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MASTER THESIS

The Potential of Microalgae in Feed for Atlantic salmon (*Salmo salar* L.)

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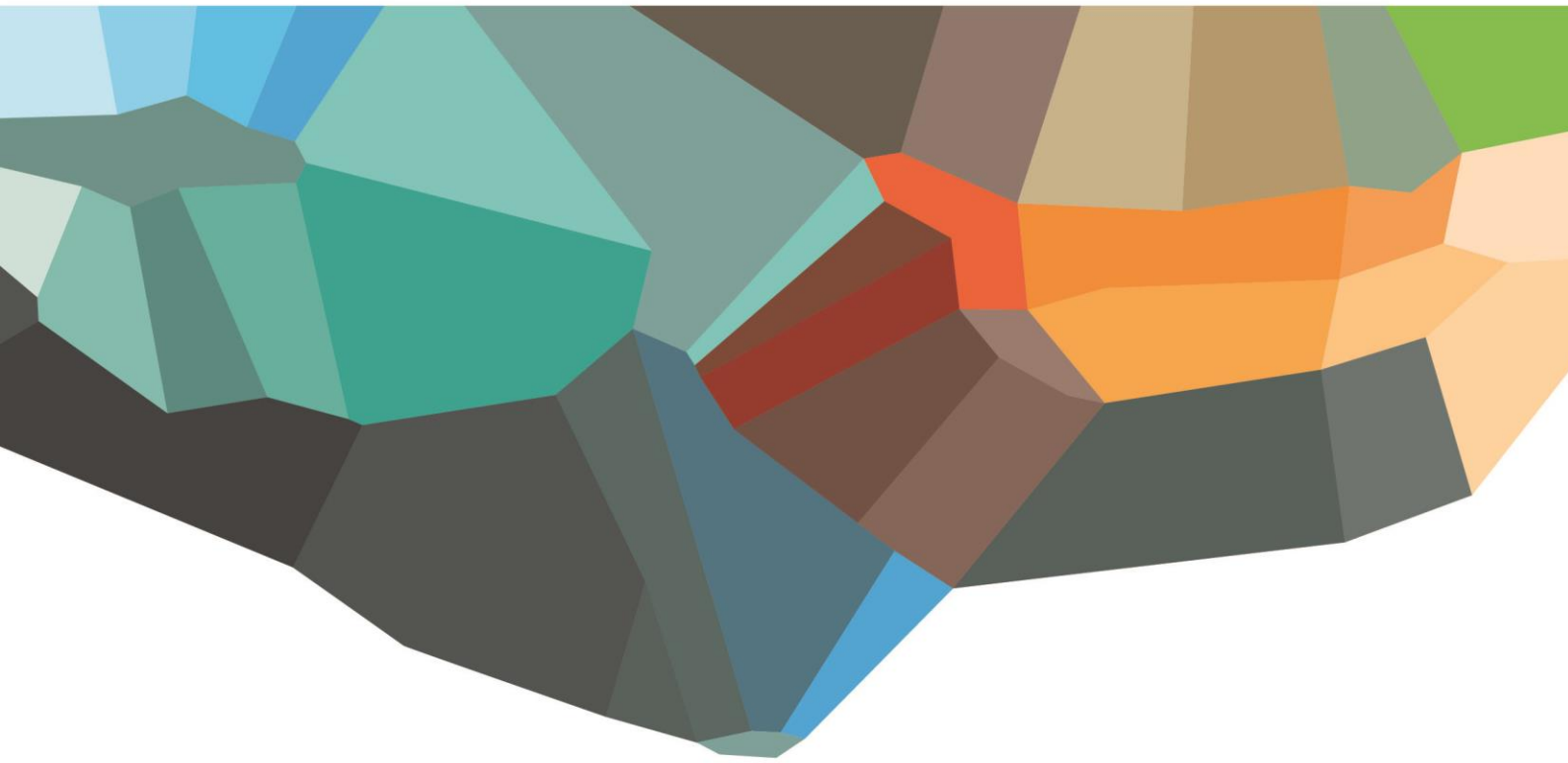

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“I can do all things through Him who gives me power” (Philippians 4:13)

Abstract

Fish meal and fish oil are limited resources. Dependency of these resources represents a major constraint for sustainable growth of aquaculture production. Currently, plant ingredients are used to replace fish meal and oil in aquafeeds. There is a growing interest for use of microbial biomass in fish feed. The aim of the experiment was to investigate apparent nutrient digestibility (ADC) of dry matter (DM), protein and ash of the three microalgae *Nanofrustulum* (C3), *Desmodesmus* (C4) and *Nannochloropsis* (C1).

Two digestibility trials were carried out with Atlantic salmon. First experiment, referred to as pre-study (P), aimed to investigate ingredient digestibility of the three algae, diluting a fish meal based control diet with 30% test ingredient (70:30 ratios). The three feeds were produced in the feed lab using cold pelleting process. Second experiment, denoted as the main experiment (M), had two purposes a) to verify results from P and b) to investigate ADC's of DM, protein and ash for whole diet at 10% and 20% inclusion level of C1 and C4, using diets made from commercial cooking extrusion process. Digestibility studies were carried out with Atlantic salmon, approximately 1600 gr in the P-study and for experiment M the size of the fish was 436 gr for the verification part and 523 gr for the whole diet nutrient digestibility part. Nutrient digestibility of ingredients was calculated using three equations based on Glencross *et al*. (2007).

Results from P showed that ADC of protein for C3, C1 and C4 ranged from 96-110%, 73-76% and from 54-68%, respectively, for the three equations used to calculate digestibility. The ADC of DM ranged from: 73-74%, 45-46% and from 29-35%, and ash ranged from 61-113%, 36-49% and from 44-51% respectively, for the three microalgae. Significant differences were noted in ADC among the microalgae. Overall, highest ADC for protein, DM and ash ($P< 0.05$) was observed for C3 while no significant differences were noted between C4 and C1.

For experiment M, ADC's of protein and DM were in the same range as values in experiment P while ADC of ash was higher. The ADC of protein for C1 and C4 ranged from 64-74% and from 66-74%, respectively, for the three equations used to calculate digestibility. The ADC of DM ranged from 55-63% and from 47-48%, and ash ranged from 76-121%, and from 76-99%, respectively, for the two microalgae. Significant differences were noted in ADC's of protein, DM and ash between C1 and C4 in experiment M, depending of equations used for digestibility estimations.

Inclusion level of C4 did not affect ADC's of protein and DM, but a significant effect was noted on digestibility of ash. Lowest digestibility of ash was observed for control diet (-11%), followed by 10% inclusion rate (2%) and 20% inclusion (15%).

It can be concluded that nutrient digestibility varies among different strains of microalgae. Based on ADC values, the C3 revealed the greatest potential as a feed ingredient followed by the C1, though no large differences were observed between the C1 and C4. The microalgae should also be tested in long-term feeding experiments with Atlantic salmon to evaluate the potential of the different candidates.

Key words: Apparent digestibility coefficient; Atlantic salmon; Fish meal; Microalgae meal

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1. Introduction

1.1. Aquaculture production

Feeding the growing world population is a challenge. A current report by FAO (2014) reported that number of hungry people in the world is still unacceptably high. At least 805 million people, or one in nine, worldwide do not have enough food to eat. From 1995 through 2009 world hunger increased substantially as a result of high commodity prices and economic turbulence (FAO, 2009). As the global economy recovers, the number of undernourished people is estimated to go down somewhat, but still remain at an unacceptable high level.

Since 2003, fish accounted for approximately 16% of the consumed animal protein worldwide and in some Asian countries the proportion ranges as high as 30 – 50% (Rana *et al*., 2009). About 1 billion people rely on seafood as their main source of proteins and there are several reasons why demand for seafood is expected to increase over time.

The world population is estimated to count 9.6 billion people by 2050. In order to feed this growing population more food need to be produced from marine environment. Last three decades, capture fisheries production increased from 69 million to 93 million tonnes; at the same time, world aquaculture production increased from 5 million to 63 million tonnes (FAO, 2013). Fish globally represents about 16.6% of animal protein supply and 6.5% of all protein for human consumption.

Aquaculture is the fastest-growing animal-food-producing sector and is even growing faster than the population growth. The growth rate in farmed fish production from 1980 to 2010 has resulted in an increase of the average annual per capita consumption of fish. The consumption of fish or farmed fish has increased almost seven times, from 1.1 kg in 1980 to 8.7 kg in 2010, at an average rate of 7.1% per year (FAO, 2012). Fish aquaculture production worldwide expanded at an average annual rate of 6.2% in the period 2000–2012, more slowly than in the periods 1980–1990 (10.8%) and 1990–2000 (9.5%) (FAO, 2014). It is expected that growth in aquaculture will relieve pressure on wild fish stocks and will also allow wild populations to recover. Most world fish stocks are now fished at or beyond capacity. The growth in demand for seafood can therefore not be met by the world's capture fisheries, but must come from aquaculture farming and cultivation. Global aquaculture production reached an all-time high production of 90.4 million tonnes (live weight equivalent)

in 2012 (US$144.4 billion), including 66.6 million tonnes of food fish and 23.8 million tonnes of aquatic algae, respectively (FAO, 2014).

For 2012, more than 86% of world fish production (capture and aquaculture) was utilised for direct human consumption (FAO, 2014). The remaining 14% was used for non-food purposes, of which 75% was reduced to fish meal (FM) and fish oil (FO).

Aquaculture is a key alternative to capture fisheries and an important economic activity, with significant growth and job creation potential in many countries. According to FAO (2014), aquaculture sector interventions have proved to be most successful to alleviate poverty. Aquaculture sector employ around 18.9 million people (more than 96% in Asia). Employment in the sector has grown faster than the growth of the world's population. In 2012, it represented 4.4% of the 1.3 billion people economically active in the broad agriculture sector worldwide (2.7% in 1990). Overall fisheries and aquaculture assured the livelihoods of 10–12% of the world's population. The number of people engaged in fish farming at the global levels has since 1990, increased at higher annual rates than that of those engaged in capture fisheries.

It is expected that aquaculture will continue to grow, intensify and diversify. The expansion of aquaculture has primarily been due to research and development breakthroughs, compliance with consumer demands and improvements in aquaculture policy and governance (NCFS, 2012). Many countries have followed an aggressive policy trying to increase the supply of seafood, either for export or for home consumption, or both.

FAO (2010) has stated that the global aquaculture sector's long-term ability to achieve economic, social and environmental sustainability "depends primarily on continued commitment by governments to provide and support a good governance framework for the sector". The main stakeholders in the aquaculture industry are investors, public authorities, researchers and civil society organizations. All the stakeholders have important roles to play, but the key to success is how these four groups interact, constituting an aquaculture system. The story of the Norwegian salmon industry demonstrates the close cooperation between farmers, researchers and public authorities, while civil society organizations have acted as critical correctives, forcing more sustainable practices over time.

1.2. Aquaculture farming systems and feeding practices

About 600 aquatic species are raised in captivity worldwide in a variety of farming systems (FAO, 2012). Asia accounted for 89% of world aquaculture production by volume in 2010, up from 87.7% in 2000 (FAO, 2014). Farming of finfish and crustaceans is carried out in

extensive and intensive farming systems. In extensive production systems fish can be raised in earthen ponds, pens and cages, rice field or small water bodies, at low (extensive) to moderate (semi intensive) densities and farming input levels. Utilizing simple culture technologies and minimal inputs, these systems have been used for centuries.

The net contribution of these traditional aquaculture systems can be great as they offer many benefits, including food security in developing nations (FAO, 2002). Like the “green revolution” of agriculture in the last century, the current “blue revolution” will take aquaculture to an industrial mode of food production. An emerging trend is increased farming of high-value carnivorous fish species in intensive farming systems, threatening environmental and social sustainability.

Faming carnivorous fish species such as shrimp and salmonids are rapidly expanding. Intensive farming practice is accused for damaging ocean and coastal area through habitat destruction, waste disposal, introduction of exotic species and pathogen invasions, and depletion of wild fisheries stocks (Naylor *et al*., 1998). Naylor *et al*. (2000) also reported that production of one kilogram of carnivorous fish typically uses two to five kilograms wild caught fish processes into fish meal and fish oil for feed.

More sustainable integrated systems can also be used for production of high value fish, such as salmon and shrimp (Naylor *et al*., 2000). In Chile, for example, salmon has been farmed along with a type of red alga that removes large amounts of dissolved nitrogen and phosphorous wastes from salmon cages (Troell *et al*., 1997). From an environmental point of view, the environmental costs of waste discharges can be reduced by making sewage treatment mandatory, and produce salmon in integrated systems that reduce the waste stream.

Feed is generally perceived to be a major constraint to aquaculture development. One-third of all farmed fish production, 20 million tonnes, is currently produced without additional feeding (FAO, 2012). Feed for cultured fish species, range from use of simple agriculture by product (e.g., rice bran) to a combination of ingredients in the form of a mash or pellet. Aquaculture feed is changing rapidly. Knowledge about nutrient requirement is needed as well as in depth knowledge about alternative ingredients that can be combined to meet nutrient requirement.

1.3. Norwegian Atlantic salmon farming

Farming of salmonids in Norway started at the 1970s. The industry has grown from production of less than 1000 tonnes in 1971 to more than 1 million tonnes in 2013 (Statistic

Norway, 2014). The conditions for production of salmonids are unique in Norway thanks to a long protected coastline, accessible areas and a clean sea with a high water replacement rate and good water quality, providing good biological prerequisites for aquaculture production. Norway manages some of the world's largest and most productive coastal and sea areas. The Norwegian seafood federation (FHL, 2011) reported that Norway has 90.000 square kilometres of sea within its sea baseline. This means that Norway has it unique potential for aquaculture production. Number of sites for the main aquaculture counties in Norway is shown in Table 1.

Table 1: Number of sites by county[1)]

	2014	2013	2012	2011	2010	2009[2)]	2008	2007	2006
County	Antall *No.*	Antall *No.*	Antall *No.*	Antall *No.*	Antall *No.*	Antall *No.*	Antall *No.*	Antall *No.*	Antall *No.*
Finnmark	72	67	69	67	62	62	74	83	88
Troms	109	111	117	116	110	107	103	123	106
Nordland	211	205	206	203	196	197	192	236	198
Nord-Trøndelag	60	63	66	69	69	71	76	78	85
Sør-Trøndelag	92	93	91	97	94	80	91	94	110
Møre og Romsdal	87	88	90	100	107	105	110	142	126
Sogn og Fjordane	87	86	82	81	96	99	106	116	115
Hordaland	188	191	196	200	203	197	211	240	230
Rogaland	74	73	71	74	73	64	63	74	66
Vest-Agder	12	12	11	11	11	11	9	9	9
Aust-Agder	2	2	2	2	2	3	3	3	3
Øvrige fylker	0	0	0	0	0	0	0	0	0
Totalt/*Total*	994	991	1001	1020	1,023	996	1,038	1,198	1,136

Source: Fiskeridir.no/English/statistic/

1) *Only commercial production*

2) *Figures per 27 January 2010*

Total aquaculture area is approximately 420 km^2 (FHL, 2013). Even though the number of aquaculture sites are reduced since 2007 (Table 1), each site is now bigger than previously and several farms have been moved out to deeper waters and more exposed locations. In total these sites cover an area less than the size of Andøya Island and use less than 0.5% of the total sea area within the base line.

