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2020

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CTAQUA internship under the projects INTEGRATE, BIOSEA &SIMBA

Pedro Diogo Faria Nascimento

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Relatório de Estágio de Mestrado apresentado à Faculdade de Ciências da Universidade do Porto Mestrado em Recursos Biológicos Aquáticos

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Mestrado em Recursos Biológicos Aquáticos

Departamento de Biologia

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Resumo

Este trabalho foi desenvolvido com base no estágio realizado nas instalações do Centro Tecnológico de Aquacultura da Andaluzia (CTAQUA), no âmbito dos projetos INTEGRATE, BIOSEA e SIMBA. Neste estágio tive a possibilidade de trabalhar em várias vertentes, com diferentes espécies. Numa fase inicial, prestei auxílio no processo de monitorização de um sistema IMTA em que foram preparadas, regularmente, amostras dos diferentes cultivos (algas, ostras e peixes) e dos componentes abióticos do sistema (água e sedimentos) para posterior análise. Os índices de condição das ostras foram calculados e a temperatura/estação do ano foi o fator que parece ter determinado a variação ocorrida.

Mais tarde, fiquei responsável pelo acompanhamento do cultivo de macroalgas (sobretudo *Ulva ohnoi*) em diferentes sistemas (culturas stock; tanques exteriores; fotobiorreatores), tendo-se verificado que a densidade inicial tem um papel preponderante nos registos da taxa de crescimento específico (SGR). Além disso, no sistema composto pelos fotobiorreatores, foi desenvolvido um teste com *U. ohnoi* que procurou avaliar a influência da temperatura sobre fatores como o SGR e o conteúdo de cinzas. Neste sentido, relativamente ao SGR, foi constatada a ausência de significância entre os tratamentos (com aquecimento vs. sem aquecimento) na primeira e segunda semana. No entanto, na terceira semana da experiência registaram-se diferenças estatisticamente significativas. Por outro lado, os valores médios do conteúdo de cinzas foram apenas medidos para a terceira semana, não se tendo observado diferenças significativas entre os tratamentos.

A análise dos dados (disponibilizados pela VITO) relativos a três experiências independentes (ensaio com diferentes salinidades; ensaio com diferentes fontes de azoto; ensaio com diferentes concentrações de fosfato) foi efetuada com o intuito de avaliar o efeito dos tratamentos sobre os níveis de cinza e macronutrientes (lípidos, proteínas e açúcares) da *U. ohnoi*. Para o ensaio de salinidades, os resultados obtidos mostraram a presença de diferenças significativas em todos as variáveis dependentes, exceto no conteúdo de cinzas. No caso do ensaio com diferentes fontes de azoto, todas as variáveis apresentaram diferenças significativas. Relativamente ao ensaio com diferentes concentrações de fosfato, apenas foram detetados valores estatisticamente significativos nas variáveis conteúdo de lípidos e conteúdo de proteína, enquanto que as cinzas e açúcares não registaram diferenças significativas.

No que diz respeito aos ensaios com o robalo europeu (*Dicentrarchus labrax*), numa primeira fase realizou-se um teste nutricional em que se procurou avaliar 4 dietas de composição distinta (dieta controlo; dieta A com extratos de Spirulina e *Ulva*; dieta B com extratos de Spirulina e *Saccharina*; dieta C com extratos de Spirulina, *Ulva* e *Saccharina*). Esta avaliação teve por base a determinação de parâmetros como a taxa de crescimento específico (SGR), a taxa de conversão alimentar (FCR), a sobrevivência (SV), o fator de condição de Fulton (K), o índice hepatossomático (HSI) e o índice viscerosomático (VSI). Os resultados obtidos mostraram um valor de SGR ligeiramente maior e significativo para a dieta controlo, um FCR menor para o controlo e dieta B e um K menor para a dieta C. Os restantes parâmetros não apresentaram diferenças significativas entre as dietas. No término da experiência realizaram-se ensaios de imunidade e procedeu-se à análise histológica do intestino. Em comparação com a dieta controlo, as dietas A e B registaram valores significativamente superiores para todos os parâmetros de imunidade avaliados (lisozima, superóxido dismutase e atividade da antiprotease). O score intestinal foi estabelecido para identificar o estado do intestino associado a cada dieta, não tendo havido diferenças estatisticamente significativas. Finalmente, alguns dos peixes alimentados com estas dietas foram submetidos a um teste de stress. As transaminases (AST-GOT e ALT-GPT), a glucose, os triglicéridos e o lactato desidrogenase (LDH) foram os indicadores de stress utilizados. Os valores de glucose registados (1h pós-stress) foram os mais elevados para as dietas A e B.

Palavras-chave: Alface do mar; Cinzas; Dietas; Ensaios de imunidade; Histologia do intestino; IMTA; Macronutrientes; Microbioma; RAS; Robalo; SGR; Stress.

Abstract

This work was developed based on the internship carried out in the facilities of the Andalusian Aquaculture Technology Center (CTAQUA), in the scope of the projects INTEGRATE, BIOSEA and SIMBA. In this internship I had the possibility to work in several areas, with different species. In an initial stage, I assisted in the process of monitoring an IMTA system in which samples of the different cultivated organisms (algae, oysters and fish) and the abiotic components of the system (water and sediments) were regularly prepared for later analysis. The condition indices of oysters were calculated and temperature/season was the factor that seems to have determined the variation that occurred.

