloropsis* sp. and *Desmodesmus* sp., and *Scenedesmus* sp. have not been investigated in the studies described in this thesis. Other authors have described them; *Nannochloropsis gaditana* has bilayered cell wall—a cellulosic inner wall protected by an outer algaenan layer (Scholz et al., 2014, Becker, 2007). The members of the genus *Desmodesmus* sp. have characteristic ornamental cell walls; the outermost layer may be spiny, granulated or dented (Kaur et al., 2012). The outer cell wall of *Scenedesmus* sp. is made up of a chemically inert biological polymer sporopollenin (Staehelin and Pickett-Heaps, 1975). The inner cell wall mainly consists of hemicellulose and cellulose polymers (e.g. *Scenedesmus obliques*; Voigt et al. (2014) as well as pectin (e.g. *S. pannonicus*; Staehelin and Pickett-Heaps (1975). Based on the abovementioned cell wall characteristics and their biochemical composition we presume that the intact cell walls of *Scenedesmus* sp. can adversely affect the nutrient digestibility in Atlantic salmon. A number of feeding experiments performed with salmonids, have also indicated that digestibility of nutrients contained in single-cell organisms such as bacteria (Aas et al., 2006), yeast (Storebakken et al., 2004, Berge et al., 2013) and microalgae (Tibbetts et al., 2017), may be impacted by their rigid cell walls. Pre-treatments including disruption of cell walls in biomass could significantly improve nutrient utilization by Atlantic salmon (Storebakken et al., 2004, Tibbetts et al., 2017). Cost-effective processing technologies should be developed to disrupt cell walls, concentrate nutrient levels, and improve nutrient availability of microalgae; to achieve commercial acceptance in salmon feed (Teuling et al., 2017, Tibbetts et al., 2017). A variety of disruption methods has been reported for cell disruption of microalgae. They are mechanical and non-mechanical treatments: mechanical treatment employs solid-shear forces (bead milling, high speed homogenization), liquid-shear forces (micro-fluidization, high pressure homogenization), energy transfer through waves (ultra-sonication and microwave), as well as currents (pulsed electric field) while non-mechanical treatment uses chemical and enzymatic methods (Günerken et al., 2015). For industrial use, the chosen method should be amenable to scale up without high costs. Extrusion is a thermomechanical process that combines high temperature (120–130°C), high

pressure (20–30 bar) and shear forces (Sørensen, 2012), that could have a potential in large scale commercial use for pre-processing of microalgae. Extrusion has been reported to be effective in cell disruption of the microalga *Nannochloropsis oceanica*, making intracellular nutrients more accessible, which in turn is likely to improve the digestibility of nutrients (Wang et al., 2018). The findings in the present thesis suggest that extrusion has potential to improve utilization of nutrients in microalgae.



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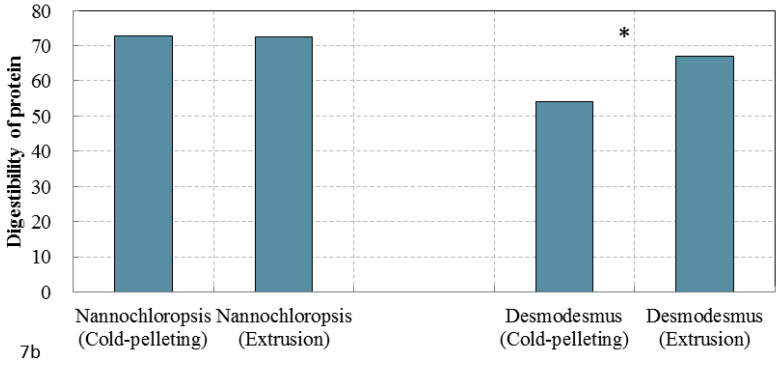

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Figure 7a, 7b. A comparison of cold-pelleting and extrusion processing on digestibility of dry matter and protein in *Nannochloropsis* sp. and *Desmodesmus* sp. fed to Atlantic salmon

3.2.2 Use of feed additives to improve utilization of microalgae feeds

We have observed that digestibility of nutrients from microalgae (*Nannochloropsis*) feeds were generally lower compared to the reference feeds (**Paper I, II** and **III**). In **Paper IV** we investigated the potential of feed additives to improve digestibility and utilization of diets with *Nannochloropsis oceanica*. The feed additives were Digestarom PEP MGE150 and ZEOFeed. Based on available information, the Digestarom PEP MGE150 contains a blend of essential oils from oregano, anise, and citrus peel, and the main active compounds are carvarol, thymol, anethol, and limonene (Rodrigues et al., 2018, Peterson et al., 2014). The beneficial effects of plant essential oils on the performance and health of cultured fish have been investigated during the last two decades (Sutili et al., 2018). However, there are only a few earlier reports on use of commercial products such as Digestarom in fish feed (Rodrigues et al., 2018, Peterson et al., 2014). Previous fish studies have shown that 0.02% Digestarom PEP MGE150 supplementation in fish feed does not improve digestibility of dry matter and protein, growth performance and FCR in channel catfish (*Ictalurus punctatus*) and gilthead seabream (*Sparus aurata*) (Rodrigues et al., 2018, Peterson et al., 2014). But supplementation of Digestarom Poultry increased the protein and lipid digestibility, body weight gain and lowered the FCR of broiler chickens (Murugesan et al., 2015). Studies with rainbow trout also showed that supplementation with 0.1% Digestarom PEP 1000 (containing 1.2% carvacrol) or 0.1% Digestarom PEP MGE 1000 (containing 0.6% thymol) improved feed efficiency compared to control diet, although, body weight gain was unaffected (Giannenas et al., 2012). Koppe et al. (2015) investigated the performance of Atlantic salmon fed 0.05-0.1% carvacrol in diets; they authors observed higher growth rate and feed efficiency ratio as well as lipid digestibility. The results from **Paper IV** indicate that supplementing 0.06% Digestarom PEP MGE150 has no effect on digestibility, growth and feed utilization of Atlantic salmon. Furthermore, we did not detect any significant differences in final body weight, weight gain, specific growth rate, thermal growth coefficient, feed intake and feed conversion ratio compared to *Nannochloropsis* control diet.

The main component in ZEOFeed is clinoptilolite. Clinoptilolite is a natural zeolite that contains silica and alumina, and has a microporous structure (EFSA, 2013).

Clinoptilolite is currently listed in the European Union Register of Feed Additives, and the maximum concentration that can be used in salmon feeds is 2% (EFSA, 2013). Previous studies with gilthead sea bream reported that clinoptilolite can improve growth rate and feed efficiency, and the optimum inclusion level was 2.7% of diet (Kanyılmaz et al., 2015). Other studies have reported that use of zeolite (bentonite and mordenite) improved the growth and feed utilization in rainbow trout (Eya et al., 2008). It is assumed that the improved growth and nutrient utilization is related to the detoxifying effects (by trapping toxic heavy metals, toxins, biomines and ammonia) of zeolites (Ghasemi et al., 2018). However, to the best of our knowledge, the effects of clinoptilolite on growth of Atlantic salmon have not been investigated earlier. Supplementation of algal-diets with 1% ZEOFeed (clinoptilolite) in salmon feed did not improve growth and feed utilization in the present study. The differential responses observed in earlier studies and ours (**Paper IV**) could be attributed to fish species, sources of the additives, duration of feeding period or supplementing levels of the additives. Future studies with ZEOFeed and Digestarom should possibly consider other incorporation levels, or perhaps the duration of administration of these products.

4 Conclusions

Microalgae can be employed as feed ingredients for Atlantic salmon. Extrusion technology can be used to improve nutrient utilization of microalgae.

- ✧ The salmon readily accepted the tested microalgae as a feed ingredient without any adverse effect on feed intake.
- ✧ The microalga *Nannochloropsis* sp. was more digestible than *Desmodesmus* sp. when fed to Atlantic salmon
- ✧ Incorporation of microalgae at 10% in both fish meal-based and plant-protein based salmon feeds did not have any negative effect on growth, feed utilization, condition indices, health parameters and proximate composition of Atlantic salmon.
- ✧ The increased PUFAs content of whole body Atlantic salmon fed *Nannochloropsis oceanica* and one of the feed additives and of those fed *Scenedesmus* sp. is noteworthy from nutritional point of view.
- ✧ Thermo-mechanical processing (extrusion) can be used as a cost-effective method to improve digestibility of nutrients from microalgae.

5 Outlook for future research

The Norwegian salmon farming industry that has a strong market position is expected to follow the projected growth of global aquaculture production. Availability of sustainable feed ingredients can ensure the growth of salmon aquaculture. Though microalgae have the potential to be feed ingredients in salmon feeds, only a few have been successfully commercialized. Incorporating protein-rich microalgae in salmon feeds can be a challenge because of the variability in nutrient bioavailability, presence of large portion of indigestible complex carbohydrates, and high production and processing costs of the algal biomass. Thorough research should be conducted to understand their effects on growth, health and product quality of farmed salmon as well as the effects on feed production process parameters, processability and technical properties of salmon feed. Besides, technological developments in the areas of industrial scale-up, processing methods are needed to provide the industry with nutrient-dense, cost-effective microalgae products.

6 References

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Paper II

PLOS ONE

RESEARCH ARTICLE

Nannochloropsis oceania-derived defatted meal as an alternative to fishmeal in Atlantic salmon feeds

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Abstract

Defatted microalgal biomass derived from biorefinery can be potential feed ingredients for carnivorous fish. The present study investigated the growth, feed intake:gain and health parameters in Atlantic salmon fed for 84 days with defatted *Nannochloropsis oceania* as a fishmeal replacer. Fish fed feeds containing the algal biomass (at 10 and 20% inclusion, alga groups) were compared with groups that consumed alga-devoid feeds (control group). The fish that received 20% alga tended to have reduced weight gain and specific growth rate. Condition factor, feed conversion ratio and feed intake of this fish group were significantly different when compared with the control group. Hepatosomatic and viscerosomatic indices, whole body and fillet proximate composition were not affected by the dietary treatments. Digestibility of dry matter, protein, lipid, ash and energy, as well as retention of lipid and energy of the fish that received feed with 20% alga meal were also significantly different from those of the control group. Serum superoxide dismutase activity of the 10% alga-fed fish was significantly higher compared with the control fish. Although alga feeding did not cause any distal intestinal inflammation, the intestinal proteins that were altered upon feeding 20% algal meal might be pointing to systemic physiological disturbances. In conclusion, feeds with 20% alga had a negative effect on feed intake, FCR, lipid and energy retention and health of the fish. The defatted *Nannochloropsis oceania* can be used at modest inclusion levels, around 10%, without negative effects on the performance of Atlantic salmon.

Introduction

Marine microalgae are unicellular organisms, and they are rich in high-quality protein, essential amino acids, polyunsaturated fatty acids, sugars, polysaccharides, vitamins, minerals and pigments [1]. Certain microalgal varieties are already marketed for human consumption. However, microalgae biomass is hardly exploited commercially as aquafeed components,

agencies had no role in the design, analyses or writing of this article.

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Abbreviations: ADC, apparent digestibility coefficients; Ahsg, Alpha-2-HS-glycoprotein-like; AOAC, Association of Official Analytical Chemists; Apoa1-1, Apolipoprotein A-I-1 precursor; CAT, catalase; *cath1* and *cath 2*, *cathelicidin 1* and *2*; CF, Condition factor; Crt, Creatine kinase B-type isoform X2; Dld, Dihydrolipoyl dehydrogenase, mitochondrial-like; DM, dry matter; Ef2, Elongation factor 2; EPA, eicosapentaenoic acid; FCR, Feed conversion rate; FDU, Forsøksdyrutvalget; Flr, Flavin reductase; ForTek, Feed Technology Center; FPC, fillet proximate composition; HSI, hepatosomatic index; igt, *immunoglobulin T*; il10, *interleukin 10*; il17d, *interleukin 17d*; il1b, *interleukin 1b*; ISO, International Organisation for Standardisation; LC-MS/MS, liquid chromatography and tandem mass spectrometry; Lei, Leukocyte elastase inhibitor-like; MS-222, Tricaine methane sulphonate; NMBU, Norwegian University of Life Sciences; nrf2, *nuclear factor erythroid 2-related factor*; NS-EN ISO, Norsk standard; P5cdh, Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial-like; PBS, Phosphate buffered saline; PUFA, Polyunsaturated fatty acids; Ret, Retentions SGR, specific growth rate; SOD, super oxide dismutase; sod1, *superoxide dismutase1*; TAC, total antioxidant capacity; TGC, thermal unit growth coefficient; tgfb, *transforming growth factor beta*; Tpi, Triosephosphate isomerase; VSI, viscerosomatic index; WBC, whole body proximate composition.

primarily due to their unavailability in large volumes and high price of the marketed products. Researchers are developing strains that contain more lipids, nutrients as well as bioactive compounds [2], and "biocrude" oil, and residual protein-rich fractions are co-products of cultivated microalgae [3]. Thus, the biofuel and the defatted biomass that is rich in protein will be available in large amounts in the near future.

Nannochloropsis is a candidate that is exploited for biofuel production because of their high lipid content [4]. The lipid content may vary from 1 to 40% of dry matter (DM) in certain strains, and under special culture conditions the level can go up to 85% [5]. In *Nannochloropsis*, eicosapentaenoic acid (EPA) is the dominant fatty acid [6], and this characteristic makes the microalga a potential partial fish oil replacer in fish feeds [7]. Feeding salmon with plant oils can reduce the levels of EPA and DHA in the fish flesh [8]. Atlantic salmon can endogenously convert long-chain PUFAs from their dietary sources, due to the presence of desaturase and elongase genes [9–11]. The presence of fatty acyl elongase *elovl2* helps the fish to elongate C20 and C22 fatty acids [11], suggesting the ability of salmon to utilize the PUFAs derived from microalgal lipids. On the other hand, the protein-rich biomass of *Nannochloropsis* can be a potential fishmeal replacer because of its nutrient content. Many research groups have reported the suitability of defatted microalgae as feed ingredients in the feeds of aquatic animals. Kiron et al. [12] showed that 10% of fishmeal protein in the feeds for Atlantic salmon post smolts can be replaced with the protein from defatted *Nanofrustulum*; without negatively affecting the growth performance, feed performance and body composition of the fish. Patterson and Gatlin III [13] also reported that up to 10% crude protein from fishmeal and soy protein concentrate in the feeds for red drum could be replaced with lipid-extracted algae meal (derived from *Navicula* sp., *Chlorella* sp. and *Nannochloropsis salina*); without negatively affecting the growth, feed utilization, protein and energy retention of the fish.

Although the potential of defatted biomass to support the growth of aquatic animals was demonstrated in earlier experiments, each alga strain needs to be tested on each target species. The results from our earlier experiments on Atlantic salmon have indicated that the digestibilities of protein from *Desmodesmus* and *Nannochloropsis* were not different [14, 15]. However, experiments on a mammalian carnivore model, mink, have pointed out that the digestibility of protein from different microalgae vary widely [16]. Digestibility coefficient estimation is however only the first step to evaluate the bioavailability of nutrients for growth and therefore, the observations need to be verified through long-term feeding experiments [17]. It is also important to assess the effects of the tested feed ingredients on the health of the animals.

In the present study, the effect of the replacement of fishmeal with *Nannochloropsis oceania* (*N. oceania*) biomass in the feeds of Atlantic salmon was evaluated on the growth performance, feed utilization and intestinal health of the fish. An 84-day feeding trial was conducted to examine the growth and feed performance, antioxidant status, expression of genes and proteins, and micromorphology of the distal intestine of Atlantic salmon.

Materials and methods

Experimental design and feeds

The study, approved by the National Animal Research Authority (FDU: Forsøksdyrutvalget ID—5887) in Norway, consisted of three groups: a control group (1C- offered control feed), and 2 algal groups [offered feed with 10% (1L) and 20% (1H) alga meal]. The algal meal is the biomass from *N. oceania*, a product obtained after biofuel extraction (Cellana, San Diego, USA). Chemical composition of the algae biomass is presented in S1 Table. The content of elements, amino acids, fatty acid composition, neutral detergent fiber (NDF) and acid detergent fiber (ADF) was reported by Gatrell et al. [18].

Ingredients and chemical composition of the feeds are presented in Table 1. The control feed was based on fishmeal while algal biomass replaced 100 and 200 g fishmeal. All other ingredients except fish oil were kept constant. Fish oil was reduced in the 1H feed to keep a constant crude protein:energy ratio. The extruded experimental feeds were produced by the Feed Technology Center (ForTek), Norwegian University of Life Sciences (NMBU), Ås, Norway. The extrusion equipment used for producing the feeds has been described earlier by Sørensen et al. [19, 20]. The feed ingredients were first mixed in a portable mixer (40L, IdeCon AS, Norway), and then fed to the extruder using a Coperion Key-Tron feeder (Type T32, Coperion K-Tron International, New Jersey, USA). This feeder was calibrated to directly deliver the mixture, at an input rate of 54–55 kg mash/h, into the extruder barrel. The screw configuration was optimised to improve the mixing efficiency and feed quality, and for efficient utilisation of mechanical energy even at a lower feeding rate. Conditioning was initiated in the second section of the extruder barrel by adding both steam and water. The temperature profile for conditioning the two algal feeds in the five section extruder barrel was 39–40, 90–91, 121–123, 106–107, 66–67 °C, respectively. The control feed was produced at slightly higher temperature ranges: 37–38, 99–105, 127–127, 109–113 and 82–91 °C. The conditioned material was passed through four 2 mm dies, resulting in pellets with 2.0–2.1 mm diameter and 3.8–4.2 mm length. The operating pressures used for making the 1C, 1L and 1H feeds were 21, 25 and 31 bar, respectively, and torque fluctuated between 334–351, 315–366 and 342–381 Nm, respectively. Pellets were collected and conveyed pneumatically to an NMBU-FORBERG fluidized bed dryer (Forberg, Oslo, Norway) and dried to the final DM content of 936–942 g kg DM^{-1} (Table 1) in small experimental batch dryers (10 kW heater, 2550 m^3h^{-1} fan capacity, keeping the product temperature < 55 °C). The feeds were shipped to Nord University where they were stored in airtight containers at 4 °C, until they were distributed to the feeders.

Table 1. Ingredients and proximate composition of the control and the microalga-containing feeds.

Ingredients (g/1000 g)	Experimental feeds		
	1C	1L	1H
Fish meal[1]	690	590	495
Algal meal[2]	0	100	200
Wheat[3]	120	120	120
Wheat gluten[4]	50	50	50
Fish oil[1]	135	135	130
Microingredients[5]	5	5	5
Marker[6]	0.1	0.1	0.1
Proximate composition (g/1000 g)			
Dry matter	942.4	942.4	935.6
In dry matter:			
Crude protein	564.0	535.3	501.2
Crude lipid	211.0	205.9	199.9
Ash	114.2	123.4	132.5
Energy (MJ/1000 g)	23.6	23.6	23.1

[1] Nordsildmel AS, Bergen, Norway
[2] Cellana LLC, Kona, Hawaii, USA
[3] Felleskjøpet AS, Moss, Norway
[4] Gluten Vital, Alimenta AS, Oslo, Norway
[5] Vitamin and mineral mix is a proprietary formulation of Europharma, Leknes, Norway.
[6] Yttrium, Metal Rare Earth Limited, Shenzhen, China

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Fish rearing facility, fish husbandry and feeding

The feeding experiment was carried out in a flow-through system at the Research Station, Nord University, Bodø, Norway. The circular fibreglass rearing tanks (800 l and 0.9 m deep) were custom made (A-plast, Skodje, Norway); they are slightly conical with top and bottom diameters of approximately 1 m and 0.9 m, respectively. The cone-shaped bottom with approximately 22 degrees slope ensures the efficient collection of faeces and left-over feeds. The tank design is provided in S1 Fig. Every automatic feeder (ArvoTec T-drum 2000 feeder) of the rearing system is equipped with a 1 g dosing drum, control cabinets and software (ArvoTec, Huutokoski, Finland). The left-over feeds were collected from the water drains in a 17 l tank-mounted solid waste collector (Aquatic Eco-Trap, Pentair Aquatic Eco-Systems®, Fl, USA).

Atlantic salmon (*Salmo salar*) post-smolts (Aquagen strain, Aquagen AS, Trondheim, Norway) were purchased from a commercial producer (Cermaq Norway AS, Hopen, Norway) and maintained at the research station on a commercial feed until they were used for the feeding trials.

The fish used for the experiment were of mean initial weight 215.4 ± 27.1 g. Fish in 6 replicate tanks (15 fish/tank; mean biomass 3232 g/tank) were allocated to one feed group. Seawater (33 g l^{-1} salinity) that was used for rearing the fish was pumped from 250 m depth in Saltenfjorden, filtered and aerated. The rearing water temperature, ranged from 6.7 to 7.1˚C and the feeding period was 594.8 degree days. Oxygen saturation in the tanks, as measured at the outlet, was kept above 90%. A 24-h lighting regime was maintained in the rearing facility.