Norwegian salmon farms were started as family businesses, producing salmon for local consumers. Due to high profitability and prospects of further expansion, the local small scale farms were merger and restructured to big multinational companies. At the beginning, Norwegian salmon farmers had difficulties marketing their product (Robert, 1984). It was more expensive to farm salmon than to catch it in the wild, and many customers believed

that cultured salmon was of inferior quality. However, consumers have found over the years that the quality of farmed salmon is the same, or better than that of wild salmon.

In 1990s the Norwegian aquaculture industry was hit by a crisis (Pettersen and Alsos, 2007). Fish disease increased rapidly in the late 1980s, causing an adverse economic impact for many fish farmers. Moreover, liberalisation of juvenile production led to overproduction and the prices of juvenile fish dropped dramatically. With an increase in the maximum permitted production volume at grow-out farms, this led to a strong increase in production of salmon and trout. As a result, market prices fell. Pettersen and Alsos (2007) also reported that around the period 1997 to 2005 the Norwegian fish farming industry has experienced several fluctuations between golden eras and crises. The period of 1997 to 2000 was a good era for Norwegian salmon farms, which are indicated high market prices and increased profits. But in 2001, prices for salmon fell dramatically, resulting in lower profits and many bankruptcies. As a result, the degree of concentration and integration increased. In addition, the focus on efficiency and cost reduction became even stronger. But in 2005 there were a change, prices of salmon increased again resulting in increased profitability.

The Norwegian College of Fishery Science (NCFS, 2012) report that the Norwegian salmon industry is offering employment to 6.000 people and an additional 12.000 people in related support industries such as production of feed, net pens, tubes, feeders, transport, banking, slaughterers, export. The five largest Norwegian Aquaculture companies (groups) produce approximately 56% of the Norwegian Atlantic salmon and 46% of the total Atlantic salmon production in the world (Norden, 2011).

Norway is the world leader in the culture of salmon in sea-cages; 582 farms operated in coastal waters in 2008 (Kjønhaug, 2009). Approximately 310 million individual Atlantic salmon and rainbow trout were held in sea cages in Norway at any given time during 2009 (Norwegian Directorate of Fisheries, 2009). The maximum allowable stocking density in net pens is 25 kg m^{-3} in Norway (Norwegian Ministry of Fisheries and Coastal Affairs, 2008) with a normal harvest weight of 4 to 5 kg. Individual cages in the 1970s held 10.000 fish, while individual cages today can hold up to 200.000 salmonids. In practice, the largest Norwegian sites produce more than 10.000 ton of salmon biomass, constituting more than 2 million individual salmon.

A typical fish farm in Norway consists of between six and ten cages, holding 3.000 to 4.000 tonnes of fish (FHL, 2011). The cage consists of a buoyancy element on the surface and a net bag in which the fish swim. A typical net bag is between 20 to 50 metres deep; with a diameter of the net cage around 50 metres. The largest net cages have a circumference of 200 metres. The salmon are kept in net pens in the sea and fjords for 14-22 months. When

the fish weighs 4-6 kg, it is ready for slaughtering. FHL (2011) also reported that the salmon are transported by well boat to the fish-processing facility. They are then stunned, gutted, washed, sorted according to size and quality and laid on ice. After slaughtering they are processed in Norway sold to the fishmonger or sent to 100 countries around the world.

Ministry of Fisheries and Coastal Affairs (2010b) reported that, Norway seafood products from aquaculture and capture, are generating the third largest export income to Norway, after the oil/gas and mineral (Figure 1). The contribution of the seafood industry to Norway's Gross National Product (GNP) through value creation amounts to NOK 46.6 billion and accounts for employment of around 44.000 full-time equivalents (FHL, 2013).The Norwegian Seafood Council (NSC, 2015), Norway exported seafood worth NOK 68.8 billion in 2014. This was an increase by 12% or NOK 7.3 billion, since 2013. The European (EU) market increase by 16% in 2014, to reach a total value of NOK 43 billion export of salmon and trout amount to NOK 46.2 billion in 2014. The average price achieved for fresh whole salmon was NOK 41.06 per kg. In 2013, the first-hand value of Norwegian fish farming reached NOK 40 billion, up 35% from 2012. The produced quantity was 1.25 million tonnes.

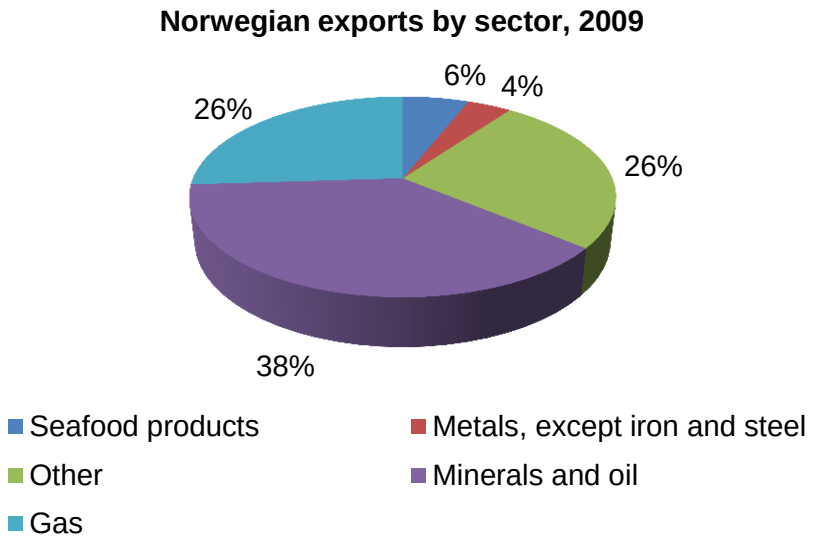


Figure 1: Major export-sectors in Norway, 2009 (Ministry of Fisheries and Coastal Affairs, 2010b).

Norwegian aquaculture has gradually undergone a number of structural and technical changes, expanded, and diversified over the year (Liu *et al*., 2010). In order to start fish farming in Norway, the company need license issued by the government. The major legislation is Aquaculture Act which has four specific focuses such as (a) growth and innovation, (b) efficiency improvement and user friendliness, (c) environmental issue and (d) relationship to the other user interests in the coastal zone. According to FHL (2011), all fish

farms in Norway have operational plans that are assessed by the Directorate of Fisheries and the Food Safety Authority.

According to NCFS (2012) Norwegian aquaculture production had a sustainable growth over a 40 years period to reach the current level. The key success factors enabling growth is based on four pillars; (a) establishment of a breeding program based on family selection (Gjedrem and Robinson, 2014) (b) fish nutrition and feed improvement (Torrissen *et al*., 2011) (c) management practices and new technology (NCFS, 2012) and (d) diseases prevention and vaccines (Jones *et al*., 2013).

Norwegian aquaculture expertise is by far large-scale farming of trout and salmon. The relatively few companies and researchers having worked outside Norway have also been involved in large-scale farming of marine as well as fresh water fish. Very few have experiences from extensive, small-scale farming at the household or village level. This is an area where Asia has superior and extensive expertise.

1.4. Fish nutrition

According to National Research Council (NRC, 2011), nutrition plays a key role in aquaculture industry by influencing growth, health, reproduction, product quality and waste generation. The growth and production of all farmed fish and shrimp species are dependent upon the intake of food containing 40 or so essential nutrients (i.e., essential amino acids, fatty acids, minerals, vitamins, etc.), the form in which these nutrients are supplied varies depending upon the farming system and feeding strategy used (Tacon, 1996). The main issue associated with nutritional value on a feed ingredient, understanding principal of the proportion of nutrients that an animal can obtain from a particular ingredient through its digestive and absorptive processes (Glencross *et al*., 2004).

Craig (2009) reported that, good nutrition in animal production systems is essential for economy in the production of a healthy product with high end quality for consumers. Fish nutrition has advanced in recent years with the development of new, balanced commercial diets that promote optimal fish growth and health. In intensive fish farming, feed cost represents 50-60% of the production costs (Torrissen *et al*., 2011). Fish nutrition has advanced dramatically in recent years with the development of new, balanced commercial diets that promote optimal fish growth and health. The development of new species-specific diet formulations supports the aquaculture industry as it expands to satisfy increasing demand for affordable, safe, and high-quality products.

1.4.1. Protein and amino acids

The basic structural component of proteins consists of amino acids. Amino acids are critical components with a fundamental role building muscles as well as other functional constituency such as enzyme (NRC, 2011). Animal protein is consists of 20 amino acids (AA). Vertebrates including fish cannot synthesize ten amino acids and must acquire these from their diets (Webster and Lim, 2002). These essential amino acids include: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The other 10 amino acids can be synthesized by fish, such as: alanine, asparagine, aspartic acid, cysteine, cysteine, glutamic acid, glutamine, glycine, hydroxyproline, proline, serine and tyrosine. Kaushik and seiliez (2010) reported that amino acids also play an important role in meeting energy (metabolic) requirement of fish and crustacean species.

Protein is usually reported as crude protein (CP). The chemical analysis is based on amount of nitrogen (N) in the protein. Content of CP can then be calculated with used of equation N X 6.25, based on the assumption that proteins contain 16% N (Mariotti *et al*., 2008). The factor 6.25 is the standard unless another factor is stated. Fish meals, as an example contain other N-containing components such as trimethylamine oxide or total volatile basic nitrogen (TMAO/TVN). Using the factor 6.25 may thus overestimates protein content in fish meal.

Protein requirements have been examined to very large number of fish and shrimp species at different life stage (NRC, 2011). Requirement of proteins of fish is influenced by several factors, such as size of fish, quality of protein, water temperature (Webster and Lim, 2002). Salmon digest protein efficiently, and 50% of the AA is absorbed in the pyloric region (Krogdahl *et al*., 1999). According to NRC (2011), protein requirement at different life stages of Atlantic salmon depends of the size of the fish. For example salmon weighing < 20 gr needs 48% of protein in the feed, 20-200 gr needs 44%, 200-600 gr needs 40%, 600-1500 gr needs 38% and > 1500 gr needs 34%. Knowledge of nutritional constraints and limitations of protein requirement is important for production of efficient feeds for the fish. This information provide simple basis for formulation of practical feeds for the different life stage of salmon.

1.4.2. Lipid

Lipids consists of a wide range of compounds grouped together simply based on their solubility in organic solvent (NRC, 2011). Lipids are important for good growth, health and reproduction (Tocher, 2003). They provide energy and essential fatty acids (EFA) to the fish and they also assist the absorption of fat-soluble vitamins (NRC, 1993).

A true lipid requirement for fish and shrimp is difficult to define because it is influence by a variety of nutritional factors (NRC, 2011). The amount of dietary lipids required is influenced by the content of protein and carbohydrate. According to Tocher (2003), animal lipids, including fish lipid can be divided into the two groups, polar lipids composed of phospholipids, and neutral lipids composed of triacylglycerol (TAG).

Current extrusion technologies allow aquafeeds to contain up to 40% oil (Miller *et al*., 2008). The natural marine diet of Atlantic salmon contains high concentrations of n-3 long chain poly unsaturated fatty acids (LC-PUFA), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), low concentrations of ω-6 PUFA. Naylor *et al*. (2009) reported that particularly for salmonid species, the essential ω-3 LC PUFA requirement exceeds that supplied by residual oil in fishmeal if dietary fishmeal levels are below 40%. However, more fish oil is used in salmonid diets to ensure healthy ω-3 LC-PUFA levels in fillets.

Atlantic salmon like other aquatic fish, are unable to synthesize fatty acids of the ω-3 and ω-6 families (Storebakken, 2002). These fatty acids must be provided in the diet. Salmon requirement for PUFA is estimated about 1% of the diet and can be met by including approximately 4% fish oil in feeds (Olsen *et al*., 1991; Yang and Dick, 1994). These fatty acids can be supplied through marine feed ingredients like fish meal or fish oil. Atlantic salmon can show nutritional 'diseases' or pathologies due to lipid imbalances (Bell *et al*., 1991; Seierstad *et al*., 2005). The main symptom of EFA deficiency from the diet is reduced growth, shock syndrome, and increased mortality (Glencross, 2009).Therefore an aquafeeds have to be supplied with ω-3 LC-PUFA as a part of the oil component.

1.4.3. Carbohydrate

It is in generally accepted that carnivore fish and shrimp do not have specific requirement of dietary carbohydrate (NRC, 2011). Inclusion carbohydrate in aquafeed is limited compared to poultry and mammals. Numerous studies have evaluated the maximum levels of carbohydrates that fish and shrimp can tolerate without physiological disorder and growth impairment, rather than level for growth.

Different fish species show different ability to digest and metabolise carbohydrates (Hemre *et al*., 2002). Carnivorous fish lower ability to utilise dietary carbohydrates than omnivorous and herbivorous fish (Enes *et al*., 2011; Enes *et al*., 2006). Excess carbohydrates reduce the growth rate accompanied by poor feed utilization (Hemre *et al*., 2002). In general, carbohydrate inclusion in carnivorous fish diets is limited to 20% (NRC, 2011). However, warm-water omnivores can use diets containing as much as 40% dietary carbohydrate because they have higher intestinal amylase activity (Dabrowski and Guderley, 2002).

Digestion of dietary starch in fish is highly variable depending on fish species, carbohydrate source, and physical state of the molecule and processing (Krogdahl *et al*., 2005). Starch in salmonid diets has to be limited to a maximum of 10% (Hemre *et al*., 2002).

1.4.4. Vitamins

Vitamins are organic compounds distinct from amino acids, carbohydrate and lipids in that they are required in trace amounts from an exogenous source (NRC, 2011).

Both qualitative and quantitative vitamins requirements of fish and shrimp have been determined by feeding chemically defined diets deficient in specific vitamins. According to Halver and Hardy (2002), salmon and trout require 15 vitamins in their diet to ensure good growth and optimal health. Vitamin deficiency may result in reduced growth, scoliosis (bent backbone symptom) and dark coloration.

Vitamins usually classified as water-soluble and fat-soluble vitamins. Water-soluble vitamins include: the B vitamins, choline, inositol, folic acid, pantothenic acid, biotin and ascorbic acid (vitamin C). Fat-soluble vitamins are A vitamins (retinols), D vitamins (cholecalciferol), E vitamins (tocopherol).

1.4.5. Minerals

Minerals are inorganic elements needed in the diet for normal body functions. Minerals are grouped into micro and macro minerals. Six minerals are important for fish. These are calcium, sodium, chlorine, magnesium, potassium and phosphorous. These macro minerals regulate osmotic balance and aid bone formation and integrity. Micro minerals are also referred to as trace minerals. Typically they are required in the diet and body at much lower concentrations than macro mineral. Usually they are required in small amounts as components of enzyme and hormones systems. Common trace minerals are copper, chromium, iodine, zinc and selenium.

Currently there is not much information on mineral requirement of aquatic species (NRC, 2011). Fish can absorb many minerals directly from the water through their gills and skin, allowing them to compensate to some extent for mineral deficiencies in their diet. At present, salmon feed is routinely supplemented with several essential elements such as Cu, I, Mn, Se, and Zn. For salmon in the freshwater aquaculture, it is also a need to supplement the diets with phosphorous to cover requirement (Storebakken, 2002).

