MASTER THESIS

BIO5002 Master Thesis in Biology and Aquaculture

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Use of thermo-mechanical pretreatment to improve utilization of the microalgae *Nannochloropsis* sp. and *Tetraselmis* sp. as feed ingredients for Atlantic salmon (*Salmo salar*).

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Ateshm Ghirmay

Abstract

Reducing the incorporation of fish meal and fish oil in diets for Atlantic salmon, without compromising the fish health and performance, is a prioritized research area for feed producers. Micro algae are emerging to be some of the most promising long-term, sustainable sources to reduce the dependence on fish meal and fish oil in aquafeed. The aim of the present study was a) to investigate the potential of two microalgae *Nannochloropsis* sp. and *Tetraselmis* sp. as replacements for fishmeal in diets for Atlantic salmon (*Salmo salar*), and b) to investigate if extrusion processing can be used as a thermo-mechanical pretreatment to improve utilization of the microalgae and c) to investigate if physical quality of pellets differed among the diets. Five experimental diets were used in the trial: one fish meal and fish oil-based control diet and 4 test diets, where the test ingredient was added at a ratio of 70:30. Test diets consisted of a fishmeal-based control feed and dry biomass of either *Nannochloropsis* (NPE) (pre-extruded), *Nannochloropsis* (NEN) *Tetraselmis* (TPE) (pre-extruded) or *Tetraselmis* (TET) were fed to Atlantic salmon (initial mean body weight 154.2g ± 26.5) for 9 weeks. The results showed that at the end of the feeding period, growth performances measured in terms of weight gain (%), SGR (% day) and TGC in fish fed algae diets were significantly lower compared to that in the CTRL group. Among the algae-diets the NPE 30 were showed the best growth performance (weight gain, SGR and TGC), almost at the same level as the CTRL group. Significant differences were observed in whole fish proximate composition among fish groups fed the different diets. Fish fed TPE 30 had significantly higher lipid and energy than other diets but lower ash content than CTRL, NEN 30 and NPE 30. Higher protein level was found in salmon fed diets CTRL, NEN 30 and TET 30 compared to fish fed the other two diets. Physical quality of diet was significantly affected by incorporation of both microalgae strains *Nannochloropsis* sp and *Tetraselmis* sp. Highest fat leakage were observed from the TPE 30 and CTRL groups. Hardness of the pellets was significantly higher in diet TPE 30. Pellet length was significantly longer in CTRL pellets. Diameter of the pellets was significantly higher in CTRL and TET30 pellets. The algae incorporated diets resulted in lower water stability than that of diet control group. In conclusion, pre-extruded *Nannochloropsis* sp. (NPE 30) can be used in feed for Atlantic salmon without compromising fish growth performances.

Keywords: Atlantic salmon Microalgae, *Nannochlopsis sp., Tetraselmis sp*, Growth, Physical quality.

Abbreviations

FAO - Food and Agricultural Organization of the United Nations

SOFIA - The State of World Fisheries and Aquaculture.

SDGs - Sustainable Development Goals

SSB - Statistik Sentralbyrå (Statistic Norway)

FKD – Fiskedirektoratet (Directorate of Fisheries Norway)

FDU - Forskrift om forsøk med dyr (National Animal Research Authority, Norway)

IFIF - International Feed Industry Federation

IFFO – The Marine Ingredients Organization

NRC - Norwegian Research Council

NOFIMA- The Norwegian Institute of Food, Fisheries and Aquaculture Research

ISO - International Organization for Standardization

AOAC – Association for Analytical Communities

GC - Gas Chromatography

EPA - Eicosapentaenoic acid

DHA - Docosahexaenoic acid

ARA - Arachidonic acid

CTRL - Control group

NEN 30 – Non- extruded *Nannochloropsis* sp.

NPE 30 – Pre-extruded *Nannochloropsis* sp.

TET 30 – Non-extruded *Tetraselmis* sp.

TPE 30 – Pre-extruded *Tetraselmis* sp.

Nanno – *Nannochloropsis* sp.

Tetra – *Tetraselmis* sp.

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1. General Introduction:

1.1. Global Aquaculture: A brief overview

Global aquaculture production has grown at an average of 3.2% per year during the course of the last 50 years (Figure 1) and it is expected to grow more rapidly than other animal food producing sectors, which may bring new challenges to sustainable use of aquatic resources and environments (FAO, 2017). Aquaculture industry is playing an increasing role in supplying animal protein for human consumption, contributing 45% to the total global aquatic animal production and over 53% to the total global fish consumption. Per capita food fish consumption has been estimated to 20.3 kg in 2015, compared to 19.7 kg in 2013 (FAO, 2017). In contrast, the wild capture fisheries landings have shown an annual decrease of 2.4% (94.7 to 92.4 million tonnes) over the same period (FAO, 2014a).

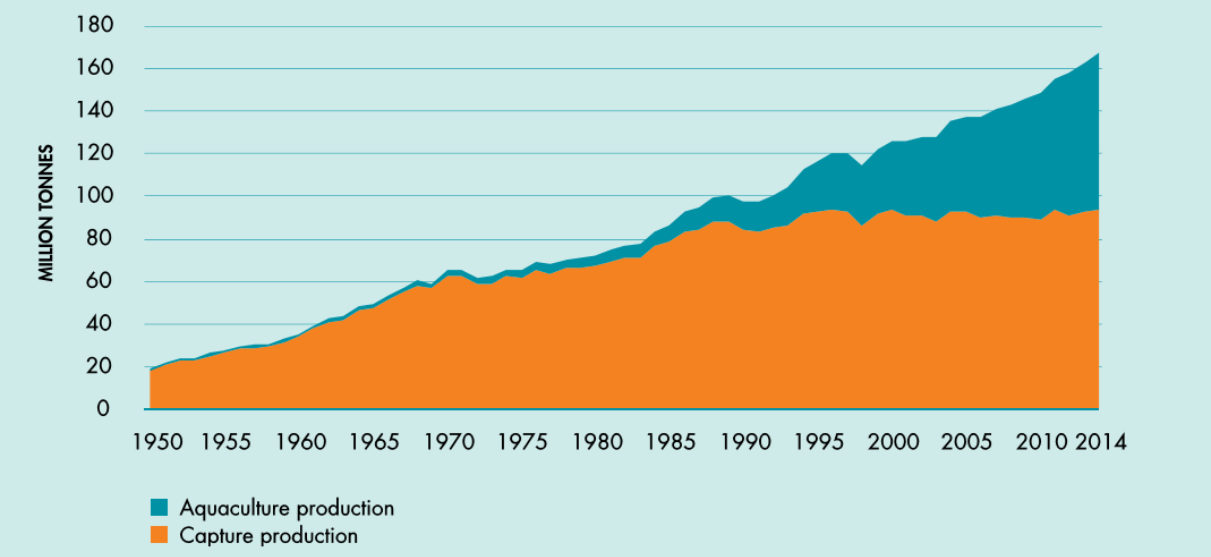


Figure 1. World capture and aquaculture production - million tonnes (Figure is collated from report of FAO, 2015).

According to SOFIA (2016), the 2030 agenda for sustainable development, it's estimated that aquaculture food fish production will reach global wild capture fish in 2030. With a growing population in the world, the Sustainable Development Goals (SDGs) will have a strong influence on fisheries and aquaculture sector. Increasing demand for fish cannot be met by wild capture simply because many fish stocks are already fully exploited. More of our food has to be produced by means of aquaculture and to the sustainable development of the sector, and one main goal clearly focuses is to conserve and sustainably use the oceans, seas and traditional marine resources (SOFIA, 2016).

1.2. Global supply of Atlantic salmon

Atlantic salmon (*Salmo salar*) was the dominating diadromous fish produced in 2015 (2.38 million tonnes or 48%) and is considered a high value product in many markets around the world. The total supply of Atlantic salmon to the market was 3.5 million in 2014 (Fiskeridirektoratet, 2016), of which farmed fish supplied 66%. The market supply of Atlantic salmon was 3.5 million tonnes. The two main producers Norway and Chile accounted for 28 and 17 percent of the global supply, respectively (FAO, 2015). Salmon farming is the most highly developed form of large-scale intensive aquaculture owing to its productivity growth and technological change since the industry started back in the 1970′s in Norway (FAO, 2004). Better growth performance, substantial improvements of the production technologies, breeding program, feed development and vaccine development have driven Atlantic salmon production to competitive level against other fish species produced at lower cost.

1.3. Atlantic salmon production in Norway

Seafood is Norway's second largest export product, after oil and gas (Gabrielsen & Juriks, 2013). Norway has a long coastline and rich marine resources. Throughout history, traditional fishing of Atlantic salmon (*Salmo salar*) has long held significant social, cultural and economic importance for Norwegians. However, the wild resource of Atlantic salmon has such, a steady decline during the last few decades. Today's salmon farming industry started out in Norway in 1970s by the government as a means to lift up the livelihood of rural fishing communities facing depressed economies due to declining wild fisheries (Hjelt, 2000; Sønvisen, 2003). The start was challenging, and the industry has been through a remarkable growth with increased production volumes and improved technology, husbandry practice and management. The emergence of salmon farming has changed the dynamics of salmon sectors as well as the whole seafood industry both in Norway and worldwide. The industry has gradually developed from a "one man – one licence" industry through mergers and restructures into a global industry with several large multinational companies (Liu et al, 2011). In 2015, the first-hand value of Norwegian fish farming came to NOK 46.7 billion. The produced quantity was 1.39 million tonnes (SSB, 2016). Salmon farming in Norway is governed by the Ministry of Fisheries and Coastal Affairs and is primarily administered by the Directorate of Fisheries. The Norwegian Food Safety Authority is also responsible for the animal health, food safety and quality to promote profitability and competitiveness of the industry within the framework of a sustainable development (FKD, 2009).

1.4. Sustainability of Salmon

Sustainability of carnivore fish production has been debated (Torrissen et al, 2011; Ytrestøyl et al, 2015). Questions adding sustainability are spurred on the use of marine ingredients in the feed, use of land to produce feed ingredients, fish diseases and environment issues. Producing food of carnivorous fish such as Atlantic salmon by intensive aquaculture is in many ways comparable to intensive agricultural meat production based on grain and oilseeds as feedstuffs like poultry and pig. Research have shown that carbon foot print of farmed salmon (2.9 kg) of carbon per kg of edible product is significantly lower than terrestrial meat production of poultry(3 kg), pork (5.9) and beef (30kg) (Winther et al, 2009). Atlantic salmon are also very efficient in retaining protein and energy, compared to terrestrial animals (Torrissen et al, 2011). Several sustainability indicators, such as the simple fish in/fish out ratio (FIFO), forage fish dependency ratio (FFDR) (Ytrestøyl et al, 2015), marine nutrient dependency ratio and nutrient retention (Papatryphon et al, 2005) have shown substantial improvements since the start of salmon farming.

1.5. Animal feed development

Feed is the single most important economic factor governing the success of the commercial aquaculture production. Feed constitutes 40-60% of the total production cost of carnivorous fish (Tacon & Metian, 2015). The aquafeed market has grown exponentially in the last decade, whereas the fish meal production has been remarkably stable or declining. This has resulted in increased demand for fishmeal with prices rising by almost 300% in the past 10 years (Origin Oil, 2014). Feed is generally perceived to be a major constraint to successful aquaculture development. In 2016, over 1 billion tonnes of animal feed were produced globally. The total aquafeed production estimated at 4% of this total global animal feed production, accounted for 40 million tonnes (Alltech, 2017; IFIF, 2016). Production of carnivorous fish in intensive farming systems is largely dependent upon the use of compounded feed. In 2012, about 70 % (35.7 million tonnes) of the production were direct fed fishes and it was estimated that about 68% of these fishes were dependent upon the use of commercially manufactured aquaculture feeds. The consumption of these species was estimated at 39.6 million tonnes for 2012 and is expected to grow to 87.1 million tonnes by 2025 (Figure 2), assuming an increasing demand of feed of 10.3% per year since 2000 (Tacon & Metian, 2015). To reach the predicted growth of world aquaculture by 2050, the supply of feed inputs should also have to grow at similar rates to meet the demand.

