INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO

MESTRADO EM BIORRECURSOS




Dietas funcionais para peixes de aquacultura

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Julho de 2020

P.PORTO

Functional diets for aquaculture fish

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Dissertação submetida como requisito parcial para a obtenção do grau de Mestre em Biorrecursos

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08/07/2020

Agradecimentos

Inicialmente, queria dedicar um enorme agradecimento às minhas duas orientadoras e à minha diretora de mestrado. À Professora Luísa Valente, porque sem a sua ajuda era impossível ter entrado no LANUCE, por sempre me ter dado apoio e ajuda sempre que necessário e por me ter mostrado uma nova área pela qual ganhei um enorme gosto. À professora Cristina Matos, por toda a sua disponibilidade sempre que eu precisava de alguma coisa, por ter ido comigo no primeiro dia em que fui conhecer o CIIMAR e por todo o apoio e carinho que sempre me demonstrou. Finalmente, à minha diretora do mestrado, a professora Simone Morais, por ter sido incansável na altura em que expressei o meu desejo de realizar um estágio numa nova área e por me ter aberto as portas e feito perceber que o nosso curso pode ser imenso e que nos fornece capacidades para sermos muito versáteis.

Gostaria de agradecer à minha família por todo o apoio que me foi dado no decorrer desta jornada. Foram as pessoas que estiveram sempre do meu lado em todas as etapas da minha vida e que me ajudaram imenso nesta etapa final do meu mestrado.

De seguida, agradeço ao meu namorado que apoiou a minha decisão desde o início de querer realizar a minha tese de mestrado num local completamente diferente do que estava habituada e numa área que me era maioritariamente desconhecida.

Estou muitíssimo agradecida a todas as pessoas que conheci desde que comecei o meu estágio no CIIMAR. Ao Tiago que, com toda a sua paciência, me acolheu de forma fantástica desde o primeiro dia e que me ajudou incansavelmente durante a duração de todo o ensaio. À Alexandra, porque sem ela não tinha forma de saber como me orientar dentro do laboratório e por ser a pessoa que respondeu a todas as minhas questões mal estas surgiam. À pessoa com quem trabalhei mais diretamente, a Cátia, que trabalhou sempre a meu lado, apoiámo-nos e conhecemo-nos mutuamente e a amizade que construímos foi uma das melhores coisas que ganhei no decorrer desta experiência.

Finalmente, um enorme agradecimento a todo a gente que fui conhecendo, não só do LANUCE mas do CIIMAR em geral, que me foi ajudando e ensinando coisas novas ao longo deste percurso. Fui imediatamente muito bem recebida por toda a equipa do laboratório e por isso fico muito agradecida. E em último, mas não menos importante, agradeço à Daniela e ao Umberto que tantos fins de semana partilharam comigo e à Inês, por ter sido uma das pessoas mais prestáveis que alguma vez conheci e por tanto me ter ajudado.

Abstract

This study aimed to test the effects on growth performance, body composition and feed conversion of European seabass Bass (*Dicentrarchus labrax*) fed a blend of macro and microalgae. For this purpose, 4 isoproteic experimental diets (50.0% on a dry basis), isolipidic (16.2% dry base) and isoenergetic (20.9 KJ/g dry basis) diets were produced with different inclusion rates (0%, 2%, 4% and 6%) of a mixture of 2 microalgae (*Chlorella* sp. and *Nannochloropsis* sp.) and two macroalgae (*Gracilaria* sp. and *Ulva* sp.). To evaluate the effects of the diets, a growth trial was carried out in triplicate groups of fish with an average ad weight and length of 11.3 ± 2.7 g and 10.5 ± 1.0 cm, respectively, distributed by 12 tanks (46 fish per tank). Fish were fed three times a day by automatic feeders until apparent satiety. The conditions of temperature, pH and salinity of the water were controlled daily in order to meets species needs.

Obtained results showed that the blend inclusion in the diets caused a significant increase ($P<0.05$) in the weight and final length of the fish, and the highest value was presented by the fish that were fed the diet with 6% inclusion rate (62.9 g and 17.7 cm, respectively). There was also a significant increase in the condition factor, voluntary feed intake, specific growth rate and protein efficiency ratio in fish fed Algae 6%. The highest FCR was registered in fish fed the control diet (1,35 ± 0,06) and the lowest in the ones fed with 4% of algae supplementation (1.22 ± 0.011). A significant increase ($P<0.001$) of hepatosomatic index was observed from a value of 0.953 corresponding to the control up to 1.42 for the diet with 6% supplementation. There were no significant differences ($P\geq0.126$) in whole body ash and protein content, but lipids and body energy showed a significant increase ($P<0.001$) in fish fed the algal blend. The algae blend caused a significantly higher ($P\leq0.002$) gain of dry matter, lipids, protein and energy when compared to the control diet (0% inclusion). There was a significant increase ($P<0.05$) in all retention parameters (dry matter, lipids and energy) except for protein retention that did not present significant variations among diets. In conclusion, it is perceptible that algae supplementation proved to bring beneficial effects for European seabass in terms of growth, feed intake, feed conversion rate and nutrient retention. However, it was also notorious that 6% inclusion rate caused a greater body fat gain which may lead to organoleptic changes, so in the future it would be advisable to use inclusion levels below 6%.

Keywords: Aquaculture; European seabass; functional diets; growth; macroalgae; microalgae; protein sources;

Resumo

Este trabalho teve como objetivo testar o efeito da inclusão de uma mistura de macro e microalgas em dietas para juvenis de Robalos europeu *(Dicentrarchus labrax*) ao nível da performance de crescimento, composição corporal e capacidade de conversão alimentar. Para este efeito foram produzidas 4 dietas experimentais isoproteicas (50,0% em base seca), isolipídicas (16,2% base seca) e isoenergéticas (≈20,9 KJ/g base seca) com diferentes taxas de inclusão de uma mistura de 2 microalgas (*Chlorella* sp. *e Nannochloropsis* sp.) e duas macroalgas (*Gracilaria* sp. *e Ulva* sp.). As taxas de inclusão da mistura de algas foram 0%, 2%, 4% e 6% sendo cada dieta testada em triplicado. Para avaliar os efeitos das rações, foi realizado um ensaio de crescimento em que foram utilizados 552 espécimes (peso e comprimento médio de 11,3 ± 2,7 g e 10,5 ± 1,0 cm, respetivamente) distribuídos por 12 tanques (46 peixes por tanque), alimentados três vezes ao dia por alimentadores automáticos até saciedade aparente. As condições de temperatura, pH e salinidade da água foram controladas diariamente de modo a corresponderem às necessidades da espécie.

Os resultados obtidos mostraram que a inclusão da mistura de algas nas dietas causou um aumento significativo ($P<0.05$) no peso e comprimento final dos peixes, sendo que o maior valor foi apresentado pelos peixes que foram alimentados com a ração com 6% de taxa de inclusão (62.9 g e 17.7 cm, respetivamente). Houve também um aumento significativo no fator de condição, no consumo de ração e na taxa de crescimento específico em todos os peixes que consumiram a ração com algas. O maior rácio de conversão alimentar foi apresentado pelos peixes alimentados com a ração de controlo (1,35 ± 0,06) e o menor pelos peixes alimentados com 4% de inclusão (1.22 ± 0.011). Em relação ao rácio de eficiência proteica, os peixes alimentados com a dieta com 4% de inclusão de algas apresentaram o maior valor. Verificou-se um aumento significativo ($P<0{,}001$) dos índices hepatosomático (desde um valor de 0,953 correspondente ao controlo até 1,42 para a dieta com 6% de suplementação) e viscerosomático à medida que a taxa de inclusão das algas aumentou. Na composição corporal, há a sublinhar que não se registaram diferenças significativas ($P\geq0{,}126$) no conteúdo de cinzas e proteína corporais, por outro lado, a humidade, os lípidos e a energia corporal apresentaram alterações significativas ($P<0{,}001$). A humidade diminuiu com o aumento de inclusão de algas mas os lípidos e a energia corporal sofreram um aumento à medida que a taxa de inclusão aumentava. A mistura de algas provocou um ganho de matéria seca, lípidos, proteína e energia quando comparado com o controlo (0% de inclusão) ($P\leq0{,}002$). Verificou-se também um aumento significativo ($P<0{,}05$) em todos os parâmetros de retenção (matéria seca, lípidos e energia) à exceção da retenção proteica que não apresentou variações significativas.

De uma forma geral, conclui-se que a suplementação das dietas com a mistura de algas provou trazer efeitos benéficos para o Robalo europeu a nível de crescimento, consumo de ração, taxa de conversão alimentar e retenção de nutrientes. No entanto, também foi notório que a taxa de inclusão a 6% provocou um maior ganho de gordura corporal podendo dar origem a alterações organoléticas, por isso, de futuro, seria aconselhável utilizar níveis de inclusão inferiores a 6%.

Palavras-chave: Aquacultura; crescimento; dietas funcionais; fontes proteicas; macroalgas; microalgas; Robalo Europeu;

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Abbreviations and Acronyms

ABW – Average Body Weight

ADC(s) – Apparent Digestibility Coefficient(s)

ANF(s) – Anti Nutritional Factor(s)

CIIMAR - Centro de Investigação Interdisciplinar Marinha e Ambiental

CP – Crude Protein

DGAV - Direção-Geral de Alimentação e Veterinária

DHA – Docosahexaenoic Acid

DM – Dry Matter

DMG – Dry Matter Gain

DMI – Dry Matter Intake

DMR – Dry Matter Retention/Consumption

EAA – Essential Amino Acids

EPA – Eicosapentaenoic Acid

EUR – Euros

FCR – Feed Conversion Ratio

FM – Fish Meal

FO – Fish Oil

GE – Gross Energy

HSI – Hepatosomatic index

HUFA – Highly Unsaturated Fatty Acids

K – Final Condition Factor

N – Nitrogen

P – Phosphorous

PER – Protein Efficiency Ratio

PUFA – Polyunsaturated Fatty Acids

RAS - Recirculation Aquatic System

SGR – Specific Growth Rate

SPC – Soy Protein Concentrate

VFI – Voluntary Feed Intake

VSI – Viscerosomatic Index

1 – Introduction

1.1 – CIIMAR

During my thesis all the experimental work was developed at CIIMAR (Centro de Investigação Interdisciplinar Marinha e Ambiental), a non-profit association dedicated to the research, spread and transfer of technology in the marine and environmental sciences. It is a leading research institution belonging to the University of Porto and provides innovative solutions and products that address today´s economic and social challenges, including marine products for industrial and medicinal use, water quality, sustainable fisheries, environmental monitoring, ecosystem preservation, and coastal management.

CIIMAR´s research is organized in three thematic areas: biotechnology, ecosystem services and aquaculture. Laboratory of Fish Nutrition, Growth and Quality (LANUCE) (http://lanuce.ciimar.up.pt/) integrates the aquaculture thematic area and its main goals are optimization of aquaculture practices aiming at an intelligent and sustainable production of fish to provide quality, safety and welfare of the animals, and meeting the market and consumer demands. One of the objectives is the inclusion and consequent appreciation of alimentary industry by-products and the inclusion of new nutrient sources for fish feeds, thus favoring a circular economy.

The ongoing projects are SEAFOOD TOMORROW, MOBFOOD, VALORMAR, ANIMAL4Aqua, CAVIAR and MARINEALGAE4aqua, which aim to achieve sustainable practices for aquaculture, quality and aquatic product safety, animal welfare, and scientific dissemination.

CIIMAR also has installations certified by DGAV (Direção Geral de Alimentação e Veterinária) for animal experimentation that allows maintenance and acclimatization of several aquatic species. It is a place where the organisms are held with specific conditions (ambient, nutritional and sanitary conditions) so that they can later be used in research and/or teaching. These optimal conditions are only possible thanks to a Recirculation Aquatic System (RAS), which includes a maintenance tank, a recirculation bomb, and a filtration system. RAS allows the conservation of the water quality according to optimal conditions of each species and is therefore essential and of great responsibility since the animals are going to be utilized in experiments and the improper functioning of the system will influence the results obtained.

1.2 – Aquaculture

The global demand for fish has been increasing over the years, not only because of the exponential growth, but also due to the recognition of its benefits of including fish into the human diet. However, the rising demand and the inadequate catching techniques have led to overexploitation of this food source which raises a concern on the part of the consumer regarding the quality of the fish and the way it is fished (REA, 2019).

It is in this context that aquaculture appears. It is a practice defined by DGRM as the culture or rearing of aquatic living beings using appropriate technics, aiming to increase their production beyond the natural capacities of the natural environment (Direção - Geral de Recursos Naturais).

Aquaculture can be divided into three production regimes: extensive, semi-intensive and intensive. The extensive regime only utilizes natural available conditions. The desired species is captured in nature or comes from reproduction units and is fed exclusively with natural feeds. This regime was utilized at the beginning of aquaculture. The second regime, semi-intensive, uses an artificial production of the specimens to obtain eggs and juveniles and feeding is done with natural feeds and by artificial dietary supplements. In the last one, intensive, all the parameters of the process are kept under continual observation, monitoring the reproduction as well as growth. In this case, the species is fed only with artificial feeds (REA, 2019).

Aquaculture has existed for many years, even though one cannot date exactly it´s beginning. Only recently it has generated growing interest. Globally, this practice had a significant development in the beginning of the XX century, when the negative effects of intensive fishing and the consequent ecologic damage started to be a concern (Rocha, 2017).

In 1970, around 3 million tons of fish were produced by aquaculture and in 2018 global aquaculture fish production reached to 82.1 million tons (FAO, 2020), demonstrating a significant increase in less than 50 years (FAO, 2018). In 2018, finfish represented the majority of aquaculture fish production (54.3 million tons), mollusks and crustaceans represented the rest (27.8 million tons) (FAO, 2020).

Figure 1.2.1 shows the evolution over the years of aquaculture production worldwide.

With the figure 1.2.1 it is possible to confirm that around the '70s the evolution of this practice started to be significant, reaching a point where fish obtained by aquaculture is greater than fish obtained through fisheries (Wright, 2018). It was agreed by the European Union that, by 2020, we should reach maximum sustainable yield, which means that all fish stocks should be exploited at sustainable levels, by taking the highest possible amount of catches without affecting long term productivity of stocks (Directorate-General for Maritime Affairs and Fisheries, 2018).

Global capture suffered an increase of 5.4% from the average registered of the last three years, reaching a record of 96.4 million tons, essentially caused by an increase of marine capture fisheries (FAO, 2020).The leading countries in fishing, regarding volume, includes Spain, Denmark, the United Kingdom and France. When combined, these account for more than 57% of EU's catches (Directorate-General for Maritime Affairs and Fisheries, 2018). In 2018, total aquaculture production of EU members was distributed by mollusks and crustaceans (around 680 thousand tons), finfish (2399 thousand tons) and other aquatic animals (3 thousand tons) as shown in figure 1.2.2 (FAO, 2020).

