Study on the development of non-fish meal and non-fish oil diet for red seabream Pagrus major using with microalgae

Doctoral Dissertation

STUDY ON THE DEVELOPMENT OF NON-FISH MEAL AND NON-FISH OIL DIET FOR RED SEABREAM *Pagrus major* USING WITH MICROALGAE

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［課程博士・論文博士共通］

博士学位論文内容要旨
Abstract

専　攻 Major	Applied Bioscience	氏　名 Name	Seong Taekyoung
論文題目 Title	Study on the development of non-fish meal and non-fish oil diet for red seabream, *Pagrus major*, using with microalgae 微細藻類によるマダイ用無魚粉･無魚油飼料の開発に関する研究		

[Introduction]

Fish meal and fish oil are known as the most suitable ingredients for the fish feed. Fish meal contains a high quantity of protein with well-balanced amino acids, and fish oil also contains high amount of n-3 long chain polyunsaturated fatty acids (LC-PUFA) which are essential for most of the marine fish species. But the price of those ingredients has been getting higher in the past decades and the usage of fish meal and fish oil in aquaculture has shown reducing trend. Therefore, to stabilize the price and lower the amount of fish oil and fish meal in aqua feed, it is necessary to find out alternative sustainable dietary ingredients for aquaculture fish species.

The several previous studies showed replacement of large amounts of the fish meal without any improper effects on growth performance and nutrient utilization, and fish meal free diet showed comparable performance with plant protein sources. However, the other studies have shown bad results on growth performance, although the experimental diets were nutritionally acceptable. Also, health problems, such as inflammation or enteritis have been observed in several aqua farmed fish species. There were several works on replacing fish oil in fish feed to vegetable oil, but there were negative effects on growth performance and notable changes in fatty acid composition of body. The major problem of plant oils is lack in n-3 LC-PUFA such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) that are essential fatty acid for most of the marine fishes.

Microalgae locate at the base of the aquatic food chain and some of the species shows well-balanced amino acid and considerable LC-PUFA. As the main producer of LC-PUFA in the marine environment, microalgae can be a promising alternative ingredient of fish feed. Previous studies suggested that lipid-rich microalgae were able to replace fish oil in fish feed and some of them contained a relatively high level of polyunsaturated fatty acids with high n-3 LC-PUFA productivity. In previous studies, *Schizochytrium* sp. appears to be a good source of LC-PUFA including DHA. In former study in 2015, it was possible to replace 100% fish oil and fish meal by a mixture of plant meals and 11% of algae meal (*Schizochytium* sp.) in a diet for red sea bream (*Pagrus major*), and no significant growth retardation was observed.

Based on previous studies, the objective through three studies was to investigate availability and possibility of microalgae as alternative source of fish derived ingredient in fish feed and to examine their growth effect and chemical transition on fish body.

Our former studies suggested that DHA rich microalgae *Schizochytrium* sp. algae meal can be appropriate substitute of fish oil and were suitable lipid source of non-fish meal and non-fish oil diet. However, in the previous study, only single dose of algae meal was used and it was impossible to determine suitable inclusion level of algae meal. So, the objective of this study was to determine the optimal formulation level of the algae meal in non-fish meal diet.

Six iso-nitrogenous (45%) and iso-lipidic (13%) experimental diets were prepared. Control diet was formulated with fish meal (40%) and fish oil (6%). Plant protein sources (soy protein concentrate, soybean meal, corn gluten meal) as substitute of fish meal were used in the second diet and fish oil was used for lipid source [NFM+FO]. In the third diet, fish oil of NFM+FO was replaced by rape seed oil [NFM+NFO] as negative control. In the other three diets, the rape seed oil in NFM+NFO diet was replaced with algae meal (*Schizochytrium* sp. powder) at 5%, 10% and 15% [AM5, AM10, AM15]. Triplicate groups of juvenile red seabream (8.8g) were fed the experimental diets for 12 weeks to near satiation.

The growth was the lowest in the fish fed NFM+NFO. It was improved by formulation of algae meal and reached to the growth level of NFM+FO in 10% algae meal group [AM10]. Lipid content of whole body in the fish on NFM+NFO group was significantly lower than the other groups. Fatty acid profile showed significant differences among dietary treatment. Fatty acid profile of liver polar lipid showed that DHA was highest in the fish fed AM10. The results might suggest that microalgae might be a candidate material for replacement of fish oil diet, and optimal level might be 5~10%.

Through the result of first study, it was possible to formulate non-fish meal and non-fish oil diet and to determine optimal substitution level. In the other hand, several previous studies suggested that various microalgae species were potential suitable protein and lipid source of non-fish oil and non-fish meal diet with desirable nutritional value. Therefore, the objective of this study was to survey proper microalgae species for non-fish meal and non-fish oil diet of red sea bream.

Six iso-nitrogenous and iso-lipidic diets were formulated (CP 45%, CL 16%). Fish oil formulated non-fish meal diet was arranged as a control diet with soybean meal, soy protein concentrate and corn gluten meal (NFM+FO). Fish oil of NFM+FO was replaced by *Nannochloropsis* meal and *Schizochytrium* meal for each of two diets (NFM+NAN, NFM+SCH). Three different species of microalgae meal (*Nannochloropsis*, *Chlorella*, *Spirulina*) was formulated in NFM+SCH diet to replace plant protein source (NAN+SCH, CHL+SCH, SPI+SCH). Duplicated groups of juvenile red sea bream (13.4g) were fed the experimental diets for 12 weeks to near satiation.

The growth was lowest in the fish fed NFM+NAN and was improved by formulation with *Schizochytrium* meal. The groups fed the other algae meal formulated diets fed groups showed the similar growth performance to NFM+FO. Algae meal formulated groups showed no significant difference in proximate composition except NFM+NAN and SPI+SCH. In fatty acid composition, DHA level of *Schizochytrium* sp. meal included diets were higher than the other diets group. Digestibility of diets group which mixed two microalgae meal got improved. The results showed the possibility of the candidate microalgae species as fish meal and fish oil substitute and might suggest that they were key ingredient for development of non-fish meal and non-fish oil diet.

The results of previous studies suggested that several microalgae species were able to replace fish meal and fish oil and availability of the microalgae species was determined in second study with improved protein and lipid digestibility. As an advanced study, the objective is to investigate effect of high level microalgae incursion and mixing microalgae species.

Six iso-nitrogenous and iso-lipidic diets were formulated (CP 47%, CL 19%). Fish oil formulated

non-fish meal diet was arranged as a control diet with soybean meal, soy protein concentrate and corn gluten meal (NFM+FO). Fish oil of NFM+FO was replaced by *Nannochloropsis* meal and *Schizochytrium* meal for each of two diets (NFM+NAN, NFM+SCH). *Nannochloropsis* algae meal was formulated in NFM+SCH diet to replace plant protein source (NAN+SCH). The other two microalgae meal (*Chlorella*, *Spirulina*) was formulated in NAN+SCH diet to replace soybean meal and corn gluten meal each (NS+CHL, NS+SPI). Duplicated groups of juvenile red sea bream (5.9g) were fed the experimental diets for 12 weeks to near satiation.

In growth result, the groups fed NAN+SCH and NS+CHL diet showed better growth performance than other diet groups but group fed NS+SPI showed the worst growth performance. Group fed NS+SPI diet showed higher moisture composition than other diet groups. These results may suggest high level microalgae incursion by mixing microalgae species could be promising device to develop non-fish meal and non-fish oil diet.

In former basic study, it was possible to substitute fish oil in non-fish meal diet with plant protein source and microalgae *Schizochytrium* sp. and in succession to the study, it was possible to reconfirm the effectiveness of *Schizochytrium* sp. and its optimal supplementation level through the 1st study. The result of the 2nd study demonstrated that mixing two microalgae species could be promising protein and lipid source. The last study identified the effectiveness of microalgae meal with successful total replacement of fish meal and fish oil by mixing three microalgae species.

Through the three studies, it was possible to develop non-fish meal and non-fish oil diet by using microalgae species without obtrusive adverse effect on growth and chemical composition of fish body. The results suggest that microalgae can be promising ingredient for fish diet.

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Chapter 1.

General Introduction and literature review

Aquaculture review

Aquaculture is the farming of fish or other aquatic organisms. Farming fresh water or saltwater species under controlling can be contrasted with commercial fishery, which is the harvesting of wild fish. According to the Food and Agriculture Organization (FAO), aquaculture is understood to mean the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated (FAO, 2017).

Global fisheries and aquaculture production peaked at about 171 million tons in 2016, and total aquaculture production was 80 million tons. Total production of capture fisheries has shown relatively static since the late 1980s, but the global aquaculture production is continuously growing for past decades. The growth rate of worldwide aquaculture has been sustained and rapid, averaging 8% per year for over 30 years, although it no longer enjoys the high annual growth rates of the 1980s and 1990s. However, aquaculture continues to grow faster than other major food production sectors. The production of aquaculture has been mostly used as human consumption, and its amount and per capita apparent consumption are showing increasing trend (FAO, 2018).

1.2 Fish diet

In animal production, good diet is essential for nutritious product. Especially in fish

farming, fish diet represents high proportion of production cost. So, developing nutritious and cost-effective diet is of great importance. To develop nutritionally balanced commercial diets, technologies in fish nutrition have also developed dramatically in past decades.

In modern aquaculture, the general meaning of diet indicates formulated diet which contains all the essential nutrients and energy for basal metabolism, growth, reproduction and health maintenance of farming fish. The diet can be widely classified as wet feed, semi-moist pelleted feed and dry pelleted feed, and hold a key post in fish farming. Especially, "formulated diet" is represented with dry pelleted feed which can be produced massively, be stored long-period and be transported long distance. Dry formed commercial diet is important for modern fish farming and it contains several advantages listed below:

- Easy maintenance and supply, it also can be used with automatic feeding machine
- Generally, dry pelleted feed can be stored with ordinary temperature, for comparatively longer time.
- Stable supply and price.
- It can easily control feed supply and its method to control production.
- By control feed supply, it is possible to reduce disease incidence rate.

With those advantages, developing formulated diet is necessary for modern aquaculture. Feed and ingredients must include essential nutrients and proper energy according to fish species which have different nutritional requirements.

1.3 Nutritional requirement and metabolism

Feeds and ingredients for feed contain essential nutrients and energy sources for fish growth, reproduction, and health. Deficiencies or excesses of essential nutrients and energy can reduce growth rates or cause emergence of diseases. Dietary requirements can be established for energy, protein and amino acids, lipids, minerals, and vitamins. In this passage, the requirements and metabolism of protein and lipid will be investigated.

Protein and amino acids

Proteins are the major organic material in fish body and it composes about 70% of dried fish body. Fish intake protein to obtain amino acids which are absorbed from the intestinal tract and distributed by the blood to the organs and tissues, and these amino acids are used by the various tissues to synthesize new protein. Proteins are synthesized with peptide bond caused by genetic information delivery system (DNA to RNA to protein) of 20 different kinds of amino acids in organism. In fish, there are 10 kinds of essential amino acids.

Proteins are composed of carbon (50 percent), nitrogen (16 percent), oxygen (21.5 percent), and hydrogen (6.5 percent), and other elements (6.0 percent). Most of fish utilize protein as main energy source; unlike most of the land animal utilize carbohydrate as main energy source. Most nitrogen as output of protein metabolism is excreted as ammonia (NH_3) from the gills of fish, and only 10 percent is excreted as solid wastes. As ammonia is excreted directly from fish, maintaining proper water quality can be a significant concern for fish farmers.

Although more than 200 amino acids occur in nature, only about 20 amino acids are common for fish nutrition. Of these, 10 are essential (indispensable) amino acids that cannot be synthesized by fish. The 10 essential amino acids that must be supplied by the diet are methionine, arginine, threonine, tryptophan, histidine, isoleucine, lysine, leucine, valine, and phenylalanine. The other non-essential (dispensable) amino acids are generally alanine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, proline, serine and tyrosine, and most of those amino acids are synthesized in fish body with enough amount. The biochemical structures of 20 main amino acids are following with figures (Figure 1).

Lipid and fatty acids

Lipid is one of the main diet sources and generally it cannot be dissolved in water but organic solvents including ether and chloroform. Lipid contains higher weight-to-energy ratio than protein or carbohydrate, which infers lipid can be important energy source, not only that lipid plays important role for source of essential fatty acids and supplier of fat soluble vitamins, also, absorbing fat soluble vitamins in fish body, consisting body tissue and cell membrane, forming of steroid hormone and bile juice, accumulating internal energy and protecting and insulating body under the skin are the crucial role of lipid.

Lipid can be divided into two groups, firstly, polar lipid, which is including phospholipid, plays principal role in constructing structure, and secondly neutral lipid, representing as triacylglycerol, mainly is used for energy and storage. Generally, lipid from animal is composed with triacylglycerol, phospholipid and cholesterol. Lipid metabolism of the fish species occurs in proximal intestine and pyloric caeca. Pancreatic enzymes from pancreas or hepatopancreas, such as triacylglycerol lipases and phospholipases play

important role in lipid metabolism. Most of the procedures of lipid metabolism are generally similar with other vertebrates. Digestion, absorption, transport, lipogenesis and beta-oxidation are the main procedures of the lipid metabolism.

Digestion and absorption: Digestion of lipid in diet taken by fish starts its proximal intestine and pyloric caeca with pancreatic enzymes. Digestion of lipid initially produces free fatty acids, cholesterol, acylglycerols mostly 2-monoacylglycerol, lyso-phospholipids and fatty alcohols. The products are solubilized or emulsified as form of lipid droplet with bile salt micelles. Intestinal mucosa cells uptake the products into the enterocytes using passive diffusion. In the intestinal mucosa cells, reesterification of free fatty acid with glycerol, partial acylglyccerols and lyso-phospholipids is occuring to reform triacylglycerol and phospholipids. Triglyceride is packaged with cholesterol, lipoproteins and other lipids into particles called chylomicrons. Chylomicrons are transported first into the lymphatic vessel that penetrates into each villus. Throughout the lymphatic system, chylomicrons can get into blood vessel. And then chylomicrons are rapidly disassembled in blood.

Transport: The products from digestion and absorption are generally hydrophobic. So, the main form of the lipid transport is lipoproteins transported in blood. There are five major groups of lipoproteins, chylomicrons, known as ultra-low-density lipoprotein, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL). Those forms of lipoprotein enable fats and cholesterol to move within the water-based solution of the bloodstream. Lipoprotein lipase decomposes lipoprotein and produces fatty acid. Intracellular fatty acid transport is conducted by fatty acid-binding protein (FABP). FABPs bind both long-chain fatty acids and

other hydrophobic ligands. Adipose cells deposit the excess lipid, some excess lipid is deposited as a layer between skin and flesh. However, some of the species, such as salmon and herring, deposit lipid in its flesh, and other species, such as cod and halibut stores lipid in liver predominantly.

Lipogenesis: Lipogenesis is the biosynthetic reaction by which acetyl coenzyme A (acetyl-CoA) is converted to fatty acids. Mitochondria form the acetyl-CoA from the oxidate decarboxylation of carbohydrate source or protein source. First, fatty acid synthetase (FAS) multienzyme complex in cytosol produces the saturated fatty acids 16:0 and 18:0. Monounsaturated fatty acids are produced by microsomal stearoyl CoA (SCD) or Δ9-desaturase, producing 18:1n-9 and 16:1n-7. Fatty acid elongases produce longer chain saturated and monounsaturated fatty acids such as 20:0 and 20:1n-9. But, monounsaturated fatty acids cannot be desaturated more, because polyunsaturated fatty acid (PUFA) cannot be *de novo* synthesized in marine fishes, so it should be consumed by diet. PUFA can be more elongated or desaturated with specific fatty acyl desaturase (Fads) and elongation of very long fatty acids protein (Elovl) enzymes. The synthesized fatty acids are esterified into complex lipids of membrane phospholipids and triacylglycerol. Through lipogenesis and subsequent triglyceride synthesis, the energy can be efficiently stored in the form of fats.

The possible biosynthetic pathways for long-chain polyunsaturated fatty acids (LC-PUFA) in fish attached below. Delta 4,6,8-desaturase and elongation of very long chain fatty acids protein 2,4,5 (Elovl 2,4,5,) operate the synthesis of fatty acid. These biosynthesis pathways are not same for every fish species. (Figure 2)

Beta-oxidation: One of the major energy source of fish is fatty acid catabolism. The

fatty acid catabolism occurs in cellular organelles, mitochondria, and peroxisomes. Once the fatty acid is inside the cytosol, it is activated by long chain acyl-CoA synthetase. The fatty acid reacted with adenosine triphosphate (ATP) and produces a fatty acyl adenylate, inorganic pyrophosphate and adenosine monophosphate (AMP). Acyl-CoA is transferred to the acyl-arnitine by carnitine palmitoyl transferase, located on the cytosolic faces of the outer and inner mitochondrial membranes. With the form of fatty acyl carnitine esters, beta-oxidation occurs by cleaving two carbons every cycle to form acetyl-CoA.

The process consists of 4 steps:

1. Dehydrogenation by flavin adenine dinucleotide (FAD): A long-chain fatty acid is dehydrogenated to create a Trans double bond between C2 and C3. This is catalyzed by acyl CoA dehydrogenase to produce trans-delta 2-enoyl CoA. It uses FAD as an electron acceptor and it is reduced to FADH2. [Acyl-CoA + Acyl-CoA dehydrogenase → Trans-Δ2-enoyl CoA]
2. Hydration: Trans-Δ2-enoyl CoA is hydrated at the double bond to produce L-3-hydroxyacyl CoA by enoyl-CoA hydratase. [Trans-Δ2-enoyl CoA + Enoyl CoA hydratase → L-3-hydroxyacyl CoA]
3. Oxidation by nicotinamide adenine dinucleotide (NAD): L-3-hydroxyacyl CoA is dehydrogenated again to create 3-ketoacyl CoA by 3-hydroxyacyl CoA dehydrogenase. This enzyme uses NAD as an electron acceptor. [L-3-hydroxyacyl CoA + 3-hydroxyacyl-CoA dehydrogenase → 3-ketoacyl CoA]
4. Thiolysis: Thiolysis occurs between C2 and C3 (alpha and beta carbons) of 3-ketoacyl CoA. Thiolase enzyme catalyzes the reaction when a new molecule of

coenzyme A breaks the bond by nucleophilic attack on C3. This releases the first two carbon units, as acetyl CoA, and a fatty acyl CoA minus two carbons. The process continues until all of the carbons in the fatty acid are turned into acetyl CoA. [3-ketoacyl CoA + Beta-ketothiolase → Acyl-CoA + Acetyl-CoA]

This process continues until the entire chain is cleaved into acetyl CoA units. The final cycle produces two separate acetyl CoAs, instead of one acyl CoA and one acetyl CoA. For every cycle, the Acyl CoA unit is shortened by two carbon atoms. Concomitantly, one molecule of FADH2, NADH and acetyl CoA are formed. The produced NADH is used for the metabolic energy in the form of ATP through the process of oxidative phosphorylation.

Because marine vertebrate species cannot synthesize LC-PUFA through endogenous biosynthesis, PUFA including docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) must be provided in the diet. By contrast, freshwater species seem to have sufficient biosynthetic capability to produce DHA, EPA, ARA from their precursors including linolenic acid (18:3n-3) and linoleic acid (18:2n-6) by delta 5, 6 desaturases and elongases (M. Izquierdo, 2005). Fish oil contains high levels of PUFA, and the cold water fish have a more nutritional requirement for n-3 fatty acids whereas some warm water fish can be satisfied with mixture of n-6 and n-3 fatty acids. In the cold temperature, phospholipids of n-3 fatty acids allow more flexibility than those of n-6 and n-9 fatty acid group in cell membrane. These facts indicate the fatty acid requirement of fish maybe related to the effect of unsaturation on the melting point of a lipid (FAO, 1980).

According to previous studies, the requirement essential fatty acid (EFA) of larvae and early juvenile red sea bream was satisfied when the 1.0-1.6% of DHA or 2.3% of EPA

and 2.1% of n-3 PUFA (with 1.0% DHA) in diet (Yone & Fujii, 1975b; Fujii & Yone, 1976; Furuita et al., 1996). Also, for juvenile red sea bream, the fatty acid requirement was 0.5% of n-3 LC-PUFA, EPA with 0.5% of DHA or 1% of EPA in diet. The results of the previous research have shown that DHA has twice efficiency to EPA for larvae and juvenile red sea bream. (Yone & Fujii, 1975a, 1975b; Fujii & Yone, 1976; Watanabe et al., 1989; Takeuchi et al., 1990; Furuita et al., 1996; M. S. Izquierdo, 1996)

1.4 Fish meal and fish oil

Traditionally in fish diet, fish meal and fish oil have been the most important ingredients. These come mainly from the processing of fish from the wild catch, usually pelagic species that are generally not suited for human consumption. Fish used for producing fish meal set a lower price than those used for human consumption. The fishery for producing fish meal is largely enacted in the Pacific Ocean, off the coast of Peru and Chile. In addition, the North Atlantic another important supplying source of fish for fish meal and fish oil.

Fish meal derives from fish body and it has a brown color and flour-like appearance. This material is made with certain procedures including cooking, pressing, drying and grinding the fish. The fish oil is one of by-products of those procedures and it proves to be a rich source of energy and fatty acids for fish, including n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are the most important fatty acids in marine fish.

According to the FAO investigation, the global fish meal and fish oil demand have increased with expanding aquaculture industry as the fish meal and fish oil are still considered the most nutritious and most digestible ingredients for farming fish feeds. However, the world fish meal production is already static for the limit of the fishery production and the price of fish meal and fish oil was unstable, has soared for recent decades. Recently, their inclusion rate in compound feeds for aquaculture is showing downward trend as they are used more selectively than before. (FAO, 2018) (Figure 3)

1.5 Alternative ingredients

Fish meal has been the major protein source with 30-70% protein contents, with excellent nutritional properties including well balanced amino acid composition and essential fatty acid profile. But the price of the fish meal has increased rapidly, thus, alternative protein sources have received attention for past years.

Fish oil is an excellent lipid source, especially in terms of n-3 LC-PUFA and PUFA (Rice, 2009). Peruvian anchovy and menhaden contain greater abundance of EPA and DHA. Fish oil especially body oil are rich in triacylglycerols (TAGs), which comprise the major component of stored fats, generally contributing in excess of 90% of the total fatty acid composition. The nutritional composition of fish oils is largely dependent by their fatty acid composition, particularly in aquaculture diets.

Plant Protein sources

Various plant protein sources including soybean, rape seed, canola, sunflower, cotton seed, wheat, corn, peas, beans, peanut etc. have been commonly used for plant protein sources. There are factors for considering plant protein sources for feed formulation.

Energy density: If chosen plant protein source have high carbohydrate content, it is not proper diet source for carnivorous species.

Amino acid content: Generally, plant protein sources show deficient of essential amino acids, such as lysine and methionine. Also, plant protein sources show less balanced amino acid contents than fish meal. (Gatlin et al., 2007)

Anti-nutritional factor: Plant diet source may contain various anti-nutritional factors, including protease inhibitors, phytates, glucosinolates, saponins, tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids, canavanine, antivitamins, and phorbol esters. For the utilization of the plant source, those factors must be controlled. (Francis et al., 2001)

There are previous studies about effects of fish meal substitution have been reported. For the cyprinids and tilapia, the research on replacing large amount of fish meal was successful without any improper effects on growth performance and nutrient utilization (Kaushik, 1995; Rodehutscord et al., 1995; Espe et al., 2006, 2007). Also, research on rainbow trout, fish meal free diet showed comparable performance with combination of plant and animal protein sources (Lee et al., 2002; Barrows et al., 2007). However, the other studies have shown impaired growth performance, although the experimental diets were

nutritionally acceptable. In addition, health problems, such as inflammation or enteritis were observed in salmon and rainbow trout (Baeverfjord & Krogdahl, 1996; Bureau et al., 1998). In a 12-week trial in sea bream, there were growth reductions when fed diets containing 50-100% plant protein ingredients due to remarkable reduction in feed intake (Gómez-Requeni et al., 2004). In contrast, sea bass fed up to 98% of dietary protein as plant meals showed no reduction in feed intake or growth performance after 12-week feeding experiment (Kaushik et al., 2004). Above mentioned have shown that economical feeds can be formulated without fish meal, but it may cause nutrient deficiencies, poor growth performance with reduced feed intake.

Insect protein sources

Most of the wild fish and crustaceans eat insects as a part of their natural diet. Insect protein source including cricket, mealworm, and black soldier fly larvae meal are considered to be novel feed ingredients that can increase aquaculture feed production. According to previous studies, insect meal could replace between 25% and 100% of soybean meal or fish meal in the animals diets with no adverse effects. (FAO, 2013)

Black soldier fly larvae are one of the most investigating ingredients. It is omnivorous insects that can turn common organic waste into protein and also they contain high-value feed source, rich in protein and fat. They contain about 40-44% crude protein (CP), Ca (7.56%) and most essential amino acid with higher level than that of soybean meal. It is also not a potential carrier of disease (van Huis, 2013).

Poultry Byproduct Meal (PBM)

Meat by-product represented with poultry by-product meal has been known as an animal protein source which has a potential to replace fishmeal. Previous studies revealed that the PBM could replace fish meal at high levels up to 100% (Nengas et al., 1999; Takagi et al., 2000; Gaylord & Rawles, 2005) and it has fine digestibility with several fish species (D. P Bureau et al., 1999; Yang et al., 2006). PBM has considered as a potential fish meal alternatives for feed of carnivorous fish species such as rainbow trout because of its relatively high protein content and lower price compared to fishmeal (Shapawi et al., 2007).

Vegetable oil source

Vegetable oils are considered as the most sustainable substitute for fish oil due to its steadily increasing production, high availability and stable prices (Fountoulaki et al., 2009). The most commonly used vegetable oils used for fish feed production was oil from oilseed including soy bean, linseed, rapeseed, sunflower, palm, corn, echium and olive (Turchini et al., 2009).

The major difference between fish oil and vegetable oil is fatty acid composition. Generally, vegetable oils do not contain long chain fatty acids, especially longer than C18 and also, it has been known that the fatty acid compositions of vegetable oil are mostly dominated by 16:0, 18:0, 18:1n-9 and 18:2n-6, although the major fatty acid varies.

Despite the limitations in fatty acid composition, it is possible to replace fish oil with vegetable oil when essential fatty acids for farming fish are sufficiently present in the diets. There have been previous studies performed to investigate the effects of complete and partial replacement of dietary fish oil with vegetable oil with fresh water species including catfish,

carp, Atlantic salmon, rainbow trout and Arctic charr; and marine species including gilthead sea bream, European sea bass, turbot and cod (Turchini et al., 2009, 2010).

Soybean, linseed and rapeseed oil appear to be a promising lipid source for gilthead sea bream (*Sparus aurata*). The growth was considerable and the feed costs were saved when it was used as partial dietary substitute for fish oil in aquacultural feeds (El-Kerdawy & Salama, 1997; Wassef et al., 2008). Palm oil formulated diets of Atlantic salmon and rainbow trout showed considerable growth and feed utilization efficiency equivalent levels of fish oil formulated diet (Torstensen et al., 2000; Rosenlund et al., 2001; Caballero et al., 2002). Also, the partial substitution of olive oil in European sea bass, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) showed equivalent growth rates with fish oil diet groups (Caballero et al., 2002; Torstensen et al., 2004; Mourente et al., 2005). Refined canola oil was also considered as a substitute of fish oil and showed successful total replacement of fish oil without negative effect on fish growth (Huang et al., 2007).

1.6 Microalgae

Microalgae are classified as aquatic relatives of plants and they get propagated in aquatic environment where the cells have sufficient access to light, carbon dioxide and other nutrients (Rosenberg et al., 2008). As an aquatic life, microalgae can grow in a wide range of habitats, unlike terrestrial plants which require relatively wide space with fertile land or irrigation. Microalgae have been successfully commercialized as production of nutritional supplements, antioxidants, cosmetics, natural dyes and PUFA (Spolaore et al., 2006).