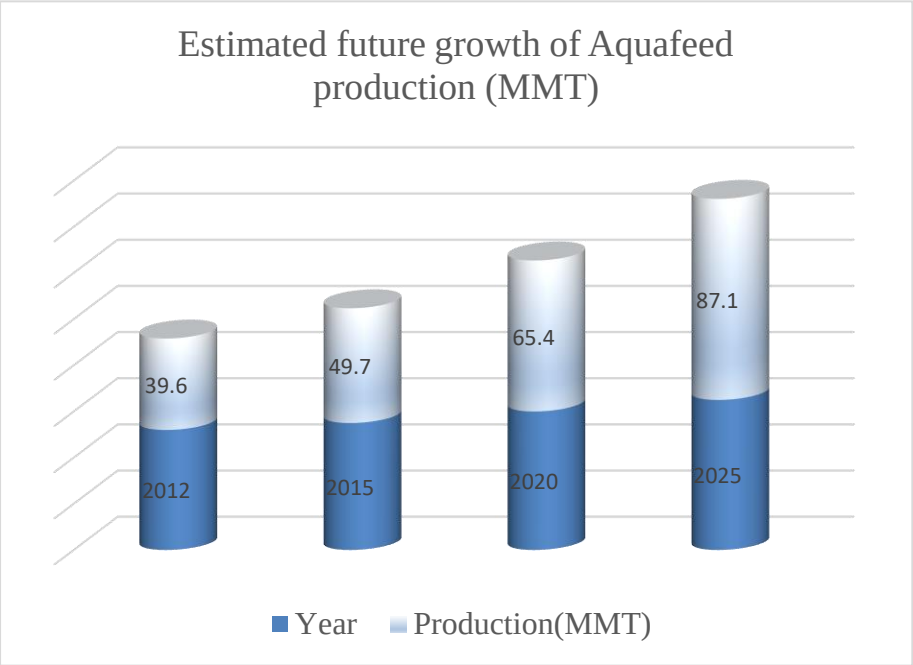


Figure 2. Total use of commercial aquaculture feeds in 2012, and the expected growth in demand for 2015, 2020, and 2025 (adopted from (Tacon & Metian, 2015)).

1.5.1. Extrusion technology to produce fish feeds

Feed manufacturing technology is combining knowledge of technology, chemistry and nutrition to produce cost effective feed for animal. The feeds are supposed to support good growth, animal health and product quality. With the industrialization of salmon aquaculture in Norway in the early 1970's, feed companies started to explore extrusion technology for manufacturing of salmonid feed. Salmonids were fed wet and moist diets before the introduction of extrusion technology (Talbot & Rosenlund, 2002). Extrusion processing is used in commercial production of fish feed. The process involves a combination of elevated temperature (120-130^{o}C), high pressure (20-30 Bar) and shear forces to transform ingredients into a dough before the pellets are shaped in a die (Alam et al, 2016). The extrusion system consists of holding bin, dosing unit, a pre-conditioner and the extruder barrel housing with one or two rotating screws (single or twin-screw extruder). The holding bin secures enough feed material for the process. A feeder doses the dry mix to the pre-conditioner. In the pre-conditioner, steam and water is added to heat and moisten the dry ingredients. The purpose of the moisture and heat is to active natural binders in the feed (starch and protein). The pre-conditioned mash is conveyed to the extruder where the temperature-induced transformation takes place in the extruder barrel, as mash is conveyed towards the outlet. A die at the outlet shapes the pellets and a knife assembly cuts the pellets at the outlet of the extruder. Use of this technology allows production of durable

high energy diets containing up to 40 % lipid. Use of high energy diets have contributed to improved feed utilization, reduced dietary protein content, improved growth performance, increased digestibility of energy and improved feed conversion ratios in farmed Atlantic salmon (Carter & Hauler, 2000).

Physical quality of feeds is usually defined as the ability of processed feed, either pelleted or granulated to withstand handling without creating excessive amount of fines (Aarseth et al, 2006; Sørensen, 2009). Some of the most important physical characteristics of feeds for salmonids are measured as: durability, breaking strength, bulk density, sinking velocity, fat absorption capacity and gelatinization of starch (Samuelsen, 2013; 2016; Sørensen, 2012). Physical quality of feed varies with ingredient composition and processing condition and may interfere with feed intake, nutrient digestibility and influence subsequent animal performance and can certainly influence final profitability. Manipulating and changing processing condition such as moisture, temperature, retention time or pressure in the extruder, influences pellet quality. Feed ingredients has also a strong impact on physical quality of feed (Kaliyan & Morey, 2009; Sørensen, 2012).

1.6. Feed ingredients in aquaculture with main focus on Atlantic salmon

The production of high quality aquaculture feeds starts with the selection of high quality ingredients. Quality of ingredients depends on its digestibility, palatability, utilization and health interference (Glencross et al, 2007).

1.6.1. Fishmeal and fish oil

Fishmeal and fish oil have traditionally been used as major ingredients in salmon feed (Sørensen et al, 2011). Fishmeal has well balanced amino acid profile and fish oil has favourable composition of long chain omega-3 (n-3) polyunsaturated fatty acid (EPA and DHA) (NRC, 2011; Tacon & Metian, 2008; Ytrestøyl et al, 2015). Fishmeal has high palatability which ensures efficient feed intake. The absence of carbohydrates and anti- nutritional factors ensures good digestibility and availability of nutrients. Fish meal also enhance fish growth and improve fish health significantly (Miles & Chapman, 2015) compared to plant ingredients.

The fish meal and fish oil in salmon feed has been reduced during the course of 1990 – 2013. In 1990, 90% of the ingredients in Norwegian salmon feed were of marine origin and was reduced to 30% in 2014 (Ytrestøyl et al, 2015). The marine ingredients are mainly replaced with plant protein and oils.

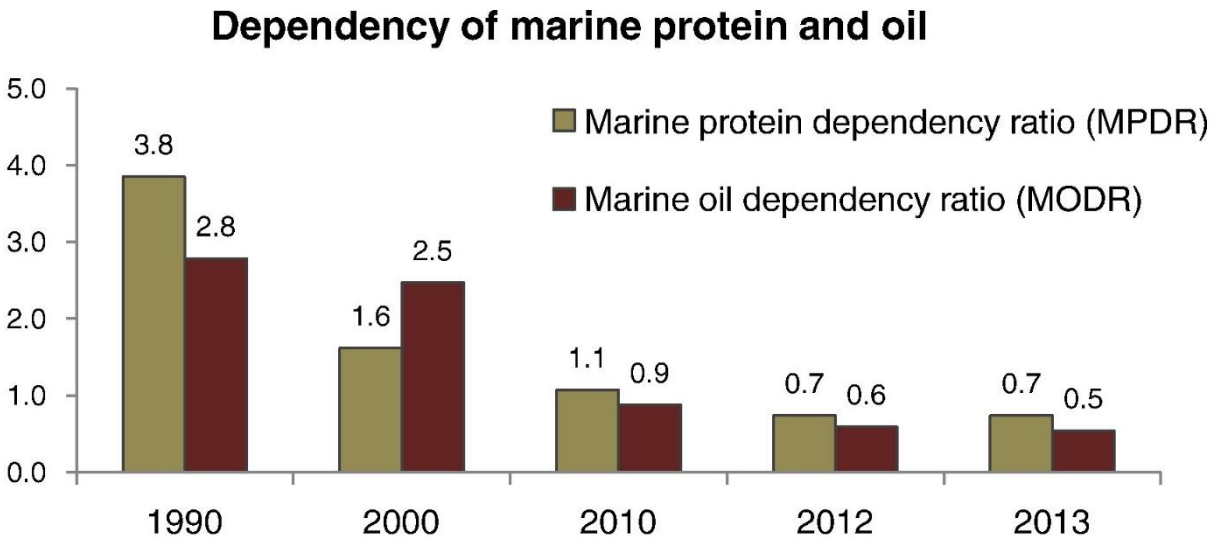


Figure 3. Dependency of marine protein and oil from forage fish in Norwegian salmon farming from 1990 to 2013. The trend is a reduction in using of marine protein and oil (Ytrestøyl et al, 2015).

1.6.2. Plant ingredients for Atlantic salmon feeds

Soybean meal, pea protein concentrate, wheat gluten and sunflower expeller are the major plant ingredients used in Norwegian aquaculture (Table 1). Fish oil is mainly replaced with rapeseed oil (Ytrestøyl et al, 2015). In contrast to fishmeal and fish oil, world production of plant derived ingredient has increased over the past two decades as a result of their abundant availability and favorable price (Rosenlund et al, 2016). Growth in the salmon production enabled an extensive development of new alternative feed plant ingredients to replace fishmeal and fish oil (Sørensen et al, 2011; Ytrestøyl et al, 2015). Compared to fishmeal, most ingredients contain lower levels of protein, higher levels of indigestible carbohydrates, imbalanced amino acid profile and may contain antinutritional factors (ANF) and/or antigens. Plant oil does not contain polyunsaturated fatty acids EPA and DHA. The long-term plant oil on fish production, health, and product quality are still unknown (Ringø et al, 2009; Sissener et al, 2016).

Table 1. The major ingredients used in production of Norwegian salmon feed in 2013 (Extracted from (Ytrestøyl et al, 2015)).

		Feed ingredient	Total amount used (tonnes)	% inclusion (of total diet)
Plant ingredients	**Protein sources**	Soy protein concentrate	346,730	21.3
		Sunflower expeller	97,354	6
		Wheat gluten	97,137	5.8
		Fava beans	30,753	1.9
		Pea protein	12,936	0.8
		Maize gluten	12,509	0.8
	Oil sources	Rapeseed oil	298,991	18.3
Marine ingredients	**Protein sources**	Fish meal	317,241	19.5
	Oil	Fish oil	182,579	11.2

1.6.3. Terrestrial animal by products

Animal by-products are obtained from by-products of major livestock and poultry ventures. By-products from terrestrial animals include blood meal, hydrolysed feather meal and poultry by product meal, have the potential to be used in aquafeed (Sørensen et al, 2011). Generally, they are safe, contain no anti-nutritional compounds and represent a protein-rich alternative. Poultry meal and feather meal hydrolysate have an essential amino acid profile comparable to fishmeal, but lower methionine, lysine, histidine and tryptophan (Naylor et al, 2009). According to Hertrampf and Piedad-pascual (2012), protein content is high in these ingredients. Poultry by-product has shown great potential as a promising protein sources for carnivorous fish. However, due to the rendering process, protein quality of by-products may be reduced compromising nutrient bioavailability (Naylor et al, 2009).

1.7. Microalgae

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow almost anywhere (In aquatic as well as on terrestrial environments), representing a large variety of species living in a wide range of environmental conditions. By estimate, more than 50,000 species exist, out of which, around 30,000, have been studied and analyzed (Richmond, 2008). Microalgae can be either phototropic, heterotrophic or a mix of these two, called mixotrophic.

In spite of a large number of species, only a very few is cultivated and approved for use in the food chain. Only ingredients available in certain amounts can be evaluated as potential feed ingredients. e.g. *Tetraselmis* and *Nannochloropsis.*

Tertraselmis sp.(chlorophyceae) are unicellular flagellate characterized by their cordiform and elliptical shape with a ranging size from 4 to 8 µm. They are highly recommended for fish larvae when combined with *Nannochloropsis* and *Isochrysis*. *Tetraselmis* sp. are an excellent source of EPA and linoleic acid, essential fatty acids for larvae development and growth performance. They are also rich in certain amino acids, vitamins and pigments (Phyto bloom, 2013).

Nannochloropsis sp. (Eustigmatophyceae) are small unicellular free floating green algae. They are spherical in shape and has a diameter ranging from 2 to 4 µm. *Nannochlopsis* are used as natural food resources for zooplankton (Rotifers, Artemia) in the food chain and filter feeders (Molluscs and crustacean larvae) (Becker, 2007). *Nannochloropsis* have capacity to accumulate a large quantity of lipids (Moody et al, 2014) and are good source of EPA and ARA which both are crucial for survival and larvae development, improving stress and disease resistance. They are also important source of certain vitamins, pigments and amino acids such as arginine, histidine, isoleucine, leucine, valine and methionine (Phyto bloom, 2013).

1.7.1. Chemical composition and nutritional value of microalgae

The chemical composition of microalgae reflects their nutritional potential. There are numerous reports on chemical composition of different microalgae (Table 2) (Becker, 2004; Brown, 2002; Shields & Lupatsch, 2012; Tibbetts et al, 2015; Yaakob et al, 2014).

Table 2. General composition of different algae (% of dry matter). Adapted from Becker (2007).

Algae	Protein	Carbohydrates	Lipids
Anabaena cylindrica	43-56	25-30	4-7
Aphanizomenon flos-aquae	62	23	3
Chlamydomonas rheinhardii	48	17	21
Chlorella pyreinoidosa	57	26	2
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Porphyridium cruentum	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	10-14
Spirogyra sp.	6-20	33-64	11-12
Arthrospira maxima	60-71	13-16	6-7
Spirulina platensis	46-63	14-18	4-9
Synechococcus sp.	63	15	11

The application of microalgae as a feed ingredient depends on detailed information about chemical composition, nutrient digestibility, as well as nutrient bioavailability (Skrede et al,

2011). Nutritional value may also depend on the various culture conditions, shape, size and digestibility (Cheirsilp & Torpee, 2012; Durmaz et al, 2009; Fernández-Reiriz, 1989). Chemical composition of some commercially important microalgae are comparable with ingredients commonly used in the aquafeed industry (Table 3).

Table 3. Typical composition of commercially available feed ingredients and microalgae species (% of dry matter). Shah et al. (2017).