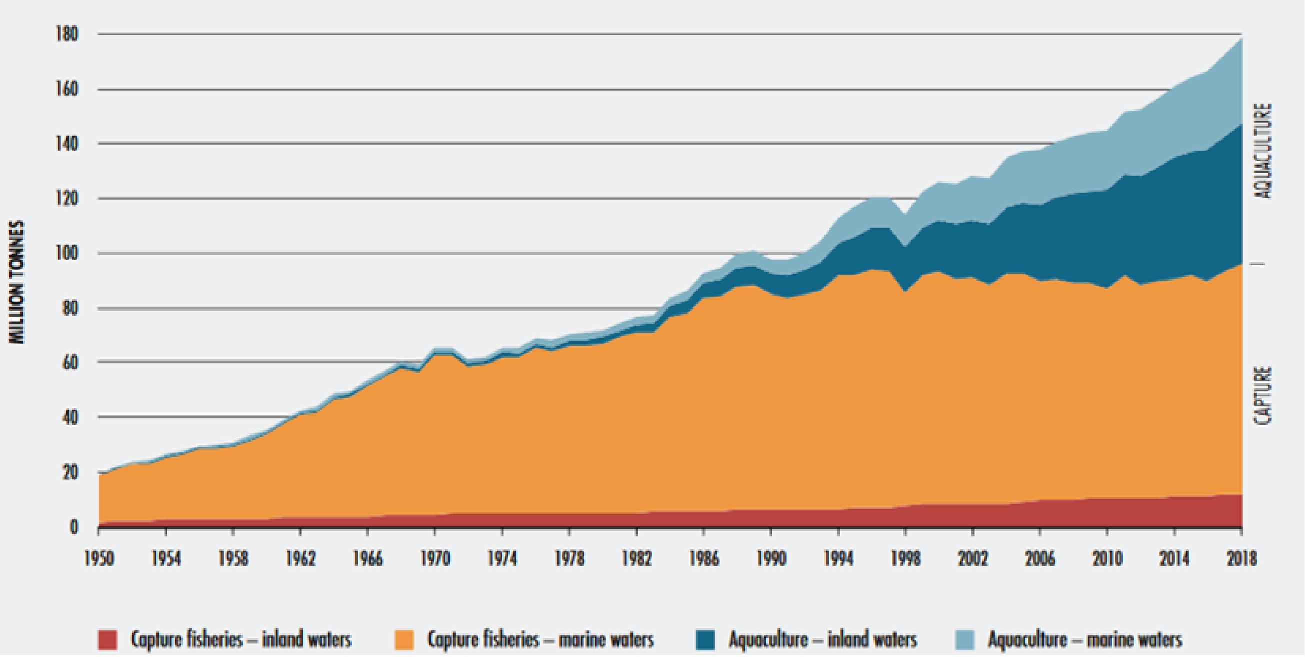
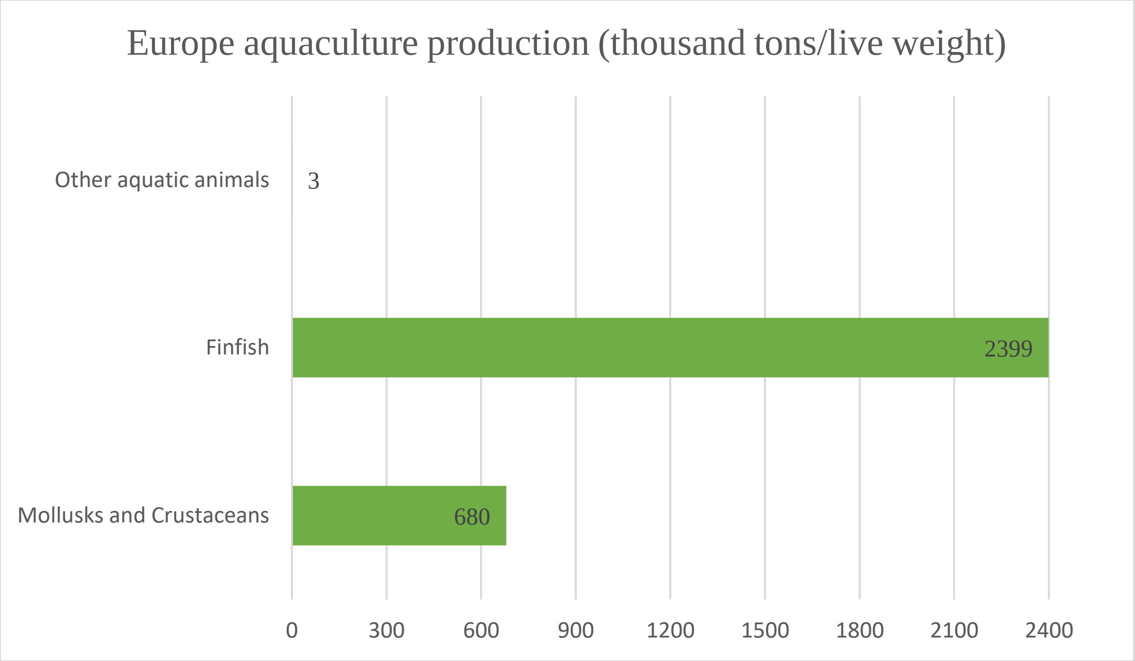


Figure 1.2.1 - Comparison at a global level between the fish quantities captured and the aquaculture fish production over the years. Retrieved from (FAO, 2020)



.Figure 1.2.2 - European Union aquaculture production per product type in 2018. Adapted from (FAO, 2020)

Regarding aquaculture worldwide, EU aquaculture represents 1.66% (FAO, 2020) in terms of volume (1 319 492 tons live weight) with Spain, the United Kingdom, France, Italy and Greece as the main producing countries (EUMOFA, 2020a). Table 1.2.2 shows some fish species that were produced in the European Union in 2018.

Table 1.2.2 - Aquaculture fish produced species in the European Union in 2018. Adapted from (EUMOFA, 2020a)

Species	**Volume (tons/ live weight)**
Salmon	169 587
Trout	187 859
Bluefin tuna	28 189
European seabass	86 236
Gilthead seabream	92 107
Carp	77 198
Freshwater catfish	9 462
Common sole	130
Turbot	10 521

1.2.1 - Aquaculture in Portugal

The main focus of Aquaculture in Portugal is the production of marine fish and mollusks, with the mainland being the major producer. During the last centuries, Portugal practiced very rudimental aquaculture techniques. Those techniques were essentially the imprisonment of juvenile fish that were brought by the tides in lagoons for them to grow only to be caught when reaching the desired size. This technique was essentially familiar until the end of the XIX century and only at the beginning of the XX century started to become an activity with commercial potential that could contribute to the country's economy. Nowadays aquaculture is a government’s strategic sector, given that the national market is a high consumer of fish, with Portugal being the third-largest fish consumer in the world (55 Kg per person/year). The first and second largest consumers are Iceland and Japan, namely (Brito, 2016).

In 2018 Portugal contributed with 13 512 tons of fish and 98 569 thousand of EUR corresponding to aquaculture production of bivalves and mollusks, crustaceans, flatfish, freshwater fish, salmonids, and other marine fish. Table 1.2.1.1 presents some of the species produced in Portugal and their respective quantities in tones for live weight (EUMOFA, 2020a).

Despite the development of this practice, fisheries always had a key role in the country’s tradition and economy (Brito, 2016) and the production of marine species through aquaculture suffered a major development only in the '90s, focusing on the production of Gilt-head sea bream (*Sparus aurata*) and European Seabass (*Dicentarchus labrax*). In comparison with other countries of the EU, Portugal has shown a slow progress regarding aquaculture, occupying the 16th place in the total production ranking contributing to the total production of fish in the EU with less than 1% in volume (Directorate-General for Maritime Affairs and Fisheries, 2018).

Table 1.2.1.1 – Some species produced by aquaculture in Portugal in 2018 and respective quantities. Adapted from (EUMOFA, 2020a; Directorate-General for Maritime Affairs and Fisheries, 2018)

Produced Species	**Value (thousand of EUR)**	**Volume (tons/ live weight)**
European seabass	4 014	456
Slamonids	1 827	661
Gilt-head seabream	6 335	1 081
Other marine fish	373	34
Bivalves and mollusks	62 512	8 430

In 2017, national production corresponded only to 7.6% of total fish consumed, which shows that until this date aquaculture had not been much considered by consumers as an alternative to fish from fishing activities (REA, 2019). However, this can also be explained by the fact that the national production is not enough to meet the great consumption of fish in Portugal and for that reason the majority of consumed fish corresponds to imported fish. In 2019 was imported 172 168 tons of fish from China, Vietnam and India mostly (EUMOFA, 2020b). Figure 1.2.1.1 shows the evolution of aquaculture production in Portugal until 2018 and Figure 1.2.1.2 displays the species and respective produced quantities, in that year. It was registered a total of 13992 tons of aquaculture produced fish in Portugal, with the main fraction (9 400 tons) correspondent to mollusks and crustaceans followed by marine fish (3 860 tons) and brackish and fresh water fish (698 tons) (PORDATA, 2020).

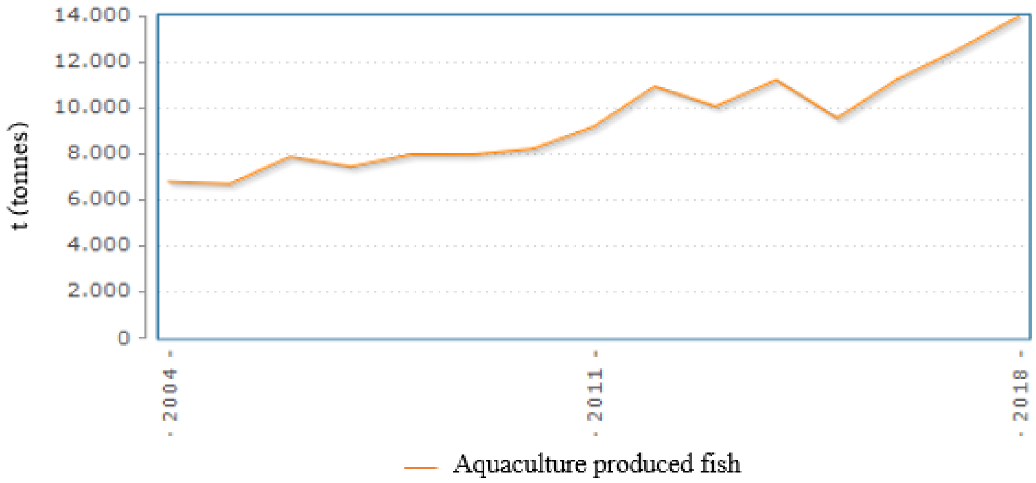


Figure 1.2.1.1 - Evolution of aquaculture production in Portugal. Adapted from (PORDATA, 2020)

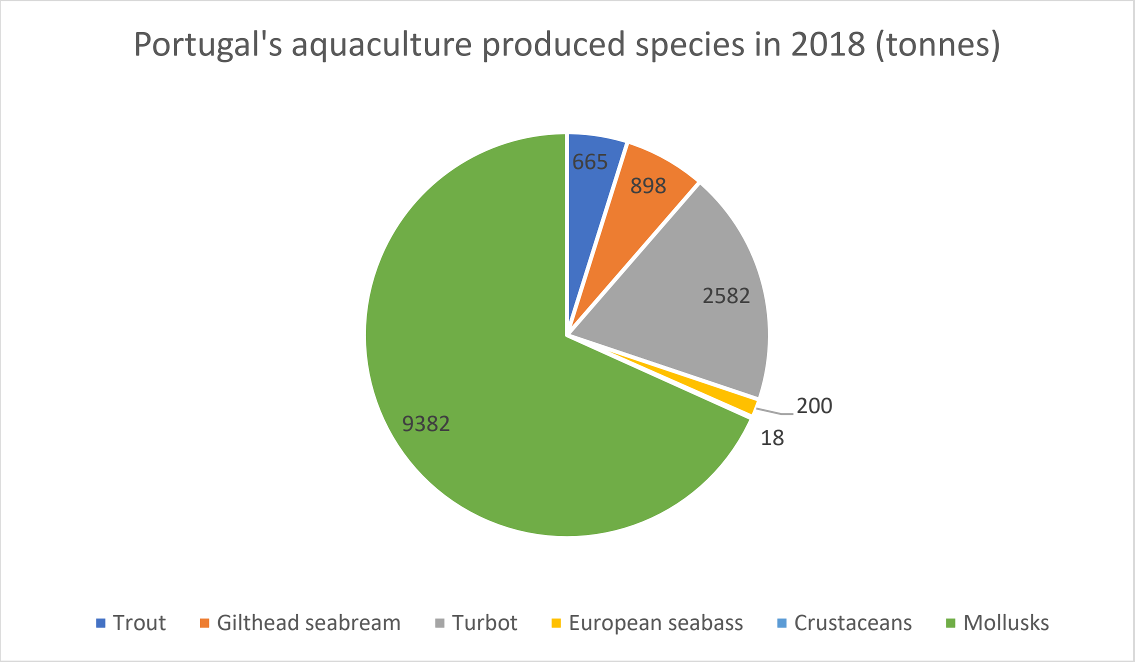


Figure 1.2.1.2 - Portugal´s aquaculture produced species and their respective quantities in tonnes (PORDATA, 2020)

1.3 – *Dicentrarchus labrax* (European seabass)

European seabass is a member of the Moronidae family (Abbate *et al.*, 2012) and can be found along the Atlantic Coast from the English Channel to Senegal (IPMA) living near the coasts and estuaries that are rich in microorganisms and has been created through aquaculture for a long time now (Directorate-General for Maritime Affairs and Fisheries, 2018)

Fish of this species can reach 1 meter long, its flanks are grayed out and the belly can be white or yellowish. It is usual for juveniles to have black spots on the upper part that disappear before they reach adulthood. The life cycle of the European Sea bass (Figure 1.3.1) is divided into four main phases: eggs and larvae, juveniles, adolescents, and adults (Dando and Demir, 1985). It can reach maturity between 4 and 7 years and can continue to reproduce until they are 20 years old.

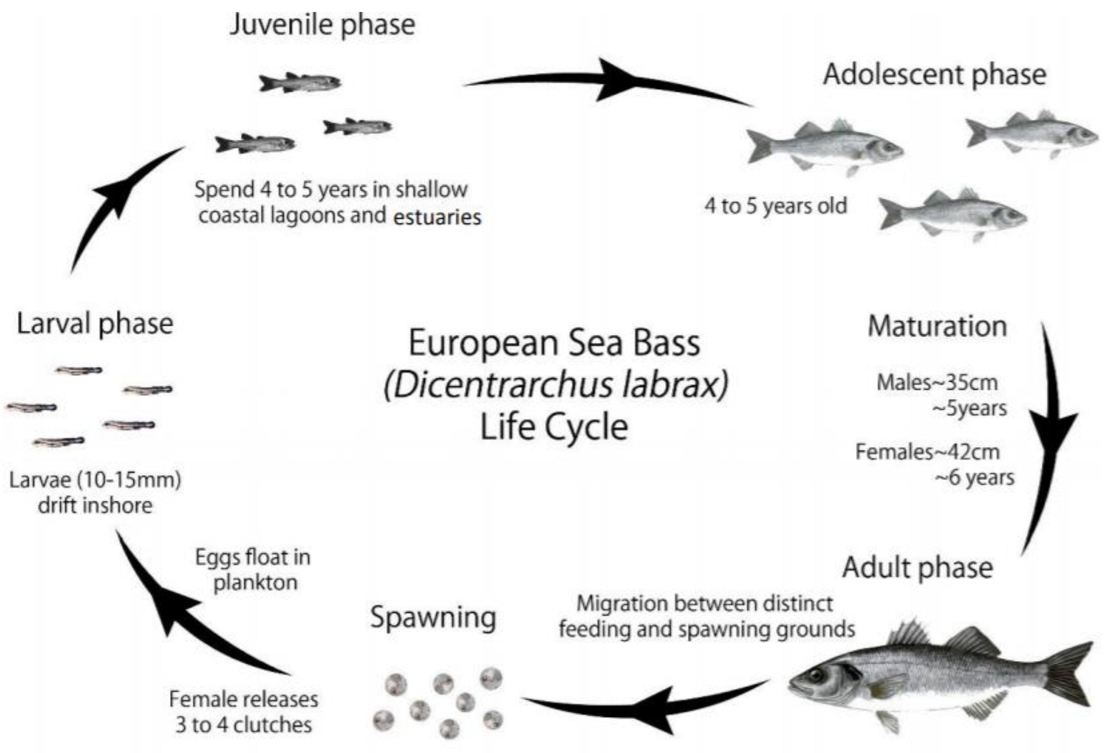


Figure 1.3.1 – Illustration of the European seabass life cycle. Adapted from (Carroll, 2014)

In nature, adult seabass dwell in coastal waters up to 100 meters deep but are most common in shallow waters. It is a eurythermal being and is, therefore, able to withstand large temperature variations (5-28°C), although it develops better at temperatures above 18°C. It is also an euralian, meaning that can support very large variations of salinity (3 – 35‰), even being able to survive in freshwater (FAO, 2019). It is a part of the bonefish family, indicative that they have a bone skeleton formed by skull, spine, and a set of bones with support function of pectoral and pelvic fins. The external morphology of the European seabass is illustrated in Figure 1.3.2.