For aquaculture, microalgae have been associated with nutrition as food additive to basic nutrients, with various biological activities. Microalgae have been used for larval nutrition for aquacultural species, including larval and juvenile bivalves, and for the larvae of some crustacean and fish species as live prey (Muller-Feuga, 2000). The most frequently used species are *Chlorella*, *Spirulina*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema* and *Thalassiosira*. According to the previous study, combination of different algal species provides better balanced nutrition and improves animal growth better than a diet composed of only one algal species (Spolaore et al., 2006; Becker, 2007; Hemaiswarya et al., 2011).

In order to use microalgae in aquaculture, there are several provisions need to meet. Easy culturing and nontoxic constituent with high nutritional qualities need to be addressed. Protein and lipid content of microalgae can be a major factor to determine the nutritional value of microalgae. In previous study, microalgae demonstrated its possibility as a potential source of single cell protein (Fabregas & Herrero, 1985). LC-PUFA, including EPA, AA and DHA are the most considerable factors in lipid. As I mentioned in previous chapter, some fatty acids are essential for many marine finfish and n-3 LC-PUFA are essential for larvae of many marine species (Izquierdo, 1996).

Microalgae locate at the base of the aquatic food chain, and main producer of LC-PUFA in the marine environment. Therefore, it can be a promising alternative to the ingredients of fish feed in which n-3 LC-PUFA are essentially contained. There were previous studies suggested that lipid rich microalgae were able to be replaced fish oil in fish

feed and some of them contained relatively high level of PUFA (Lewis et al., 1999; Handayani et al., 2011), with high n-3 LC-PUFA productivity.

One of the microalgae species, *Schizochytrium* sp. showed high lipid content, including relatively high DHA level. The microalgae already used as live feeds for rotifers and brine shrimp (*Artemia*) as a rich source of n-3 and n-6 fatty acid (Barclay & Zeller, 1996; Yamasaki et al., 2007). Lipid of the algae has been shown successful replacement of fish oil in the diet for several marine species including red sea bream (Seong et al., 2019) Atlantic salmon (Miller et al., 2007; Sprague et al., 2015), giant grouper (García-Ortega et al., 2016), channel cat fish (Li et al., 2009) and jade perch (Hoestenberghe et al., 2016). In those studies, *Schizochytrium* sp. appears to be a good source of LC-PUFA including DHA. It also showed beneficial effect on egg production, egg weight, yolk color and blood lipid profiles of chicken layers, with enhanced DHA and EPA concentrations in eggs (Park et al., 2015). Supplement of *Schizochytrium* sp. in dairy cow diet increased DHA content as well (Franklin et al., 1999).

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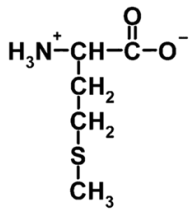
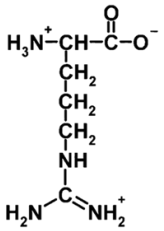
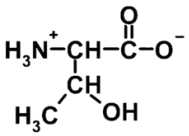
1.9 Figures

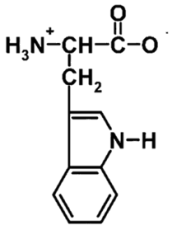
Figure 1. Structures of indispensable and dispensable amino acids

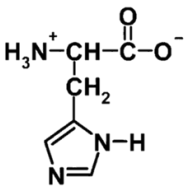
Indispensable amino acids

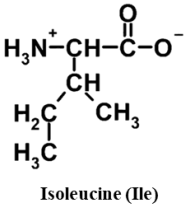

Methionine (Met)

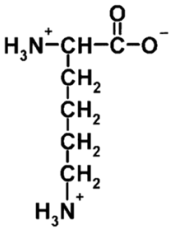

Arginine (Arg)

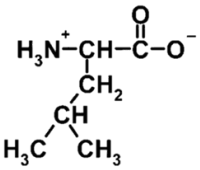

Threonine (Thr)

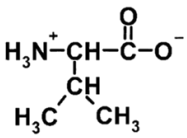

Tryptophan (Trp)

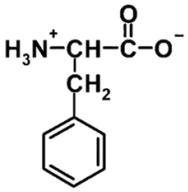

Histidine (His)


Isoleucine (Ile)

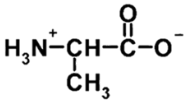

Lysine (Lys)

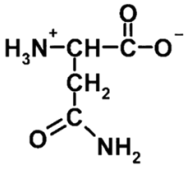

Leucine (Leu)


Valine (Val)


Phenylalanine (Phe)

Dispensable amino acids


Alanine (Ala)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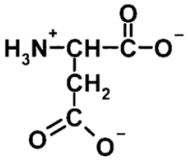
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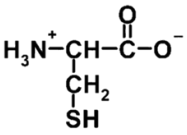
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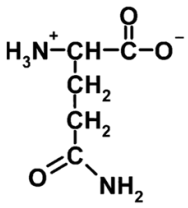
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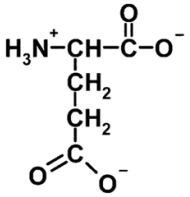
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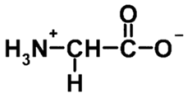
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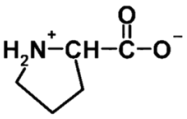
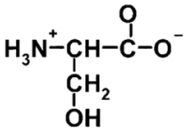

Proline (Pro)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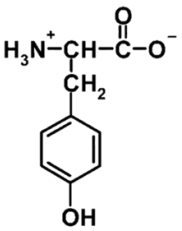

Throsine (Tyr)

Figure 2. The possible biosynthetic pathways for long chain poly unsaturated fatty acids (LC-PUFA) in fish

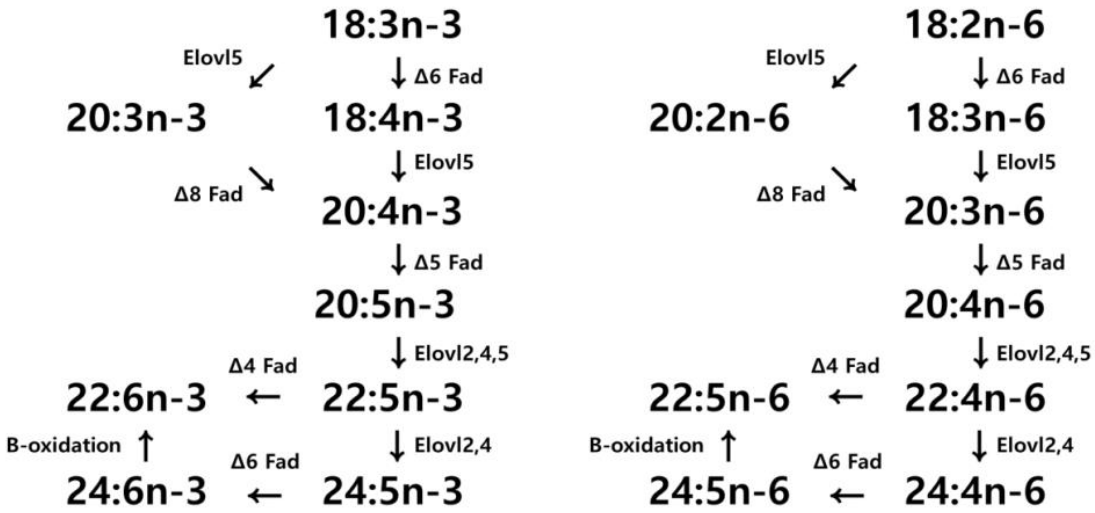
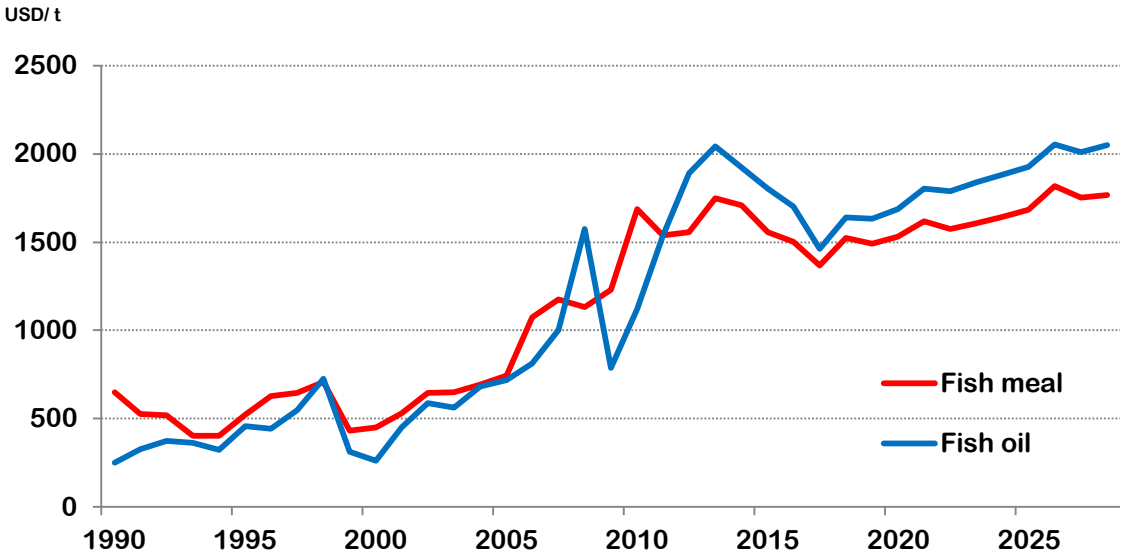


Figure 3. Fish meal and fish oil price (OECD-FAO 2019)



Chapter 2.

Non-fish meal and non-fish oil diet development for red seabream, *Pagrus major*, with microalgae *Schizochytrium* sp.

2.1 Objective of study

Fish derived products are known as the most suitable ingredients for the aquaculture diet. Representatively, fish meal contains a high quantity of protein with well-balanced amino acids, and fish oil also contains high amount of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) which are essential for most of the marine fish species. But the price of those ingredients has been getting higher in the past decades and showing unstable trend with fluctuating supply (FAO, 2016).

Plant protein source is a promising substitute of fish meal, and there have been several studies to replace fish meal with a plant protein source. There were positive results in cyprinids, tilapia, rainbow trout and sea bass (Kaushik, 1995; Rodehutscord et al., 1995; Lee et al., 2002; Kaushik et al., 2004; Espe et al., 2006; Barrows et al., 2007), however, the other studies have shown bad results on growth performance, although the experimental diets were nutritionally acceptable. Also, health problems, such as inflammation or enteritis were observed in salmon and rainbow trout (Baeverfjord and Krogdahl, 1996; Bureau et al., 1998). In feeding trial in sea bream, growth reductions with remarkable feed intake reduction were observed. (Gómez-Requeni et al., 2004). Studies have shown that economical feeds can be formulated without fish meal, but it may cause nutrient deficiencies, poor growth performance with reduced feed intake (Baeverfjord and Krogdahl, 1996; Francis et al., 2001; Gómez-Requeni et al., 2004).

One of the most prospective substitutes of fish oil is vegetable oil. There were several works on replacing fish oil in fish feed to vegetable oil, but there were negative effects on growth performance and showed notable changes in fatty acid composition of body

(Bell et al., 2001; Huang et al., 2007; Fountoulaki et al., 2009; Turchini et al., 2011). The major problem of plant oils is lack of essential fatty acids (EFAs) for most of marine species, represented with n-3 LC-PUFA such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).

Microalgae locate at the base of the aquatic food chain, and the main producer of LC-PUFA in the marine environment. Previous studies suggested that lipid-rich microalgae were able to replace fish oil in fish feed and some of them contained a relatively high level of PUFA with high n-3 LC-PUFA productivity (Lewis et al., 1999; Handayani et al., 2011). One of the heterotrophic microalgae species, *Schizochytrium* sp. showed high lipid content, including relatively high DHA. The microalgae already used as live feeds for rotifers and brine shrimp (*Artemia*) as a rich source of n-3 and n-6 PUFA (Barclay and Zeller, 1996; Yamasaki et al., 2007). Microalgae, *Schizochytrium* sp. has been demonstrated as successful fish oil alternative in the diet for several marine species including red sea bream (Seong et al., 2019), Atlantic salmon (Miller et al., 2007; Sprague et al., 2015), channel catfish (Li et al., 2009), giant grouper (García-Ortega et al., 2016) and jade perch (Hoestenberghe et al., 2016). In those studies, *Schizochytrium* sp. appears to be a good source of LC-PUFA including high DHA. Nonetheless, there are very little information in terms of the availability of algae oil as fish oil alternative in a practical diet for marine fish. Very recently, it was possible to replace 100% fish oil and fish meal by a mixture of plant meals and algae meal (*Schizochytium* sp.) in a diet for red sea bream and no significant growth retardation was observed (Seong et al., 2019). Also, better feed conversion ratio was recorded in the algae meal group than fish derived product used diet group (Seong et al., 2019). These results strongly suggested that

usefulness of algae meal as oil sources for non-fishmeal and non-fish oil diet for marine fish. However, in the previous experiment, only single dose of algae meal (11% in diet) was used, so there need more detailed studies on suitable inclusion level of *Schizochytrium* sp. is required (Seong et al., 2019). In addition, the *Schizochytrium* algae meal contains a high level of DHA in polar lipid. So, examine the fatty acid composition of the polar lipid of fish fed algae meal is of great interest. Therefore, the objective of this study was to investigate the effect of non-fishmeal and non-fish oil diet formulating different inclusion levels of DHA-rich microalgae *Schizochytrium* sp. meal on growth, feed performance and fatty acid composition of red sea bream.

2.2 Materials and method

Experimental microalgae

Powder type algae meal *Schizochytrium* sp. produced in Alltech Co. Ltd (Lexington, Kentucky, U.S.A) was used in the current study. Algae meal contained around 13% protein and 68% lipid. [Table 2]. The *Schizochytrium* sp. algae meal showed a relatively higher level of saturates with approximately 60% of palmitic acid (16:0) in fatty acid composition of total lipid. Low levels of total monoenes and n-6 LC-PUFA contained, but algae meal contains high level of n-3 LC-PUFA and the most of the LC-PUFA composed of DHA. [Table 3]

Experimental diets

Six iso-nitrogenous (45%) and iso-lipidic (13%) experimental diets were prepared. The control diet was formulated with 40% of fish meal and 6% of fish oil. Plant protein sources (soy protein concentrate, soybean meal, corn gluten meal) were formulated in the non-fish meal diets. In the second diet, fish oil was used for lipid source [FO]. Fish oil of FO was replaced by rapeseed oil in the third diet as a negative control [NFO]. In the other three diets, the rapeseed oil in the NFO diet was replaced with algae meal (*Schizochytrium* sp. powder) at 5%, 10% and 15% each [AM5, AM10, AM15]. Except for control diet, 2.2% of amino acid premix (Lysine 1: Methionine 0.5: Threonine 0.5: Tryptophan 0.2) was supplemented to compensate for the lack of indispensable amino acid content to improve dietary quality (Takagi et al., 2001). 0.5% of taurine was also supplemented to the non-fish meal diet to meet the nutritional requirement (Matsunari et al., 2008a, 2008b). 1% of bonito peptide was supplemented to enhance the palatability of feed. SSF (solid-state fermentation) enzyme mixture was used in non-fish meal used diets to reduce the negative effect of the anti-nutritional factor of plant protein source (Moura et al., 2012; Hanini et al., 2013). [Tables 1 & 2]

Dietary fatty acid composition differs with different lipid sources. Control diet contains the highest n-3 LC-PUFA among diets. NFO diet contained the scant amount of EPA and DHA. The other algae meal formulated diets showed higher levels of saturated fatty acid than the other three diets and most of their LC-PUFA was comprised of DHA. [Table 3]

The animal experiment was conducted according to the guideline of the Animal Experiment Treaty of the Tokyo University of Marine Science and Technology. The experiment was conducted at the Laboratory of Fish Nutrition, Tokyo University of Marine

Science and Technology, Tokyo, Japan. Juvenile red sea bream (*Pagrus major*) were obtained from Marua Suisan Co. Ltd. (Kamijima, Japan) and commercial larval feed (Ambrose, Feed One Co. Ltd., Tokyo, Japan) was fed for 2 weeks. NFO diet was fed for 1 week before the beginning of the experiment to acclimate to the experimental diet. Following this, 306 fish (average weight 8.8 ± 2.5 g) were distributed randomly into eighteen 60-L glass tanks that were placed in a re-circulating system filled with artificial seawater (Sea Life® , Tokyo, Japan; salinity, 30‰). The water temperature was maintained at 22.9 ± 1.3 ℃. NO_2 and NO_3 levels were monitored by a colorimetric test kit (Kyoritsu Chemical-Check lab Co., Tokyo, Japan) and confirmed within a safe level. A 12-h light/12-h dark photoperiod regime was employed during the study. Triplicate groups of fish were fed by hand to apparent satiation three times daily (09:00, 12:00 and 16:00) for 12 weeks. The fish tanks were cleaned every day after the first feeding time and half of the water was renewed twice a month to maintain the acceptable water quality.

Fish weighing and sampling

The fish weighing had been conducted a day before the first day and every 3 weeks during the feeding experiment. All fish in each group were anesthetized (2- phenoxyethanol at 0.5 ml/L) and weighed individually. Before a day of the experiment, 10 fish were randomly taken from the pool before introduction into experimental tanks for the chemical analysis of initial fish. Also, on the last day of the 12 weeks experiment, five fish from each tank were sampled randomly and ground with a centrifugal mill (Retsch, Haan, Germany) for chemical analysis. Before sampling for chemical analysis, fish were kept in starvation for 24 hours to

avoid the effect of diet resided in gastrointestinal tract on the chemical composition of fish. Another six fish were also sampled randomly from each group for the hepatosomatic index and assessment of liver lipid analysis. The samples were stored at −30 ℃ until analysis.

Biochemical analysis

Dehydration of moisture was conducted by oven drying each sample at 110 ℃ for 4h and then weighing each sample at one-hour intervals until a constant weight was obtained. Ash content was determined by ashing each dried sample in a porcelain crucible using a muffle furnace at 600 ℃ overnight (Woyewoda et al., 1986). Crude protein content was determined by the Kjeldahl procedure using a Kjeltec Auto Sampler System 1030 Analyzer (Foss Ltd., Tokyo, Japan). Percent nitrogen was multiplied by 6.25 to obtain an estimate of percent protein.

As for lipid content, the total lipids were extracted by using chloroform/methanol (2:1, v/v) according to the methods of Folch et al (1957). Total lipids extracted from the whole body and liver of fish were separated into neutral and polar lipid fractions via silica cartridges (Sep-Pack, Waters Co., Milford, U.S.A.), as described by Juaneda and Rocquelin (1985). For the fatty acid analysis, fatty acid methyl esters (FAME) were prepared according to Christie and Han (2010). The FAMEs were analyzed by a gas chromatograph (GC2025; Shimadzu Co., Tokyo, Japan) equipped with a hydrogen flame ionization detector and a silica capillary column (L × I.D., 30 m × 0.32 mm, df = 0.25μm Supelcowax 10 Fused Silica Capillary GC column; Supelco, Bellefonte, PA, USA).

Data calculation and statistical analysis

The growth performance of the fish was calculated by the following formulae:

Wet weight gain (WG)(g)

= (final mean wet weight (FW)(g)−initial mean wet weight (IW)(g))

Food conversion ratio (FCR)

= Dry feed intake (g)/WG (g)

Protein efficiency ratio (PER) (g/g)

= WG (g)/total protein intake (g)

Specific growth rate (SGR)(%bodyweight/day)

= [(ln FW (g) − ln IW (g))/time (days)]×100

Survival rate (SR) (%)

= (number of fish in each group remained/initial number of fish)×100

Daily feed intake (DFI) (%)

= (Dry feed intake)/[(IW (g) +FW (g))/2×time (days)]×100

To test the result of the experiment, all data were statistically analyzed by One Way Analyses of Variance (ANOVA; Sigma Stat 3.0, SPSS, Chicago, U.S.A.). Tukey's test was

used to detect significant differences among means ($P<0.05$).

2.3 Results

Growth performance

There were significant differences in growth performances and feed utilization efficiencies between the NFO and the other diet groups. Except for the NFO diet group, the other groups including control diet group had similar values in final weight (FW), weight gain (WG), feed conversion ratio (FCR) and specific growth rate (SGR) at the end of the 84-day feeding period. The survival rate (SR) showed no significant differences among each diet group. The result of daily feed intake (DFI) among the diets was no significant difference, but the amount of diet intake was definitely low in the NFO diet group. There was a significant difference in protein efficiency ratio (PER) between the control diet and NFO diet fed group, but there was no significant difference in the other diet fed group. FO diet fed group showed the highest hepatosomatic index (HSI) among all six diets. [Table 4]

Proximate composition of fish carcass

Fish groups fed all six different diets showed different proximate composition by the diets they were fed. The moisture content of the NFO diet group showed the highest and significant difference with the control diet group, it also showed the lowest lipid content among the experimental groups. Lipid content of algae meal-diet groups showed no

significant difference among the algae meal-diet fed groups, but it showed significantly lower lipid content than the control diet group. [Table 5]

The lipid composition of the whole body and liver is shown in Table 6. In the whole body, the polar lipid content of each diet group showed no significant difference, but there was a significant difference in neutral lipid content. In the NFO diet group, fish showed the lowest neutral lipid content ($P < 0.05$). In the fish liver, it also showed no significant difference in the polar lipid content, but it was shown a significant difference in the neutral lipid content. Similar to the neutral lipid content of the whole body, the NFO diet fed fish group showed significantly lower neutral lipid content ($P < 0.05$).

Fatty acid profile

The fish fed six different diets showed a significant difference in the fatty acid profile of total lipid from whole body. It was shown that a similar trend with the dietary fatty acid except for the NFO diet group. Fatty acid composition of the saturates, monoenes and n-6 polyunsaturated fatty acids (PUFA) in the fish carcass of control, FO and AM diet fed fish groups reflected the diet dietary fatty acids. In the n-3 PUFA composition of the total fish body, the accumulation of n-3 LC-PUFA including eicosatetraenoic acid (20:4n-3), EPA (20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) and DHA (22:6n-3) was detected. AM15 diet group showed the highest n-3 LC-PUFA levels among the experimental diet fed fish group. NFO diet fed group showed the lowest rate of myristic acid (14:0), linolenic acid (18:2n-6), alpha-linolenic acid (18:3n-3) and eicosatetraenoic acid (20:4n-3), but it showed

the highest composition on stearic acid (18:0). [Table 7]

The result of the fatty acid profile of the total lipid of the fish liver showed a similar trend with the fatty acid composition in whole body of the fish. Each group showed a similar amount of total saturates, monoenes and n-6 PUFA. Total n-3 PUFA content in total lipid in the fish liver was lower than fatty acid composition in the fish body. The other groups except for the NFO diet group reflected the fatty acid profile of the diets. [Table 8]

As the result of polar lipid fatty acid analysis in the fish body, there was a significant difference in every fatty acid except stearic acid (18:0), gadoleic acid (20:1n-11) and total PUFA. Fatty acid profile of polar lipid in the fish body showed lower total monounsaturated fatty acid and n-6 PUFA level but higher n-3 PUFA level than that of total lipid in fish body. 18:1/n-3 LC-PUFA ratio which implies a deficiency of EFA when its numerical value is more than 1 was also calculated. NFO diet fed fish group showed the highest ratio. [Table 9]

The fatty acid profile of the polar lipid in the liver showed a similar trend of the fatty acid composition of polar lipid in the whole body of fish. It showed a higher trend in total saturated fatty acid and total monounsaturated fatty acid levels. In n-3 LC-PUFA composition, AM10 diet fed fish showed the significantly highest among the diets fed groups. [Table 10]

2.4 Discussion

The EFA requirement of juvenile red sea bream has been known as 1% EPA and 0.5% DHA in diet, respectively (Takeuchi et al., 1990). In this experiment, most of the experimental diets met the EFA requirement with more than 0.5% of DHA content in diet

except the NFO diet.

As a result of growth performance, there was no significant difference between fish fed control and algae meal diets. Our previous study also showed similar growth of fish fed fishmeal based diet supplemented with fish oil and algae meal formulated non-fishmeal and non-fish oil diets (Seong et al., 2019). In our previous experiment, the formula of the control diet and the FO diet were the same with the present study and the algae meal diet was mostly the same as the AM10 diet in the present study (Seong et al., 2019). Fish fed the NFO diet showed the lowest growth performance probably because of low feed intake of the group. This result possibly suggests that juvenile red sea bream may distinguish the type of lipid source in the diet and that affects the palatability of the fish diet which has been reported in rainbow trout (Geurden et al., 2005).

Significant difference was observed in moisture and lipid content of the whole body. Fish fed NFO diet showed the highest moisture content but the lowest lipid content ($P < 0.05$). There might be strong accordance with growth performance, the NFO group stored less lipid than the other groups because of lower feed intake. The algae meal group showed lower lipid content in the whole body than the control and this was similar with previous study (Seong et al., 2019). This result may indicate that the lipid of algae meal is not accumulated in the fish body than the other lipid sources.

The polar and neutral lipid content in whole body and liver of fish showed a similar trend. In the whole body and liver, there was no significant difference in polar lipid content among the groups, but there was significant difference in the neutral lipid content among the groups. The results may suggest an upper limit of polar lipid content in whole body and liver. Fish fed

algae meal diet showed a significantly lower neutral lipid content in the whole body than fish fed control and FO diets. This could be due to low neutral lipid content in *Schizochytrium* sp. meal [Table 6]. On the contrary, FO, AM5 and AM10 groups showed higher hepatic neutral lipid content than other groups including control. Fish fed AM15 diet showed the lowest neutral lipid content which appeared to be affected by full dietary lipid substitution by algae meal which contains little neutral lipid. These results also suggested that neutral lipids in diet can be easily assimilated and stored in fish liver.

It has been known that n-3 LC-PUFA is the most important fatty acid for marine finfish species. But they have limited or no ability to desaturate and elongate 18:3n-3 to n-3 LC-PUFA as well as 18:2n-6 to 22:5n-6 via 20:4n-6 because of low or absent Δ6- and Δ5-desaturase activities (Bell et al., 1994; Montero et al., 2004; Izquierdo et al., 2005). Therefore, the result of fatty acid composition on the total lipid of fish body and liver were strongly influenced by the different fatty acid compositions of each six diets. Unlike other groups, it did not reflect the fatty acid profile of the diet in fish fed NFO diet. That might occur due to low growth performance caused by lower feed intake. Considering fatty acid composition in the diet and total lipid in whole body, DHA and n-3 LC-PUFA have been accumulated in the fish body with increasing dietary AM inclusion. Also, the total fatty acid of fish carcass suggested that dietary saturated fatty acid was preferentially used as a source of energy in the red sea bream particularly in AM 10 and AM15 groups. In the algae meal group, 1.72-2.15 fold higher DHA was observed in fatty acid composition of total lipid in whole body than those of dietary fatty acid. In contrast, 1-1.9 fold higher DHA was detected in fatty acid composition of hepatic total lipid of the fish. These results seem to suggest that dietary DHA

in algae meal seems to be accumulated in extrahepatic tissues of fish fed AM.