Feed ingredients	Protein (%)	Lipid (%)	Carbohydrate (%)	Ash (%)	Gross energy (MJ/kg-1	References
Fish meal	63	11	-	15.8	20.1	(Shields & Lupatsch, 2012)
Soybean	44	2.2	39	6.1	18.2	
Chlorella sp.	52	7.5	24.3	8.2	19.3	(Shields & Lupatsch, 2012)
Dunaliella salina	49-57	6-8	4-32	-	-	
Scenedesmus obliqus	50-56	12-14	10-52	-	-	Becker (2007)
Arthrospira platensisc	55.8	14.2	22.2	7.8	22.7	Tibbets et al. (2015a,b,c,d)
*Tetraselmis chuii (*PLY-429)	46.5	12.3	25	16.2	19.9	Tibbets et al. (2015a,b,c,d)

1.7.2. Microalgal protein and amino acid composition

Microalgal may represent a potential protein source because of their good quality protein profiles comparable with that of other conventional high-quality plant proteins (Becker 2007) (Table 3). There are several studies on the utilization of biomass of *Arthrospira sp., Chlorella sp., Scenedesmus sp., Nanofrustulum sp., and Tetraselmis suecica* partial substitutes for fishmeal protein in the diet of various omnivorous and carnivorous fish species (Badwy et al, 2008; Blazencic, 2007).

Amino acids in fish feed are important for protein synthesis, feed utilization, growth, stress response, immunity and survival (Li et al, 2009). Amino acid composition of some microalgae are comparable with fish meal (Table 4) (Ryckebosch et al, 2014). Studies with a large array of microalgae have shown that amino acid pattern are quite similar among species, though minor differences are present. The essential amino acids lysine and methionine may be at the same level, or lower than fish meal (Brown, 1991; Roy & Pal, 2015).

Table 4. Amino acid composition of several microalgae compared with fish meal

	C. gracilis [1]	*N. Oceania* [2]	*Isochrysis galbana* [2]	*Phaeodactylum tricornutum* [2]	N. closterium [1]	High quality fish meal [1]
Lysine	6.3	4.8	3.1	4.2	5.7	6.8
Methionine	2.4	1.8	2.5	2.0	1.6	2.5
Tryptophan	1.6	1.7	2.5	1.3	1.4	0.7
Threonine	4.5	3.6	4.6	3.7	5.5	3.5
Valine	5.9	4.6	6.1	4.6	6.2	4.0
Isoleucine	5.8	3.5	5.1	3.8	5.0	3.7
Leucine	7.2	6.7	9.2	6.2	8.1	6.2
Phenylalanine	6.7	3.9	5.7	4.2	6.9	3.3
Arginine	6.6	4.9	4.1	4.4	6.6	5.4
Histidine	2.4	ND	ND	ND	1.4	ND

ND- not determined

[1] Brown (1999)

[2] Skrede et al. (2011)

1.7.3. Microalgal lipids and fatty acid composition

Microalgae mainly produce fatty acids with chain lengths of 16 and 18 carbon atoms, but some species can make fatty acids of up to 24 carbon atoms in length. They are mainly present in the form of glycerolipids. These glycerolipids in turn mainly consist of phospholipids, glycolipids, and triacylglycerol (TAG) (Brown & Farmer, 1994). Fatty acid profile of microalgae consists saturated, monounsaturated and polyunsaturated fatty acids (PUFA). Fatty acid composition may differ among different algal species (Table 5). The fatty acid composition can be manipulated with light intensity, culture media, temperature, and pH (Daroch et al, 2013), the cell cycle, lipid class composition and membrane fluidity of microalgae (Napolitano, 1999). However, Brown (2002) reported that there is correlation between different algal taxa and fatty acid composition. Microalgae may be a promising source of PUFA in fish diets. The content of highly unsaturated fatty acids (HUFA), in particular eicosapentaenoic acid (20:5n-3, EPA), arachidonic acid (20:4n-6, ARA), and docosahexaenoic acid (22:6n-3, DHA), is of major importance in the evaluation of the nutritional composition of an algal species to be used as food for marine organisms. Compared with other micro algal classes, chlorophytes (e.g.

Scenedesmus sp.) usually has deficiency in PUFA while eustigmatophytes (e.g. *Nanochloropsis* sp.) and cryptomonads (e.g. *Chryptomonas* sp.) are rich in PUFAs (Brown, 2002).

Table 5. Fatty acid composition of selected microalgae types (Napolitano, 1999)

	Oscillatoria sp.	***Scenedesmus sp.***	***Cladophora sp.***	***Navicula sp.***	***Chryptomonas sp.***	***Peridinium sp.***
14:0	2.1	1.4	0.5	3	5	6.9
16:0	18.5	12.7	39.3	16	10	28.8
16:1ω7	22.6	-	1.3	31	9	1.6
C16 PUFA	-	-	0.5	17	1	-
18:0	1.6	0.6	0.3	1	8	0.6
18:1ω9	1.9	11.6	12.9	2	16	29
18:1ω7	0.5	0.4	1.1	-	-	-
18:2ω6	11.4	12.9	10.7	1	26	0.5
18:3ω6	-	1.3	0.7	-	-	0.1
18:3ω3	24.6	22.2	12.2	1	5	0.2
18:4ω3	-	3.3	-	-	-	5.2
20:0	-	0.2	0.3	-	-	-
20:1ω9	-	0.3	0.6	-	-	-
20:4ω6	-	2.8	-	-	-	2.0
20:5ω3	-	-	2.3	26	-	7.8
22:5ω3	-	-	-	-	-	-
22:6ω3	-	-	0.02	-	-	11

1.7.4. Microalgal Carbohydrates and Microalgal cell walls

Carbohydrates of algae are found in the form of starch, cellulose, sugars and other polysaccharides. Microalgae cells are surrounded by a dynamic, complex, carbohydrate-rich cell wall which may represent 10% of the algal dry matter. The cell wall is a robust structure that completely encloses the cytoplasm and allows the cell to increase its turgor pressure without bursting (Demozych, 2011). The cell wall exerts considerable biological and biomechanical control over individual cells and organisms, thus playing a key role in their environmental interactions (Popper et al, 2011). The biochemical composition of cell walls varies amongst algal groups (Hu & Richmond, 2004). Most carbohydrates are found in the cell walls. The thick cell wall of microalgal can prevent digestion, absorption of nutrients from the

cell. Besides, type and quality of extracellular polysaccharides of certain microalgae can also interfere with nutrient absorption (Baker, 1975; Roy & Pal, 2015).

1.7.5. Other biochemical components from microalgae

Microalgae are also rich in biomolecules such as pigments, and nutraceutical compounds. Pigments such as astaxanthin, lutein, and beta carotenoids play a crucial role for skin and flesh coloration of rainbow trout and salmonids (Del campo et al, 2007). Today synthetic astaxanthin is added to salmon feeds to enhance the fish color of the salmon flesh (Ambati et al, 2014; Shah et al, 2016). Beta 1-3 glucan from microalgae has initiated host defense mechanisms and improved both specific and nonspecific immunity of fish species such as Rohu (Misra et al, 2006) and rainbow trout (Skov et al, 2012). Incorporation of ascorbic acid rich algae such as *Chaetoceros gracilis, T. pseudonana* in salmonid feed resulted in improved reproductive performance, reduce oxidative damage and disease resistance (Sandnes et al, 1984).

1.7.6. Potential and challenge using microalgae as aqua feed ingredient

Micro algae in fish feed have shown enhanced skin coloration, improved carcass quality, diseases and stress tolerance, decreased nitrogen output into the environment (Borowitzka, 1997). Other advantages of using microalgae as feed ingredients is that they can grow in a wide range of habitats, some species have several-fold higher biomass production than plants, they can divide fast with simple nutritional requirements, accumulation of useful metabolites, and availability is independent of wild fish harvesting for fishmeal (Hemaiswarya et al, 2011). Current challenges to use microalgae in aquafeed is the rigid cell walls of some microalgae (e.g., Chlorella), that limit nutrient bioavailability to the fish (Øverland et al, 2010). The high production costs of microalgae remain a constraint to the aquaculture industry. The collection, drying of microalgae require considerable time and effort. Inappropriate drying may adversely affect their nutritional and physical properties and greatly reduce their value as feeds (Skrede et al, 2011). Another drawbacks of microalgal ingredients is the selection of best microalgae for culturing, designing efficient systems for culturing and efficient harvesting of micro algae (Chen & Walker, 2011). Marine microalgae may contain high amount of salt, which may limit the use in feed compared with fishmeal.

1.7.7. Micro algae as feed ingredient

Studies on the use of micro algae as a possible ingredient for aquafeed for different fish species has been increasing exponentially. Several recent reviews (Hemaiswarya et al, 2011; Roy & Pal, 2015; Yaakob et al, 2014; Ytrestøyl et al, 2015) have indicated high potential for microalgae as a bulk feed stuff for aquaculture feeds. However, each species of micro algae

represents a novel ingredient and need to be thoroughly tested on the target species to evaluate the potential to support or/and growth performance, nutrient utilization and to ensure animal health and safety of the fish. Tilapia (*Oreochromis mossambicus*) fed with *Spirulina maxima* at 20% inclusion increased feed utilization, growth performance and nutrient utilization (Olvera-Novoa et al, 1998). Digestibility varies among different strains. Atlantic salmon (*Salmo salar*) fed with *Spirulina* sp. at 30% inclusion level showed protein digestibility of 84.7% (Burr et al, 2011), whereas 6% inclusion of *Phaeodactylum tricornutum* at in Atlantic salmon diet (Sørensen et al, 2016) showed protein digestibility of 90%. This indicate that inclusion level of various algae species has to be carefully optimized. According to shi et al. (2016), different processing conditions of feeds may affect the digestibility and overall feed utilization. Gibel carp fed extruded feeds showed higher digestibility of dry matter and protein compared with fish fed pelleted feeds, particularly those of feeds with low fishmeal. Gong et al. (2017) also found that the inclusion of extruded defatted *Nannochloropsis* sp. biomass was more digestible than the cold pelleted *Desmodesmus* sp. biomass in diets fed to Atlantic salmon (*Salmo salar*).

1.8. The objective of the study

This study was designed to investigate effect of double extrusion (thermo mechanical treatment) on nutrient digestibility. Unfortunately, Yttrium oxide (Y_2O_3) the inert marker to calculate apparent digestibility coefficients was, by a mistake not included in the experimental diets. Hence, the aim of the study was diverted to investigate the effect of experimental diets on growth, proximate composition of whole body, fatty acid composition of fillet and physical quality of feed.

The hypothesis of the present study was that incorporation of microalgae *Nannochloropsis* sp. and *Tetraselmis*.sp. in diets for Atlantic salmon had no negative effects on growth performance and whole body proximate composition. The aims of the study were 1) to examine the effect of replacing fishmeal with processed or non-processed *Nannochloropsis* sp. and *Tetraselmis*.sp. in feeds for Atlantic salmon on growth performance, fatty acid composition of fillet and whole body proximate composition. 2) to investigate the effect of *Nannochloropsis* sp. and *Tetraselmis*.sp. on physical quality of the feed.

2. Material and Methods

2.1. Fish and experimental setup

The feeding experiment was conducted at the Mørkvedbukta research station, Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway.The trial had a duration of 9

weeks (60 days). The feeding trials were carried out in 20 circular fiber glass tanks (800 l and 0.9 m deep) in a flow- through sea water system. The tanks were maintained under consistent 24-hour photoperiod regime. The sea water was pumped from the deep (250m) basin of Saltenfjorden, Bodø with a salinity of 35‰ and supplied to the system. Flow rates of 1000L per hour were maintained through the experimental period. Dead fish were removed, and weight was recorded. The water temperature during the experimental period had a mean of 8.6°C ± 0.21, ranging from 8.0°C to 9.0°C. Dissolved oxygen was measured in the outlet, with a mean saturation of 86.8% ± 3.10, ranging between 81.6% and 95.4%, (Figure 4). Water quality parameters such as oxygen and temperature were measure using a hand held OxyGuard Handy Polaris 2 Portable DO Meter (Oxyguard International A/S, Denmark).

Experimental fish of Atlantic salmon (*Salmo salar)* post-smolts (AquaGen R*E QTL) were purchased from a commercial producer (Cermaq Norway AS, Hopen, Norway) in june 2017. Fish were acclimated and maintained at the research station for more than two months before the experiment started. The fish were fed commercial feeds until they were used for the feeding trials. At the start of the experiment, a group of 780 Atlantic salmon (initial mean body weight 154.17g ± 26.48) and (initial mean fork length 24.21cm ± 1.40) were weighed, and randomly allocated to 20 tanks (39 fish/tank). The experiment was approved by the National Animal Research Authority (FDU, ID-5887, Norway).