The experimental feeds were fed to fish during the experimental period of 84 days. The feeding procedure aimed at maximum voluntary feed intake by all groups of fish. Fish were fed two meals per day; first meal from 08.00–09.00 and second meal from 14.00–15.00. The left-over feeds were collected immediately after the two feeding sessions.

Fish sampling

Fish handling and sampling procedures were in accordance with the protocols approved by the FDU. Before weighing and sampling, the fish were anaesthetised with MS-222 (Tricaine methane sulphonate; Argent Chemical Laboratories, Redmond, USA; 80 mg/l). The fish for sample collection at the start and at termination of the experiment were euthanized by a sharp blow to the head. Fish that were not removed for sampling at termination of the experiment were returned to the fish holding facility, to be used for other purposes, thereby adhering to the principle of reducing the number of fish sacrificed for the study.

Individual weight and length were taken at the start and end of the experiment. Initial samples for whole body and fillet chemical composition were obtained from 6 and 12 fish, respectively. For the end-of-the-study sampling, blood was drawn from the caudal vein of 3 fish/tank to assess the haematocrit values and to collect serum for enzyme assays. Thereafter, these fish were dissected and the visceral organs (without heart and kidney) and the liver were removed for calculating the viscerosomatic and hepatosomatic indices. The fillet of these 3 fish were collected, sealed in plastic bags and kept frozen at -40˚C until they were used for analysing the fillet proximate composition (FPC). Distal intestinal samples intended for molecular studies were snap frozen in liquid nitrogen and transferred to a -80˚C freezer. Histology samples of the distal intestine were rinsed with PBS and fixed in phosphate-buffered formaldehyde solution. Whole body of 6 fish from each tank were collected (after the term for faeces collection), sealed in plastic bags and frozen at -40˚C to determine the whole body proximate composition (WBC).

Faecal samples were collected after the feeding trial, from the remaining 12 fish/tank. For determining the digestibility of the feeds, the fish were stripped two times (one week time interval between strippings), employing the procedure described by Austreng [21]. The samples from fish in one tank were pooled and kept frozen.

Chemical analysis

The WBC of the initial samples were analysed individually (n = 6 fish), while those of the final samples were analysed after pooling the 6 fish from one tank (obtained at the end of the experiment). For the analysis of FPC of the initial samples, fillets from 2 fish (4 fillets) were pooled (n = 6). The FPC of the final samples were determined after pooling 6 fillets that were obtained from 3 fish from a tank (described previously).

Fish whole body and fillet samples were thawed and homogenized prior to chemical analysis (dry matter, ash, nitrogen, crude lipid) and energy determination. Faecal samples were freeze-dried prior to chemical analysis (dry matter, ash, nitrogen, crude lipid and yttrium) and energy determination. Faecal matter of fish from two tanks of the same group were pooled to secure enough material for the analysis.

Dry matter was determined by oven drying (105˚C for 20 h) to constant weight (ISO 6496–1999), crude protein by Kjeldahl method (N × 6.25; Kjeldahl Auto System, Tecator Systems, Höganäs, Sweden; ISO 5983–1987), crude lipid by Soxhlet method with acid hydrolysis (Soxtec HT6, Tecator, Höganäs, Sweden; AOAC Method 954.02), ash by incineration in a muffle furnace at 540˚C for 16 h (ISO 5984–2002), and energy by bomb calorimetry (IKA C200 bomb calorimeter, Staufen, Germany; ISO 9831–1998), yttrium by inductive coupled plasma mass spectroscopy (ICP-MS; performed at Eurofins, Moss, Norway; NS-EN ISO 11885). The proximate composition, energy and yttrium content of the samples were measured in duplicates.

Antioxidant markers

The antioxidant status of the fish was evaluated by performing different assays, employing the serum aliquots. The total antioxidant capacity (TAC), catalase (CAT) activity and super oxide dismutase (SOD) activity were determined using kits from Cell Biolabs (STA-360, Cell Biolabs Inc., San Diego, CA, USA), Cayman Chemicals (707002, Cayman Chemicals, Ann Arbor, MI, USA), and Cell Biolabs (STA-340, Cell Biolabs Inc.), respectively. The protocols are described in our previous paper [22].

Gene expression

The mRNA levels of antioxidant-related (*superoxide dismutase1—sod1*; *nuclear factor erythroid 2-related factor—nrf2*), gut mucosa-related (*immunoglobulin T—igt*), inflammation-related (*interleukin 1b—il1b*; *interleukin 10—il10*; *interleukin 17d—il17d*; *transforming growth factor beta—tgfb*), antimicrobial (*cathelicidin 1* and *2—cath1*, *cath2*) genes were assessed in this study.

The total RNA from the frozen tissues were extracted using E-Z 96 Total RNA Kit (OMEGA Bio-Tek Inc, USA) following the instructions from the manufacturer. To quantify the mRNA level of a particular gene, samples from 12 fish/group (2 from each tank) were considered. The list of primers of the genes [22–24] and the detailed protocol for the analysis [22] are described in our previous publications. List of primers of the differentially expressed and reference genes are presented in Table 2.

Table 2. List of primers for the differentially expressed genes and the reference genes.

Gene name	Sequence(5'-3')	Amplicon size (bp)	PCR efficiency (%)	GenBank accession numbers
Target genes				
sod1	CCACGTCCATGCCTTTGGR-F	141	95.3	AY736282.1
	TCAGCTGCTGCAGTCACGTT-R			
il17d	CTTGTCTCCCTGGGCATACAG-F	201	112.7	EU689087.1
	CAATATGCCTCGGGTATGAACCT-R			
igt	CAACACTGACTGGAACAACAAGGT-F	97	107.7	GQ907004
	CGTCAGCGGTTCTGTTTTGGA-R			
Reference genes				
ef1ab	TGCCCCTCCAGGATGTCTAC-F	59	96	BG933853
	CACGGCCCACAGGTACTG-R			
rpl13	CGCTCCAAGCTCATCCTCTTCCC-F	79	96.4	BT048949.1
	CCATCTTGAGTTCCTCCTCAGTGC-R			
rps29	GGGTCATCAGCAGCTCTATTGG-F	167	94.5	BT043522.1
	AGTCCAGCTTAACAAAGCCGATG-R			
ubi	AGCTGGCCCAGAAGTACAACTGTG-F CCACAAAAAGCACCAAGCCAAC-R	162	92.7	AB036060.1

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Protein expression

The distal intestinal proteomes of the three groups (n = 6/group) of fish were analysed employing two-dimensional electrophoresis and liquid chromatography and tandem mass spectrometry (LC-MS/MS). The protein extraction and the 2-DE were carried out as described previously [24]. The LC-MS/MS work was undertaken at the Tromsø University Proteomics Platform, Tromsø, Norway. Gel analysis and protein identification were performed as detailed in our previous paper [22].

Distal intestinal morphology

Approximately 5 μm sections were prepared from the distal intestinal samples of 6 fish per group. The sections were stained with Alician Blue-Periodic Acid Shiff's reagent and the photomicrographs were prepared as described in our previous papers [22, 24].

Calculations and statistical analysis

The apparent digestibility coefficients (ADCs) of DM, protein, lipid, ash and energy were calculated using the following equation [25]:

$$\mathrm{ADC}_{nutrient\ or\ energy} = \left[1 - \left(\frac{Marker_{feed} \times Nutrient_{faeces}}{Marker_{faeces} \times Nutrient_{feed}}\right)\right] \times 100 \quad (1)$$

$$\mathrm{ADC}_{dry\ matter} = \left[1 - \left(\frac{Marker_{feed}}{Marker_{faeces}}\right)\right] \times 100 \quad (2)$$

where $Marker_{feed}$ and $Marker_{faeces}$ are the contents of the marker (% dry matter) in the feed and faeces, respectively, and $Nutrient_{feed}$ and $Nutrient_{faeces}$ are the nutrient contents (% dry matter) in the feed and faeces.

Specific growth rate (SGR) and thermal unit growth coefficient (TGC) were calculated based on mean weights, employing the equations:

$$\mathrm{SGR} = [\frac{(ln\ W_1 - lnW_0)}{t}] \times 100 \quad (3)$$

$$\mathrm{TGC} = \frac{(W1^{1/3} - W0^{1/3}) \times 1000}{d^o} \quad (4)$$

W0 is the initial weight, W_1 is the final weight, and t is the time (days), and d^o is the total number of degree days.

The organosomatic indices namely, hepatosomatic index (HSI), viscerosomatic index (VSI) and condition factor (CF) were calculated using the formulae:

$$\mathrm{HSI} = \left(\frac{Liver\ weight\ (g)}{Final\ body\ weight\ (g)}\right) \times 100 \quad (5)$$

$$\mathrm{VSI} = \left(\frac{Viscera\ weight\ (g)}{Final\ body\ weight\ (g)}\right) \times 100 \quad (6)$$

$$\mathrm{CF} = \frac{Body\ weight\ (g)}{Fish\ fork\ length^3(cm)} \times 1000 \quad (7)$$

Feed conversion rate, FCR, was calculated using the formula:

$$\mathrm{FCR} = \frac{Dry\ matter\ feed\ intake(g)}{Weight\ gain(g)} \quad (8)$$

Retentions (Ret) of nitrogen (or energy) were calculated for each tank employing the following formula:

$$\mathrm{Ret} = \left(\frac{[(FB \times N_f) - (IB \times N_i)]}{(DM\ Feed\ intake \times N\ feed)}\right) \times 100 \quad (9)$$

where IB and FB are the initial and final biomass and N is the concentration of the nitrogen (or energy) in fish (subscripts i and f represent initial and final samples, respectively) or feed.

Statistical analyses were carried out using Graphpad Prism 6 (Graphpad Software Inc., La Jolla, CA, USA). Normality and equal variance of the data were tested before performing one-way ANOVA. Tukey's multiple comparisons test was employed to detect the significant differences between the means of interest. Kruskal-Wallis test followed by Dunn's multiple comparisons test was employed in the case of non-parametric data, to understand the differences between the study groups. The differences between groups were considered significant at $P < 0.05$, and differences at $0.10 > P > 0.05$ suggests a trend.

Results

Growth and feed performance

The fish had good health and growth during the experimental period; mortality was not recorded and the final weights of fish in the 3 groups were approximately twice that of their initial weights. Weight gain (P = 0.09) and SGR (P = 0.09) tended to differ among the feeding groups (Table 3). The fish fed the 20% alga-feed tended to have lower weights and SGR compared to that of the control fish. The FI of the 1H group was significantly higher compared to

Table 3. Survival, growth and feed utilization of Atlantic salmon fed the control or microalga feeds for 84 days.

	1C	1L	1H	ANOVA P-value
Survival (%)	100	100	100	
Initial weight (g)	214.5 ± 3.1	213.8 ± 2.7	218.0 ± 2.8	0.76
Final weight (g)	429.0 ± 12.2	420.2 ± 13.3	407.8 ± 12.1	0.50
Weight gain (%)	100.2 ± 5.1	96.3 ± 3.4	86.9 ± 3.2	0.09
SGR (% day^{-1})	0.82 ± 0.03	0.80 ± 0.02	0.74 ± 0.02	0.09
TGC	2.61 ± 0.10	2.54 ± 0.08	2.35 ± 0.08	0.12
FI (% BW day^{-1})	0.68 ± 0.01^{b}	0.70 ± 0.01^{ab}	0.75 ± 0.02^{a}	0.01
FCR	0.81 ± 0.02^{bc}	0.86 ± 0.02^{b}	1.00 ± 0.06^{a}	0.01
PER	2.20 ± 0.06	2.18 ± 0.06	2.03 ± 0.10	0.24

Specific growth rate–SGR. Thermal growth coefficient–TGC. Feed intake–FI. Feed conversion ratio–FCR. Protein efficiency ratio–PER. Different superscripts (a, b, c) in a row indicate statistically significant differences (P < 0.05) among groups. n = 6 tanks, values from 15 fish/tank, mean ± SEM

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that of the 1C group, and the FCR of the 1H group was significantly higher than those of the 1L and 1C groups. The TGC and PER of the alga-fed fish were lower (P>0.05) than the values of the 1C group.

Organosomatic indices and hematocrit values

The CF of the study groups ranged between 2.16 and 2.34 (Table 4). Fish of the 1H group had significantly lower CF compared to that of the 1C group. HSI and VSI of the 1L and 1H groups were similar to those of the control group (Table 4). The hematocrit values were significantly different among the three study groups (Table 4). The values of the 1H and 1C differed significantly from each other, while 1L ranked in between.

Chemical composition

The proximate composition of whole body and fillet is presented in Table 5. Moisture, protein, lipid and ash contents of the whole body and fillet of the fish from the 3 groups did not vary significantly. However, the lipid content in the whole body of the 3 groups at the end of the feeding trial was lower than that of the initial fish. The lipid content in the fillet was lower compared to that in the whole body.

Protein, lipid and energy retention

Protein, lipid and energy retention in Atlantic salmon from the three study groups are presented in Table 6. Protein retention of the three groups were not significantly different. Lipid and energy retention values of the 1H group were significantly lower compared to the respective values of the 1C group.

Table 4. Organosomatic indices and hematocrit values of Atlantic salmon fed the control or microalga feeds for 84 days.

	1C	1L	1H	ANOVA P-value
Condition factor	2.34 ± 0.04^{a}	2.29 ± 0.04^{ab}	2.16 ± 0.04^{b}	0.023
Hepatosomatic index	1.27 ± 0.02	1.24 ± 0.01	1.24 ± 0.03	0.563
Viscerosomatic index	8.13 ± 0.09	8.28 ± 0.12	8.33 ± 0.15	0.511
Hematocrit	47 ± 1^{b}	50 ± 2^{ab}	54 ± 2^{a}	0.049

Different superscripts (a, b) in a row indicate statistically significant differences (P < 0.05) among groups. n = 6 tanks, values from 6 fish/tank, mean ± SEM)

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Table 5. Proximate composition (g/100 g dry matter) of Atlantic salmon fed control or microalgae feeds for 84 days.

	Initial	Final		
		1C	1L	1H
Whole body				
Moisture	69.2 ± 0.2	68.7 ± 0.2	68.5 ± 0.2	69.2 ± 0.3
Protein	54.7 ± 0.4	55.5 ± 0.5	55.6 ± 0.6	56.3 ± 0.6
Lipid	37.3 ± 0.4	36.4 ± 0.2	36.3 ± 0.5	35.8 ± 0.5
Ash	6.7 ± 0.2	6.0 ± 0.1	6.3 ± 0.2	6.4 ± 0.1
Fillet				
Moisture	74.3 ± 0.2	74.4 ± 0.2	74.2 ± 0.1	74.2 ± 0.1
Protein	77.6 ± 0.3	78.8 ± 0.4	79.0 ± 1.0	80.6 ± 0.7
Lipid	18.2 ± 0.4	14.5 ± 0.7	14.8 ± 1.0	14.9 ± 0.4
Ash	5.6 ± 0.1	5.5 ± 0.2	6.1 ± 0.3	5.5 ± 0.1

n = 6 tanks, proximate composition values from pooled samples from 3 fish (for fillet) and 6 fish (for whole body); mean ± SEM

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Digestibility

The ADCs of protein, ash and energy in the three feeds were significantly different (Table 7). The ADC's of DM and lipid in the alga feeds were significantly different from those of the 1C group.

Antioxidant status

The antioxidant markers such as the TAC and CAT activities in the serum of the 1C, 1L and 1H groups were similar (Fig 1). However, the serum SOD activity of the 1L group was significantly higher than that of the 1C group.

Intestinal health status

Gene expression: The mRNA level of *il17d* was apparently higher ($P<0.1$) in the 1L group compared to the level in the control group (Fig 2). In addition, *sod* was apparently higher ($P<0.1$) in the 1L group compared to the expression in the 1H group. On the other hand, the mRNA levels of *igt* were similar in the study groups. The mRNA levels of *il1b*, *il10*, *tgfb*, *nrf2*, *cath1* and *cath2* were below the detection range.

Protein expression: Comparison of the distal intestinal proteome of the fish from the different groups revealed that the expression of 7 proteins were altered by the algal feeding—Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial-like (P5cdh), Dihydrolipoyl dehydrogenase, mitochondrial-like (Dld), Leukocyte elastase inhibitor-like (Lei), Creatine kinase B-type isoform X2 (Crt), Elongation factor 2 (Ef2), Triosephosphate isomerase (Tpi), Flavin reductase (Flr) (Fig 3, S2 Fig, Table 8). On the other hand, the proteins Alpha-2-HS-glycoprotein-like (Ahsg) and Apolipoprotein precursor (Apoa1-1) were significantly underexpressed,

Table 6. Retention of protein, lipid and energy of Atlantic salmon fed control or microalga feeds for 84 days.

	1C	1L	1H	ANOVA P-value
Protein	39.5 ± 1.4	39.8 ± 0.7	36.2 ± 1.9	0.19
Lipid	66.7 ± 2.1^{a}	64.6 ± 4.0^{ab}	53.4 ± 3.9^{b}	0.04
Energy	46.0 ± 1.4^{a}	43.6 ± 1.8^{ab}	36.7 ± 2.4^{b}	0.01

Values are given as mean ± SEM; n = 6 replicate tanks. Different superscripts (a, b) in a row indicate statistically significant differences ($P < 0.05$) among groups.

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Table 7. Apparent digestibility coefficients (%) of dry matter, protein, ash and energy in the control and microalga feeds.

	1C	1L	1H	P-value
Dry matter	76.0 ± 0.3[a]	71.6 ± 0.4[b]	70.7 ± 0.4[b]	<0.01
Protein	87.9 ± 0.1[a]	85.0 ± 0.4[b]	83.4 ± 0.2[c]	<0.01
Lipid	92.6 ± 0.3[a]	88.6 ± 0.6[b]	87.8 ± 0.3[b]	<0.01
Ash	15.5 ± 1.1[a]	20.8 ± 1.7[b]	30.6 ± 1.2[c]	<0.01
Energy	85.9 ± 0.3[a]	81.5 ± 0.5[b]	79.2 ± 0.3[c]	<0.01

Values are given as mean ± SEM; n = 3, faeces were pooled per tank. Different superscripts (a, b, c) in a row indicate statistically significant differences (P < 0.01) among groups.

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0.5 fold and 0.7-fold respectively, in the 1H group when compared with their expression in the 1L group. Flr was significantly overexpressed (1.7-fold) and Ckt was underexpressed (0.6-fold) in the 1L group when compared to its expression in the other study groups. Tpi was overexpressed (1.8-fold) in the 1L group compared to the expression in the 1C group. Dld (2.5-, 2.8-fold) P5cdh (2.5-, 2.8-fold) were overexpressed in the 1H group compared to the 1C and 1L groups. Ef2 (0.6-fold) and Pfn2 (0.6-fold) were underexpressed in the 1H group compared to the other study groups. Lei (0.5-fold) protein was underexpressed in the 1H group compared to the expression in the 1C group.

Distal intestinal micromorphology: Feeding the microalga-feeds did not alter the architecture of the distal intestine (Fig 4). Furthermore, there were no signs of inflammation in the intestine.

Discussion

In the present study, the potential of defatted biomass of the microalga *N. oceania* to be an ingredient in the feeds for Atlantic salmon was assessed based on the growth, nutrient digestibility, feed utilization and health parameters.

Growth and feed performance

The performance of the fish was good throughout the experimental period and SGR was higher than expected, based on growth tables that consider both fish size and water temperature [21]. The SGR values obtained in the present study were slightly lower than those reported earlier [22, 26]. The TGC values in the present experiment were higher than those obtained by Sørensen et al. [15], but were in the same range as reported by Hatlen et al. [26]. Feed conversion rate was in line with studies performed on yeast- or microalga-fed Atlantic salmon of

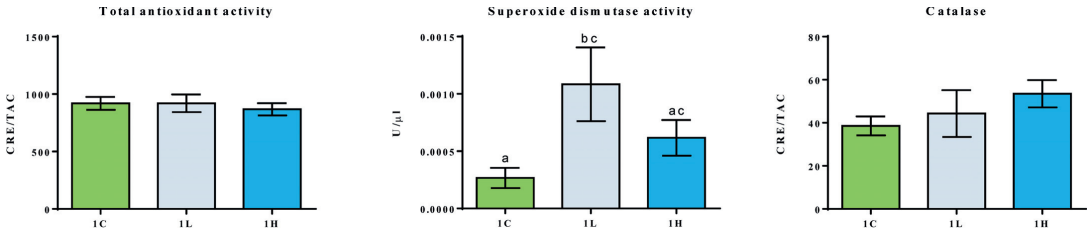

Fig 1. Serum antioxidant capacity of 1C, 1L and 1H groups. Values are expressed as mean ±SEM, n = 6 tanks.Different letters indicate significant differences (P<0.05) between the study groups.