Fatty acid composition of polar lipids in the fish body generally showed different trends from those of the total lipid of the whole body and dietary lipids. The result seems to suggest selective utilization and retention of specific fatty acids. For instance, a higher total saturates level was observed in fatty acid composition of polar lipid from whole body than those of the total lipid in the whole body and dietary lipid although the difference was not significant. Palmitic acid (16:0) and stearic acid (18:0) showed higher accumulation, but myristic acid (14:0) content was lower than those of the total lipid of whole body and dietary lipid. All groups showed generally lower monoenes content in hepatic polar lipid than those of total lipid of the whole body and dietary lipid. This result suggests that most of the monounsaturated fatty acid was stored as neutral lipid. Total n-6 LC-PUFA content in polar lipid of the whole body was lower than those in total lipid of whole body and dietary lipid. On the other hand, total n-3 PUFA contents in dietary lipid, total lipid of whole body and hepatic polar lipid were different. 18:3n-3, 18:4n-3 and 20:4n-3 contents in hepatic polar lipid were lower but EPA, DPA, and DHA contents were higher or similar with total lipid of whole body and dietary lipid. These results may suggest that the LC-PUFA in diets have been utilized selectively and accumulated preferentially as forms of polar lipid in fish body. The fatty acid composition of the polar lipid in fish liver showed higher saturated fatty acid but lower mono-unsaturated fatty acid composition than that of the polar lipid in the whole body of fish, suggesting selective accumulation and consumption of these fatty acids. But the total n-6 PUFA and n-3 PUFA content showed a similar trend. The ratio of 18:1n-9/Σn-3 LC-PUFA in the fatty acid composition of polar lipid of the whole body has considered as an

EFA index for red sea bream (Fujii and Yone, 1976) and gilthead bream (Kalogeropoulos et al., 1992). When the ratio more than 1 is considered as EFA deficiency associated with poor growth performance and feed efficiency. Except for fish fed NFO diet whose ratio was 1, the other groups showed negative growth performance with the ratio less than 1.

In conclusion, DHA-rich microalgae *Schizochytrium* sp. meal is a suitable substitute for fish oil for the juvenile red sea bream diet, and the optimal supplementation level could be 5 to 10% in diet. By using microalgae meal, it could be possible to develop non-fish meal and non-fish oil diet for marine fish, not only red sea bream. Also, future work should include the investigation of histological and immunological reactions by the reason of increased DHA level in the fish body with the inclusion of the algae meal.

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2.5 Experimental tables

Table 1. Formula of the experimental diets for red sea bream (g kg^{-1})

	Experimental diets					
Ingredients	Control	FO	NFO	AM5	AM10	AM15
Peruvian anchovy meal	400	0	0	0	0	0
Soy protein concentrate	0	140	140	120	110	100
Soybean meal	110	200	200	200	200	200
Corn gluten meal	110	350	350	366	366	366
Schizochytrium sp. meal [a]	0	0	0	50	100	150
Wheat flour	100	6	6	0	0	0
Celluose	54	0	0	0	0	0
Alpha-starch	60	60	60	60	60	60
Fish oil [b]	60	80	0	0	0	0
Rapeseed oil	50	40	120	80	40	0
Vitamin premix [c]	30	30	30	30	30	30
Vitamin E	1	1	1	1	1	1
Choline chloride	5	5	5	5	5	5
Mineral premix [d]	10	10	10	10	10	10
Calcium phosphate	10	40	40	40	40	40
Amino acid (Lys1, Met0.5, Thr0.5, Try0.2)	0	22	22	22	22	22
Bonito peptide	0	10	10	10	10	10
SSF Enzyme mix [e]	0	1	1	1	1	1
Taurine	0	5	5	5	5	5
Total	1000	1000	1000	1000	1000	1000

[a] *Schizochytrium* sp. algae meal (Alltech Inc., Lexington, Kentucky, U.S.A).

[b] Cod liver oil (Kanematsu Shintoa Foods Co. Ltd, Tokyo, Japan).

[c] Vitamin mixture composition (unit kg^{-1}) : Vitamin D_3, 2420000 IU; Vitamin K_3, 6050 mg; thiamin, 3025 mg; riboflavin, 3630 mg; pyridoxine, 2420 mg; cyanocobalamine, 6 mg; L-ascorbic acid, 368900 mg; nicotinic acid, 24200 mg; D-pantothenic acid, 6050 mg; inositol,

121 000 mg; D-biotin, 363 mg; folic acid, 908 mg; para-aminobenzoic acid, 3025 mg.

[d] Mineral mixture compostition (g/kg^{-1}) : Sodium chloride 50; magnesium sulphate 745; iron(III) cirtrate n-hydrate 125; trace element mix 50; cellulose 30 [trace element mixture contains ($g\ kg^{-1}$) : zinc sulphateheptahydrate 353; manganese sulphate 162; copper(II) sulphate pentahydrate 31; aluminum chloride hexahydrate 10; cobalt chloride hexahydrate 10; cobalt chloride 3; potassium iodate 1; cellulose 440].

[e] SSF (Alltech Inc., Lexington, Kentucky, U.S.A).

Table 2. Proximate composition of the AM and experimental diets (% dry weight basis except for % moisture)

	AM[a]	Control	FO	NFO	AM5	AM10	AM15
Moisture	1.5	3.1	2.4	2.1	1.9	2.1	2.2
Protein	12.5	43.5	44.0	46.7	46.0	46.6	47.0
Lipid	67.7	14.5	13.1	13.4	14.8	13.1	14.0
Ash	3.7	8.8	7.1	7.0	7.1	7.2	7.2

[a] Algae meal : *Schizochytrium* sp. algae meal (Alltech Co., Lexington, Kentucky, U.S.A)

Table 3. Fatty acid composition of the AM and diets (area%)

	AM[a]	Control	FO	NFO	AM5	AM10	AM15
14:0	6.0	3.8	3.3	0.0	1.5	3.1	5.1
16:0	59.7	12.7	11.8	6.4	20.3	37.5	55.3
18:0	1.4	2.3	1.8	1.5	1.7	1.5	1.6
ΣSFA[*]	67.0	18.8	16.9	7.9	23.4	42.1	61.9
16:1n-7	0.1	4.0	4.2	0.2	0.3	0.2	0.2
18:1n-9	0.0	30.2	29.4	54.9	39.8	22.8	4.9
20:1n-9	0.0	2.7	3.5	0.0	0.0	0.0	0.1
20:1n-11	0.0	2.1	2.2	0.8	0.5	0.2	0.0
22:1n-11	0.0	4.9	4.9	0.0	0.0	0.0	0.1
ΣMUFA[**]	0.1	43.2	44.3	55.9	40.7	23.3	5.2
18:2n-6	0.1	13.0	17.5	25.6	20.5	18.1	12.2
20:4n-6	0.0	0.3	0.2	0.0	0.2	0.3	0.5
Σn-6 PUFA[***]	0.1	13.3	17.6	25.6	20.7	18.4	12.7
18:3n-3	0.0	3.2	2.9	6.0	4.4	2.6	0.6
18:4n-3	0.1	1.2	1.2	0.0	0.0	0.0	0.1
20:4n-3	0.3	0.3	0.3	0.1	0.1	0.1	0.2
20:5n-3	0.2	4.8	4.1	0.0	0.2	0.1	0.2
22:5n-3	0.0	0.6	0.5	0.0	0.1	0.1	0.0
22:6n-3	22.0	4.9	3.2	0.1	4.3	7.3	12.1
Σn-3 PUFA[***]	22.7	15.1	12.3	6.2	9.1	10.1	13.2
22:4n-9	0.0	0.1	8.7	4.4	6.1	6.1	6.9
ΣPUFA[***]	22.7	28.4	29.9	31.8	29.8	28.5	25.9
Σn-3 LCPUFA[****]	22.7	11.8	9.3	0.2	4.7	7.5	12.6

[a] Algae meal : *Schizochytrium* sp. algae meal (Alltech Inc., Lexington, Kentucky, U.S.A).

[*] Saturated fatty acid.

[**] Monounsaturated fatty acid.

[***] Polyunsaturated fatty acid.

[****] Long-chain polyunsaturated fatty acid.

Table 4. Growth performance and feed utilization efficiencies of red sea bream fed experimental diets over 12 weeks (mean±SD, n=3)

	Control	FO	NFO	AM5	AM10	AM15
IW(g)	8.8 ± 2.5	8.8 ± 2.5	8.8 ± 2.5	8.8 ± 2.5	8.8 ± 2.5	8.8 ± 2.5
FW(g)	51.5 ± 11.8^{a}	42.6 ± 15.1^{a}	13.1 ± 1.0^{b}	36.9 ± 6.2^{a}	46.3 ± 5.1^{a}	43.0 ± 3.2^{a}
WG(%)	587.4 ± 136.5^{a}	486.0 ± 169.4^{a}	148.9 ± 11.4^{b}	423.1 ± 69.6^{a}	527.2 ± 58.4^{a}	489.1 ± 35.8^{a}
FCR	1.3 ± 0.1^{b}	1.5 ± 0.3^{b}	3.3 ± 0.7^{a}	1.5 ± 0.5^{b}	1.2 ± 0.1^{b}	1.3 ± 0.2^{b}
PER	1.9 ± 0.2^{a}	1.5 ± 0.4^{ab}	0.6 ± 0.1^{b}	1.3 ± 0.6^{ab}	1.5 ± 0.2^{ab}	1.5 ± 0.4^{ab}
SGR	2.1 ± 0.3^{a}	1.8 ± 0.4^{a}	0.5 ± 0.1^{b}	1.7 ± 0.2^{a}	2.0 ± 0.1^{a}	1.9 ± 0.1^{a}
SR(%)	68.6 ± 12.3^{a}	56.9 ± 14.8^{a}	66.7 ± 6.8^{a}	54.9 ± 18.0^{a}	58.8 ± 10.2^{a}	58.8 ± 21.2^{a}
DFI(%)	2.2 ± 0.2^{a}	2.4 ± 0.3^{a}	1.6 ± 0.1^{a}	2.7 ± 0.9^{a}	2.5 ± 0.3^{a}	2.5 ± 0.5^{a}
HSI	1.3 ± 0.1^{ab}	1.8 ± 0.3^{a}	1.1 ± 0.1^{b}	1.6 ± 0.2^{ab}	1.3 ± 0.2^{b}	1.0 ± 0.1^{b}

Values with different superscrip letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

Table 5. Proximate composition of the whole body of the fish fed the experimental diet (% wet weight basis, mean±SD, n=3)

	Control	FO	NFO	AM5	AM10	AM15
Moisture	66.6 ± 2.8^{b}	69.9 ± 2.4^{ab}	74.0 ± 1.7^{a}	71.9 ± 0.6^{ab}	70.3 ± 4.6^{ab}	72.4 ± 0.9^{ab}
Ash	4.8 ± 1.8^{a}	4.5 ± 1.4^{a}	6.6 ± 1.2^{a}	3.8 ± 0.9^{a}	4.1 ± 0.5^{a}	3.8 ± 0.8^{a}
Protein (w.b)	17.3 ± 0.8^{a}	15.7 ± 0.8^{a}	17.1 ± 1.5^{a}	16.2 ± 1.6^{a}	17.2 ± 1.3^{a}	17.3 ± 0.7^{a}
Lipid (w.b)	11.3 ± 0.4^{a}	8.8 ± 1.3^{ab}	2.4 ± 0.2^{c}	5.0 ± 1.5^{b}	7.3 ± 1.9^{b}	6.8 ± 1.5^{b}

Values with different superscrip letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c)...

Table 6. Polar and neutral lipid content in AM, whole body and liver of fish (% wet weight basis ,mean±SD, n=3)

		Lipid in whole fish body					
	AM*	Control	FO	NFO	AM5	AM10	AM15
Polar (%)	64.63	1.10±0.23[a]	0.88±0.18[a]	1.06±0.08[a]	1.27±0.39[a]	0.85±0.45[a]	0.70±0.19[a]
Neutral (%)	0.03	10.24±0.23[a]	7.88±0.18[b]	1.33±0.08[d]	6.47±0.39[c]	6.40±0.45[c]	6.06±0.19[c]

	Lipid in fish liver					
	Control	FO	NFO	AM5	AM10	AM15
Polar (%)	1.94±0.21[a]	2.66±0.24[a]	2.51±0.14[a]	1.51±0.70[a]	2.21±0.65[a]	1.60±0.18[a]
Neutral (%)	14.18±0.21[b]	18.33±0.24[a]	4.65±0.14[c]	17.77±0.70[a]	18.72±0.65[a]	8.02±0.18[c]

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Algae meal: *Schizochytrium* sp. algae meal (Alltech Inc., Lexington, Kentucky, U.S.A).

Table 7. Fatty acid composition of total lipid from whole body of fish (area %, mean±SD, n=3)

	Control	FO	NFO	AM05	AM10	AM15
14:0	2.5 ± 0.0^{a}	2.5 ± 0.1^{a}	1.9 ± 0.2^{b}	1.2 ± 0.1^{c}	1.8 ± 0.3^{b}	2.5 ± 0.2^{a}
16:0	14.3 ± 0.3^{c}	15.2 ± 0.9^{c}	15.2 ± 0.9^{c}	14.8 ± 1.0^{c}	19.3 ± 0.5^{b}	27.1 ± 1.7^{a}
18:0	3.9 ± 0.0^{b}	4.3 ± 0.4^{b}	5.9 ± 0.8^{a}	3.4 ± 0.4^{b}	3.7 ± 0.2^{b}	3.6 ± 0.3^{b}
ΣSFA*	21.0 ± 0.3^{c}	22.2 ± 1.3^{bc}	23.2 ± 1.6^{bc}	19.6 ± 1.4^{d}	25.0 ± 1.0^{b}	33.4 ± 1.9^{a}
16:1n-7	3.6 ± 0.1^{a}	3.2 ± 0.0^{b}	1.9 ± 0.2^{c}	1.1 ± 0.0^{d}	1.2 ± 0.2^{d}	1.1 ± 0.2^{d}
18:1n-9	28.5 ± 0.6^{b}	26.4 ± 1.2^{b}	26.6 ± 2.9^{b}	36.3 ± 1.6^{a}	26.2 ± 1.3^{b}	9.5 ± 1.0^{c}
20:1n-9	3.2 ± 0.0^{b}	3.7 ± 0.2^{a}	0.6 ± 0.1^{c}	0.2 ± 0.2^{d}	0.2 ± 0.1^{d}	0.1 ± 0.1^{d}
20:1n-11	2.1 ± 0.0^{a}	2.1 ± 0.0^{a}	1.5 ± 0.1^{b}	1.4 ± 0.1^{bc}	1.1 ± 0.1^{c}	0.4 ± 0.1^{d}
22:1n-11	2.8 ± 0.1^{b}	3.1 ± 0.1^{a}	0.5 ± 0.1^{c}	0.2 ± 0.0^{d}	0.2 ± 0.1^{d}	0.1 ± 0.1^{d}
ΣMUFA**	40.2 ± 0.6^{a}	38.5 ± 1.5^{a}	31.1 ± 3.3^{b}	39.1 ± 1.5^{a}	28.8 ± 1.0^{b}	11.2 ± 1.2^{c}
18:2n-6	13.0 ± 0.2^{d}	16.6 ± 0.7^{bc}	15.9 ± 2.0^{cd}	20.9 ± 1.0^{a}	19.3 ± 1.3^{ab}	16.8 ± 1.0^{bc}
20:4n-6	0.4 ± 0.0^{c}	0.4 ± 0.1^{c}	0.8 ± 0.2^{b}	0.4 ± 0.0^{c}	0.7 ± 0.1^{b}	1.1 ± 0.0^{a}
Σn-6 PUFA***	14.0 ± 0.2^{c}	17.5 ± 0.7^{b}	17.4 ± 1.8^{b}	21.7 ± 1.0^{a}	20.4 ± 1.2^{ab}	18.2 ± 1.1^{b}
18:3n-3	2.5 ± 0.0^{b}	1.9 ± 0.1^{bc}	1.7 ± 0.4^{c}	3.4 ± 0.4^{a}	2.4 ± 0.3^{bc}	0.8 ± 0.0^{d}
18:4n-3	1.0 ± 0.1^{a}	0.9 ± 0.0^{a}	0.3 ± 0.0^{b}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}
20:4n-3	0.6 ± 0.0^{a}	0.5 ± 0.0^{a}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.3 ± 0.0^{c}	0.4 ± 0.0^{b}
20:5n-3	4.7 ± 0.2^{a}	3.7 ± 0.1^{b}	2.3 ± 0.1^{c}	0.9 ± 0.1^{d}	1.1 ± 0.2^{d}	0.9 ± 0.2^{d}
22:5n-3	1.6 ± 0.0^{a}	1.2 ± 0.0^{b}	1.3 ± 0.2^{ab}	0.5 ± 0.2^{c}	0.4 ± 0.1^{c}	0.3 ± 0.1^{c}
22:6n-3	7.1 ± 0.3^{d}	6.4 ± 0.8^{d}	10.9 ± 2.6^{c}	7.4 ± 0.6^{d}	14.4 ± 1.2^{b}	26.1 ± 0.3^{a}
Σn-3 PUFA***	17.5 ± 0.5^{bc}	14.6 ± 0.8^{cd}	16.7 ± 2.3^{bc}	12.5 ± 0.5^{d}	18.7 ± 1.2^{b}	28.7 ± 0.3^{a}
ΣPUFA***	31.4 ± 0.5^{d}	32.0 ± 1.2^{cd}	34.1 ± 0.6^{c}	34.2 ± 0.5^{c}	39.0 ± 0.1^{b}	46.9 ± 1.3^{a}
Σn-3 LCPUFA****	15.0 ± 0.5^{b}	12.6 ± 0.9^{bc}	15.0 ± 2.7^{b}	9.1 ± 0.8^{c}	16.3 ± 1.5^{b}	27.9 ± 0.3^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 8. Fatty acid composition of hepatic total lipid in fish (area %, mean±SD, n=3)

	Control	FO	NFO	AM5	AM10	AM15
14:0	1.8 ± 0.2^{ab}	1.9 ± 0.0^{a}	1.5 ± 0.1^{b}	0.7 ± 0.0^{c}	1.0 ± 0.0^{b}	2.0 ± 0.1^{a}
16:0	13.5 ± 0.6^{c}	13.0 ± 0.6^{c}	13.4 ± 0.6^{c}	12.6 ± 0.6^{c}	17.2 ± 0.5^{b}	27.8 ± 1.7^{a}
18:0	3.5 ± 0.3^{b}	2.4 ± 0.2^{b}	6.2 ± 1.4^{a}	1.9 ± 0.1^{b}	2.8 ± 1.1^{b}	3.9 ± 0.5^{b}
ΣSFA*	18.9 ± 1.1^{bc}	17.4 ± 0.9^{cd}	21.3 ± 2.2^{b}	15.2 ± 0.8^{d}	21.2 ± 1.7^{b}	33.8 ± 2.3^{a}
16:1n-7	4.0 ± 0.1^{a}	3.6 ± 0.1^{a}	1.8 ± 0.3^{b}	0.8 ± 0.1^{d}	1.1 ± 0.3^{cd}	1.4 ± 0.2^{bc}
18:1n-9	31.0 ± 1.4^{b}	30.0 ± 3.0^{b}	20.1 ± 2.3^{c}	38.5 ± 1.3^{a}	26.7 ± 2.9^{b}	9.4 ± 1.2^{d}
20:1n-9	2.8 ± 0.2^{a}	3.2 ± 0.5^{a}	0.7 ± 0.3^{b}	0.1 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
20:1n-11	2.7 ± 0.5^{a}	2.4 ± 0.7^{a}	1.2 ± 0.3^{ab}	1.3 ± 0.4^{ab}	1.6 ± 0.8^{ab}	0.5 ± 0.1^{b}
22:1n-11	1.9 ± 0.2^{a}	2.1 ± 0.2^{a}	0.4 ± 0.2^{b}	0.0 ± 0.0^{c}	0.1 ± 0.1^{c}	0.0 ± 0.0^{c}
ΣMUFA**	42.3 ± 2.4^{a}	41.3 ± 4.5^{a}	24.3 ± 3.4^{b}	40.6 ± 1.8^{a}	29.5 ± 4.1^{b}	11.3 ± 1.6^{c}
18:2n-6	13.4 ± 0.7^{c}	19.6 ± 2.0^{b}	18.3 ± 2.7^{bc}	26.8 ± 0.8^{a}	22.3 ± 2.4^{ab}	17.6 ± 1.1^{bc}
20:4n-6	0.4 ± 0.0^{bc}	0.3 ± 0.1^{c}	0.9 ± 0.2^{ab}	0.4 ± 0.0^{c}	0.9 ± 0.2^{ab}	1.3 ± 0.3^{a}
Σn-6 PUFA***	14.7 ± 0.8^{c}	20.8 ± 2.3^{b}	20.3 ± 3.6^{b}	27.8 ± 1.1^{a}	24.2 ± 2.9^{ab}	19.7 ± 1.5^{bc}
18:3n-3	2.3 ± 0.2^{b}	2.0 ± 0.1^{b}	2.0 ± 0.2^{b}	3.8 ± 0.2^{a}	2.2 ± 0.2^{b}	0.7 ± 0.1^{c}
18:4n-3	0.4 ± 0.1^{a}	0.4 ± 0.0^{a}	0.2 ± 0.1^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
20:4n-3	0.5 ± 0.3^{ab}	0.7 ± 0.1^{a}	0.2 ± 0.1^{bc}	0.1 ± 0.1^{c}	0.2 ± 0.0^{bc}	0.3 ± 0.0^{bc}
20:5n-3	3.7 ± 0.4^{a}	2.9 ± 0.5^{a}	2.0 ± 0.2^{b}	0.3 ± 0.0^{c}	0.5 ± 0.2^{c}	0.8 ± 0.0^{c}
22:5n-3	2.0 ± 0.2^{a}	1.5 ± 0.1^{b}	1.1 ± 0.2^{c}	0.2 ± 0.0^{d}	0.3 ± 0.0^{d}	0.4 ± 0.0^{d}
22:6n-3	5.3 ± 0.4^{c}	4.5 ± 1.8^{c}	7.4 ± 2.0^{c}	4.3 ± 0.2^{c}	13.0 ± 3.1^{b}	22.7 ± 2.3^{a}
Σn-3 PUFA***	14.2 ± 1.6^{bc}	11.9 ± 2.7^{bc}	13.0 ± 2.8^{bc}	8.6 ± 0.5^{c}	16.2 ± 3.5^{b}	24.9 ± 2.4^{a}
Total PUFA***	28.8 ± 2.4^{c}	32.7 ± 5.0^{bc}	33.3 ± 6.4^{bc}	36.4 ± 1.6^{abc}	40.4 ± 6.4^{ab}	44.6 ± 3.9^{a}
Σn-3 LCPUFA****	11.9 ± 1.4^{b}	10.0 ± 2.6^{bc}	11.0 ± 2.6^{b}	4.8 ± 0.4^{c}	14.0 ± 3.3^{b}	24.2 ± 2.4^{a}
18:1/Σn-3 LCPUFA****	2.6 ± 1.0^{b}	3.0 ± 1.1^{b}	1.8 ± 0.9^{b}	8.0 ± 3.4^{a}	1.9 ± 0.9^{b}	0.4 ± 0.5^{c}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 9. Fatty acid composition in the polar lipid from whole body of fish (area %, mean±SD, n=3)

	Control	FO	NFO	AM5	AM10	AM15
14:0	0.5 ± 0.1^{a}	0.4 ± 0.1^{a}	0.3 ± 0.0^{b}	0.2 ± 0.0^{b}	0.3 ± 0.0^{b}	0.4 ± 0.1^{a}
16:0	20.4 ± 1.1^{bc}	19.9 ± 0.4^{bc}	16.8 ± 0.1^{c}	20.0 ± 0.9^{bc}	22.8 ± 1.5^{b}	28.6 ± 3.3^{a}
18:0	7.1 ± 0.1^{a}	6.7 ± 0.2^{a}	7.3 ± 0.7^{a}	6.2 ± 0.3^{a}	6.6 ± 0.3^{a}	7.0 ± 0.4^{a}
ΣSFA*	28.1 ± 1.2^{bc}	27.2 ± 0.2^{bc}	24.6 ± 0.8^{c}	26.6 ± 0.7^{bc}	29.8 ± 1.5^{b}	36.2 ± 3.8^{a}
16:1n-7	1.0 ± 0.1^{a}	0.9 ± 0.0^{a}	0.6 ± 0.0^{b}	0.3 ± 0.0^{c}	0.4 ± 0.0^{c}	0.6 ± 0.0^{b}
18:1n-9	15.3 ± 0.7^{c}	15.0 ± 1.3^{c}	21.3 ± 0.3^{a}	18.0 ± 0.1^{b}	12.9 ± 0.6^{d}	7.0 ± 0.3^{e}
20:1n-9	0.9 ± 0.1^{a}	1.0 ± 0.1^{a}	0.2 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
20:1n-11	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n-11	0.3 ± 0.0^{b}	0.4 ± 0.0^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
ΣMUFA**	17.5 ± 0.9^{b}	17.2 ± 1.4^{b}	22.1 ± 0.3^{a}	18.3 ±0.1^{b}	13.3 ± 0.6^{c}	7.6 ± 0.4^{d}
18:2n-6	10.1 ± 0.5^{cd}	14.0 ± 0.4^{b}	17.8 ± 1.5^{a}	17.4 ± 0.9^{a}	12.2 ± 1.0^{bc}	8.9 ± 0.3^{d}
20:4n-6	1.0 ± 0.1^{a}	1.0 ± 0.1^{a}	1.0 ± 0.1^{ab}	0.8 ± 0.1^{bc}	0.6 ± 0.0^{c}	0.3 ± 0.0^{d}
Σn-6 PUFA***	11.2 ± 0.6^{cd}	15.0 ± 0.4^{b}	18.9 ± 1.6^{a}	18.2 ± 0.9^{a}	12.8 ± 1.1^{bc}	9.1 ± 0.3^{d}
18:3n-3	0.9 ± 0.1^{bc}	0.7 ± 0.0^{cd}	1.0 ± 0.1^{ab}	1.2 ± 0.1^{a}	0.6 ± 0.1^{d}	0.2 ± 0.0^{e}
18:4n-3	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
20:4n-3	0.3 ± 0.0^{a}	0.4 ± 0.0^{a}	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
20:5n-3	7.3 ± 0.2^{a}	6.4 ± 0.3^{b}	2.1 ± 0.1^{c}	1.1 ± 0.1^{d}	1.0 ± 0.1^{d}	0.8 ± 0.0^{d}
22:5n-3	2.3 ± 0.1^{a}	1.9 ± 0.1^{b}	1.3 ± 0.1^{c}	0.4 ± 0.1^{d}	0.3 ± 0.0^{d}	0.2 ± 0.0^{d}
22:6n-3	21.0 ± 0.8^{b}	19.7 ± 2.1^{b}	17.8 ± 1.6^{b}	21.4 ± 1.4^{b}	29.1 ± 2.7^{a}	32.2 ± 3.1^{a}
Σn-3 PUFA***	32.0 ± 0.9^{a}	29.3 ± 1.9^{ab}	22.3 ± 1.4^{c}	24.2 ± 1.4^{bc}	31.1 ± 2.7^{a}	33.5 ± 3.2^{a}
Σ PUFA***	43.2 ± 1.0^{a}	44.3 ± 1.9^{a}	41.2 ± 0.6^{a}	42.4 ± 0.8^{a}	43.9 ± 1.8^{a}	42.6 ± 3.3^{a}
Σn-3 LCPUFA****	31.1 ± 0.9^{a}	28.6 ± 2.0^{ab}	21.3 ± 1.5^{b}	23.0 ± 1.5^{b}	30.5 ± 2.8^{a}	33.3 ± 3.2^{a}
18:1/Σn-3 LCPUFA****	0.5 ± 0.0^{c}	0.5 ± 0.1^{c}	1.0 ± 0.1^{a}	0.8 ± 0.1^{b}	0.4 ± 0.1^{c}	0.2 ± 0.0^{d}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 10. Fatty acid composition of the hepatic polar lipid in fish (area %, mean±SD, n=3)