Macro minerals of phosphorus and calcium are closely related to the development and maintenance of the skeletal system. The stability of the vertebrae is maintained by a solid

phase of calcium phosphate (Lall and McCrea, 2007). Dietary available P of fish ranged between 0.4% and 0.8% of diet (NRC, 1993) with the exception of Japanese eel (0.3%) and haddock (0.96%). Calcium deficiency is not common in fish. Phosphorus is one of the minerals for which the dietary requirement is highest, estimated to be ≈ 10 gr kg^{-1} (1%) of a fishmeal-based diet for Atlantic salmon, *Salmo salar* (Åsgard and Shearer, 1997). Phosphorus deficiency signs include reduced growth, decreased feed efficiency, reduced bone mineralization and skeletal abnormalities (Lall and McCrea, 2007). Common skeletal deformities include curved spines and soft bones in Atlantic salmon (Baeverfjord *et al*., 1998). Sodium content in fish varies a great deal, depending on the species and variety. Especially, Atlantic salmon contain fewer than 60 mg of sodium per 100 gr (Atanasoff *et al*., 2013). Potassium is important for building muscle, metabolizing protein and carbohydrate, balances water and acid in the blood and body tissues. Farmed Atlantic salmon have only 384 mg, while wild Atlantic salmon has 628 mg of potassium. Magnesium is an important element for organisms and oxidative phosphorylation, as well activates many enzymes (Öksüz, 2012). Magnesium content of farmer salmon was determined as 32.6 mg/100 gr.

1.4.6. Energy

Energy is not nutrient but is released during metabolic oxidation of protein, lipids and carbohydrate (Webster and Lim, 2002). Fish need energy to live, which is obtained from oxidizing chemical bonds.

The nutritional value of a dietary ingredient is in part dependant on its ability to supply energy (Craig, 2009). According to Webster and Lim (2002), there are a number of factors that could affect the energy requirement of fish, such as: physical activity, temperature, fish size, growth rate, species and food consumption. Fish must be fed diets containing appropriate amounts of energy. The optimum ratio of protein to energy must be determined separately for each fish species (Craig, 2009). Excess energy relative to protein content in the diet may result in high lipid deposition. In addition Craig (2009) also reported that a diet with inadequate energy content can result in reduced weight gain because the fish cannot eat enough feed to satisfy their energy requirements for growth.

1.5. Modern fish feed contain a wide range of ingredients

The development of new specific diet formulations replacing fish meal and fish oil protein and lipids from alternative resources, have supported the growth of aquaculture industry. Norden (2011) reported that the fish feed plays an important role in the value chain. Control of the quality of raw materials is thus crucial for food safety. Control of ingredient are also

important for production of high quality feed types that ensure optimal growth for different fish species farmed under a variety of different conditions.

Modern aquaculture feeds are now being formulated based on digestible amino acid basis (Sørensen *et al*., 2002). According to Glencross *et al*. (2007), the ability of fish to digest nutrients from a specific ingredient, varies depending on a number of factors. Thus, when ingredients are evaluated for use in aquaculture feeds, there are several important knowledge components that should be understood to enable the judicious use of a particular ingredient in feed formulation. This includes information on (1) ingredient digestibility, (2) ingredient palatability and (3) nutrient utilization and interference with health and product quality. Ideally, the science of nutrition should endeavour to gain knowledge on the nutritional implication of using novel ingredients, and once this knowledge is gained, it can be applied in commercial feed formulation.

1.5.1. Fish meal and fish oil

Marine resources are usually unique ingredients because they are an excellent source of all the main nutrients required by the fish. Usually they are providing high quality animal protein and essential amino acids, minerals and vitamins, lipids, including essential polyunsaturated fatty acids (PUFA) of the n-3 series (Hertrampd and Piedad-Pascual, 2000; Cowey, 1975).

Rapid expansion in aquaculture industry has resulted in increased demand of high quality aquafeeds. The fishmeal and fish oil industry use around 20-33 million tonnes of fish annually together with 4-6 million tonnes of by-products and trimmings (Shepherd *et al*., 2005; Ministry of Fisheries and Coastal Affairs, 2009b). Global landings of forage fish, and hence, the supply of fishmeal and fish oil have been fairly stable the last 25 years, as indicated by (Figure 2). From these raw materials, production of fishmeal varies between 4.5 and 7.5 million tonnes, while fish oil production fluctuates 0.85-1.67 million tonnes (Tacon and Metian, 2009). The global aquaculture industry is by far the largest consumer, accounting for about 60% of fishmeal and 81% of fish oil respectively (World ocean review, 2013).

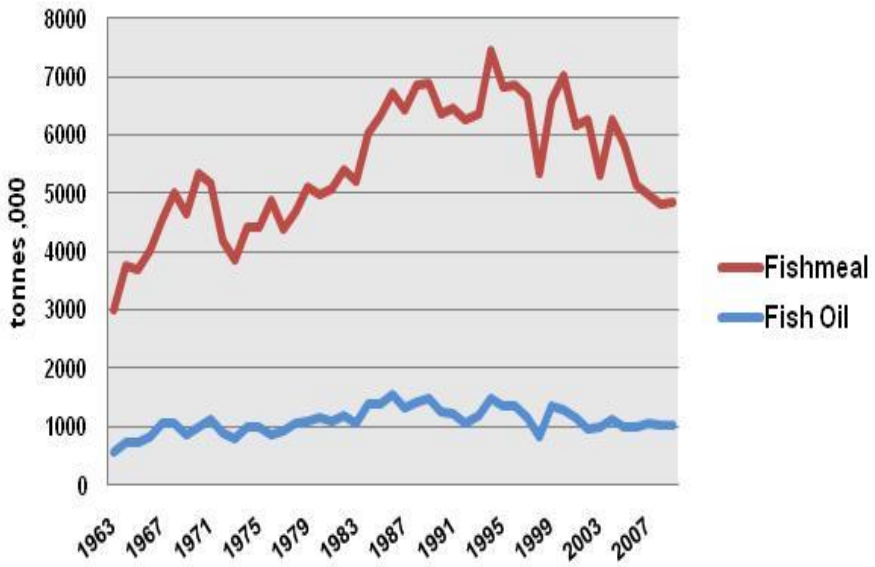


Figure 2: Global production of fishmeal and fish oil 1963-2009 (Jackson, 2010a).

Fishmeal and fish oil are produced in large industrial facilities, which involves grinding and boiling the whole fish. Centrifuges are used to separate, dewater and dry the resultant mass (World ocean review, 2013). Fish meal has traditionally been the principal source of protein in the diet of farmed carnivorous fish and represents the largest operating costs (Naylor *et al*., 2009). Fishmeal is generally composed of 70% protein, 10% ash, 9% fat and 8% water (Blanco *et al*., 2007). Amino acid profile, digestibility and palatability can vary depending on the raw material used and how it is processed (Blanco *et al*., 2007). Fish oil was originally used as an ingredient in paints, lubricants, soaps, printing inks and the tanning of animal hides (Tacon and Metian, 2009). Today fish oil is mainly used in the production of salmonids (World ocean review, 2013). Fish oil is rich in polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), commonly referred to as omega-3 (Blanco *et al*., 2007). The *n*-3 PUFA are required in higher concentrations in fish diets compared to *n*-6 PUFA. The *n*-3 PUFA are constituents of the major fatty acids in fish cell membranes (Sargent *et al*., 1999).

Aquaculture industry has been depending on fishmeal and fish oil, however this source is finite. In fact, there is a global decrease of dietary fish meal and fish oil inclusion levels in commercial aquafeeds, due to the increasing prices of these commodities since 2000 (Tacon and Metian, 2008). World ocean review (2013) reported that the price for fishmeal has increased considerably as a result of strong demand in the importing countries, especially China. On the other hand, Naylor *et al*. (2009) reported that the ratio of wild fish input via industrial feeds to total farmed fish output (excluding filter feeders) has fallen by more than one-third from 1.04 in 1995 to 0.63 in 2007. The decline is mainly explained by

expanding volume of omnivorous fish produced. Since fish meal and fish oil are limited, the demand for aquafeeds will certainly outstrip the global production of these products.

Catching of fish for reduction to fish meal and fish oil, is currently accounting for 67% while trimmings and by products from fish caught for human consumption is providing 33% of the material for fish meal and fish oil production. Total global capture production of 93.7% million tons in 2011 was second-highest ever. Global fish meal and fish oil production from marine capture fisheries has been decreasing at annual average rates of 1.7% and 2.6%, respectively, during the course of 1994-2009 (FAO, 2012). The highly variable recruitment dynamics of teleost fish used for the production of fish meal and fish oil, make prediction of fish stock over time difficult (EU Parliament, 2004). Thus a guidelines was published in 2009 (FAO, 2011) for sustainable management of fisheries and harvest of stock used for aqua feed production.

Norway has had a great expansion of salmon farming and is now the largest importer of fish oil; while China, Japan and Taiwan are the largest importers of fish meal (World ocean review, 2013). In 2009, the Norwegian marine fisheries amounted to 2.7 million tonnes of which 1.7 million tonnes were pelagic fish and 670.000 tonnes cod fish (Norden, 2011). About 540.000 tonnes were reduced to fish meal and fish oil. FAO (2010b) reported that main species for fish meal and fish oil production are anchoveta, capelin, sprat, herring, blue whiting, sandeel, trimming and other species (Table 2). All are harvested in North Atlantic waters, with the exception of anchoveta, which is harvested in the South Pacific outside Peru (Sheperd *et al*., 2005).

Table 2: Main species used for fish meal production in Norway

Species	2007	2008	2009
Anchovy	22	2	43
Blue Whiting	27	21	7
Capelin	4	2	1
Herring	20	19	20
Sandeel	4	11	5
Sprat	9	6	5
Trimmings & other species*	14	18	19

(Helland, 2010; skretting, 2010b)

*Trimmings are mostly from processing of herring. Other species refers to a wide variety of species included at a low level

Sørensen *et al*. (2011) reported that herring and mackerel in used to a large extent for human consumption, and less catches are available for reduction to meal and oil. Other species such as blue whiting, sand eel and Norway pout has become more important in fish

meal and fish oil production. Also the category “other” has increased, and this category includes trimmings from fish for human consumption. The use of by-products in fish meal production is also increasing world-wide. International fishmeal and fish oil organisation (IFFO) predicted that the amount of ingredients coming from by-product has reached over 25 % of global production, and Norway 22% of Norwegian production (Chamberlain, 2011).

In Norway, most of the raw materials are taken care of, but still there are a good potential both to increase the volumes and further value adding (Olafsen *et al*., 2014). Marine by-products add substantial value to the seafood industry, and many companies have a special focus on product development of marine ingredients. There are restrictions in the use of by product from aquaculture because the main aquaculture production in Norway is Atlantic salmon. By products from salmonids cannot be used in feed for Atlantic salmon.

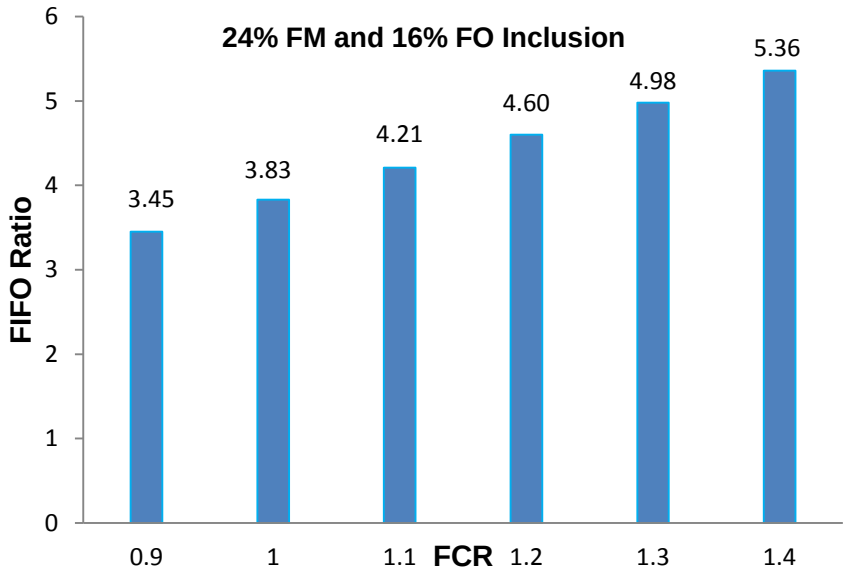
There are currently five producers of fish feed in Norway: EWOS, Skretting, BioMar, Marine Harvest and PolarSeed. Marine Harvest started feed production about one and half year ago. These companies have in common that they specialize mainly in salmon feed, but a small quantities of feed for other species is also produced.

Salmon feed has been dominated by marine raw materials. Inclusion rate of fishmeal has made up 40-60% of the feed, while fish oil has had an inclusion level of about 20-30% (Gillund and Myhr, 2010). These resources have satisfied the nutritional requirements of salmon, while at the same time have provided high levels of ω-3. Fish meal and fish oil has a nutritional profile which approximates closest to the known dietary requirements of salmonids, and as such usually has a high biological value and digestibility for salmonids compared with other non-marine animal feedstuffs (Tacon and Metian, 2008).

In 2010, Norwegian salmon feed industry consumed 257.167 tonnes of fish meal and 165.277 tonnes of fish oil from reduction fisheries, plus 68.292 tonnes and 53.396 of fish meal and fish oil respectively, produced from trimmings and silage. Ytrestøyl *et al*. (2014) recently reported that Norwegian salmon industry in 2012 used 53% fish oil and 20% fishmeal available on the world market.

Reducing aquacultures reliance on marine resources in the future will depend on improving FCRs (Feed conversion rations) and reductions in fishmeal and fish oil inclusion rates (Naylor *et al.,* 2009). The fish in:fish out (FIFO) is mainly dictated by feed conversion ratio and inclusion rate at fish meal and fish oil. For example, a reduction in FCRs in farming of Atlantic salmon from 1.4 to 1.0 will lead an improvement in FIFO from 5.4 to 3.8 (Figure 3). Use of the terminology FIFO as a sustainability measure is however, debated because different authors use different assumptions for calculating the value.

Figure 3: Sensitivity of FIFO to changes in FCR



Source: Adopted from Naylor *et al*. (2009)

The FIFO is much more sensitive to changes in fish oil inclusion than fishmeal inclusion because oil level in fish varies to a greater extent. In other words, the amount of forage fish used to produce feeds for salmon is driven by the need for fish oil rather than fishmeal.

The FCRs in Norwegian salmon production ranged between 1.0 - 1.4, with an average 1.2, in 2008 (Tacon and Metian, 2008). This is a bit lower than the global average of 1.25. Changed feed conversion rates occur on a continuous basis. Some stakeholders state that the FCRs and FIFO ratios are too narrow and provide a perception on reality that is miscalculated. Naylor *et al*. (2009) also reported that calculating FIFO ratios is complicated by the fact that feeds for some species, like salmon and trout, are high in fish oil, whereas feeds for other species, such as tilapia and carp, contain fishmeal but very little fish oil.

Farming salmon is one of the most resource-efficient ways of animal farming for food. Approximately 1.15 kg of feed produces 1 kg of salmon, and this feed comes from 2 to 2.5 kg wild fish (FHL, 2011). In comparison, wild salmon have to eat 10 kg of fish to grow 1 kg. The global FIFO ratio is currently at an average of 4.9:1 (2006), meaning that 4.9 kg of wild fish was needed to produce 1 kg farmed salmon (Tacon and Metian, 2008). For Norwegian salmon farming, the FIFO ratio was lower than the global average at 2.27:1, indicating that salmon from Norway is produced more sustainably than the global average (Tacon and Metian, 2008).