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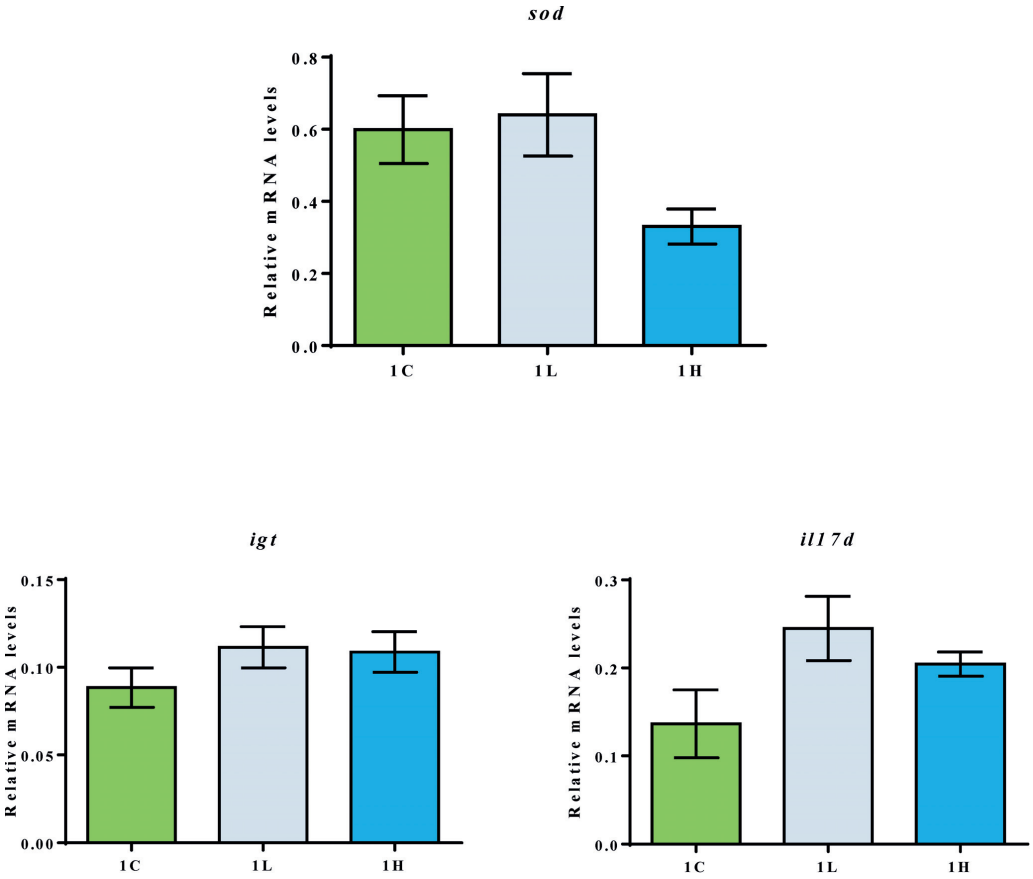

Fig 2. Relative mRNA levels of *sod*, *il17d*, *igt* in the distal intestine of 1C, 1L and 1H groups. The distal intestinal gene expression of the three groups (n = 12 fish) at the end of the 84-days feeding period. Values are presented as mean ± SEM, n = 6 tanks, 2 fish/tank.

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sizes comparable to that used in the present study [26, 27]. The dry matter intake (for each kg wet weight gain) of fish fed the highest inclusion level of defatted *N. oceania* was almost 24% greater than that of the control group. Higher feed intake and reduced feed conversion was also reported in other studies investigating microalgae in feeds for Atlantic salmon [28] and European sea bass [29]. The higher feed intake of the 1H group may be a compensation for the slightly lower lipid and energy content in the feeds with 20% alga meal inclusion. The experimental feeds were designed to keep a constant crude protein / energy ratio among the feeds. However, the calculated digestible protein (DP) / digestible energy (DE) values were 25, 24 and 23 for the 1C, 1L and 1H, respectively. These values were within the range 20–24 g DP / DE, which is suggested as optimal for young Atlantic salmon [30]. Increased feed intake to compensate for the low energy content in the feed is a strategy adopted by fish to secure

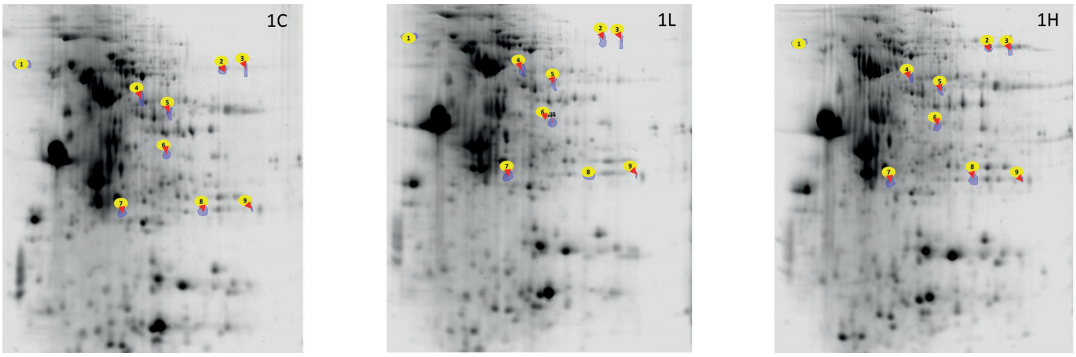

Fig 3. Representative 2-DE gels showing the spots of proteins from the distal intestine of Atlantic salmon. The spots 1–9 corresponds to Ahsg, P5cdh, Dld, Lei, Crt, Ef2, Apoa1-1, Tpi, and Flr, respectively (see Table 8). n = 6 fish/group.

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growth [31, 32]. The increased feed intake, noted in our study for Atlantic salmon that received 20% *N. oceania*, indicates the palatability of the ingredient. In contrast, other researchers have reported that microalgae may have negative effects on feed intake in fish. Atlantic cod juveniles offered feeds containing 140g/kg mixed biomass of microalgae dominated by *Isochrysis* sp., had reduced feed intake and growth [33]. We cannot make direct comparisons between varieties of algae or even production batches of one particular algae as their biochemical profiles largely depend on the nutrient availability and growing conditions [6].

Digestibility of protein in the fishmeal-based reference feed is in line with other studies [26, 27, 34–37]. However, the overall reduction in digestibility of protein, lipids and energy in the

Table 8. Differentially expressed distal intestinal proteins of Atlantic salmon fed microalga feeds for 84 days.

Spot no.	Protein name	Apparent pI/ MW (kDa)	Peptide sequenced[a]
1	Alpha-2-HS-glycoprotein-like, Ahsg	3.0/88.5	**YALNQIDDIK VVTAVEGDCDVVLR ESLFAIMEVGR**
2↑	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial-like, P5cdh	7.9/88.6	**NEPILGFNEGSPER AADIISGPK TVVQAEIDAAAELIDFFR HAVELESQQPLDSDGSTNTMLYR QVAQNLDVYK SADVQSVVTGTIR STGSIVAQQPFGGAR**
3↑	Dihydrolipoyl dehydrogenase, mitochondrial-like, Dld	8.16/87.8	**NQVTATAEDGSMQVINSK RPDGQIDVAVEAAAGGK NLGLDTVGLELDNR VPSIYAIGDVIAGPMLAHK FPFAANSR**
4↓	Leukocyte elastase inhibitor-like, Lei	6.0/68.8	**TGNVFYSPLSISSALAMVSLGAR ATDNVHVGFNK GAPYALSLANR LYGEQSYQFVETFLGDTK KHYNAELEAVDFK HYNAELEAVDFK NLLAEGVVDHLTR LVLVNAIYFK FKESSTSDALFK ESSTSDALFK NLVEWTRPDMMDTVEVQVGLPK FKLEESLDLK SDFSGMSPNNDLVLSK AFVEVNEEGTEAAGATAAIMMMR**
5↓	Creatine kinase B-type isoform X2, Crt	6.7/60.7	**ILTPAIYER ELLDPIIEDR MSVEALDSLSGDLK GTGGVDTAAVGGTFDISNADR LGFSEVELVQMVVDGVK GQSIDDLMPAQK**
6↓	Elongation factor 2, Ef2	6.6/40.3	**AKPFPDGLAEDIEK EGVLCEENMR TAIVVAETR**
7	Apolipoprotein A-I-1 precursor, Apoa1-1	5.6/29.1	**AALNMYIAQVK SIDLLDDTEYK SIDLLDDTEYKEYK SLAPYTTVFGTQLADATATVR AKIEPVVEEMR IEPVVEEMR VAVNVEETK LMPIVEIVR LMPIVEIVR TLAAPYAEEYKEQMFK**
8↑	Triosephosphate isomerase, Tpi	7.6/28.8	IGVAAQNCYK **GGAFTGEISPAMIK** VVLAYEPVWAIGTGK **ANVSEAVANSVR DVDGFLVGGAALKPEFVDIINAK**
9↑	Flavin reductase, Flr	8.1/29.2	**TMQGQDAVIIILGTR LLPVTEDHDR ESGLDFVAVMPPHIDDNFPLTEK**

[a]Unique peptides are in bold; ↓ indicates underexpression and ↑ indicates overexpression in the algal groups compared to the control group

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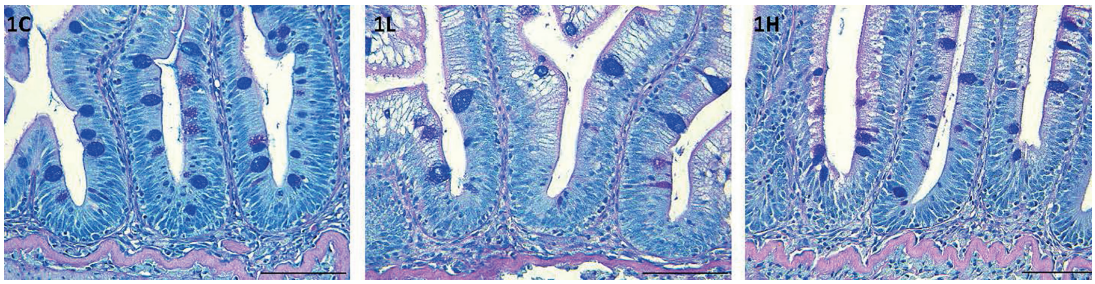

Fig 4. Photomicrographs of the distal intestine of 1C, 1L and 1H groups at the end of the 84-days feeding period. Scale bar: 50μm.

https://doi.org/10.1371/journal.pone.0179907.g004

alga feeds may explain the lower weight gain despite the higher feed intake. Some authors have reported similar reductions in nutrient digestibilities upon microalgal feeding [28, 29], while higher microalgal nutrient digestibilities have also been recorded [38]. The digestibility of DM and protein in feeds with 20% *N. oceania* is comparable to those obtained by Gong et al. [14], who estimated digestibility of DM and protein of *Nannochloropsis* sp. to be 63% and 72%, respectively. These values are significantly lower than digestibility values of fishmeal fed to salmonids [37, 39, 40], but greater than the protein digestibility (36%) of *Nannochloropsis* in mink [16]. The ADC of lipid and energy showed greater reduction with algae inclusion in the feed compared to the protein. In keeping with these observations, reduced lipid digestibility was reported at 6% inclusion of *Schizohytrium* sp. [27] and 10% inclusion of the yeast *Yarrowia lipolytica* [26]. The reduction in digestibility of lipid and energy in the present study could be due to the high content of complex indigestible cell wall carbohydrates in the microalga [41]. Fish has a limited capacity to digest carbohydrates, in particular the indigestible non-starch polysaccharides [42, 43]. Physical treatment including disruption of cell walls in biomass from *Y. lipolytica* caused significantly improved nutrient utilization by Atlantic salmon [44]. Improved ash digestibility noted in the present study is in line with our earlier findings [14]. Interestingly, these findings suggest improved utilization of minerals in the *N. oceania*-incorporated feeds.

Protein retention in Atlantic salmon in the present experiment was lower compared to that reported by Hatlen et al. [26]. Energy retention in fish fed the control feed was similar to that in the above publication, but in our case the values decreased with increasing intake of the alga. These findings suggest that protein and energy in defatted *N. oceania* are less utilized compared to other single cell protein sources such as the yeast *Y. lipolytica* [26].

Changes in biochemical composition of fish

Neither the whole body, nor fillet proximate composition of Atlantic salmon was affected by the intake of the alga meal. The proximate composition varies with life stages of the fish and is also influenced by endogenous factors such as genetics, size and sex, as well as exogenous factors such as feed composition, feeding frequency and environment [45]. The lipid content of the experimental fish was in the same range as that of similar sized salmon [15, 46]. The higher protein and lower lipid content in fillet compared to whole body are noteworthy results. Another interesting finding is the 19% reduction in lipid content in the fillet of the final samples. This finding was unexpected because of the high correlation between lipid content and size of fish [45]. However, similar results were obtained in other studies too–experiments with

Atlantic salmon that were fed *Desmodesmus* [22], trials on Atlantic salmon and common carp fed *Nanofrustulum* [12]. Alne et al. [46] reported that the feeding rate, growth rate and feed utilization of S0 smolt (transferred to sea 8–10 months after hatching) were reduced compared to the performance of S1 smolt that were transferred to sea in the following spring. Thus the CF, muscle fat and retention of energy of S0 smolts were lower compared to S1 smolts. The smolt used in the present experiment was S0 and the drop in lipid content may be explained by physiological changes taking place in the fish related to season. Furthermore, in our case the feed intake of the 1H group was significantly higher than that of the 1C group.

General physiological status

The organosomatic indices HSI and VSI of the fish fed on feeds with and without alga meal did not vary significantly, and the values were comparable to that reported for Atlantic salmon fed the microalga *Desmodesmus* [22]. *Schizochytrium limacinum* also did not alter the HSI of longfin yellowtail *Seriola rivoliana* [47]. However, previous studies have reported that *Spirulina* feeding can elevate the VSI levels in sturgeon *Acipenser baeri* and parrot fish *Oplegnathus fasciatus* [48, 49].

The increased hematocrit value for the alga-fed groups indicate a positive effect of *Nannochloropsis*. Similar increase was noted in young rockfish, *Sebastes schlegeli*, fed sea mustard (*Undaria pinnatifida*) [50]. A non-significant increase in hematocrit values was also noted in red sea bream, *Pagrus major*, fed *Ulva pertusa* meal [51]. The high oxygen demand to metabolize large amounts of feeds ingested by the 1H group might be the reason for this increase in hematocrit levels. Hematocrit values in fish are tightly associated with environmental parameters such as temperature and oxygen concentration in the rearing water. However, we do not expect the influence of these factors as the fish groups were maintained under identical controlled conditions.

The antioxidant status was determined to understand the alterations in the physiological capacities of the fish. The serum SOD activity in the fish fed on 10% alga-containing feed was higher compared to that in the fish fed on alga-devoid feeds. On the contrary, such an increase was not detected in the fish fed on 20% alga-containing feeds. In our earlier study on *Desmodesmus*, a similar trend in SOD activities was noted [22]. Furthermore, the mRNA level of *sod* was apparently higher in the 10% alga-fed group compared to the level in the 20% alga-fed group. The increased SOD activity may indicate improved antioxidant defence in fish receiving moderate amounts of the alga meal, but this has to be verified through additional investigations.

Intestinal health condition

In order to confirm the suitability of the alga meal as a feed component, it is necessary to evaluate the intestinal health of the fish through morphological and molecular observations. We examined the expression of selected marker genes related to inflammation and intestinal immune system. Among those studied, the level of the pro-inflammatory gene *il17d* in the 10% alga fed group was apparently higher compared to the level in the control group. However, there were no signs of distal intestinal inflammation in the alga-fed groups in this study, as well as in our previous study [22] using *Desmodesmus*. In inflamed distal intestine of Atlantic salmon, the mRNA levels of *igt* were apparently higher [24], but in the present study the gene expression in the fish from different groups were similar. These findings, taken together, suggest that the microalgal biomass tested do not induce inflammatory reactions in the distal intestine of the fish. Plant ingredients in feeds can trigger inflammatory reactions and aberrations in the distal intestinal structure of Atlantic salmon [52–54]. The n-6 fatty acids in plant-

derived feed ingredients can cause intestinal inflammation, and the mid-intestinal folds of Atlantic salmon were shortened by feeding with olive oil, rapeseed oil or soybean oil [55].

To further elucidate the effect of the algal product, we compared the distal intestinal proteomes of salmon that received the different experimental feeds. Nine of the identified proteins were impacted by the alga feeding. This included the protein Apoa1 that has antimicrobial properties in fish [56–58]. The protein was underexpressed in the 1H group when compared to its expression in the 1L group, implying that higher inclusion of the alga meal may affect the defence mechanisms of the fish. Furthermore, we noticed the underexpression of the protein Ahsg in the 1H group. The reduction in levels of the glycoprotein AHSG in the serum of protein-energy-malnourished children was linked to stunted growth, and compromised defence ability [59]. The low Ahsg expression coincided with the lower growth in the 1H group. In our previous report too, feeding *Desmodesmus* led to the underexpression of Ahsg in the distal intestine of Atlantic salmon [22].

Two energy metabolism-related proteins were overexpressed (Flr and Tpi) and one was underexpressed (Ckt) in the distal intestine of the 1L group. The overexpression of Flr and Tpi and the underexpression of Ckt may have benefitted the 1L group. It should be noted that in the 1H group, the lipid and energy retention was significantly lower compared to those of the control group. Dld which is also associated with energy metabolism was significantly overexpressed in the 1H group. On the contrary, lower energy digestibility was associated with the underexpression of Dld when Atlantic salmon was fed on *Desmodesmus* [22].

Two other proteins—Ef2, Lei—were underexpressed and a third one—P5cdh—was overexpressed in the distal intestine of the 1H group. P5CDH is one of the two mitochondrial enzymes that helps the oxidation of proline to glutamate leading to an increase in intracellular reactive oxygen species [60]. Eukaryotic elongation factor 2 (eEF2) mediates the GTP-dependent movement of the ribosome during protein synthesis [61], and increase in oxidative stress has been correlated to decrease in eEF2 [62]. The overexpression of P5cdh and underexpression of Ef2 in the IH group could be pointing to oxidative stress in the fish. This group also had apparently lower SOD activity levels and lower *sod* expression. Leukocyte elastase inhibitor (LEI), also called serpin B1, is a member of the serine protease inhibitors [63]. It is reported that during wound healing LEI expression is increased [64, 65]. Although the Lei-like protein was overexpressed in the distal intestine of the 1H group, we did not observe intestinal damage.

Conclusions

The results indicate that the defatted microalgae *N. oceania* can be used at modest inclusion levels–a level close to 10%–without negative effects on weight gain and specific growth rate and health parameters.

Supporting information

S1 Table. Proximate compostition of defatted microalgae biomass used in feed.
(DOCX)

S1 Fig. The design of the experimental fish tank.
(TIFF)

S2 Fig. The volumes of the differentially expressed proteins in the 2-DE gels. * Different letters above the bar graphs indicate statistically significant differences. Values are presented as mean ± SEM.
(TIF)

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Funding acquisition: Mark Huntley, Viswanath Kiron.

Investigation: Mette Sørensen, Yangyang Gong, Fridrik Bjarnason, Ghana K. Vasanth, Dalia Dahle, Viswanath Kiron.

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Visualization: Mette Sørensen, Yangyang Gong, Fridrik Bjarnason, Viswanath Kiron.

Writing – original draft: Mette Sørensen, Yangyang Gong, Fridrik Bjarnason, Viswanath Kiron.

Writing – review & editing: Mette Sørensen, Mark Huntley, Viswanath Kiron.

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Supporting information

S1 Table. Proximate composition of defatted microalgae biomass used in feed.

S1 Fig. The design of the experimental fish tank.

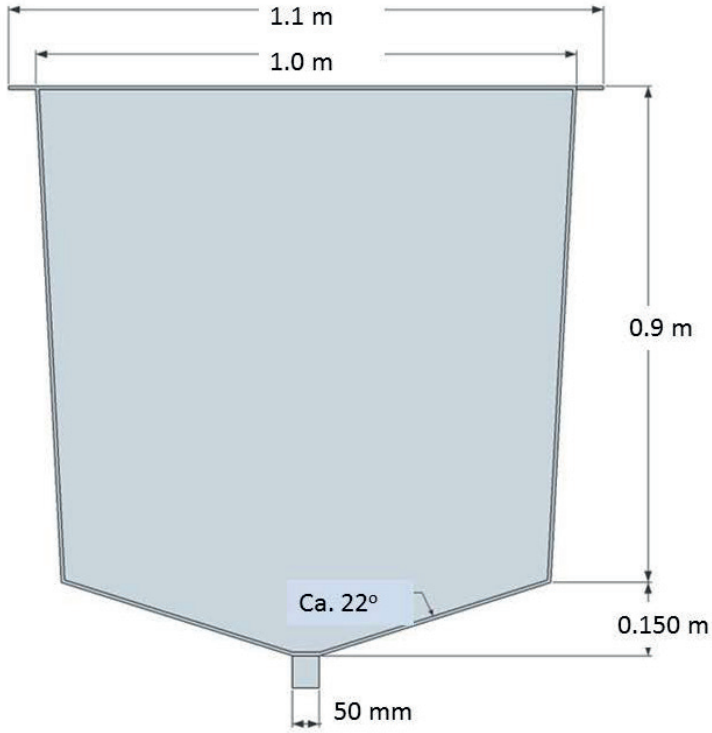
S2 Fig. The volumes of the differentially expressed proteins in the 2-DE gels.