	Control	FO	NFO	AM5	AM10	AM15
14:0	0.8 ± 0.0^{a}	0.5 ± 0.1^{b}	0.6 ± 0.1^{ab}	0.2 ± 0.1^{d}	0.3 ± 0.1^{cd}	0.4 ± 0.0^{bc}
16:0	24.0 ± 1.0^{b}	19.6 ± 1.8^{bc}	15.9 ± 0.5^{c}	15.6 ± 3.0^{c}	24.9 ± 3.4^{b}	36.5 ± 3.5^{a}
18:0	8.7 ± 0.5^{ab}	9.2 ± 0.9^{ab}	10.2 ± 1.9^{a}	7.3 ± 0.6^{b}	7.8 ± 0.5^{ab}	9.2 ± 0.5^{ab}
ΣSFA*	33.6 ± 1.4^{b}	29.4 ± 2.8^{bc}	27.1 ± 2.3^{bc}	23.3 ± 3.4^{c}	33.1 ± 3.7^{b}	46.2 ± 3.5^{a}
16:1n-7	1.1 ± 0.1^{a}	0.7 ± 0.0^{b}	0.5 ± 0.2^{bc}	0.2 ± 0.1^{d}	0.3 ± 0.1^{cd}	0.3 ± 0.0^{cd}
18:1n-9	10.0 ± 0.8^{bc}	10.7 ± 0.9^{abc}	13.5 ± 3.1^{ab}	14.1 ± 0.6^{a}	8.5 ± 0.4^{c}	3.3 ± 0.4^{d}
20:1n-9	0.5 ± 0.1^{b}	0.7 ± 0.1^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.1 ± 0.1^{c}
20:1n-11	0.9 ± 0.1^{ab}	1.1 ± 0.2^{a}	0.8 ± 0.2^{ab}	0.8 ± 0.1^{ab}	0.5 ± 0.1^{bc}	0.1 ± 0.1^{c}
22:1n-11	0.5 ± 0.0^{b}	0.6 ± 0.1^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
ΣMUFA**	13.0 ± 0.9^{ab}	13.8 ± 1.3^{a}	14.9 ± 3.3^{a}	15.0 ± 0.7^{a}	9.3 ± 0.4^{b}	3.8 ± 0.4^{c}
18:2n-6	7.8 ± 0.6^{c}	11.4 ± 0.4^{abc}	14.7 ± 4.0^{ab}	15.8 ± 1.0^{a}	10.4 ± 0.7^{bc}	7.2 ± 0.5^{c}
20:4n-6	2.3 ± 0.1^{b}	2.3 ± 0.3^{b}	3.5 ± 0.9^{ab}	2.7 ± 0.2^{ab}	3.6 ± 0.9^{ab}	3.9 ± 0.4^{a}
Σn-6 PUFA***	11.1 ± 0.5^{b}	14.8 ± 0.4^{b}	19.1 ± 3.4^{a}	19.6 ± 1.1^{a}	14.8 ± 1.0^{b}	11.8 ± 0.5^{b}
18:3n-3	0.8 ± 0.1^{b}	0.7 ± 0.1^{b}	0.8 ± 0.2^{b}	1.2 ± 0.1^{a}	0.6 ± 0.0^{b}	0.1 ± 0.0^{c}
18:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n-3	0.4 ± 0.0^{a}	0.5 ± 0.2^{a}	0.0 ± 0.0^{b}	0.1 ± 0.1^{b}	0.1 ± 0.0^{b}	0.1 ± 0.1^{b}
20:5n-3	7.0 ± 0.3^{a}	7.5 ± 0.5^{a}	3.1 ± 0.3^{b}	1.4 ± 0.3^{c}	1.1 ± 0.2^{c}	0.8 ± 0.1^{c}
22:5n-3	2.2 ± 0.3^{a}	2.1 ± 0.4^{a}	1.3 ± 0.2^{b}	0.2 ± 0.1^{c}	0.1 ± 0.0^{c}	0.0 ± 0.0^{c}
22:6n-3	23.4 ± 2.0^{bc}	24.5 ± 1.1^{bc}	20.4 ± 3.5^{c}	27.4 ± 2.9^{ab}	32.8 ± 2.4^{a}	28.3 ± 1.2^{ab}
Σn-3 PUFA***	33.9 ± 2.3^{a}	35.3 ± 1.6^{a}	25.5 ± 3.5^{b}	30.3 ± 3.3^{ab}	34.7 ± 2.3^{a}	29.2 ± 1.1^{ab}
Σ PUFA***	44.9 ± 1.9^{ab}	50.1 ± 2.0^{a}	44.6 ± 2.6^{ab}	49.9 ± 4.3^{a}	49.4 ± 3.2^{a}	41.0 ± 1.2^{b}
Σn-3 LCPUFA****	33.1 ± 2.4^{ab}	34.7 ± 1.6^{a}	24.7 ± 3.6^{c}	29.1 ± 3.2^{bc}	34.1 ± 2.3^{a}	29.1 ± 1.1^{bc}
18:1/Σn-3 LCPUFA****	0.3 ± 0.0^{bc}	0.3 ± 0.0^{bc}	0.5 ± 0.2^{a}	0.5 ± 0.0^{ab}	0.3 ± 0.0^{c}	0.1 ± 0.0^{c}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Chapter 3.

Non-fish meal and non-fish oil diet development for red seabream, *Pagrus major*, by mixing two microalgae species

3.1 Objective of study

To minimize use of fish meal in aquaculture for continued growth and sustainability of the aquaculture industry, it is necessary to develop more cost-effective and sustainable ingredients. There have been several studies to replace fish meal with plant-derived protein sources to promote sustainable aquaculture. For the cyprinids and tilapia, the research on replacing large amount of fish meal without any improper effects on growth performance and nutrient utilization (Kaushik, 1995; Rodehutscord et al., 1995; Espe et al., 2006, 2007). Also, research on rainbow trout, fish meal free diet showed comparable performance with combination of plant and animal protein sources (Lee et al., 2002; Barrows et al., 2007). However, the other studies have showed reduced growth performance, although the experimental diets were nutritionally acceptable. Furthermore, health problems, such as inflammation or enteritis have been observed in salmon, rainbow trout, totoaba (*Totoaba macdonaldi*), common carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), and turbot (*Scophthalmus maximus*) (Bureau, Harris, & Cho, 1998; Yamamoto et al., 2008; Urán et al., 2009; Hedrera et al., 2013; Gu, Bai, Zhang, & Krogdahl, 2016; Fuentes-Quesada et al., 2018). In a 12 week trial in sea bream, there were growth reductions when fed diets containing 50-100% plant protein ingredients according to remarkable reduction in feed intake (Gómez-Requeni et al., 2004). In contrast, a 12 week experiment was in 2004, sea bass fed up to 98% of dietary protein as plant meals showed no reduction in feed intake or growth performance (Kaushik et al., 2004).

Microalgae have been known as a rich source of essential fatty acids, amino acids, vitamins (especially vitamin A, vitamin E, niacin, thiamine, and ascorbic acid), minerals, and

carotenoid pigments and shown to be beneficial as a feed ingredient for marine organisms (Ahlgren et al., 1992; Walker and Berlinsky, 2011). Use of microalgae in fish diet has been focused because of their high protein content and favorable fatty acid profiles. Unlike terrestrial plant proteins, microalgae are relatively high in n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) such as docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) , which are essential for growth of fish and have beneficial effects on human health including imparting neurological, cardiovascular, and anticancer benefits to humans (Harper and Jacobson, 2005; Peet and Stokes, 2005; Brasky et al., 2010). There was an experiment with DHA-rich microalgae *Schizochytrium* to replace fish oil and to develop vegetarian diet for red sea bream (*Pagrus major*). The algae meal diet fed fish showed similar growth performance with fish derived product used diet fed fish (Seong et al., 2019). Among the microalgae species, *Chlorella* and *Spirulina* have been received attention due to their high protein content (Dillon et al., 1995; Badwy et al., 2008; Hemaiswarya et al., 2011) and demonstrated their possibility of fish meal substitution. *Nannochloropsis* has known as a microalga which containing both protein and lipid with rich in EPA (Hemaiswarya et al., 2011; Haas et al., 2016; Gbadamosi and Lupatsch, 2018). Through the studies, *Nannochloropsis* algae meal showed its effectiveness and potential as fish meal and fish oi l substitute.

The purpose of the present study was to investigate the value of incorporating microalgae (*Nannochloropsis*, *Chlorella*, and *Spirulina*) as a substitute of fish derived product in the non-fish meal and non-fish oil diets of juvenile red sea bream.

3.2 Materials and method

Experimental microalgae

Microlgae *Nannochloropsis*, *Chlorella*, *Spirulina* and *Schizochytrium* have been used as experimental subjects. *Nannochloropsis* held both protein and lipid, which are around 53% and 27%. *Chlorella* and *Spirulina* accommodated relatively high protein (59% and 71% each) and low lipid (15% and 13% each). *Schizochytrium* algae meal contained around 11% protein and 51% lipid [Table 2]. *Nannochloropsis* was purchased through Pacific Trading (Tokyo, Japan) and the other three microalgae were the product of Marine Tech (Aichi, Japan).

Nannochloropsis, *Chlorella* and *Spirulina* contains considerable amount of amino acids, including indispensable amino acids [Table 3]. The three protein-rich microalgae showed considerably little amount of taurine.

As the lipid sources, *Nannochloropsis* and *Schizochytrium* algae meal contained substantial content of n-3 long chain polyunsaturated fatty acid (n-3 LC-PUFA). The *Nannochloropsis* algae meal showed relatively high saturates and monoenes with approximately 25% of palmitoleic acid (16:1n-7) in fatty acid composition of total lipid in diet. High EPA (35.2% in lipid) was also shown in fatty acid composition of the *Nannochloropsis* and the EPA was the main content of the n-3 LC-PUFA. The *Schizochytrium* algae meal showed a relatively higher level of saturates with approximately 60% of palmitic acid (16:0) in fatty acid composition of total lipid. Low levels of total monoenes and n-6 LC-PUFA contained, but *Schizochytrium* meal contains high level of n-3 LC-PUFA and the most of the LC-PUFA composed of DHA (36.2% in lipid) [Table 4].

Experimental diets

Six iso-nitrogenous and iso-lipidic diets were formulated (CP 45%, CL 16%). Fish oil formulated non-fish meal diet was arranged as a control diet with soybean meal, soy protein concentrate and corn gluten meal (FO). Fish oil of FO diet was replaced by *Nannochloropsis* meal and *Schizochytrium* meal for each of two diets respectively (NAN, SCH). Three different species of microalgae meal (*Nannochloropsis*, *Chlorella* and *Spirulina*) was formulated in SCH diet to replace plant protein source (NS, CS, SS). In every diet, 2.2% of amino acid premix (Lysine 1: Methionine 0.5: Threonine 0.5: Tryptophan 0.2) was supplemented to compensate for the lack of indispensable amino acid content to improve dietary quality (Takagi et al., 2001). 0.5% of taurine was also supplemented to the non-fish meal diet to meet the nutritional requirement (Matsunari et al., 2008a, 2008b). 1% of bonito peptide was supplemented to enhance the palatability of feed. SSF (solid-state fermentation) enzyme mixture was used in non-fish meal used diets to reduce the negative effect of the anti-nutritional factor of plant protein source (Moura et al., 2012; Hanini et al., 2013) [Tables 1 & 5].

Total amino acid composition of the diets showed different according to the diet formula. However, there were minor differences in indispensable amino acid among diets. Dietary fatty acid composition differs with dietary lipid sources. FO diet contains low total n-6 PUFA, but the highest n-3 PUFA and n-3 LC-PUFA among diets. NAN diet showed small amount of DHA but high EPA. In contrast to NAN diet, *Schizochytrium* formulated diet except NS showed scant amount of EPA but fairly high DHA. FO and NS diets showed both

high amount of DHA and EPA. [Table 7]

Feeding expeiment

The animal experiment was conducted according to the guideline of the Animal Experiment Treaty of the Tokyo University of Marine Science and Technology. The experiment was conducted at the Laboratory of Fish Nutrition, Tokyo University of Marine Science and Technology, Tokyo, Japan. Juvenile red sea bream (*Pagrus major*) were obtained from Marua Suisan Co. Ltd. (Kamijima, Japan) and commercial larval feed (Ambrose, Feed One Co. Ltd., Tokyo, Japan) was fed for 2 weeks. SCH diet was fed for 1 week before the beginning of the experiment to acclimate to the experimental diet. Following this, 180 fish (average weight 13.4 ± 3.2 g) were distributed randomly into twelve 60-L glass tanks that were placed in a re-circulating system filled with artificial seawater (Sea Life® , Tokyo, Japan; salinity, 30‰). The water temperature was maintained at 21.0 ± 1.0 °C. NO_2 and NO_3 levels were monitored by a colorimetric test kit (Kyoritsu Chemical-Check lab Co., Tokyo, Japan) and confirmed within a safe level. A 12-h light/12-h dark photoperiod regime was employed during the study. Triplicate groups of fish were fed by hand to apparent satiation three times daily (09:00, 12:00 and 16:00) for 12 weeks. The fish tanks were cleaned every day after the first feeding time and half of the water was renewed twice a month to maintain the acceptable water quality.

Fish weighing and sampling

The fish weighing had been conducted a day before the first day and every 4 weeks during the feeding experiment. All fish in each group were anesthetized (2- phenoxyethanol at 0.5 ml/L) and weighed individually. Before the experiment, 10 fish were randomly taken from the pool for the chemical analysis of initial fish. Also, on the last day of the 12 week experiment, five fish from each tank were sampled randomly and ground with a centrifugal mill (Retsch, Haan, Germany) for chemical analyses. Before sampling for chemical analyses, fish were kept in starvation for 24 hours to avoid the effect of diet resided in gastrointestinal tract on the chemical composition of fish. Another six fish were also sampled randomly from each tank for the hepatosomatic index and assessment of liver lipid analysis. The samples were stored at −30 ℃ until analysis. The fecal materials were continuously siphoned through glass trap column of each tank by the water flow after the last feeding of a day. After collection, the feces were centrifuged at 3,000 rpm for 5 min, and the precipitate was freeze-dried and ground for determination of chromic oxide concentration and nutrient content (Watanabe et al., 1996).

Biochemical analysis

Dehydration of moisture was conducted by oven drying each sample at 110 ℃ for 4h and then weighing each sample at one-hour intervals until a constant weight was obtained. Ash content was determined by ashing each dried sample in a porcelain crucible using a muffle furnace at 600 ℃ overnight (Woyewoda et al., 1986). Crude protein content was determined by the Kjeldahl procedure using a Kjeltec 2400 (Foss Ltd., Tokyo, Japan). Percent nitrogen was multiplied by 6.25 to obtain an estimate of percent protein.

As for lipid content, the total lipids were extracted by using chloroform/methanol (2:1, v/v) according to the methods of Folch et al (1957). Total lipids extracted from the whole body and liver of fish were separated into neutral and polar lipid fractions via silica cartridges (Sep-Pack, Waters Co., Milford, U.S.A.), as described by Juaneda and Rocquelin (1985). For the fatty acid analysis, fatty acid methyl esters (FAME) were prepared according to Christie and Han (2010). The FAMEs were analyzed by a gas chromatograph (GC2025; Shimadzu Co., Tokyo, Japan) equipped with a hydrogen flame ionization detector and a silica capillary column (L × I.D., 30 m × 0.32 mm, Supelcowax 10 Fused Silica Capillary GC column; Supelco, Bellefonte, PA, USA).

Data calculation and statistical analysis

The growth performance of the fish was calculated by the following formulae:

Wet weight gain (WG) (%)

= (final mean wet weight (FW)(g) / initial mean wet weight (IW)(g)) × 100

Food conversion ratio (FCR)

= Dry feed intake (g) / (final mean wet weight (FW) (g) − initial mean wet weight (IW) (g))

Protein efficiency ratio (PER) (g/g)

= WG (g)/total protein intake (g)

Specific growth rate (SGR) (%bodyweight/day)

= [(ln FW (g) − ln IW (g))/time (days)]×100

Survival rate (SR) (%)

= (number of fish in each group remained/initial number of fish) × 100

Daily feed intake (DFI) (%)

= (Dry feed intake)/ [(IW (g) +FW (g))/2×time (days)] × 100

Apparent digestibility coefficients (ADC) (%)

= 100 − (1 − ((Cr_2O_3 in diet/ Cr_2O_3 in feces) × (nutrient in feces/nutrient in diet)))

To test the result of the experiment, all data were statistically analyzed by One Way Analyses of Variance (ANOVA; Sigma Stat 3.0, SPSS, Chicago, U.S.A.). Tukey's test and LSD test were used to detect significant differences among means.

3.3 Results

Growth performance

There were significant differences in growth performances and feed utilization efficiencies in the result of FW, WG, SGR and DFI at the end of the 84-day feeding period. NAN diet group showed significantly lower FW, WG and SGR than FO diet group. DFI was the lowest in NS diet group and it showed significant difference between SCH diets fed group. There were no significant differences in FCR, PER, SR and HSI. Even there was no significant difference in FCR, the highest value was shown in NAN diet fed group. [Table 8]

Proximate composition of fish carcass

Fish groups fed all six different diets showed different proximate composition by the dietary treatment but there were no significant differences in moisture, ash and protein composition. In lipid content, NAN diet group showed significant difference with FO and CS diet fed group, they showed significantly lower lipid content than the other diet groups. [Table 9]

Polar and Neutral lipid content

The polar lipid contents in the whole fish body showed no significant difference among all tested groups as well as those in liver. However, significant differences were found in the neutral lipid content obtained from both whole body and liver. In particular, the highest and lowest neutral lipid contents were obtained from FO and NAN diet groups, lipid contents were obtained from FO and NAN diet groups, respectively, in the whole fish body, while the lowest was obserbed in SCH diet group in the liver. [Table 10]

Total amino acid composition of fish carcass

In total amino acid composition of whole body of fish, there was no significant difference in indispensable amino acids except tryptophan and taurine. CS diet fed group showed significantly lower tryptophan content than NAN diet fed group and FO group

showed significantly lower taurine content than the other diets. NAN, CS and SS diet group showed the highest taurine level there were significant differences in dispensable amino acid composition, in taurine and aspartic acid. In aspartic acid composition, SS diet group showed significantly higher content than FO, NS and CS diet fed fish.

Fatty acid profile

The fish fed six different diets showed significant differences in the fatty acids profile of total lipid from whole body. Fatty acid composition of the total saturates showed significant difference between SS and NAN diet group. In n-6 PUFA, CS diet group showed the significantly highest linoleic acid (18:2n-6). NAN diet group showed the lowest total n-3 PUFA with the lowest DHA (22:6n-3) composition in total lipid of whole body of fish. NAN diet fed fish also showed significantly lower total saturates than the other diet groups and higher monoenes fatty acids. *Schizochytrium* formulated diets group except NS group showed the significantly lowest EPA (20:5n-3) and the highest DHA [Table 12].

The result of the fatty acid profiles of the total lipid of the fish liver in FO and NAN groups showed a similar trend with the fatty acid composition in whole body of the fish by contrary, *Schizochytrium* formulated diets group showed lower saturates and monoenes fatty acids level and higher n-6 PUFA level. n-3 PUFA including EPA and DHA showed similar trend with total lipid of fish body fatty acids composition. Total n-3 LC-PUFA composition was lower than fatty acid composition in the fish body [Table 13].

As the result of polar lipid fatty acid analysis in the fish body, there was a significant

difference in most of fatty acids except stearic acid (18:0), eicosenoic acid (20:1n-9), cetoleic acid (22:1n-11) and total PUFA. Fatty acids profile of polar lipid in the fish body showed lower total monounsaturated fatty acids and n-6 PUFA level but higher saturated fatty acids n-3 PUFA level than that of total lipid in fish body. n-3 LC-PUFA composition including EPA, docosapentaenoic acid (DPA) (22:5n-3) and DHA increased. 18:1/n-3 LC-PUFA ratio which implies a deficiency of EFA when its numerical value is more than 1 was also calculated. NAN diet fed fish group showed the highest ratio [Table 14].

The fatty acid profile of the polar lipid in the liver showed a similar trend of the fatty acid composition of polar lipid in the whole body of fish. It showed a higher trend in total saturated fatty acid and total monounsaturated fatty acid levels. Total monoenes and n-6 PUFA showed higher trend than fatty acid composition of the hepatic total lipid in fish. In contrary, total saturated fatty acids and n-3 PUFA including n-3 LC-PUFA showed increased composition. In n-3 LC-PUFA composition, NAN diet fed fish showed the significantly lowest among the diets fed groups. Also, 18:1/n-3 LC-PUFA ratio of NAN group showed the highest and the numerical value was more than 1 [Table 15].

Digestibility

In ADC of protein, NS diet fed fish showed the significantly highest and FO group showed the lowest digestibility. Also, NS diet group showed the highest lipid digestibility and no significant difference from FO diet group. The diet group which formulated with more than two microalgae species showed higher digestibility than the diet groups formulated with

one microalga [Table 16].

There were significant differences in ADC of total amino acid in both of indispensable and dispensable amino acids, including lysine, threonine, tyrosine, glutamic acid and aspartic acid. NS and SS groups showed significantly higher ADC than FO diet group in indispensable amino acids ADC. Algae meal formulated diets showed significantly higher or same ADC in dispensable amino acids [Table 17]

ADC of fatty acids in total lipid of diets showed significance in most of the fatty acids. Only one microalga formulated diets, NAN and SCH, showed the lowest digestibility in overall fatty acids ADC. Whereas, diets formulated two microalgae meal showed better digestibility, especially NS and SS diet group. NAN formulated diets group showed better EPA digestibility than SCH diet group, however, it was significantly lower than that of FO group. *Schizochytrium* formulated diets showed high DHA digestibility, significantly no difference with DHA digestibility of FO diet fed fish [Table 18].

3.4 Discussion

Fulfilling dietary requirement of red sea bream is one of the top priority tasks in development of novel ingredient-used diet. Total amino acids requirement of juvenile red sea bream has been proved as Arg 3.5, His 1.4, Ile 2.2, Leu 4.2, Met+Cys 2.2, Phe+Tyr 4.1, Thr 1.8, Trp 0.6, Val 2.5 % of dietary protein (Forster and Ogata, 1998). The EFA requirement of juvenile red sea bream has been known as 1% EPA and 0.5% DHA in diet, respectively (Takeuchi et al., 1990). In this experiment, most of the *Schizochytrium* algae meal formulated diets met the EFA requirement of red sea bream with more than 0.5% of DHA content in diet but NAN diet, which used only 10% of *Nannochloropsis*, did not met the EFA requirement.

As a result of growth performance, there was no significant difference between fish fed control and the other algae meal diets except NAN diet group in FW, WG and SGR. The previous study (Seong et al., 2019) showed similar growth between fish fed non-fish meal diet supplemented with fish oil and non-fishmeal and non-fish oil diets formulated by algae meal. In the previous experiment, the formula of the diet formulated with fish oil was the same as the FO diet of present study, and the diet formulated with 5% of algae meal was also same with the SCH diet in the present study. Fish fed the NAN diet showed the lowest growth performance probably because of high FCR of the group. Despite no significant difference in FCR, NAN diet group showed the highest performance, which indicates feed efficiency is lower than the other diet groups.

There was no significant difference in proximate composition of the whole body. However, fish fed NAN diet showed the lowest lipid content ($P < 0.05$). The NAN group stored less lipid than the other groups because of higher FCR and there might be a strong

accordance with growth performance. The algae meal group seems lower lipid accumulation in the whole body than FO group but there was no significant difference. This was similar to previous studies, and the result may indicate that the lipid of algae meal is rarely accumulated in the fish body than the other lipid sources (Seong et al., 2019).

The polar and neutral lipid content in whole body and liver of fish showed a similar trend. In the whole body and liver, there was no significant difference in polar lipid content among the groups, but there was significant difference in the neutral lipid content. The results may suggest an upper limit of polar lipid content in whole body and liver and the additional accumulated lipid was the form of neutral lipid. Fish fed algae meal diet showed a significantly lower neutral lipid content in the whole body and liver. This could be due to low total lipid content in fish body of NAN diet group and low neutral lipid content in *Schizochytrium* sp. meal which showed similar in the previous study (Seong et al., 2019). These results also suggested that neutral lipids in diet can be easily assimilated and stored in fish body and liver.

For aquacultural marine species, n-3 LC-PUFA is considered as the most important fatty acids. However, most of the marine species have limited or no ability to desaturate and elongate 18:3n-3 to n-3 LC-PUFA as well as 18:2n-6 to 22:5n-6 via 20:4n-6 because of low or absent Δ6- and Δ5-desaturase activities (Bell et al., 1994; Montero et al., 2004; Izquierdo et al., 2005). The result of fatty acids composition on the total lipid of fish body and liver demonstrated their poor ability of n-3 LC-PUFA synthesis, as well as they were strongly influenced by the different fatty acid compositions of each six diets. In the fatty acids compo sition in the diet and total lipid in whole body and liver, n-3 LC-PUFA including EPA, DPA

and DHA seemed to be highly accumulated, in comparison with dietary fatty acid composition. *Schizochytrium* algae meal formulated diets showed lower saturated and monoenes fatty acids composition in hepatic total lipid than that of total lipid from whole body of fish and it indicates selective lipid reposition of fish liver (Takama et al., 1994). In the each of *Schizochytrim* and *Nannochloropsis* algae meal formulated groups, 1.19-1.22 folds of DHA and 0.54-0.59 folds of EPA was observed in fatty acid composition of total lipid in whole body than those of dietary fatty acid. In contrast, fish oil formulated diet showed higher value with 1.27 fold of DHA and 0.68 fold of EPA. The proportions of DHA in hepatic total lipid in fish and diet total lipid showed divergent trend, FO diet group showed lower proportion with 1.00 fold and *Schizochytrium* diets group showed 1.07 to 1.16 folds higher proportions. *Nannochloropsis* algae meal formulated groups showed lower proportions of hepatic total lipid and diet total lipid in EPA than FO diet group (0.39-0.51 in *Nannochloropsis* formulated diet groups and 0.60 in FO group).

Similar to the previous study, higher total saturates level was observed in fatty acid composition of polar lipid from whole body than those of the total lipid in the whole body and dietary lipid in fatty acid compositions of each diet groups. All groups showed generally lower monoenes content in hepatic polar lipid than those of total lipid of the whole body and dietary lipid. This result suggests that most of the monounsaturated fatty acid was stored as neutral lipid. Total n-6 LC-PUFA content in polar lipid of the whole body was mostly lower than those in total lipid of whole body and dietary lipid. On the other hand, total n-3 PUFA contents in dietary lipid, total lipid of whole body and hepatic polar lipid showed generally higher in whole body and hepatic polar lipid than those of total lipid of the whole body and

dietary lipid. Especially, EPA, DPA, and DHA contents were higher than total lipid of whole body and dietary lipid. The results may suggest that the LC-PUFA in diets have been utilized selectively and accumulated preferentially as forms of polar lipid in fish body (Huang et al., 2007). The results of saturated and mono-unsaturated fatty acid composition of the polar lipid in whole body of fish and liver are also suggesting selective accumulation and consumption of the fatty acids. Total n-6 PUFA content showed a similar trend, but n-3 PUFA content of hepatic polar lipid in fish showed generally higher than the content of whole body polar lipid. The ratio of 18:1n-9/Σn-3 LC-PUFA in the fatty acid composition of polar lipid of the whole body and hepatic polar lipid have been considered as an EFA index for red sea bream (Fujii and Yone, 1976) and gilthead bream (Kalogeropoulos et al., 1992). When the numerical ratio more than 1 is considered as EFA deficiency associated with poor growth performance and feed efficiency. Except for fish fed NAN diet, whose ratios were 1.8 and 1.9 in fatty acid composition of polar lipid from whole body and hepatic polar lipid, the other groups showed the ratio less than 1with no negative effects on fish growth.

Two algae meal formulated diets showed the better or similar ADC in protein and it may related with significantly higher digestibility in total amino acid including lysine, tyrosine, glutamic acid and aspartic acid. Also, digestibility of lipid and fatty acids of total lipid in two algae meal diets showed generally higher or similar than that of FO diet group. These result may suggest that formulating diets with the mixed two microalgae species may increase nutritional value (Spolaore et al., 2006; Becker, 2007; Hemaiswarya et al., 2011) and raised the digestibility of the diets.