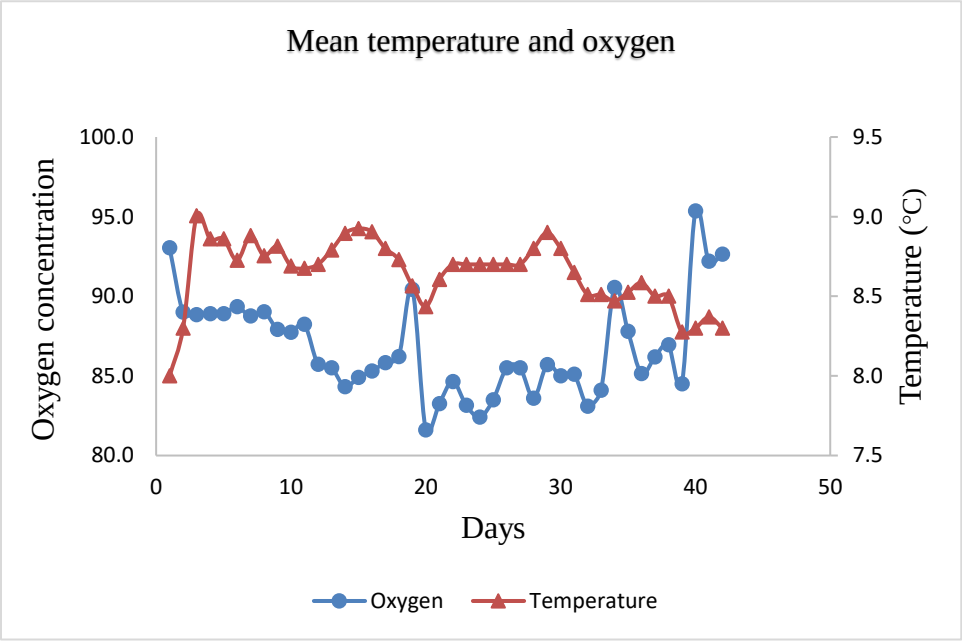


Figure 4. Mean dissolved oxygen and temperature throughout the experiment. Temperature and oxygen was inversely related to each other.

2.2. Experimental design and diets

The feeding experiment was designed to investigate the nutrient digestibility of two different microalgae. The microalgae were either added to the feed *as is* (extruded), or the algae received a thermo-mechanical pretreatment with use of an extruder (pre-extruded). Five experimental diets were used in the trial: one control diet (CTRL) and 4 test diets (Tables 6 and 7). The test diets consisted of 70% CTRL diet and 30% dry biomass of either *Nannochloropsis* (NPE) (pre-extruded), *Nannochloropsis* (NEN) (extruded), *Tetraselmis* (TPE) (pre-extruded) or *Tetraselmis* (TET) (extruded). Each of the diets were fed to fish in 4 replicate tanks and 39 fish per tank. The allocation of experimental feed to the different tanks are shown in (Figure 5).

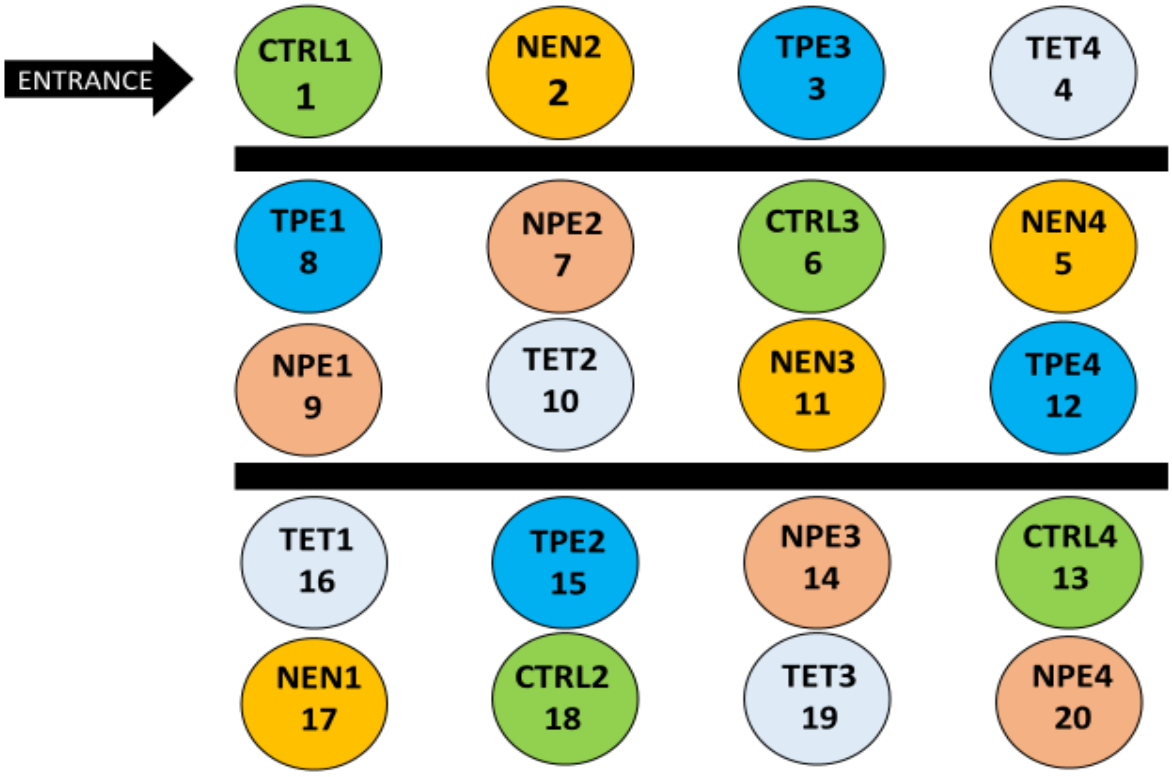


Figure 5. Allocation of feed to the experimental units. Each of the treatment groups consisted 4 replicates (CTRL: Control diet, NEN: *Nannochloropsis* extruded diet, NPE: *Nannochloropsis* pre-extruded diet, TET: *Tetraselmis* extruded diet and TPE: *Tetraselmis* pre-extruded diet).

The compositions of the diets were formulated to meet the nutrient requirement for Atlantic salmon, to ensure the fish were not exposed to nutrient deficiencies. The goal of the experiment was originally to evaluate nutrient digestibility of *Nannochloropsis* and *Tetraselmis*, and if the digestibility could be improved by use of double extrusion. Yttrium oxide (Y_2O_3) was therefore planned as an inert marker to calculate apparent digestibility coefficients. Unfortunately, the

marker was not added to the feeds by a mistake. It was therefore not possible to calculate nutrient digestibility of the algae. The feeds were produced by Sparos, Portugal.

Table 6. Ingredient composition of the five different Experimental diets

Ingredients	**CRTL**	**NEN 30**	**NPE 30**	**TET 30**	**TPE 30**
	%	%	%	%	%
Fishmeal LT70[1]	52.000	36.400	36.400	36.400	36.400
Nannochloropsis (no process) [2]		30.000			
Nannochloropsis (pre-extruded) [2]			30.000		
Tetraselmis (no process) [2]				30.000	
Tetraselmis (pre-extruded) [2]					30.000
Wheat gluten[3]	15.000	10.500	10.500	10.500	10.500
Wheat meal[4]	8.480	5.936	5.936	5.936	5.936
Pea starch	6.000	4.200	4.200	4.200	4.200
Fish oil[5]	17.500	12.250	12.250	12.250	12.250
Vit & Min Premix PV01[6]	1.000	0.700	0.700	0.700	0.700
Yttrium oxide[7]	0.020	0.014	0.014	0.014	0.014

[1]NORVIK 70, Sopropeche, France, [2]All microalgae, Portugal, [3]ROQUETTE Frères, France [4]Casa Lanchinha, Portugal, [5]SAVINOR UTS, Portugal, [6]PREMIX Lda, Portugal,[7]Sigma-Aldrich, Spain.

Table 7. Proximate composition of the feeds based upon dry matter basis

Parameter	CTRL	NEN 30	NPE 30	TET 30	TPE 30
per 100 g dry matter					
Dry matter	92.6	92.5	91.9	93.4	90.4
Protein	52.4	47.0	47.0	44.8	45.3
Lipid	20.9	18.4	18.8	16.0	17.5
Ash	8.7	17.2	16.9	16.6	16.1
Energy (MJ/1000 g)	23.4	21.5	21.7	20.7	21.2

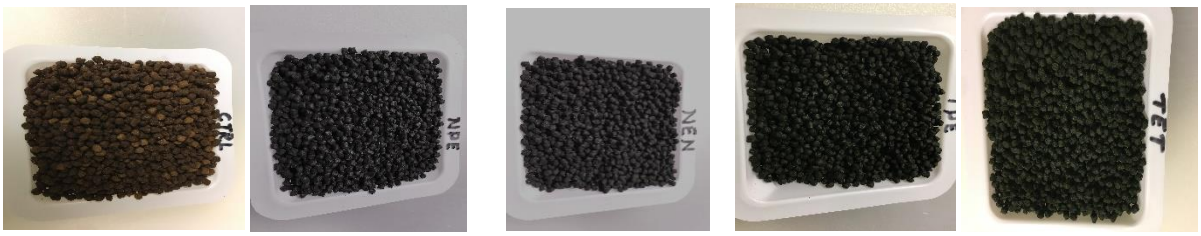


Figure 6. Physical appearance of experimental diet after extruder. The colour of the diet was light brown (CTRL), light green (NPE & NEN) and dark green (TPE & TET).

2.3. Feeding regime of fish

After stocking of the fish, the five experimental diets were randomly assigned to 20 tanks, resulting in four replicates per diet. The fish were fed the experimental diets in two feedings per day, from 08:00-09:00 in the morning and 14:00-15:00 in the afternoon using automatic feeders (Arvo Tech, Finland). Feeding rate was adjusted on a weekly basis, based on the average feed intake of the previous week, targeting 10-15% waste feed. The daily ration was approximately 1% of their biomass when digestibility experiment was started. The feeding level was gradually increased from 1% of biomass to 1.2% and 1.4% within the first 10 and 13 days of the trial, respectively. Feeders were filled when half empty, and all feeders were filled at the same time. Throughout the trial, fish behavior also was monitored.

2.4. Fish Sampling and data collection

At the end of the feeding trials, the total number, individual body weight and length of fish in each tank were measured. The fish were transferred to holding tank (500 l) and anaesthetized in a smaller tank (50 l) with tricaine methanesulfonate (MS-222) at concentration 40 mg/litter. Individual fish were stripped for faeces according to a procedure described by Austreng et al. (1978) and then placed back in their respective tank. The fish were stripped three times (two weeks' time interval between strippings). Faeces sampled from the fish in each tank were pooled to ensure enough material for chemical analysis and kept frozen at -40 ^{o}c. Final samples for whole body and fillet chemical composition were collected. Whole body of six fish from each replicate tank (n=39 for each treatment; total 234 fish) were randomly collected for final whole-body analysis and three fish from each replicate tank (n=3) for fillet chemical analysis were also collected. Sampled fish were sacrificed by giving percussive stunning to the head. Immediately after termination of experiment, all samples were transferred to the main campus at Nord University and kept frozen at -40°C until they were used for analysing.

2.5. Sample preparation

Fish from final samplings of whole body (6 fish per tank) and fillet (3 fish per tank) samples were thawed and homogenized prior to chemical analysis. Fish samples were taken out from the freezer and thawed 24 hours before the analysis of whole body proximate composition. Each fish was cut into pieces, and minced using industrial food processor (Foss tecator, 2096 homogenizer, Denmark) until it became well homogenized. Fish samples were used for analyses of protein, crude fat, ash, moisture and energy. Fillets were taken out of the freezer and thawed for approximately 3 hours. Fillets were then minced to a homogeneous sample using a home food processor (Bosch, Grinder MCM2004/02, Slovenia). These samples were used for analyses of total lipid and fatty acids composition. Approximately 100 gr of each diets were homogenized in a food processor (Retsch, Grindomix GM 200, Germany) for analyses of the experimental diet samples. Protein, moisture, ash and energy were determined from these samples. Faeces samples were freeze dried (VirTis benchtop U.S) for 72 hours in -76°C with 20 μ Bar and pooled within feed group, reducing number of replicates from twelve to four prior to the analysis of chemical analysis. These samples were used for protein, moisture, ash and energy. Proximate chemical composition was analysed in duplicate. Experimental feeds were analysed in 4 replicates per diet.

2.6. Proximate chemical analysis of whole fish, faeces and feeds

Commonly used methodologies for biochemical analysis of diets, faeces and fish samples are briefly described below.

2.6.1. Moisture analysis

Dry matter content was measured in duplicate by using oven drying at 105 °C for 20 hours to constant weight (ISO 6496-1999). Then dried samples were taken in to desiccator for 20 minutes. Approximately 5 g of the homogenized fish samples and/ or diet (1 g for faeces) were weighted into the steel cups. Loss in weight represented moisture content.

Moisture (%) = {(weight before drying-weight after drying/weight before drying)} *100

2.6.2. Ash content

Samples were taken in crucible with cover and placed in a muffle furnace at 550 °C for about 16 hours until sample weight becomes constant (ISO 5984–2002).

Ash (%) = (weight of ash/sample weight) *100

2.6.3. Protein analysis

Approximately 1 g of the homogenized fish samples (0.3 g for faeces and 0.5 g for feeds) and 2 tablets of catalyser (K_2SO_4:$CuSO_4$) were digested with 15 ml of concentrated H_2SO_4 for 50 min at 420 °C. Then digested samples were cooled down for about 30 min, 75 ml of distilled water was added, and samples were distilled using (Kjeldhal auto system, Tecator Systems, Höganäs, Sweden; ISO 5983-1987). Crude protein was calculated from content of N x factor 6.25.