S1 Table. Proximate composition of defatted microalgae biomass used in feed.

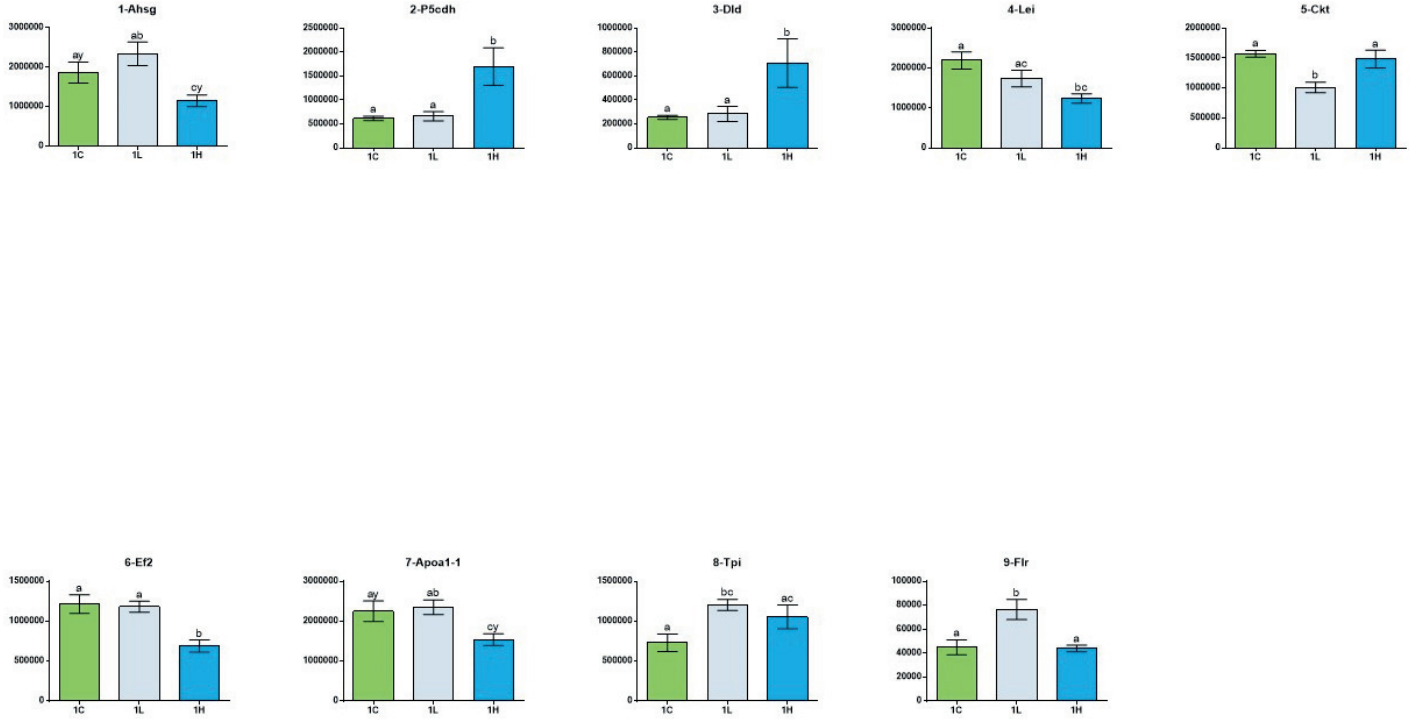
Content[1]	
Moisture	2.2
In dry matter (g/100g)	
Crude protein	43.0
Crude lipid	2.5
Ash	23.5
Carbohydrate[2]	28.8
Energy KJ g^{-1}	19.0

[1] Analyses of 4 samples of defatted algae biomass.

[2]Carbohydrates were calculated by differences (100-moisture-crude protein-crude lipid-ash)



S1 Fig. The design of the experimental fish tank.



S2 Fig. The volumes of the differentially expressed proteins in the 2-DE gels.

* Different letters above the bar graphs indicate statistically significant differences. Values are presented as mean ± SEM.

Paper III

Microalgae *Scenedesmus* sp. as a potential ingredient in low fishmeal diets for Atlantic salmon (*Salmo salar* L.)

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Abstract

Salmonid feeds can be formulated with high quality microalgae to maintain sustainability in the aquaculture industry. But, the suitability of different microalgae species as potential feed ingredients needs to be documented to enable ready acceptance by the farming industry. The aim of the present study is to investigate the potential of the microalga *Scenedesmus* sp. as a major ingredient in low fishmeal feeds of Atlantic salmon. Three feeds were formulated with *Scenedesmus/*fishmeal, at inclusion levels of 0/10, 10/5 and 20/2.5% (CT, SCE 10 and SCE 20, respectively); to investigate the effect of the ingredient on the weight gain, growth rate, feed conversion ratio, nutrient retention and chemical composition and nutrient digestibility in Atlantic salmon. In addition, the physical characteristics of feeds were investigated to assess the impact of the alga-incorporation on the quality of the feeds. Fish (initial average weight of 229 g) in 6 replicate tanks were fed one of the experimental feeds for 65 days. The results showed that fish fed SCE 20 had significantly lower weight gain, specific growth rate, thermal growth coefficient and feed conversion ratio than the CT group which did not receive the microalga. Furthermore, the condition factor and protein efficiency ratio of the microalga-fed groups were lower than the CT group. Hepatosomatic and viscerosomatic indices of the groups did not differ significantly. Ash and protein content of whole fish fed SCE 20 were significantly higher, but dry matter, lipid, and energy of this group were lower than either the CT or the SCE 10 group. Retention of lipid and energy of all groups differed significantly, while that of protein was significantly different in the *Scenedesmus*-fed groups. Compared to the CT feed, digestibility of dry matter, protein, and energy in the algal feeds were significantly reduced. The highest fat leakage observed for the feed devoid of the alga and the hardness of the SCE 20 feed points to the better physical stability of the alga-containing feeds. Higher contents of n-3 fatty acids and PUFAs were found in the whole body of fish fed SCE 10. In conclusion, *Scenedesmus* sp. can be incorporated in low fishmeal diets for Atlantic salmon, at inclusion levels below 10%.

Keywords: Microalgae, *Scenedesmus* sp., Atlantic salmon, Apparent Nutrient Digestibility, Feed Conversion Ratio; Fatty Acid Composition

Introduction

Global aquaculture production is increasing year-over-year (FAO, 2018) and to sustain the trend in the future, industry should depend on high quality feed ingredients. Considering sustainability issues and high price of fishmeal and fish oil, the European fish feed industry has reduced its reliance on marine ingredients by employing more plant ingredients (Shepherd et al., 2017; Ytrestøyl et al., 2015). Consequently, feed sustainability measured in terms of fish in:fish out has improved considerably (Bendiksen et al., 2011; Crampton et al., 2010; Sanden et al., 2011; Ytrestøyl et al., 2015). According to Tacon and Metian (2015), more than 70% of the global aquaculture production depends on formulated feeds or feed input. The need for high quality ingredients will increase with the growth of the aquaculture sector. Therefore, future feed ingredients should be derived from sustainable and scalable sources. Furthermore, the use of plant ingredients should not jeopardize human food security. High levels of plant oils in feeds have changed the lipid profile in the flesh of farmed Atlantic salmon (Sprague et al., 2016). Since 2006, the contents of 18:2n-6 (linoleic acid, LA), 18:3n-6 (γ-linolenic acid, GLA) and C18:1n-9 (oleic acid, OA) has increased while C20:5n-3 (eicosapentaenoic acid, EPA) and C22:6n-3 (docosahexaenoic acid, DHA) have been reduced (Sprague et al., 2016). This reduction in EPA and DHA levels in the fish flesh is raising concerns about the nutritional benefits of Atlantic salmon. As microalgae are primary producers of EPA and DHA in the food web, there is increasing interest for their use in supplementing fish feeds (Kousoulaki et al., 2015; Sørensen et al., 2016).

Microalgae can play a pivotal role in both freshwater and marine aquaculture because they contain high quality protein and can accumulate EPA and DHA. All essential amino acids are present in microalgae, though the level of individual amino acids may vary with growth medium composition and environmental conditions (Brown, 1991; Safafar et al., 2016). Although strain- and species-specific variations in the fatty acid composition are evident, some microalgae may be promising sources of PUFA, especially EPA and DHA (Lang et al., 2011). *Scenedesmus* sp. is a commercially available microalga, and it is grown in photobioreactors. The content of protein, lipid and carbohydrate in *Scenedesmus obliqus* dry matter is in the range 50-56%, 12-14% and 10-17%, respectively (Becker, 2007). Palmitic acid (16:0), OA, LA and α-linolenic acid (18:3n-3, ALA) are the dominant fatty acids in *Scenedesmus* sp. (Tibbetts et al., 2015).

Growth, feed utilization and nutrient digestibility of carnivorous fish fed microalgae depends on the microalgal type (Burr et al., 2011; Gong et al., 2018; Kiron et al., 2016; Vizcaíno et al., 2014) as well as inclusion level (Sørensen et al., 2016; Sørensen et al., 2017). Therefore, the effects of potential fishmeal replacements have to be evaluated by conducting feeding and digestibility trials with candidate microalgae.

Sørensen et al. (2016, 2017) have already shown the potential of other microalgae as fishmeal replacements in feeds for Atlantic salmon. The aim of the present study was to investigate the weight gain, growth rate, feed conversion ratio, nutrient retention, chemical composition of whole body and nutrient digestibility of Atlantic salmon fed low fishmeal diets where microalga *Scenedesmus* partly replaced fishmeal, a mix of plant protein concentrates and wheat.

Material and methods

Experimental design and feeds

The feeding trial was approved by the National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway. The animal handling procedures were according to approved protocols.

The test microalgae *Scenedesmus* sp. (5.6% moisture, 45.7% protein, 9.1% fat, 15.8% fiber and 8.3% ash) used in the feeds was cultured in closed photobioreactors, dewatered by centrifugation and spray drying at Algafarm (Pataias, Portugal) and commercialized by Allmicroalgae – Natural Products® (Lisbon, Portugal). The study comprised three experimental diets: a control diet (CT) with a low level of fishmeal (10%) and relatively high levels of soy, pea and potato protein concentrates (1:1:1 blend), wheat gluten and corn gluten as major protein sources; a diet containing 10% *Scenedesmus* and 5% fishmeal (SCE 10); and a diet with 20% *Scenedesmus* and 2.5% fishmeal (SCE 20) (Table 1). In order to balance the protein, lipid, carbohydrates and energy contents of the feeds, the gradual increase of the microalgae incorporation level was made at the expenses of fishmeal, but implied also some minor changes on the level of the various plant protein sources and a pronounced reduction of wheat meal. In all diets, the major lipid source was a blend of fish oil and rapeseed oil (1:1). All diets were supplemented with crystalline amino acids (L-histidine and DL-methionine) and inorganic phosphate. Diets contained also 0.02% yttrium oxide as an inert marker for digestibility measurements.

The experimental extruded diets were manufactured by SPAROS LDA (Olhão, Portugal). All powder ingredients were mixed accordingly to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 400 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 3.0 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate (78 kg/h), screw speed (235 rpm), water addition (approximately 295 ml/min), temperature barrel 1 (28-31ºC), temperature barrel 3 (118-121°C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (700 mbar, for approximately 50 sec) (model PG-10VCLAB, Dinnissen, The Netherlands). Immediately after coating, diets were packed in sealed plastic buckets and shipped to the research site.

Fish and experimental set up

The experimental fish, Atlantic salmon (*Salmo salar*), were obtained from a commercial producer (Sundsfjord Smolt, Nygårdsjøen, Norway) and maintained at the Research Station, Nord University, Bodø, Norway for approximately 4 months before the start of the feeding trial. At the start of the experiment, a total number of 450 fish

(Initial weight 229 ± 3.8 g, total length 27.0 ± 0.2 cm) (mean ± SD) were randomly allocated to the experimental units (n = 6 tanks per treatment group) .

The feeding experiment was carried out in a flow-through system. In total, 18 circular fiberglass tanks (800 L and 0.9 m deep) were used for the study. Each tank was supplied with 740 L of water pumped from Saltenfjorden, from a depth of 250 m. During the experiment, water flow rate was maintained at 1000 L per hour, and the average temperature and salinity of the rearing water were 7.4 ± 0.1°C and 35 ‰, respectively. Oxygen saturation was always above 85% saturation measured at the water outlet. A 24-h photoperiod was maintained throughout the experimental period.

Feeding regime

The fish were fed ad libitum using automatic feeders (Arvo Tech, Finland); two feedings per day were administered from 08:00-09:00 in the morning and 14:00-15:00 in the afternoon. The fish was fed 10% in excess based on the feed intake of the previous week. Approximately 30 min before each feeding, all the tanks were flushed to remove faeces from the tanks and minimize the risk of contaminating uneaten feed with faeces. The uneaten feeds were removed from the feed collection traps of each tank shortly after every feed delivery. These leftover feeds were stored at -20°C and the amount gathered weekly was later oven-dried at 110°C to determine the dry matter that was used for calculating the actual feed consumption of the fish.

Fish sampling and data collection

At the beginning and end of the experiment, all fish (450) were individually weighed and their total lengths recorded. Before handling, fish were anesthetized using tricainemethanesulfonate (MS 222, 160 mg/L). From the initial stock, 6 fish were sampled to assess the initial chemical composition of the fish. Upon termination of the experiment, 6 fish per tank were pooled to assess the final chemical composition. These fish were packed in plastic bags, immediately frozen and kept at -20°C until analyses. The fecal matter from the remaining fish in the tanks was obtained by stripping individual fish. The fish and fecal samples were immediately transferred to -20 °C storage prior to further analyses.

Biochemical analyses

The frozen fish samples were thawed for approximately 24 h at 4^0C, and each fish was homogenized using an industrial food processor (Foss Tecator, 2096 homogenizer, Denmark) before analyzing the whole body chemical composition. Frozen fecal samples were freeze dried (VirTis benchtop, U.S.A.) for 72 h at -76°C and at a pressure of 20 bar. The freeze-dried fecal samples from two tanks of a particular feed group

were pooled prior to the analysis of their chemical composition. The chemical composition of the feed pellets was also determined.

The moisture, protein, ash, and energy contents of the fish, feed and freeze-dried faeces were determined as described below. Total dry matter content was determined by oven drying the samples at 105°C for 24 h until consistent results were obtained (ISO 6496-1999). Protein content was analyzed by using the Kjeldahl method (Kjeltech Auto Analyser, Tecator, Höganäs, Sweden, Crude protein = N × 6.25, ISO 5983–1987). Ash content was measured gravimetrically by combusting the samples using a flame at 550°C for 12-16 h until constant weights were registered (ISO 5984–2002). Energy content was measured using a bomb calorimeter (IKA, c200, GmbH & Co. KG, Germany) (ISO 9831–1998).

Total lipid content of the fish was determined by the ethyl-acetate extraction method. Eurofins® (Moss, Norway) analysed the total lipid content of the faeces, employing the Soxhlet method with acid hydrolysis (Soxtec HT 6209, Tecator, Höganäs, Sweden: modified AOAC method 954.020). Fatty acid composition of fish and feed was measured by gas chromatography (GC) of methyl-ester derivatives in the samples. For this, the homogenized samples were lyophilized for 72 h before the lipids were extracted and analyzed in duplicate. Total lipid from the samples was extracted based on the method of Bligh and Dyer (1959). The fatty acid methyl esters (FAMEs) were prepared following the AOCS Official Method Ce 1b-89. FAMEs were separated and quantitated using a Scion 436 GC (Bruker, USA) equipped with a flame ionization detector, a splitless injector and a DB-23 column (Agilent Technologies, USA). Standard mixtures of FAMEs were used for identification and quantitation of common fatty acids in samples (GLC-473, Nu-Chek Prep, Elysian, MN, USA). Yttrium contents in both faeces and feeds were analyzed by Eurofins (Moss, Norway) as described by Sørensen et al. (2016).

Physical quality of feed

The method described by Sørensen et al. (2011) was employed to analyze susceptibility of pellets to leak fat, which may reduce the nutrient quality of feeds during storage or in automatic feeders.

Pellet hardness was determined by using TA-XT2 analyzer (Stable Micro Systems Ltd, Surrey, England). Feed pellets (n = 120) from a particular feed group were randomly selected and their hardness values were determined in 6 replicates (20 pellets per replicate). Each pellet was placed horizontally and hardness was measured using a cylindrical probe (SMP/0.5, 1.2 cm width) at 60% compression rate and at a velocity of 1 mm sec^{-1}. Hardness value was registered in Newtons (N), as the peak force during the first compression.

Pellet length was measured using Vernier caliper (Biltema® Art. 16-105). 120 feed pellets from each feed group were randomly selected and analyzed in 6 replicates (20 pellets per replicate). Pellet diameter was measured using a TA-XT2 analyzer (Stable Micro Systems Ltd, Surrey, England).

To determine the physical stability of the feeds in water, pellet samples from each feed group were placed into a pre-weighed embedding cassette (M 512 Macrosette™, Simport®, Canada, 40.1 x 28.5 x 13 mm). Briefly, 3 g of pellets were incubated in a water bath (Julabo™, SW22, Seelbach, Germany) at 25°C. Four shaking regimes were employed to determine the pellet stability: 100 shakings of the cassette per minute over 15, 30, 45 and 60 min. The test was carried out in 6 replicates for each treatment. After incubation, cassettes were placed on tissue paper and gently dried and placed in a pre-heated oven at 80°C for 48 h. Residual dry matter weight of each cassette was determined after drying. The weight difference of dry matter before and after incubation, divided by dry matter weight of the feeds before incubation was calculated to determine the pellet stability.

Calculations and statistical analysis

Fish growth performance was analyzed using the following equations.

$$\text{Weight gain (\%)} = \left(\frac{W_f - W_i}{W_i}\right) \times 100$$

Where, W_f = final body weight of fish (g/fish) and W_i = initial body weight of fish (g/fish)

$$\text{Specific Growth Rate (\% day}^{-1}) = \left(\frac{\text{Ln}\,(W_f) - \text{Ln}\,(W_i)}{\text{No. of feeding days}}\right) \times 100$$

$$\text{Feed intake (\% BW day}^{-1}) = \frac{\text{Daily feed intake in dry basis (g)}}{\sqrt{W_f \times W_i}} \times 100$$

$$\text{Feed conversition ratio (FCR)} = \frac{\text{Total feed intake in dry basis (g)}}{\text{Weight gain (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Weight gain (g)}}{\text{Total protein ingested (g)}}$$

$$\text{Thermal growth coefficient (TGC)} = \frac{(W_f)^{1/3} - (W_i)^{1/3}}{(T \times d)} \times 1000$$

where T is the temperature in °C and d is time in days.

$$\text{Hepato} - \text{somatic index (\%)} = \frac{\text{Liver weight of fish (g)}}{W_f\,(g)} \times 100$$

$$\text{Viscero} - \text{Somatic Index (\%)} = \frac{\text{Visceral Weight (g)}}{W_f\,(g)} \times 100$$

$$\text{Condition factor (g/cm}^3) = \frac{W_f(g)}{FL^3\,(cm)} \times 100$$

where FL (cm) = Fork length of fish

Apparent Digestibility Coefficient (ADC) and nutrient and energy retention were calculated according to following equations.

$$\text{ADC}\ (\%) = [1 - \frac{(\text{marker in feed}\ \times \text{nutrient in faeces})}{(\text{marker in the faeces}\ \times \text{nutrient in feeds})}] \times 100$$

$$\text{Nutrient (or Energy) retention efficiency}(\%) = \frac{(W_f \times N_f\,(\text{or}\ E_f\ ) - W_i \times N_i\,(\text{or}\ E_i\ )}{\text{NI (or EI)}} \times 100$$

where N_f=final nutrient content of the body; N_i=initial Nutrient content of the body, E_i=Initial Energy content of the body, E_f=Final Energy content of the body, NI=Nutrient intake or EI=Energy Intake. Retention of a digested nutrient was calculated based on values for each tank:

$$\text{Nutrient (or Energy) retention efficiency}_{\text{digested}}(\%) = \frac{\text{Nutrient (or Energy) retention}(\%)}{\text{ADC}(\%)} \times 100$$

In this study, tank was used as the experimental unit. Statistical analyses were performed by using R v3.3.1 (R Development Core Team, 2016), employing packages stats v3.5.0 (R Development Core Team, 2016) and dunn.test package (v1.3.5) (Dinno, 2016). Data were checked for normality by the Kolmogorov-Smirnov test. For parametric data, one way analysis of variance (ANOVA) was performed after checking for equal variance using Bartlett's test. Tukey's multiple comparison test was used to identify the significant differences among the means of the 3 groups. For non-parametric data, the Kruskal-Wallis test, followed by Dunn's multiple comparison test, was performed to decipher the significant differences between the groups. A significance level of $p<0.05$ was chosen to indicate the differences.