In conclusion, formulating non-fish meal, non-fish oil diet for red sea bream with

microalgae species was promising with considerable growth result, significantly no difference with FO diet group except NAN diet group which was insufficient in EFA requirement in diet. As it showed in previous study, DHA-rich microalgae *Schizochytrium* sp. meal is a suitable substitute for fish oil for the juvenile red sea bream diet, and it could also be used with the other microalgae species. By using two microalgae meal, it seems to organize better nutritional value than only one microalga used diets. These results imply that the possibility of development of non-fish meal and non-fish oil diet highly covered with mixed microalgae species.

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3.6 Experimental tables

Table 1. Formula of the experimental diets for red sea bream (g kg^{-1})

	Experimental diets					
Ingredients	FO	NAN	SCH	NS	CS	SS
Soy protein concentrate	140	120	120	120	120	120
Soybean meal	200	200	200	150	100	200
Corn gluten meal	350	290	350	310	260	160
Nannochloropsis meal[a]	0	100	0	100	0	0
Chlorella meal[b]	0	0	0	0	200	0
Spirulina meal[c]	0	0	0	0	0	200
Schizochytrium meal[d]	0	0	50	50	50	50
Wheat flour	6	6	6	6	6	6
Alpha-starch	55	55	55	55	55	55
Fish oil[e]	90	0	0	0	0	0
Rape seed oil	30	100	90	80	80	80
Amino acid (Lysin1, Methionine 0.5)	22	22	22	22	22	22
Vitamin premix[f]	30	30	30	30	30	30
Vitamin E	1	1	1	1	1	1
Choline chloride	5	5	5	5	5	5
Mineral premix[g]	10	10	10	10	10	10
Calcium phosphate	40	40	40	40	40	40
Bonito peptide	10	10	10	10	10	10
SSF Enzyme mix[h]	1	1	1	1	1	1
Taurine	5	5	5	5	5	5
Cr_2O_3	5	5	5	5	5	5
Total	1000	1000	1000	1000	1000	1000

[a] *Nannochloropsis* algae meal (Pacific Trading Co., Ltd, Tokyo, Japan)

[b] *Chlorella* algae meal (Marine Tech Inc., Aichi, Japan)

[c] *Spirulina* algae meal (Marine Tech Inc., Aichi, Japan)

[d] *Schizochytrium* algae meal (Marine Tech Inc., Aichi, Japan).

[e] Cod liver oil (Kanematsu Shintoa Foods Co. Ltd, Tokyo, Japan).

[f] Vitamin mixture composition (unit kg^{-1}) : Vitamin D_3, 2420000 IU; Vitamin K_3, 6050 mg; thiamin, 3025 mg; riboflavin, 3630 mg; pyridoxine, 2420 mg; cyanocobalamine, 6 mg; L-

ascorbic acid, 368900 mg; nicotinic acid, 24200 mg; D-pantothenic acid, 6050 mg; inositol, 121 000 mg; D-biotin, 363 mg; folic acid, 908 mg; para-aminobenzoic acid, 3025 mg.

[g] Mineral mixture compostition (g/kg^{-1}) : Sodium chloride 50; magnesium sulphate 745; iron(III) cirtrate n-hydrate 125; trace element mix 50; cellulose 30 [trace element mixture contains ($g\ kg^{-1}$) : zinc sulphateheptahydrate 353; manganese sulphate 162; copper(II) sulphate pentahydrate 31; aluminum chloride hexahydrate 10; cobalt chloride hexahydrate 10; cobalt chloride 3; potassium iodate 1; cellulose 440].

[h] SSF (Alltech Inc., Lexington, Kentucky, U.S.A).

Table 2. Proximate composition of the algae meals (% dry weight basis except for % moisture)

	Nannochlorpsis[a]	*Chlorella*[b]	*Spirulina*[c]	*Schizochytrium*[d]
Moisture	2.1	3.9	4.3	2.2
Protein	52.9	59.2	70.9	11.3
Lipid	27.2	14.6	13.0	50.9
Ash	8.4	4.7	6.3	8.4

[a] *Nannochloropsis* algae meal (Pacific Trading Co., Ltd, Tokyo, Japan)

[b] *Chlorella* algae meal (Marine Tech Inc., Aichi, Japan)

[c] *Spirulina* algae meal (Marine Tech Inc., Aichi, Japan)

[d] *Schizochytrium* algae meal (Marine Tech Inc., Aichi, Japan).

Table 3. Total amino acids composition of the microalgae (in diet %)

	Nannochlorpsis[a]	*Chlorella*[b]	*Spirulina*[c]	*Schizochytrium*[d]
Arg	3.12	3.74	4.40	0.69
Lys	3.52	4.61	3.26	0.43
His	0.95	1.11	1.02	0.17
Phe	2.32	2.60	2.98	0.38
Leu	4.42	4.87	5.72	0.71
Ile	1.81	1.38	2.99	0.29
Met	1.11	0.86	1.48	0.27
Val	2.46	2.23	3.04	0.40
Thr	2.58	2.56	3.47	0.48
Trp	0.89	0.69	0.71	0.29
Tau	0.06	0.02	0.07	0.29
Cys	0.09	0.18	0.15	0.07
Cysta	0.01	0.16	0.18	0.37
Ala	3.92	5.24	5.95	0.67
Tyr	2.00	2.19	3.13	0.23
Gly	2.97	3.51	3.65	0.55
Glu	6.41	7.56	10.88	2.16
Ser	2.47	2.65	3.73	0.57
Asp	5.02	5.61	6.93	1.07
Pro	4.89	2.91	2.77	0.78

[a] *Nannochloropsis* algae meal (Pacific Trading Co., Ltd, Tokyo, Japan)

[b] *Chlorella* algae meal (Marine Tech Inc., Aichi, Japan)

[c] *Spirulina* algae meal (Marine Tech Inc., Aichi, Japan)

[d] *Schizochytrium* algae meal (Marine Tech Inc., Aichi, Japan).

Table 4. Fatty acids composition of the microalgae (area %)

	Nannochlorpsis[a]	*Chlorella*[b]	*Spirulina*[c]	*Schizochytrium*[d]
14:0	4.9	0.2	0.4	10.6
16:0	16.8	18.4	52.0	27.8
18:0	0.3	0.3	0.9	0.6
ΣSFA[*]	22.0	18.9	53.3	39.0
16:1n-7	25.1	0.4	2.6	0.1
18:1n-9	2.8	2.3	2.0	0.1
20:1n-9	0.0	0.1	0.0	0.0
22:1n-11	0.0	0.0	0.1	0.0
ΣMUFA[**]	27.9	2.9	4.7	0.1
18:2n-6	3.0	49.1	19.8	0.0
20:4n-6	5.1	0.0	0.0	1.9
Σn-6 PUFA[***]	8.0	49.1	19.8	2.0
18:3n-3	0.1	2.2	0.3	0.0
18:4n-3	0.0	0.0	0.0	0.3
20:4n-3	0.0	0.0	0.0	0.9
20:5n-3	35.2	0.1	0.0	1.3
22:5n-3	0.0	0.3	0.0	0.5
22:6n-3	0.0	0.0	0.0	36.2
Σn-3 PUFA[***]	35.3	2.6	0.3	39.2
ΣPUFA[***]	43.3	51.6	37.1	41.2
Σn-3 LCPUFA[****]	35.2	0.4	0.0	39.2

[a] *Nannochloropsis* algae meal (Pacific Trading Co., Ltd, Tokyo, Japan)

[b] *Chlorella* algae meal (Marine Tech Inc., Aichi, Japan)

[c] *Spirulina* algae meal (Marine Tech Inc., Aichi, Japan)

[d] *Schizochytrium* algae meal (Marine Tech Inc., Aichi, Japan).

[*] Saturated fatty acid.

[**] Monounsaturated fatty acid.

[***] Polyunsaturated fatty acid.

[****] Long-chain polyunsaturated fatty acid.

Table 5. Proximate composition of the experimental diets (% dry weight basis except for % moisture)

	FO	NAN	SCH	NS	CS	SS
Moisture	7.4	4.2	7.6	5.9	7.7	2.5
Protein	48.0	47.6	47.2	46.9	47.2	48.4
Lipid	17.3	15.7	16.3	17.9	18.3	17.6
Ash	6.3	7.0	6.4	6.8	6.9	7.0

Table 6. Total amino acids composition of the diets (in diet %)

	FO	NAN	SCH	NS	CS	SS
Arg	2.40	2.92	2.56	2.54	2.79	3.21
Lys	3.70	4.39	4.48	4.46	4.78	4.53
His	0.96	1.15	0.93	1.10	0.96	1.01
Phe	3.02	3.07	2.82	2.87	2.77	2.96
Leu	6.23	6.18	5.80	5.50	5.40	5.25
Ile	1.51	1.66	1.38	1.47	1.45	1.51
Met	1.83	2.02	2.00	2.17	2.09	2.21
Val	1.67	1.94	1.57	1.78	1.77	1.65
Thr	1.84	2.11	1.92	1.99	2.06	2.17
Trp	0.61	0.66	0.76	0.62	0.46	0.65
Tau	0.73	0.86	1.24	0.49	0.95	1.00
Cys	0.49	0.48	0.42	0.38	0.39	0.35
Cysta	0.46	0.43	0.53	0.41	0.52	0.35
Ala	3.77	3.98	3.65	3.62	3.89	4.27
Tyr	2.38	2.45	2.28	2.23	2.20	2.39
Gly	2.00	2.37	2.18	2.29	2.49	2.75
Glu	11.26	11.41	11.46	10.60	10.18	11.50
Ser	2.98	3.13	3.08	2.92	2.85	3.36
Asp	4.75	5.36	5.25	5.10	5.15	6.11
Pro	4.58	4.50	4.07	6.40	3.55	3.51

Table 7. Fatty acids composition of the diets (area %)

	FO	NAN	SCH	NS	CS	SS
14:0	3.8	0.6	1.8	2.4	1.8	1.8
16:0	12.3	7.8	10.3	11.3	11.5	13.5
18:0	2.0	1.6	1.6	1.5	1.4	1.5
ΣSFA*	18.0	10.0	13.7	15.2	14.7	16.9
16:1n-7	4.3	2.5	0.2	2.3	0.2	0.4
18:1n-9	26.9	43.0	41.0	35.5	36.5	37.0
20:1n-9	3.7	0.1	0.0	0.4	0.0	0.0
22:1n-11	1.2	0.4	0.6	0.5	0.5	0.5
ΣMUFA**	39.9	46.0	41.7	38.7	37.2	37.9
18:2n-6	20.2	27.8	26.2	23.8	27.3	22.5
20:4n-6	0.3	0.4	0.2	0.1	0.3	0.4
Σn-6 PUFA***	20.5	28.2	26.4	24.0	27.6	22.9
18:3n-3	2.7	5.9	5.2	4.5	4.6	4.8
18:4n-3	1.4	0.0	0.0	0.0	0.0	0.1
20:4n-3	0.4	0.0	0.0	0.1	0.1	0.1
20:5n-3	5.3	4.6	0.1	4.1	0.2	0.2
22:5n-3	0.7	0.2	0.2	0.2	0.2	0.1
22:6n-3	4.8	0.1	6.7	6.4	6.0	7.1
Σn-3 PUFA***	15.3	10.8	12.2	15.1	11.1	12.6
ΣPUFA***	35.7	39.0	38.7	39.1	38.7	35.5
Σn-3 LCPUFA****	12.5	4.9	7.0	10.7	6.5	7.8

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 8. Growth performance and feed utilization efficiencies of red sea bream fed experimental diets over 12 weeks (mean±SD, n=2)

	FO	NAN	SCH	NS	CS	SS
IW(g)	13.3±0.1	13.4±0.1	13.4±0.1	13.4±0.1	13.4±0.1	13.3±0.1
FW(g)	58.0±0.8^{a}	29.4±2.1^{b}	37.2±2.8^{ab}	40.7±18.4^{ab}	40.1±3.9^{ab}	43.1±8.3^{ab}
WG(%)	435.6±6.1^{a}	219.9±16.5^{b}	277.7±18.5^{ab}	303.5±138.5^{ab}	300.5±30.3^{ab}	322.3±64.0^{ab}
FCR	1.33±0.28^{a}	1.81±0.12^{a}	1.54±0.03^{a}	1.52±0.58^{a}	1.31±0.02^{a}	1.26±0.26^{a}
PER	2.29±0.09^{a}	2.12±0.08^{a}	1.92±0.16^{a}	2.04±0.75^{a}	2.33±0.15^{a}	2.30±0.29^{a}
SGR	1.75±0.02^{a}	0.94±0.09^{b}	1.21±0.08^{ab}	1.26±0.56^{ab}	1.31±0.12^{ab}	1.38±0.24^{ab}
SR(%)	76.67±4.71^{a}	96.67±4.71^{a}	76.67±14.14^{a}	63.33±51.85^{a}	93.33±0.00^{a}	86.67±0.00^{a}
DFI(%)	1.67±0.10^{ab}	1.60±0.07^{ab}	1.79±0.08^{a}	1.39±0.28^{b}	1.60±0.14^{ab}	1.58±0.12^{ab}
HSI	1.42±0.06^{a}	1.30±0.01^{a}	1.37±0.01^{a}	1.28±0.00^{a}	1.33±0.12^{a}	1.29±0.01^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (LSD test, a>b>c).

Table 9. Proximate composition of the whole body of the fish fed the experimental diets (% wet weight basis, mean±SD, n=2)

	FO	NAN	SCH	NS	CS	SS
Moisture	70.1±0.6^{a}	73.2±0.2^{a}	71.7±2.0^{a}	71.5±3.8^{a}	70.1±0.7^{a}	72.2±0.5^{a}
Ash	4.2±0.2^{a}	4.6±0.5^{a}	4.7±0.2^{a}	4.5±0.7^{a}	4.8±0.8^{a}	4.6±0.3^{a}
Protein (w.b)	17.5±0.1^{a}	16.7±1.1^{a}	16.9±0.5^{a}	17.2±0.1^{a}	17.4±0.8^{a}	17.2±0.1^{a}
Lipid (w.b)	8.6±0.0^{a}	5.4±0.2^{c}	6.7±1.1^{abc}	7.0±1.7^{abc}	7.8±0.8^{ab}	6.1±0.4^{bc}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c)...

Table 10. Polar and neutral lipids content in whole body and liver of fish (% wet weight basis, mean±SD, n=3)

	Lipid in whole fish body					
	FO	NAN	SCH	NS	CS	SS
Polar (%)	1.41±0.10^{a}	0.84±0.12^{a}	1.07±0.17^{a}	1.07±0.43^{a}	1.12±0.14^{a}	1.13±0.29^{a}
Neutral (%)	7.16±0.10^{a}	4.52±0.12^{d}	5.66±0.17^{bc}	5.89±0.43^{b}	6.70±0.14^{a}	5.02±0.29^{cd}

	Lipid in fish liver					
	FO	NAN	SCH	NS	CS	SS
Polar (%)	3.06±1.60^{a}	2.79±1.38^{a}	2.96±0.85^{a}	1.63±0.34^{a}	2.36±0.21^{a}	2.14±1.10^{a}
Neutral (%)	13.67±1.60^{a}	11.19±1.38^{abc}	8.79±0.85^{c}	9.70±0.34^{bc}	12.47±0.21^{ab}	12.39±1.10^{ab}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

Table 11. Total amino acids composition of whole body of fish (in dry fish body %, mean±SD, n=2)

	FO	NAN	SCH	NS	CS	SS
Arg	3.60±0.13^{a}	3.83±0.21^{a}	3.79±0.12^{a}	3.76±0.11^{a}	3.58±0.11^{a}	3.77±0.18^{a}
Lys	4.93±0.11^{a}	5.22±0.24^{a}	4.88±0.46^{a}	5.14±0.05^{a}	4.91±0.26^{a}	5.27±0.36^{a}
His	1.30±0.02^{a}	1.28±0.11^{a}	1.37±0.08^{a}	1.32±0.06^{a}	1.30±0.05^{a}	1.31±0.07^{a}
Phe	2.40±0.03^{a}	2.61±0.05^{a}	2.48±0.13^{a}	2.41±0.04^{a}	2.46±0.11^{a}	2.54±0.08^{a}
Leu	4.18±0.09^{a}	4.46±0.13^{a}	4.37±0.26^{a}	4.26±0.05^{a}	4.21±0.21^{a}	4.52±0.15^{a}
Ile	1.50±0.05^{a}	1.50±0.29^{a}	1.52±0.14^{a}	1.42±0.12^{a}	1.43±0.24^{a}	1.48±0.12^{a}
Met	1.66±0.05^{a}	1.80±0.12^{a}	1.64±0.10^{a}	1.67±0.03^{a}	1.57±0.14^{a}	1.61±0.20^{a}
Val	1.89±0.08^{a}	2.02±0.29^{a}	1.99±0.20^{a}	1.92±0.01^{a}	1.94±0.20^{a}	1.90±0.13^{a}
Thr	2.55±0.06^{a}	2.68±0.13^{a}	2.66±0.14^{a}	2.64±0.01^{a}	2.53±0.11^{a}	2.71±0.08^{a}
Trp	0.57±0.11^{ab}	0.77±0.12^{a}	0.70±0.11^{ab}	0.52±0.01^{ab}	0.43±0.10^{b}	0.58±0.19^{ab}
Tau	1.09±0.04^{b}	1.37±0.08^{a}	1.22±0.06^{ab}	1.23±0.03^{ab}	1.38±0.16^{a}	1.44±0.04^{a}
Cys	0.09±0.02^{a}	0.14±0.03^{a}	0.08±0.03^{a}	0.09±0.02^{a}	0.11±0.01^{a}	0.13±0.02^{a}
Cysta	0.28±0.04^{a}	0.13±0.07^{a}	0.20±0.10^{a}	0.15±0.09^{a}	0.13±0.08^{a}	0.28±0.01^{a}
Ala	4.34±0.08^{a}	4.49±0.15^{a}	4.45±0.15^{a}	4.48±0.03^{a}	4.27±0.18^{a}	4.69±0.26^{a}
Tyr	1.85±0.04^{a}	1.98±0.08^{a}	1.94±0.10^{a}	1.90±0.03^{a}	1.84±0.13^{a}	1.93±0.16^{a}
Gly	4.65±0.17^{a}	4.89±0.35^{a}	4.67±0.13^{a}	4.96±0.19^{a}	4.62±0.52^{a}	4.60±1.47^{a}
Glu	8.95±0.12^{a}	9.59±0.09^{a}	9.32±0.37^{a}	9.1±0.06^{a}	8.84±0.25^{a}	9.15±1.09^{a}
Ser	2.90±0.03^{a}	3.14±0.07^{a}	2.12±1.42^{a}	3.00±0.03^{a}	2.85±0.07^{a}	3.17±0.06^{a}
Asp	6.24±0.08^{b}	6.61±0.19^{ab}	6.48±0.33^{ab}	6.36±0.11^{b}	6.17±0.11^{b}	6.88±0.11^{a}
Pro	2.72±0.41^{a}	3.00±0.52^{a}	2.97±0.22^{a}	2.97±0.10^{a}	2.57±0.39^{a}	3.06±0.71^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

Table 12. Fatty acids composition of total lipid from whole body of fish (area %, mean±SD, n=2)

	FO	NAN	SCH	NS	CS	SS
14:0	2.4±0.2^{a}	1.2±0.1^{b}	1.6±0.2^{ab}	1.8±0.1^{ab}	1.3±0.4^{b}	1.5±0.1^{b}
16:0	12.6±0.3^{abc}	10.6±0.6^{c}	12.2±0.7^{bc}	12.3±0.5^{abc}	12.8±0.7^{ab}	14.3±0.3^{a}
18:0	2.8±0.2^{a}	3.3±0.3^{a}	3.0±0.3^{a}	2.6±0.2^{a}	2.7±0.0^{a}	2.9±0.1^{a}
ΣSFA*	17.8±0.3^{ab}	15.1±1.0^{b}	16.8±1.2^{ab}	16.7±0.8^{ab}	16.8±1.0^{ab}	18.7±0.1^{a}
16:1n-7	3.5±0.1^{a}	2.5±0.0^{b}	1.1±0.3^{c}	2.2±0.1^{b}	0.9±0.4^{c}	1.1±0.1^{c}
18:1n-9	27.8±0.0^{c}	39.3±1.9^{a}	35.5±1.9^{ab}	35.2±0.5^{ab}	34.1±1.4^{b}	34.7±0.5^{ab}
20:1n-9	3.2±0.1^{a}	0.7±0.0^{b}	0.5±0.1^{b}	0.3±0.1^{b}	0.4±0.2^{b}	0.4±0.0^{b}
20:1n-11	2.1±0.1^{a}	1.4±0.1^{b}	1.3±0.0^{bc}	1.0±0.0^{cd}	1.1±0.1^{cd}	1.0±0.0^{d}
22:1n-11	3.1±0.1^{a}	0.6±0.0^{b}	0.3±0.3^{b}	0.3±0.1^{b}	0.3±0.2^{b}	0.4±0.0^{b}
ΣMUFA**	39.7±0.1^{b}	44.4±2.0^{a}	38.7±1.2^{b}	39.1±0.4^{b}	36.8±0.6^{b}	37.5±0.5^{b}
18:2n-6	19.0±0.1^{c}	21.8±0.8^{ab}	21.7±1.2^{abc}	21.1±0.5^{abc}	23.7±0.8^{a}	20.0±0.0^{bc}
20:4n-6	0.3±0.0^{c}	0.6±0.0^{b}	0.5±0.1^{b}	0.9±0.1^{a}	0.5±0.0^{bc}	0.6±0.0^{b}
Σn-6 PUFA***	19.4±0.1^{c}	22.4±0.7^{ab}	22.2±1.1^{ab}	21.9±0.4^{ab}	24.2±0.8^{a}	20.6±0.0^{bc}
18:3n-3	2.2±0.1^{b}	3.3±0.2^{a}	3.2±0.3^{a}	3.3±0.2^{a}	3.2±0.0^{a}	3.3±0.0^{a}
18:4n-3	0.7±0.0^{a}	0.2±0.0^{b}	0.2±0.0^{b}	0.1±0.0^{b}	0.2±0.1^{b}	0.2±0.0^{b}
20:4n-3	0.4±0.1^{a}	0.1±0.0^{b}	0.2±0.0^{b}	0.2±0.0^{b}	0.2±0.0^{b}	0.2±0.0^{b}
20:5n-3	3.6±0.0^{a}	2.5±0.1^{b}	0.9±0.2^{c}	2.4±0.2^{b}	0.7±0.3^{c}	0.8±0.0^{c}
22:5n-3	1.2±0.1^{a}	0.8±0.1^{b}	0.5±0.1^{bc}	0.5±0.0^{bc}	0.3±0.1^{c}	0.4±0.0^{c}
22:6n-3	6.1±0.0^{b}	2.6±0.8^{c}	8.3±0.4^{ab}	7.6±0.1^{ab}	7.3±0.1^{ab}	8.6±1.2^{a}
Σn-3 PUFA***	14.1±0.1^{a}	9.5±0.6^{b}	13.3±0.4^{a}	14.1±0.5^{a}	11.9±0.6^{ab}	13.3±1.2^{a}
ΣPUFA***	33.5±0.0^{ab}	32.0±0.2^{b}	35.6±0.6^{a}	36.1±0.9^{a}	36.1±0.2^{a}	33.9±1.2^{ab}
Σn-3 LCPUFA****	12.0±0.1^{a}	6.2±0.8^{c}	10.1±0.7^{ab}	10.8±0.3^{ab}	8.7±0.6^{bc}	10.1±1.2^{ab}
18:1/Σn-3 LCPUFA****	2.3±0.0^{b}	6.4±1.0^{a}	3.6±0.4^{b}	3.3±0.1^{b}	3.9±0.4^{b}	3.5±0.4^{b}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 13. Fatty acids composition of hepatic total lipid in fish (area %, mean±SD, n=3)

	FO	NAN	SCH	NS	CS	SS
14:0	2.1±0.2^{a}	0.6±0.1^{c}	1.4±0.1^{ab}	1.5±0.3^{ab}	1.3±0.5^{bc}	1.1±0.2^{bc}
16:0	13.3±0.6^{ab}	8.6±0.8^{d}	11.3±0.6^{bc}	12.1±0.8^{abc}	11.2±0.7^{c}	13.4±0.9^{a}
18:0	3.3±0.2^{a}	2.5±0.5^{ab}	2.4±0.2^{b}	2.5±0.2^{ab}	1.9±0.2^{b}	2.2±0.5^{b}
ΣSFA*	18.7±1.1^{a}	11.6±1.3^{c}	15.1±0.7^{b}	16.2±1.3^{ab}	14.4±1.3^{bc}	16.7±1.4^{ab}
16:1n-7	4.2±0.5^{a}	2.7±0.2^{b}	1.0±0.2^{c}	2.3±0.3^{b}	0.5±0.0^{c}	0.7±0.1^{c}
18:1n-9	28.9±4.8^{b}	40.9±3.1^{a}	31.4±1.4^{b}	32.2±1.9^{b}	31.1±2.5^{b}	33±1.9^{b}
20:1n-9	2.3±0.1^{a}	0.0±0.0^{b}	0.1±0.1^{b}	0.0±0.1^{b}	0.0±0.1^{b}	0.3±0.4^{b}
20:1n-11	2.2±0.1^{a}	1.2±0.2^{b}	1.1±0.1^{b}	1.1±0.3^{b}	1.0±0.3^{b}	1.1±0.1^{b}
22:1n-11	1.6±0.3^{a}	0.2±0.2^{b}	0.2±0.1^{b}	0.2±0.0^{b}	0.1±0.1^{b}	0.1±0.0^{b}
ΣMUFA**	39.2±4.2^{ab}	45.0±2.9^{a}	33.7±1.1^{b}	35.9±1.8^{b}	32.7±2.7^{b}	35.1±2.0^{b}
18:2n-6	18.6±0.3^{d}	25.7±1.4^{b}	26.1±0.3^{b}	22.5±0.3^{c}	28.8±0.9^{a}	22.4±1.6^{c}
20:4n-6	0.4±0.0^{d}	0.6±0.1^{cd}	0.9±0.0^{b}	1.2±0.1^{a}	0.7±0.0^{bc}	0.9±0.1^{b}
Σn-6 PUFA***	19.0±0.3^{e}	26.3±1.4^{bc}	27.0±0.3^{ab}	23.7±0.4^{cd}	29.6±0.9^{a}	23.4±0.1.5^{d}
18:3n-3	1.9±0.2^{b}	3.2±0.3^{a}	3.6±0.5^{a}	3.1±0.2^{a}	3.7±0.3^{a}	3.2±0.3^{a}
18:4n-3	0.4±0.1^{a}	0.1±0.0^{b}	0.1±0.1^{b}	0.1±0.1^{b}	0.1±0.0^{b}	0.1±0.0^{b}
20:4n-3	0.6±0.0^{a}	0.1±0.0^{c}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{bc}	0.1±0.0^{bc}
20:5n-3	3.2±0.4^{a}	1.8±0.4^{b}	0.6±0.1^{c}	2.1±0.1^{b}	0.4±0.0^{c}	0.4±0.0^{c}
22:5n-3	1.6±0.2^{a}	0.4±0.1^{c}	0.3±0.1^{cd}	0.9±0.1^{b}	0.2±0.0^{cd}	0.2±0.0^{d}
22:6n-3	4.8±1.0^{b}	0.6±0.1^{c}	7.8±0.3^{a}	6.9±1.4^{ab}	6.5±0.2^{ab}	8.4±1.2^{a}
Σn-3 PUFA***	12.4±1.5^{a}	6.2±0.9^{b}	12.5±0.6^{a}	13.2±1.5^{a}	11.0±0.2^{a}	12.3±0.9^{a}
Total PUFA***	31.4±1.7^{c}	32.5±1.5^{c}	39.5±0.5^{ab}	37.0±1.8^{abc}	40.5±0.8^{a}	35.6±1.1^{bc}
Σn-3 LCPUFA****	10.5±1.4^{a}	3.0±0.5^{b}	8.9±0.2^{a}	10.2±1.5^{a}	7.3±0.2^{a}	9.1±1.2^{a}
18:1/Σn-3 LCPUFA****	2.8±0.7^{b}	13.6±3.1^{a}	3.5±0.2^{b}	3.2±0.4^{b}	4.3±0.3^{b}	3.7±0.6^{b}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 14. Fatty acids composition in the polar lipid from whole body of fish (area %, mean± SD, n=3)