Dicentrarchus labrax is a carnivore fish, feeding essentially of crustaceans, mollusks, and other smaller fish. As adults, they are usually solitary hunters and hunt other fish while juveniles’ group in shoals searching for small crustaceans and mollusks (Lobo, Pereira, Gonçalves, Peixoto, and Ozório, 2018).

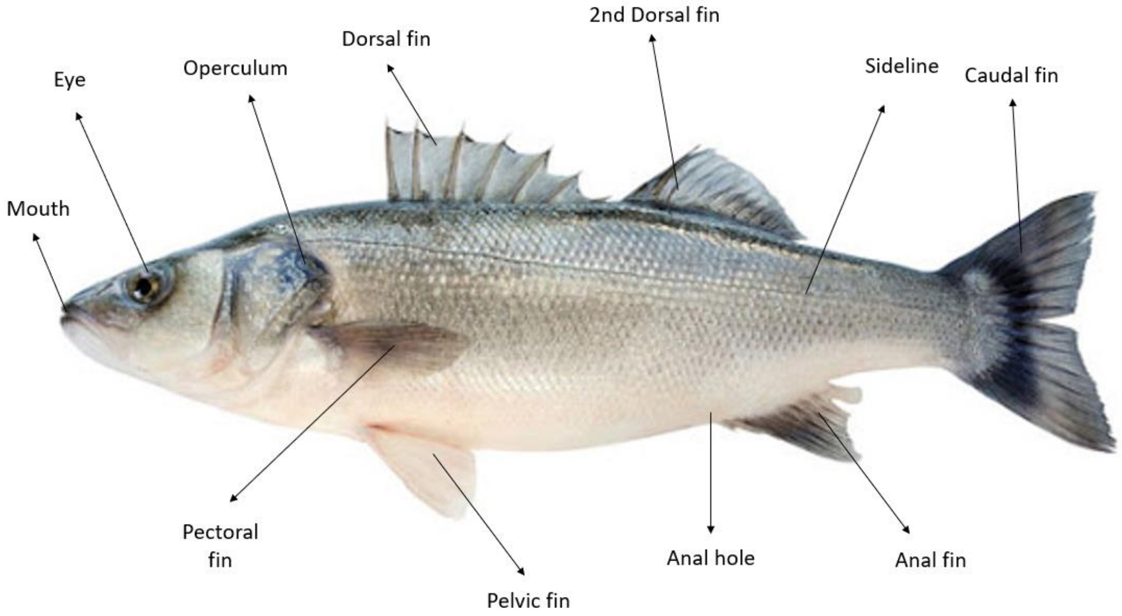


Figure 1.3.2 - External morphology of European seabass

Initially, this species was grown in coastal lagoons before the interest in mass production began in the ‘60s. This was the first non-salmonid marine species to be commercially cultivated in Europe and is presently one of the most important fish at a commercial level in the Mediterranean areas. Currently, the largest producers are Turkey, Greece, Italy, Spain, Croatia, and Egypt (FAO, 2019). Concerning commercial exploitation, Seabass has a high commercial value due to the quality of its fillet and is the third most-produced marine species by aquaculture in Portugal, next to turbot (*Psetta maxima*) and gilthead bream (*Sparus aurata)* (Ferreira, 2019) as previously shown in Figure 1.2.1.2.

1.4 – European Sea bass Nutritional Requirements

The adequate knowledge of the nutritional requirements of a fish species is essential for the correct formulation of high-quality diets able to promote optimal growth rates and minimize feed waste, thus contributing to the sustainable development of the feed-making industry (Oliva-Teles, 2000). Although European Seabass is a very important species in Aquaculture, especially in Mediterranean countries, there are very few studies quantifying its nutritional requirements (Campos *et al*., 2019).

Currently, sea bass feeds are made up of dry pellets with optimal amounts of proteins and lipids, which correspond to 50% and about 12% of the diet, respectively. When production conditions are well controlled, the efficiency of food conversion (fresh weight gained by dry weight of the food ingested) is between 0.24 and 0.74 (Peres, 2000; Rocha, 2017).

Proteins

Reflecting its carnivorous nature, this species has a very high protein requirement (Fournier *et al*., 2002), but protein necessities of fish vary accordingly to the size and age of the fish. A smaller or juvenile fish requires a higher concentration of protein than larger or older fish. This is because, as fish reach maturity, they decrease their growth rate, and consequently there is a reduction in body protein synthesis (Peres, 2000). For this species, it was initially estimated that protein requirement was of 52-60% of the diet (Metailler, Aldrin, Messager, Mevel, and Stephan, 2009) but later, thanks to better methodologies, it was found that for juveniles, protein requirements could be less than what was initially estimated (43- 48% of the diet) (Dias *et al*. 1998; Peres e Oliva-Teles, 1999)

The amount of protein present in the diets is highly important since all fish require a source of essential amino acids (EAA) to ensure maintenance and growth. All fish require the same ten essential amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Wilson, 1986) and two others that are considered as conditionally indispensable, cystine and tyrosine, meaning that they can only be synthesized from methionine and phenylalanine, so if there is a supply of these precursors there is no need of adding cystine and tyrosine to the diets (Cabano, 2017). Table 1.4.1. shows the required amounts of some of the essential amino acids.

Considering the high protein requirement, the fact that this species is one of the most important marine fish species produced in Mediterranean countries and the high costs of aquaculture feeds (>50% of total aquaculture costs) (Rana *et al.*, 2009) reinforce the need for alternative protein sources for ensuring an aquaculture sustainable production (Campos *et al.*, 2018).

Table 1.4.1. -Required amounts of essential amino acids (expressed in g per 16 g of nitrogen) for European seabass. Adapted from (Webster and Lim, 2002)

Essential amino acid	**Requirements (g/ 16 g N)**
Arginine	4.1
Lysine	4.8
Threonine	2.6
Tryptophan	0.5
Methionine + cysteine	4.4

Lipids

Regarding lipids, these are essential in fish feeds, since they play a fundamental role in the proper functioning of cellular operations, maintaining the integrity of membrane structures and are a source of essential fatty acids, among other important functions. Some of the fatty acids of interest in fish feeds are linoleic acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid, and arachidonic acid (Peres and Oliva-Teles, 1999). Fish are the main source of essential fatty acids in human's diets. Therefore, the adequate amount of these compounds in fish is very important since they play a key role in human's cerebral and cardiovascular health (Gebauer *et al.*, 2006; Swanson *et al.*, 2012).

It is recommended a certain level of fat in diets (18-20%) for maximum protein efficiency, because fat has a protein-sparing effect, meaning that the referred level of dietary fat can increase protein utilization efficiency (Campos *et al.*, 2019).

Fatty acid requirements vary according to the species and their habitat. In the case of this marine species, in a juvenile phase, it is recommended a minimum of 0.7% / dry diet of LC-HUFA with a DHA/EPA ratio of 1.5/1 (Peres and Oliva-Teles, 1999; Skalli and Robin, 2004). Lipid deposition in fish occurs in different parts of the body such as in the liver, muscle, and adipose tissue (Peres, 2000). The place where greater deposition occurs will vary according to the species and the habitat. In the case of the European seabass, the liver is the place where greater lipid deposition occurs (McClelland *et al.*, 1995; Peres, 2000).

Minerals and Vitamins

As far as the needs of minerals and trace elements of the European Sea bass are concerned, very little is known. About minerals, it is known that fish need phosphorus for their normal growth and the mineralization of bones. Although fish can absorb minerals directly from water, the main source of phosphorus is in the diet, since the concentration of this mineral in water is too low to meet nutritional requirements (Cabano, 2017). When it comes to carnivorous fish reared in aquaculture, the main source of phosphorus is fishmeal and it was estimated that this mineral should correspond to 0.65% of the diet by dry weight (Oliva-Teles and Pimentel-Rodrigues, 2004).

Quantitative data on vitamin requirements is very scarce, but it is assumed that they are not very different from those established for salmonid species. Existing data for European Seabass suggests that vitamin C requirements are less than 50 mg/kg, vitamin E about 500 mg/kg (Webster and Lim, 2002), and vitamin A 35 mg/Kg (Vázquez and Muñoz-Cueto, 2014).

Carbohydrates and Energy

Like other animals, fish do not have carbohydrate requirements, but the absence of these compounds leads to the utilization of proteins and lipids to obtain energy in order to synthesize biological components normally derived from carbohydrates. So, it is important to have a source of carbohydrates in feeds. It has been admitted that, for European Seabass juveniles, the rate of inclusion of carbohydrates in diets should not exceed 20% (Enes *et al.*, 2011) and it was already proved by Alliot *et al.*(1979) that high carbohydrate dietary levels (>30% dietary starch) can impair growth.

Like any other animal, fish require energy to maintain cellular activity, growth, and reproduction. This energy is obtained through the oxidation of organic compounds resulting from digestion and absorption of food. Although when the energy supply of the diet is insufficient, body reserves (proteins, lipids and carbohydrates) are employed for that purpose. Energy requirements differ according to metabolic activity (influenced by age, habitat, and physiological state) (Peres, 2000). Energy and protein requirements are very complex because of their link to each other. The goal of formulating a diet able to induce a protein-sparing effect is to provide enough carbohydrates and fats to avoid the conversion of protein into energy. With this, proteins can be utilized for other life-supporting roles (e.g. building and repairing tissues and making antibodies or enzymes) and prevent it from being used to obtain energy, utilizing primarily carbohydrates and fats for that purpose. Besides that, proteins are much more expensive than carbohydrates or lipids thus the importance of reducing cost on protein usage. However, fish always prefer utilizing protein as long as it is available.

It is essential to maintain a balance between protein and energy in the diets, thus allowing the optimization of protein utilization and decreasing the loss of nitrogen compounds into the environment (Peres and Oliva-Teles, 1999). There is no growth without protein, but neither is without the correct amount of energy. It has been shown that for the same amount of digestible energy (DE), larger fish require lesser amount of dietary protein than smaller fish (Lupatsch, 2005). As an example, it was shown that the required dietary digestible protein to digestible energy ratio (DP/DE) decreased from 25 to 20 mg DP/ kJ DE when comparing seabass with 50 g and 300 g, respectively (Campos, 2019), and from 28.8 to 23.8 DP/DE when comparing 18.4 g and 56.0 g seabass, respectively (García-Alcázar *et al.*, 1994).

1.5 – Fish feeds

The feeding of carnivorous aquaculture fish is based on fish oil (FO) as the main source of fatty acids and fishmeal (FM) as the main source of essential amino acids. FM is the most important proteins source in fish feeds because of its adequate amino acid profile, high palatability, and a great source of essential fatty acids and minerals (Campos *et al.*, 2018).

Fishmeal is very utilized in feeds for numerous species (e.g. pigs, ruminants, poultry, farmed fish, etc.) for its known increase of productivity and improvement of feed conversion. Besides being a rich source of high-quality proteins, FM is also rich in important minerals like phosphorous, calcium, selenium, B vitamins (choline and niacin) and have relatively high energy content, all essential for fish (FAO, 1986). FM has an excellent amino acid balance that reflects directly amino acid balance in fish, so it is no surprise that this ingredient meets directly the nutritional requirements of carnivorous species (Auchterlonie, 2017).

FM and FO are produced through fish that are caught and do not meet the requirements to be directly used for human feeding (Pike and Jackson, 2010). These two products can be obtained from different species of fish, generally small pelagic fish (e.g. sardines, herrings, capelin) (Auchterlonie, 2017) serving as raw material, which is comprised of three main fractions: solids, water, and oil. Nowadays, fishmeal supply is not produced exclusively from fisheries and there is even a crescent supply coming from processing of seafood byproducts where frames, heads, viscera, and other trimmings are used to produce important marine ingredients. According to FAO (2016) 25-35% of fish meal global supply comes from by-products. This is an excellent way of valuing these products preventing its waste.

Fish oil is present in fishmeal and is constituted by many triglycerides of fatty acids with phospholipids, glycerol, ethers, and wax esters (FAO, 1986). FO is the major source of highly unsaturated fatty acids (HUFA), namely EPA and DHA that are responsible for the optimal growth and health of fish. These HUFA are also associated with the prevention of cardiovascular and inflammatory diseases, mental health, and normal brain development in humans. Such fatty acids are widely used as a food supplement, not only in the fish feed but also for many other animal species. In the case of aquaculture, having a source of HUFAs is extremely important, therefore the utilization of FO, because marine fish have a low ability to synthesize these fatty acids, so they need to consume microscopic algae rich in these compounds, or else feed on fish that consume these algae (Stamey *et al.*, 2012).

Considering the steady decline in wild fish fishing and the increasing demand for fresh fish and fish feed, there has been a rapid decrease in the availability of fish meal and oil. Consequently, their price has increased, to the point where the cost of aquaculture feeds accounts for 40 to 70% of the total cost of fish produced (Henry *et al.*, 2015). And for this reason, is of high interest to look for an alternative to these ingredients that is sustainable, low cost and easy to obtain. The stagnation of FM and FO production together with the rising demand for finfish feeds has led feed manufacturers to seek other alternatives that are economically and environmentally sustainable that could replace these two feed constituents (Wan *et al*, 2019). The replacement of FM and FO is a complicated task, but has already been tested, as it is necessary to find viable substitutes to include in aquafeeds to meet all the necessary fish nutritional requirements (Campos *et al.*, 2019). However, for these alternatives to be accepted, they must assure fish growth, overall health and keep fillet quality while meeting consumer expectations (Wan *et al.*, 2019).

1.6 – Alternative protein sources

Alternative source proteins need to check some requirements. They need to be nutritionally suitable for important aquaculture species and be accessible in the market with competitive prices and it is preferable if they do not compete with human food chain (Council, 2011). For this reason, several alternatives have been considered and these can be divided in four major groups: land vegetables, animals (animal by-products, invertebrates and insects), microbial (yeasts and bacteria) and algae (macro and microalgae).

1.6.1 - Land vegetable protein sources

Land vegetables (e.g. wheat meal, soybean protein concentrate, peas) (Cabano, 2017) are the most commonly used alternative because of their high availability and competitive prices, however, it also competes directly with animal and human food. Also, vegetable-based competitors do not contain the essential compounds at the same levels as animal protein sources, mainly because they reflect the physiological needs of the plant of a terrestrial environment (Auchterlonie, 2017). Despite the common usage of plant protein in fish feeds, many of them do not present the adequate levels of essential compounds (e.g. lacking one or more essential amino acids or HUFA) to meet fish requirements that may lead to specific amino acid supplementation (Cabano, 2017). Another important factor is that vegetable-based material contains anti-nutritional factors (ANFs) (e.g. substances that exist as protection of being eaten) like protease inhibitors, lectins, and allergens (Francis *et al.*, 2001). Plants also contain fibers and another compound that cannot be metabolized by many carnivorous species, so their implementation in feed requires additional processing prior use (e.g. soy protein concentrate (SPC)), and consequently additional energy costs (Auchterlonie, 2017).