Results

Chemical composition and quality of pellets

The chemical composition of the feeds (dry matter basis) is given in Table 2. Fatty acid composition of the feeds is provided in Table 3. Palmitic acid in the feed increased with increasing inclusion of *Scenedesmus*. The fatty acids α-Linolenic acid (ALA) and Eicosapentaenoic acid (EPA) were higher in the SCE 20 feed while Docosahexaenoic acid (DHA) decreased with increasing inclusion of the alga.

Physical characteristics of the experimental feeds are given in Table 4. The color of the CT feed was light-brown, and those of the alga-incorporated feeds were light (SCE 10) and dark black (SCE 20) (Figure 1). Fat leakage was least from the SCE 20, though this feed appeared to have an oilier surface than the other feeds. Hardness values of the feeds varied from approximately 23 to 40 N. The SCE 20 had significantly higher hardness, while no differences were noted between CT and SCE 10. Length of pellets varied from 4.1 to 4.4 mm. The SCE 20 feed had significantly shorter pellets than CT, while SCE 10 tended to be longer than the SCE 20 but shorter than the CT.

Results of the pellet stability test are shown in Figure 2. The lowest stability ($P<0.05$) was observed for the CT feed compared with the SCE 10 and SCE 20 at 15, 45 and 60 min. The stability of SCE 10 and SCE 20 were not significantly different.

Apparent digestibility coefficients (ADC)

Digestibility values of dry matter, lipid, and energy decreased with increasing inclusion level of the alga-fed groups (SCE 10 and SCE 20) were significantly different compared to the CT group (Table 5). Digestibility of protein in fish fed the SCE 20 was significantly lower compared to the CT group, but no significant differences were noted between SCE 10 and the CT group. ADCs of ash of all three groups were negative but increasing the inclusion of microalgae did not make the values significantly different.

Growth performance

The weight gain, growth rate, feed intake, feed conversion ratio, protein efficiency ratio, and condition indices (condition factor and somatic indices) are given in Table 6. The fish grew from an initial average weight of 229.1 g to a final average body weight of 447.0 g during the experimental period of 85 days. Significant reduction in the final mean body weight, weight gain, specific growth rate, and thermal growth coefficient was noted in fish fed the SCE 20, compared to the fish in the CT group. No differences in feed intake were found among dietary treatments. Feed conversion ratios of the fish fed the algae feeds were poorer than the control group. As for the protein efficiency ratio, fish fed the CT feed had higher values than groups fed SCE 10 and SCE 20. Condition factor was significantly higher in fish fed the control feed than fish fed the

Scenedesmus-incorporated feeds. No significant differences were recorded between the hepato-somatic and viscero somatic indices of the three study groups.

Nutrient retention

Retention efficiency of lipid, protein, and energy is given in Table 7. Retention efficiency of lipid in the three feed groups differed significantly, with the highest retention detected in fish fed the CT feed and lowest in those fed the SCE 20 feed. Fish fed the SCE 20 also showed significantly lower retention of protein and energy compared to CT fed groups, while fish fed SCE 10 tended to have values lower than the CT group, but higher than the SCE 20 group. Retention efficiency of digested lipid differed significantly, and the lowest value was found in the SCE 20 group and highest in the CT group. The retention efficiency of digested protein and energy of the SCE 20 group was lower than that of the CT group. No differences in retention efficiency of digested protein and energy were noted for the fish fed CT vs. SCE 10.

Chemical composition of fish

The chemical composition of fish from the initial population and those sampled at the termination of the experiment are presented in Table 8. Values from the initial population were excluded from the statistical analysis.

At the end of the experimental period, protein was highest in fish fed SCE 20 and lowest in those fed SCE 10, while the lipid content was significantly lower in fish fed SCE 20 compared with the other two groups. The ash content was significantly lower in the CT fed fish and highest in fish fed SCE 20, and the energy was significantly higher in CT and lowest in fish fed SCE 20.

Fatty acid composition of whole body

Fatty acid composition of the whole body is given in Table 9. The saturated fatty acids (ΣSFAs) tended (P=0.092) to decrease with increasing inclusion level of algae in the feeds, though significant reduction was observed for stearic acid, C18:0. Monounsaturated fatty acids were not significantly different among feed groups. Linoleic acid (LA), C18:2 n-6 dominated the n-6 fatty acids and LA was lower in fish fed the CT feeds than those fed the algal feeds, but a significant difference was noted only between the CT and SCE 10. The ALA (P=0.050), EPA (P=0.070) and DHA (P=0.097) were higher in fish fed *Scenedesmus*-containing feeds compared to those on the control feed. This resulted in an overall higher content of Σn-3 PUFAs and ΣPUFAs in the whole body of fish fed algae feeds, though significantly higher content was noted only for those fed SCE 10.

Discussion

Experimental feeds

Most studies performed to investigate the suitability of microalgae for Atlantic salmon have employed high fishmeal feeds (Kiron et al., 2012; Kiron et al., 2016; Kousoulaki et al., 2016; Kousoulaki et al., 2015; Sørensen et al., 2016; Sørensen et al., 2017). The present study was designed to investigate the potential of the microalga *Scenedesmus* sp. in high plant protein–low fishmeal feeds The fishmeal inclusion level in the control feed of the present experiment was based on an earlier study in which Atlantic salmon grew from 137 g to approximately 400 g on feeds containing 10 or 30% fishmeal (Kousoulaki et al., 2009). Although the authors did not observe any differences in weight gain or feed utilization they emphasized the importance of the quality of the fishmeal when its inclusion level is low (Kousoulaki et al., 2009). Later studies with rainbow trout have shown that marine protein ingredients (krill products) can be incorporated at 5% level, but to avoid negative effects on growth and feed utilization the protein quality must be secured by supplementation of amino acids (Zhang et al., 2012). In the present experiment, we have seen a nonsignificant reduction in growth and feed utilization in SCE 10 compared to the control group. Reducing fishmeal to 2.5% in combination with 20% of the microalga *Scenedesmus* sp. significantly compromised growth and feed utilization compared to fish fed the SCE 20.

Protein content of the microalga was lower while lipid content was comparable to a high-quality fishmeal. To balance the lipid component, fish oil and rapeseed oil were slightly reduced with the incorporation of the microalga in the feed. Palmitic acid, oleic acid, LA and ALA are the dominant fatty acids in *Scenedesmus* sp. (Tibbetts et al., 2015). The fatty acid composition of the experimental feeds was mainly reflected by the composition of fish oil and rapeseed oil, but LA and ALA content were slightly higher in the SCF 20 feeds.

The differences in the pellet quality observed in the present study could be due to the ingredients and processing parameters in the extrusion process, as reported by Sørensen (2012). Furthermore, Samuelsen et al. (2018) has indicated that for better extruder performance, feed hardness and durability, the optimal inclusion level of high lipid microalgae such as *Schizochytrium* sp. is 13.2%. Fat leakage was higher in the CT feeds; this can possibly be explained by the microstructure and the ingredient composition of the feed. Earlier studies have indicated that different pellet microstructure is dependent on the feed ingredients (Draganovic et al., 2013; Sørensen et al., 2009).

Hardness values observed in the present experiment were higher than those recorded by Morken et al. (2012), but lower than the values reported by Samuelsen et al. (2018). The hardness of the pellets is positively correlated with pellet diameter (Samuelsen et al., 2018). Diameter of the pellets from the different feed types used in

the present experiment were similar, but was lower than those employed in other studies, e.g. 8-11 mm (Samuelsen et al., 2018). The hardness of pellets may be affected by the functional components such as carbohydrate fractions, starch source, amount of starch, as well as the type of the plant protein ingredients in the feeds (Sørensen, 2012). Although the starch and non-starch polysaccharides contents were not analyzed in the experimental diets, the content and composition probably varied widely. Increasing the content of non-starch polysaccharides result in harder pellets (Hansen and Storebakken, 2007; Sørensen et al., 2011).

The stability of the CT feeds was lower than the SCE 10 and SCE 20 feeds, at all the assessed time points, except for 30 min. Water stability values recorded in the present study were higher than those reported by Aas et al. (2011). Higher pellet stability has been associated with reduced feed intake in rainbow trout (Aas et al., 2011). However, in the present experiment we did not observe any significant differences in feed intake.

Apparent digestibility coefficients

In general, with the incorporation of the microalga the ADC values of dry matter, protein and lipid were reduced significantly. The results are in line with findings reported for Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) fed diets containing 30% *Scenedesmus dimorphus* (Teuling et al., 2017). Overall, the ADC values of protein were lower than those reported for 10 and 20% incorporation of *Desmodesmus* sp. (Kiron et al., 2016) or *Nannochloropsis oceania* (Sørensen et al., 2017) in feeds for Atlantic salmon. Lipid digestibility was also lower in the present study than that reported by Kiron et al. (2016) and Sørensen et al. (2017). Therefore, nutrient digestibility, depends on the microalgal type. The variation in ADC values of protein, lipid and energy of different microalgae species was reported earlier by us and others (Gong et al., 2018; Skrede et al., 2011; Teuling et al., 2017).

The microalgae used in the present study were centrifuged and spray-dried without any further processing. The cell walls of the alga were assumed to be more intact, in contrast to the oil-extracted microalgae biomass used in the studies of Kiron et al. (2016) and Sørensen et al. (2017). This could be one reason for the lower nutrient digestibility recorded in this study compared to our above-mentioned studies. Teuling et al. (2017) reported that 10 min bead milling of *Scenedesmus*, *Chlorella* and *Nannochloropsis* can disrupt 11-39% of the algal cell walls and significantly increase the soluble protein fraction of the algae, which in turn is likely to improve protein digestibility. Teuling et al. (2019) confirmed that there is a high correlation between nutrient digestibility and the accessibility of nutrients from the microalga *Nannochloropsis gaditana* by Nile tilapia. The authors also observed different effects on cell wall integrity and digestibility by using various pre-treatments. The difference in digestibility of the *Desmodesmus* sp. (Kiron et al., 2016) and *N. oceania* (Sørensen et al.,

2017) and the *Scenedesmus* sp. in the present experiment could be attributed to the discrepancies in pretreatment-induced nutrient availability.

The negative digestibility of ash may be associated with drinking of seawater (Thodesen et al., 2001). The digestibility value of the hardest feed in the present study, SCE 20 feed decreased further compared to the CT feed. Gastro-evacuation time for pellets with higher value for hardness or water stability will be longer (Aas et al., 2011), and during such circumstances, fish may drink seawater to soften the pellets or prevent dehydration (Sørensen et al., 2016). This results in high intake of elements present in seawater. The ash digestibility values were lower than those reported by Sørensen et al. (2016, 2017).

Growth Performance of the fish

There were no mortalities during the course of the experiment and the fish performed well. The present findings suggest that in spite of relatively low levels of fishmeal in the experimental diets (2.5-10%), the overall growth performance and feed utilization were similar to those reported by Kiron et al. (2016), or even better compared to Atlantic salmon of comparable size fed fishmeal-based feeds (Sørensen et al., 2017). However, inclusion of *Scenedesmus* up to 20% in the 2.5% fishmeal diet could not sustain the growth and feed utilization of fish. Feeding Atlantic salmon with 20% *Desomdesmus* sp. (Kiron et al., 2016) or 10% defatted *Nannochloropsis oceania* (Sørensen et al., 2017) had no negative effect on final mean body weight, weight gain, specific growth rate, and thermal growth coefficient – in these studies fishmeal inclusion level was 10%. On the other hand, weight gain and specific growth rate of Atlantic salmon were negatively affected when fish were fed 11% *Schyzochrytrium* sp. (Sprague et al., 2016) or 12% *Phaeodactylum tricornutum* (Sørensen et al., 2016). The responses, however, also depend on fish size, microalgae species, ingredient and chemical composition of feeds, as well as the nutrient digestibility and physical quality (e.g. hardness) of feeds (Glencross et al., 2007).

Feed conversion ratio recorded in the present experiment was in line with the results of Kiron et al. (2016). Fish fed the SCE10 and SCE 20 feed had significantly higher feed conversion ratio compared with the CT group, but lower than the values reported by both Burr et al. (2012) and Sprague et al. (2015). Poor feed conversion ratio recorded for the SCE 10 and SCE 20 feed may indicate lower bioavailability of nutrients from the microalga compared with the CT feed. However, feed intake of all the study groups was not significantly different, suggesting that incorporation of the microalga had no negative effect on palatability. In contrast to our findings, Palmegiano et al. (2009) reported increased feed intake and improved feed conversion ratio when 70% *Isochryris* sp. was fed to gilthead sea bream (*Sparus aurata*) juveniles.

Condition indices are used to evaluate the general well-being or fitness of fishes (Bolger and Connolly, 1989). Condition indices were not affected in the present study; this result is corroborated by the study of Vizcaíno et al. (2014), in which the authors fed gilthead sea bream (*Sparus aurata*) 12 and 20% *Scenedesmus almeriensis*.

Protein efficiency ratio was significantly lower in the algae-fed fish compared to the fish fed the CT feed. However, values were within the 2-2.7% range reported in other studies in which Atlantic salmon were fed microalgae-incorporated feeds (Kiron et al., 2012; Kiron et al., 2016; Norambuena et al., 2015). The reduced protein efficiency ratio obtained in our study could be due to the low bioavailability of nutrients from the microalgal feeds.

Energy and nutrient retention efficiency

Protein, lipid, and energy retention efficiencies were reduced in fish fed the microalga-containing feed; protein and lipid values in the present experiment were higher than those reported by Sørensen et al. (2016) and Aas et al. (2015). Energy retention efficiency was in line with values (42-50%) reported by Sørensen et al. (2016). The reduced retention of digested lipid and protein from the diet SCE 20 indicates that the utilization of lipid and protein from the microalga might be lower than that from LT fishmeal and other high quality plant ingredients.

Chemical composition of the fish

Earlier studies have reported changes in the chemical composition of fish fed microalgae feeds (Dallaire et al., 2007; Mustafa et al., 1994). Although weight gain, protein efficiency ratio as well as protein retention of fish fed the SCE 20 feed was lower compared to the other study groups, whole body protein content was high in this fish group. As for the whole body lipid content, the apparently higher ($p>0.05$) values observed in fish fed the SCE 10 feeds cannot be explained based on the feed lipid content, as reported by others (Dallaire et al., 2007; Watanabe, 1982). The lower lipid content in fish fed the SCE 20 feed can be explained by lower utilization of energy. Consequently, only marginal differences were observed in whole body energy level of the feed groups. Whole body lipid content of fish in the present study was higher than values (29-32%) reported for Atlantic salmon fed microalgae feed (Kiron et al., 2012; Kiron et al., 2016; Norambuena et al., 2015; Sørensen et al., 2016).

The ash content of fish in the present study was in line with the value reported for fish fed with microalgae (Kiron et al., 2016). The non-significant higher whole-body ash values observed in the algae fed fish were noteworthy and suggest improved utilization of the elements in fish fed the algae incorporated feeds.

In spite of the low fishmeal level and 50% replacement of fish oil with rapeseed oil, the calculated content of EPA + DHA was 2.6%, 2.7% and 2.0% of the CT, SCE 10 and

SCE 20 feeds, respectively. These levels are in the nutritional requirement range recently suggested by Bou et al. (2017a, 2017b, 2017c) . When Atlantic salmon are fed feeds devoid of fishmeal or fish oil, the requirement of 1% EPA + DHA (National Research Council, 2011) seems to be too low. The significantly increased contents of LA, ALA and EPA in the whole body of fish fed SCE 10 feed points to an improved utilization and deposition of fatty acids. However, higher incorporation of the microalgae did not result in any significant differences in the fatty acids. In salmonid fish, the fatty acid composition of the flesh is closely related to the composition in feed (Sprague et al., 2016; Teimouri et al., 2016). The increased Σn-6 FAs content in whole body of fish fed *Scenedesmus* feed was mainly attributed to the higher content of LA in the feed. The increase in Σn-3 FAs and ΣPUFA observed in fish fed the SCE 10 feeds is also noteworthy. The modest increase in whole body EPA and DHA, in spite of reduced content of DHA in the SCE 10 and SCE 20, may have been stimulated by slightly higher LA and ALA in the microalgae. The pathways are well known for the endogenous production of EPA and DHA from n-3 or n-6 C_{18} PUFA (Tocher, 2015). Earlier studies have shown that substrate-specific acyl elongases and desaturases can be modulated by the dietary fatty acid composition to stimulate the production of EPA and DHA from ALA (Tocher et al., 2003; Zheng et al., 2005). Furthermore, it has been shown that high levels of dietary EPA and in particular DHA reduce endogenous production of EPA and DHA (Bou et al., 2017a; Thomassen et al., 2012). The CT-fed fish had lower EPA and DHA content while the microalga-fed fish had similar or higher values compared to the initial EPA and DHA content. The tendency of increased EPA and DHA content as well as increased PUFA contents of Atlantic salmon induced by an ingredient such as *Scenedesmus* sp. is favorable from a nutritional point of view.

Conclusion

The present study indicates that incorporation of microalgae *Scenedesmus* sp. of up to 10% in low fishmeal diet did not affect the feed intake, growth and chemical composition of salmon. However, the inclusion of the microalga, particularly at 20% in low fishmeal diets, significantly reduced the digestibility, nutrient retention efficiency and feed conversion ratio in Atlantic salmon. *Scenedesmus* sp. at 10% in the diet improved the total n-3 PUFAs and total PUFAs content in salmon. Inclusion of the microalga up to 10% also did not significantly alter the physical quality of the diet.

The microalga *Scenedesmus* has the potential to be used as feed ingredient in diets for Atlantic salmon. However, novel, cost-effective methods for cell wall destruction may be essential for increasing the bioavailability of nutrients.

Declarations

Abbreviations

CT – Control group
DHA - Docosahexaenoic Acid
EPA – Eicosapentaenoic Acid
FAO – Food and Agricultural Organization of the United Nations
IFFO – The Marine Ingredients Association
PUFA – Poly Unsaturated Fatty Acid
SCE 10 – Low alga group
SCE 20 – High alga group
SFA – Saturated fatty acids

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Availability of data and materials

All the data are presented in the article. Any additional information required from the authors will be available upon request.

Author's contributions

Yangyang Gong: Execution; Investigation; Methodology; Writing original draft

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Mark Huntley: Conception; Project administration; Review and editing
Zackary Johnson: Conception; Project administration; Review and editing
Jorge Dias: Methodology; Review and editing
Mette Sørensen: Conception; Design of experiment; Execution, Writing the manuscript
Viswanath Kiron: Conception; Design of experiment; Execution, Writing the manuscript

Ethical approval and consent to participate

The National Animal Welfare Authority (Mattilsynet) approved the conduct of animal experiment and the animals were handled according to the sanctioned protocols. All persons associated with the project scientifically are authors on the paper and have approved the final version of the manuscript submitted for review. All data gathered during the study formed the basis of this manuscript and is presented in its entirety.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1: Physical appearance of the three different feeds. Control (CT), SCE 10, SCE 20: low fishmeal control diet, *Scenedesmus* 10% diet, *Scenedesmus* 20% diet, respectively.

Figure 2: Water stability test for CT, SCE 10 and SCE 20 feeds. Control (CT), SCE 10, SCE 20: low fishmeal control diet, *Scenedesmus* 10% diet, *Scenedesmus* 20% diet, respectively. Four shaking regimes were employed to determine the pellet water stability: 100 shakings of the cassette per minute over 15, 30, 45 and 60 minutes. Water stability values are expressed as percentage of dry matter that is retained from the initial dry weight. Error bars depict standard deviations.