2.6.4. Crude lipid content

Crude lipid content was determined using Ethyl Acetate to extract the lipid. Approximately 10 g of homogenized fish sample was weighted into a porcelain cup and 20 g of Na_2SO_4 was added to the same cup. Samples were ground together to dry powder and transferred into a bottle with 50 ml of Ethyl Acetate. samples were placed on homogenizer for one hour and the homogenate were transferred to separatory funnel filtered through Whatman filter paper (41, CAT NO. 1441-150) to a measuring cylinder. 20 ml of the sample were transferred to a weighed glass cup on water bath until all the water removed. Glass cups were then dried using the oven at 105°C for 15-20 min to remove all moisture. Weight of the dried samples were used to measure the crude lipid content of the samples.

2.6.5. Energy content

Gross energy was determined in duplicate with a bomb calorimeter (IKA, c200, GmbH & Co. KG, Germany) (ISO 9831–1998). Approximately 0.5g of homogenized fish sample and/or faeces were pelleted and placed on calorimetry. Energy released from each pellet was recorded.

2.6.6. Yttrium and lipid content of faeces and feeds

Yttrium and lipid content of freeze dried faeces and feed samples were analysed by Eurofins®. Yttrium content was determined by employing inductive coupled plasma mass spectroscopy (ICP-MS) by Eurofins (Moss, Norway) (NS-EN ISO 11885).

The lipid content of the feeds and faecal samples were determined by Soxhlet method with acid hydrolysis (Soxtec HT 6209, Tecator, Höganäs, Sweden modified; AOAC method 954.020).

2.6.7. Total lipid and fatty acids

Extraction of total lipids from freeze dried fish fillet samples were analysed according to Bligh and Dyer (1959) method. Approximately 0.05 g fish fillet samples were homogenized with 0.08ml of distilled water, 1ml of chloroform and 2ml of methanol by homogenizer for 1 minute. Another 1ml of chloroform were added and homogenized again for 30 sec. 1ml of distilled

water was added to the homogenate and were centrifuged for 10 minutes, 4000rpm at 4^{o}C. The volume of chloroform, methanol and water were maintained in the proportions 1:1:0.08, respectively. The lower phase which contained chloroform and lipid fraction was collected to a kimax tube and evaporated the chloroform completely with nitrogen gas at ReactiVap (Thermo fisher). 0.5ml of the sample was transferred to a pre-weighed glass crucibles and solvents were removed using grant heater at 40 °C. Dried lipid was used to determine the content of lipids in the sample by weight difference. The total lipid was calculated as follows:

Total lipid (%) = {lipid weight(g)/ dried sample weight(g)} *100

Next to the extraction, hydrolysis of lipid was analysed according to Metcalfe et al. (1961) method. 1ml of 0.5M NaOH-methanol was added to the dry sample and heated at grant heater for 15 minutes at 100^{o}C. Samples were then cooled for 5 minutes using ice. 2ml, 12% BF3-methanol was added to the samples. Samples were again heated for another 5 minutes at 100^{o}C and then cooled for 4 minutes.1ml of hexane was added to the sample and heated for 1 minute and samples were cooled for 3 minutes. After that, 3ml, saturated NaCl in distilled water was added to the sample. After two-three minutes, added two times 0.5ml hexane, shaken vigorously and stood for evaporation. 0.5ml of hexane layer (from the upper phase) was transferred in to a pre-weighed glass vial to evaporate the solvents at 40^{o}C at grant heater. The weight of vial was measured after evaporation to calculate the concentration of the sample. Finally, sample was diluted with hexane (50:950), respectively and stood for GC.

Dietary fatty acid contents were identified with the gas chromatograph SCION 436-GC equipped with a wax embedded column, part number CP7713, CP-WAX 52 CB 25m x 0,25mm x 0,20µm (Agilent Technologies) and by reference of known standard (Fame mix2, Absolute standards, Inc. Fatty acids were measured by peak integration and expressed as relative area percentage on the total fatty acid area by using a software Compass CDS, Bruker Co-operation.

2.7. Physical quality of diet

2.7.1. Hardness and diameter

Strength at rupture (hardness) of the extruded pellet was recorded diametral compression using TA-XT2 (Stable Micro Systems Ltd, Surrey, England) analyser. Feed pellets (N = 20) from each diet were randomly selected. The feed pellets from each diet group were placed horizontally and force at rapture was measured by pressing a cylindrical probe (SMP/0.5, 1.2 cm width) onto the pellets to achieve 60% compression at constant speed of 1mm sec^{1}. The hardness of pellets was recorded automatically by stable micro computer program (TA-XT2)

in Newton. Diameter was measured on the same pellets that are used for the hardness analysis. The diameter of the pellets was automatically measured by the texture analyser.

2.7.2. Pellet length

Length of the pellets was manually measured by use of an electronic Vernier calliper (Biltema® Art. 16-105) in mm. 100 pellets in four replicates for each diet were randomly selected and analysed for the length of the diets.

2.7.3. Fat leakage

Fat leakage was tested as the loss of oil from diets. Approximately 100 g of each diet were weighed in a plastic tray with two layers of Whatman gel blotting paper (Grade GB003, 30x60 cm) were incubated at 40 °C for 24 h in heating oven. Final weight of the diet was recorded to determine the fat leakage and calculated as fat leakage in %. four replicates for each diet were performed in the analysis.

2.7.4. Water stability

Approximately 3g sample of pellets from each diet group were placed into pre-weighted embedding cassette (M 512 Macrosette™, Simport®, Canada). Pellet were randomly taken, and test were carried out in 4 replicates for each treatment. Cassettes containing pellets were placed on to a beaker with a 600 ml of water. Beakers were then incubated in a water bath (Julabo™, SW22, Seelbach, Germany) at a temperature of 25 °C and subjected to 100 rpm shakings per minute at different time period 30, 60 and 90 min. After each trial, cassettes were placed on paper tissues and gently dried. All the cassettes were then placed on pre-heated oven at 80 °C for 48h. Pellet residual dry matter weight of each cassette were determined after drying. Water stability was calculated as weight difference of dry matter before and after incubation, divided by dry matter weight of the feeds before incubation.

2.8. Calculations and statistical analysis

Fish growth performance was calculated based on following equations:

Weight gain (WG %) = (final wt – initial wt) × 100/initial wt

where, Final wt=final body weight of fish (g/fish) and initial wt =initial body weight of fish (g/fish)

Specific growth rate (SGR, % day^{-1}) = {Ln (final wt) – Ln (initial wt)/days} × 100

Thermal growth coefficient (TGC) = {(final wt)$^{1/3}$ – (initial wt)$^{1/3}$/(Txt)} x 100

where T is temperature in °C and t is time in days.

All data were subjected to statistical analysis using SPSS 24.0 IBM software package for Windows. For parametric data, one-way variance (ANOVA) was used to analyse the data. The data were tested for normality (Shapiro–Wilk normality test) and equality of variance (Levene's test). Tukey's multiple comparison test were used to identify significant difference among the means. For non-parametric data Kruskal-Wallis test was performed followed by Dunn's multiple comparison test. Significant differences between microalgae *Nannochloropsis* sp. and *Tetraselmis* sp. values contained extruded and non-extruded feeds and interaction between algae and extrusion were identified by employing the two-way variance (ANOVA). Correlation analysis was performed by using Pearson correlation coefficient (SPSS 24.0 IBM software package for windows). The difference between treatment was considered significant at $p<0.05$ and $0.05<p<0.01$ considered as tendency.

3. Result

All the experimental feeds were well accepted by Atlantic salmon. The experimental fish were in good condition, and no mortality was observed during the course of the experiment.

3.1. Growth performance of the fish

The fish growth performance results are summarized in (Table 8). The fish grew from an average initial weight of 154.2 g to an average final weight of 291.5 g, a 137.3 g body weight gain during the course of the 9 week feeding trial. Significant reduction of body weight gain (g) was noted in fish groups fed NEN 30, NPE 30, TET 30 and TPE30, compared with CTRL group. Growth performances in terms of weight gain (%), SGR (% day) and TGC in fish fed NEN 30, TET 30 and TPE30 were also significantly lower compared to the CTRL group. Fish fed the NPE 30 tended to have higher weight gain (%), SGR (% day) and TGC compared with those fish fed NEN 30, TET 30 and TPE30. Fish fed the TET 30 had the lowest final mean body weight (g), weight gain (%), SGR (% day) and TGC compared to those fed CTRL feed. However, weight gain (%), SGR (% day) and TGC were not significantly different among fish fed NEN 30, TET 30 and TPE 30 diets.

Table 8. Growth performance of Atlantic salmon

Parameter	CTRL	NEN 30	NPE 30	TET 30	TPE 30	P value
Growth parameter						
Initial mean body weight (g)	154.4±0.13	154.0±0.1	153.9±0.35	154.4±0.17	154.3±0.19	0.342
Final mean body weight (g)	307.8±1.62^{a}	288.9±4.49^{b}	292.9±1.73^{b}	282.9±3.41^{b}	285.1±3.58^{b}	0.001
Weight gain (%)	99.4±1.02^{a}	87.6±3.15^{b}	90.3±1.48^{ab}	83.3±2.16^{b}	84.8±2.41^{b}	0.001
Specific growth rate (% day)	1.2±0.01^{a}	1.0±0.03^{b}	1.1±0.01^{ab}	1.0±0.02^{b}	1.0±0.02^{b}	0.001
Thermal growth coefficient	2.7±0.02^{a}	2.4±0.07^{b}	2.5±0.03^{ab}	2.3±0.05^{b}	2.4±0.05^{b}	0.001

Values are expressed as mean ± SD (n=4 replicates). Values in the same row with different superscript letters show significant differences ($p<0.05$)

3.2. Whole body proximate composition

The whole body proximate composition of experimental groups at termination of the experiment is presented in (Table 9). Highest protein content was found in salmon fed diet CTRL, but the values were not significantly different from the protein content in fish fed diets NEN 30 and TET 30. Fish fed NPE 30 and TPE 30 had lower protein content than fish fed diets CTRL, NEN 30 and TET 30. Content of lipid was significantly higher in fish fed diet TPE 30 than all other groups, while no differences were observed among fish fed diets CTRL, NEN 30, NPE 30 and TET 30. Ash content was significantly higher in fish fed diets NEN 30 and NPE 30. Fish fed the CTRL diet tended to have lower ash content compared with those fed the diets NEN 30 and NPE 30. Ash content was significantly lower in fish fed diets TET 30 and TPE 30. Energy content was significantly higher in fish fed the diets TET 30 and TPE 30. Fish fed the diets CTRL, NEN 30 and NPE 30 had significantly lower energy level than fish fed TET 30 and TPE 30.

Table 9. Whole body proximate composition

Parameter	CTRL	NEN 30	NPE 30	TET 30	TPE 30	P.value
Dry matter (g/kg)	287.8 ± 0.09^{c}	288.4 ± 0.19^{ab}	296.3 ± 0.19^{a}	292.8 ± 0.16^{abc}	295.7 ± 0.28^{ab}	0.009
per 1000 g dry matter						
Protein	625.5 ± 0.42^{a}	610.8 ± 0.45^{ab}	603.9 ± 0.29^{b}	614.1 ± 0.6^{ab}	596.1 ± 0.43^{b}	0.009
Lipid	324.7 ± 0.54^{b}	317.2 ± 0.53^{b}	320.3 ± 0.3^{b}	319.5 ± 0.57^{b}	349.8 ± 0.54^{a}	0.002
Ash	67.8 ± 0.01^{ab}	70.2 ± 0.01^{a}	69.8 ± 0.01^{a}	61.3 ± 0.01^{bc}	60.3 ± 0.01^{c}	0.004
Energy	25.6 ± 0.07^{b}	25.6 ± 0.02^{b}	25.6 ± 0.06^{b}	26.1 ± 0.11^{a}	26.3 ± 0.13^{a}	0.001

Values are expressed as mean ± SD (n=4 replicates). Values in the same row with different superscript letters indicate significant differences ($p<0.05$)

Table 10. Content of total lipid and fatty acids in fish fillet after feeding the experimental diets for 9 weeks.