Tacon and Metian (2008) reported that plant ingredients such as rapeseed, soybean, corn and gluten can replace fish meal. Gillund and Myhr (2010) stated that, new resources that

replace of fish meal and fish oil must possess the optimal as an ingredient in salmon feed, fulfil the nutritional requirements, and the feed has to be sustainable and economically viable for the company. Another important aspect is that these feed resources do not exist in Norway, and need to be imported from other country. The production and transportation of food crops also have socio-economic effects that should not be neglected. Naylor *et al.* (2009) state that to be a viable alternative for fishmeal or fish oil, a candidate ingredient must possess certain characteristics, including nutritional suitability, readily availability, and ease of handling, shipping, storage, and use in feed production. Furthermore, candidate ingredients should be consumer acceptance, minimal pollution and ecosystem stress, and human health benefits. Finally, competitive pricing is essential for the adoption of non-fish alternatives in feeds.

Research progress has resulted in a substantial reduction of fishmeal in modern feeds for species such as Atlantic salmon. Until recently, 25% appeared to be the limit below which performance suffered, in terms of growth rate and feed conversion ratio (Obach, 2012). Today, feed producers such as Skretting can formulate fish feed with levels of fishmeal as low as 5-10%. Fish meals are replaced solely by vegetable raw materials or by a combination of vegetable raw materials and non-ruminant processed animal proteins.

To promote sustainable aquaculture, several studies have been carried out in recent years to replace fish meal and fish oil. Sørensen *et al*. (2011) reported that, the most important protein and lipid ingredients used by Norwegian fish feed industries in 2010 were: soy protein concentrate, fish meal, wheat gluten, sunflower meal, pea protein concentrate, faba beans, rapeseed oil and fish oil. According to Gillund and Myhr (2010) salmon diets in Norway are currently based on about 40% vegetable ingredients and about 60% marine resources (Figure 4). Marine inclusion levels will vary among companies and regions due to prices, policies and availability.

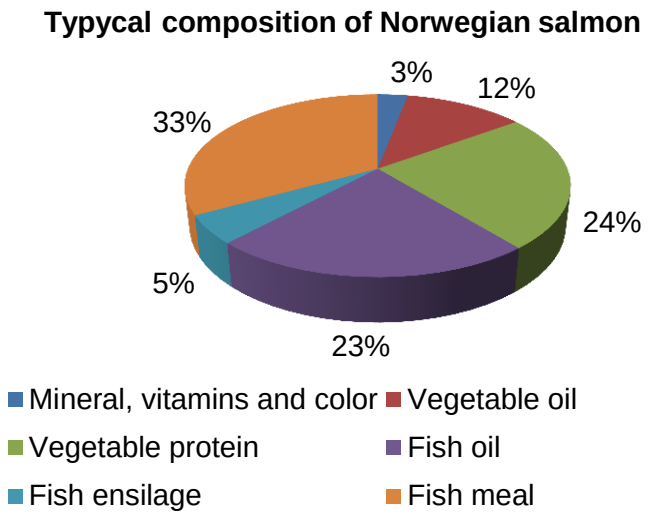


Figure 4: Typical composition of salmon feed (Gillund and Myhr, 2010)

A current report from Nofima (Ytrestøyl *et al*., 2014) showed that the average of Norwegian salmon diet in 1990 contained 65% fish meal and 24% fish oil and that it had come down to 19% and 11%, respectively, in 2013. The FIFO for fish oil was reduced from 7.2 down to 1.7 in 2013, and FIFO for fish meal was reduced from 4.4 to 1.0 in the same year. At these low levels, salmon farming is a net producer of marine protein, in others words more fish protein is produced than what is used to make the feed (Bendiksen *et al*., 2011).

1.5.2. Plant ingredients

Fishmeal has always been a relatively expensive feed ingredient compared to soybean meal, with a cost remaining relatively constant in the past at 2 to 2.5 times higher (Asche and Tveteras, 2004). This is the key driver for adopting more sustainable protein and oil sources for aquaculture feeds. Plant ingredients are also good candidates because of their abundance and relatively low cost.

Plant-based proteins in aquafeeds need to possess certain nutritional characteristics, such as low levels of fiber (especially nonsoluble carbohydrates), starch, and antinutrients. Moreover, the ingredients also should contain a relatively high protein content, favourable amino acid profile, high nutrient digestibility, and reasonable palatability. According to Gatlin *et al*. (2007), plant ingredients are the most promising alternative sources to fish meal and fish oil in fish feed. Particularly, varieties such as grain legume, pulse and cereal thanks to their global availability and competitive price. Use of plant oil may also be advantageous in terms of their low contents of saturated fatty acid making them well suited in salmon feed used at low ambient temperature.

Norwegian salmon aquaculture composition has changed over the past two decades towards use of plant based ingredients (Sørensen *et al*., 2011). Since 1990 the ingredient composition in Norwegian fish feed has switched from marine resources to a feed dominated by plant ingredients. The most important protein and lipid ingredients used by Norwegian fish feed industries in 2010 were: soy protein concentrate, fish meal, wheat gluten, sunflower meal, pea protein concentrate, faba beans, rapeseed oil and fish oil. In near future the alternatives will also include canola, lupins and distillers dried grains with soluble (Sørensen *et al*., 2011). Among the ingredients that are being investigated as alternatives to fish meal, soybean meal (SBM) is one of the basal ingredients in commercial aquafeed, because of its good quality, low cost, steady availability and SBM is a good source of dietary protein and phospholipids (Hanel *et al*., 2007). According to Sørensen *et al*. (2011) plant oil used today is mainly rapeseed oil (low erucic acid). Also small amounts of the palm oil and soybean oil may be used. The search for new very long chain (VLC) ω-3 fatty acid containing oils as alternatives to fish oil is urgent. Potential sources are suggested, but no immediate solution is found.

The use of soybean as a protein source has been examined for many commercially important fish species. For example, Xu *et al*. (2012) reported that 57.64% fish meal could be replaced by soy protein isolate without significantly affecting the WGR (weight gain rate), FCR and survival rate of juvenile Amur sturgeon (*Acipenser schrenckii*), and this fish is able to effectively utilize appropriate levels of soy protein isolate as the main protein ingredient in diet. Acar *et al*. (2013) investigated the replacement of fish meal with 40% soybean meal in diets for banded Sea bream (*Diplodus vulgaris*). At 40% there were no serious effects on growth performance; feed conversion rate, specific growth rate and serum biochemical variables.

Overall, the drawbacks for using alternative plant protein sources in diets for carnivorous fish is related to their lower levels of protein and higher levels of carbohydrate, and unfavourable amino acid profile (Hemre *et al*., 2009). They also contain anti-nutritional and has an impaired effect on palatability that can compromise nutritional value and restrict the use carnivorous fish (Francis *et al*., 2001; Drew *et al*., 2007b).

Several studies also with Atlantic salmon have shown that substituting the fish meal with plant protein ingredient reduced growth performance of Atlantic salmon (Refstie *et al*., 1998; 2000; Storebakken *et al*., 1998; Carter and Hauler, 2000; Krogdahl *et al*., 2003; Opstvedt *et al*., 2003; Bostock *et al*., 2010). Research on use of plant oil in salmon diet; have shown that the level of ω-3 in muscle lipids is significantly reduced (Bell *et al*., 2001; Olsvik *et al*., 2007).

Further, high levels of plant lipids may have negative effect on fish health. Reduction of ω-3 may damage the reputation of salmon as healthy food for humans.

1.5.3. Use of land animal by-products in feed

Numbers of studies have shown that many terrestrial by products have potential in aqua feeds. A study carried out by Forster and Dominy (2006), reported that rendered animal by-products can replace a significant portion of fishmeal in diets for shrimp without significant reduction of growth. Two main varieties of rendered terrestrial animal by-product are meat and bone meals (MBM), obtained mainly from cattle, swine; or poultry by-product meals (PBM) primary derived from poultry meat meals, feathers meal and eggs meal. Sørensen *et al*. (2011) reported that animal by-products from terrestrial animals, such as bone, meat, skin, and feathers are resources that have potential to be used in the diets for fish. Poultry by-product had the greatest potential as a promising well balance protein ingredient for carnivorous fish. The protein quality of by-products can also be reduced by processing of the meals because intensive heat is used for hygienic reasons as well for drying. For instance, feather meal has a low protein digestibility because of disulphide bonds. Feather meal therefore has to be processed in order to increase digestibility.

Naylor *et al*. (2009) reported that, animal by-product meals have a more complete amino acid profile, and some of them contain high levels of available lysine and phosphorous. Animal by product are inexpensive per kg of crude protein than fishmeal. Animal lipids are also inexpensive but they are high in saturated fats (Naylor *et al*., 2009). Animal lipids have low digestibility at cold temperatures and must be blended with polyunsaturated fats to facilitate digestion. Lipid from poultry may, however, not be suitable to replace fish oil in diets for salmonids living in cold water, at least not in the coldest month of the year because high melting point (Turchini *et al.*, 2009). High ash content in land based animal by-products may also reduce nutritive value. Ash content can reduced by developing processing practices and thereby improve quality and digestibility of the meal.

1.5.4. Single Cell Protein (SCP)

Since the early fifties, intense efforts have been made to explore new alternate protein source as a food supplements to relieve shortage of proteins to a growing world population (Becker, 2007). Single cell protein (SCP) is a term applied to a wide range of unicellular organism such as yeast, microalgae, bacteria, and fungi produced, on waste biomass or other sources of energy.

A major advantage for the use of SCP is that the technology exists to produce industrial quantities under controlled and environmentally safe conditions. In addition, the composition of many microorganisms can be manipulated to produce higher levels of protein and lipid, by enriching grown media with specific essential amino acids or fatty acids (Kangas *et al*., 1982; Tan and Johns, 1991; Sanchez *et al*., 1995; Day and Tsavalos, 1996).

Compared with conventional plant and animal feed proteins, micro-organisms offer numerous advantages as protein producers (Tacon, 1987). For example, their production is based on carbon which is available in large quantity. Under optimum culture conditions they can double cell mass and nutritional composition controlled by genetic manipulation etc.

In addition to SCP monocultures for protein production, mixed SCP cultures can be grown as activated sludge (e.g., mixed suspension of bacteria, algae and yeast in specific waste streams such as brewery waste, human sewage, and paper processing waste. Over the last few years, there has been a growing interest for production of unicellular organisms. Microbial ingredients from bacteria, yeast and microalgae are new ingredients that have a potential in diets for salmonids (Sørensen *et al*., 2011).

a. **Bacterial meal-Bioprotein**

Meal made from bacterial biomass produced on natural gas is a new feed ingredient with a proximate composition and amino acid profile similar to high-quality fish meal, making it interesting as a fish meal substitute (Skrede *et al*., 1998). BioProtein had a great potential in feed for fish and domestic animals (European Commission, 2003). The current status is that BioProtein was approved by the EU (Regulation (EC) No 767/2009). Revision of the EU regulations concerning microbial protein sources may facilitate further development and use of such products as feed ingredients, for use in feed for salmon and to some extent for domestic animals.

Øverland *et al.* (2010) reported that bacterial proteins represent a potential future nutrient source for terrestrial animal as well as fish. Bacterial proteins can grow rapidly on substrates with minimum dependence on soil, water, and climate conditions. Bacterial meal (BM) derived from natural gas fermentation, is utilising a bacteria culture containing mainly the methanotroph *Methylococcus capsulatus* (Bath). The BM is a promising source of protein based on criteria such as amino acid composition, digestibility, animal performance and health. Future research challenges include modified downstream processing to produce value added products, and improved understanding of factors contributing to nutrient availability and animal performance.

A comparative study was carried out by Skrede *et al*. (1998) to evaluated digestibility of amino acids in bacterial meal by several terrestrial animals as well as fish. Digestibility of individual amino acids in BM varied considerably: high digestibility was found for lysine and arginine, while digestibility of cysteine was low. There were significant correlations between ileal amino acid digestibility in pigs and total tract digestibility in mink (r = 0.985), chickens (r = 0.987) and salmon (r = 0.944). The digestibility of crude fat in BM was estimated at 87.0% in salmon and 90.5% in mink.

Storebakken *et al*. (2004) reported that juvenile Atlantic salmon is less tolerant of dietary bacteria protein meal during the first feeding stage than at later stages during the freshwater period. A study carried out by Aas *et al*. (2006) found that salmon, *Salmo salar* fed diets containing 18% and 36% bacterial protein had faster growth rates than those fed a 100% fish meal control diet. In another study carried out by Berge *et al*. (2007), growth rates in salmon fed diets containing 10% and 20% bacterial meal was similar to the control group fed 100% fish meal as protein source in the control diet.

b. **Yeast**

Yeast is a single cell organism that can ferment sugars to alcohol. Yeast is already available at the world market (Salnur *et al*., 2009), and has lower price than many other ingredients. Lee and Kim (2001) considered yeast as a cheap dietary supplement as they are easily produced on an industrial level from a number of carbon-rich substrate by-products.

Yeast is reported to have no adverse effect on nutrient digestion of cows and fish growth (Oliva-Teles and Goncalves, 2001). Yeast has been identified as part of the normal microbiota of both wild and farmed fish, and their role in fish health and nutrition has been addressed in the literature. Yeast can be used either alive to feed live food organisms or after processing as a feed ingredient. Yeast seems to have an important role for development of digestible tract in fish (Navarrete and Ramírez, 2014).

Different strains were sprayed to pellets fed to Sea bass larvae (Tovar *et al*., 2002) These authors showed that *Debaryomyces hansenii* enhanced maturation of the digestive tract in marine fish larvae. It was suggested that the beneficial effect on the digestive tract was due to high secretion of spermine and spermidine by the yeast. Another Study carried out by Harikrishnan *et al*. (2011) investigated the effect of dietary administered *Saccharomyces cerevisiae* in fish. Yeast supplemented diets stimulated growth, feed efficiency, blood biochemistry, survival rate, and non-specific immune responses in Uronema marinum-infected Olive flounder (*Paralichthys olivaceus*). The use of brewer's yeast at probiotic

levels (up to 2%) has proven to have a positive effect on the performance and welfare in several fish species, such as African catfish *Clarias gariepinus* (Essa *et al*., 2011).

c. Microalgae

Microalgae play a vital role in aquaculture. They are consequently used as larval feeds in intensive aquaculture, because these microalgae are available on the ingredients market (Henry, 2012). Microalgae such as *Spirulina*, *Chlorella* and *Dunaliella* can be produced by low-cost open-pond technologies and are marketed as dry powders, and their nutritional profiles are well-documented.

Microalgae are used as natural food resources for zooplankton in the food chain. Becker (2007) reported that the high protein content in some algae species is one of the main reasons to consider them as an unconventional source of proteins. They are capable of synthesizing all amino acids; they can be good sources of the essential ones. Becker (2013) reported that microalgae are numerous, and have high protein content, high lipids, vitamins, rich in eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA). Their biomass is a rich source of nutrients such as ω-3 and ω-6 fatty acids. It has great potential as an alternative ingredient for sustainable aquaculture feeds.

Microalgae incorporated into fish feed formulations have shown promising results. *Chlorella* or *Scenedesmus* fed to tilapia (Tartiel *et al*., 2008) showed that growth performance, FCRs and protein productive value were significantly ($P<0.05$) higher in fish fed diets containing 50% of both *Chlorella spp* and *Scenedesmus spp*, whereas fish feed diets containing 75% algae had significance lower performance ($P<0.05$). Combination fed *Nannochloropsis* sp and *Isochrysis* sp. replaced fish meal protein in isonitrogenous to Atlantic cod (Walker and Berlinsky, 2011). At conclusion no differences in survival, FCRs, viscerosomatic indices, and ω-3 and ω-6 fatty acids in the muscle were found among the treatment groups. Microalgae also enhanced the colour of the flesh of salmonids (Hemaiswarya *et al*., 2011).