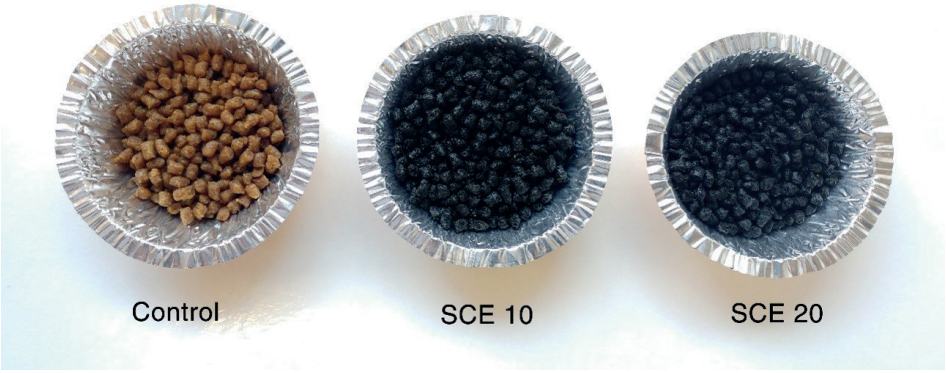
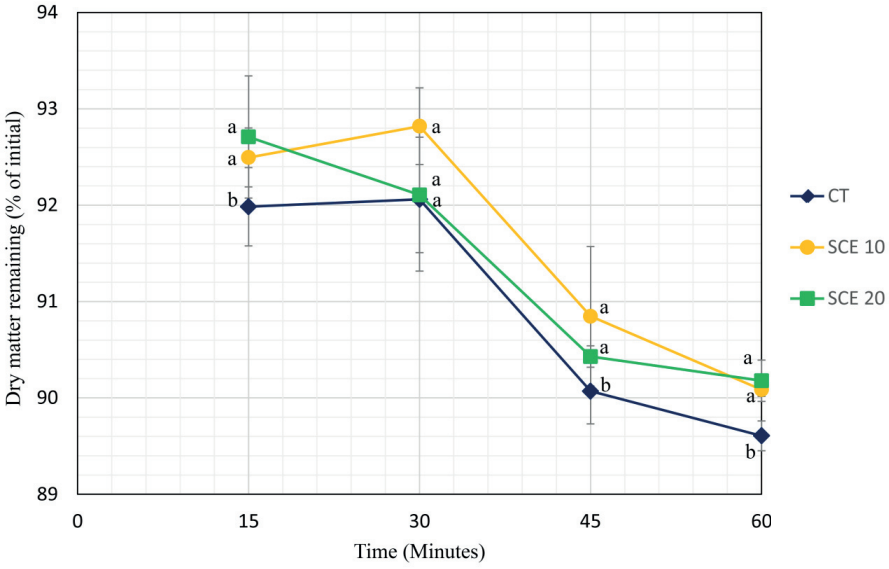


Figure 1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Dry matter remaining (% of initial)
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30
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Time (Minutes)
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CT
SCE 10
SCE 20

Figure 2

Tables

Table 1: Ingredient composition (%) of the control (CT), low algae (SCE 10) and high algae (SCE 20) feeds

Ingredients	CT %	SCE 10 %	SCE 20 %
Fishmeal 70 LT FF (SKAGEN) [a]	10.0	5.0	2.5
Scenedesmus sp. – (Allma®) [b]	0.0	10.0	20.0
Soy protein concentrate (SOYCOMIL®) [c]	12.0	11.7	10.9
Pea protein concentrate [d]	12.0	11.7	10.9
Potato concentrate [e]	12.0	11.7	10.9
Wheat Gluten [f]	8.5	8.3	7.7
Corn gluten [g]	7.0	6.8	6.3
Wheat meal [h]	14.5	11.0	7.6
Fish oil (SAVINOR) [i]	10.0	9.8	9.5
Rapeseed oil [j]	10.0	9.8	9.5
Vitamin & Mineral Premix PV01 [k]	1.0	1.0	1.0
Soy lecithin [l]	0.5	0.5	0.5
MCP [m]	2.0	2.0	2.0
L-Histidine [n]	0.1	0.1	0.1
DL-Methionine [o]	0.3	0.3	0.3
Yttrium oxide [p]	0.02	0.02	0.02

a Sopropeche, France
b Allmicroalgae, Portugal
c ADM, The Netherlands
d ROQUETTE Frères, France
e AVEBE, The Netherlands
f ROQUETTE Frères, France
g COPAM, Portugal
h Casa Lanchinha, Portugal
i SAVINOR UTS, Portugal
j Henry Lamotte Oils GmbH, Germany
k PREMIX Lda, Portugal.
l Lecico P700IPM, LECICO GmbH, Germany
m Fosfitalia, Italy
n Ajinomoto Eurolysine SAS, France
o Evonik Nutrition & Care GmbH, Germany
p Sigma-Aldrich, Spain

948 **Table 2**: Analyzed proximate composition (%) of the feeds

Parameter	CT	SCE 10	SCE 20
Moisture	6.3	6.2	6.9
In dry matter, %			
Protein	49.2	49.3	48.9
Lipid	21.1	22.5	21.0
Ash	5.8	5.6	5.9
Energy (KJ g^{-1})	24.5	24.8	24.9

949 CT: Control; SCE 10: incorporation of 10% *Scenedesmus* in the diet; SCE 20:
950 incorporation of 20% *Scenedesmus* in the diet. Values are expressed as mean of 4
951 replicate samples per diet.

952 **Table 3**: Analyzed fatty acid composition (% of total fatty acids) of the experimental
953 feeds

Fatty acid %	CT	SCE 10	SCE 20
C14:0	3.11	2.90	2.48
C15:0	0.42	0.40	0.53
C16:0	13.05	13.62	14.21
C16:1n-7	3.53	3.43	2.86
C18:0	3.26	3.38	3.87
C18:1n-9	36.46	37.06	36.61
C18:1n-7	3.43	3.48	3.29
C18:2n-6	14.33	14.06	15.08
C18:3n-6	0.35	0.34	0.21
C18:3n-3	4.94	4.69	6.33
C20:0	0.37	0.36	0.65
C20:1n-9	3.45	3.43	1.83
C20:5n-3	3.35	3.29	4.15
C20:4n-6	0.72	0.64	0.50
C22:6n-3	9.12	8.85	7.27
C24:0	0.12	0.08	0.14
Saturates (SFAs)	20.33	20.74	21.88
Monounsaturates (MUFAs)	46.87	47.40	44.59
n-6 PUFAs	15.40	15.04	15.79
n-3 PUFAs	17.41	16.83	17.75
PUFAs	31.81	31.87	33.54

954 CT: Control; SCE 10: incorporation of 10% *Scenedesmus* in the diet; SCE 20:
955 incorporation of 20% *Scenedesmus* in the diet. Values are expressed as mean value of
956 2 replicate samples per diet.
957 SFAs, Saturated fatty acids; MUFAs, Monounsaturated fatty acids; n-6 PUFAs, Omega-6
958 polyunsaturated fatty acids; n-3 PUFAs, Omega-3 polyunsaturated fatty acids; PUFAs,
959 Polyunsaturated fatty acids
960
961
962

963 **Table 4**: Physical characteristics of the experimental feeds

Parameter	CT	SCE 10	SCE 20	p value
Fat leakage (%)	6.2 ± 0.6^{a}	5.3 ± 0.3^{b}	3.9 ± 0.4^{c}	< 0.001
Hardness (N)	22.9 ± 4.8^{b}	22.2 ± 5.0^{b}	39.6 ± 8.1^{a}	< 0.001
Length (mm)	4.4 ± 0.5^{a}	4.2 ± 0.5^{ab}	4.1 ± 0.6^{b}	<0.001
Diameter (mm)	3.0 ± 0.2	3.0 ± 0.1	3.1 ± 0.2	0.4634

964 CT: Control; SCE 10: incorporation of 10% *Scenedesmus* in the diet; SCE 20:
965 incorporation of 20% *Scenedesmus* in the diet. Fat leakage is expressed as mean ± SD
966 (n=6 replicates). Hardness, length and diameter is reported as an average value of 6
967 means ± SD, where each mean value is an average of 20 pellets. Values in the same
968 row with different superscript letters indicate significant difference ($p<0.05$)
969

970 **Table 5**: Apparent digestibility coefficients (ADC, %) of dry matter, lipid, protein, ash
971 and energy in the experimental feeds

Parameter	CT	SCE 10	SCE 20	p value
Dry matter	67.6 ± 0.8[a]	62.5 ± 0.2[b]	54.5 ± 3.1[c]	<0.001
Lipid	90.9 ± 0.2[a]	88.1± 0.4[b]	79.4 ± 1.8[c]	0.001
Protein	82.3 ± 1.1[a]	77.6 ± 0.9[a]	69.2 ± 3.4[b]	< 0.001
Ash	-22.9 ± 8.6	-31.6 ± 8.4	-42.9 ± 7.1	0.061
Energy	77.6 ± 0.4[a]	72.6 ± 0.1[b]	63.8 ± 2.5[c]	< 0.001

972 CT: Control; SCE 10: incorporation of 10% *Scenedesmus* in the diet; SCE 20:
973 incorporation of 20% *Scenedesmus* in the diet. Values are expressed as mean ± SD (n=6
974 replicate tanks). Values in the same row with different superscript letters indicate
975 significant difference ($p<0.05$)
976
977

978 **Table 6**: Weight gain, growth rate, feed conversion ratio, and somatic indices of
979 Atlantic salmon for the experimental period

Parameter	CT	SCE 10	SCE 20	p value
Growth parameter				
Initial body weight (g)	228.4 ± 4.6	230.8 ± 2.2	228.1 ± 4.1	0.418
Final body weight (g)	473.6 ± 47.7^{a}	451.0 ± 23.4^{ab}	416.7 ± 21.8^{b}	0.030
Weight gain (%)	107.1 ± 17.2^{a}	95.4 ± 10.3^{ab}	82.6 ± 7.2^{b}	0.013
Specific growth rate (% day^{-1})	1.12 ± 0.13^{a}	1.03 ± 0.08^{ab}	0.93 ± 0.06^{b}	0.014
Feed conversion ratio	0.76 ± 0.09^{c}	0.88 ± 0.04^{b}	0.97 ± 0.04^{a}	<0.001
Protein efficiency ratio	2.69 ± 0.23^{a}	2.36 ± 0.11^{b}	2.13 ± 0.12^{b}	<0.001
Thermal growth coefficient	3.48 ± 0.47^{a}	3.19± 0.27^{ab}	2.8 ± 0.22^{b}	0.015
Condition indices				
Hepato-somatic index (%)	1.6 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	0.781
Viscero-somatic-Index (%)	10.1 ± 1.2	10.4 ± 0.9	11.1 ± 1.4	0.282
Condition factor (g cm^{-3})	1.42 ± 0.04^{a}	1.35 ± 0.02^{b}	1.32 ± 0.03^{b}	<0.001

980 CT: Control; SCE 10: incorporation of 10% *Scenedesmus* in the diet; SCE 20:
981 incorporation of 20% *Scenedesmus* in the diet. Values are expressed as mean ± SD (n=6
982 replicate tanks). Values in the same row with different superscript letters show
983 significant differences ($p<0.05$)
984

985 **Table 7**: Nutrient retention efficiency (%) of lipid, protein and energy (gross) and
986 retention efficiency of the digested nutrients (%) in Atlantic salmon fed the
987 experimental diets

Parameter	CT	SCE 10	SCE 20	p value
Gross				
Lipid	85.7 ± 2.9^{a}	73.0 ± 3.5^{b}	63.0 ± 5.1^{c}	< 0.001
Protein	47.6 ± 3.9^{a}	41.1 ± 2.5^{b}	37.8 ± 2.1^{b}	< 0.001
Energy	49.6 ± 2.7^{a}	43.1 ± 3.7^{b}	36.4 ± 2.1^{c}	< 0.001
Digested				
Lipid	99.2 ± 5.6^{a}	85.4 ± 6.6^{b}	73.2 ± 4.8^{c}	0.020
Protein	62.3 ± 8.7^{a}	54.8 ± 6.8^{ab}	49.8 ± 4.1^{b}	< 0.001
Energy	69.1 ± 4.8^{a}	61.4 ± 7.1^{a}	51.2 ± 4.2^{b}	< 0.001

988 CT: Control; SCE 10: incorporation of 10% *Scenedesmus* in the diet; SCE 20:
989 incorporation of 20% *Scenedesmus* in the diet. Values are expressed as mean ± SD (n=6
990 replicate tanks). Values in the same row with different superscript letters indicate
991 significant difference ($p<0.05$)
992
993
994

995 **Table 8**: Chemical composition of the whole body (g kg^{-1} dry matter) of Atlantic salmon
996 at the end of the feeding period

Parameter	Initial	CT	SCE 10	SCE 20	p value
Moisture (g kg^{-1})	71.3	68.7 ± 5.6^{ab}	68.5 ± 4.7^{a}	69.3 ± 3.4^{b}	0.017
g kg^{-1} dry matter					
Protein	593.0	556.2 ± 12.3^{ab}	546.4 ± 13.3^{b}	565.6 ± 7.3^{a}	0.032
Lipid	332.6	373.1 ± 8.6^{a}	374.2 ± 7.0^{a}	357.0 ± 4.9^{b}	<0.001
Ash	66.3	56.2 ± 3.3^{b}	58.5 ± 3.2^{ab}	63.7 ± 4.8^{a}	0.012
Energy (KJ g^{-1})	25.8	26.6 ± 0.1^{a}	26.2 ± 0.6^{ab}	26.0 ± 0.2^{b}	0.029

997 CT: Control; SCE 10: incorporation of 10% *Scenedesmus* in the diet; SCE 20:
998 incorporation of 20% *Scenedesmus* in the diet. Values are expressed as mean ± SD (n=6
999 replicate tanks). Values in the same row with different superscript letters indicate
1000 significant difference ($p<0.05$)
1001
1002

Table 9: Fatty acid composition (% of total fatty acids) in fish at the start (initial) and at the end of the feeding period

Fatty acid %	Initial	CT	SCE 10	SCE 20	p value
Saturates (SFAs)					
C14:0	4.06	3.11 ± 0.47	2.96 ± 0.15	3.05 ± 0.23	0.716
C15:0	0.37	0.44 ± 0.03	0.47 ± 0.08	0.43 ± 0.05	0.501
C16:0	12.56	13.79 ± 0.47	13.57 ± 0.26	13.36 ± 0.26	0.123
C18:0	3.18	3.62 ± 0.07^{a}	3.45 ± 0.17^{ab}	3.33 ± 0.08^{b}	0.002
C20:0	0.37	0.42 ± 0.06	0.45 ± 0.13	0.33 ± 0.06	0.087
C24:0	0.11	0.12 ± 0.03	0.12 ± 0.04	0.12 ± 0.03	0.954
ΣSFAs	20.65	21.50 ± 0.86	21.02 ± 0.53	20.61 ± 0.47	0.092
Monounsaturates (MUFAs)					
C16:1n-7	4.29	3.41 ± 0.20	3.22 ± 0.34	3.50 ± 0.12	0.154
C18:1n-9	34.91	37.46 ± 0.89	36.95 ± 0.84	36.88 ± 1.07	0.509
C18:1n-7	3.42	3.45 ± 0.06	3.37 ± 0.10	3.42 ± 0.06	0.217
C20:1n-9	5.16	3.70 ± 0.06	2.95 ± 0.88	3.49 ± 0.15	0.065
ΣMUFAs	47.78	48.03 ± 0.81	46.49 ± 1.97	47.28 ± 1.14	0.204
n-6 PUFAs					
C18:2n-6	13.98	13.95 ± 0.17^{a}	14.54 ± 0.42^{b}	14.28 ± 0.21^{ab}	0.010
C18:3n-6	0.36	0.31 ± 0.11	0.34 ± 0.11	0.36 ± 0.05	0.689
C20:4n-6	0.82	0.63 ± 0.08	0.64 ± 0.06	0.70 ± 0.05	0.183
Σn-6 PUFAs	15.15	14.89 ± 0.30^{a}	15.52 ± 0.38^{b}	15.34 ± 0.20^{ab}	0.008
n-3 PUFAs					
C18:3n-3	4.49	4.48 ± 0.29^{a}	5.18 ± 0.71^{b}	4.88 ± 0.25^{ab}	0.050
C20:5n-3	3.61	2.91 ± 0.15	3.58 ± 0.76	3.24 ± 0.21	0.070
C22:6n-3	8.32	8.19 ± 0.30	8.22 ± 0.34	8.65 ± 0.48	0.097
Σn-3 PUFAs	16.42	15.58 ± 0.55^{a}	16.97 ± 1.22^{b}	16.77 ± 0.89^{ab}	0.041
ΣPUFAs	31.57	30.47 ± 0.68^{a}	32.49 ± 1.56^{b}	32.11 ± 1.00^{ab}	0.017
n-3/n-6	1.08	1.05 ± 0.04	1.09 ± 0.06	1.09 ± 0.05	0.202

CT: Control; SCE 10: incorporation of 10% *Scenedesmus* in the diet; SCE 20: incorporation of 20% *Scenedesmus* in the diet. SFAs, Saturated fatty acids; MUFAs, Monounsaturated fatty acids; n-6 PUFAs, Omega-6 polyunsaturated fatty acids; n-3 PUFAs, Omega-3 polyunsaturated fatty acids; PUFAs, Polyunsaturated fatty acids. Values are expressed as mean ± SD (n=6 replicate tanks). Values in the same row with different superscript letters indicate significant difference ($p<0.05$)

Paper IV

Effect of feed additives on the utilization of pre-extruded microalgae *Nannochloropsis oceanica* fed to Atlantic salmon *Salmo salar*

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Abstract

Rigid cell walls of microalgae prevent efficient digestibility and utilization of nutrients when incorporated into carnivore fish diets. Thermo mechanical processing – with use of extrusion technology – is an efficient scalable technology that improves nutrient utilization. It is also hypothesized that certain feed additives can further improve nutrient digestibility and feed utilization of microalgae in feed. The aim of the study was to investigate if incorporation of pre-extruded *Nannochloropsis oceanica* had an effect on nutrient digestibility, growth and feed utilization, and if feed additives can be used to improve feed utilization. Pre-extruded microalga incorporated at 10% in the feed. Four low fish meal diets were formulated; control diet without the microalga *Nannochloropsis oceanica* (CO), a diet containing 10% of the microalga (NC), and two diets containing 10% of the microalga supplemented with either 0.06% Digestarom (ND) or 1% ZEOFeed (NZ). Fish (initial average weight of 227.3 ± 3.97 g) in 5 replicate tanks were fed one of the experimental diets for 68 days. The results showed that the apparent digestibility of dry matter in the NC and NZ groups were significantly higher compared to the control group (CO). Digestibility of lipid was significantly lower and digestibility of ash was higher in the alga-fed groups (NC, ND and NZ) compared to the control group (CO). No significant differences of the final weight, weight gain, specific growth rate, thermal growth coefficient, feed conversion ratio, feed intake and protein efficiency ratio was noted in fish fed the experimental feeds, compared with the control group (CO). The whole body proximate composition of Atlantic salmon was not affected by the intake of the alga meal and the additives. The present study indicates that incorporation of 10% pre-extruded microalgae *Nannochloropsis oceanica* in plant-based commercial-like diets did not affect the growth, feed utilization and whole body proximate composition of salmon. The results also reflected no beneficial effect of the feed additives on growth and feed utilization at their respective levels in salmon feed. However, an increased content total PUFAs of Atlantic salmon fed NZ was noteworthy and warrants further investigation.

KEYWORDS

Microalgae, Extrusion, Feed additives, *Nannochloropsis oceanica*, Atlantic salmon, Utilization, Digestarom, ZEOFeed

Introduction

The Norwegian aquaculture production has increased from around 150, 000 tons in the 1990s to more than 1.3 million tons today (Ytrestøyl *et al.* 2015). The Norwegian aquaculture industry is dominated by Atlantic salmon, accounting for around 95% of total volume produced in 2017 (SSB 2018). It is estimated that the production of salmonids in Norway can reach 5 million tons by 2050 and have a six-fold increase in sales value (Olafsen *et al.* 2012). To meet the growth potential of the Norwegian salmon aquaculture sector the demand for feed by 2050 will be 6 million tons (Olafsen et al. 2012). The feed has to be produced from sustainable sources of high quality that meet the nutrient requirment, promote good health for the fish and high product quality for the consumer of the fish.

Based on chemical composition, some microalgae have potential as feed ingredients for Atlantic salmon (Shields and Lupatsch 2012, Becker 2007) because they are good sources of amino acids, n-3 Polyunsaturated fatty acids (PUFAs) and astaxanthin (Shah *et al.* 2018). However, only a few is successfully commercialized and used in salmon feeds. Heterotrophic microalgae *Schizochytrium* as a good source of n-3 -PUFAs, in particular the Docosahexaenoic Acid (DHA), and may be a good replacement of fish oil (Sprague *et al.* 2017, Sprague *et al.* 2015, Kousoulaki *et al.* 2015), while photoautotrophic microalgae *Haematococcus* have the capacity to natural accumulate astaxanthin and may represent a good alternative to synthetic astaxanthin (Griffiths *et al.* 2016).