1.6.2 - Animal protein sources

Insects

Insects have been receiving increasing attention as a viable animal feed dur to a variety of advantages, such as high nutritional value (high energy and protein content, bioactive compounds with known immune-boosting properties (Sogari *et al.*, 2019). Insects have an amino acid profile that meets fish needs (PROteINSECT, 2016) and can improve feed acceptance as they are a part of the natural diet of many species (Makkar, 2018). They are also very promising from an environmental point of view having low emission of greenhouse gases and there is no need for large areas to produce them (van Huis and Oonincx, 2017). Insects have hence been increasingly used to substitute conventional protein and fat sources. However, they present some disadvantages: can have low of some essential amino acids that need to be supplemented in aquafeeds, fatty acids profile totally lacks HUFA (Harinder *et al.*, 2014; Tran *et al*, 2015), the presence of chitin, an antinutritional factor, affects nutrient absorption in fish, have low content of some minerals (e.g. phosphorous and calcium) (Tran *et al.*, 2015). There are already a few insect species authorized to be used in aquaculture, but their still high prices and low consumer acceptance are still problematic (Sogari *et al.*, 2019). Nevertheless, growing demand will decrease the price of an insect meal.

Animal by-products

Animal by-products (ABPs) such as processed animal proteins (PAPs), animal fats, poultry meal, blood meal, feather meal (Cabano, 2017) are important protein sources (European Comission, 2013). In 2001, the utilization of terrestrial animal by-products in animal feeds was forbidden in EU due to problems associated with BSE. However, in 2013 processed animal protein from Category 3 (parts of slaughtered animals that are fit for human consumption but aren't utilized for food production) were declared safe for utilization and in the following years animal by-products were slowly reintroduce in animal feeds (Cabano, 2017).

Agrofood by-products are very abundant, have high market availability and competitive prices and their use largely contributes to a circular economy. PAPs have high protein and amino acid content, a low fiber content adequate to carnivorous species, good phosphorous availability (contributing to pollution reduction originated from faeces), don´t have ANFs, and some studies already proved that the substitution of fishmeal by animal by-products is possible without impairing growth (SONAC, 2001). Yet, the ABPs have low consumer acceptance because of consumers awareness related to zoonosis (e.g. bovine spongiform encephalopathy (BSE) or *salmonella*) (FAO, 2004).

1.6.3 - Microbial protein sources

Unicellular protein sources (yeasts, bacteria, fungi) are a non-conventional source that has been gaining interest over the years and can be directly used as a live feed or utilized as feed supplements. Microorganisms can provide essential amino acids, fatty acids, proteins, vitamins, and even exogenous enzymes than can help in the digestion process (Onianwah *et al.*, 2019). A bacterial meal presents favourable amino acid content, bioactive components, high-quality protein (around 70% crude protein) and has no health risks (Overland *et al.*, 2010). Yeasts can present 50-60% of proteins, and are produced from green carbons, have a favourable amino acid profile and provide good taste to feeds (Overland and Skrede, 2017). In a previous study, it was shown that the inclusion of 15% of brewer's yeast tilapia feeds promotes growth without affecting body composition (Ozorio *et al.*, 2012). The main disadvantage of such biomass is related to its limited production volume, still high costs and the easy possibility of being infected. Nevertheless, bioreactor is recently to be developed and using bioengineering methods this biomass may gain great relevance in the nearest future.

1.6.4 – Algae

Algal biomass can be seen as a functional feed additive able to provide benefits at an environmental and economic level and that can promote fish health and growth (Soto *et al.*, 2015). Algae (macro and microalgae) are still not very available in the market in large volumes, and still have a high price. However, they have a great consumer's acceptance, are rich sources of ω-3 and ω-6 fatty acids and can be considered as functional feeds. Algae as a sustainable aquafeed ingredient is a strong possibility for the future because its production can be achieved without requiring expensive arable land as they can be collected from coastal areas or farmed (Wan *et al.*, 2019).

Algae are a group of photosynthetic organisms, which grow easily in various environments, such as saltwater, sweet, brackish, and even wastewater. It is estimated that there are about 200 000 species of algae, however currently, only about 200 species are used. These organisms are rich in lipids, proteins, carbohydrates, minerals, vitamins, pigments, and bioactive compounds like polyphenols that can be used in various applications such as food, dietary supplements, biofertilizers, source of biofuels (ethanol or biodiesel) or even in the cosmetic and pharmaceutical industry. The protein content of different algae is presented in Table 1.6.4.1 and compared to conventional sources used in aquafeeds.

Table 1.6.4.1 - Protein content of different algae and some conventional feedstuffs (% Dry Matter)

Macroalgae [1]	**Protein (%DM)**
Porphyra yezoensis	43.6
Ulva sp.	15-25
Enteromorpha sp.	20.7
Microalgae [1]	
Chlorella pyrenoidosa	57
Spirulina plantesis	61-64
Chlorella vulgaris	51-58
Conventional protein sources	
Fish meal[3]	68-71
Soy protein concentrate[4]	68-72

Soy[1]	37
Corn[1]	10
Corn gluten meal [2]	70-73
Wheat[1]	14
Rapeseed protein concentrate [2]	35

[1] Adapted from (Rajauria, Cornish, Ometto, Msuya, and Villa, 2015); [2] Retrieved from (Cabano, 2017); [3] Retrieved from (Cruz, 1997); [4]Retrieved from (Altschul and Wilcke, 2013)

Various algae species were already tested in different fish species as protein or lipid sources to replace dietary FM or FO or even as feed additives. Table 1.6.4.2 illustrates some algae that were already used in the diets of different fish species and the main results obtained. Within the great number of existing algae, later on a focus will be placed on those that were utilized in this study (*Gracilaria* sp., *Ulva* sp., *Chlorella* sp. and *Nannochloropsis* sp.).

Table 1.6.4.2 - Algae used in feeds for some fish species

Algae	**Algae type**	**Applied Species**	**Main Results**	**Reference**
Gracilaria bursa-pastoris; Ulva rígida;	Macroalgae	*Dicentrarchus labrax* (European seabass)	Carcass composition was similar among treatments. Inclusion of these algae up to 10% caused no negative consequences in growth performance, nutrient utilization, or body composition	(Valente *et al.*, 2006)
Gracilaria cornea	Macroalgae	*Dicentrarchus labrax* (European seabass)	In fish fed *Gracilaria cornea* 10% growth performance was significantly reduced but FCR increased *Gracilaria cornea* should be limited to 5% of the diet	
Ulva sp; *Gracilaria* sp; *Fucus* sp	Macroalgae	*Dicentrarchus labrax* (European seabass)	Tested at 2.5 and 7.5% supplementation levels, improvement of immune and antioxidant response without compromising growth performance	(Peixoto *et al.*, 2016)
Tisochrysis lutea; Tetraselmis suecica	Microalgae	*Dicentrarchus labrax* (European seabass)	The replacing of 45% protein and 36% lipid of fish meal by microalgae didn´t affect growth but caused a decline in	(Cardinaletti *et al.*, 2018)

			feed digestibility, greenish pigmentation of the skin	
Gracilaria vermiculophylla	Macroalgae	Oncorhynchus mykiss (Rainbow trout)	*Gracilaria vermiculophylla* can be included in diets for this specie up to 5%, highest fillet color intensity and juiciness, flesh iodine levels doubled	(Valente *et al.*, 2015)
Spirulina Platensis; Chlorella vulgaris	Microalgae	*Clarias gariepinus* (African catfish)	Growth performance boosted, an increase of white and red blood cells	(Raji *et al.*, 2018)
Nannochloropsis salina	Microalgae	*Oreochromis niloticus* (Nile tilapia)	Higher ω-3 polyunsaturated and ω -3/ ω -6 fatty acid ratio found in fish fed *N. salina* diet and better nutrient utilization	(Gbadamosi and Lupatsch, 2018)
Nannochloropsis sp.; Desmodesmus sp.	Microalgae	*Salmo salar* (Atlantic salmon)	*Nannochloropsis sp.* was more digestible than *Desmodesmus sp.*	(Gong *et al.*, 2018)
Gracilaria gracilis; Nannochloropsis oceanica	Macro and Microalgae	*Dicentrarchus labrax* (European seabass)	*G. gracilis* and *N. oceanica* utilized single (8% inclusion level) or blended (4% inclusion of each alga) can partially replace fish meal in Seabass diets	(Batista *et al.*, 2020)

1.6.4.1- Macroalgae

Macroalgae or seaweed are multicellular plant-like organisms (eukaryotic and autotrophic) and are taxonomically divided into three major groups: Chlorophyta (green algae, more than 1800 species), Ochrophyta–Phaeophyceae (brown algae, about 2000 species) and Rodophyta (red algae, over 7200 species) being spread around the globe. Macroalgae usually live attached to rocks or other solid substrates in coastal areas (Guiry, 2019; Leandro *et al.*, 2019).

In general, their chemical composition is characterized by a low lipid content (0,3 - 7,2 g/100 g dry weight), moderate protein content (10-30 g/100 g dry weight) but high level of polysaccharides (mainly pectin, alginic acid, agar and carrageenan), minerals and vitamins (Vizcaíno *et al.*, 2016). Their chemical composition varies according to the species, harvesting season, geographic origin, environmental status, amongst other parameters (Matanjun *et al.*, 2008). They are biological resources with high nutritional value due to their secondary metabolites, such as carotenoids, terpenoids, vitamins, amino acids, and all kinds of polysaccharides (MarineBiotech, 2019) with important bioactivities (e.g. antioxidant, anti-inflammatory, anticoagulant, etc.) which shows that they need to be more sustainably explored to become a new source of these bio compounds (Leandro *et al.*, 2019).

The pigments produced by algae are essentially carotenoids that are antioxidants (meaning that they offer essential protection at cellular level against free radicals and prevent lipid peroxidation) (Dineshbabu *et al.*, 2019). Accordingly, to Brown *et al.* (1999), aquaculture feeds that contain carotenoids in their constitution make it possible to improve fish health, their development and survival rate. Various species of macroalgae and microalgae have been used in fish feeds formulation in order to assess their nutritional value and many have already shown to be beneficial. When it comes to choosing the algae for fish feeding studies, algae often they to be chosen by convenience, which means low cost and commercial availability (Towers, 2013). For proteins, algae also show to be a good option for supplementing feeds, as they are producers of all amino acids, in particular the ten essential amino acids required for fish (Lum *et al.*, 2013). Green macroalgae have a higher protein content than brown macroalgae, ranging between 11-26% of dry biomass. Sometimes the high concentrations of these compounds turn seaweeds in a good alternative to protein-rich ingredients (SPC or fishmeal) already used in animal food (García-Vaquero, 2018).

There are already studies showing that protein sources from an animal source can be replaced in whole or in part by protein sources of plant origin for various fish species, sometimes presenting an equal or greater performance compared to that of fish fed with animal feed (Boscolo *et al.*, 2001). Most algae currently used as a feed's supplement have higher protein amounts than conventionally used protein sources such as soybeans, corn, or wheat (Rajauria *et al.*, 2015).

Focusing on the macroalgae utilized in this work, *Gracilaria* sp. (Figure 1.6.4.1.1) is a red seaweed (Rodophyta) rich in polysaccharides and it's very used for agar production. It is a species very rich in polyphenols and sulphated polysaccharides, that are known to reduce oxidative stress (Fidelis *et al.*, 2014). Araújo *et al.* (2015) suggested that diets supplemented with this species could prevent the oxidation of packaged fish, increasing its shelf-life. Moreover, seaweeds belonging to the Rodophyta family are quite rich in photosynthetic pigments such as chlorophyll-*a* and carotenoids (β- carotene, lutein, and zeaxanthins) (Cardoso *et al.*, 2014), and may represent an interesting natural alternative for replacing or reducing the utilization of artificial colorants in feeds (Peixoto *et al.*, 2019). It was recently observed that *Gracilaria gracilis* is a good ingredient to partially replace fishmeal in European seabass diets. However, a pre-treatment step is required in order to avoid negative effects in fish nutrient digestibility (Batista *et al.*, 2020a).

Figure 1.6.4.1.1 - Gracilaria sp.

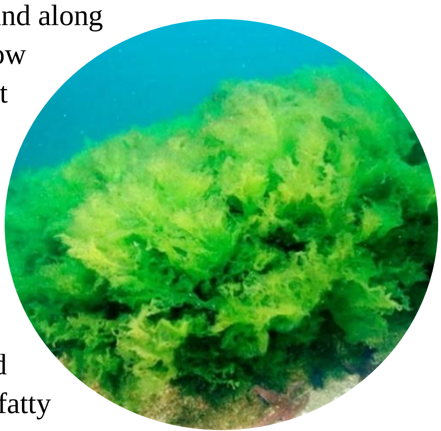


Figure 1.6.4.1.2 – Ulva sp.

Ulva sp. (Figure 1.6.4.1.2) is a green macroalga (Chlorophyta) found along the Mediterranean coasts. It is a fast-growing species and with a low production cost (Vizcaíno *et al.*, 2016). Is a very common species that emerge preferentially in eutrophic places rich in phosphate, ammonium, and phytoplankton (Guiry, 2019). According to Taboada *et al.* (2010) *Ulva rigida* is a good protein source (178 g/Kg) with adequate levels of of essential amino acids, fat content (9g/kg where 24.2% are ω-3 and 10.1% ω-6 are polyunsaturated fatty acids) and high soluble polysaccharides content (426 g/kg) (Taboada *et al.*, 2010). Furthermore, it is also the most common edible green seaweed utilized in human nutrition for being a good source of protein, essential fatty acids, and fiber. It was even used in bioremediation of some low scale aquaculture systems (Pavão, 2014).

1.6.4.2 – Microalgae

Microalgae or microphytes are aquatic microscopic single cells found in marine or freshwater systems (Coêlho *et al.*, 2019) and can be prokaryotic or eukaryotic (Barkia *et al.*, 2019). They can grow as individual cells or as small colonies (Coêlho *et al.*, 2019) and can be applied in biofuel´s production, incorporated in health supplements, in pharmaceuticals and in the cosmetic industry, in wastewater remediation processes and atmospheric CO_2 mitigation (Figure 1.6.4.2.1). This group of organisms has enormous biodiversity and about 40000 species are already described or analyzed (Safi *et al.*, 2014).

These organisms are a great source of vitamins such as A, B1, B2, B6, B12, C, E, K, biotin, and folic acid. They are also very rich in minerals such as calcium, phosphorus, magnesium, potassium, sodium, zinc, iron and copper, and these compounds usually constitute 2.2 to 4.8% of the dry weight of algae, depending on the species and their growth conditions. Lipids present in algae have a high content of ω-3 fatty acids and polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA, 20:5ω-3), docosahexaenoic acid (DHA, 22:6ω-3) and linoleic acid (Dineshbabu *et al.*, 2019). Some microalgae are so rich in protein content that basically half of their biomass corresponds to proteins. For example, most strains of *Spirulina* and some strains of *Chlorella* and *Nannochloropsis* have protein content ranging from 40 to 65% (Vaz *et al.*, 2016).

Microalgae are a good source of biological compounds like polysaccharides, lipids, vitamins, proteins (Table 1.6.4.2.1) pigments, and antioxidants. Microalgae can become a renewable and sustainable feedstock for biofuels and other products of interest and its growth and potential can always be enhanced with genetic engineering techniques (Khan *et al.*, 2018).

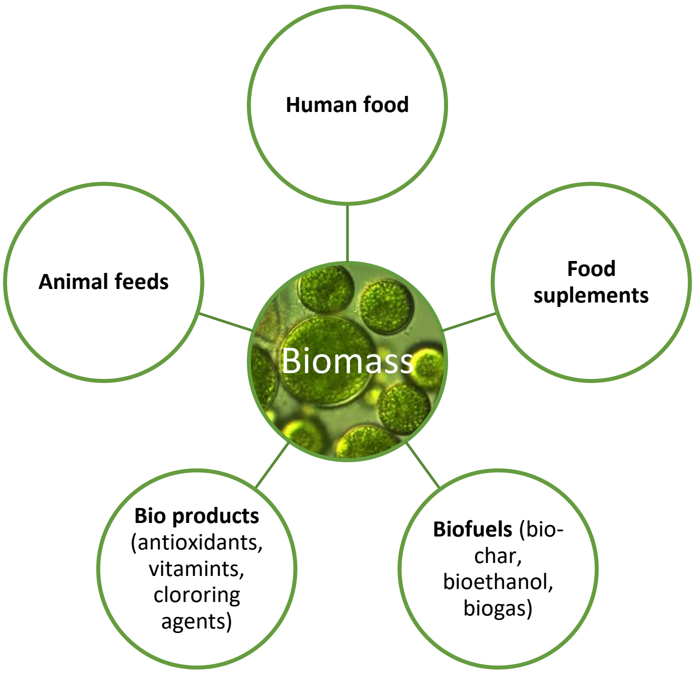


Figure 1.6.4.2.1 – Products that can be obtained from microalgae. Adapted from (Khan et al., 2018)

Microalgae need to fulfill a key number of attributes so they can be used in aquaculture. For this reason, a great amount (several hundred) of species have been tested but only a few are utilized. They must have an appropriate size for ingestion, rapid growth rate, be stable enough to resist to a certain temperature, light or nutrient fluctuations that may occur in hatchery systems, have a good nutrient composition and absence of toxins that may be transferred to the food chain (Brown, 2002).