1.6. Potential and challenge using microalgae in aquaculture

Microalgae represent potential sources of both protein and lipids in aquafeeds. Cultivated microalgae are already used in hatchery production of many farmed finfish, shellfish and other commercially important aquaculture species (Shields and Lupatsch, 2012). By contrast, macroalgae are less widely used in aquaculture, although they do provide an important source of nutrition for certain farmed invertebrates, such as Sea urchins and Abalone.

In recent years many studies have been undertaken in order to evaluate microalgae as fish feed. Microalgae have been used fresh or dried algae meal has been used in partial replacement of fishmeal protein in pelleted diets (FAO, 2009). According to Borowitzka (1997) many studies provide an excellent data-base for the selection of algae species for use in aquaculture. However, knowledge about biochemical composition is not enough. Bioavailability of nutrients needs to be tested in targeted species. Bioavailability of nutrient is tested in digestibility experiments in combination with growth experiments.

Uni Miljø (2012) reported that temperate and sub-tropical species such as *Isochrysis sp*, *I. galbana*, *Tetraselmis suecica* and *Nannochloropsis oculata* are widely use feed organisms in cold waters. Today, algae species isolated from cold waters are hardly in use. Therefore, new and more suitable species isolated from local areas for use in cold waters are highly demanded. Some attempts have been made on isolating new species from cold waters, but they so far not been implemented in intensive production.

1.6.1. Genera and species of microalgae used in aquaculture

Most microalgae are photoautotrophs, meaning that they use light energy to produce chemical energy and convert inorganic carbon (CO_2) into sugars and organic compounds (ProAlgae, 2013). Another group, called heterotrophs, can grow without light and use organic carbon compounds as both energy and carbon source.

ProAlgae (2013) also reported that the microalgae biodiversity are estimated to hundred thousand species. Out of the 35.000 microalgae species which are described, only a very few are commercially produced at the moment: the cyanobacteria *Spirulina* and *Aphanizomenon flos-aquae*, the thraustochytrids *Ulkenia* sp. and *Schizochytrium* sp., and the eukaryote algae *Crypthecodinium cohnii*, *Chlorella* sp., *Dunaliella salina*, *Haematococcus pluvialis*, *Euglena* sp. and *Odontella aurita*. In terms of volume, the three genera *Spirulina*, *Chlorella* and *Cryptecodinium* are contributing to the biggest volumes. About half of microalgae productions are dedicated to products with whole microalgae and the other half to production of extracts. Table 3 provides a non-exhaustive list of most commonly used strains and their area application in aquaculture.

Table 3: Groups, genera and species of major microalgae strains used in aquaculture and their areas of application

Group	*Genus*	*Species*	*Area of application*
Cyanobacteria	Arthrospira	platensis	FFI
Chlorophyta	Tetraselmis	suecica, chui	B, CL
	Chlorella	sp., vulagaris, minutissima, virgina, grossii	R, FFI
	Dunaliella	sp., tertiolecta, salina	FFI
	Haematococcus	pluvialis	FFI
Eustigmatophyceae (Phylum Heterokontophyta)	Nannochlropsis	sp., oculata	R, GW
Labyrinthulea	Schizochytrium	sp.	RAD
(Phylum Heterokonta)	Ulkenia	sp.	RAD
Bacillariophyta (diatoms)	Chaetoceros	calcitrans, gracilis	B, CL
	Skeletonema	costatum	B, CL
	Thalassiosira	pseudonana	B, CL
	Nitzschia	sp.	GU
	Navicula	sp.	GU
	Amphora	sp.	GU
Haptophyta	Pavlova	lutheri	B
	Isochrysis	galbana,add.galbana'Tahiti' (T-Iso)	B, W
Dinophyta (dinoflagellates)	Crypthecondium	chonii	RAD

Key: FFI formulated feed ingredient; B bivalve molluscs (larvae/postlarve/broodstock); C crustacean larvae (shrimps, lobster); R rotifer live prey, RAD rotifer and artemia live prey (dry product form); GU gastropod molluscs and sea urchins; GW'green water' for finfish (Source Shields and Lupatsch, 2012).

1.6.2. Chemical composition and nutritional value of microalgae

The knowledge about chemical composition of feed-species has a key role for mariculture (Thompson *et al*., 1996; Laing and Psimopoulos, 1998; Southgate *et al*., 1998; Leonardos and Lucas, 2000; Rivero-Rodríguez *et al*., 2007). The nutritional value of microalgae depends mainly on their chemical composition and cellular structure, which are influenced to a certain degree by culture conditions (Becker, 2004). Metabolic studies have been carried out to confirm microalgae as a novel source of protein. Furthermore, Becker (2004) also reported that the quality of most of the algae examined is equal or even superior to that of other conventional high-quality plant proteins. Many analysis of gross composition of different microalgae have been published in the literature. In order to give a general

overview on the major constituent, selected data of various microalgae strains are presented in Table 4.

Table 4: General composition of different algae (% of dry matter)

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

Source: Adapted from Becker (2007)

The application of microalgae as a future nutrient source for the aquafeed industry depends on detailed information about as chemical composition and nutrient digestibility (Skrede *et al*., 2011). The chemical content of microalgae varies with species, age and with changes in environmental conditions (Fernández-Reiriz *et al*., 1989) like temperature (Durmaz *et al*., 2009) and light intensity (Cheirsilp and Torpee, 2012), and culture conditions (Araújo and Garcia, 2005) such as photo bioreactors or open pond production (Banerjee *et al*., 2011; Huerlimann *et al*., 2010). A batch culture is in continuous chemical change because of the interaction with the medium. The chemical composition of a given species may vary widely under different growth conditions, and such changes may be related to the growth phase of the culture (Fernández-Reiriz *et al.,* 1989; Lourenço *et al*., 1997).

Microalgae species can vary significantly in their nutritional value, and nutritional value may also change under different culture conditions (Shield and Lupatsch, 2012). Future commercial viability of microalgae will depend on available quantity, quality (composition) and cost in relation to currently used commodity materials. Use of microalgae as raw materials will be based on evaluation of nutritive value, balance of amino acid, lipids and quality of fatty acids, absence of anti-nutritional factors, and digestibility of proteins, availability and cost. Other factors that influence the nutritional value of a microalgae, includes size, shape, digestibility (related to cell wall structure and composition), biochemical

composition (e.g., nutrients, enzymes, toxins if present) and the requirements of the animal feeding on the algae (Brown, 2002).

Microalgae have received particular interest in the aquaculture feeds due to their nutrient profile. Assuming that technology is developed to improve nutrient bioavailability from microalgae, it is likely to expect that microalgae lipids and proteins can have a great potential in aquafeed. According to Shields and Lupatsch (2012), the bioavailability of microalgae nutrients need to be explored in long term growth experiments in order to evaluate the potential of microalgae as feed ingredient.

Microalgae contain high contents of essential amino acids. The amino acid content of the microalgae examined was strikingly similar in composition, irrespective of algal class, which suggests that protein quality also was similar (Brown *et al*., 1997). Skrede *at al*. (2011) evaluated three microalgae of *N. Oceanica*, *P. tricornutum* and *I. galbana* in the diet of mink. The result showed that the three strains had an amino acid pattern similar to fish meal. Brown *et al*. (1997) analysed 40 species of microalgae from seven algae classes and reported that "all species had similar amino acid composition, and were rich in the essential amino acids".

Microalgae are in particular interesting for aquaculture feed, because some species have high contents of HUFA, in particular of EPA and DHA (Sijtsma and de Swaaf, 2004; Guschina and Harwood, 2006; Mendes *et al*., 2009). Unlike terrestrial crops, algae also can directly produce HUFA such as arachidonic acid (AA, 20:4n-6) (Porphyridium), eicosapentaenoic acid (EPA, 20:5n-3) (*Nannochloropsis*, *Phaeodactylum*, *Nitzschia*, *Isochrysis*, *Diacronema*) and docosahexaenoic acid (DHA, 22:6n-3) (*Crypthecodinium*, *Schizochytrium*). Lipids from microalgae can be used as high-valued DHA and HUFA ingredients in aquaculture feed. The total amount and relative proportion of lipid can be affected by nutritional and environmental factor, and also nutrient limitation. The content of EPA for most microalgae can be in the range 7-34% (Brown, 2002). Prymnesiophytes (eg. *Pavlova* spp. and *Isochrysis* sp. and cryptomonads are relatively rich in DHA (0.2 to 11%), whereas eustigmatophytes (*Nannochloropsis* spp.) and diatoms have the highest percentages of arachidonic acid (0 to 4%). Chlorophytes (*Dunaliella* spp. and *Chlorella* spp.) are deficient in both C20 and C22 PUFAs, although some species have small amounts of EPA (up to 3.2%). Because of this PUFA deficiency, chlorophytes generally have low nutritional value and are not suitable as a single species diet (Brown *et al*., 1997). Prasinophyte species contain significant proportions of C20 (*Tetraselmis* spp.) or C22 (*Micromonas* spp.) - but rarely both. Relatively few studies have been carried out to date to evaluate microalgae lipids in feeds for farmed fish (Atalah *et al*., 2007; Ganuza *et al*., 2008).

However, some studies have been carried out to evaluate nutrient digestibility in microalgae. A digestibility study was carried out with mink evaluating three microalgae species *Nannochloropsis oceanica*, *Phaeodatylum tricornutum* and *Isochrysis galbana* (Skrede *et al*., 2011). The protein digestibility determined by linear regression was 35.5%, 79.9% and 18.8% respectively. Among these three algae, *P. tricornutum* showed the highest potential as a protein ingredient. In another study with *Spirulina* fed to Atlantic salmon, apparent digestibility of protein was estimated to 84.7% (Burr *et al*., 2011). A combination of dried *Nannochloropsis* sp. and *Isochrysis* sp. were used to replace 0, 15, or 30% of dietary fish meal protein in feed for Atlantic cod (Walker and Berlinsky, 2011). The results from this growth experiment, showed no significant differences in survival, feed conversion ratios, viscerosomatic indices, and omega-3 and omega-6 fatty acids in the muscle among the treatment groups. However, a reduction in feed intake and growth was observed with increasing inclusion level of microalgae attributed to palatability problems.

Successful inclusion of microalgae in aquaculture feeds has been reported in feed for sturgeon. Palmegiano *et al*. (2005) investigated the use of *Spirulina* spp. as a nutrient source in diets for growing sturgeon (*Acipenser baeri*). Three isoproteic and isoenergetic diets were formulated with an increasing level of Indian strain *Spirulina* (SP 40%, SP 50% and SP 60%); the diets were tested against a control without microalgae. The results showed that *Spirulina* inclusion improved growth and that an inclusion level of 50% gave the greatest growth rate, a better favourable feed conversion rate and the highest protein efficiency. Kiron *et al*. (2012) examined two marine algal products (e.g., *Nanofrustulum* and *Tetraselmis*) for their suitability as fish meal protein substitutes in feeds for the three aquaculture species Atlantic salmon, Common carp and Pacific white shrimp. Growth performance and feed utilization of these species fed the algae meal did not differ from the group fed control, indicating that algal meal is an effective replacement of fish meal.

1.7. Evaluation of microalgae as feed ingredients

1.7.1. Principle of digestible study

Digestibility of a nutrient can be defined as the proportion absorbed from a feed (NRC, 2011). Modern aquaculture diets are routinely formulated based on the digestible nutrient and energy criteria (Cho and Kaushik, 1990). Knowledge about the digestibility of ingredients is a basic requirement for formulating diets. Ideally the nutrient requirements of fish and the nutrient concentration of a foodstuff should be expressed in units of availability so that least-cost formulations can optimize the balance between nutrient requirements and the cost of feeds. According to Shields and Lupatsch (2012), knowledge about nutrient digestibility should be evaluated for each species of fish.

Digestibility experiments with fish are usually carried out *in vivo* by adding an indigestibility marker to the feed at a known amount, collecting faecal matter, by suitable method and analysing the ration between nutrient and marker in the faecal matter (NRC, 2011). A number of different markers have been tested for use in digestibility studies. Chromic oxide (Cr_2O_3) is perhaps the most commonly used markers, while trivalent metal oxides such as La_2O_3 , Y_2O_3 and Yb_2O_3 , can substitute Cr_2O_3 in digestibility studies with salmonids, and be used at low concentration without affecting accuracy (Austreng *et al*., 2000).

Fish faeces can be collected in different ways (Belal, 2005); (a) collecting faeces deposited in the aquarium, (b) siphoning the faeces, (c) the Guelph system, (d) the mechanically rotating filter screen, (e) dissecting the fish gut, (f) manual stripping from abdominal cavity, (g) anal suctioning. The major challenges with methods a, b, c, and d is leaching of water soluble nutrients into the water. Leaching of nutrients, protein in particular, will result in over estimation of the digestibility. The other methods (e, f and g) were developed to overcome the problem of leaching by collecting faeces directly from the intestinal tract of fish. According to Bureau and Cho (1999) established techniques collect faeces from the lower part of the intestine, mainly by stripping, suction or intestinal dissection. Suction and intestinal dissection method, leads to an underestimation of the digestibility of nutrients (protein in particular). One explanation was that faeces collected with these methods are contaminated with endogenous material.

Fish faeces are composed of the undigested feed components and the endogenous residues of body origin (Bureau and Cho, 1999). These residues are the remains of mucosal cells, digestive enzymes, mucoproteins and other secretions released into the digestive tract by the fish. The faeces nitrogen derived from the fish itself is referred to as endogenous nitrogen gut losses (ENL). There is interest in quantifying the ENL in order to be able to calculate the “true” digestibility of protein and amino acids in feeds and ingredients. In order to estimate ENL the fish need to eat a protein free diet (Bureau and Cho, 1999). Fish does not eat feed devoid of protein; it is therefore difficult to calculate meaningful estimates of ENL. There is evidence that the amount of ENL produced by fish receiving a protein-free diet differs significantly from that of fish fed diets containing protein. Several other dietary constituents (fiber, antinutritional factors) can enhance ENL (Nyachoti *et al*., 1997). For these reasons, accuracy of “true” protein digestibility coefficients calculated using estimates of ENL obtained from fish fed protein-free diets is disputed. Further, Nyachoti *et al.* (1997) also reported that accurate estimation of ENL may require the use of more sophisticated techniques.

It should be noted that in fish maintaining a high feed intake, the contribution of ENL to total faeces nitrogen is probably small. Under these conditions, the difference between the "true" and apparent digestibility of protein is insignificant (Bureau and Cho, 1999). If feed intake is low, or poor growth is observed in a digestibility trial, faeces may contain a high proportion of ENL, and could produce unreliable estimates of apparent digestibility.

1.7.2. Principle of growth experiment

Nutrient digestibility does not reveal the full potential of an ingredient to support growth or retained nutrients in the flesh. Information from digestibility studies need to be combined with long term growth experiments.

In fish, feed intake determines the weight gain. Traditionally feed intake has been of less concern when the fishmeal inclusion was high because of good palatability of the ingredients. High voluntary feed intake in a condition for maximise in the growth potential (Espe *et al*., 2012). However, replacing fishmeal with plant protein ingredients, the acceptability of the feed may be compromised resulting in reduced growth performance (Kaushik *et al*., 1995; Fournier *et al*., 2004; Glencross *et al*., 2004; Dias *et al*., 2005). Consequently, one of the greatest challenges in the formulation of high plant protein diets for farmed Atlantic salmon has been to secure that the fish accept the feed offered to them equally well as the fishmeal based diets. Palatability of an ingredient is important serve good feed intake.