Fish fillet	CTRL	NEN 30	NPE 30	TET 30	TPE 30	P value
Total lipid	28.9 ± 1.11^{b}	35.9 ± 0.34^{a}	33.6 ± 0.74^{ab}	32.5 ± 1.14^{ab}	32.4 ± 1.69^{ab}	0.008
% of total fatty acids						
C14:0	3.5 ± 0.04^{ab}	3.4 ± 0.03^{b}	3.6 ± 0.02^{a}	3.5 ± 0.03^{ab}	3.4 ± 0.02^{b}	0.002
C16:0	14.5 ± 0.18^{bc}	14.3 ± 0.17^{c}	16.1 ± 0.26^{a}	15.1 ± 0.27^{abc}	15.5 ± 0.17^{ab}	< 0.001
C18:0	3.3 ± 0.04	3.2 ± 0.04	3.4 ± 0.06	3.3 ± 0.10	3.3 ± 0.05	0.147
C19:0	2.6 ± 0.21^{bc}	2.6 ± 0.09^{bc}	2.5 ± 0.05^{c}	3.2 ± 0.07^{ab}	3.7 ± 0.22^{a}	< 0.001
∑SFA	23.9 ± 0.17^{b}	23.6 ± 0.14^{b}	25.6 ± 0.27^{a}	25.1 ± 0.39^{a}	25.9 ± 0.12^{a}	< 0.001
C16:1	3.8 ± 0.03^{c}	4.5 ± 0.05^{b}	5.2 ± 0.04^{a}	3.7 ± 0.04^{c}	3.8 ± 0.08^{c}	< 0.001
C18:1n-11	24.1 ± 0.50^{ab}	25.4 ± 0.22^{a}	23.2 ± 0.42^{b}	24.6 ± 0.48^{ab}	25.8 ± 0.25^{a}	0.002
C18:1n-9	3.3 ± 0.05	3.3 ± 0.02	3.2 ± 0.01	3.3 ± 0.04	3.2 ± 0.02	0,256
C20:1n-9	4.2 ± 0.16^{b}	4.8 ± 0.01^{a}	4.5 ± 0.03^{ab}	4.6 ± 0.07^{ab}	4.6 ± 0.07^{ab}	0.006
C22:1n-9	4.2 ± 0.03^{c}	4.7 ± 0.03^{a}	4.4 ± 0.04^{b}	4.4 ± 0.07^{b}	4.4 ± 0.01^{b}	< 0.001
∑ MUFA	39.6 ± 0.54^{c}	42.7± 0.20^{a}	40.5± 0.46^{bc}	40.5 ± 0.61^{bc}	41.8± 0.22^{ab}	0.001
C18:2n-6	7.7 ± 0.20^{b}	8.6 ± 0.16^{a}	8.0 ± 0.17^{ab}	8.2 ± 0.16^{ab}	8.3 ± 0.15^{ab}	0.015
C20:5n-3	5.9 ± 0.26^{b}	6.0 ± 0.27^{ab}	7.3 ± 0.17^{a}	5.5 ± 0.60^{b}	5.9 ± 0.08^{b}	0.006
C22:5n-3	2.4 ± 0.12^{a}	2.2 ± 0.02^{ab}	2.4 ± 0.07^{a}	2.1 ± 0.01^{bc}	1.9 ± 0.02^{c}	< 0.001
C226n-3	20.5 ± 0.46^{a}	16.9 ± 0.22^{c}	16.3 ± 0.18^{c}	18.6 ± 0.45^{b}	16.2 ± 0.30^{c}	< 0.001
∑ PUFA	36.4 ± 0.64^{a}	33.7 ± 0.09^{bc}	33.9 ± 0.23^{bc}	34.4 ± 0.79^{b}	32.3 ± 0.12^{c}	< 0.001
∑ n-6	7.7 ± 0.20^{b}	8.6 ± 0.16^{a}	8.0 ± 0.17^{ab}	8.2 ± 0.16^{ab}	8.3 ± 0.15^{ab}	0.015
∑ n-3	28.8 ± 0.78^{a}	25.1 ± 0.24^{b}	26.0 ± 0.36^{b}	26.2 ± 0.82^{b}	24.00 ± 0.27^{b}	< 0.001
∑ EPA + DHA	26.4 ± 0.70^{a}	22.9 ± 0.25^{b}	23.6 ± 0.30^{b}	24.1 ± 0.83^{b}	22.1 ± 0.27^{b}	< 0.001
∑ n-6/n-3	0.27 ± 0.01^{b}	0.34 ± 0.01^{a}	0.31 ± 0.01^{ab}	0.32 ± 0.01^{a}	0.35 ± 0.01^{a}	0.001

Values are expressed as mean ± SD (n=4 replicates). Values in the same row with different superscript letters indicate significant differences ($p<0.05$)

SFA - Saturated fatty acids.

MUFA - monounsaturated fatty acids.

PUFA - polyunsaturated fatty acids.

n-6 - omega-6 polyunsaturated fatty acids.
n-3 - omega-3 polyunsaturated fatty acids.
EPA + DHA - Eicosapentaenoic acid and Docosahexaenoic acid
n-6/n-3 - ratio of n-6 PUFA/n-3 PUFA

The total lipid content and fatty acid composition of salmon fillet after 9 weeks of feeding with the experimental diets are presented in (Table 10). Highest total lipid level was found in fillet of salmon fed diet NEN 30, but the values were not significantly different from the lipid level in the group fed diets NPE 30, TET 30 and TPE 30. The CTRL showed the lowest lipid content. The highest content of SFA in the flesh lipids (Table 10) was found in fillet of salmon fed diet TPE 30, though values were not significantly different from fatty acid levels in the group fed diets NPE 30 and TET 30. SFA content was significantly lower in fish fed diets CTRL and NEN 30. Percentage of 18:0 were not found to be affected by the dietary treatment. With respect to MUFAs in the fillet, fatty acids were noted significantly higher in fish fed diet NEN 30. Fish fed diets NPE 30, TET 30 and TPE 30 tended to have lower fatty acid content compared with those fed diet NEN 30. The CTRL resulted in lower fatty acid levels. Percentage of C18:1n-9 was not affected by the dietary treatments. Fish fed the CTRL showed significantly higher PUFA content compared to the other groups. Fish fed TPE 30 resulted in lower PUFA content, while fish fed NEN 30, NPE 30 and TET 30 ranked in between. Total content of n-6 fatty acids in the fillet were significantly higher in fish fed diet NEN 30, while fish fed diets NPE 30, TET 30 and TPE 30 tended to be lower than NEN 30 and higher than CTRL. With respect to the n-3 fatty acids and EPA+DHA, fish fed the CTRL diet showed significantly higher content of n-3 fatty acids and EPE+ DHA in the fillet compared to fish fed other diet groups. Diets NEN 30, NPE 30, TET 30 and TPE 30 resulted in lower EPA+DHA content, while no differences were observed among them. Ratios of n-6 to n-3 fatty acids in the fillet were found significantly higher in fish fed diets NEN 30, TET 30 and TPE 30. Fish fed diet NPE 30 tended to be lower than NEN 30, TET 30 and TPE 30, but higher than CTRL.

3.3. Feed quality

3.3.1. Physical characteristics of experimental feeds

Physical characteristics of the experimental feeds are shown in (Table 11). physical quality differed among the experimental diets. Fat leakage was significantly higher in diet TPE 30, though it was not significantly different compared to the diet CTL group. Hardness of the pellets was significantly higher in diet TPE 30 compared to the rest of the diet groups while diets CTRL and NPE 30 resulted in lower hardness. Pellet length was significantly longer in CTRL pellets

while NPE 30 and TPE 30 resulted in significant shorter pellets. The NEN 30 and TET 30 ranked in between. Diameter of the pellets was significantly higher in diets CTRL and TET30 compared to NEN 30, NPE 30 and TPE 30 diet groups.

Results of the water stability of diets is shown in (Figure 7). Water stability was measured after 30- 60 and 90 min in a shaking water bath. Significant ($p<0.05$) differences in water stability were found at all three test intervals. The CTRL feed showed significantly highest water stability at all sampling points, while TET 30 tended to be less water stable than CTRL but more stable than the other three experimental diets. No significant differences were noted among NPE 30 and TPE 30 during the course of experiment. The NEN 30 feed tended to have lower water stability compared with NPE 30 and TPE 30 at 30 minutes. TPE 30 diet had numerically lowest water stability at 90 minutes compared to NEN 30 and NPE 30 diets, but no significant differences were noted.

Table 11. Physical characteristics of the experimental feed

Parameter	CTRL	NEN 30	NPE 30	TET 30	TPE 30	P value
Fat leakage (%)	5.6 ± 0.18^{a}	5.2 ± 0.02^{b}	4.3 ± 0.07^{c}	4.1 ± 0.09^{c}	5.9 ± 05^{a}	< 0.001
Length (mm)	3.4 ± 0.01^{a}	2.8 ± 0.01^{b}	2.5 ± 0.01^{c}	2.7 ± 0.01^{b}	2.5 ± 00^{c}	< 0.001
Hardness (N)	13.4 ± 0.22^{c}	15.9 ± 0.39^{b}	13.4 ± 0.19^{c}	18.1 ± 0.38^{b}	21.4 ± 1.0^{a}	< 0.001
Diameter (mm)	2.7 ± 0.05^{a}	2.5 ± 0.01^{b}	2.4 ± 0.01^{b}	2.6 ± 0.02^{a}	2.5 ± 0.03^{b}	< 0.001

Values are expressed as mean ± SD (n=4 replicates). Values in the same row with different superscript letters indicate significant differences ($p<0.05$)

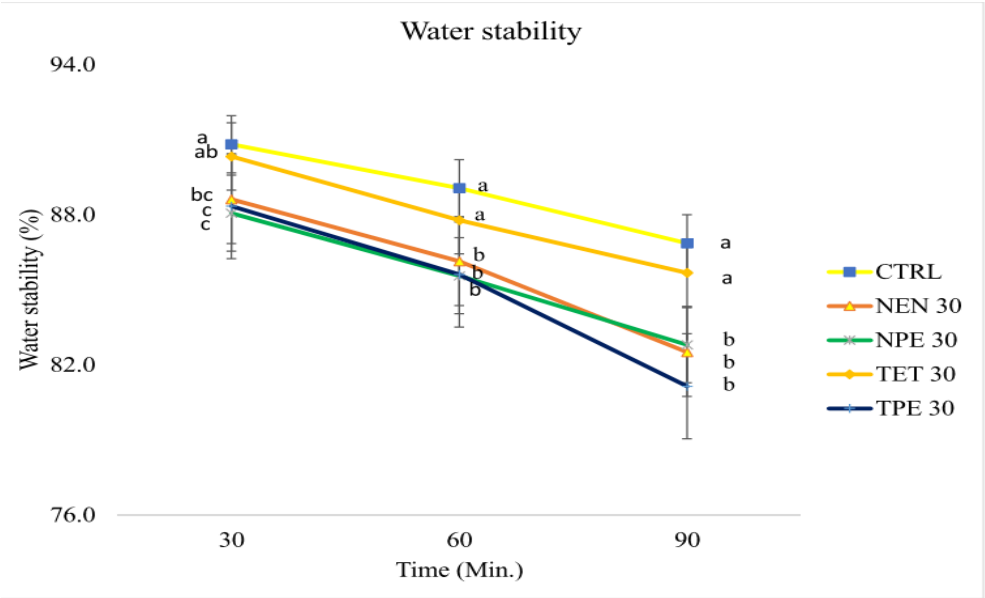


Figure 7. Water stability test for CTRL, NEN 30, NTP 30, TET 30 and TPE 30 feeds.

3.3.2. Correlation

Correlation coefficients between fish growth parameters and physical quality of the feeds are shown in Table 12. The WG was positively correlated with SGR, TGC and length of the pellets and negatively correlated with hardness. Length was positively correlated with SGR and TGC. There was a significantly negative correlation between Hardness and WG, SGR, TGC as well as length. Significant positive correlation was observed among water stability at 30, 60, 90 and length as well as diameter, but negatively correlated with hardness. Fat leakage was not correlated with any other physical quality parameters or growth of the fish.

Table 12. Correlation among growth parameters and physical quality of the feeds of Atlantic salmon

	Variables	1	2	3	4	5	6	7	8	9	10
1	WG	-									
2	SGR	1.00**	-								
3	TGC	1.00**	1.00**	-							
4	Length	0.69**	0.68**	0.69**	-						
5	FL	0.27	0.29	0.27	0.25	-					
6	Hardness	-0.57**	-0.543*	-0.56*	-0.50*	0.28	-				
7	Diameter	0.35	0.33	0.34	0.82**	0.11	-0.23	-			
8	WS 30	0.33	0.30	0.32	0.66**	0.01	-0.24	0.84**	-		
9	WS 60	0.43	0.42	0.43	0.66**	0.14	-0.07	0.79**	0.90**	-	
10	WS 90	0.39	0.36	0.39	0.77**	-0.13	-0.29	0.84**	0.88**	0.88**	-

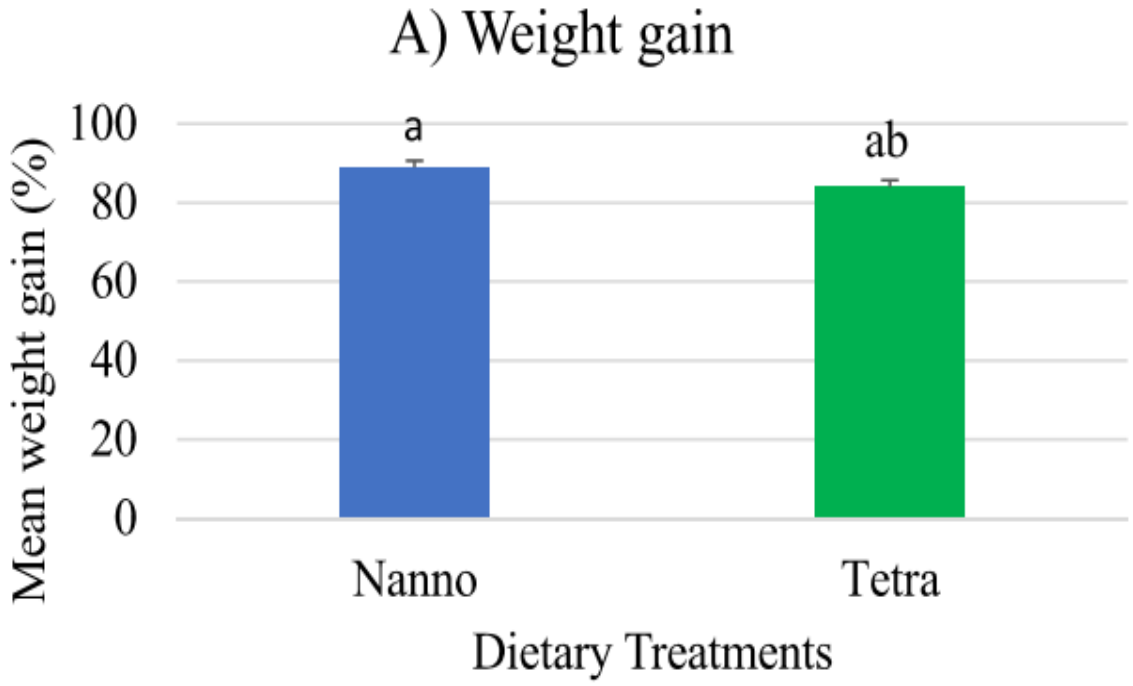
**. Correlation is significant at the 0.01 level.