Chlorella sp. and *Nannochloropsis* sp. were the microalgae used in the formulation of European seabass diets in this study. *Chlorella* sp. (Figure 1.6.4.2.2) grows in freshwater and was the first photosynthetic microorganism isolated and cultivated in pure culture. It is a spherical unicellular eukaryotic green alga that present a thick cell wall (100-200 nm) as its main characteristic (Sydney *et al.*, 2014) and with sizes ranging between 2 and 10 μm in diameter (Liu, 2014). It is already very used in human alimentation and when it comes to animal feed this microalga has been very utilized and showed interesting results like enhancing growth and life expectancy in some species (Chacón-Lee and González-Mariño, 2010; Gouveia *et al.*, 2002) and also showed a protective effect against heavy metals and other harmful compounds by reducing the oxidative stress and increasing antioxidant activity in some tested animals (Safi *et al.*, 2014).

Chlorella sp. all the essential amino acids indicating that it can be a good protein substitute for fish feeds as alternative to current protein sources and it is very rich in ω-3 long-chain polyunsaturated fatty acids (Enyidi, 2017) .When mature, it can produce essential and non-essential amino acids and has a protein content that ranges between 42 and 58% of biomass dry weight (quantities that vary according to growth conditions) (Safi *et al.*, 2014). During optimal growth conditions, lipids can reach 5-40% per dry weight of biomass and its fatty acid profile is more suitable for nutritional uses since it is rich in polyunsaturated fatty acids (linoleic acid $C_{18:2}$, linoleic acid $C_{18:3,}$ and eicosapentaenoic acid $C_{20:5}$) (Safi *et al.*, 2014). This microalga also has high vitamin content including vitamins A, E, C and B. Altogether, these are key elements for cell growth, antioxidant activity, improvement of blood circulation, between other important biological roles (Safi *et al.*, 2014; Salvador *et al.*, 1998). Batista *et al.*(2020b) showed that algae nutrient acessibility can be improved by employing physical methods to modify *Chlorella vulgaris* cellular integrity, resulting in a better digestibility of this alga by European seabass.

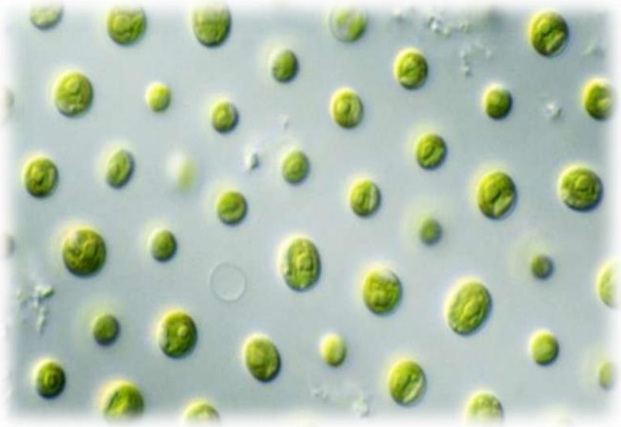


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Figure 1.6.4.2.2 - Chlorella sp.

Nannochloropsis sp. (Figure 1.6.4.2.3) belongs to the class Eustigmatophyceae. It is a marine unicellular microalga, worldwide distributed, with a spherical form and a diameter between 2-5 µm. This microalga is commonly used in aquaculture since it has high protein, amino acids, and fatty acids content, in specific, its high content of EPA that exceeds other microalgae (Jorge, 2016; Li *et al.*, 2015). According to Grimi *et al.* (2014) *Nannochloropsis* is composed by 38% carbohydrates, 18% lipids, 29% crude proteins, 17,4% fatty acids, 0,29% chlorophylls, 0,06 carotenoids and 3% microelements. In this species, PUFA can reach up to 65-70% of total dry weight (Khatoon *et al.*, 2014). This species is used in aquaculture as a source of lipids and it is very appealing to the sector because of its easy culture, and lack of toxicity (Khatoon *et al.*, 2014), it´s also known as a good source of high-quality protein (Kilian *et al.*, 2011), vitamin E and pigments (chlorophyll, astaxanthin, zeaxanthin, and canthaxanthin) (Elnabris, 2012). Earlier this year, Batista *et al.* (2020a) preformed a trial with seabass to understand the potential of *Nannochloropsis oceanica* and *Gracilaria gracilis* (single or blended) as natural dietary ingredients for this species, concluding that 8% of *Nannochloropsis oceanica* inclusion in

European seabass diets did not cause negative effects in fish growth, feed efficiency or whole body composition .

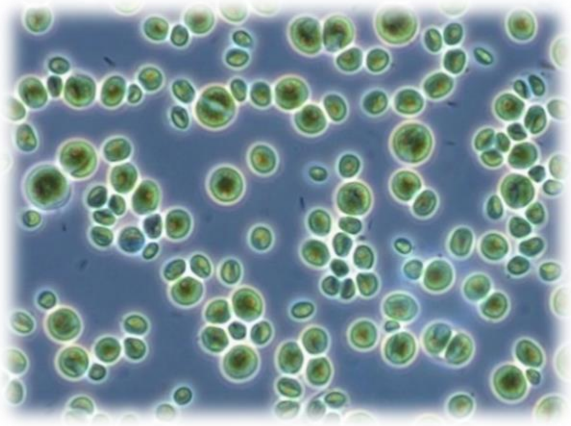

Figure 1.6.4.2.3 - Nannochloropsis sp.

1.7 - Aim of this study

This work aims to evaluate the effect of increasing inclusion levels (0, 2, 4 and 6%) of a blend of two macroalgae (*Gracilaria* sp. and *Ulva* sp.) and two microalgae (*Chlorella* sp. and *Nannochloropsis* sp.) to replace plant protein sources in diets for European seabass juveniles. The experimental diets were fed to triplicate groups of fish until apparent satiety for a period of 3 months. The objective of the thesis is to evaluate the maximum possible inclusion level of the algae blend able to assure a good fish growth performance, feed utilisation and whole-body composition.

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2 - Materials and methods

2.1 – Ethics statement

The present study was performed by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority DGV-Portugal and conducted according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

2.2 - Experimental diets

Four isoproteic (50.0% dry mass), isolipidic (16.2% dry mass) and isoenergetic (≈20.9 kJ/g dry mass) diets were formulated with increasing quantities of an algal blend (ALGAESSENCE) taking into consideration all the nutritional requirements of European sea bass. The blend was a mixture of two macroalgae (*Gracilaria* sp. and *Ulva* sp.) and two microalgae (*Chlorella* sp. and *Nannochloropsis* sp.). A control diet was formulated without the algae blend (Algae 0%) and compared with three experimental diets containing 2, 4 or 6% of the blend (Algae 2%, Algae 4% and Algae 6%, respectively) that was inlcuded at the expense of wheat gluten and whole peas. Diets were formulated and extruded by SPAROS Lda. (Olhão, Portugal) and pellets had 2.0 mm. The ingredients and the proximal composition of the experimental diets are presented in Table 2.2.1.

Table 2.2.1 – Formulation and proximate composition of the experimental diets

Ingredients (%)	Algae 0%	Algae 2%	Algae 4%	Algae 6%
Fishmeal LT70	12.5	12.5	12.5	12.5
Soy protein concentrate	30	30	30	30
Wheat gluten	11	10.5	10.1	9.6
Corn gluten	12.5	12.5	12.5	12.5
Soybean meal 48	10	10	10	10
Whole peas	8.28	6.88	5.28	3.78
Fish oil	13.2	13.1	13.1	13.1
Vitamin and mineral premix	0.5	0.5	0.5	0.5
Monocalcium phosphate	2	2	2	2
Yttrium oxide	0.02	0.02	0.02	0.02
Algae blend [1]	0	2	4	6
Proximate composition (% DM)				
Dry matter (% DM)	99.28	98.57	99.30	97.80

Ash	7.42	7.86	8.30	8.64
Protein	52.58	52.87	52.47	52.99
Fibre	2.90	2.81	2.71	2.53
Lipids	15.48	15.12	15.25	15.38
Starch	5.57	5.06	4.58	3.87
Energy (kJ/g)	21.77	21.54	21.65	21.80

[1] Algae blend composition (%DM): Ash – 25.14; Protein – 34.67; Lipids – 5.42; Starch – 2.17;

2.3 - Growth trial

The growth trial was conducted in the Fish Culture Experimental Unit of CIIMAR (Matosinhos, Portugal) with juvenile European Seabass (*Dicentrarchus labrax*) obtained from a commercial fish farm (Acuinuga, S.L., Spain). After transported, all fish were kept in quarantine for 22 days and fed a commercial diet obtained from AQUASOJA, Sorgal S.A., Portugal (49% crude protein and 20% crude fat, DM basis) before the growth trial for acclimation to the new rearing conditions.

After this period, a total of 552 specimens were individually weighed (g) and measured (total length, cm) and randomly distributed among 12 tanks cylindrical fiberglass tanks (Figure 2.3.1) of 160 L water capacity within a saltwater recirculation system with a water inflow of 6 L min^{-1} in each tank. Homogeneous groups of 46 fish per tank were established with an initial mean body weight of fish was 11.3 ± 2.7 g and mean body length 10.5 ± 1.0 cm. Fish were acclimatized to the system for 2 weeks and fed by hand 2% of their live weight a standard commercial diet from AQUASOJA, Sorgal S.A. After this period, the experimental diets were randomly assigned to triplicate groups of fish (initial body weight of 11.3 ± 2.7 g). Fish were fed by automatic feeders (Figure 2.3.2) to apparent visual satiation 3 times a day (9:00, 13:00 and 17:00 hours), seven days a week for 97 days. The amount of feed supplied to each tank was adjusted daily based on the presence or absence of uneaten feed in each tank.

Figure 2.3.1 - Experimental room and fiberglass tanks utilized

Figure 2.3.2 - Automatic feeders utilized

Feed consumption and some water parameters (temperature, pH, salinity and dissolved oxygen) were recorded daily, while other water parameters such as ammonia and nitrites were recorded every two days. It was established a natural photoperiod and the light conditions were similar for every tank (twelve hours of light and twelve hours of darkness). The water temperature was maintained at 20.9 ± 0.4°C, dissolved oxygen (DO) at 8 mg/L, pH around 8, water salinity at 35.8 ± 1.3‰, ammonia <0.05 mg/L and nitrites <0.5 mg/L.

2.4 – Fish Samplings

At the beginning of the trial, nineteen fish from the initial fish stock were euthanized by an overdose of anaesthesia with 2 – phenoxyethanol for evaluation of initial fish whole body composition.

An intermediate sampling was conducted at week 5, in which fish were bulk weighed to monitor weight gain and register feed consumption. At the end of the growth trial (12-week period), all fish were individually weighed (g) and measured (total length, cm) after a 24-h fasting period. Twelve fish from each tank were euthanized by an overdose of anaesthesia (2 – phenoxyethanol, 1 mL/15 L water). From those twelve fish, six were stored at -80°C for evaluation of whole-body composition; the other six fish were immediately sampled for tissue collection (liver and viscera) and determination of HSI and VSI.

2.5 - Chemical analysis

Experimental diets and carcass were ground and homogenized before analysis. Proximate composition analysis was performed in duplicate. All samples were analysed for dry matter (105°C for 24 hours). Ash content was analysed by combustion in a muffle furnace (Nabertherm L9/11/ B170, Bremen, Germany; 500°C for 6 hr).

Crude protein was analysed using a Leco nitrogen analyzer (Model FP-528; Leco Corporation, St. Joseph, USA), and protein was estimated as N × 6.25. Crude fat content was analysed by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany). Gross energy was determined in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany).

2.6 – Data analysis

Growth indexes were calculated with the following formulas:

ABW (Average Body Weight) (g) = (initial weight + final weight) /2

SGR (Specific growth rate) =100 x (LN final weight – LN initial weight) / nº trial days

VFI (Voluntary Feed Intake) (g/100g/day) = 100 x feed intake (g/fish) / g ABW / nº trail days

FCR (Feed Conversion Ratio) = feed consumption (g MS /fish) / (final weight (g) – initial weight (g))

K (Final Condition Factor) = 100 x final weight / final length^3

HSI (Hepatosomatic index) (%) = 100 x (liver weight / final fish weight)

VSI (Viscerosomatic index) (%) = 100 x (viscera weight / final fish weight)

DMI (dry matter intake) (g/100g ABW/day) = 100 x feed consumption (g MS /fish) / (initial weight (g) + final weight (g) /2) / nº trail days (%/fish/day)

Nutrient Intake (g/100g ABW/day) = dry matter intake (g/100g ABW/day) x % diet nutrient x **%**

Energy Intake (kJ/100g ABW/day) = dry matter intake (g/100g ABW/day) x diet energy (kJ/g)

Ingested protein (g) = feed intake (g DM /fish) x diet protein (% DM) x **%**

PER (Protein Efficiency Ratio) = weight gain (g) / ingested protein by fish (g)

DMG (Dry matter gain, g/ kg ABW/dia) = [(final weight x final composition % MS %) – (initial weight x initial composition % MS)] / ABW (kg) / nº trial days

Nutrient Gain (g/ kg ABW/dia) = [(final weight (g) x % final composition nutrient (%WW) x **%**) – (initial weight (g) x % initial composition nutrient (%WW) x **%**)] / ABW (kg) / nº trial days

Energy Gain (kJ/ kg ABW/dia) = [(final weight (g) x energy of final composition (%WW) (kJ/g)) – (initial weight (g) x energy of initial composition (%WW) (kJ/g)] / ABW (kg) / nº trial days

DMR (Dry matter retention/consumption) (g DM / fish) = (final weight x % dry matter of final fish composition) – (initial weight x % dry matter of initial fish composition) / consumption

Nutrient retention (nutrient retention/consumption) **=** 100 x ((final weight x nutrient of final fish composition (%WW)) – (initial weight x nutrient initial fish composition (%WW)) / (feed intake (g DM /fish) x diet nutrient (%DM))

2.7 – Statistical analysis

Data analysis was performed with the software program IBM SPSS Statistics 26.0 (SPSS Inc., Chicago, IL, USA). A significance level of 5% ($P<0.05$) was used for all comparisons. Homogeneity of variances was checked by performing Levene’s test. When homogeneity was verified, data was compared by one-way analysis of variance (ANOVA) and means were compared using post-hoc Tukey’s honest significant difference test (Tukey HSD test). The differences between groups were considered significant if $P<0.05$ and a tendency if $0.05<P<0.1$.

3 – Results

3.1 - Growth Performance and Feed Efficiency

The effects of the dietary treatments on body weight after the 12-week trial is represented in Figure 3.1.1. Fish increased their weight from an initial weight of 11.3 ± 2.7 g to a final weight of 37.3 ± 2.39 g in fish fed the Algae 0% diet, 54.4 ± 6.21 g for fish fed the Algae 2% diet, 56.6 ± 4.54 g for fish fed the Algae 4% diet and 62.9 ± 2.16 g for fish fed the Algae 6% diet. In the initial sampling there were no significant differences between diets (P=1.000). At the end of the experiment it was noticeable that body weight of fish fed with Algae 6% was significantly higher (P<0.001) than fish fed the remaining diets. Fish fed with Algae 2% and Algae 4% had similar final body weight, but lower than fish fed Algae 6%. Moreover, fish fed with Algae 0% (control diet) had a significantly lower body weight compared to all dietary treatments.