Replacement of fish meal and plant ingredients currently used in salmon feeds with microalgae remains a challenge. Single cell microalgae are diverse in terms of chemical composition and cell wall structure, and thus need thoroughly testing to ensure safe use as well as to understand their effects on growth, feed utilization, nutrient digestibility, animal health and product quality as well as feed quality (Glencross *et al.* 2007, Ringø *et al.* 2009). Our previous studies have shown that the microalgae such as *Nannochloropsis oceanica* can be used at modest inclusion levels, around 10%, without negative effects on the performance and health of salmon (Sørensen *et al.* 2017). However, we have observed that nutrient digestibility values (e.g. lipid) of microalgae-incorporated feeds were lower compared to the fish-meal-based reference feeds in Atlantic salmon (Sørensen et al. 2017, Gong *et al.* 2018). It is assumed that the mechanical and chemical properties of the cell walls of certain microalgae could hinder intracellular nutrient accessibility, leading to a decreased nutrient digestibility and feed utilization (Teuling *et al.* 2017, Tibbetts *et al.* 2017, Teuling *et al.* 2018). Some cost-effective processing technologies are required to disrupt cell walls and improve

nutrient availability of microalgae to achieve commercial acceptance in salmon feed (Teuling et al. 2017, Tibbetts et al. 2017, Teuling et al. 2018). Extrusion has been found effective in cell disruption of *Nannochloropsis* for the extraction of intracellular valuables (Gong et al. 2018, Wang *et al.* 2018). Besides, it has been reported that feed additives such as essential oils and clinoptilolite may improve the feed intake, weight gain and the feed conversion ratio of farmed fish species (Kanyılmaz *et al.* 2015, Ghasemi *et al.* 2018). Most studies performed to evaluate the potential of microalgae in diets for Atlantic salmon were using diets high in fish meal and fish oil (Kiron *et al.* 2012, Kiron *et al.* 2016, Sørensen et al. 2017). More research is needed to understand the nutritional value of microalgae in commercial like diets; i.e. diets high in plant and low in marine ingredients. The aim of the present study was twofold: 1. To investigate the potential of a thermo-mechanical processed (extruded) microalgae *Nannochloropsis oceanica* as an ingredient in high plant-low marine feed fed to Atlantic salmon and 2. The potential of using two different feed additives to improve the nutrient digestibility and utilization of the feeds with microalgae incorporated.

Material and methods

Experimental design and diets

The experiment was conducted to investigate both the nutrient digestibility and growth performance of Atlantic salmon, and the study was approved by the National Animal Research Authority (FDU: Forsøksdyrutvalget ID-5887) in Norway.

The test microalga *Nannochloropsis oceanica* (2.8% moisture, 36.6% protein, 14.3% lipid, 9.4% fiber, 22.8% ash, 17.5 KJ g^{-1} of energy, 2.1% lysine and 0.9% methionine) used in the diets was cultured in closed photobioreactors at Allma®, Lisbon, Portugal. The microalgae were pre-processed prior to mixing with other ingredients and processed into the experimental feed. The pre-extrusion of algae was carried out with the following procedure: powder algae *Nannochloropsis oceanica* (98.5%) was blended with wheat meal (1.5%) in a double-helix mixer (model 500L, TGC Extrusion, France). The mixture were shaped into pellets (2.0 mm diameter size) in a pilot-scale twin-screw extruder (model BC45, CLEXTRAL, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate 65 kg/h; screw speed 243 rpm;, steam addition at conditioner 3%; water addition at extrusion barrel 1 295 ml/min; temperature barrel 3 112-113°C; moisture level at die exiting 26%. Extruded algae pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). The chemical composition of pre-extruded *Nannochloropsis oceanica* was 3.3% moisture, 36.4% protein, 14.2% lipid, 9.3% fiber, 22.6% ash, 17.4 KJ g^{-1} of energy, 2.0% lysine and 0.9% methionine.

Four diets were formulated to be grossly isoproteic (43% of dry matter) and isolipidic (29% of dry matter). Ingredient composition is shown in Table 1, chemical and amino acid composition is shown in Table 2 and fatty acids are shown in Table 3. Four low fish meal diets were employed in the current study; the control diet containing 15% fish meal and no *Nannochloropsis oceanica* (CO), a diet containing 7.5% fish meal and 10% of the microalgae (NC), and the other two diets consisting of NC + 0.06% Digestarom PEP MGE150 (Biomin GmbH, Getzersdorf, Austria) (ND), or NC + 1% ZEOFeed (ZEOCEM AS, Bystré, Slovakia) (NZ).

The experimental extruded diets were manufactured by SPAROS LDA (Olhão, Portugal). All powder ingredients and pre-extruded algae pellets were mixed according to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 400 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 3.0 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate (80-89 kg/h), screw speed (235-244 rpm), water addition (approximately 230 ml/min), temperature barrel 1 (34-36ºC), temperature

barrel 3 (124-127°C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (700 mbar, for approximately 50 sec) (model PG-10VCLAB, Dinnissen, The Netherlands). Immediately after coating, diets were packed in sealed plastic buckets and shipped to the research site.

Fish and feeding

Atlantic salmon (*Salmo Salar*) post-smolts were obtained from Cermaq, Hopen, Bodø, Norway (Aquagen strain, Aquagen AS, Trondheim, Norway) and maintained at the Research Station, Nord University, Bodø, Norway for approximately 5 months. At the start of the experiment, a total number of 600 fish with initial weight 227.3 ± 3.97 g were randomly allocated to the experimental units (n=30 fish per tank).

The feeding experiment was carried out in a flow-through system. In total, 20 circular fiberglass tanks (800 L) were used for the study. Each tank was supplied with sea water pumped from Saltfjorden, from a depth of 250 m. During the experiment, water flow rate was maintained at 1000 L per hour, and the average temperature and salinity of the rearing water were 7.5°C and 35 ‰, respectively. Oxygen saturation was always above 85% saturation measured in the outlet water. A 24-h photoperiod was maintained throughout the feeding period. The fish were fed ad libitum using automatic feeders (Arvo Tech, Finland); administrated in two feedings per day, from 08:00-09:00 in the morning and 14:00-15:00 in the afternoon. After each feeding, the uneaten feeds that settled in the steel wire mesh of each experimental tank were collected.

Fish sampling and data collection

At the beginning and end of the experiment, all the fish (600) were individually weighed and their lengths were recorded. Before handling, fish were anesthetized using tricainemethanesulfonate (MS 222, 140 mg/L). At termination of the experiment, six fish per tank were pooled to assess the final chemical composition. These fish were packed in plastic bags, immediately frozen and kept at -40 °C until analyses. Three fish from each tank were weighed, dissected and the visceral organs (without heart and kidney) and liver from each fish were removed and weighed for calculation of organosomatic indexes. Faeces were collected from the remaining fish in the tanks. Fecal matter was obtained from individual fish by stripping and pooled to obtain enough material for chemical analysis.

Chemical analyses

The fish samples from each tank was homogenized using an industrial food processor (Foss Tecator, 2096 homogenizer, Denmark) before analyzing the whole body proximate composition. As for the faecal samples, the frozen materials were freeze dried (VirTis benchtop, U.S.A.) for 72 h.

The fish, experimental feeds and freeze-dried faeces were finely ground by mortar and pestle and homogenized prior to analyses of dry matter (105°C for 20 hr) (ISO 6496:1999), crude protein (Kjeldahl Auto System, Tecator Systems, Höganäs, Sweden) (ISO 5983:1987), crude lipid (Soxtec HT6, Tecator, Höganäs, Sweden) (ISO 6492:1999), ash (incineration in a muffle furnace at 540°C for 16 hr) (ISO 5984:2002) and energy (IKA C200 bomb calorimeter, Staufen, Germany) (ISO 9831:1998). The amino acid analyses were performed according to ISO 13903:2005. Yttrium in both faeces and feeds was analyzed by employing inductive coupled plasma mass spectroscopy (ICP-MS) by Eurofins (Moss, Norway) (NS-EN ISO 11885). All the samples were analyzed in duplicate.

Total lipid content of the fish was determined by ethyl-acetate extraction method. Total lipid content of the faeces was analyzed employing the Soxhlet method with acid hydrolysis (Soxtec HT 6209, Tecator, Höganäs, Sweden: modified AOAC method 954.020), by Eurofins® (Moss, Norway). Fatty acid composition of fish and feed was measured by gas chromatography (GC) of methyl-ester derivatives in the samples. For this, the homogenized samples were lyophilized for 72 h before the lipids were extracted and analyzed in duplicate. Total lipid from the samples was extracted according to the method of Bligh and Dyer (1959). The fatty acid methyl esters (FAMEs) were prepared according to the AOCS Official Method Ce 1b-89. FAMEs were separated and quantitated using a Scion 436 GC (Bruker, USA) equipped with a flame ionization detector, a splitless injector and a DB-23 column (Agilent Technologies, USA). Standard mixtures of FAMEs were used for identification and quantitation of common fatty acids in samples (GLC-473, Nu-Chek Prep, Elysian, MN, USA).

Calculations and statistical analysis

Fish growth performance was analyzed using the following equations:

$$\text{Weight gain } (\%)(\text{WG}) = \left(\frac{W_f - W_i}{W_i}\right) \times 100$$

$$\text{Feed intake } (\%\ \text{BW day}^{-1}) = \left(\frac{\text{Daily feed intake in dry basis (g)}}{\sqrt{W_f \times W_i}}\right) \times 100$$

$$\text{Specific Growth Rate (\% day}^{-1}\text{)(SGR)} = \left(\frac{\text{Ln (W}_\text{f}\text{)} - \text{Ln (W}_\text{i}\text{)}}{\text{d}}\right) \times 100$$

$$\text{Feed conversition ratio (FCR)} = \frac{\text{Total feed intake in dry basis (g)}}{\text{Weight gain (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Weight gain (g)}}{\text{Total protein ingested (g)}}$$

$$\text{Thermal growth coefficient (TGC)} = \frac{(\text{W}_\text{f})^{1/3} - (\text{W}_\text{i})^{1/3}}{(\text{T} \times \text{d})} \times 1000$$

$$\text{Hepato} - \text{somatic index (\%)(HSI)} = \frac{\text{Liver weight of fish (g)}}{\text{W}_\text{f}\text{(g)}} \times 100$$

$$\text{Viscero} - \text{Somatic Index (\%)(VSI)} = \frac{\text{Visceral Weight of fish (g)}}{\text{W}_\text{f}\text{(g)}} \times 100$$

$$\text{Condition factor (g/cm}^3\text{)(CF)} = \frac{\text{W}_\text{f}\text{(g)}}{\text{FL}^3} \times 100$$

where, W_f = final body weight of fish (g/fish), W_i = initial body weight of fish (g/fish), T is the temperature in °C and d is feeding days, FL = Fork length of fish (cm)

Apparent Digestibility Coefficient (ADC) of nutrients and dry matter were calculated according to following equations:

$$\text{ADC}_{\,nutrient} = \left[1 - \left(\frac{Marker_{feed} \times Nutrient_{faeces}}{Marker_{faeces} \times Nutritent_{feed}}\right)\right] \times 100$$

$$\text{ADC}_{\,dry\ matter} = \left[1 - \left(\frac{Marker_{feed}}{Marker_{faeces}}\right)\right] \times 100$$

where $Marker_{feed}$ and $Marker_{faeces}$ represent the marker content (% dry matter) of the feed and faeces, respectively, and $Nutrient_{feed}$ and $Nutrient_{faeces}$ represent the nutrient contents (% dry matter) in the feed and faeces.

All statistical analyses were performed using SPSS 22.0 software package for Windows. The data were tested for normality (Shapiro–Wilk normality test) and equality of variance (Levene's test). For parametric data, one way analysis of variance (ANOVA) was performed after checking for equal variance. Tukey's multiple comparison test was used to identify the significant differences among the means of the dietary groups. For non-parametric data, Kruskal-Wallis test, followed by Dunn's multiple comparison test, was performed to decipher the significant differences between the groups. A significance level of $p < 0.05$ was chosen to indicate the differences.

Results

Experimental diets

All the experimental diets were formulated and balanced for amino acids and other main essential nutrients. The dietary amino acid (AA) composition was balanced to match AA requirements of Atlantic salmon through the dietary supplementation of several crystalline amino acids (lysine, methionine, threonine and tryptophan). The content of lysine and methionine was 2.7-3.0%, 0.7-0.8% of diet (dry basis), respectively. Besides, the content of EPA + DHA was similar among the diets (2.7-2.9% of dry basis).

Apparent digestibility coefficients of feeds

Digestibility of DM, protein, lipid and ash showed significant differences among the four feeds ($p < 0.05$) (Table 4). The DM digestibility was significantly lower in CO-fed fish compared to the algae incorporated diets, while no differences were noted among the algae incorporated diets. Protein digestibility was significantly highest in fish fed the NC and lowest in those fed ND. Lipid digestibility was highest in fish fed CO, while no differences were observed among the algae fed groups. Digestibility of ash in algae fed fish showed positive values, while fish fed CO showed negative value ($p < 0.05$).

Growth and feed utilization

The growth and feed utilization are given in Table 5. The fish grew from an initial average weight of 227.3 g to a final mean body weight of 419.6 g during the experimental period of 68 days. There were no significant differences in final weight, weight gain, specific growth rate, thermal growth coefficient, feed conversion ratio, feed intake and protein efficiency ratio among the different groups. Condition factor was similar in fish fed the CO feed than fish fed the alga-incorporated feeds. Neither were there any significant differences in condition factor or viscero-somatic indices (VSI) among the 4 dietary groups. Hepato-somatic index (HSI) ranged between 1.1-1.2, with the highest value in fish fed ND and lowest value for the NC groups ($p < 0.05$).

Proximate composition of whole body

The proximate composition of fish from the groups sampled at the termination of the experiment is presented in Table 6. At the end of the experimental period, no

significant differences were found in protein, lipid or ash content among the dietary groups. The energy content was significantly higher in NZ and lowest in fish fed NC ($p < 0.05$).

Fatty acid composition of fish whole body

The fatty acid composition of fish whole body is given in Table 7. For the individual fatty acid, Linoleic acid (LA), C18:2 n-6 dominated the n-6 fatty acids and LA was lower in fish fed the CO diets than those fed the algal diets ($p < 0.05$). The eicosapentaenoic acid (EPA), C20:5n-3 was found to be slightly higher in fish fed NZ than fish fed the control feed (CO) ($p = 0.056$). The ΣSFAs was significantly higher in fish fed CO compared with fish fed NZ ($p < 0.05$). The ΣMUFAs and Σn-3 PUFAs of the four groups were not significantly different. The Σn-6 PUFAs were significantly lower in fish fed CO compared to other groups ($p < 0.05$). The ΣPUFAs were significantly higher in fish fed NZ compared to other groups ($p < 0.05$).

Discussion

Apparent digestibility coefficients of diets

The digestibility of protein, lipid and ash of the reference control feed used in the present trial were similar or even higher compared to fish-meal-based feed reported in our previous studies (Sørensen et al. 2017, Kiron et al. 2016). The digestibility of protein and lipid in the microalgae-incorporated feeds in the present study also showed higher values than those reported for 10% and 20% incorporation of *Nannochloropsis oceanica* in Atlantic salmon (Sørensen et al. 2017). This observation indicates that pre-processing of the microalgae disrupted the cell walls making protein readily available for digestive enzymes (Teuling et al. 2018). This is in line with earlier studies, reporting that extrusion was effective in cell wall disruption of microalgae of *Nannochloropsis oceanica*, making intracellular nutrients more accessible (Gong et al. 2018, Wang et al. 2018). IIncorporation of microalgae (NC) even improved digestibility of dry matter and ash comapred to the control group (CO). Increased digestibility of ash was also observed in Nile tilapia and African catfish when they were fed *Nannochloropsis gaditana* (Teuling et al. 2017). Earlier studies have also reported reduced digestibility of lipid with increasing content of dietary saturated fatty acids (SFAs) (Kousoulaki *et al.* 2016, Kousoulaki et al. 2015). Salmonids have limited capacity to digest SFAs at low temperature and increasing dietary SFAs levels as well (Ng *et al.* 2004, Menoyo *et al.* 2003, Menoyo *et al.* 2007). The SFAs were similar among diets (Table 3) and is therefore not a likely explanation of the reduced lipid digestibility noted for the microalgae incorporated diets. Lipid digestibility also relates to the positioning of the fatty acids on the triacylglycerol (TAG) (Nielsen *et al.* 2005, Mu and Høy 2004). However, the positioning of the SFAs in the tested microalgal TAG are unknown, and the effect of positioning on lipid digestibility warrants further investigation. Reduction in lipid digestibility with incorporation of *Nannochloropsis Oceanica* is most likely explained by the carbohydrate composition of the e cell walls (Teuling et al. 2017, Tibbetts et al. 2017, Glencross *et al.* 2012). Microalgae have complex carbohydrate such as cellulose, pectins and hemicelluloses (Scholz *et al.* 2014, Baudelet *et al.* 2017). Carnivore fish has no capacity to digest non-starch polysaccharides (NSPs) and thus they act as non-nutritive filler in the feed (Krogdahl *et al.* 2005, Irvin *et al.* 2016). Besides, studies have shown that NSPs have negative effects on lipid and energy digestibilities of fish feed (Espinal-Ruiz *et al.* 2014, Irvin et al. 2016, Leenhouwers *et al.* 2006, Refstie *et al.* 1999, Aslaksen *et al.* 2007). Aslaksen et al. (2007) and Lekva *et al.* (2010) found a linear reduction in digestibility of lipid with increasing cellulose level (0-18%) in diets for Atlantic salmon and Atlantic cod (*Gadus morhua* L.). The non-starch

polysaccharides from cereals and legumes have been shown to disturb fat micelle formation and increase viscosity of gut contents leading to a reduced gastric emptying rate, in which may affect fat digestion in farmed fish (Espinal-Ruiz et al. 2014, Refstie et al. 1999, Leenhouwers et al. 2006, Overland *et al.* 2009, Sinha *et al.* 2011).

Growth Performance and feed utilization of the fish

Atlantic salmon readily accepted the experimental diets and there were no mortalities during the course of the experiment. The overall growth performance and feed utilization were similar to earlier studies on Atlantic salmon (Hatlen *et al.* 2012, Austreng *et al.* 1987), or even better compared to Atlantic salmon of comparable size fed fish meal-based feeds (Sørensen et al. 2017, Kiron et al. 2016). Feeding Atlantic salmon with 10% pre-extruded *Nannochloropsis oceanica* had no negative effect on feed intake, final mean body weight, weight gain, specific growth rate, and thermal growth coefficient. The present findings suggest that if the feeds are carefully balanced for essential amino acids and other essential nutrients, fish meal incorporation can be reduced to 7.5% or even lower without compromising the growth (Kousoulaki *et al.* 2013, Kousoulaki *et al.* 2018). In contrast to Sørensen et al., 2017 who reported higher feed intake when salmon were fed defatted *Nannochloropsis oceanica*, no differences were observed in feed intake in the present trial. These findings are in line with Kiron et al. (2012) and Sprague et al. (2015). They reported no effect on feed intake when Atlantic salmon were fed *Nanofrustulum* sp. or *Tetraselmis* sp. at 10% inclusion rate, or *Schizochytrium* sp. at 11% inclusion level. In contrast, other researchers have reported that microalgae may have negative effects on feed intake in fish. Atlantic salmon fed diets containing 12% dried whole cells microalgae *Phaeodactylum tricornutum* had reduced feed intake (Sørensen *et al.* 2016). The growth of the fish in the present experiment were in line with results reported by Kiron et al. (2012). They reported no effect on growth and feed conversion ratio when Atlantic salmon were fed *Nanofrustulum* sp. or *Tetraselmis* sp. at 10% inclusion rate. Other studies have reported negative effects on growth and/or feed conversion ratio when Atlantic salmon were fed diets with *Desmodesmus* sp. (10/20% inclusion level), *Schyzochrytrium* sp. (11% inclusion level), or *Phaedactylum tricornutum* at an inclusion rate of 12% (Sprague et al. 2015, Kiron et al. 2016, Sørensen et al. 2016). Taken together, the contrasting results suggest that direct comparison of microalgae varieties across experiments are difficult and results need careful interpretation. The responses in the fish depend on the fish species and size, feed formulation, nutritional contents of diets and their availability (Glencross et al. 2007, Jobling 2016).

Supplementation of the diets with the two feed additives ZEOFeed or Digestarom PEP MGE150 in salmon feed could did not improve growth and feed utilization in the present study. The beneficial effects of various plant essential oils on the growth and health of cultured fish have been investigated through last decades (Sutili *et al.* 2018). The Digestarom PEP MGE150 contains essential oils from oregano, anise, and citrus peel and the main active compounds are carvarol, thymol, anethol, and limonene (Rodrigues *et al.* 2018, Peterson *et al.* 2014). Previous studies showed that supplementing 0.02% Digestarom PEP MGE150 in fish feed did not improve digestibility of dry matter and protein, growth performance and FCR in channel catfish (*Ictalurus punctatus*) and gilthead seabream (*Sparus aurata*) (Rodrigues et al. 2018, Peterson et al. 2014). But supplementation of Digestarom to diets of broiler chickens and other terrestrial animals increased the apparent total tract nutrient digestibility (Murugesan *et al.* 2015). Studies in rainbow trout showed that supplementing 0.1% Digestarom PEP 1000 (containing 1.2% carvacrol) or 0.1% Digestarom PEP MGE 1000 (containing 0.6% thymol) improved FCR compared to control diet (Giannenas *et al.* 2012). Koppe *et al.* (2015) found an numerically higher growth rate and feed efficiency ratio as well as lipid digestibility in Atlantic salmon fed diet containing 0.05-0.1% carvacrol. The main component in ZEOFeed is clinoptilolite. Previous studies with gilthead sea bream suggested that use of clinoptilolite (2.7% of diet) could improve growth rate and FCR (Kanyılmaz et al. 2015). It has also been reported that use of zeolite (5-10% bentonite or 2.5% mordenite) improved the growth and feed utilization in rainbow trout (Eya *et al.* 2008). It is assumed that these benefits are likely to be mainly related to the detoxifying effects of zeolites (Ghasemi et al. 2018). The different effects of these two feed additives among other reported findings and our results may be explained by different fish species, supplementing levels of the additives, duration of feeding period, et al. A longer experimental trial and/or species-specific optimal dose needs to be conducted to further evaluate the benefits of the feed additives.