For most of the nutrient utilization studies, growth is used as the response variable (Glencross *et al*., 2007). Growth can simply be defined as the difference between initial and final live weight. Technically, three most routinely used growth rate assessment are daily gain (DG), daily growth coefficient (DGC) and specific growth rate (SGR). Further, growth rate parameter gaining use is the thermal growth coefficient (TGC). DG is merely the live weight gain and given in units of gr day^{-1}. DGC is calculated based on percentage of the one-third root transformation of final (w_f) and initial body weight (w_i) live over time (t). Whereas, SGR is calculated based on the percentage of the natural logarithm transformation of final body weight (w_f) and initial (w_i) live weight over time. The point using growth rate is essential to try and standardize the assessment of performance across experiments. Kaushik (1998) were using DGR and SGR for growth assessment of non-salmonids species, the results shown that SGR did not revealed a shown transformation of growth compared to DGR. In that study, DGR provided more uniform rate across the entire fish live weight.

1.8. The objective of study

The aim of the current study was to evaluate the potential of alternative microalgae in feed for Atlantic salmon. Three microalgae were tested in digestibility studies, *Nanofrustulum* (C3), *Desmodesmus* (C4) and *Nannochloropsis* (C1).

2. Materials and Methods

Two experiments were designed to investigate digestibility of dry matter, protein and ash, for three different microalgae strains in diets for Atlantic salmon. First experiment referred to as pre-study, and second experiment is called the main experiment.

2.1. Pre-study

2.1.1. Experimental design, test ingredients and diets

The microalgae employed in the study were provided by the Algae Consortium funded by the US Department of Energy and produced at the pilot facilities of Cellana in Hawaii.

Digestibility of three microalgae were determined in a pre-study using experimental diets produced locally employing cold pelleting technology at the research station Mørkvedbukta, University of Nordland. Digestibility of dry matter (DM), protein and ash of the three microalgae strains *Nannochloropsis* (C1), *Nanofrustulum* (C3) and *Desmodesmus* (C4) the results were determined in Atlantic salmon (*Salmo salar* L.). The three microalgae containing feeds were tested against a control based on fish meal. Four experimental diets were formulated for the pre-study. A reference diet devoid of algae was diluted with 30% biomass (test ingredient). Ingredient composition of reference diet and test diet are shown in Table 5. Yttrium was used as an inert marker and was incorporated at the same inclusion level for the reference and test diets. The chemical composition of the microalgae is presented Table 6.

Table 5: The ingredient composition of the experimental diets (%)

Ingredients	Reference diet	Test diet		
	P-Control	P-C3	P-C4	P-C1
Fish meal[1]	75.3	52.7	52.7	52.7
Fish oil[2]	12	8.4	8.4	8.4
Mineral[3] and vitamin[4] premix	0.7	0.5	0.5	0.5
Potato starch[5]	12	8.4	8.4	8.4
Microalgae C3	0	30	0	0
Microalgae C4	0	0	30	0
Microalgae C1	0	0	0	30
Yttrium oxide (Y_2O_3)[6]	0.01	0.01	0.01	0.01
Total	100	100	100	100

[1]Bodø Sildoljefabrikk AS, Bodø, Norway
[2]Bodø Sildoljefabrikk AS, Bodø, Norway
[3]Mineral mix (gr kg^{-1}): $MgSO_4$ – 2.477, KH_2PO_4 – 1.008, ZnSO4 – 0.220, $FeSO_4$ – 0.249, $MnSO_4$ – 0.031, $CuSO_4$ – 0.013, $CoCl_2$ – 0.002, Na_2SeO_4 – 0.0012 (Kiron *et al*., 2012)
[4]Proprietary formulation of Skretting Aquaculture Research Center, Stavanger, Norway.
[5]Swely gel 700, Lyckeby Culiner, AB, Filkinge, Sweden
[6]Metal Rare Earth Limited, Shenzhen, China

Table 6: Chemical composition of the ingredients for pre-study (%)

Ingredients	Dry matter	Protein	Ash
Microalgae C3	92.9	17.0	48.8
Microalgae C1	97.8	42.9	23.3
Microalgae C4	88.6	26.9	16.0

The reference diet contained 54.2% of crude protein, and crude fat 20.3% with an estimated gross energy 22.7 MJ/kg. Test diet contained crude protein were 43.2%, 46.7% and 50.6% in microalgae C3, C4 and C1 respectively. Crude fat in test diet ranged from 15.3% (microalgae C4) to 20.3% (fish meal). Chemical composition of all four experimental diets is presented in Table 7.

Table 7: Chemical composition of the experimental diets used in the pre-study (%)

	P-Control	P-C3	P-C1	P-C4
Dry Matter (%)	96.3	95.9	96.6	96.2
Crude Protein (%)	54.2	43.2	50.6	46.7
Crude Fat (%)	20.3	15.7	16.4	15.3
Ash (%)	11.2	22.1	14.6	13.0
Gross energy (MJ/Kg)	22.7	19.0	21.6	21.0

Experimental feeds used in pre-study were produced at UiN feed laboratory according to standard procedure well established in the lab. The different steps are explained.

a. Mixing of dry "macro ingredients"

All macro ingredients i.e., fishmeal, potato starch and test algae were mixed thoroughly. The mixers run for 3 minutes, at low speed to keep the dust low. During the 3 minutes mixing cycle, the mixers were stopped 3 times and the content in the bowl were turned with the hands, to ensure good mixing also in the dead zones of the mixer.

b. Preparing the premix of vitamins and minerals

To ensure a homogenous mix of micro elements, a premix was made of vitamins, minerals and marker. Vitamin, mineral premix + yttrium oxide (marker) were weighed in two separate containers. The micro ingredient was premixed with approximately 3% of dry macro ingredient (fish meal, fish oil and potato starch). The capacity of the mixer allowed batches of 5 kg per mixing cycle. Approximately 3% (150 gr) of the mixed macro ingredients was split in three containers, to allow dilution of the micro ingredients in three steps. The micro ingredients were first diluted in 50 gr by mixing thoroughly with a spatula; this initial macro-micromix was then mixed thoroughly to the next 50 gr of the macromix and finally the current macromix to the leftover macromix.

c. Mixing macro ingredients and premix

The vitamin and mineral premix was mixed with the rest of the macro ingredient, batch in the mixer. The mixer was run for 5 times; five minutes each run (repeat 5 times). Each 5 minutes, the mixer was stopped completely and hands were used to turn the content in the bowl, so that dry ingredients at the bottom got to the top before the mixer was started again. This procedure was repeated in total five times.

d. Final mixing step with oil and water

When the dry ingredients were mixed thoroughly, water and oil was added in the proportion 2.5 kg water (50% water) to 5 kg of dry ingredient in bowl. The mixer was run for 60 seconds before oil was added and finally the mixer was run for 60-90 seconds. The procedure was slightly modified for the microalgae feed. Feed with algae required 3 kg of water (60%). Water was added in two cycles with oil in between. 1.5 kg water was added in each cycle and the mixer run 1.5 minutes before oil was added. The mixture was forced through the dies of a meat mincer (Sirman TC 22), 6-7 mm die openings and shaped (pelletizing). The pelletized product came out as long spaghetti threads. They were collected and dried in a dryer for 24 hours (60^0C). The next day, feed were crushed and packed in plastic bags (200 gr each) and stored in cooling room (2^0C).

2.1.2. Fish and experimental conditions

Atlantic salmon (*Salmo salar* L.) was used in the pre-study, with an average weight approximately 1600 gr. The fish were distributed and acclimated for 2 weeks prior to start the experiment. The fish was stocked in indoor 1100 L tanks (1 X 0.925 X 1.295 m^3) with 20 fish per tank. In the pre-study each diet was fed to six replicate tanks (18 tanks), except for diet C4 that were carried out in triplicate due to limiting number of tanks available.

The experimental tanks were kept indoor in a constant environment and under continuous light. The tanks were equipped with a flow-through system supplying sea water at a rate of 0.5 Litter sec^{-1}. The water was taken from a depth of 250 meter in Saltfjorden. The tanks were flushed on the alternate days to remove the uneaten feed and faecal matter. 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). The pre-study was carried out during the course of May-July 2014. The water quality parameters temperature and oxygen among tanks with an average at 8.2^0C in the day time, and oxygen saturation was average 80-92% among tanks.

2.1.3. Feeding and sampling

For the pre-study, fish in each tank were fed 200 gr of feed each day. Feeding started 2 o'clock pm. The daily ration was approximately 1% of their biomass when experiment was started. The feed were dispensed using programmed automatic feeders, hanging above the tank. The fish were fed experimental diets for 3 weeks prior to stripping of the faeces.

Before stripping fish from each tank was collected using a scoop net. The fish were transferred to a 50 litters holding tank and anaesthetized with tricaine methanesulfonate (MS-222) at concentration 40 mg/litter. Fish was taken out of the water and faeces were collected from anesthetised fish by applying gentle pressure to the abdomen of the fish, approximately over the distal intestine, to expel its faecal contents according to a procedure described by Austreng *et al*. (1978). Faeces from each tank treatment including the control were collected in a tube and weighed, labelled and stored in freezer (-40^{0}C) for chemical analysis. In order to get enough faeces for dry matter, protein and ash analysis, faeces from two and two tanks were pooled. As a consequence numbers of replicates were reduced from 6 to 3. After faeces collection, fish were sacrificed by a blow to the head.

Nutrient digestibility was measured on faeces collected from experimental unit. Faeces from all fish in one tank (experimental unit) were pooled. At the end of collection period, pooled faeces from each tank were freeze dried prior to chemical analysis.

2.1.4. Chemical analysis

All chemical analysis in the pre-study was carried out by Eurofins, a laboratory accredited by the Norwegian National Accreditation body.

a. Dry matter

Dry matter of feed was determined by drying samples in an electric furnace maintained at 105°C for 20 hours. The method used based on EN14918/15400/ISO1928.

b. Protein

The faeces sample were convection oven at 55^{0}C for 24 hours before storage ground with mortar and pestle and kept at room temperature 4^{0}C for subsequent analysis. Feed samples (3 strains algae diets and the control feed) were finely ground in a hammer mill using a 1 mm screen. Crude protein of feed and faeces were measured with Kjeldahl method (NS/EN ISO/IEC 17025:2005) and multiplying N by 6.25 (Total N X 6.25).

c. Ash

Ash (minerals content) is obtained burning the feed or ingredient sample in a muffle furnace for 16 hours at 540^0C. At this temperature all organic matter in the sample is burned, leaving behind ash or an inorganic mineral salt (NS/EN ISO/IEC 17025:2005).

d. Yttrium

For analysis of marker in diets and faeces, freeze-dried samples of 150-200 mg were combusted at 550°C overnight in glass scintillation vials. When cooled, 5 ml of HCl:HNO_3, 2:l (v/v) was added and the samples were boiled until colourless. When cooled, a few drops of water were added; the sample was dissolved in 1.25 ml HNO (concentrated) and diluted to 25 ml with distilled water. The concentration was measured using an ICAP-AES spectrometer (Model 1100, Thermo Jarrell/Ash, Franklin, MA, USA) at the Eurofins Environment Testing Lab, Norway /Moss.

2.1.5. Calculations and Statistical analysis

a. Apparent digestibility coefficient calculations

The apparent digestibility Coefficient (ADC) (%) is an important parameter for evaluation of the nutritional quality of a feed ingredient. The ADC can be used to disclose the potential for an ingredient or diet to be utilized by an animal. It is expressed as a percentage of the quantity of food ingested which is not excreted as faeces.

ADC of the dietary nutrients and energy were calculated as proposed by Glencross *et al*. (2007) as follows:

$$ADC_{diet} = 1 - \left(\frac{Marker_{diet} \text{ X } Nutrient_{feces}}{Marker_{faeces} \text{ X } Nutrient_{diet}}\right) \quad (1)$$

In this equation (1), $Marker_{diet}$ and $Marker_{faeces}$ represent marker content in the diet and in the faeces, respectively. Value range typically from 0 to 1, and to achieve the percentage of ADC of the diet, the equation should be multiply by 100.

For measuring the apparent digestibility coefficients (ADC) of the three microalgaes, a reference diet was used. The control diet was diluted with 30% test ingredient (microalgae). Nutrient digestibility of the test ingredient was calculated using equation 2.

The following equation, which has been arranged for the calculation of the ADC test ingredients:

$$Nutr.AD_{ingredient} = \left(ADN_{test} - \frac{ADN_{basal} \text{ X } 70\%}{30\%}\right) \quad (2)$$

The $Nutr.AD_{ingredient}$ is the apparent digestibility of a given nutrient or energy from the test ingredient which is included at 30% in the test diet. ADN_{test} is the apparent digestibility of the nutrient of interest in the test diets, while ADN_{basal} is apparent digestibility of the same nutrient from the same basal diet, which makes up 70% of the test diet.

A progression of the equation (2) was reported by Sugiura *et al*. (1998). They used the following equation to calculate $Nutr.AD_{ingredient}$ of a given nutrients from the test ingredient included in the test diet at 30%:

$$Nutr.AD_{ingredient} = \frac{[AD_{test} \times Nutr_{test} - (AD_{basal} \times Nutr_{basal} \times 70\%)]}{(30\% \times Nutr_{ingredient})} \quad (3)$$

The AD_{test} and AD_{basal} is the apparent digestibility of the test diet and the basal diet calculated in equation (2). The $Nutr_{igredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the levels of the nutrient of interest in the ingredient, test and basal diet, respectively.

Forster (1999) claimed that the equation (3) did not, account for the relative contribution of the nutrient from the reference diet and the test ingredient to the combined diet, and is inappropriate for use in estimating nutrient digestibility. Therefore, he suggested the following equation should be used, that take into calculation for the relative nutrient contribution of the reference diet and the test ingredient to the combined diet.

$$Nutr.AD_{ingredient} = \frac{[(70\% \times Nutr_{basal} + Nutr_{ingredient} \times 30\% \times AD_{test} - (70\% \times Nutr_{basal} \times AD_{basal})]}{Nutr_{ingredient} \times 30\%} \quad (4)$$

In this equation (4), $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the levels of the nutrient of interest in the ingredient, test diet and basal diet respectively (Forster, 1999).

According to Glencross *et al*. (2007) equations 3 and 4, are the more appropriate ones for determining ingredient digestibility, because they account for the relative contributions of the test ingredient and reference diet to energy or nutrient digestibility being investigated.

b. Statistical analysis

The nutrient digestibility data was subjected to a one-way analysis of variance. Statistical significance was chosen at a 0.05 probability level and the results are presented as means ± SD (standard deviation of the mean). The means were compared by the Duncan's multiple range tests. All statistical data analyses were carried out using IBM SPPS Statistics 19.0 (IBM SPSS Statistics Inc., Chicago, IL)

2.2. Main experiment

Experiment was run as of February 24 to April 11, 2015. The aim of the second experiment was to:

a. To verify results from the pre-study for two of the microalgae C1 and C4
b. To investigate ADC's of DM, protein and ash from whole diet at 10% and 20% inclusion level of C4, using diets made with cooking extrusion process

2.2.1. Experimental design, test ingredients and diets

Two strains of microalgae C1 and C4 were tested against a fish meal to determine the dry matter, protein, and ash. Different diets were produced for the experiments in the verification study and inclusion level parts, respectively. For verification study, reference diet was diluted with the test diet in the ratio 70:30, as earlier described for the pre-study. Ingredient composition is shown in Table 8. For inclusion level study, C4 was added 10% and 20% inclusion level, respectively. Ingredient composition is shown in Table 9. The design for verification study used triplicate tanks (9 tanks) for microalgae *Nannochloropsis* (C1) and *Desmodesmus* (C4), as well as inclusion level 10% and 20% to test again fish meal. The chemical composition of the ingredients for verification study and inclusion level study is presented Table 10.