In Figure 3.1.2 it is represented the effect of the different dietary treatments on body length. There was an increase in body length from an initial length of 10.5 ± 1.0 cm to a final length of 15.6 ± 1.37 cm in fish fed the Algae 0% diet, 17.1 ± 1.47 cm for fish fed the Algae 2% diet, 17.1 ± 1.62 cm for fish fed the Algae 4% diet and 17.7 ± 1.53 cm for fish fed the Algae 6% diet. There were no significant differences (P=0.833) between diets at the initial sampling. In the final sampling, likewise body weight, it was notorious that fish fed with Algae 6% showed a significantly higher (P<0.001) length when compared with other three diets. Moreover, fish fed with Algae 2% presented no significantly differences when compared with Algae 4%. Fish fed the control diet were the smallest.

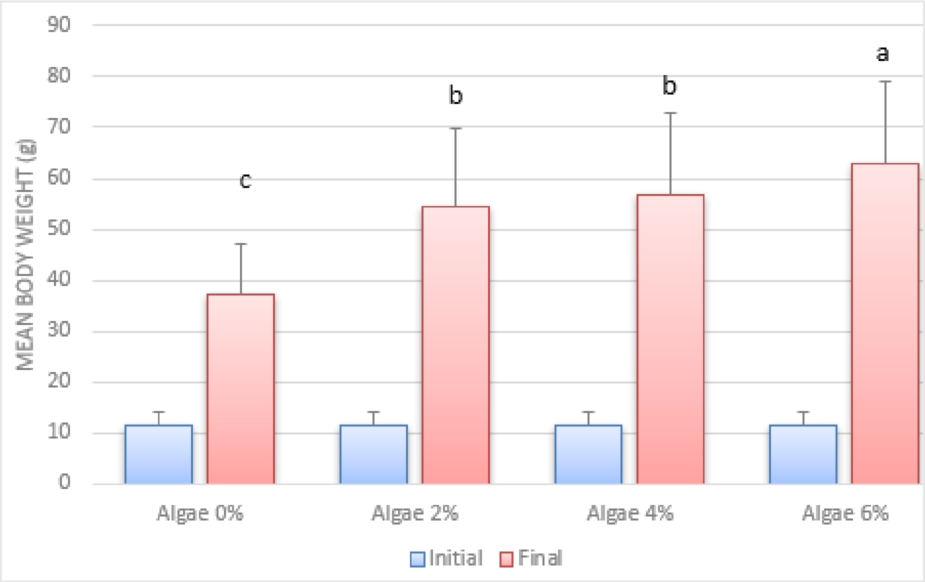

Figure 3.1.1 – Mean body weight (g) during trial period. Data shown is divided in initial and final weightings. Means and standard deviation (N ≈120 for final weighting and N=138 for initial weighting) are represented and within the same sampling day, data represented with different superscript letters are significantly different (P<0.05).

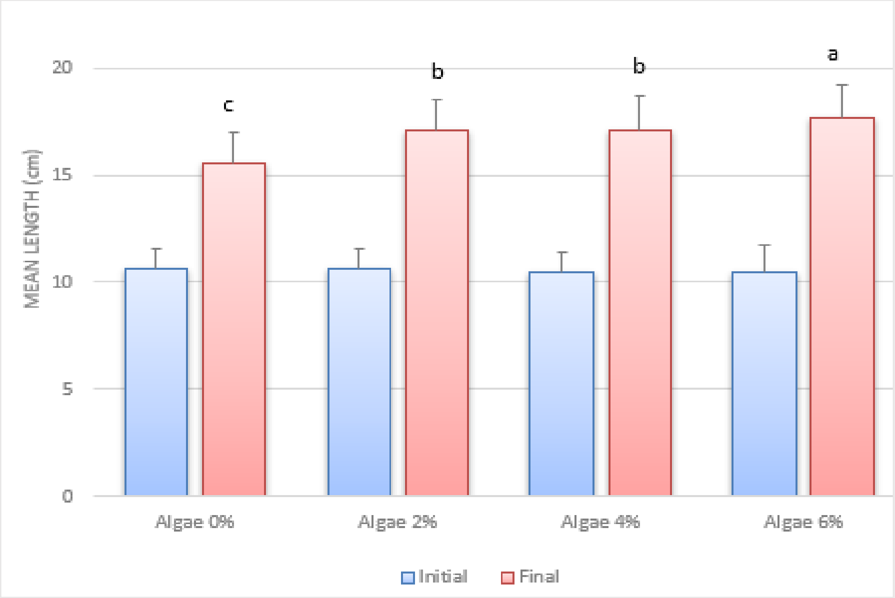

Figure 3.1.2 – Mean length (cm) during trial period. Data shown is divided in initial and final measurements. Means and standard deviation (N≈120 for final measurement sampling and N=138 for initial measurement) are represented and within the same sampling day, data represented with different superscript letters are significantly different (P<0.05).

In Figure 3.1.3 the differences registered in the final condition factor (K) are displayed. Final condition factor in fish fed with Algae 6% was significantly higher (*P*<0.001) than fish fed with Algae 2% and Algae 0%, but no significant differences were found when compared with fish fed with Algae 4%. In addition, it was possible to verify that K of fish fed with Algae 0% was significantly lower than all others.

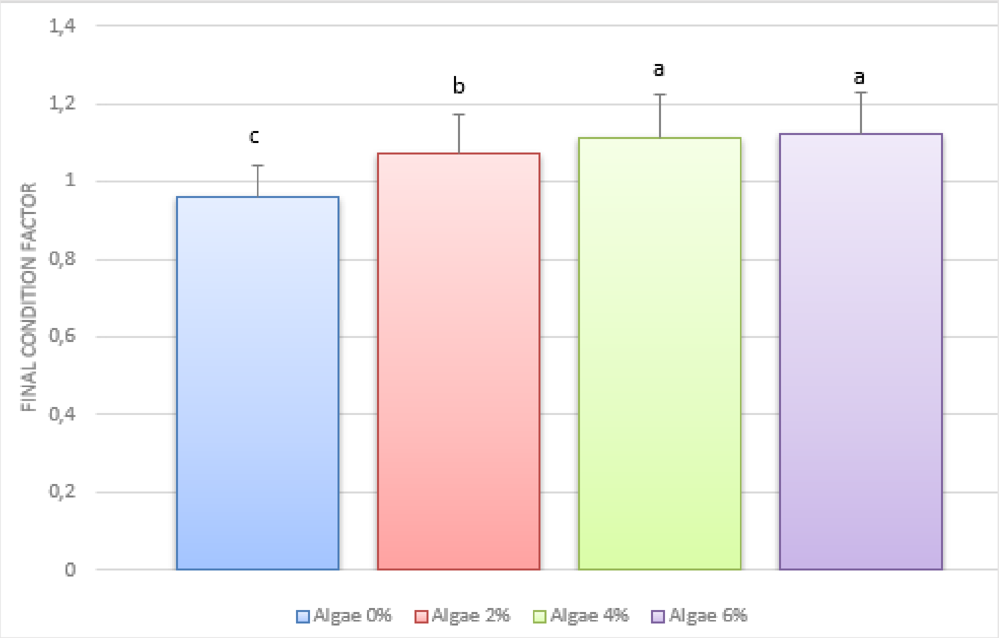

Figure 3.1.3 – Final condition factor (k) registered at the end of the trial. Means and standard deviation (N≈120) are represented and data represented with different superscript letters are significantly different (P<0.05).

Voluntary feed intake (VFI) of fish (Figure 3.1.4) was of 1.49 ± 0.03 g for fish fed with Algae 0% diet, 1.67 ± 0.07 g for fish fed with Algae 2% diet, 1.68 ± 0.07 g for fish fed with Algae 4% diet and 1.80 ± 0.05 g for fish fed with Algae 6% diet. There were no significant differences between fish fed Algae 2, 4 and 6%, but those fed with Algae 0% showed a significantly lower (*P*=0.001) voluntary feed intake when compared with the remaining diets.

Regarding nutrient (Table 3.1.1), all parameters were significantly affected (*P*=0.001) by algae presence (Table 3.4.2). Protein intake presented a significantly lower value in fish fed the control diet (Algae 0%) and a higher value in fish fed with Algae 6%, with no significant differences between algae-containing diets.

Lipid intake increased concomitantly with increasing levels of algae. The highest intake was registered in Algae 6% fed fish (2.77± 0.070 g/Kg ABW/day) and the lowest lipid intake was registered with Algae 0% (2.30 ± 0.041 g/Kg ABW/day). There were no significant differences between fish fed Algae 2% and 4% and those fed Algae 4% had similar lipid intake as those fed Algae 6%. Finally, for energy intake the highest value was again recorded when fish were fed Algae 6% diets and the lowest for fish fed with control diet. There were no significant differences between fish fed Algae 2% with fish fed Algae 4% and both ingested more lipids than those fed the control diet.

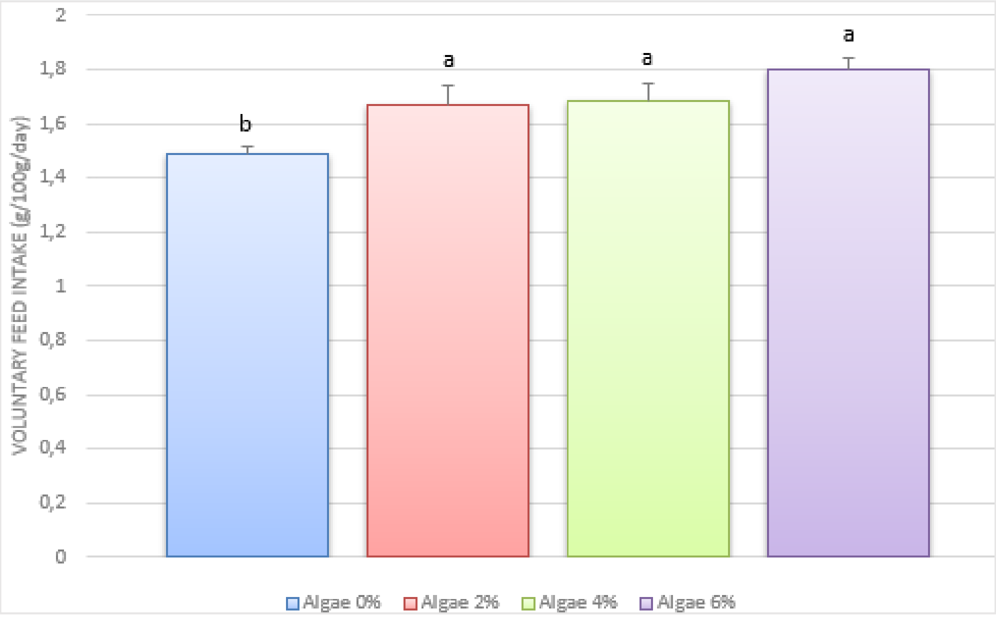

Figure 3.1.4 – Voluntary feed intake registered during the experimental period. Means and standard deviation (N=3) are represented and data represented with different superscript letters are significantly different (P<0.05).

Table 3.1.1 – Nutrient intake of experimental fish. Means and standard deviation (N=3) are represented and data represented with different superscript letters are significantly different (P<0.05).

Intake	Algae 0%	Algae 2%	Algae 4%	Algae 6%
Protein (g/kg ABW/day)	7.8 ± 0.1^{b}	8.8 ± 0.4^{a}	8.8 ± 0.4^{a}	9.5 ± 0.2^{a}
Lipids (g/kg ABW/day)	2.3 ± 0.0^{c}	2.5 ± 0.1^{bc}	2.6 ± 0.1^{ab}	2.8 ± 0.1^{a}
Energy (kJ/kg ABW/day)	324 ± 5.7^{c}	359 ± 15.5^{b}	363 ± 14.6^{ab}	392 ± 9.9^{a}

Specific growth rate (SGR) registered between experimental diets is exhibited in Figure 3.1.5. The presence of the algae blend caused a significantly increase ($P<0.001$) of specific growth rate in all fish fed the algae blend, with fish fed with Algae 6% being the ones with the highest SGR (1.77 ± 0.04). It was noticeable that fish fed with Algae 0% had a specific growth rate significantly lower ($P<0.001$) than fish fed with all the diets containing the blend and there were no significant differences between fish fed algae diets.

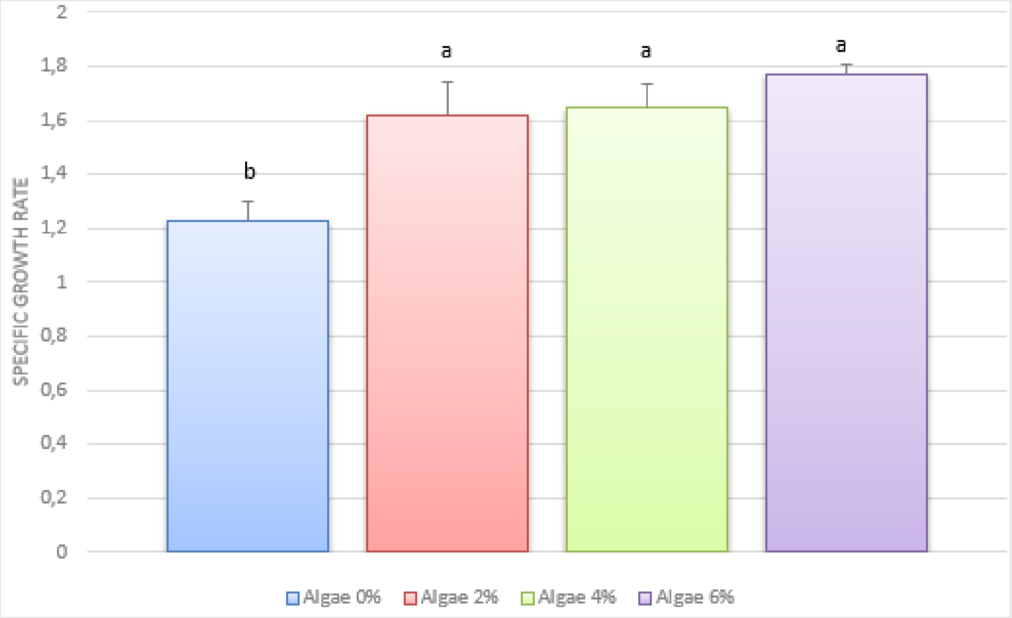

Figure 3.1.5 – Specific growth rate registered during the experimental period. Means and standard deviation (N=3) are represented and data represented with different superscript letters are significantly different (P<0.05).

Figure 3.1.6 shows that Algae 2 and 4% caused a significant effect (P=0.021) in feed conversion ratio (FCR). The highest value was registered with Algae 0% (1.35 ± 0.06) diet and the lowest with Algae 4% (1.22 ± 0.011). For this reason, best feed conversion ratio (meaning that fish need to be fed a higher amount of feed in order to have the same weight gain as other diets with lower FCR) was presented by fish fed Algae 4%. The diet 6% inclusion caused no significant differences when comparing with all the other diets and no significant changes were observed when comparing fish fed Algae 2% and fish fed with Algae 4%.