Proximate composition of the fish

The whole body proximate composition of Atlantic salmon was not affected by either the intake of the microalgae or the feed additive. Whole body protein of fish in the present study was lower and lipid content of fish was higher than values (protein 55-58%, lipid 29-37%) reported for Atlantic salmon fed microalgae feed (Kiron et al. 2016, Sørensen et al. 2017). The proximate composition can vary with life stages of the fish and is also influenced by endogenous factors such as genetics, size and sex, as well as exogenous factors such as feed composition, feeding frequency and environment

(Shearer 1994). The ash content of the fish in the present study was in line with the values reported for fish fed microalgae diet (Kiron et al. 2016, Sørensen et al. 2017, Sørensen et al. 2016).

Fatty acid composition of the fish

In salmonid fish, the fatty acid compositions of the flesh are closely related to the composition in diet (Sprague *et al.* 2016, Teimouri *et al.* 2016). The significantly increased contents of linoleic acid (C18:2n-6, LA) in the whole body of fish fed algal diet points to an effective utilization of and deposition of the fatty acid from diets. The increased content of Σn-6 FUFAs in whole body of fish fed algal diet was mainly attributed to the higher content of LA and Arachidonic acid (C20:4n-6, ARA) of total fatty acids. The increase in ΣPUFAs observed in fish fed the NZ diets is also noteworthy. Fatty acid profiles of rainbow trout were also reported to be improved by supplementing 1-3% zeolite (clinoptilolite) in diets (Danabas 2011). The higher content of ΣPUFAs have been contributed by the slightly higher levels of LA and α-linolenic acid (C18:3n-3, ALA), ARA and EPA of total fatty acids in the whole body. The increased PUFAs content of the whole fish induced by an ingredient such as *Nannochloropsis oceanica* and feed additive such as ZEOFeed (clinoptilolite) is favorable and warrants further investigation.

Conclusion

The present study indicates that incorporation of 10% pre-extruded microalgae *Nannochloropsis oceanica* in plant-based commercial-like feeds did not affect the growth, feed utilization and whole body proximate composition of salmon. However, microalgal inclusion significantly reduced the digestibility of lipid in Atlantic salmon. The results reflected no beneficial effect of the feed additives at their respective levels in salmon feed. The increased PUFAs of Atlantic salmon induced by *Nannochloropsis oceanica* combined with ZEOFeed is favorable from nutritional point of view.

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TABLES

TABLE 1. Ingredient composition (%) of the four experimental diets

Ingredients	CO	NC	ND	NZ
Fish meal 70 LT FF (NORVIK) [1]	15.00	7.50	7.50	7.50
Nannochloropsis Extruded [2]	-	10.00	10.00	10.00
Soy protein concentrate [3]	16.00	16.00	16.00	16.00
Pea protein concentrate [4]	10.00	10.00	10.00	10.00
Wheat gluten [5]	11.30	13.00	13.00	13.24
Wheat meal [6]	9.44	7.04	7.04	5.80
Faba beans [7]	7.00	7.00	7.00	7.00
Fish oil (SAVINOR) [8]	10.00	9.05	10.00	9.05
Rapeseed oil [9]	15.00	15.00	15.00	15.00
Vitamin & Mineral Premix INVIVO [10]	1.00	1.00	1.00	1.00
Lutavit C35 [11]	0.03	0.03	0.03	0.03
Lutavit E50 [12]	0.05	0.05	0.05	0.05
Choline chloride [13]	0.20	0.20	0.20	0.20
Monocalcium phosphate [14]	2.00	2.90	2.90	2.90
Calcium carbonate [15]	2.22	0.00	0.00	0.00
L-lysine [16]	0.40	0.60	0.60	0.60
L-threonine [17]	0.20	0.30	0.30	0.30
L-tryptophan [18]	0.04	0.11	0.11	0.11
DL-methionine [19]	0.10	0.20	0.20	0.20
Yttrium oxide [20]	0.02	0.02	0.02	0.02
Digestarom [21]			0.06	
ZEOFeed [22]				1.00

CO: Plant based control diet; NC: *Nannochloropsis oceanica* 10% diet; ND: *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% diet; NZ: *Nannochloropsis oceanica* 10% + ZEOFeed 1% diet

1 NORVIK 70: 70.3% crude protein (CP) 5.8% crude fat (CF), Sopropeche, France

2 Allmicroalgae, Portugal

3 Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands

4 NUTRALYS F85F: 78% crude protein, 1% crude fat, ROQUETTE Frères, France

5 VITAL: 80% CP, 7.5% CF, Roquette Frères, France

6 Wheat meal: 11.7% CP, 1.6% CF, Casa Lanchinha, Portugal

7 Faba beans: 28.5% CP; 1.2% CF, Ribeiro & Sousa Cereais, Portugal

8 SAVINOR UTS, Portugal

9 Henry Lamotte Oils GmbH, Germany

10 PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings

11 ROVIMIX STAY-C35, DSM Nutritional Products, Switzerland

12 ROVIMIX E50, DSM Nutritional Products, Switzerland

13 ORFFA, The Netherlands

14 MCP: 21.8 % phosphorus, 18.4 % calcium, Fosfitalia, Italy

15 CaCO3: 40% Ca, Premix Lda., Portugal

16 Biolys: 54.6% Lysine, Evonik Nutrition & Care GmbH, Germany

17 ThreAMINO: 98% L-Threonine, Evonik Nutrition & Care GmbH, Germany

18 TrypAMINO: 98% Tryptophan, Evonik Nutrition & Care GmbH, Germany

19 DL-Methionine for Aquaculture: 99% Methionine, Evonik Nutrition & Care GmbH, Germany

20 Sigma Aldrich, USA

21 BIOMIN Holding GmbH, Austria

22 ZEOCEM, Slovak Republic

TABLE 2. Chemical composition of the four experimental diets (% of dry matter)

	CO	NC	ND	NZ
Proximate composition				
Dry matter	94.98	94.06	94.79	95.35
% of dry matter				
Protein	44.43	43.06	42.30	42.89
Lipid	29.48	28.17	30.28	29.47
Ash	8.90	8.85	9.04	9.63
Energy (KJ g^{-1}) [1]	23.8	23.0	23.5	23.3
Amino acids (% of dry matter)				
Alanine	1.9	1.7	1.8	1.8
Arginine	2.8	2.6	2.6	2.5
Aspartic acid	4.1	3.6	3.9	3.8
Cysteine	0.5	0.5	0.6	0.6
Glutamic acid	9.5	9.0	9.4	9.2
Glycine	2.1	1.8	1.9	1.8
Histidine	1.0	0.9	1.0	0.9
Leucine	3.4	3.1	3.2	3.2
Lysine	3.0	2.7	2.8	2.8
Isoleucine	1.8	1.7	1.8	1.7
Methionine	0.8	0.8	0.8	0.7
Phenylalanine	2.2	2.1	2.1	2.1
Proline	3.1	3.0	2.9	2.9
Serine	2.3	2.1	2.1	2.1
Threonine	1.9	1.8	1.9	1.8
Tryptophan	0.5	0.6	0.6	0.6
Tyrosine	1.5	1.4	1.4	1.4
Valine	2.0	1.9	2.0	2.0

CO: Plant based control diet; NC: *Nannochloropsis oceanica* 10% diet; ND: *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% diet; NZ: *Nannochloropsis oceanica* 10% + ZEOFeed 1% diet

1 The gross energy content of feeds was not analyzed but calculated based on 23.7, 39.5 and 17.2 KJ g^{-1} for protein, lipids and starch, respectively.

803 **TABLE 3.** Fatty acid composition (% of total fatty acids) of the experimental diets

Fatty acids	CO	NC	ND	NZ
C14:0	2.8	2.7	2.7	2.7
C15:0	0.3	0.2	0.2	0.2
C16:0	10.2	9.9	10	9.9
C16:1 n-7	3.2	3.4	3.4	3.4
C17:0	0.3	0.2	0.2	0.2
C18:0	2.3	2.2	2.2	2.2
C18:1 n-9	39.1	39.9	40.0	40.1
C18:2n-6	14.3	14.5	14.4	14.4
C18:3n-3	6.0	6.1	6.1	6.1
C18:3 n-6	0.1	0.1	0.1	0.1
C18:4n-3	0.9	0.8	0.9	0.9
C20:0	0.5	0.5	0.5	0.5
C20:1 n-9	1.6	1.5	1.5	1.5
C20:2n-6	0.1	0.2	0.2	0.2
C20:4 n-6	0.4	0.4	0.4	0.4
C20:4n-3	0.3	0.2	0.2	0.2
C20:5n-3	5.5	5.7	5.6	5.6
C22:0	0.3	0.3	0.3	0.3
C22:1	1.6	1.4	1.4	1.4
C22:5 n-6	0.1	0.1	0.1	0.1
C22:5 n-3	0.7	0.7	0.7	0.7
C22:6n-3	4.5	4.0	4.0	4.0
C24:0	0.1	0.1	0.1	0.1
C24:1 n-9	0.3	0.3	0.3	0.3
SFAs	16.8	16.3	16.4	16.3
MUFAs	45.9	46.6	46.7	46.8
PUFAs	33.2	33.0	32.8	32.8
Σn-6 PUFAs	15.2	15.5	15.3	15.4
Σn-3 FUFAs	18.0	17.6	17.5	17.5
n-3/n-6	1.19	1.14	1.14	1.14
EPA+DHA	10.0	9.7	9.6	9.6

804 CO: Plant based control diet; NC: *Nannochloropsis oceanica* 10% diet; ND: *Nannochloropsis oceanica* 10%

805 + Digestarom PEP MGE150 0.06% diet; NZ: Nannochloropsis oceanica 10% + ZEOFeed 1% diet

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TABLE 4. Apparent digestibility coefficients (ADC %) of dry matter, lipid, protein, ash and energy in Atlantic salmon fed the experimental diets

	CO	NC	ND	NZ	p value
Dry matter	63.3 ± 0.52^{b}	67.5 ± 0.41^{a}	65.3 ± 0.34^{ab}	66.1 ± 0.89^{a}	0.008
Protein	87.8 ± 0.11^{ab}	88.5 ± 0.07^{a}	86.5 ± 0.54^{b}	87.9 ± 0.60^{ab}	0.032
Lipid	94.3 ± 0.28^{a}	91.3 ± 0.04^{b}	91.1 ± 0.32^{b}	91.9 ± 0.52^{b}	0.002
Ash	-24.0 ± 2.05^{b}	12.9 ± 2.66^{a}	13.9 ± 1.06^{a}	7.7 ± 0.18^{a}	<0.001

CO: Plant based control diet; NC: *Nannochloropsis oceanica* 10% diet; ND: *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% diet; NZ: *Nannochloropsis oceanica* 10% + ZEOFeed 1% diet

Values are expressed as mean ± SD (n=5 replicates). Values in the same row with different superscript letters indicate significant difference ($p < 0.05$)

TABLE 5. Growth performance, feed utilization and somatic indices of Atlantic salmon for experimental period

	CO	NC	ND	NZ	p value
Growth parameter					
IBW(g)	227.94 ± 5.93	228.51 ± 1.82	225.27 ± 1.48	227.31 ± 4.24	0.628
FBW (g)	422.77 ± 22.16	415.05 ± 25.01	417.28 ± 21.08	423.26 ± 11.20	0.898
WG (%)	85.44 ± 7.80	81.61 ± 10.41	86.23 ± 4.74	85.21 ± 8.28	0.802
FI (% BW day^{-1})	0.83 ± 0.05	0.84 ± 0.05	0.82 ± 0.03	0.83 ± 0.02	0.836
SGR (% day^{-1})	0.91 ± 0.63	0.87 ± 0.08	0.90 ± 0.66	0.91 ± 0.38	0.774
FCR	0.90 ± 0.01	0.95 ± 0.05	0.89 ± 0.04	0.89 ± 0.02	0.109
PER	2.49 ± 0.05	2.39 ± 0.14	2.53 ± 0.12	2.52 ± 0.07	0.140
TGC	2.74 ± 0.21	2.64 ± 0.28	2.72 ± 0.22	2.76 ± 0.12	0.815
Somatic Indices					
HSI	1.16 ± 0.03^{ab}	1.10 ± 0.59^{b}	1.19 ± 0.06^{a}	1.15 ± 0.02^{ab}	0.042
VSI	8.22 ± 2 .27	8.30 ± 2.72	8.55 ± 0.50	8.38 ± 0.51	0.635
CF	1.41 ± 0.03	1.42 ± 0.03	1.44 ± 0.03	1.42 ± 0.03	0.332

CO: Plant based control diet; NC: *Nannochloropsis oceanica* 10% diet; ND: *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% diet; NZ: *Nannochloropsis oceanica* 10% + ZEOFeed 1% diet

IBW, Initial body weight; FBW, Final body weight; WG, Weight gain; FI, Feed intake; SGR, Specific growth rate; FCR, Feed conversion ratio; PER, Protein efficiency ratio; TGC, Thermal growth rate; HSI, Hepato-somatic index; VSI, Viscero-Somatic Index; CF, Condition factor

Values are expressed as mean ± SD (n=5 replicates). Values in the same row with different superscript letters show significant differences ($p < 0.05$)

TABLE 6. Proximate composition of the whole fish on a dry matter basis (%)

	CO	NC	ND	NZ	p value
Protein	50.26 ± 0.35	50.72 ± 1.06	50.67 ± 0.64	50.65 ± 0.79	0.762
Lipid	41.94 ± 1.08	42.22 ± 1.65	39.26 ± 3.38	39.14 ± 2.14	0.075
Ash	5.40 ± 0.14	5.75 ± 0.38	5.60 ± 0.42	5.53 ± 0.15	0.366
Energy (KJ g^{-1})	29.05 ± 0.17^{ab}	28.82 ± 0.14^{b}	28.99 ± 0.10^{ab}	29.14 ± 0.23^{a}	0.048

CO: Plant based control diet; NC: *Nannochloropsis oceanica* 10% diet; ND: *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% diet; NZ: *Nannochloropsis oceanica* 10% + ZEOFeed 1% diet

Values are expressed as mean ± SD (n=5 replicates). Values in the same row with different superscript letters indicate significant difference ($p < 0.05$)

TABLE 7. Fatty acid composition (% of total fatty acids) of the whole fish

Fatty acids	CO	NC	ND	NZ	P value
C14:0	2.78 ± 0.08[a]	2.82 ± 0.04[a]	2.80 ± 0.12[a]	2.62 ± 0.04[b]	0.005
C15:0	0.24 ± 0.05	0.22 ± 0.04	0.22 ± 0.04	0.20 ± 0.00	0.532
C16:0	10.86 ± 0.11[a]	10.78 ± 0.11[a]	10.70 ± 0.22[ab]	10.52 ± 0.04[b]	0.009
C17:0	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	1.000
C18:0	2.70 ± 0.07[a]	2.58 ± 0.04[b]	2.62 ± 0.04[ab]	2.60 ±0.70[ab]	0.028
C20:0	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	0.30 ± 0.00	1.000
C22:0	0.14 ± 0.05	0.14 ± 0.05	0.16 ± 0.05	0.18 ± 0.04	0.585
ΣSFAs	17.34 ± 0.19[a]	17.14 ± 0.15[ab]	17.08 ± 0.37[ab]	16.78 ± 0.08[b]	0.010
C16:1n-7	3.20 ± 0.00[b]	3.32 ± 0.04[a]	3.30 ± 0.70[a]	3.20 ± 0.00[b]	<0.001
C18:1n-9	37.30 ± 0.22	37.36 ± 0.32	37.40 ± 0.29	37.58 ± 0.30	0.472
C20:1n-9	3.42 ± 0.10	3.38 ± 0.04	3.38 ± 0.13	3.42 ± 0.10	0.862
C22:1n-9	3.04 ± 0.15	2.98 ± 0.15	2.96 ± 0.20	2.96 ± 0.13	0.846
C24:1n-9	0.50 ± 0.00[a]	0.42 ± 0.04[b]	0.44 ± 0.05[ab]	0.50 ± 0.00[a]	0.004
ΣMUFAs	47.52 ± 0.16	47.60 ± 0.14	47.60 ± 0.14	47.72 ± 0.10	0.203
C18:2n-6	11.82 ± 0.11[b]	12.12 ±0.08[a]	12.10 ± 0.21[a]	12.22 ± 0.13[a]	0.003
C18:3n-6	0.22 ± 0.04	0.24 ± 0.05	0.24 ± 0.05	0.22 ± 0.04	0.848
C20:2n-6	0.90 ± 0.00	0.90 ± 0.07	0.88 ± 0.04	0.92 ± 0.04	0.629
C20:3n-6	0.30 ± 0.00	0.30 ± 0.00	0.32 ± 0.04	0.30 ± 0.00	0.418
C20:4n-6	0.30 ±0.00[b]	0.40 ± 0.00[a]	0.36 ± 0.05[a]	0.40 ± 0.00[a]	<0.001
C22:5n-6	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	1.000
Σn-6 PUFAs	13.86 ± 0.13[b]	14.20 ± 0.07[a]	14.16 ± 0.19[a]	14.30 ± 0.21[a]	0.002
C18:3n-3	4.18 ± 0.08	4.26 ± 0.11	4.26 ± 0.11	4.30 ± 0.07	0.299
C18:4n-3	1.02 ± 0.04	1.04 ± 0.11	1.00 ± 0.07	1.00 ± 0.00	0.778
C20:3n-3	0.30 ± 0.00	0.32 ± 0.04	0.30 ± 0.00	0.30 ± 0.00	0.418
C20:4n-3	0.80 ± 0.00	0.76 ± 0.05	0.78 ± 0.04	0.76 ± 0.05	0.455
C20:5n-3	2.86 ± 0.05	2.94 ± 0.05	2.98 ± 0.08	3.02 ± 0.13	0.056
C22:5n-3	1.20 ± 0.00	1.20 ± 0.00	1.20 ± 0.00	1.24 ± 0.05	0.083
C22:6n-3	6.82 ± 0.13	6.60 ± 0.20	6.58 ± 0.22	6.64 ± 0.20	0.233
Σn-3 FUFAs	17.20 ± 0.00	17.08 ± 0.13	17.12 ± 0.16	17.26 ± 0.08	0.097
ΣPUFAs	31.06 ± 0.08[b]	31.30 ± 0.07[b]	31.28 ± 0.21[b]	31.60 ± 0.18[a]	<0.001
n-3/n-6	1.24 ± 0.00[a]	1.21 ± 0.01[b]	1.21 ± 0.01[b]	1.21 ± 0.01[b]	0.011
EPA+DHA	9.68 ± 0.08	9.54 ± 0.20	9.56 ± 0.19	9.66 ± 0.13	0.449

CO: Plant based control diet; NC: *Nannochloropsis oceanica* 10% diet; ND: *Nannochloropsis oceanica* 10% + Digestarom PEP MGE150 0.06% diet; NZ: *Nannochloropsis oceanica* 10% + ZEOFeed 1% diet

Values are expressed as mean ± SD (n=5 replicates). Values in the same row with

different superscript letters indicate significant difference ($p < 0.05$)

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The need for high quality ingredients is expected to increase with the growth of the salmon aquaculture industry. Microalgae are of great interest because they are primary producers of poly-unsaturated fatty acids in the marine environment, and some species can be good sources of protein, lipid and bioactive components for fish feed. This PhD thesis examined the potential of microalgae *Desmodesmus, Nannochloropsis* and *Scenedesmus* in diets for Atlantic salmon. Apparent digestibility coefficients were determined for *Desmodesmus* and *Nannochloropsis*, and effects of different inclusion levels were evaluated in terms of digestibility of main nutrients and energy as well as their effects on growth, feed utilization, whole body chemical composition and intestinal health of the fish. Thermal-mechanical treatment (extrusion) or feed additives were also studied to examine their ability to improve nutrient utilization. The overall conclusion from the thesis was that palatability of tested microalgae fed to salmon was good and they can be incorporated up to 10% in both fish meal-based and plant-protein-based salmon feeds. Extrusion can be used as a cost-effective method to improve digestibility of nutrients from microalgae. Additional evidences of improvement of nutrient utilization from microalgae would help to fully understand the potential of different types of microalgae as ingredients for Atlantic salmon feeds.

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