Table 8: The ingredient composition of the experimental diets for verification study (%)

Ingredients	Reference diet	Test diet	
	V-Control	V-C1	V-C4
Fish meal	70.3	49.2	49.2
Fish oil	12	8.4	8.4
Wheat	12	8.4	8.4
Wheat gluten	5	3.5	3.5
Mineral and vitamin premix[1]	0.7	0.5	0.5
Microalgae C4	0	30	0
Microalgae C1	0	0	30
Yttrium oxide (Y_2O_3)[2]	0.01	0.01	0.01
Total	100	100	100

[1] Proprietary formulation of Polar Feeds, Norway

[2] Metal Rare Earth Limited, Shenzhen, China

The feed ingredients were sourced from different suppliers by Fortek

Table 9: The ingredient composition of the experimental diets for inclusion study (%)

Ingredients	I-Control	I-C4 L	I-C4 H
Fish meal	69.0	60.0	51.0
Fish oil	13.5	12.5	11.5
Wheat	12.0	12.0	12.0
Wheat gluten	5.0	5.0	5.0
Mineral and vitamin premix[1]	5.0	5.0	5.0
Yttrium oxide (Y_2O_3)[2]	0.02	0.02	0.02
Microalgae C4	0	10	20

[1]Proprietary formulation of Polar Feeds, Norway

[2]Metal Rare Earth Limited, Shenzhen, China

The feed ingredients were sourced from different suppliers by Fortek

Table 10: Chemical composition of the microalgae used in verification study and inclusion level study (%)

Ingredients	Dry matter	Protein	Ash
Microalgae C1	97.8	42.1	23.0
Microalgae C4	88.6	26.9	16.1

The chemical composition of the experimental diets for verification (30%) replacement and 10% and 20% inclusion level study is presented in Table 11 and 12.

Table 11: Chemical composition of the experimental diets for verification study (%)

	V-Control	V-C1	V-C4
Dry Matter (%)	95.7	92.7	94.0
Crude Protein (%)	52.1	47.8	44.5
Crude Fat (%)	19.0	17.2	15.0
Ash (%)	10.8	14	12.7
Gross energy (MJ/Kg)	22.2	20.4	20.4

Table 12: Chemical composition of the experimental diets for inclusion level study (%)

	I-Control	I-C4 L	I-C4 H
Dry Matter (%)	96.5	92.4	92.9
Crude Protein (%)	51.9	46.6	44.4
Crude Fat (%)	19.4	18.3	18.1
Ash (%)	10.7	10.6	11.3
Gross energy (MJ/Kg)	22.4	21.4	21.1

2.2.2. Fish and experimental conditions

For the verification study, fish had an average weight at 435.60 ± 16.6 gr (mean ± SD) and were stocked 50 fish per tank in indoor 1100 L tanks. The inclusion level study had a fish with an average weight of 533.65 ± 21.4 gr (mean ± SD), and 34 fish were stocked in each tank. Atlantic salmon used in the main experiment was acclimated for 4 days prior to start the experiment. The verification study was started on winter time (February-April 2015).

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. The water temperature was stable 5-6^{0}C in the day time, and oxygen saturation was 83-85%.

The experimental tanks were kept indoor in a constant environment and under continuous light. The tanks were equipped with a flow-through system supplying sea water at a rate of 0.5 Litter sec^{-1}. The water was taken from a depth of 250 meter in Saltfjorden. The tanks were flushed on the alternate days to remove the uneaten feed and faecal matter.

2.2.3. Feeding and sampling

The main experiment was carried out using triplicate tanks for the dietary treatments. Feeds were dispensed using programmed automatic feeders, hanging above the tank. The fish were fed experimental diets for 11 days prior to stripping of the faeces. The daily ration was approximately 1% of the biomass throughout the experimental period.

Faecal collection was carried out at day 11 of the experiment. Before stripping fish from each experimental unit were collected using a scoop net. The fish were transferred to a 50 litters holding tank and anaesthetized with tricaine methanesulfonate (MS-222) at concentration 40 mg/litter. Fish was taken out of the water and faeces were collected from anesthetised fish by applying gentle pressure to the abdomen of the fish, approximately over the distal intestine, to expel its faecal contents according to a procedure described by Austreng *et al*. (1978). Faeces from each tank treatment including the control were collected in a tube and weighed, labelled and stored in freezer (-40^{0}C) for chemical analysis. In order to get enough faeces for digestible determination faeces from the tanks of the same treatment were pooled prior to chemical analysis. After faeces collection, fish were sacrificed by a blow to the head.

2.2.4. Chemical analysis

In the verification study feed and faeces dry matter, protein and ash analysed by UiN lab., except for yttrium analysed by Eurofins. The procedure used for the chemical analysis was the same as earlier described for the pre-study**.**

2.2.5. Calculation and statistical analysis

Calculation of apparent digestibility coefficient (ADC) and same as the pre-study**.**

3. Results

3.1. Results of pre-study

The chemical composition varied slightly among the experimental diets in the pre-study (Table 7) because chemical composition of the microalgae varied (Table 6). Feed pellets had a non-homogeneous size because the feeds produced at campus were crumbled without any sifting.

The pre-study went well and was carried out without any mortality during the experiment. The apparent digestibility coefficients (ADC's) of dry matter, protein and ash for the reference and test diets calculated according to equation 1 and are presented in (Table 13). Significantly differences among diets were noted for ADC of DM, protein and ash. The ADC's of DM in control diet (P-CO) and diet P-C3 were significantly higher than diet P-C1 and diet P-C4. The digestibility of protein was significantly highest in diet P-C3, followed by control diet (P-CO), diet P-C1 and diet P-C4. The digestibility of ash for diet P-C3 was also significantly higher than diet P-C1 and diet P-C4, while lowest digestibility for ash was found in Control diet (P-CO).

Table 13: Calculation of ADC's diet of dry matter, protein and ash (mean ± SD) for the pre-study based on equation 1

	P-Control	P-C3	P-C1	P-C4
Dry matter	76.12 ± 0.70^{a}	75.15 ± 4.81^{a}	66.91 ± 1.84^{b}	62.87 ± 0.44^{b}
Protein	87.96 ± 0.52^{b}	90.25 ± 2.01^{a}	84.48 ± 0.59^{c}	81.93 ± 1.02^{d}
Ash	15.43 ± 2.88^{c}	44.81 ± 10.09^{a}	25.50 ± 5.69^{b}	26.15 ± 5.75^{b}

^{a,b,c,d} different superscript among rows denote significant differences at <0.05

Data calculated using equation 1, were used in equation 2 for calculation of ADC's for dry matter, protein and ash of the test feed ingredients. The results are presented in Table 14. The apparent digestibility for dry matter, protein and ash of microalgae *Nanofrustulum* (C3) were significantly higher than microalgae *Nannochloropsis* (C1) and *Desmodesmus* (C4).

Table 14: Apparent digestibility coefficients (%) of dry matter, protein and ash of the test ingredients calculated with equation 2 in pre-study

	C3	C1	C4
Dry matter	72.87 ± 16.02^{a}	45.40 ± 6.14^{b}	31.95 ± 1.47^{b}
Protein	95.59 ± 6.69^{a}	76.38 ± 1.96^{b}	67.85 ± 3.40^{b}
Ash	113.36 ± 33.65^{a}	48.99 ± 18.97^{b}	51.16 ± 19.17^{b}

^{a,b} different superscript among rows denote significant differences ($P < 0.05$)

The ADC's of ingredients calculated using 3 are presented in Table 15. There were significantly higher digestibility of dry matter and protein of *Nanofrustulum* (C3) compared to *Nannochloropsis* (C1) and *Desmodesmus* (C4). However, ash showed no significant differences among microalgae *Nanofrustulum*, *Nannochloropsis* and *Desmodesmus.*

Table 15: Apparent digestibility coefficients (%) of dry matter, protein and ash of the test ingredients calculated with use of equation 3 in pre-study

	C3	C1	C4
Dry matter	74.45 ± 16.53[a]	45.38 ± 6.07[b]	34.48 ± 1.60[b]
Protein	110.17 ± 17.01[a]	72.83 ± 2.31[b]	60.51 ± 4.55[b]
Ash	59.33 ± 15.23[a]	35.93 ± 11. 88[a]	45.48 ± 15.53[a]

[a,b] different superscript among rows denote significant differences ($P < 0.05$)

Table 16 showed the results of Nutrient apparent digestibility for the test ingredients calculated with use of equation 4. There was significantly higher digestibility for dry matter, protein and ash of microalgae *Nanofrustulum* than *Nannochloropsis* and *Desmodesmus*. However, no differences were noted between *Nannochloropsis* and *Desmodesmus*.

Table 16: Apparent digestibility coefficients (%) of dry matter, protein and ash of the test ingredients in pre-study calculated with use of equation 4

	C3	C1	C4
Dry matter	72.78 ± 16.43[a]	45.74 ± 6.08[b]	29.28 ± 1.56[b]
Protein	107.28 ± 16.95[a]	74.25 ± 2.32[b]	53.60 ± 5.82[b]
Ash	60.53 ± 15.50[a]	36.78 ± 12. 07[b]	43.60 ± 15.12[b]

[a,b] different superscript among rows denote significant differences ($P < 0.05$)

3.2. Results of main experiment

3.2.1. Verification study

The whole diet ADC's of dry matter, protein and ash were calculated according to equation 1. There were significant differences in digestibility among the diets (Table 17). The ADC of dry matter in control diet (V-CO) and *Nanofrustulum* diet (V-C1) were significantly higher than *Desmodesmus* diet (V-C4). The ADC of protein in control diet (V-CO) was significantly higher than *Nanofrustulum* diet (V-C1) and *Desmodesmus* diet (V-C4). The ADC of ash in *Nanofrustulum* (V-C1) was significantly higher than *Desmodesmus* diet (V-C4) and control diet (V-Control), and *Desmodesmus diet* (V-C4) significantly higher than control diet (V-CO) ($P<0.05$).

Table 17: Calculation of ADC's diet of dry matter, protein and ash (mean ± SD) calculated with equation 1 for the main study

	V- Control	V-C1	V-C4
Dry matter	69.18 ± 1.02[a]	67.33 ± 0.46[a]	62.84 ± 2.69[b]
Protein	85.58 ± 0.20[a]	82.24 ± 0.19[b]	82.01 ± 0.61[b]
Ash	7.87 ± 2.21[c]	41.87 ± 1.25[a]	34.57 ± 4.67[b]

[a,b,c] different superscript among rows denote significant differences at P <0.05

Using the data from equation 1, the ADC's of dry matter, protein and ash for test ingredients were calculated with use of equation 2. Apparent digestibility coefficients of dry matter, protein and ash of the test ingredients for Atlantic salmon are presented in Table 18. The ADC's of dry matter in *Nannochloropsis* (C1) was significantly higher than *Desmodesmus* (C4). No significantly differences in ADC's of protein and ash were found between *Nannochloropsis* and *Desmodesmus*.

Table 18: Apparent digestibility coefficients (%) of dry matter, protein and ash of the test ingredients in verification study calculated with use of equation 2

	C1	C4
Dry matter	63.02 ± 1.52[a]	48.04 ± 8.98[b]
Protein	74.43 ± 0.65[a]	73.67 ± 2.03[a]
Ash	121.2 ± 4.16[a]	96.87 ± 15.58[a]

[a,b] different superscript among rows denote significant differences ($P < 0.05$)

The results of apparent nutrient digestibility calculated based on equation 3 for test ingredient are presented in Table 19. No significant differences were observed in the ADC's of dry matter, protein and ash for microalgae *Nannochloropsis* (C1) and *Desmodesmus (C4)* (P>0.05).

Table 19: Apparent digestibility coefficients (%) of dry matter, protein and ash for the test ingredients calculated using equation 3 in the verification study

	C1	C4
Dry matter	54.83 ± 1.44	48.03 ± 9.52
Protein	64.07 ± 0.74	65.83 ± 3.35
Ash	76.21 ± 2.53	79.08 ± 12.36

The ADC's of the test ingredient of the test ingredient calculated with use of equation 4 is presented in Table 20. Significant differences were observed between the two microalgae

diets. The ADC's of dry matter and protein of *Nannochloropsis* (C1) was significantly higher than *Desmodesmus* (C4). Digestibility of ash were not different between the two microalgae *Nannochloropsis* and *Desmodesmus* (P>0.05).

Table 20: Apparent digestibility coefficients (%) of dry matter, protein and ash of the test ingredients in verification study calculated with use of equation 4

	C1	C4
Dry matter	63.12 ± 1.49[a]	46.87 ± 9.47[b]
Protein	72.58 ± 0.76[a]	65.88 ± 3.35[b]
Ash	79.07 ± 2.61[a]	76.44 ± 12.00[a]

[a,b] different superscript among rows denote significant differences (*P < 0.05*)

3.2.2. Inclusion level study

Mortality was observed in one tank fed I-C4 H feed. In total 8 fish died or were put to death because of cataract or generally poor performance. Apparent digestibility coefficients of dry matter, protein and ash of the test diets for Atlantic salmon are presented in Table 21. There were no significant differences in the ADC's for dry matter and protein among the diets. The ADC for ash was significantly higher in the diet I-C4 H, followed by diet I-C4 L and lowest in I-Control diet.

Table 21: Apparent digestibility coefficients (%) of dry matter, protein and ash of the test diets calculated with use of equation 1 in inclusion level study

	I-Control	I-C4 L	I-C4 H
Dry matter	64.95 ± 2.04[a]	62.54 ± 1.34[a]	64.64 ± 1.36[a]
Protein	84.04 ± 1.02[a]	82.91 ± 0.83[a]	83.26 ± 0.70[a]
Ash	-10.87 ± 3.11[c]	1.97 ± 3.72[b]	15.49 ± 3.44[a]

[a,b,c] different superscript among rows denote significant differences (*P < 0.05*)

4. Discussions

4.1. Feed production: cold pelleted versus extruded pellets

Feed productions for pre-study and main experiment employed two different production technologies. For the pre-study a cold pelleting technique was used. After mixing of all ingredients, the dough was shaped in spaghetti like long strings. Before freezing of the feed, strings were broken into pellet like structures. The shape and size of pellets were irregular compared to the feeds used in the main experiment. Extruded pellets were used for the main experiment. These pellets had more regular shape compared to those used in the pre-study. There may also have been differences in water stability, hardness and durability between the pellets produced by cold pelleting and extrusion. Early studies have shown that pellet quality may have an impact on feed intake and utilization of the diet (Glencross *et al*., 2011; Baeverfjord *et al*., 2006; Oehme, 2013).

4.2. The ADC's Pre-study and Main experiment

4.2.1. Pre-study

Currently, only a few digestibility trials have been carried out with use of microalgae in fish feeds (Pereira *et al.,* 2012; Sarker *et al.,* 2015). The present study was therefore carried out to determine apparent digestibility coefficients of dry matter, protein and ash in three algal products (*Nanofrustulum* sp., *Nannochloropsis* sp. and *Desmodesmus* sp.), to evaluate the potential of their use in Atlantic salmon feed.