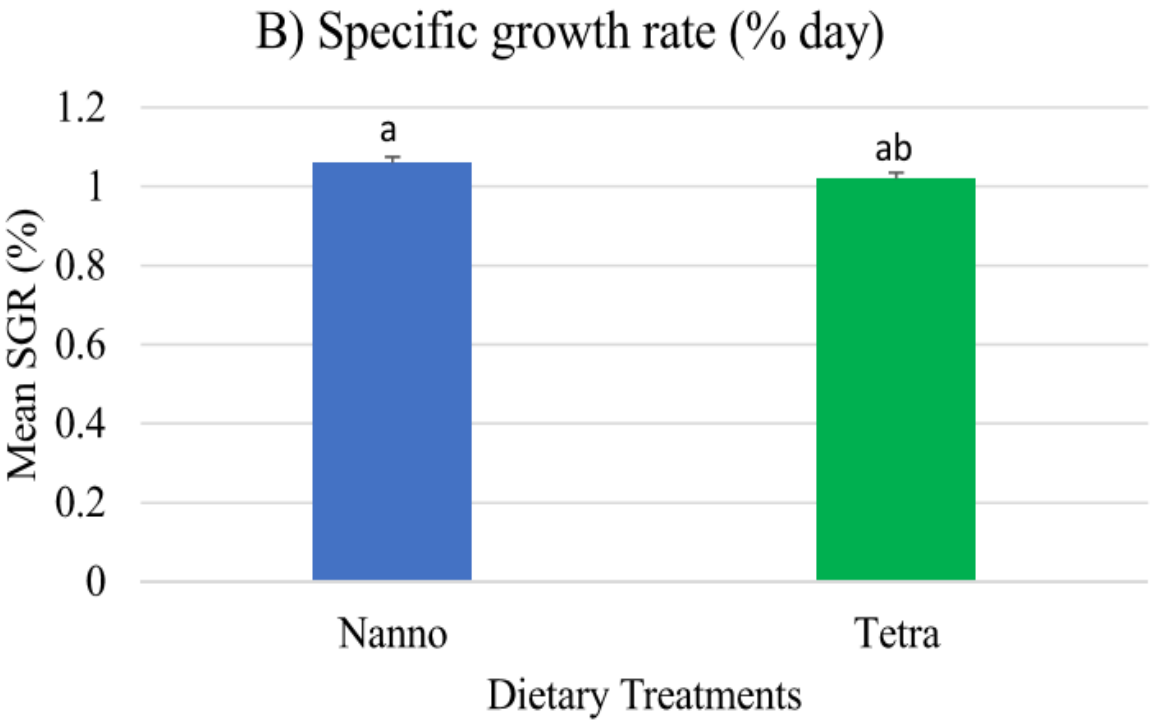
*. Correlation is significant at the 0.05 level.

(WG – Weight gain, SGR – Specific Growth Rate, TGC – Thermal Growth Coefficient, FL – Fat Leakage, WS 30 - Water Stability at 30 minutes, WS 60 - Water Stability at 60 minutes, WS 90 - Water Stability at 90 minutes)

3.3.3. The effect of algae and processing of microalgae

The effect of microalgae and pre-processing was investigated in a Two-way ANOVA with interaction. The results showed that fish fed the *Nannochlopsis* tended to have higher weight gain (WG) ($P = 0.060$), SGR ($P = 0.068$) and TGC ($P= 0.065$) than fish fed the *Tetraselmis* There were no significant effect of processing on the growth parameters (WG) ($P = 0.396$), SGR ($P = 0.406$) and TGC ($P= 0.422$).


A) Weight gain
Mean weight gain (%)
100
80
60
40
20
0
a
ab
Nanno
Tetra
Dietary Treatments


B) Specific growth rate (% day)
Mean SGR (%)
1.2
1
0.8
0.6
0.4
0.2
0
a
ab
Nanno
Tetra
Dietary Treatments

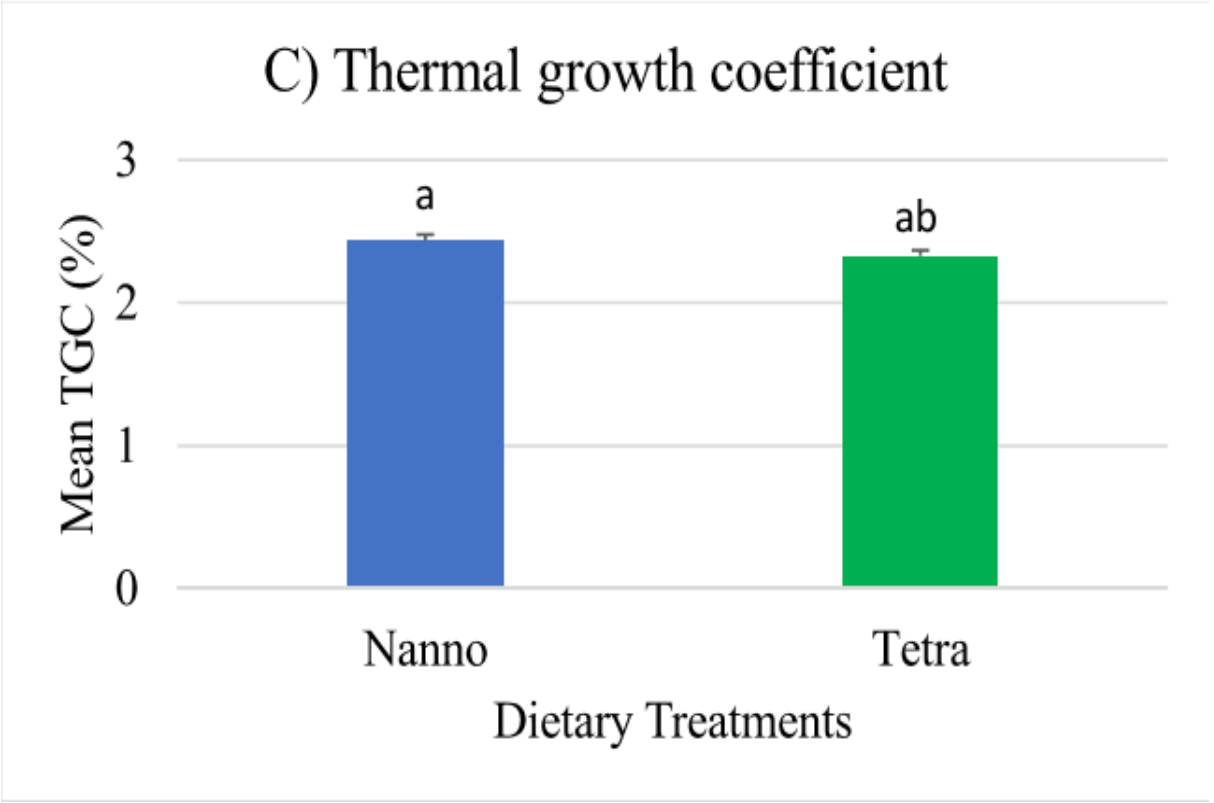


Figure 8. Effect of microalgae and processing on Weight gain (a), Specific growth rate (b) and Thermal growth coefficient (c) for fish fed microalgae during the course of a 9-week experiment. Values are expressed as mean ± SEM; n = 4 replicate tanks. Different letters above column, if any, represent significant differences ($P < 0.05$) between groups

Table 13. Effect of microalgae (*Nannochloropsis sp*. and *Tetraselmis sp*.) and processing on fatty acid composition of fillet

	Algae			Extrusion			Algae* processing Interaction				
							Nanno		Tetra		
	Nanno	Tetra	P.value	Non	Pre	P.value	Non	Pre	Non	Pre	P.value
SFA	24.6	25.5	0.01	24.4	25.8	0.000	23.6	25.6	25.1	25.9	0.017
MUFA	41.6	41.0	0.136	41.5	41.1	0.351	42.7	40.5	40.2	41.8	0.000
PUFA	33.8	33.5	0.404	34.2	33.1	0.011	33.7	34.0	34.7	32.3	0.003
n-3	25.6	25.3	0.525	25.8	25.0	0.084	25.1	26.0	26.5	24.0	0.002
n-6	8.3	8.2	0.897	8.4	8.1	0.158	8.6	8.0	8.2	8.3	0.025
EPA+DHA	23.3	23.3	0.919	23.7	22.9	0.075	22.9	23.6	24.5	22.1	0.004
n-6/n-3	0.33	0.33	0.720	0.33	0.33	0.905	0.34	0.31	0.31	0.35	0.004

Two-way ANOVA.

There were no significant effect of microalgae or processing on the content of MUFAs, PUFAs, n-3, n-6, EPA + DHA and n-6/n- except for the content of PUFAs (P= 0.011) (Table 13). PUFA was significantly higher in flesh when the fish were fed Tetra than in Nanno. SFA was significantly lower in flesh when the fish were fed Nanno than in Tetra and it was highly affected by processing (P= 000). The interaction between algae and processing was also significantly different for all fatty acids in the fish fillet.

Table 14. Effect of microalgae and processing on physical quality of the experimental diets

	Algae			Extrusion			Algae* processing Interaction				
							Nanno		Tetra		
	Nanno	Tetra	P.value	Non	Pre	P.value	Non	Pre	Non	Pre	P.value
Fat leakage	4.6	5.0	0.000	4.5	5.1	0.000	5.0 [b]	4.3 [c]	4.0 [c]	6.0 [a]	0.00
Length	2.7	2.6	0.007	2.8	2.5	0.000	2.8 [a]	2.5 [b]	2.7 [a]	2.5 [b]	0.820
Hardness	14.6	19.8	0.000	17.0	17.4	0.534	15.9 [b]	13.4 [a]	18.1 [b]	21.4 [a]	0.00
Diameter	2.5	2.6	0.000	2.6	2.5	0.000	2.5 [b]	2.4 [b]	2.7 [a]	2.5 [b]	0.034

Two-way ANOVA.

The main effect and interaction of microalgae and processing on physical quality of diets are presented in (Table 14).

There were significant effects of the two microalgae *Nannochloropsis* sp and *Tetraselmis* sp. on all physical quality parameters. The Tetra pellets had more fat leakage, shorter pellets, higher hardness and greater diameter. Processing of the algae also had significant effect on all parameters except for hardness (P=0.534). The pre-extruded pellets had more fat leakage, shorter pellets, higher hardness and shorter diameter. The interaction effect between algae and processing was also significantly different for all physical quality parameters except for length (P= 0.820)

4. Discussion

Utilization of nutrients in single cell ingredients, such as microalgae, may be limited by rigid cell walls. Studies have reported that there is a large diversity in the cell wall structures of microalgae and cyanobacteria: from peptidoglycan cell walls to cellulosic cell walls. To break rigid cell wall and release the internal components of microalgae, multiple cell disruption techniques have been tested in earlier studies. According to Middelberg (1995), most of the cell disruption methods developed for use with non-photosynthetic microorganisms can be applied to microalgae. Disruption methods greatly enhances the bioavailability and the assimilation of the intracellular products from the cells (Vanthoor-Koopmans et al, 2013). Generally, the techniques are categorized in to mechanical, chemical method or use of enzymes. Chemical processes may have a negative effect on the functionality of the components (Schwenzfeier et al, 2011). Alternatively, mechanical processes could be applied as mild cell disruption method (Günerken et al, 2015; Schwenzfeier et al, 2011). Proteins from *Tetraselmis* sp. were extracted and purified after cell disintegration by bead milling (Schwenzfeier et al, 2011). Similarly, agitation of microalgal biomass in presence of glass and ceramic beads (0.5 mm bead diameter) in bead mills has been used to disrupt cells of *Scenedesmus obliquus, S. platensis* and *Monodus subterraneous* (Chisti & Moo-Young, 1986). Bermejo Roma et al. (2001) reported that ultrasonication of suspended microalgal cells is not applicable to large-scale use. However, it can be used to disrupt small amounts of biomass. Different cell disruption processes have been used for recovering astaxanthin from encysted cells of *Haematococcus pluvialis* (Mendes-Pinto et al, 2001). Mendes-Pinto et al. (2001) reported that biomass that had been autoclaved or mechanically disrupted in a high-pressure homogenizer (including treatment with acid, alkali, and enzymes), yielded three times more astaxanthin than biomass treated with other methods. Alkali is an effective method of lysing the cell wall and can be used to isolate free fatty acids from microalgae, but should not be used for sensitive products such as proteins (Giménez et al, 1998). Other research has also shown that upon completely breaking the cells of the Arthrospira (*spirulina*) platensis, up to 85% of protein could be extracted (Devi et al, 1981). Ingredient processing (harvesting and drying) and feed manufacturing (mixing and extruding) may also affect cell wall integrity (Palinska & Krumbein, 2000; Scholz et al, 2014). The present experiment tested two different microalgae, added to the feed *as is* (extruded), or with use of extrusion as an additional pre-treatment before mixing in to the diet (pre-extruded). The effect of using microalgae on growth performance, whole body proximate composition, fatty acid of fillet and physical quality of feed were tested in feeding experiment for Atlantic salmon.