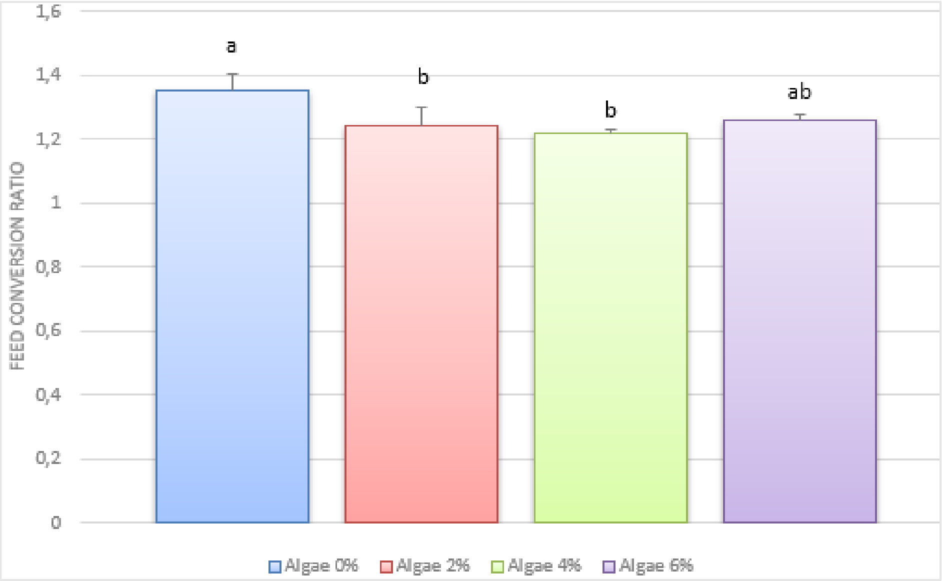

Figure 3.1.6 – Feed conversion ratio registered during the experimental period. Means and standard deviation (N=3) are represented and data represented with different superscript letters are significantly different (P<0.05).

Protein efficiency ratio (PER) of fish (Figure 3.1.7) was significantly (*P*=0.024) affected by the presence of the algae blend. Fish fed with Algae 4% showed a significantly higher (*P*=0.024) protein efficiency ratio than those fed with Algae 0%. There were no significant differences between fish fed with Algae 2, 4 and 6%.

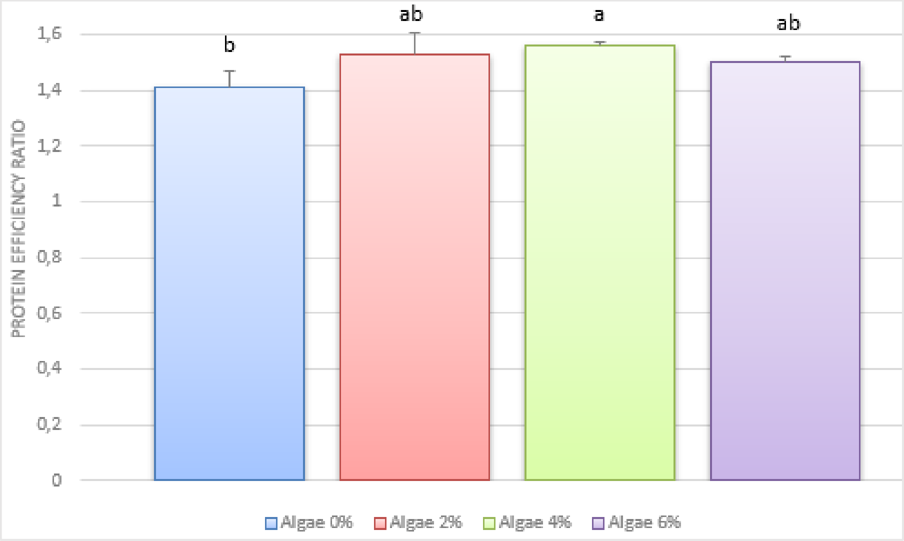

Figure 3.1.7 – Protein efficiency ratio during trial period. Means and standard deviation (N=3) are represented and data represented with different superscript letters are significantly different (P<0.05).

3.2 – Somatic indices

Both hepatosomatic (HSI) and viscerosomatic (VSI) indices, exhibited in Figures 3.2.1 and 3.2.2, respectively, showed a significantly increase (*P*<0.001) with the algae blend inclusion. Hepatosomatic index ranged from 0.95 ± 0.17 (Algae 0%) to 1.42 ± 0.23 (Algae 6%) presenting no statistical differences between fish fed with the algae blend; the viscerosomatic index also showed the same tendency with an increase with the dietary inclusion of algae, ranging from 6.14 ± 1.215 (Algae 0%) to 8.38 ± 1.379 (Algae 6%).

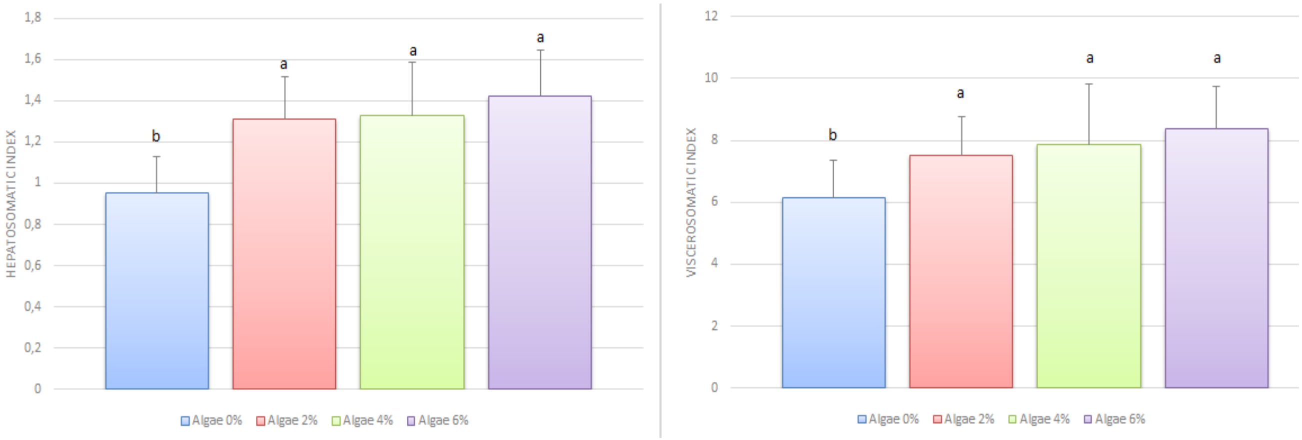

Figure 3.2.1 and 3.2.2 – Hepatosomatic and viscerosomatic index, respectively, during trial period. Means and standard deviation (N=36) are represented and data represented with different superscript letters are significantly different (P<0.05).

3.3 – Whole body composition

Proximal composition of the experimental fish is represented in Table 3.3.1. There was a decrease in humidity and ash content in all fish at the end of the experimental trial. Protein, lipids, and energy increased. Regarding humidity, fish fed with the control diet showed significantly higher value (*P*<0.001) than all the other diets, followed by Algae 2% and Algae 6%. Fish fed with Algae 4% showed no significant differences when compared with Algae 2 and 6%.

Ash content, at the end of the experiment, revealed no significant differences (*P*=0.126) between diets and whole-body protein there were no significant differences verified (*P*=0.128). A significant increase of lipids with the increase of algae inclusion occurred in comparison with the control diet. Fish fed Algae 6% with Algae 4% did not differ significantly. Whole body energy increased concomitantly with increasing algae inclusion that differed significantly from fish fed the control diet. Fish fed Algae 0% had a significantly lower (*P*<0.001) energy content when compared to those fed with algal diets.

Table 3.3.1 - Whole body composition (% wet weight basis) of experimental fish. Means and standard deviation (N=3) are represented and data represented with different superscript letters are significantly different (P<0.05).

Whole body composition (%WW)[1]	**Initial**	**Algae 0%**	**Algae 2%**	**Algae 4%**	**Algae 6%**
Humidity	70.5	69.0 ± 0.1^{a}	64.0 ± 0,7^{b}	62.8 ± 0,6^{bc}	62.3 ± 0,5^{c}
Ash	4.9	4.5 ± 0.7	4.1 ± 0.1	4.2 ± 0.2	3.7 ± 0.1
Protein	16.6	18.2 ± 0.1	17.9 ± 0.3	18.5 ± 0.4	18.3 ± 0.1
Lipids	7.5	8.8 ± 0,6^{c}	13.8 ± 0.3^{b}	15.0 ± 0.7^{ab}	15.8 ± 0.5^{a}
Energy (kJ/g)	6.8	7.3 ± 0.3^{b}	8.9 ± 0.2^{a}	9.2 ± 0.3^{a}	9.3 ± 0.1^{a}

1 – WW - wet weight;

3.4 - Nutrient gain

By examining nutrient and energy gain at the end of the experimental trial (Figure 3.4.1 and 3.4.2) it was possible to see that all three parameters were significantly affected by the presence of the algae blend ($P \leq 0.002$). Dry matter, protein and energy gain were significantly higher in fish fed with Algae than in those fed the control diet. However, there were no significant differences between fish fed with feed containing the algae. Lipid gain was also significantly improved in fish fed algae-rich diets, particularly on those fed with 4 and 6% algae.

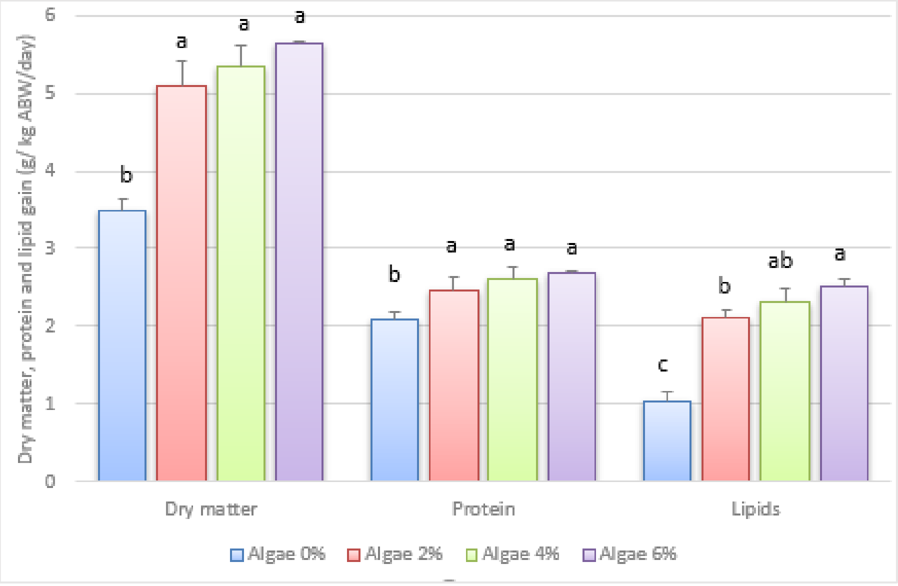

Figure 3.4.1 -Dry matter, protein, and lipid gain during the experimental trial. Means and standard deviation (N=3) are represented and data represented with different superscript letters are significantly different (P<0.05).

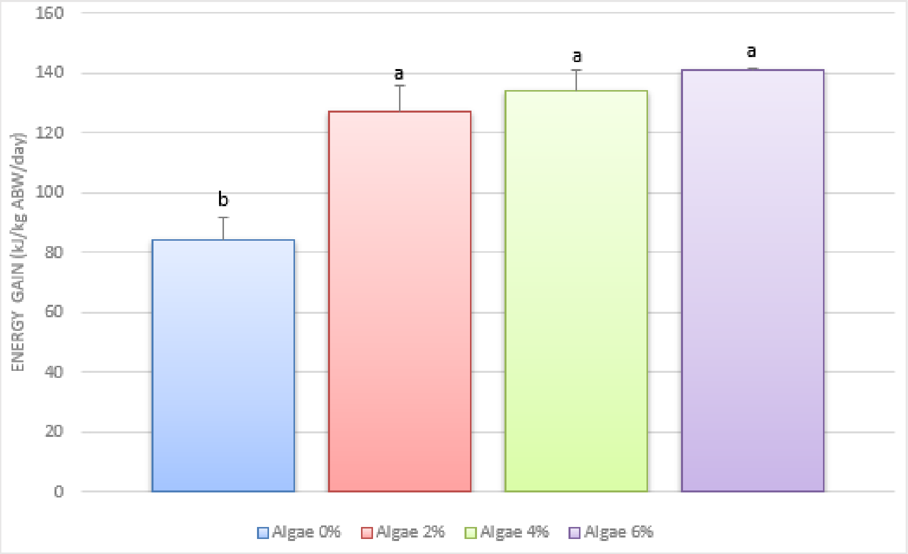

Figure 3.4.2 - Energy gain during the experimental trial. Means and standard deviation (N=3) are represented and data represented with different superscript letters are significantly different (P<0.05).

3.5 - Retention/consumption

Dry matter, protein, lipids, and energy retention according to the different treatments is depicted in Figure 3.5.1. The increase level of algae inclusion caused a significant effect (*P*<0.001) on dry matter, lipids, and energy retention, however, protein retention was not significantly affected (*P*=0.099). When looking to dry matter, lipids, and energy retention values it was possible to notice that there were no significant differences between the three experimental diets containing the algae blend. When it comes to protein retention the lowest value recorded was in fish fed with Algae 0% (26.6 ± 1.2%) and the highest in fish fed Algae 4% (29.5 ± 0.8%). So, it is possible to tell that with the exception of protein retention, all other parameters showed significantly higher retention for fish fed the algal blend with no significant differences between Algae 2, 4 and 6%.

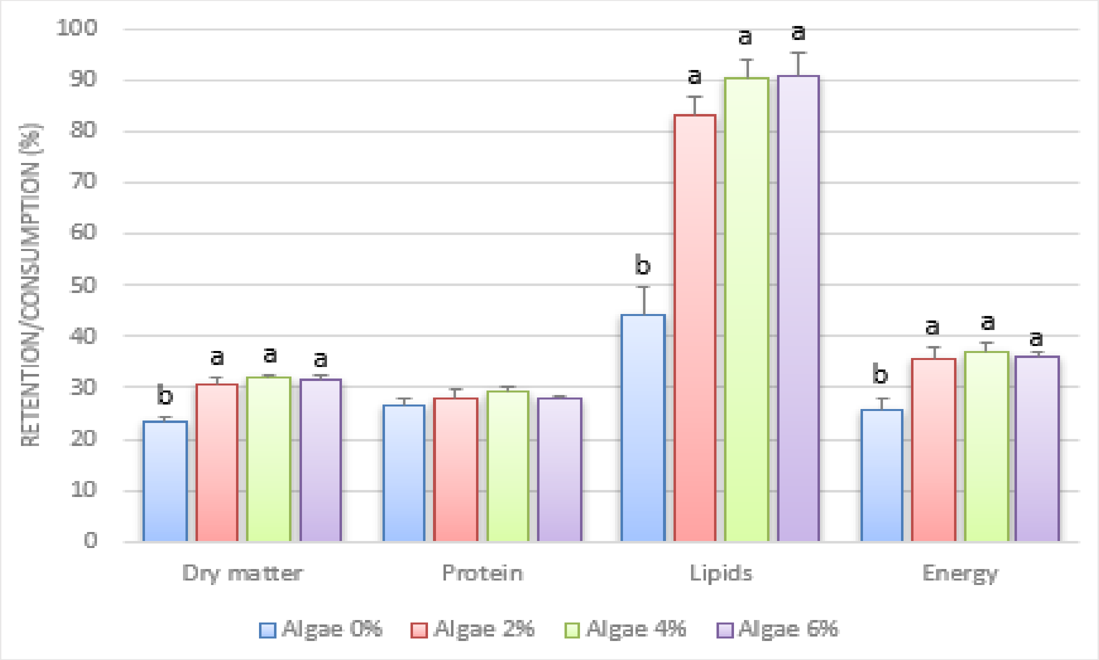

Figure 3.5.1 – Retention of dry matter, protein, lipids, and energy on fish fed with experimental diets. Means and standard deviation (N=3) are represented and data represented with different superscript letters are significantly different (P<0.05).