	FO	NAN	SCH	NS	CS	SS
14:0	0.5±0.0^{a}	0.2±0.0^{c}	0.3±0.0^{bc}	0.4±0.0^{ab}	0.3±0.1^{bc}	0.2±0.1^{bc}
16:0	17.1±1.3^{ab}	15.8±0.3^{b}	16.3±0.6^{b}	17.4±0.2^{ab}	17.0±0.8^{ab}	19.0±1.5^{a}
18:0	6.1±0.4^{a}	6.2±0.3^{a}	5.9±0.3^{a}	5.6±0.2^{a}	5.8±0.3^{a}	5.8±0.1^{a}
ΣSFA*	23.6±1.5^{a}	22.2±0.6^{a}	22.5±0.8^{a}	23.4±0.4^{a}	23.1±1.1^{a}	25.0±1.5^{a}
16:1n-7	1.2±0.1^{a}	0.8±0.1^{b}	0.2±0.1^{c}	0.7±0.0^{b}	0.3±0.1^{c}	0.3±0.1^{c}
18:1n-9	16.5±0.3^{b}	23.2±0.2^{a}	18.3±1.5^{b}	18.7±0.2^{b}	18.1±1.1^{b}	19.0±1.4^{b}
20:1n-9	1.1±0.0^{a}	1.1±0.1^{a}	1.2±0.2^{a}	1.1±0.0^{a}	0.9±0.1^{a}	0.6±0.5^{a}
20:1n-11	1.3±0.0^{a}	0.3±0.5^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.1±0.2^{b}	0.5±0.2^{b}
22:1n-11	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.0±0.0^{a}
ΣMUFA**	20.0±0.4^{b}	25.4±0.6^{a}	19.8±1.3^{b}	20.7±0.2^{b}	19.4±1.2^{b}	20.4±1.6^{b}
18:2n-6	15.2±0.4^{c}	22.8±0.4^{a}	16.2±1.4^{bc}	14.3±0.3^{c}	18.1±1.1^{b}	15.5±1.1^{c}
20:4n-6	1.3±0.0^{c}	2.4±0.1^{a}	1.6±0.1^{b}	2.4±0.1^{a}	1.5±0.1^{bc}	1.7±0.1^{b}
Σn-6 PUFA***	16.4±0.4^{c}	25.3±0.4^{a}	17.7±1.4^{bc}	16.7±0.3^{c}	19.5±1.1^{b}	17.2±1.0^{bc}
18:3n-3	0.8±0.0^{b}	1.6±0.0^{a}	1.0±0.2^{ab}	0.7±0.5^{b}	1.2±0.2^{ab}	1.0±0.1^{ab}
18:4n-3	0.1±0.1^{a}	0.1±0.0^{ab}	0.1±0.0^{ab}	0.1±0.0^{ab}	0.1±0.0^{ab}	0.0±0.0^{b}
20:4n-3	0.3±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}
20:5n-3	5.2±0.3^{a}	4.4±0.1^{b}	0.7±0.1^{d}	2.5±0.1^{c}	0.6±0.0^{d}	0.6±0.1^{d}
22:5n-3	2.3±0.3^{a}	1.7±0.2^{b}	0.5±0.1^{c}	0.7±0.0^{c}	0.3±0.0^{c}	0.4±0.1^{c}
22:6n-3	17.3±2.3^{a}	6.7±1.2^{b}	21.7±1.3^{a}	19.3±0.2^{a}	19.7±2.0^{a}	20.3±2.7^{a}
Σn-3 PUFA***	26.1±2.9^{a}	14.5±1.0^{b}	24.1±1.3^{a}	23.4±0.2^{a}	22.0±1.8^{a}	22.4±2.8^{a}
Σ PUFA***	42.5±3.2^{a}	39.8±1.2^{a}	41.8±0.2^{a}	40.1±0.2^{a}	41.5±2.2^{a}	39.6±1.8^{a}
Σn-3 LCPUFA****	25.3±2.9^{a}	13.0±1.0^{b}	23.0±1.5^{a}	22.7±0.4^{a}	20.8±2.1^{a}	21.3±2.9^{a}
18:1/Σn-3 LCPUFA****	0.7±0.1^{b}	1.8±0.1^{a}	0.8±0.1^{b}	0.8±0.0^{b}	0.9±0.1^{b}	0.9±0.2^{b}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 15. Fatty acids composition of the hepatic polar lipid in fish (area %, mean±SD, n=3)

	FO	NAN	SCH	NS	CS	SS
14:0	0.6±0.1^{a}	0.2±0.0^{d}	0.3±0.0^{c}	0.4±0.0^{b}	0.3±0.0^{c}	0.3±0.0^{c}
16:0	20.6±1.3^{a}	16.0±2.0^{b}	19.5±1.5^{ab}	20.9±0.8^{a}	20.1±1.1^{a}	22.9±0.8^{a}
18:0	7.5±0.2^{a}	7.7±0.5^{a}	7.1±0.6^{ab}	6.6±0.6^{abc}	5.9±0.0^{c}	6.3±0.2^{bc}
ΣSFA*	28.7±1.6^{a}	23.9±2.3^{b}	27.0±1.9^{ab}	27.9±0.7^{ab}	26.3±1.1^{ab}	29.4±1.0^{a}
16:1n-7	1.0±0.1^{a}	0.7±0.0^{b}	0.2±0.1^{d}	0.5±0.0^{c}	0.2±0.0^{d}	0.2±0.0^{d}
18:1n-9	11.0±0.5^{d}	22.8±0.5^{a}	15.9±1.1^{b}	14.0±1.3^{bc}	13.4±0.0^{c}	13.6±1.0^{bc}
20:1n-9	0.6±0.0^{a}	0.0±0.0^{b}	0.0±0.1^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}
20:1n-11	1.2±0.0^{a}	1.3±0.0^{a}	0.9±0.1^{b}	0.9±0.1^{b}	0.7±0.0^{bc}	0.7±0.0^{c}
22:1n-11	0.5±0.0^{a}	0.1±0.0^{b}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}
ΣMUFA**	14.3±0.7^{c}	24.9±0.4^{a}	16.9±1.2^{b}	15.4±1.4^{bc}	14.2±0.1^{c}	14.4±0.9^{c}
18:2n-6	11.1±0.3^{d}	22.8±0.8^{a}	15.4±0.4^{b}	11.7±0.4^{d}	16.2±0.3^{b}	13.3±0.3^{c}
20:4n-6	2.1±0.1^{b}	3.4±0.7^{a}	3.1±0.5^{ab}	4.0±0.4^{a}	3.0±0.1^{ab}	3.4±0.4^{a}
Σn-6 PUFA***	13.2±0.2^{d}	26.2±0.9^{a}	18.5±0.7^{b}	15.7±0.7^{c}	19.3±0.4^{b}	16.7±0.4^{c}
18:3n-3	0.7±0.1^{c}	1.6±0.1^{a}	1.0±0.1^{b}	0.9±0.1^{bc}	1.1±0.0^{b}	1.0±0.0^{b}
18:4n-3	0.0±0.0^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}
20:4n-3	0.5±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}	0.2±0.0^{b}
20:5n-3	6.6±0.3^{a}	5.9±1.0^{a}	1.0±0.2^{c}	3.5±0.1^{b}	0.7±0.0^{c}	0.8±0.1^{c}
22:5n-3	2.7±0.1^{a}	1.8±0.4^{b}	0.3±0.0^{d}	0.8±0.1^{c}	0.2±0.0^{d}	0.2±0.0^{d}
22:6n-3	19.8±1.0^{a}	4.2±0.5^{b}	21.5±3.6^{a}	22.9±2.1^{a}	24.4±0.9^{a}	23.9±1.6^{a}
Σn-3 PUFA***	30.4±1.4^{a}	13.6±1.8^{c}	23.9±3.8^{b}	28.2±2.1^{ab}	26.6±1.0^{ab}	26.1±1.6^{ab}
Σ PUFA***	43.5±1.2^{a}	39.9±2.7^{a}	42.4±4.1^{a}	43.9±1.7^{a}	45.9±1.2^{a}	42.8±1.9^{a}
Σn-3 LCPUFA****	29.6±1.4^{a}	12.0±1.8^{c}	23.0±3.8^{b}	27.3±2.2^{ab}	25.5±1.0^{ab}	25.0±1.6^{ab}
18:1/Σn-3 LCPUFA****	0.4±0.0^{b}	1.9±0.3^{a}	0.7±0.1^{b}	0.5±0.1^{b}	0.5±0.0^{b}	0.5±0.1^{b}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 16. Apparent digestibility coefficients of protein and lipid in diets (%, mean±SD, n=3)

	FO	NAN	SCH	NS	CS	SS
Protein	94.9±0.1^{c}	95.1±0.2^{bc}	94.8±0.3^{c}	96.1±0.0^{a}	95.4±0.1^{b}	95.1±0.0^{bc}
Lipid	92.5±0.5^{a}	89.4±0.9^{bc}	89.0±0.4^{c}	92.6±0.4^{a}	90.9±0.7^{b}	92.5±0.6^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

Table 17. Apparent digestibility coefficients of total amino acids in diets (%, mean±SD, n=3)

	FO	NAN	SCH	NS	CS	SS
Arg	96.43±0.34^{a}	97.05±0.37^{a}	95.91±0.92^{a}	96.79±0.31^{a}	96.31±0.62^{a}	97.15±0.41^{a}
Lys	98.07±0.33^{b}	98.25±0.15^{ab}	98.22±0.09^{ab}	98.66±0.21^{a}	95.83±0.08^{c}	98.26±0.25^{ab}
His	94.64±1.60^{a}	95.92±0.98^{a}	94.33±0.98^{a}	96.28±0.38^{a}	95.13±0.58^{a}	96.42±0.66^{a}
Phe	94.37±0.20^{a}	95.09±0.32^{a}	94.32±0.74^{a}	94.96±0.83^{a}	94.26±0.13^{a}	95.44±0.96^{a}
Leu	93.50±0.62^{a}	95.01±0.30^{a}	94.60±0.25^{a}	94.21±1.61^{a}	94.67±0.23^{a}	96.17±0.45^{a}
Ile	94.94±1.23^{a}	95.80±0.97^{a}	93.72±0.35^{a}	96.07±0.94^{a}	95.51±0.18^{a}	94.64±0.39^{a}
Met	97.94±0.51^{a}	98.68±0.19^{a}	98.43±0.66^{a}	98.53±0.34^{a}	98.07±0.15^{a}	99.09±0.41^{a}
Val	94.49±1.20^{a}	95.5±0.14^{a}	94.26±0.50^{a}	95.44±1.26^{a}	94.53±0.22^{a}	96.12±0.19^{a}
Thr	93.84±0.19^{c}	94.67±0.07^{bc}	94.05±0.35^{c}	95.06±0.55^{ab}	94.32±0.18^{bc}	95.90±0.31^{a}
Trp	95.89±2.05^{a}	97.8±1.13^{a}	97.9±1.61^{a}	99.23±0.25^{a}	94.75±6.10^{a}	98.40±0.68^{a}
Tau	98.45±1.31^{a}	98.51±0.29^{a}	99.14±0.49^{a}	96.50±2.78^{a}	97.42±0.36^{a}	97.02±0.81^{a}
Cys	93.97±0.98^{a}	94.47±0.96^{a}	90.84±0.66^{a}	93.47±1.76^{a}	93.16±1.04^{a}	94.25±3.36^{a}
Cysta	98.58±1.62^{a}	97.66±0.72^{a}	98.51±1.47^{a}	98.68±1.00^{a}	98.49±0.53^{a}	96.48±1.20^{a}
Ala	93.59±0.36^{a}	95.24±0.21^{a}	94.88±0.32^{a}	94.44±1.14^{a}	95.08±0.20^{a}	94.02±0.90^{a}
Tyr	93.58±0.39^{b}	94.62±0.48^{ab}	94.13±0.40^{ab}	94.33±1.32^{ab}	94.16±0.30^{ab}	95.46±0.06^{a}
Gly	93.13±0.90^{a}	94.24±0.68^{a}	93.35±0.59^{a}	94.18±0.68^{a}	93.26±0.28^{a}	92.64±0.55^{a}
Glu	99.11±0.02^{b}	99.30±0.04^{a}	99.31±0.04^{a}	99.26±0.13^{ab}	99.34±0.08^{a}	99.32±0.04^{a}
Ser	93.79±0.55^{a}	94.68±0.52^{a}	94.84±0.46^{a}	94.92±0.59^{a}	94.62±0.22^{a}	95.01±0.59^{a}
Asp	94.62±0.70^{b}	95.41±0.34^{ab}	95.19±0.35^{ab}	95.96±0.43^{a}	95.15±0.22^{ab}	95.22±0.32^{ab}
Pro	95.38±0.55^{a}	97.44±0.69^{a}	96.96±0.21^{a}	94.93±3.07^{a}	93.67±1.48^{a}	93.96±4.33^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

Table 18. Apparent digestibility coefficients of fatty acids in diets (%, mean±SD, n=3)

	FO	NAN	SCH	NS	CS	SS
14:0	94.6±0.5^{a}	88.4±0.1^{d}	91.5±1.0^{bc}	92.6±0.5^{b}	91.5±0.7^{bc}	90.3±0.8^{c}
16:0	89.7±0.4^{a}	84.9±0.2^{c}	84.4±0.9^{c}	89.5±0.1^{a}	87.5±0.5^{b}	90.7±0.0^{a}
18:0	82.9±0.3^{b}	75.0±0.3^{e}	76.7±0.2^{d}	84.8±0.2^{a}	81.2±1.0^{c}	81.3±0.1^{c}
ΣSFA*	90.1±0.3^{a}	83.6±0.2^{c}	84.4±0.8^{c}	89.5±0.2^{a}	87.4±0.2^{b}	89.8±0.1^{a}
16:1n-7	95.5±0.2^{a}	89.3±0.3^{c}	83.4±1.6^{d}	91.1±0.3^{bc}	89.6±0.0^{c}	92.6±0.4^{b}
18:1n-9	94.3±0.1^{a}	89.8±0.2^{c}	90.0±0.6^{c}	94.4±0.1^{a}	93.3±0.1^{b}	94.6±0.1^{a}
20:1n-9	93.5±0.1^{b}	97.8±3.8^{a}	ND	100.0±0.0^{a}	ND	ND
20:1n-11	82.3±1.1^{b}	75.5±4.0^{c}	82.7±0.4^{b}	89.2±1.1^{a}	86.6±1.0^{ab}	89.4±0.4^{a}
22:1n-11	86.5±1.2^{a}	ND	ND	ND	ND	ND
ΣMUFA**	93.4±0.1^{b}	89.7±0.3^{c}	89.9±0.6^{c}	94.2±0.1^{a}	93.2±0.1^{b}	94.5±0.1^{a}
18:2n-6	93.2±0.2^{c}	91.6±0.3^{e}	91.2±0.3^{e}	94.8±0.1^{a}	92.5±0.0^{d}	94.2±0.0^{b}
20:4n-6	94.0±0.5^{a}	84.6±0.9^{b}	85.9±0.4^{b}	62.7±2.4^{c}	92.0±0.9^{a}	95.6±2.9^{a}
Σn-6 PUFA***	93.2±0.2^{b}	91.5±0.3^{d}	91.2±0.3^{d}	94.6±0.1^{a}	92.5±0.0^{c}	94.2±0.0^{a}
18:3n-3	93.9±0.2^{bc}	93.1±0.3^{cd}	92.5±0.2^{d}	95.3±0.2^{a}	94.3±0.7^{ab}	94.9±0.4^{ab}
18:4n-3	96.5±0.3^{a}	64.9±11.6^{b}	ND	ND	ND	94.3±2.9^{a}
20:4n-3	96.0±0.1^{a}	ND	ND	93.1±0.4^{b}	94.1±0.6^{b}	95.8±0.5^{a}
20:5n-3	96.2±0.3^{a}	92.5±0.2^{b}	81.9±1.4^{c}	93.6±0.1^{ab}	91.7±2.3^{b}	92.0±1.6^{b}
22:5n-3	95.3±0.3^{a}	90.4±1.2^{b}	94.3±3.2^{ab}	96.9±0.9^{a}	96.3±1.2^{a}	96.1±1.3^{a}
22:6n-3	94.5±0.4^{a}	36.7±12.9^{b}	91.4±0.7^{a}	95.1±0.2^{a}	92.4±0.6^{a}	94.6±0.4^{a}
Σn-3 PUFA***	95.3±0.3^{a}	92.0±0.2^{c}	91.6±0.3^{c}	94.7±0.1^{a}	93.2±0.4^{b}	94.7±0.4^{a}
Σ PUFA***	94.0±0.1^{b}	91.6±0.1^{d}	91.3±0.3^{d}	94.6±0.1^{a}	92.7±0.1^{c}	94.4±0.2^{ab}
Σn-3 LCPUFA****	95.4±0.3^{a}	90.9±0.7^{c}	91.1±0.5^{c}	94.5±0.0^{a}	92.5±0.6^{b}	94.6±0.4^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Chapter 4.

Non-fish meal and non-fish oil diet development for red seabream, *Pagrus major*, by mixing several microalgae species

4.1 Objective of study

Microalgae have been known as a rich source of essential fatty acids, amino acids, vitamins (especially vitamin A, vitamin E, niacin, thiamine, and ascorbic acid), minerals, and carotenoid pigments and shown to be beneficial as a feed ingredient for marine organisms (Ahlgren et al., 1992; Walker and Berlinsky, 2011). It has been noted that microalgae can play an important role in aquaculture with high quality protein with indispensable amino acids and lipid with n-3 LC-PUFA including EPA and DHA (Brown, 1991; Becker, 2007; Handayani et al., 2011). Because of the various species with various proportions of microalgae, employment of microalgae species in fish feed needs to be done selectively with diverse inclusion level. Therefore, the effects of potential fishmeal replacements have to be evaluated by conducting feeding and digestibility trials with candidate microalgae (Gong et al., 2019).

One heterotrophic microalgae species, *Schizochytrium* sp., showed high lipid content, including relatively high DHA. Recently, 100% replacement of fish oil and fish meal by a mixture of plant meals and 11% algae meal (AM; *Schizochytrium* sp.) in a diet for red sea bream (*Pagrus major*) was succeeded with no significant growth retardation (Seong et al., 2019). Previous studies have shown that the other microalgae *Nannochloropsis* can be used at 10% in diet without negative effects on the growth performance and health of salmon (Sørensen et al., 2017). However, Nutrient digestibility of the algae meal incorporated feeds was lower than the fish-meal-based reference feeds (Gong et al., 2017; Sørensen et al., 2017). There were several studies to utilize microalgae *Chlorella* in aquaculture feed such as Crucian carp (Shi et al., 2017), African catfish (Enyidi, 2017) and Nile tilapia (Badwy et al., 2008). Through the studies, diets formulated with *Chlorella* algae meal showed acceptable

growth performance and feed utilization. There also have been several experiments to utilize the other microalgae *Spirulina* with diverse fish species including tilapia (Olvera-Novoa et al., 1998), Indian major carp (Nandeesha et al., 2001), rainbow trout (Teimouri et al., 2013) and parrot fish (Kim et al., 2013). *Spirulina* meal showed remarkable possibility of fish meal replacement with noticeable growth result.

The purpose of the present study was the further study to investigate the value of incorporating mixed microalgae (*Nannochloropsis*, *Chlorella*, and *Spirulina*) as a substitute of fish derived product in the development of non-fish meal and non-fish oil diets of juvenile red sea bream, with high inclusion level.

4.2 Materials and method

Experimental microalgae

Microlgae *Nannochloropsis*, *Chlorella*, *Spirulina* and *Schizochytrium* have been used as experimental subjects. *Nannochloropsis* held both protein and lipid, which are around 51% and 27%. *Chlorella* and *Spirulina* accommodated relatively high protein (57% and 69% each) and low lipid (16% and 12% each). *Schizochytrium* algae meal contained around 14% protein and 54% lipid [Table 2]. *Nannochloropsis* was purchased through Pacific Trading (Tokyo, Japan) and Schizochytrium was provided by Alltech Inc (Lexington, Kentucky, U.S.A). The other two microalgae were the product of Marine Tech (Aichi, Japan).

Nannochloropsis, *Chlorella* and *Spirulina* contain considerable amount of amino acids, including indispensable amino acids [Table 3]. The three protein-rich microalgae showed lack of taurine.

As the lipid sources, *Nannochloropsis* and *Schizochytrium* meal contained substantial content of n-3 LC-PUFA. The *Nannochloropsis* algae meal showed relatively high saturates and monoenes with approximately 22% of palmitoleic acid (16:1n-7) in fatty acid composition of total lipid in diet. High EPA (39.5% in lipid) was also shown in fatty acid composition of the *Nannochloropsis* and the EPA was the main content of the n-3 LC-PUFA. The *Schizochytrium* algae meal showed a relatively higher level of saturates with approximately 54% of palmitic acid (16:0) in fatty acid composition of total lipid. Low levels of total monoenes and n-6 LC-PUFA contained, but algae meal contains high level of n-3 LC-PUFA and the most of the LC-PUFA composed of DHA (28.3% in lipid) [Table 4].

Experimental diets

The experimental diets were six and formulated iso-nitrogenously and iso-lipidically (CP 47%, CL 19%). A control diet was formulated with fish oil with non-fish meal ingredients including soybean meal, soy protein concentrate and corn gluten meal (FO). Two different algae meal *Nannochloropsis* meal and *Schizochytrium* meal were used each of two diets for substitute of fish oil in FO (NAN, SCH). *Nannochloropsis* meal and *Schizochytrium* meal were blended and formulated in a non-fish meal and non-fish oil diet as lipid and protein source (NS). Two species of microalgae meal (*Chlorella* and *Spirulina*) was formulated in NS diet to replace plant protein source (NSC, NSS). 2.2% of amino acid premix (Lysine 1: Methionine 0.5: Threonine 0.5: Tryptophan 0.2) was supplemented to each diets to compensate for the lack of indispensable amino acid content and to improve dietary quality (Takagi et al., 2001). Also, 0.5% of taurine was supplemented to the every diet to meet the nutritional requirement (Matsunari et al., 2008a, 2008b). To reinforce palatability of feed, 1% of bonito peptide was supplemented to each diet. SSF (solid-state fermentation) enzyme mixture was also used in diets to relieve the negative effect of the anti-nutritional factor of plant protein source (Moura et al., 2012; Hanini et al., 2013) [Tables 1 & 5].

There were minor differences in total amino acid composition of the diets according to the diets formula [Table 3]. However, fatty acid composition of each diet showed considerable differences with different dietary lipid sources and fatty acid composition of the formulated microalgae were highly affected on each diet. NAN and SCH showed high monoenes fatty acids and n-6 PUFA but little or no DHA or EPA, respectively. FO and the other diets which formulated with more than two microalgae contain both of EPA and DHA

with high total n-3 PUFA [Table 7].

The animal experiment was followed the guideline of the Animal Experiment Treaty of the Tokyo University of Marine Science and Technology. The experiment was conducted at the Laboratory of Fish Nutrition, Tokyo University of Marine Science and Technology, Tokyo, Japan. Juvenile red sea bream (*Pagrus major*) were purchsed from A-marine Kindai Co. Ltd. (Wakayama, Japan) and commercial larval feed (Ambrose, Feed One Co. Ltd., Tokyo, Japan) was fed for 2 weeks. Before the beginning of the experiment, SCH diet was fed for 1 week to acclimate to the experimental diet. For beginning of the experiment, 384 fish (average weight 5.9 ± 2.3 g) were randomly distributed into twelve 60-L glass tanks. The re-circulating system filled with artificial seawater (Sea Life®, Tokyo, Japan; salinity, 30‰) was used for fish rearing and the water temperature was maintained at 21.0 ± 1.0 ℃. To confirm water quality in safe level, NO_2 and NO_3 levels were monitored by a colorimetric test kit (Kyoritsu Chemical-Check lab Co., Tokyo, Japan). Three-times of feeding (09:00, 12:00 and 16:00) were conducted to duplicate groups of fish by hand to apparent satiation for 75days. To maintain the acceptable water quality, the fish tanks were cleaned every day after the first feeding time and half of the water was replaced twice a month.

Fish weighing and sampling

Every 4 weeks and the last day of the experiment, the fish weight of fish had been measured. For the fish weighing, all fish in each group were anesthetized (2- phenoxyethanol at 0.5 ml/L) and weighed individually. 10 fish were randomly taken from the pool before

introduction into experimental tanks before a day of the experiment, for the chemical analysis of initial fish. Also, five fish from each tank were sampled randomly on the last day of the 75 days experiment, and sampled fish were ground with a centrifugal mill (Retsch, Haan, Germany) for chemical analysis. To avoid the biochemical effect of diet resided in gastrointestinal tract, fish were kept in starvation for 24 hours before the each sampling for chemical analyses. For the hepatosomatic index and assessment of liver lipid analyses, another six fish were also sampled randomly from each tank. The samples were stored at −30 ℃ freezer until analysis. The fecal materials were collected continuously for three weeks before the end of feeding trial. Glass trap column of each tank siphoned feces by the water flow after the third feeding of a day to before the first feeding. The collected feces were used for determination of chromic oxide and nutrients content and treated through centrifuging (3,000 rpm for 5 min), freeze-drying and grounding (Watanabe et al., 1996).

Biochemical analysis

was conducted by

Each sample was oven dried at 110 ℃ for 4h to dehydrate moisture. After the drying, each sample was weighed at one-hour intervals until a constant weight was obtained. Ash content was determined by incinerating each dried sample in a porcelain crucible using a muffle furnace at 600 ℃ overnight (Woyewoda et al., 1986). Crude protein content was measured by the Kjeldahl method with Kjeltec 2400 (Foss Ltd., Tokyo, Japan). The total lipids were extracted by using chloroform/methanol (2:1, v/v) to determine lipid content,

according to the methods of Folch et al (1957). The extracted total lipids from the whole body and liver of fish were separated into neutral and polar lipid fractions via silica cartridges (Sep-Pack, Waters Co., Milford, U.S.A.), as described by Juaneda and Rocquelin (1985). Fatty acid methyl esters (FAME) were prepared for the fatty acid analysis according to Christie and Han (2010). The prepared FAMEs were analyzed by a gas chromatograph (GC2025; Shimadzu Co., Tokyo, Japan) equipped with a hydrogen flame ionization detector and a silica capillary column (L × I.D., 30 m × 0.32 mm, Supelcowax 10 Fused Silica Capillary GC column; Supelco, Bellefonte, PA, USA).

Data calculation and statistical analysis

The growth performance of the fish was calculated by the following formulae:

Wet weight gain (WG) (%)

= (final mean wet weight (FW)(g) / initial mean wet weight (IW)(g)) × 100

Food conversion ratio (FCR)

= Dry feed intake (g) / (final mean wet weight (FW) (g) − initial mean wet weight (IW) (g))

Protein efficiency ratio (PER) (g/g)

= WG (g)/total protein intake (g)

Specific growth rate (SGR) (%bodyweight/day)

= [(ln FW (g) − ln IW (g))/time (days)]×100

Survival rate (SR) (%)

= (number of fish in each group remained/initial number of fish) × 100

Daily feed intake (DFI) (%)

= (Dry feed intake)/ [(IW (g) +FW (g))/2×time (days)] × 100

Apparent digestibility coefficients (ADC) (%)

= 100 − (1 − ((Cr_2O_3 in diet/ Cr_2O_3 in feces) × (nutrient in feces/nutrient in diet)))

To test the result of the experiment, all data were statistically analyzed by One Way Analyses of Variance (ANOVA; Sigma Stat 3.0, SPSS, Chicago, U.S.A.). Tukey's test was used to detect significant differences among means (P<0.05).