In general, the dry matter (DM) digestibility varied among the microalgae tested, with significantly higher value for *Nanofrustulum* C3 (73-74%), compared to *Nannochloropsis* C1 (45-46%) and *Desmodesmus* C4 (29-35%). The ADC of C3 is within the range of values reported for ADC's of DM for other feed ingredients such as soybean meal (71.2-74.9%) in salmonids fed (Sugiura *et al.,* 1998). Burr *et al.* (2011) reported an ADC of 70.8% for canola protein concentrate and 69.6% for soybean meal fed to Atlantic salmon. These values are also comparable to ADC of DM in rapeseed meal fed to Rainbow trout (70.8%; Burel *et al.,* 2000). The DM ADC of C3 was also in the same range as earlier reported for meat and bone meals fed to Rainbow trout. Bureau *et al.* (1999) reported ADC of DM between 61% and 76% for different meat and bone meals fed to Rainbow trout. Cheng and Hardy (2003) reported a DM ADC between 67.6% and 75.2% for brewer's dried yeast fed to Rainbow trout. The ADC's of DM observed for C1 and C4 were lower than reported values for typical protein ingredients tested in salmonids. These values were more in line with DM ADC values for carbohydrate rich ingredients. The DM ADC's of wheat middling and wheat flour fed to Coho salmon were calculated to be 38.3% and 37.5%, respectively (Sugiura *et al.,* 1998). In comparison to other studies with microalgae fed to fish, DM ADC's of the three microalgaes

were lower than *Spirulina* fed to Atlantic salmon. Burr *et al.* (2011) reported DM ADC's for *Spirulina* algae when fed to Arctic charr and Atlantic salmon of 77.9% and 82.1%, respectively. Higher DM ADC's were also reported for Nile tilapia fed *Spirulina* sp. (79.7%), *Chlorella* sp. (73.4%) and S*chizochytrium* sp. (81.8%; Sarker *et al.,* 2015).

The protein digestibility for C3 was in line with plant ingredients containing relatively high protein content, such as corn gluten meal (94.2%), barley protein concentrate (96.3%), soybean meal (93.7%) and lupin protein concentrate (108.7%) fed to Atlantic salmon (Glencross *et al.,* 2004; Burr *et al.,* 2011). It is also similar to herring meal (94.7%), wheat gluten (99.6%) and poultry-BP meal (94.2%) when fed to Coho salmon (Sugiura *et al.,* 1998). High protein digestibility is also reported for anchovy fish meal (97%), spray-dried porcine plasma (99.7%) and soy protein isolate (97.8%) fed to Rainbow trout (Cheng and Hardy*,* 2003; Glencross *et al.,* 2004; Gaylord and Barrows*,* 2008). Similar results were obtained from pea protein concentration fed to Atlantic salmon, showed increased from 90 to 97% (Carter *et al*., 1999).

A positive relationship is often reported between protein content and protein digestibility (e.g., Glencross *et al.,* 2010). However, the protein content of C3 was low compared to C1 and C4, only 17%. At 30% inclusion, constituted with 5.1% of protein to the diet. The high protein digestibility noted for C3 can be explained by the fish meal dominated protein content. High ADC of protein was reported by Carter *et al*. (1999) for wheat flour, wheat gluten and soybean meal when the partial inclusion of protein from these ingredients were 1.8%, 11.5% and 6.9%, respectively. The protein digestibility for wheat flour ranged from 89.99-94.88%, for wheat gluten it was ranged from 96.33-100%, while soybean meal ranged from 92.79-100%.

ADC of protein for C1 and C4 was lower than other protein ingredients currently used to replace fish meal in diets for Atlantic salmon, but in line with protein digestibility of some reported seaweeds (65.5%-79.5%) for Rainbow trout (Pereira *et al.,* 2012), and diatom (75.2%) fed to *Holothuria scabra* jaeger (Orozco *et al.,* 2014). Burr *et al.* (2011) reported a high ADC protein (88.2% and 84.7%) for *Spirulina* algae when fed to Arctic charr and Atlantic salmon, respectively. Sarker *et al.* (2015) also showed a high value of ADC protein for *Spirulina* sp. (86.1%), *Chlorella* sp. (80.0%) and *Schizochytrium* sp. (81.7%) fed to Nile tilapia. The low capacity of the carnivorous salmon to digest microalgae protein may be due to limitation in digesting cell wall components compared to herbivorous fish, such as tilapia. Nile tilapia has a gastrointestinal tract adapted to digest plant ingredients. For example it has a pH close to 1 in the stomach for efficient digestion of nutrients from the cell wall (Ekpo and

Bender, 1989). Salmonid has a short gastrointestinal tract equipped with digestive enzymes directed towards digestion of proteins rather than carbohydrates.
Values for ADC's of ash for C3, C1 and C4, 59-113%, 36-49% and 44-51%, respectively, are in line with ash digestibility reported for Nile tilapia fed *Spirulina* sp. (68.5%) and *Chlorella* sp. (56.6%; Sarker *et al.,* 2015).

The significantly lower in ADC's of dry matter, protein and ash for C1 and C4 compared to those of C3, could be due to different construction of cell walls among the three microalgae. Cell walls are reported to reduce astaxanthin availability in red yeast (*Phaffia rhodozyma;* Storebakken *et al.,* 2004) as well as nutrient digestibility in bacterial meal (Aas *et al*., 2006). In terms of algae, C3 belongs to diatom, while C1 and C4 belong to green algae. Green algae such as *Nannochloropsis* have a rigid and thick cell wall with *N-acetylglucosamine* containing polymer in the cell walls, while diatoms consist of glucose that is more easily utilized (Brown *et al*., 1997; Becker, 2007; Gerken *et al.,* 2012; Marshall *et al.,* 2010). The carnivore Atlantic salmon may is most likely not capable to digest high amount of polysaccharides (Krogdahl *et al*., 2003; Torstensen *et al*., 2008). The complex structure and high contents of non-starch polysaccharides in C1 and C4 can thus inhibit enzymatic activities, reducing digestibility and nutrient absorption in the Atlantic salmon.

4.2.2. Main experiment

Nutrient digestibility of *Nannochloropsis* (C1) and *Desmodesmus* (C4) showed the same range in the pre-study and verification study.
In comparison to the pre-study, the verification study gave higher ADC's of ash, slightly higher ADC of DM, while ADC of protein showed the same range for the two studies. The higher digestibility of DM may be explained by feed processing method. Cheng and Hardy (2003) also reported that use of extrusion processing increased DM digestibility for Rainbow trout. Extrusion processing result in physical and chemical changes of the feed ingredients, and may further influence digestibility of nutrients for the feed ingredients and the diets. Improved nutrient utilization of soybean meal and lupin after extrusion processing has been reported in feed for Rainbow trout (Bangoula *et al.,* 1993; Francis *et al.,* 2001; Robinson *et al*., 2001; Cheng and Hardy*,* 2003; Barrows *et al*., 2007).

Lack of improved protein digestibility in the extruded diets for the C4 and C1 diets are also in line with Glencross *et al*. (2011), who reported no benefit of the extrusion process on the digestibility of nitrogen or the sum of amino acid in lupin kermel meal, as well as soybean meal when fed to Rainbow trout. Most likely, the extrusion process is not vigorous enough to improve availability of protein for proteases in the digestive tract. For algae, Janczyk *et al*.

(2005) also reported that the crude protein digestibility and biological value of green microalgae *C. vulgaris* fed to rats was enhanced by ultrasonication technique. These authors suggested that improved digestibility was mainly related to the breakdown of cell walls, increasing enzyme: subtract contact. Another explanation was increased nutrient utilization because of a reduction in antinutritional components. The increase in ADC's of DM for the microalgae in main experiment compared to those of in pre-study is most likely explained by chemical and physical changes in the feed.

4.2.3. Inclusion level study 10% and 20%

Digestibility of DM and protein was not affected by inclusion level of the C4. However, protein digestibility was slightly higher at 10% and 20% inclusion than values estimated for the microalgae in the pre-study and verification study. The ADC of protein for C4 of included at 10% and 20% was in line with herring meal fed to Atlantic salmon (82.6-85.1%; Anderson *et al*., 1995). Ash digestibility was in general lower compared to the values observed in the pre-study and verification part of the main study, but improved with inclusion level of C4 in the feed. Negative digestibility values for the control diet indicate that drinking rate may have differed among the diets and low values for the other two. Differences in drinking rate may be explained by pellet quality. Pellet quality varies with processing technology (Lundblad *et al*., 2011). Pelleted feed is less water stable than extruded pellets (Venou *et al*., 2009; Hilton *et al*., 1981). The cold pelleted diets used in the pre-study may thus have disintegrated faster in the stomach, requiring less water to solubilize the feed. However, the high ash digestibility observed for the fish fed the extruded diet in the verification part of main study, does not support the hypothesis that extrusion per se cause a main difference in drinking rate.

4.3. Differences equation to calculate nutrient digestibility

Using equation 1 for calculation of nutrient digestibility is limited to whole diet nutrient digestibility. This equation can't be used to assess digestibility of the single ingredients. For this purpose Cho and Slinger (1979) developed equation 2. Equation 2 has been used by many laboratories to calculate the ADC of nutrients in test ingredients. The equation is based on a partial replacement of a reference diet with a test ingredient in the ratio 70:30. This equation assumes that the nutrient digestibility of the combined diet is the weighted average of the nutrient digestibility of the reference diet and the test diet. Equation 3 takes into consideration the relative nutrient contribution from the reference diet and the test ingredient. Equation 3 is thus slightly modified (Sugiura *et al*., 1998) by adding nutrient ingredient, nutrient test, and nutrient basal components in this equation. For equation 2, the weighting is based on the relative proportions of the reference diet and the test ingredient, whereas, in equation 3 the weighting is based on the nutrient contribution of each of those

components. According to Forster (1999) the weighting in equation 3 is reflecting that test ingredients with higher or lower levels of the nutrient will have concomitantly greater or lesser influence on the calculated nutrient digestibility of the combined diet. The extent, to which the values for the digestibility coefficients obtained from equation 2 and 3 differ, thus depends upon the nutrient content and digestibility of the test ingredient (Forster, 1999). Equation 4 is a modification of equation 3, but slightly modified of this equation were by adding $Nutrient_{ingredient}$ and $Nutrient_{basal}$ value.

For whole diet protein digestibility in the pre-study, C3 improved digestibility of whole diet, whereas C1 and C4 gave a reduction. High digestibility of C3 was also reflected in a higher digestibility of this algae compared to C1 and C4 in the pre-study. Calculating ADC's of protein and DM with equation 2 gave the same results compared to equation 3 and 4 for C1 and C4 for both experiments. For C3 (were only used in pre-study). Equation 2 gave ADC's of protein below 100% while use equations 3 and 4 gave values higher than 100%. Digestibility of ash was highest for equation 2 compared to equations 3 and 4 in both experiments for C3 in pre-study and C1 in main experiment. The main explanation for the high ash digestibility using equation 2 is that this equation did not give the real nutrient contribution of the reference diet and the test ingredient. As pointed out by Foster (1999) and Sugiura *et al*. (1998) the weighting in this equation is based on the relative proportions of the references diet and test ingredient. This is mathematically incorrect since it did not account for the real nutrient contribution of the reference diet and the test ingredient.

The ADC protein of C3 in equation 3 (110%), were slightly higher than equation 2 (96%), but comparable to equation 4 (107%). The ADC's of DM and ash gave the same results for equation 3 and 4 both experiments, while ADC protein was higher for the pre-study. As pointed out by (Sugiura *et al*., 1998) equation 3 provided better resolution and gave significant differences in protein digestibility between ingredients. The C3 protein digestibility is in line with Glencross *et al*. (2004, 2005), who reported protein digestibility values of 98–107% for soy protein concentrate (SPC) and 98% for soy protein isolate in two trials with Rainbow trout. Protein digestibility higher than 100% is often reported for ingredients with low protein content, when the partial contribution of the protein is low in the diet (Carter *et al*., 1999). ADC values for protein greater than 100% was for example reported for wheat gluten fed to Rainbow trout (Glencross and Hawkins, 2004). Sugiura *et al*. (1998) reported an ADC of protein in wheat gluten at 100% for Coho salmon and Rainbow trout. These findings were confirmed by Gaylord and Barrows (2008), who reported an ADC of protein at 100% for wheat gluten fed to Rainbow trout. Equation 3 and 4 are appropriate for determination of ingredient digestibility, because these two equations include contribution

from test ingredient and reference diet. Our results clearly demonstrated that ADC values obtained using these two equations did not differ between the pre-study and main experiment.

Negative ash digestibilities and protein digestibility greater than 100 is challenging the assumption that digestibility coefficients should be between 0 and 100% (Glencross *et al*., 2007). This could be attributed to amongst others, analytical errors for markers or nutrients, poor mixing of the marker, non-representative samples of diets or faeces, interaction among feed ingredients.

4.4. Faecal collection

The accuracy of ADC's depends largely on procedure used to collect faeces, which ideally should represent normal defaecation. A number of methods have been used to collect faecal matter in salmonids such as intestinal dissection, stripping, anal suction, mechanically rotating screen (Choubert system) and faecal collection column (Guelph system), each one having its own advantages and drawbacks. Stripping of faeces in the present study was performed after fish were adapted to the experimental diets, and the handling stress was no longer detrimental to the performance of the fish. During collection of faeces, care was taken to reduce experimental error. Fish was stripped using a moderate stripping pressure, and faeces were only once drawn from the distal intestine. Handling of the fish was also carried out in a careful manner to avoid contamination with mucus from the intestine and urine. Austreng (1978) observed that the faecal stripping technique can underestimate protein and energy digestibility compared to the column collection technique or the automated faeces collection device. Gaylord and Barrows (2008) suggested that underestimation of nutrient digestibility employing faecal stripping technique most likely is explained by induction of defecation prior to complete digestion or contamination with other body fluids. The same authors claimed that overestimation may occur with the settlement techniques because of leaching of water soluble nutrients into the water. They also reported that the degree to which the stripping technique underestimates or, in contrast, the two faecal settlement techniques overestimate protein and energy digestibility, is still debated. In conclusion, use of the faecal stripping technique represents a conservative estimate of digestibility of protein and energy. Krogdahl *et al*. (1999) reported nutrient digestibility with use of dissection. The advantage of dissection is that nutrient absorption can be studied along the gastrointestinal tract from different sections. It is also more convenient if the same intestines are used for other purposes. Care needs to be taken to avoid contamination of digest from anterior part of the intestine. Lower protein digestibility has been reported when faeces were collected with dissection compared to stripping, indicating contamination of faeces from anterior parts of the gastrointestinal tract (Percival *et al*., 2001).

5. Conclusions

It can be concluded that nutrient digestibility varied among the three different strains of microalgae. Based on ADC values, the *Nanofrustulum* (C3) revealed the greatest potential as a feed ingredient followed by the *Nannochloropsis* (C1), though no large differences were observed between the *Nannochloropsis* (C1) and *Desmodesmus* C4. The present study also showed that *Desmodesmus* (C4) can be used up to 20% inclusion in feed for Atlantic salmon without negative effects on digestibility of DM, protein and ash. However, in order to reveal the full potential of these three microalgae in feed for Atlantic salmon, they have to be tested in long-term feeding experiment to assess effects on growth performance, health and product quality.

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