4 – Discussion

Several studies have been made reporting that algae inclusion in fish feeds, until certain amounts (<10% of the diet), leads to positive effects like growth performance improvement, increase of carcass quality, intestinal micro biota or even increase of feed utilization efficiency (Batista *et al.*, 2020; Valente *et al.*, 2006). On the other hand, high inclusion rates can result in a negative effect. Previous studies showed that an inclusion rate of *Ulva* sp. higher than 10% (until 20% of the diet) caused a negative effect on fish growth and feed efficiency (Norambuena *et al.*, 2015) in fish species such as rainbow trout, gilthead sea bream and Nile tilapia (Azaza *et al.*, 2008; Wassef *et al.*, 2005a) which can be justified by the presence of certain ANFs (Oliveira *et al.*, 2009). However, considering literature already available it is believed that the results from algae inclusion in fish feeds vary with inclusion rate and utilized species (Valente *et al.*, 2006).

According to the literature, it is usually found only microalgae (Ali, *et al.*, 2019; Atalah *et al.*, 2007; Hasanein *et al.*, 2018; Raji *et al.*, 2018) or macroalgae species (Dernekbaşı, 2009; Moutinho *et al.*, 2018; Mustafa *et al.*, 1995; Valente *et al.*, 2006). However, to the best of my knowledge, only a very recent study from our group has evaluated a blend of *Gracilaria gracilis* and *Nanochloropsis oceanica* in diets for European seabass (Batista *et al.*, 2020).

In the present study body weight and length increased in fish fed algal diets which supports previous studies showing that algae inclusion in aquafeeds can promote fish growth. For example, when *Gracilaria* inclusion rates of 3, 6, 9 and 12% were tested in rainbow trout juveniles, results showed that fish growth increased with increasing inclusion rate (Sotoudeh and Jafari, 2017). Also, it was previously documented by Gouveia *et al.* (2002) that the utilization of *Chlorella vulgaris* as a carotenoid source enhanced growth and life expectancy in gilthead seabream. However, Valente *et al.* (2006) and Cardinatelli *et al.* (2018) could not observe significant effects on growth of seabass fed diets with 5% and 10% of *Gracilaria bursa-pastoris*, *Ulva rigida* and *Gracilaria cornea* and graded levels of a blend of *Tisochrysis lutea* and *Tetraselmis suecica* supplementation. Younis *et al.* (2018) reported that high inclusion rates (20, 40 and 60%) of *Gracilaria arcuata* in Nile tilapia feeds had a negative impact in fish final weight and in almost all growth performance parameters. However, when 50 and 75% of *Spirulina platensis* or *Chlorella vulgaris* were used in African catfish diets fish growth was improved (Raji *et al.*, 2018). It seems that the effects of algae inclusion in fish growth performance differ according to the fish species, the algae species and also the inclusion level. So, the results obtained in the present study showed that with low inclusion levels, such as the ones here utilized, growth performance can in fact be positively affected. Moreover, Valente *et al.* (2019) tested an inclusion level up to 15% of *Nannochloropsis* sp. defatted microalgae meal in diets for European seabass without affecting growth performance. Wahbeh (1997) suggested that a mixture of different algae species could originate an adequate supply of certain compounds (e.g. all essential amino acids) leading to a growth performance increase. But in a very recent study, a blend of 4% of *N. oceanica* and 4% of *G. gracilis* was tested in diets for seabass without resulting in any significant changes in growth when compared with the control diet or with a 8% inclusion of each single alga (Batista *et al.*, 2020). However, the present results show that a more complex blend could significantly increase seabass growth.

Final condition factor gives information about the relation between length and weight of fish and provides an idea about fish robustness, fatness and its general welfare condition (Abdel-

Hakim *et al.*, 2010). The increase of K here observed was also registered by Batista *et al.* (2020) when including *G. gracilis and N.s oceanica* up to 8% in seabass diets. Mustafa *et al.* (1995) and Bai *et al.* (2001) showed that the utilization of 5% *Ulva* meal in diets for Red sea bream, and low inclusion levels (0,0.5, 1.0, 1.5, 2.0 and 4%) of *Chlorella* in Korean rockfish, caused non-significant changes in final condition factor. Contradictory results were obtained by Sorensen *et al.* (2017) who observed a decrease in Atlantic salmon condition factor with increasing levels of *Nannochloropsis*. The discrepancy in the results obtained in the present study, and the ones found in literature may be a result of the different fish species used, or even be caused by the interaction between different algae species used in each experiment.

In this study, European seabass increased voluntary feed intake (VFI) with the algae inclusion rate indicating that the algae blend was very well accepted by fish. Likewise, Hashim and Mat Saat (1992) observed that snakehead fish fed with a diet with 5% inclusion of *Ulva* sp. increased feed consumption, suggesting that this alga could have an attractant effect for snakehead. The same can perhaps be true for European seabass. However, the dietary inclusion of seaweeds, namely *Ulva* sp. and *Gracilaria* sp. (2.5 and 7.5% inclusion) in European seabass showed no significant differences in voluntary feed intake (Peixoto *et al.*, 2016). The presence of *Nannochloropsis* was shown to improve VFI in several fish species suggesting a good palatability of this alga (Sorensen *et al.*, 2017). Nevertheless, it was recently observed that the dietary inclusion of 8% *N. oceanica* or a blend of 4% *N. oceanica* and 4% *G. gracilis* in seabass could not affect VFI (Batista *et al.*, 2020). Likewise, a recent work with Nile tilapia fed a diet supplemented with *N. salina* showed no significant differences in the amount of feed consumed when compared with non-supplemented feeds (Gbadamosi and Lupatsch, 2018). Furthermore, the inclusion of *Chlorella* at a 5% inclusion level enhanced voluntary feed intake of koi fish (Khani *et al.*, 2017). It remains to be ascertained what algae species contributed mostly for the presently observed increased feed intake, or it was a synergetic effect of the tested algae blend. Above all, it was clearly demonstrated an increased appetence for the algae-rich diets.

The increased VFI also resulted in a significant increase in nutrient and energy intake. Different results were found in literature that reported non-significant changes in European seabass fed defatted *Nannochloropsis* (Valente *et al.*, 2019), or even a significantly decrease in protein intake when *Ulva lactuca* was tested in rainbow trout (Dernekbaşı, 2009) or non-significative changes when was tested in European seabass. Likewise, in rainbow trout, low levels of *Gracilaria* inclusion (<10%) resulted in no significant differences in protein intake, but increased lipid and energy intake when compared to the control diet (Araújo *et al.*, 2016). This corresponds with the present findings that evidence increased lipid and energy intake with increasing of algal blend.

European seabass fed the algal biomass up to 4% resulted in improved feed utilisation (lower FCR). Peixoto *et al.* (2016) concluded that feed supplementation with *Gracilaria* sp. and *Ulva* sp. at low levels (up to 7.5%) for European seabass showed no effects on FCR when compared with the non-supplemented feed. Considering that there are no studies with an algae blend like this one, it can be assumed that results obtained may be due to the different inclusion rates or the presence of *Chlorella* sp. and *Nannochloropsis* sp. The inclusion of *Chlorella vulgaris* together with *Spirulina platensis* was already tested in seabass feed utilization resulting in no significant differences between fish fed diets supplemented with algae and the control (Hasanein *et al.*, 2018). But when *Chlorella vulgaris* was utilized alone in African catfish diets (inclusion levels of 0, 5, 15 and 25%) the lowest FCR was observed with the highest inclusion rate (Enyidi, 2017), suggesting that *Chlorella* can be capable of improving feed conversion ratio in those fish. Moreover, a trial made with 3, 6, 9 and 12% of a *Gracilaria* sp. demonstrated that FCR of rainbow trout decreased until reaching 6% inclusion of algal biomass (Sotoudeh and Jafari, 2017). This suggests that there are different results according to fish species utilized, the algae tested and their inclusion level. On the other hand, the nutritional value of the algae utilized in the various studies may also not have been the same which may explain this big difference between results.

Protein efficiency ratio (PER) of fish was significantly affected by the presence of the algae blend, being highest in fish fed 4% algae. These results support previous studies that reported that the presence *Nannochloropsis* (Qiao *et al.*, 2019) and *Ulva* (Ergün *et al.*, 2008) The inclusion of *Chlorella* vulgaris also improved PER in African catfish (Enyidi, 2017), however, in European seabass, the inclusion of low levels (2.5 and 7.5%) of *Gracilaria* sp. and *Ulva* sp. feeds did not affect protein efficiency ratio (Peixoto *et al.*, 2016). So, the presence of *Chlorella*, *Gracilaria*, *Nannochloropsis* and *Ulva* in fish diets may be what caused the significantly higher PER observed in fish fed with algal diets.

SGR significantly increased with the algae blend containing diets, supporting previous studies showing that a 3, 5 and 7% inclusion of *Nannochloropsis* sp. in Nile tilapia feeds, caused a significantly better ($P<0.005$) SGR for fish fed with 7% supplemented feeds (Ali *et al.*, 2019). Likewise, Qiao *et al.* (2019) reported increased SGR in juvenile turbot fed diets containing 2.5, 5, 7.5 and 10% of *Nannochlorospsis* inclusion. The dietary inclusion of 5% *Ulva* also enhanced specific growth rate in Nile tilapia juveniles (Ergün *et al.*, 2008). According to the above cited authors, the positive effect registered on specific growth rate can be justified by the presence of *Ulva* sp. and *Nannochloropsis* sp. in the utilized diets. In fact, that fish fed with algal diets presented a higher voluntary feed intake and also a better feed utilization, particularly in the case of Algae 2 and 4% that presented the best FCR results.

The increase of hepatosomatic index with increasing algae inclusion suggests an increased lipid deposition in liver. These results were not expected since the presence of algae in the fish feeds normally causes a decrease or a non-significant change of this indices (Badwy *et al.*, 2008; Tulli *et al.*, 2012; Younis *et al.*, 2018). Valente *et al.* (2016) concluded that hepatosomatic index of rainbow trout fed with *Gracilaria vermiculophylla* up to 10% varied from 0.89 to 1.01 among diets. The same was registered later by Qiao *et al.* (2019) when testing four different *Nannochloropsis* inclusion levels (2.5, 5, 7.5 and 10%) in turbot juveniles feeds. Enyidi (2017) showed that when *Chlorella vulgaris* was utilized in diets for African catfish, HSI tended to decrease as the inclusion rate increased. In addition, the utilization of *Ulva* meal in rainbow trout feeds caused a non-significant effect on viscerosomatic and hepatosomatic indices when low inclusion levels (5% and 10%) were utilized (Güroy *et al.*, 2012). The same was recorded earlier when *Chlorella* powder was included in juvenile Korean rockfish feeds at different inclusion levels (0, 0.5, 1, 1.5, 2 and 4%) (Bai *et al.*, 2001). In conclusion the increased HSI presently observed suggests metabolic adaptations to increased inclusion levels of the algal blend which resulted in increased whole-body lipid content in those fish. In fact, a significant increase of lipids with the increase of algae inclusion occurred in comparison with the control diet. Ali *et al.* (2019) also observed that a 7% *Nannochloropsis* inclusion level resulted in the highest lipid content in Nile tilapia. Likewise, increased whole-body lipid content was observed with the dietary inclusion of Ulva meal up to 5% in *Sparus aurata* diets (Wassef *et al.*, 2005b). However, most studies found in literature reported a decrease in whole body lipid content when utilising algae containing diets

(Araújo *et al.*, 2015; Güroy *et al.*, 2007; Sorensen *et al.*, 2017; Valente *et al.*, 2006; Zhu *et al.*, 2015)

Contrarily to whole body lipids, body humidity decreased when algae inclusion increased. This was also reported by Badwy *et al.* (2008) when utilizing *Chlorella* sp. in Nile tilapia feeds, and the same was reported later when the same fish species was fed with *Nannochloropsis salina* (Gbadamosi and Lupatsch, 2018). Ash content presented no significant differences when most results found in previous fish trials testing algae supplemented feeds shows a general increase (Ergün *et al.*, 2008; Gbadamosi and Lupatsch, 2018; Sorensen *et al.*, 2017). Ash content in fish is related to the minerals (e.g. calcium, magnesium, zinc, phosphorous) present in solid body parts (Chaimongkol and Boonyaratpalin, 2001). So, the absence of variations in body ash content in this study, suggests that even though dietary ash content increase with the algae inclusion degree, there were no differences in mineral gain or retention.

There were no significant differences in whole-body protein confirming previous studies utilizing *Nannochloropsis* (Sorensen *et al.*, 2017), *Gracilaria* (Araújo *et al.*, 2016; Morshedi *et al.*, 2017) and *Ulva* (Wassef *et al.*, 2005b) in different fish species. Energy suffered an increase with increasing algae inclusion and fish fed Algae 6% presented the highest energy content (9.32 kJ/g) but no significant differences were recorded between algal treatments. Likewise, Valente *et al.* (2019) showed no significant differences in seabass whole body energy when utilizing increasing levels of *Nannochloropsis* (5, 10 and 15%).

Nutrient and energy retention and gain were significantly affected by the dietary inclusion of algae, whilst protein retention remained unaffected. It was verified that the presence of the algae blend stimulated fish's appetite leading to an increase of feed consumption and better growth. Which means that there was also a greater lipid and protein consumption that resulted in increased nutrient gain and retention. As a result, it was verified a higher whole-body and liver fat deposition, implying metabolic changes caused by the dietary inclusion of algae. Wassef *et al.* (2013) observed that a 5% of *Ulva* meal inclusion improved European seabass nutrient retention. Likewise, 10% of *Ulva* inclusion in Senegalese sole diets increased dry matter, protein and energy gain (Moutinho *et al.*, 2018). Also, an experimental trial conducted with rainbow trout showed that protein and lipid retention increased with increasing levels of *Gracilaria* inclusion up to 9% (Sotoudeh and Jafari, 2017). In fact, it was hypothesized that seaweed supplementation could improve lipid accumulation and mobilization (Nakagawa, 1997) and dietary protein assimilation (Yone *et al.*, 1986). Nonetheless, Valente *et al.* (2019) observed no significant differences regarding protein, lipids and energy gain in European seabass when feeding fish with defatted *Nannochloropsis* sp. as protein source. Also, when utilizing *Nannochloropsis* as a fishmeal alternative in Atlantic salmon diets (Sorensen *et al.*, 2017) observed no significant differences ($P>0.05$) regarding protein retention and a significant decrease in lipids and energy retention up to 10% inclusion.

In this study, lipid intake increased with increasing dietary intake, which lead to greater whole -body fat deposition and increased HSI. Adequate lipid supply results in improved growth rates, FCR and nutrient utilization (Yiğit *et al.*, 2002), although excessive lipid can negatively affect feed intake and utilization of other nutrients (Hemre and Sandnes, 2001), however this was not observed in this study. Furthermore, excessive dietary lipid intake can increase HSI, VSI and whole-body lipid content caused by an increase in lipid deposition (Ergün *et al.*, 2008). This can negatively affect the fish product quality by enabling lipidic peroxidation and affecting fillet organoleptic features like texture, flavour, and colour (Larsen *et al.*, 2011).

5 – Conclusions

The objective of this 12 weeks’ growth trial was to understand how growth performance, feed utilization and body composition of European seabass were affected by the different algae containing diets. Three inclusion rates were tested (0%, 2%, 4% and 6%) in triplicates (12 tanks with 46 fish each). Water parameters (temperature, pH, and salinity) were kept under control in order to maintain species requirements.

In the end of the feeding trial, it was possible to observe that nutrient intake and growth performance were positively affected by algae inclusion and fish fed with 6% algae presented significantly higher values. However, the highest FCR and PER were recorded for the control diet and the lowest value in fish fed Algae 4%

Algae inclusion improved lipid and energy retention and gain that was reflected in increased lipid and energy whole body content, that was particularly high in fish fed with Algae 6%. The same trend was observed in HSI and VSI whilst ash and protein whole body content remained unchanged.

In conclusion, the algae blend proved to positively affect European seabass growth potential, and when included up to 4% decreased the FCR. Moreover, the increased lipid content with increasing levels of the algae blend may affect fish organoleptic features and requires further evaluation.

6 – References

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