4.3 Results

Growth performance

Significant differences were observed in growth performances and feed utilization efficiencies at the end of the 75-day feeding period in the result of FW, WG, SGR and DFI. FW, WG and SGR were significantly lower in NSS diet group than NS and NSC diet groups. NSC diet group showed the significantly highest DFI. However, there were no significant differences shown in FCR, PER, SR and HSI. The result of FCR showed no significant difference however, it is NSS diet fed group showed the definitely highest value. [Table 8]

Proximate composition of fish carcass

There were no significant differences in moisture, ash and protein composition. In contrast, NSS diet group showed significantly lower lipid composition than the other diet groups and showed significant difference with FO and NAN diet fed groups. [Table 9]

Polar and Neutral lipid content

The polar lipid content of lipid in whole fish body and liver showed no significant difference in each group. However, there were significant differences in neutral lipid content. NSS diet group showed the lowest neutral lipid content in fish body and liver. In contrast, NAN diet group showed the significantly highest neutral lipid content. [Table 10]

Total amino acid composition of fish carcass

There was significant difference in the result of total amino acid composition of whole body of fish. In indispensable amino acids, there were significant differences in lysine, histidine, phenylalanine, leucine and tryptophan. There also were significant differences in dispensable amino acid composition, in alanine, tyrosine, glutamic acid, serine and aspartic acid. NSS diet group showed generally higher amino acid composition than the other diets groups. [Table 11]

Fatty acid profile

Except stearic acid (18:0), there were significant differences in every fatty acids profile of total lipid from whole body. Most of the fatty acid composition showed similar trend with the dietary fatty acid. NAN diet group showed the significantly lowest fatty acid composition of the total saturates and NSS group showed the highest. Monoenes fatty acid was generally higher than the other fatty acids group. On the contrary, n-3 PUFA was lower than the other fatty acids group. Total n-3 PUFA of NS, NSC and NSS showed the significantly highest with high EPA and DHA content. Accordingly, diets formulated with more than two microalgae groups showed the significantly higher LC-PUFA than single microalgae formulated diets. NAN and SCH diets fed fish showed significantly lower total saturates and n-3 PUFA than the other diet groups and higher monoenes fatty acids and n-6 PUFA [Table 12].

The fatty acid profile of the total lipid of the fish liver seems similar to the fatty acid composition in whole body of the fish. Exceptionally, NSS diets group showed higher total saturates and total n-3 PUFA level. Diets formulated with more than two microalgae groups showed the significantly higher total n-3 PUFA and n-3 LC-PUFA than single microalgae formulated diets [Table 13].

There was a significant difference in most of fatty acids except stearic acid (18:0) and total PUFA in the result of polar lipid fatty acid analysis in the fish body. Lower total monounsaturated fatty acids and n-6 PUFA level, and higher saturated fatty acids and n-3 PUFA than those of total lipid in fish body were observed in fatty acid composition of polar lipid in the fish body. Relevantly, n-3 LC-PUFA including EPA, docosapentaenoic acid (DPA, 22:5n-3) and DHA became increased than the fatty acid composition of total lipid in fish

body. Diets formulated with more than two microalgae groups showed the significantly higher total n-3 PUFA and n-3 LC-PUFA than single microalgae formulated diets. They also showed higher DHA composition. 18:1/n-3 LC-PUFA ratio was also calculated. NAN diet fed fish group showed the highest ratio [Table 14].

Higher total saturated fatty acid and total n-3 PUFA with increased n-3 LC-PUFA levels than the fatty acid composition of polar lipid in the whole body of fish were shown in the fatty acid profile of the polar lipid in the liver. However, total monoenes and n-6 PUFA showed lower trend. Diets formulated with more than two microalgae groups showed the significantly higher n-3 PUFA and n-3 LC-PUFA composition than single microalgae formulated diets with higher DHA than NAN group, EPA than SCH group. 18:1/n-3 LC-PUFA ratio was calculated and NAN group showed the highest. The numerical value was 1 [Table 15].

Digestibility

NS diet fed fish showed the significantly highest and NAN group showed the lowest protein digestibility. There was no significant difference in lipid digestibility. [Table 16]

ADC of total amino acid in both of indispensable and dispensable amino acids showed significant differences, except tryptophan, cysteine, cystathionine and proline. FO, NS and NSS groups showed generally higher ADC than NAN, SCH and NSC diet groups in indispensable and dispensable amino acids. NSC diet group showed generally similar amino acids digestibility with single microalgae adopted diet groups [Table 17]

There was significant difference in ADC of fatty acids in total lipid of diets, except palmitoleic acid (16:1n-7). NAN and SCH, single microalga formulated diet groups, generally showed the lowest digestibility in overall fatty acids with NSC diet group. Whereas, diets which used mixed microalgae meal showed better digestibility, except NSC diet group. The digestibility of algae meal formulated diet groups was significantly lower than that of FO group except oleic acid (18:1n-9). Algae meal formulated diets showed significantly lower DHA digestibility with FO diet fed fish [Table 18].

4.4 Discussion

There were significant differences in the result of growth performance and feed utilization efficiencies of red sea bream fed experimental diets over 75 days. More than two microalgae meal formulated diet group showed significantly no differences between fish fed control except NSS diet group in FW, WG and SGR. 2nd experiment showed similar growth performance in fish fed vegetarian diet supplemented with *Schizochytrium* meal groups which showed significantly no difference with fish oil diet group. In the previous experiment, the formula of the fish oil formulated diet and the FO diet of current experiment were the same with the present study, and the algae meal diet formulated with 5% was also same with the SCH diet in the present study. Fish fed the NSS diet showed the lowest growth performance probably because of high FCR and low PER of the group. Despite there was no significant difference in FCR and PER, NSS diet group showed the highest FCR and the lowest PER among the groups, which indicates feed efficiency was lower than the other diet groups.

There was no significant difference in proximate composition of the whole body except lipid composition. Fish fed NSS diet showed the significantly lowest lipid content ($P < 0.05$). There might be strong accordance with growth performance, the NSS group stored less lipid than the other groups because of the poor feed efficiency. SCH group showed lower lipid accumulation in the whole body than the other diet groups but there was no significant difference. In 1st and 2nd experiments, the lipid accumulation of diets employed only *Schizochytrium* or *Nannochloropsis* meal as EFA source were notably lower than the fish oil used diet, and the result may indicate that the lipid of *Schizochytrium* or *Nannochloropsis* meal is rarely accumulated in the fish body than the other lipid sources (Seong et al., 2019).

In the lipid of whole body and liver, there was no significant difference in polar lipid content among the groups, but there was significant difference in the neutral lipid content among the groups. The results may suggest an upper limit of polar lipid content in whole body and liver and the additional accumulated lipid was the form of neutral lipid. Fish fed algae meal diet showed a significantly lower neutral lipid content in the whole body than FO diet except NAN diet group. This could be due to low lipid content of NSS diet group and low neutral lipid content in *Schizochytrium* sp. meal which showed similar results in the previous studies in 1st and 2nd experiments, and suggested that neutral lipids in diet can be easily assimilated and stored in fish body. NSS diet group showed significantly lower hepatic neutral lipid content than the other diets groups, and it may cause by the low lipid composition of liver. NSC diet group contained high neutral lipid content in whole fish body and liver among the blended algae meal diet group, with strong accordance with high growth performance with significantly higher DFI.

It has been known that the total amino acids requirement of juvenile red sea bream is Arg 3.5, His 1.4, Ile 2.2, Leu 4.2, Met+Cys 2.2, Phe+Tyr 4.1, Thr 1.8, Trp 0.6, Val 2.5 % of dietary protein (Forster and Ogata, 1998), and the diets used in current study met the essential amino acid requirement. Generally NSS diet group showed significantly higher indispensable and dispensable amino acids composition than NSC diet group and it maybe cause by high dry protein content of NSS diet fed fish. Total amino acid composition of fish body did not follow the composition of diets.

Similar to the other aquacultural marine species, n-3 LC-PUFA have considered as the most important fatty acids for red sea bream. Most of the marine species including red sea bream have limited or no ability to desaturate and elongate 18:3n-3 to n-3 LC-PUFA as well as 18:2n-6 to 22:5n-6 via 20:4n-6 because of low or absent Δ6- and Δ5-desaturase activities (Bell et al., 1994; Montero et al., 2004; Izquierdo et al., 2005). EFA requirement of juvenile red sea bream has been known as 1% EPA and 0.5% DHA in diet, respectively (Takeuchi et al., 1990). In this experiment, every diet met the EFA requirement of red sea bream with more than 1% of EPA and/or 0.5% of DHA content in diet. The result of fatty acids composition on the total lipid of fish body and liver demonstrated their poor ability of n-3 LC-PUFA synthesis, as well as they were strongly influenced by the different fatty acid compositions of each six diets. In the fatty acids composition in the diet and total lipid in whole body and liver, monoenes fatty acids seemed to be accumulated, in comparison with dietary fatty acid composition. In the each of algae meal formulated groups, 1.11-1.17 folds of DHA and 0.59-0.70 folds of EPA was observed in fatty acid composition of total lipid in whole body than those of dietary fatty acid. In contrast, fish oil formulated diet showed similar or lower value

with 0.83 fold of DHA and 0.61 fold of EPA. The proportions of DHA in hepatic total lipid in fish and diet total lipid showed divergent trend, FO diet group showed same proportion with 0.83 fold and more than two microalgae used diet groups showed higher proportions with 0.93 to 1.17 folds. Single algae meal adopted groups showed lower proportions of hepatic total lipid and diet total lipid in EPA and DHA, and it was lower than the each proportions of FO diet group.

Fatty acid composition of polar lipids in the fish body generally showed different trends from those of the total lipid of the whole body and dietary lipids. The result seems to suggest selective utilization and retention of specific fatty acids (Huang et al., 2007). Similar to the previous study in 2017 and 2018, higher total saturates level was observed in fatty acid composition of polar lipid from whole body than those of the total lipid in the whole body and dietary lipid in fatty acid compositions of each diet groups. All groups showed generally lower monoenes content in whole body and hepatic polar lipid than those of total lipid of the whole body, liver and dietary lipid. This result suggests that most of the monounsaturated fatty acid was stored as neutral lipid. Total n-6 PUFA content in polar lipid of the whole body and liver was lower than those in total lipid of whole body and liver. However, n-3 LC-PUFA contents including EPA, DPA, and DHA in polar lipid of whole body and liver showed drastically increased trend than total lipid of whole body and liver. These results may suggest that the n-3 LC-PUFA in diets have been utilized selectively and accumulated preferentially as forms of polar lipid in fish body (Takama et al., 1994; Huang et al., 2007). The ratio of 18:1n-9/Σn-3 LC-PUFA in the fatty acid composition of polar lipid of the whole body and hepatic polar lipid have been considered as an EFA index for red sea bream (Fujii and Yone,

1976) and gilthead bream (Kalogeropoulos et al., 1992). When the numerical ratio more than 1 is considered as EFA deficiency associated with poor growth performance and feed efficiency. Except for fish fed NAN diet, which ratios were 1.42 in fatty acid composition of polar lipid from whole body, the other groups showed the ratio less than 1with no negative effects on fish growth.

Generally, two algae meal diet groups showed significantly no difference or higher ADC with FO diet group except NSC group which showed lower numerical value in protein digestibility. There were no significant difference in lipid digestibility, NSC diet group showed the lowest numerical value with SCH and NSS diet group. Accordingly, ADC of fatty acids in diets showed similar trend. FO and NS diet group showed significantly better performance in overall fatty acids and SCH, NSC and NSS diet group showed lower digestibility, antithetically. In spite of the low protein and lipid digestibility, NSC diet group showed the significantly highest growth. The result may cause by high DFI and that indicates the improvement of palatability in NSC diet. Digestibility of fatty acids of protein and lipid in NS diet group showed generally higher or similar than that of FO diet group. These result may suggest that formulating diets with the mixed two microalgae species may increase nutritional value (Spolaore et al., 2006; Becker, 2007; Hemaiswarya et al., 2011) and raised the digestibility of the diets.

In conclusion, formulating non-fish meal, non-fish oil diet for red sea bream with microalgae species was successful with considerable growth result, significantly no difference with FO diet group. As it showed in previous study in 2018, three microalgae species, *Nannochloropsis*, *Chlorella*, *Spirulina* and *Schizochytrium* are suitable substitute for

fish derived products for the juvenile red sea bream diet without fatal negative effects. By using more than two microalgae meal, it seems to organize better nutritional value than only one microalgae used diets. These results imply that development of non-fish meal and non-fish oil diet highly covered with high inclusion of mixed microalgae species was successful with remarkable growth performance and considerable fish body fatty acid composition. As the last step of algae meal diet development, further work should include experimental diet which formulated with both fish meal and fish oil as a control group.

4.5 References

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4.6 Experimental tables

Table 1. Formula of the experimental diets for red sea bream (g kg^{-1})

	Experimental diets					
Ingredients	FO	NAN	SCH	NS	NSC	NSS
Soy protein concentrate	140	120	120	120	120	120
Soybean meal	200	150	200	150	50	140
Corn gluten meal	350	260	350	240	140	50
Nannochloropsis meal[a]	0	200	0	200	200	200
Chlorella meal[b]	0	0	0	0	200	0
Spirulina meal[c]	0	0	0	0	0	200
Schizochytrium meal[d]	0	0	50	50	50	50
Wheat flour	6	6	6	6	6	6
Pregelatinized-starch	55	55	55	55	55	55
Fish oil[e]	90	0	0	0	0	0
Rape seed oil	30	80	90	50	50	50
Amino acid (Lys1.0, Met0.5)	22	22	22	22	22	22
Vitamin premix[f]	30	30	30	30	30	30
Vitamin E	1	1	1	1	1	1
Choline chloride	5	5	5	5	5	5
Mineral premix[g]	10	10	10	10	10	10
Calcium phosphate	40	40	40	40	40	40
Bonito peptide	10	10	10	10	10	10
SSF Enzyme mix[h]	1	1	1	1	1	1
Taurine	5	5	5	5	5	5
Cr_2O_3	5	5	5	5	5	5
Total	1000	1000	1000	1000	1000	1000

[a] *Nannochloropsis* algae meal (Pacific Trading Co., Ltd, Tokyo, Japan)

[b] *Chlorella* algae meal (Marine Tech Inc., Aichi, Japan)

[c] *Spirulina* algae meal (Marine Tech Inc., Aichi, Japan)

[d] *Schizochytrium* algae meal (Alltech Inc., Lexington, Kentucky, U.S.A).

[e] Cod liver oil (Kanematsu Shintoa Foods Co. Ltd, Tokyo, Japan).

[f] Vitamin mixture composition (unit kg^{-1}) : Vitamin D_3, 2420000 IU; Vitamin K_3, 6050 mg; thiamin, 3025 mg; riboflavin, 3630 mg; pyridoxine, 2420 mg; cyanocobalamine, 6 mg; L-

ascorbic acid, 368900 mg; nicotinic acid, 24200 mg; D-pantothenic acid, 6050 mg; inositol, 121 000 mg; D-biotin, 363 mg; folic acid, 908 mg; para-aminobenzoic acid, 3025 mg.

[g] Mineral mixture compostition (g/kg^{-1}) : Sodium chloride 50; magnesium sulphate 745; iron(III) cirtrate n-hydrate 125; trace element mix 50; cellulose 30 [trace element mixture contains ($g\ kg^{-1}$) : zinc sulphateheptahydrate 353; manganese sulphate 162; copper(II) sulphate pentahydrate 31; aluminum chloride hexahydrate 10; cobalt chloride hexahydrate 10; cobalt chloride 3; potassium iodate 1; cellulose 440].

[h] SSF (Alltech Inc., Lexington, Kentucky, U.S.A).

Table 2. Proximate composition of the algae meals (% dry weight basis except for % moisture)

	Nannochlorpsis[a]	*Chlorella*[b]	*Spirulina*[c]	*Schizochytrium*[d]
Moisture	2.1	3.6	2.8	2.6
Protein	50.7	56.8	69.2	13.5
Lipid	27.2	16.0	12.3	54.2
Ash	8.4	2.5	4.7	6.7

[a] *Nannochloropsis* algae meal (Pacific Trading Co., Ltd, Tokyo, Japan)

[b] *Chlorella* algae meal (Marine Tech Inc., Aichi, Japan)

[c] *Spirulina* algae meal (Marine Tech Inc., Aichi, Japan)

[d] *Schizochytrium* algae meal (Marine Tech Inc., Aichi, Japan).

Table 3. Total amino acids composition of the microalgae (in diet %)

	Nannochlorpsis[a]	*Chlorella*[b]	*Spirulina*[c]	*Schizochytrium*[d]
Arg	3.39	3.57	4.22	1.17
Lys	3.94	4.51	3.24	0.74
His	0.99	1.08	1.01	0.18
Phe	2.40	2.63	3.13	0.42
Leu	4.42	4.67	5.33	0.73
Ile	1.43	1.30	2.27	0.31
Met	1.32	1.21	1.74	0.27
Val	2.06	2.03	2.02	0.37
Thr	2.48	2.47	3.03	0.57
Trp	0.97	0.80	1.65	0.38
Tau	0.00	0.00	0.00	0.28
Cys	0.25	0.25	0.03	0.04
Cysta	0.11	0.13	0.19	0.22
Ala	4.31	5.22	6.26	0.74
Tyr	1.93	2.08	3.17	1.36
Gly	3.30	3.52	4.16	0.58
Glu	6.68	7.73	11.61	2.43
Ser	2.79	2.67	4.16	0.75
Asp	2.41	5.83	7.81	1.25
Pro	3.30	2.96	2.65	0.34

[a] *Nannochloropsis* algae meal (Pacific Trading Co., Ltd, Tokyo, Japan)

[b] *Chlorella* algae meal (Marine Tech Inc., Aichi, Japan)

[c] *Spirulina* algae meal (Marine Tech Inc., Aichi, Japan)

[d] *Schizochytrium* algae meal (Marine Tech Inc., Aichi, Japan).

Table 4. Fatty acids composition of the microalgae (area %)

	Nannochlorpsis[a]	*Chlorella*[b]	*Spirulina*[c]	*Schizochytrium*[d]
14:0	3.8	0.4	0.9	4.8
16:0	12.7	18.6	49.2	54.2
18:0	0.3	0.4	1.2	1.3
ΣSFA[*]	16.8	19.4	51.3	60.3
16:1n-7	22.1	0.4	1.7	0.1
18:1n-9	2.9	1.8	1.5	0.0
20:1n-9	0.0	0.1	0.0	0.0
22:1n-11	0.1	0.0	0.0	0.0
ΣMUFA[**]	25.2	2.4	3.3	0.2
18:2n-6	3.8	43.1	19.2	0.0
20:4n-6	4.5	0.0	0.2	0.8
Σn-6 PUFA[***]	8.3	43.1	37.3	6.0
18:3n-3	0.1	2.1	0.1	0.0
18:4n-3	0.1	0.0	0.0	0.0
20:4n-3	0.1	0.0	0.1	0.3
20:5n-3	39.5	0.0	0.1	0.2
22:5n-3	0.1	0.0	0.0	0.0
22:6n-3	0.1	1.2	2.9	28.3
Σn-3 PUFA[***]	40.0	3.4	3.2	29.0
ΣPUFA[***]	49.4	73.8	40.5	35.0
Σn-3 LCPUFA[****]	39.9	1.3	3.1	28.9

[a] *Nannochloropsis* algae meal (Pacific Trading Co., Ltd, Tokyo, Japan)

[b] *Chlorella* algae meal (Marine Tech Inc., Aichi, Japan)

[c] *Spirulina* algae meal (Marine Tech Inc., Aichi, Japan)

[d] *Schizochytrium* algae meal (Marine Tech Inc., Aichi, Japan).

[*] Saturated fatty acid.

[**] Monounsaturated fatty acid.

[***] Polyunsaturated fatty acid.

[****] Long-chain polyunsaturated fatty acid.

Table 5. Proximate composition of the experimental diets (% dry weight basis except for % moisture)

	FO	NAN	SCH	NS	NSC	NSS
Moisture	3.6	4.3	3.8	4.4	4.6	5.4
Protein	47.4	48.3	46.5	47.8	47.1	48.3
Lipid	18.8	20.1	19.7	19.7	21.3	19.2
Ash	6.8	7.7	6.9	7.5	8.3	7.7

Table 6. Total amino acids composition of the diets (in diet %)

	FO	NAN	SCH	NS	NSC	NSS
Arg	2.06	2.42	2.10	2.58	2.57	2.77
Lys	3.03	3.77	3.04	3.98	4.06	5.50
His	0.90	0.94	0.90	0.94	0.90	0.87
Phe	2.35	2.28	2.32	2.21	2.19	1.81
Leu	4.93	4.45	4.89	4.20	4.16	3.15
Ile	1.19	1.24	1.20	1.29	1.21	1.40
Met	1.74	1.83	1.69	1.80	1.78	2.73
Val	1.30	1.45	1.34	1.53	1.57	1.40
Thr	1.53	1.73	1.54	1.80	1.84	1.79
Trp	0.31	0.35	0.28	0.32	0.37	0.55
Tau	0.58	0.66	0.58	0.71	0.56	1.17
Cys	0.43	0.39	0.43	0.40	0.32	0.23
Cysta	0.11	0.11	0.10	0.09	0.10	0.24
Ala	3.09	3.15	3.00	2.97	3.32	2.86
Tyr	1.98	1.84	1.92	1.80	1.71	1.54
Gly	1.67	2.02	1.60	2.01	2.17	2.23
Glu	9.32	8.45	9.10	8.15	7.57	7.73
Ser	2.55	2.52	2.47	2.46	2.33	2.26
Asp	3.96	4.27	3.83	4.31	4.23	4.59
Pro	3.31	3.20	3.23	3.02	2.90	2.26

Table 7. Fatty acids composition of the diets (area %)

	FO	NAN	SCH	NS	NSC	NSS
14:0	3.5	0.8	0.9	1.9	1.9	2.2
16:0	12.1	7.1	16.3	20.6	21.1	25.3
18:0	2.1	1.5	1.6	1.5	1.3	1.4
ΣSFA*	17.7	9.4	18.8	24.0	24.2	28.9
16:1n-7	3.6	3.5	0.1	3.9	3.9	4.5
18:1n-9	25.7	42.2	42.7	28.8	25.1	26.0
20:1n-11	5.1	0.6	0.6	0.4	0.4	0.4
22:1n-11	4.6	0.0	0.0	0.0	0.0	0.0
ΣMUFA**	39.1	46.2	43.5	33.1	29.4	30.9
18:2n-6	20.2	24.2	25.2	20.3	21.6	14.9
20:4n-6	0.3	0.7	0.1	1.0	0.9	1.0
Σn-6 PUFA***	20.5	24.9	25.4	21.3	22.5	17.5
18:3n-3	2.9	4.5	4.6	3.1	3.3	3.1
18:4n-3	1.2	0.0	0.0	0.0	0.0	0.0
20:4n-3	0.4	0.0	0.0	0.1	0.1	0.1
20:5n-3	5.1	6.3	0.0	7.9	7.4	8.3
22:5n-3	0.7	0.0	0.0	0.0	0.0	0.0
22:6n-3	4.1	0.1	3.1	3.5	3.3	4.1
Σn-3 PUFA***	14.4	10.8	7.6	14.6	14.1	15.6
ΣPUFA***	34.8	35.7	33.0	35.8	39.7	33.1
Σn-3 LCPUFA****	11.4	6.4	3.1	11.4	10.9	12.5

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 8. Growth performance and feed utilization efficiencies of red sea bream fed experimental diets over 75 days (mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
IW(g)	5.9±0.0	5.9±0.0	5.9±0.0	5.9±0.0	5.9±0.0	5.9±0.0
FW(g)	41.3±1.1^{ab}	40.4±3.1^{ab}	37.6±8.1^{ab}	52.1±6.9^{a}	52.9±1.4^{a}	34.1±3.0^{b}
WG(%)	699±18^{ab}	683±53^{ab}	637±138^{ab}	881±118^{a}	896±23^{a}	578±51^{b}
FCR	0.72±0.24^{a}	0.70±0.04^{a}	0.81±0.16^{a}	0.77±0.21^{a}	0.78±0.26^{a}	0.98±0.09^{a}
PER	2.05±0.10^{a}	1.90±0.07^{a}	1.84±0.16^{a}	2.03±0.06^{a}	1.84±0.28^{a}	1.53±0.32^{a}
SGR	2.63±0.04^{ab}	2.60±0.10^{ab}	2.49±0.29^{ab}	2.93±0.18^{a}	2.96±0.03^{a}	2.37±0.12^{b}
SR(%)	73.4±2.2^{a}	78.1±13.3^{a}	70.3±6.6^{a}	73.4±15.5^{a}	76.6±19.9^{a}	73.4±15.5^{a}
DFI(%)	1.88±0.09^{b}	2.01±0.00^{ab}	1.97±0.13^{ab}	2.02±0.01^{ab}	2.32±0.18^{a}	2.25±0.14^{ab}
HSI	1.57±0.01^{a}	1.98±0.29^{a}	1.94±0.05^{a}	1.73±0.01^{a}	1.99±0.06^{a}	1.51±0.22^{a}

Values with different superscript letters in the same row were significantly different when $P<0.1$ (Tukey's test, a>b>c).