4.1. Growth performance of the fish

The experimental diets contained rather high incorporation of microalgae, due to the fact that the experiment was designed as a digestibility study.

Results obtained in the present study showed that all the microalgae incorporated diets had lower growth performance (WG, SGR, TGC) than the CTRL fed groups. However, the growth of the fish fed *Nannochloropsis* tended to be improved compared to those fed the *Tetraselmis.* The present experiment used diets with high (30%) incorporation of microalgae. This is higher levels than other studies with Atlantic salmon (Kiron et al, 2016; Sørensen et al, 2016). The reduction in growth performance is in line with other studies with mircroalgae replacing fish meal at lower incorporation levels (Burr et al, 2012; Sprague et al, 2015). Burr et al. (2012), who found that the replacement of fishmeal with *Spirulina* sp. up to 11% did not decline growth performances in Atlantic salmon (*Salmo salar*). It has also been reported that *Spirulina platensis* supplemented diets at 10% did not change growth related parameters in the diets of rainbow trout (*Oncorhynchus mykiss*), which indeed resulted in improved feed efficiency by increasing gut bacterial colonization (Teimouri et al, 2013). The reduction of growth performances in terms of weight gain (%), SGR (% day) and TGC in fish fed NEN 30, TET 30 and TPE30 observed in the current study is also in agreement with Sørensen et al. (2016), who found that replacement of fish meal with *Phaedactylum tricornutum* at an inclusion rate of 12% in the diets of Atlantic salmon had negative effects on weight gain (%) and SGR (% day) or *Schyzochrytrium* sp. at 11% inclusion (Sprague et al, 2015).

The present experiment showed lower SGR and TGC compared to studies where Atlantic salmon was fed *Schizochytrium* sp. incorporated at 10% (Kousoulaki et al, 2015) or *Phaeodactylum tricornutum* at 12 % inclusion (Sørensen et al, 2016). The TGC values were in the same range as reported by Sørensen et al. (2017). The SGR of the present experiment was higher values than Kiron et al. (2012) reported in study with Atlantic salmon fed the microalgae species *Tetraselmis* Chlorophyceae at inclusion level of 5-10%, but in line with Kiron et al. (2016) who was feeding Atlantic salmon feeds with defatted microalgae *Desmodesmus* sp. replacing 10% and 20% of the fish meal, respectively. Some studies have shown that incorporation of microalgae at higher level reduce the diet palatability, results in reduction of feed intake in Atlantic salmon (Norambuena et al, 2015). Taken together, the resulting's of the present experiment suggest that the relationship between growth parameters and microalgae diets depends on fish species, palatability and processing conditions of microalgae (Tibaldi et al., 2015).

The pellet used in present experiment were 2.5 mm in size. However, Bailey et al. (2003) investigated the effect of pellet size on Atlantic salmon growth using 14 combinations of pellet sizes (0.03–2.68 mm), and reported that Atlantic salmon can accept a range of pellet size. Accordingly, it may be suggested that the observed negative effect on growth performances in diets formulated with the inclusion of NEN 30, TET 30 and TPE 30 was likely not due pellet size.

4.2. Proximate composition of the whole fish

Changes in biochemical composition of fish fed microalgae diets, have been reported in earlier studies (Dallaire et al, 2007; Mustafa et al, 1995). The proximate composition varies with life stages of fish and is influenced by environmental and dietary factors (Shearer, 1994). Protein content of growing salmonids is determined solely by fish size (Shearer et al, 1994). Significant differences were observed in proximate composition of whole fish among the feeding groups in the present experiment. The highest protein content was in fish fed the CTRL diet while fish fed NEN 30 and TET 30 showed a tendency. Compared to the CTRL fish fed pre- extruded microalgae showed significantly lower protein content. All the algae diets had lower protein content than the CTRL, which may partly explain the lower proximate protein content. The pre-extruded microalgae were exposed for more heat treatment (double extrusion) The extrusion itself may not be negative (Sørensen et al, 2009). However, microalgae biomass was also dried after the first and second extrusion, which all these may lead to oxidized protein or maillard reaction, reducing bioavailability of nutrients in the diet (Jasour et al, 2017; 2018).

The whole-body lipid content of the experimental fish was in the same range (Kiron et al, 2016), or higher (Kiron et al, 2012; Norambuena et al, 2015; Sprague et al, 2015) than earlier studies with Atlantic salmon fed microalgae. The fish fed TPE 30 diet had lowest protein content and highest lipid content. The proximate composition showed significantly higher lipid content in fish fed TPE 30 diet, while no differences were in lipid content among fish fed the other four diets. The higher lipid content in fish fed TPE 30 diet indicate that protein quality or protein content in this diet is not sufficient to support efficient growth.

The ash content of the experimental fish was in agreement with other reported values on fish fed with microalgae (Higgs et al, 2006; Kiron et al, 2016) The higher whole-body ash values obtained in the present study were noticeable.

4.2.1. Total lipid and fatty acid composition in the fillet of Atlantic salmon

The lipid content of the experimental fish fillet was higher than other reported values on Atlantic salmon fed 10% and 20% of *Desmodesmus* sp. (Kiron et al, 2016).. In the present experiment, significant differences were noted in fatty acid composition among groups. Fatty acid composition of Atlantic salmon fillet reflects that of the diet (Grisdale-Helland et al, 2002; Sprague et al, 2015; Torstensen et al, 2005). The present study found an increase in SFAs, MUFAs, n-6 PUFAs and n-6/n-3 content in fish fillet of fish fed the algal diets. The higher content of lipid in the algal diets is partly explained by the differences in source and quality of ingredients. The content of PUFAs, n-3 PUFAs and EPA and DHA were lower in the algal diets compared to fish oil fed (CTRL) groups. The results also showed that the algae feed groups had higher saturated and mono unsaturated fatty acid contents. Results agree with Sprague et al (2015) who showed that microalgae diets increased deposition of SFAs and MUFAs. Control groups had a higher EPA and DHA content.

4.3. Physical characteristics of experimental diets

Physical characteristics of pellet for Atlantic salmon were reported in several other studies (Aas et al, 2015; 2014; Oehme et al, 2014; Sørensen et al, 2012; 2011b). Other studies have shown that pellet quality is affected by feed ingredients and extrusion process (Sørensen, 2012). However, the extrusion parameters used to produce the experimental feeds were not available. Therefore, the discussion is limited to effects of ingredients on pellet quality as it is not possible to give any conclusions about the effect of extrusion processing parameters on the physical quality of pellets. In the present study, physical quality differed among the experimental diets. Length and diameter were higher in CTRL group than the algae diet groups. Earlier studies have reported that fishmeal based feed have different microstructure than the pellets based on plant ingredients (Sørensen et al, 2009). It can therefore be expected that microstructure of the pellets in the present study differed, affecting fat leakage. Sørensen et al. (2011b) reported that fat leaking was associated with low oil absorption capacity and not with oil level. More leaking would have been expected if pellets were coated with higher levels of oil. Though oil absorption capacity was not measured in present experiment, the TPE 30 pellets seem more oily surface compared to the other feeds. This observation suggests a lower oil absorption capacity of TPE 30.

Pellet length was significantly longer in CTRL pellets while NPE 30 and TPE 30 resulted in significant shorter pellet. The NEN 30 and TET 30 ranked in between. The shorter pellets from NPE 30 and TPE 30 diets may be due to double extrusion processing conditions.

Hardness of pellet recorded in the present experiment was higher than those recorded by Morken et al. (2012), but lower than the values recorded by (Aas et al, 2011b; 2011a; Oehme et al, 2014). Hardness of the pellets was significantly higher in diet TPE 30 compared to the rest of the diet groups. The TPE 30 also had the highest moisture level compared to that of other diets. This was supported by Li. (2012), who observed higher hardness in feed pellet when moisture in feed increased from 2.5%-5.0% and 5.0%-7.5% followed by a reduction when moisture subsequently increased from 7.5%-10%. Aas et al. (2011a) reported higher pellet hardness than the obtained result of the present study. The different results can be explained by differences in pellet size and compression speed and the cylinder probe used to break the pellet. For the present experiment pellets of (2.4 – 2.7mm) was used whereas Aas et al. (2011a) used pellets (10-12mm). Lack of standardization for the methods used to analyse hardness make comparison between experiments difficult. Sørensen (2012) reported that variation in pellet hardness from 9 N – 82 N, depending on raw ingredients, production process and/or method used to analyse hardness. However, the hardness of the pellets used in the present experiment is well within the normal range reported earlier. Previous studies also indicate that hardness of pellets may be affected by functional components in the ingredients, such as carbohydrate fractions (Aslaksen et al, 2007; Kraugerud et al, 2011; Refstie et al, 2006; Sørensen et al, 2011b).

Results of the water stability test indicates that CTRL diet had highest water stability compared with the algae containing diets. Overall water stability decreased over time, in line with Aas et al. (2011b). TPE 30 group had numerically lowest water stability after 60 min and 90 min compared to the other algae diet groups, but no significant differences were noted. In contradiction to Aas et al. (2011b), who reported that pellets with the highest hardness also had the highest water stability, the present results showed that diet containing TPE 30 with the highest hardness had the lowest water stability. One explanation to the differences in water stability may be the different type and quantity of ingredients on experimental diets differ in functionality.

4.4. Correlation coefficient among growth parameters and physical characteristics

Previous studies showed that physical quality of feed varies considerably among different diets because of different ingredients (Aarseth et al, 2006; Glencross et al, 2009; Øverland et al, 2009) and processing conditions (Sørensen et al, 2009, 2010, 2011). Studies have also reported a correlation between quality of pellets and feed intake, nutrient utilization and growth of the fish (Aas et al, 2011; Baeverfjord et al, 2006; Johansson et al, 2006). In the present study, there

were no significant correlation between pellet fat leakage and growth performances. The negative correlation between hardness and length is associated with expansion during the extrusion (Sørensen et al, 2009). The expansion rate is an important factor in aquafeeds as it affects the density, hardness and oil holding capacity (Rosentrater et al, 2009), though expansion rate was not analysed in the present experiment.

Water stability was positively correlated to diameter and length. Studies in poultry have shown that, regardless of grain type larger pellet length (3mm – 6mm) resulted in an improved pellet integrity (Abdollahi et al, 2013a, 2013b). Therefore, pellet length can have a significant effect on pellet quality. On the other hand, Baeverfjord et al. (2006) found no significant difference on growth in rainbow trout fed diets with high or low water stability. The present results suggest that pellet quality was affected by the ingredient composition.

4.5. The effect of algae and processing

The two way analysis of variance showed no significant effect of microalgae or processing or combined interaction on growth parameters (Figure 8). However, there were a tendency that fish fed *Nannochloropsis* sp had higher growth compared to fish fed the *Tetraselmis* sp. The combined (algae and processing) interaction were significant different in all fatty acid groups. The effect of algae and processing together was different than what would be expected from each algae and processing alone was interesting. Two-way analysis of variance also showed significant difference between algae and processing and their combined interactions on physical quality of feeds, but No effect of processing on pellet hardness and no effect of algae and processing interaction on pellet length. Therefore, the result suggest that fatty acids contents were more affected by the combined interaction of algae and processing than by their main effect while physical quality was highly affected by the algae, processing and by their combined interactions.

5. Conclusion

Present study generally showed that growth in fish fed algae diets were lower compared to that in the CTRL group. Among the algae-diets the NPE 30 showed the best growth performance (weight gain, SGR and TGC), almost at the same level as the CTRL group. Fatty acid composition of fillet was significantly affected by incorporation of both microalgae strains. The microalgae biomass in the feed had a higher SFAs, MUFAs and n-6 than fish fed the CTRL group. The present study also showed that physical quality of diet and proximate composition of Atlantic salmon was significantly affected by incorporation of both microalgae diets. The extrusion processing did not cause significant changes in growth parameters. However, fatty

acid composition of fillet and physical quality of diets were significantly affected by the extrusion processing. More long-term feeding experiment is needed to reveal the full potential of the pre-processing as a thermo mechanical treatment and to assess the potential of *Nannochloropsis* sp and *Tetraselmis* sp microalgae in feed for Atlantic salmon.

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