Table 9. Proximate composition of the whole body of the fish fed the experimental diets (% wet weight basis, mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
Moisture	67.3±0.3[a]	67.6±1.0[a]	70.5±1.5[a]	68.1±0.3[a]	67.9±0.6[a]	71.6±3.2[a]
Ash	4.0±0.1[a]	3.8±0.1[a]	3.5±0.1[a]	3.8±1.4[a]	3.6±0.8[a]	4.3±0.0[a]
Protein (w.b)	17.8±0.7[a]	17.5±0.7[a]	17.2±0.1[a]	18.0±0.3[a]	17.2±0.1[a]	18.1±0.7[a]
Lipid (w.b)	10.8±0.9[a]	10.9±0.2[a]	9.3±1.6[ab]	10.3±0.7[ab]	10.5±0.2[ab]	6.7±2.1[b]

Values with different superscript letters in the same row were significantly different when $P<0.1$ (Tukey's test, a>b>c)

Table 10. Polar and neutral lipid content in whole body and liver of fish (% wet weight basis, mean±SD, n=2)

	Lipid in whole fish body					
	FO	NAN	SCH	NS	NSC	NSS
Polar (%)	0.83±0.04^{a}	0.89±0.13^{a}	0.79±0.01^{a}	0.83±0.10^{a}	0.90±0.06^{a}	0.77±0.13^{a}
Neutral (%)	9.97±0.04^{a}	10.01±0.13^{a}	8.52±0.01^{c}	9.48±0.10^{b}	9.57±0.06^{b}	5.95±0.13^{d}

	Lipid in fish liver					
	FO	NAN	SCH	NS	NSC	NSS
Polar (%)	2.93±0.96^{a}	2.61±2.34^{a}	1.93±0.50^{a}	1.40±0.36^{a}	2.62±0.71^{a}	2.51±1.06^{a}
Neutral (%)	23.51±0.96^{bc}	31.29±2.34^{a}	21.78±0.50^{bc}	21.97±0.36^{bc}	25.34±0.71^{b}	20.07±1.06^{c}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

Table 11. Total amino acids composition of whole body of fish (in dry fish body %, mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
Arg	4.01±0.23^{a}	4.03±0.26^{a}	4.36±0.31^{a}	4.09±0.05^{a}	3.81±0.13^{a}	4.54±0.02^{a}
Lys	5.52±0.19^{c}	5.51±0.16^{c}	6.10±0.03^{ab}	5.97±0.08^{b}	5.43±0.04^{c}	6.48±0.01^{a}
His	1.48±0.07^{abc}	1.42±0.07^{bc}	1.65±0.05^{a}	1.52±0.03^{abc}	1.38±0.00^{c}	1.58±0.02^{ab}
Phe	2.58±0.13^{bc}	2.60±0.05^{bc}	2.77±0.02^{bc}	2.81±0.04^{ab}	2.51±0.04^{c}	3.09±0.08^{a}
Leu	4.60±0.06^{bc}	4.57±0.15^{bc}	5.02±0.04^{a}	4.94±0.17^{ab}	4.48±0.02^{c}	5.34±0.06^{a}
Ile	1.64±0.00^{a}	1.64±0.15^{a}	1.84±0.05^{a}	1.93±0.17^{a}	1.62±0.24^{a}	1.79±0.14^{a}
Met	1.91±0.07^{a}	1.84±0.00^{a}	1.85±0.30^{a}	1.92±0.06^{a}	1.74±0.12^{a}	2.18±0.09^{a}
Val	2.00±0.01^{a}	2.12±0.13^{a}	2.33±0.11^{a}	2.40±0.18^{a}	1.95±0.22^{a}	2.27±0.10^{a}
Thr	2.77±0.00^{d}	2.83±0.08^{cd}	3.10±0.04^{b}	2.98±0.04^{bc}	2.74±0.01^{d}	3.29±0.02^{a}
Trp	0.99±0.01^{ab}	0.78±0.00^{b}	1.02±0.18^{ab}	0.94±0.13^{ab}	0.99±0.15^{ab}	1.25±0.03^{a}
Tau	1.34±0.02^{a}	1.33±0.02^{a}	1.38±0.10^{a}	1.29±0.11^{a}	1.56±0.21^{a}	1.66±0.11^{a}
Cys	0.10±0.00^{a}	0.15±0.02^{a}	0.14±0.03^{a}	0.13±0.05^{a}	0.10±0.00^{a}	0.15±0.01^{a}
Cysta	0.08±0.00^{a}	0.06±0.01^{a}	0.06±0.04^{a}	0.05±0.03^{a}	0.11±0.01^{a}	0.08±0.01^{a}
Ala	4.63±0.13^{b}	4.42±0.12^{b}	4.75±0.04^{ab}	4.45±0.02^{b}	4.60±0.17^{b}	5.30±0.23^{a}
Tyr	1.97±0.01^{b}	1.99±0.09^{b}	2.15±0.06^{b}	2.13±0.07^{b}	1.94±0.03^{b}	2.39±0.02^{a}
Gly	4.71±0.55^{a}	4.53±0.52^{a}	4.63±0.05^{a}	4.40±0.09^{a}	4.75±0.29^{a}	5.26±0.07^{a}
Glu	1.66±0.04^{bc}	1.63±0.01^{c}	1.77±0.02^{b}	1.69±0.00^{bc}	1.64±0.06^{bc}	1.99±0.05^{a}
Ser	3.10±0.03^{bc}	3.08±0.01^{bc}	3.29±0.08^{b}	3.12±0.01^{bc}	3.02±0.12^{c}	3.66±0.06^{a}
Asp	6.74±0.23^{b}	6.63±0.17^{b}	7.27±0.11^{b}	7.02±0.05^{b}	6.75±0.31^{b}	8.10±0.14^{a}
Pro	2.74±0.17^{a}	2.70±0.18^{a}	2.71±0.23^{a}	2.68±0.29^{a}	2.64±0.29^{a}	3.20±0.14^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

Table 12. Fatty acids composition of total lipid from whole body of fish (area %, mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
14:0	2.5±0.2^{a}	0.8±0.1^{b}	0.8±0.2^{b}	1.4±0.1^{ab}	2.3±0.9^{ab}	1.7±0.1^{ab}
16:0	13.6±1.4^{d}	10.1±0.3^{e}	13.7±0.2^{cd}	17.2±0.2^{ab}	17.1±1.5^{bc}	20.6±0.2^{a}
18:0	3.4±0.1^{a}	3.4±0.4^{a}	2.8±0.3^{a}	3.7±0.3^{a}	3.8±0.6^{a}	4.2±0.2^{a}
ΣSFA*	19.5±1.6^{cd}	14.3±0.1^{e}	17.3±0.1^{de}	22.3±0.4^{bc}	23.1±1.2^{ab}	26.4±0.1^{a}
16:1n-7	3.6±0.0^{c}	3.6±0.1^{c}	0.5±0.1^{d}	4.0±0.1^{ab}	4.0±0.1^{b}	4.4±0.2^{a}
18:1n-9	29.0±0.3^{b}	42.0±1.3^{a}	42.0±1.2^{a}	32.1±0.1^{b}	26.8±2.8^{b}	28.2±0.1^{b}
20:1n-9	3.6±0.0^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	1.1±1.5^{ab}	0.0±0.0^{b}
20:1n-11	2.4±0.1^{a}	1.7±0.5^{ab}	1.3±0.0^{b}	1.2±0.1^{b}	1.4±0.4^{b}	1.1±0.0^{b}
ΣMUFA**	38.6±0.2^{c}	47.2±0.8^{a}	43.9±1.1^{b}	37.3±0.3^{c}	33.3±0.8^{d}	33.7±0.2^{d}
18:2n-6	19.0±0.1^{bc}	22.2±1.0^{ab}	25.0±0.0^{a}	19.7±0.1^{b}	18.3±2.5^{bc}	15.3±0.2^{c}
20:4n-6	0.2±0.0^{c}	0.6±0.1^{b}	0.0±0.0^{d}	0.9±0.0^{a}	0.7±0.0^{b}	1.0±0.0^{a}
Σn-6 PUFA***	19.2±0.1^{bc}	22.8±1.0^{ab}	25.0±0.0^{a}	20.6±0.0^{b}	19.0±2.4^{bc}	16.2±0.2^{c}
18:3n-3	2.4±0.2^{a}	4.0±0.3^{a}	4.1±0.0^{a}	3.1±0.0^{a}	3.9±1.0^{a}	2.9±0.2^{a}
18:4n-3	0.7±0.0^{a}	0.1±0.1^{b}	0.0±0.0^{b}	0.1±0.1^{b}	0.2±0.3^{ab}	0.1±0.1^{ab}
20:5n-3	3.1±0.3^{b}	4.4±0.4^{a}	0.2±0.1^{c}	5.2±0.3^{a}	4.4±0.3^{a}	5.2±0.4^{a}
22:5n-3	1.1±0.1^{a}	1.0±0.2^{a}	0.0±0.0^{b}	0.9±0.1^{a}	0.9±0.1^{a}	1.0±0.0^{a}
22:6n-3	3.4±0.8^{a}	0.2±0.2^{b}	3.5±0.5^{a}	3.9±0.1^{a}	3.8±0.8^{a}	4.8±0.1^{a}
Σn-3 PUFA***	10.7±1.5^{ab}	9.7±0.3^{ab}	7.8±0.6^{b}	13.1±0.6^{a}	13.3±2.6^{a}	13.9±0.4^{a}
ΣPUFA***	29.9±1.6^{b}	32.5±1.3^{ab}	32.7±0.6^{ab}	33.7±0.6^{a}	32.3±0.1^{ab}	30.1±0.5^{ab}
Σn-3 LCPUFA****	8.3±1.3^{ab}	5.6±0.0^{bc}	3.7±0.6^{c}	10.1±0.6^{a}	9.4±1.5^{a}	11.0±0.2^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 13. Fatty acids composition of hepatic total lipid in fish (area %, mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
14:0	1.5±0.1^{a}	0.7±0.2^{bc}	0.5±0.0^{c}	0.8±0.0^{bc}	0.9±0.1^{bc}	1.1±0.2^{ab}
16:0	14.5±1.0^{abc}	11.1±0.9^{c}	12.0±0.7^{bc}	15.4±1.0^{ab}	15.9±0.3^{a}	17.6±1.3^{a}
18:0	6.3±0.5^{a}	7.0±2.3^{a}	5.0±0.9^{a}	7.6±2.3^{a}	10.9±2.0^{a}	5.7±2.7^{a}
ΣSFA*	22.3±1.3^{ab}	18.8±3.0^{b}	17.5±1.6^{b}	23.7±3.3^{ab}	27.7±1.8^{a}	24.4±1.3^{ab}
16:1n-7	3.2±0.2^{ab}	3.0±0.2^{b}	0.8±0.2^{c}	3.4±0.4^{ab}	2.7±0.1^{b}	4.4±0.7^{a}
18:1n-9	31.2±0.3^{b}	43.1±1.6^{a}	42.9±0.8^{a}	34.5±1.8^{ab}	34.9±3.5^{ab}	28.2±3.4^{b}
20:1n-9	2.8±0.0^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.8±1.1^{b}	0.0±0.0^{b}
20:1n-11	3.0±0.2^{a}	3.1±0.9^{a}	2.6±0.2^{a}	2.7±0.6^{a}	1.9±1.2^{a}	1.6±0.5^{a}
ΣMUFA**	40.1±0.1^{ab}	49.2±2.3^{a}	46.2±0.4^{a}	40.5±2.1^{ab}	40.3±3.3^{ab}	34.2±3.3^{b}
18:2n-6	14.5±1.0^{a}	14.9±3.6^{a}	21.0±1.5^{a}	13.7±3.6^{a}	12.0±3.3^{a}	14.4±2.8^{a}
20:4n-6	0.3±0.0^{cd}	0.5±0.1^{bc}	0.0±0.0^{d}	0.9±0.1^{ab}	0.7±0.1^{b}	1.2±0.1^{a}
Σn-6 PUFA***	14.8±0.9^{a}	15.4±3.7^{a}	21.0±1.5^{a}	14.6±3.6^{a}	12.8±3.5^{a}	15.6±2.9^{a}
18:3n-3	1.6±0.1^{a}	2.0±0.3^{a}	2.8±0.3^{a}	1.7±0.4^{a}	1.5±0.4^{a}	2.6±0.6^{a}
18:4n-3	0.2±0.0^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}
20:5n-3	2.3±0.2^{b}	2.4±0.2^{b}	0.1±0.1^{c}	3.6±0.5^{b}	2.8±0.1^{b}	5.6±0.9^{a}
22:5n-3	1.9±0.4^{a}	1.9±0.3^{a}	0.0±0.0^{b}	2.6±0.5^{a}	2.1±0.3^{a}	2.4±0.3^{a}
22:6n-3	3.4±0.6^{abc}	0.0±0.0^{d}	2.4±0.3^{c}	4.0±0.4^{ab}	3.1±0.1^{bc}	4.8±0.3^{a}
Σn-3 PUFA***	9.3±1.1^{bc}	6.3±0.3^{c}	5.3±0.5^{c}	11.8±1.8^{ab}	9.5±0.9^{bc}	15.3±1.5^{a}
ΣPUFA***	24.1±0.2^{a}	21.7±4.0^{a}	26.2±2.0^{a}	26.4±5.5^{a}	22.2±4.4^{a}	30.9±4.4^{a}
Σn-3 LCPUFA****	7.7±1.2^{bc}	4.3±0.0^{cd}	2.5±0.2^{d}	10.1±1.4^{ab}	7.9±0.5^{b}	12.8±0.9^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 14. Fatty acids composition in the polar lipid from whole body of fish (area %, mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
14:0	0.5±0.1^{a}	0.3±0.0^{cd}	0.1±0.0^{d}	0.3±0.0^{bc}	0.5±0.1^{ab}	0.3±0.0^{cd}
16:0	18.1±0.3^{b}	16.5±0.8^{b}	17.3±0.9^{b}	21.9±1.4^{a}	22.3±0.9^{a}	23.5±0.1^{a}
18:0	6.8±0.1^{a}	7.6±0.7^{a}	6.0±0.3^{a}	7.3±0.2^{a}	7.9±1.0^{a}	7.3±0.4^{a}
ΣSFA*	25.5±0.4^{bc}	24.4±1.5^{c}	23.5±0.6^{c}	29.5±1.6^{ab}	30.6±1.7^{a}	31.1±0.5^{a}
16:1n-7	0.3±0.0^{ab}	0.4±0.0^{a}	0.3±0.0^{abc}	0.2±0.0^{cd}	0.3±0.0^{bcd}	0.2±0.0^{d}
18:1n-9	17.4±0.7^{c}	22.7±1.1^{a}	20.1±0.2^{b}	16.9±0.1^{c}	15.4±0.3^{c}	15.8±0.6^{c}
20:1n-9	1.1±0.0^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.3±0.4^{b}	0.0±0.0^{b}
20:1n-11	1.4±0.1^{a}	1.4±0.0^{a}	1.0±0.0^{b}	1.0±0.0^{b}	0.9±0.0^{b}	0.7±0.0^{c}
ΣMUFA**	20.3±0.8^{bc}	24.5±1.1^{a}	21.4±0.2^{b}	18.2±0.1^{cd}	16.9±0.2^{d}	16.7±0.7^{d}
18:2n-6	15.0±0.5^{c}	17.0±0.3^{b}	20.3±0.5^{a}	11.4±0.5^{de}	12.2±0.2^{d}	9.9±0.2^{e}
20:4n-6	1.2±0.0^{c}	3.2±0.0^{a}	0.8±0.1^{c}	2.6±0.2^{b}	2.6±0.1^{b}	2.7±0.1^{b}
Σn-6 PUFA***	16.1±0.5^{b}	20.2±0.3^{a}	21.1±0.4^{a}	14.1±0.3^{c}	14.9±0.3^{bc}	12.5±0.3^{d}
18:3n-3	1.1±0.1^{bc}	1.7±0.0^{a}	1.5±0.1^{ab}	1.0±0.1^{bc}	1.3±0.3^{abc}	0.9±0.1^{c}
20:5n-3	6.3±0.4^{b}	9.4±0.2^{a}	0.5±0.0^{c}	6.7±0.7^{b}	6.1±0.2^{b}	5.4±1.0^{b}
22:5n-3	2.2±0.0^{b}	4.7±0.2^{a}	0.2±0.0^{d}	1.7±0.2^{c}	1.6±0.1^{c}	1.4±0.1^{c}
22:6n-3	15.8±1.3^{a}	1.8±0.1^{b}	18.7±0.3^{a}	15.5±2.0^{a}	15.2±1.0^{a}	17.7±1.4^{a}
Σn-3 PUFA***	25.3±1.8^{a}	17.7±0.5^{b}	20.9±0.3^{ab}	25.0±2.9^{a}	24.1±1.6^{a}	25.4±0.2^{a}
ΣPUFA***	41.5±2.3^{a}	37.9±0.1^{a}	42.0±0.2^{a}	39.0±2.6^{a}	38.9±1.3^{a}	38.0±0.1^{a}
Σn-3 LCPUFA****	24.3±1.7^{a}	16.0±0.5^{b}	19.4±0.4^{ab}	24.0±2.9^{a}	22.8±1.3^{a}	24.6±0.3^{a}
18:1/n-3 LCPUFA	0.72±0.08^{c}	1.42±0.11^{a}	1.03±0.03^{b}	0.71±0.09^{c}	0.68±0.05^{c}	0.64±0.03^{c}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 15. Fatty acids composition of the hepatic polar lipid in fish (area %, mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
14:0	0.4±0.0^{a}	0.3±0.0^{b}	0.1±0.0^{c}	0.2±0.0^{bc}	0.2±0.0^{bc}	0.2±0.0^{bc}
16:0	19.8±0.1^{abc}	14.9±3.0^{c}	18.2±0.2^{bc}	23.0±0.8^{ab}	21.7±1.2^{ab}	24.8±0.5^{a}
18:0	9.8±0.9^{a}	11.4±0.3^{a}	9.0±0.6^{a}	11.3±1.2^{a}	12.7±1.5^{a}	9.7±1.9^{a}
ΣSFA*	30.0±0.8^{ab}	26.6±3.2^{b}	27.3±0.4^{b}	34.5±2.1^{a}	34.7±0.3^{a}	34.7±1.3^{a}
16:1n-7	0.5±0.1^{ab}	0.4±0.0^{ab}	0.5±0.1^{a}	0.3±0.1^{ab}	0.4±0.1^{ab}	0.3±0.0^{b}
18:1n-9	9.0±0.7^{b}	19.8±3.6^{a}	13.3±0.8^{b}	8.6±0.6^{b}	8.2±0.2^{b}	7.9±0.5^{b}
20:1n-9	0.4±0.1^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}
20:1n-11	0.9±0.1^{ab}	1.7±0.7^{a}	1.0±0.1^{ab}	0.7±0.1^{ab}	0.8±0.1^{ab}	0.5±0.0^{b}
ΣMUFA**	10.8±0.8^{b}	21.9±4.4^{a}	14.8±1.0^{ab}	9.6±0.5^{b}	9.3±0.2^{b}	8.7±0.4^{b}
18:2n-6	9.8±0.2^{bc}	12.5±1.4^{ab}	15.4±0.1^{a}	7.6±1.0^{c}	8.2±1.1^{c}	6.6±0.4^{c}
20:4n-6	2.2±0.1^{b}	4.3±0.6^{a}	1.2±0.1^{b}	4.1±0.2^{a}	4.4±0.1^{a}	4.3±0.3^{a}
Σn-6 PUFA***	12.1±0.0^{b}	16.8±2.0^{a}	16.6±0.0^{a}	11.7±1.2^{b}	12.6±1.2^{ab}	10.9±0.1^{b}
18:3n-3	0.7±0.0^{b}	1.2±0.0^{a}	1.2±0.1^{a}	0.6±0.1^{b}	0.6±0.1^{b}	0.7±0.0^{b}
20:5n-3	8.1±0.2^{b}	10.5±0.4^{a}	0.8±0.1^{c}	8.0±0.5^{b}	7.6±0.5^{b}	8.5±0.1^{b}
22:5n-3	2.5±0.2^{b}	9.4±0.1^{a}	0.4±0.3^{c}	2.1±0.1^{b}	2.3±0.2^{b}	2.0±0.2^{b}
22:6n-3	25.2±2.1^{a}	0.8±0.1^{b}	25.2±0.4^{a}	21.9±1.1^{a}	21.1±1.0^{a}	24.1±1.4^{a}
Σn-3 PUFA***	36.5±2.0^{a}	21.9±0.4^{c}	27.5±0.0^{b}	32.6±1.7^{ab}	31.7±1.3^{ab}	35.3±1.6^{a}
ΣPUFA***	48.5±2.0^{a}	38.8±2.4^{b}	44.2±0.1^{ab}	44.3±3.0^{ab}	44.3±0.1^{ab}	46.1±1.7^{ab}
Σn-3 LCPUFA****	35.8±2.1^{a}	20.8±0.4^{c}	26.4±0.1^{b}	31.9±1.7^{a}	31.1±1.4^{ab}	34.5±1.6^{a}
18:1/n-3 LCPUFA	0.25±0.03^{b}	0.95±0.19^{a}	0.50±0.03^{b}	0.27±0.01^{b}	0.26±0.00^{b}	0.23±0.00^{b}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Table 16. Apparent digestibility coefficients of protein and lipid in diets (%, mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
Protein	95.7±0.2^{ab}	93.0±1.5^{b}	93.4±0.1^{ab}	96.0±0.2^{a}	93.3±0.3^{ab}	95.2±0.7^{ab}
Lipid	93.4±0.8^{a}	90.7±3.1^{a}	90.0±1.3^{a}	92.2±0.4^{a}	89.3±0.5^{a}	90.0±0.9^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

Table 16. Apparent digestibility coefficients of total amino acids in diets (%, mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
Arg	96.99±0.06^{ab}	95.07±0.07^{d}	95.81±0.27^{cd}	97.25±0.13^{a}	95.01±0.52^{d}	96.16±0.21^{bc}
Lys	98.23±0.42^{a}	97.87±0.24^{a}	97.93±0.09^{a}	98.55±0.13^{a}	95.29±0.91^{b}	98.31±0.20^{a}
His	96.26±1.08^{a}	92.87±1.85^{ab}	94.13±0.35^{ab}	95.82±0.29^{ab}	92.42±0.31^{b}	95.62±0.16^{ab}
Phe	93.70±0.45^{a}	89.66±1.70^{c}	90.18±0.28^{bc}	93.58±0.41^{a}	89.74±0.17^{c}	92.83±0.24^{ab}
Leu	92.78±0.23^{a}	87.33±0.20^{c}	89.11±0.26^{b}	93.09±0.54^{a}	87.84±0.19^{bc}	92.71±0.71^{a}
Ile	94.89±0.35^{a}	91.32±1.93^{b}	92.57±0.04^{ab}	95.63±0.49^{a}	92.32±0.28^{ab}	95.12±0.69^{a}
Met	97.37±0.19^{ab}	95.62±0.12^{c}	95.69±0.24^{c}	96.91±0.73^{bc}	96.15±0.36^{bc}	98.57±0.49^{a}
Val	94.02±0.01^{a}	91.77±0.15^{b}	92.25±0.79^{b}	95.39±0.52^{a}	91.71±0.41^{b}	94.58±0.03^{a}
Thr	94.16±0.80^{a}	91.76±0.29^{b}	92.18±0.25^{b}	95.22±0.16^{a}	91.94±0.52^{b}	94.40±0.07^{a}
Trp	96.07±0.24^{a}	96.34±1.29^{a}	94.66±2.46^{a}	93.84±3.68^{a}	95.37±1.06^{a}	94.97±0.59^{a}
Tau	98.85±0.14^{a}	98.27±0.64^{ab}	97.72±0.75^{ab}	97.45±0.26^{ab}	96.46±0.80^{b}	96.62±0.55^{ab}
Cys	95.69±0.42^{a}	90.42±2.61^{a}	90.27±0.92^{a}	94.42±5.64^{a}	89.15±0.18^{a}	85.14±5.19^{a}
Cysta	92.13±2.91^{a}	90.15±0.31^{a}	88.72±2.40^{a}	88.35±1.92^{a}	93.57±0.32^{a}	87.44±0.53^{a}
Ala	92.70±0.03^{a}	88.08±0.41^{b}	88.54±0.55^{b}	93.53±0.05^{a}	89.66±0.13^{b}	90.83±1.58^{ab}
Tyr	93.93±0.23^{a}	88.86±1.28^{c}	90.13±0.21^{bc}	93.52±0.06^{ab}	89.16±0.47^{c}	91.10±1.78^{abc}
Gly	93.05±1.94^{ab}	91.62±0.62^{ab}	91.34±0.68^{ab}	94.12±0.47^{a}	90.42±1.37^{ab}	89.05±1.03^{b}
Glu	99.05±0.03^{a}	98.31±0.06^{b}	98.49±0.03^{b}	99.12±0.02^{a}	98.38±0.01^{b}	99.04±0.08^{a}
Ser	93.89±0.51^{ab}	90.21±0.18^{d}	90.95±0.45^{cd}	94.27±0.26^{a}	89.95±0.65^{d}	92.29±0.12^{bc}
Asp	94.71±0.47^{ab}	92.33±0.45^{c}	92.99±0.18^{bc}	96.26±0.80^{a}	92.51±0.53^{c}	93.06±0.36^{bc}
Pro	94.88±2.93^{a}	89.77±1.71^{a}	89.25±2.48^{a}	94.52±0.04^{a}	95.72±5.28^{a}	95.47±1.00^{a}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

Table 17. Apparent digestibility coefficients of fatty acids in diets (%, mean±SD, n=2)

	FO	NAN	SCH	NS	NSC	NSS
14:0	95.5±0.5^{a}	90.0±2.7^{ab}	69.0±2.2^{d}	85.4±1.0^{bc}	85.7±0.4^{bc}	82.3±0.2^{c}
16:0	89.6±1.7^{a}	86.6±0.4^{ab}	73.6±1.5^{d}	84.1±0.1^{bc}	84.8±1.2^{bc}	81.5±0.5^{c}
18:0	87.4±0.1^{a}	83.9±1.0^{b}	79.0±0.6^{c}	86.0±1.4^{ab}	79.9±0.2^{c}	78.8±0.8^{c}
ΣSFA*	90.4±1.3^{a}	86.4±0.7^{b}	73.3±1.5^{d}	83.9±0.1^{bc}	84.3±1.1^{bc}	81.0±0.5^{c}
16:1n-7	95.8±0.2^{a}	92.1±1.0^{a}	78.3±1.8^{b}	94.2±1.3^{a}	93.0±0.3^{a}	93.9±0.2^{a}
18:1n-9	93.8±0.1^{c}	92.2±0.0^{d}	94.6±0.2^{b}	95.9±0.1^{a}	91.3±0.1^{e}	93.9±0.2^{c}
20:1n-9	96.2±0.2^{a}	ND	ND	ND	ND	ND
ΣMUFA**	94.7±0.0^{b}	91.6±0.1^{e}	94.1±0.1^{c}	95.4±0.0^{a}	90.9±0.1^{f}	93.5±0.2^{d}
18:2n-6	92.3±0.7^{a}	88.4±0.9^{b}	93.5±1.0^{a}	94.6±0.3^{a}	88.5±0.3^{b}	93.6±0.2^{a}
20:4n-6	98.2±2.5^{ab}	92.6±0.9^{c}	100.0±0.0^{a}	94.0±1.1^{bc}	93.3±0.7^{c}	93.6±0.2^{bc}
Σn-6 PUFA***	92.3±0.7^{a}	88.5±0.9^{b}	93.6±1.0^{a}	94.6±0.3^{a}	88.7±0.4^{b}	94.2±0.1^{a}
18:3n-3	95.2±0.2^{a}	91.4±0.4^{b}	95.4±0.8^{a}	95.2±0.1^{a}	90.6±0.4^{b}	94.5±0.2^{a}
18:4n-3	96.2±2.8^{a}	ND	ND	ND	ND	ND
20:5n-3	98.2±0.1^{a}	92.6±0.5^{b}	ND	93.3±1.8^{b}	93.5±0.5^{b}	94.7±0.1^{b}
22:5n-3	97.5±0.2^{a}	ND	ND	ND	ND	ND
22:6n-3	97.4±0.0^{b}	100.0±0.0^{a}	95.7±0.8^{bc}	95.0±0.5^{bc}	91.5±1.0^{d}	94.2±0.7^{c}
Σn-3 PUFA***	97.2±0.4^{a}	92.1±0.1^{d}	95.5±0.8^{ab}	94.2±0.8^{bcd}	92.4±0.6^{cd}	94.6±0.1^{bc}
Σn-3 LCPUFA****	98.1±0.1^{a}	92.7±0.4^{c}	95.7±0.8^{ab}	93.9±1.1^{bc}	93.0±0.6^{c}	94.6±0.2^{bc}

Values with different superscript letters in the same row were significantly different when $P<0.05$ (Tukey's test, a>b>c).

ND: Not detected

* Saturated fatty acid.

** Monounsaturated fatty acid.

*** Polyunsaturated fatty acid.

**** Long-chain polyunsaturated fatty acid.

Chapter 5.

General conclusion

In conclusion, formulating non-fish meal, non-fish oil diet for red sea bream with microalgae species was promising with considerable growth result, significantly no difference with fish oil formulated diet group if the essential nutritional value for fish has fulfilled. As it showed in previous study, DHA-rich microalgae *Schizochytrium* sp. meal is a suitable substitute for fish oil for the juvenile red sea bream diet, and through these studies, it has been demonstrated that *Schizochytrium* algae meal could be used with the other microalgae species. Three microalgae species, *Nannochloropsis*, *Chlorella*, and *Spirulina* were the suitable substitute for fish derived products for the juvenile red sea bream diet without fatal negative effects. By using more than two microalgae meal, it seems to organize better nutritional value than only one microalgae used diets.

The non-fish meal and non-fish oil diet development with high inclusion of mixed microalgae species was successful and it showed remarkable growth performance and considerable fish body fatty acid composition. Microalgae species can play important role to supply essential nutrition to aquacultural species with high nutritive value and appropriate physical properties as sustainable materials.