Later, I was responsible for monitoring the cultivation of macroalgae (mainly *Ulva ohnoi*) in different systems (stock cultures; outdoor tanks; photobioreactors), and it was found that stock density plays a predominant role in specific growth rate (SGR) records. Furthermore, in the photobioreactors, an experiment with *U. ohnoi* was developed in order to assess the influence of temperature on factors such as SGR and ash content. Regarding the SGR, was found an absence of significance between treatments (with heating vs. without heating) in the first and second weeks. However, in the third week of the experiment there were statistically significant differences. On the other hand, mean values of ash content were only measured for the third week and no significant differences between treatments were observed.

The analysis of the data (made available by VITO) for three independent experiments (test with different salinities; test with different nitrogen sources; test with different phosphate concentrations) was carried out in order to evaluate the effect of the treatments on the levels of ash and macronutrients (lipids, proteins and sugars) in *U. ohnoi*. For the salinity test, the results obtained showed significant differences in all dependent variables except ash content. In the case of the test with different nitrogen sources, all variables showed significant differences. For the test with different phosphate concentrations, only statistically significant values were detected for the variables lipid content and protein content, whereas ash and sugar showed no significant differences.

With respect to European seabass (*Dicentrarchus labrax*) trials, a nutritional test was carried out in a first stage in which 4 diets of different composition were evaluated (control diet; diet A with Spirulina and *Ulva* extracts; diet B with Spirulina and *Saccharina* extracts; diet C with Spirulina, *Ulva* and *Saccharina* extracts). This evaluation was based on the

determination of parameters such as specific growth rate (SGR), feed conversion rate (FCR), survival (SV), Fulton's condition factor (K), hepatosomatic index (HSI) and viscerosomatic index (VSI). The results obtained showed a slightly higher and significant SGR value for the control diet, a lower FCR value for the control and diet B and a lower K value for diet C. The remaining parameters showed no significant differences between diets. At the end of the experiment, immunoassays and histological analysis of the intestine were performed. In comparison with the control diet, diets A and B recorded significantly higher values for all immunity parameters evaluated (lysozyme, superoxide dismutase and antiprotease activity). The intestinal score was established to identify the state of the intestine associated with each diet, and there were no statistically significant differences. Finally, some of the fish fed these diets were subjected to a stress test. Transaminases (AST-GOT and ALT-GPT), glucose, triglycerides and lactate dehydrogenase (LDH) were the stress indicators used. The glucose values recorded (1h post-stress) were the highest for diets A and B.

Keywords: Ash; Diets; European sea bass; Histology of the intestine; Immunoassays; IMTA; Macronutrients; Microbiome; RAS; Sea lettuce; SGR; Stress.

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List of abbreviations

ALT-GPT	Alanine aminotransferase
ARA	Arachidonic acid
AST-GOT	Aspartate aminotransferase
BIOSEA	Innovative cost-effective technology for maximizing aquatic biomass-based molecules for food, feed and cosmetic applications
C	Control
CI	Condition index
CTAQUA	Andalusian Aquaculture Technology Center
DHA	Docosahexaenoic acid
DM	Dry matter
DNA	Deoxyribonucleic acid
DW	Dry weight
EPA	Eicosapentaenoic acid
ERDF	European Regional Development Fund
FAO	Food and Agriculture Organization of the United Nations
FAs	Fatty acids
FCR	Feed conversion ratio
FI	Feed intake
HP	High phosphate
HS	High salinity
HSI	Hepatosomatic index
IMTA	Integrated multi-trophic aquaculture
INTEGRATE	Integrate Aquaculture: an eco-innovative solution to foster sustainability in the Atlantic area
K	Fulton's condition factor

LC-PUFA	Long-chain polyunsaturated fatty acids
LDH	Lactate dehydrogenase
LP	Low phosphate
LS	Low salinity
MUFAs	Monounsaturated fatty acids
NaCl	Sodium chloride
NH_4^+	Ammonium
NIOZ	Royal Netherlands Institute for Sea Research
NO_2^-	Nitrite
NO_3	Nitrate
NP	Normal phosphate
PAR	Photosynthetically active radiation
PFV	Fresh meat weight
PSC	Dry shell weight
PSV	Dry meat weight
PUFAs	Polyunsaturated fatty acids
PV	Total fresh weight
RAS	Recirculating aquaculture system
RBA	Respiratory burst activity
ROS	Reactive oxygen species
SFAs	Saturated fatty acids
SGR	Specific growth rate
SIMBA	Sustainable innovation of microbiome applications in food system
SOD	Superoxide dismutase
SV	Survival

VSI	Viscerosomatic index
WP	Work package
∆W	Weight gain

1. Introduction

1.1. World aquaculture

The growth of the human population has been generating significant challenges to the supply of high-quality, nutrient-rich food whereby a population of 9.7 billion by 2050 will require an increase in the supply of food by 25%-70% (Hua *et al*., 2019). Hence, due to increased pressure over natural resources, namely aquatic species, search for new sustainable alternatives of food production is crucial and imperative. The Food and Agriculture Organisation of the United Nations (FAO) estimates that over 90% of global fish stocks are either overfished or fished to maximum sustainable levels, which poses a threat to biodiversity, to sustainability of fisheries, and to the people who depend on them for their lives and livelihoods (FAO, 2019).

Aquaculture is defined as the production of aquatic organisms, which in some way implies human intervention, with the aim of increasing their production. Globally, aquaculture has been the fastest growing animal food production sector in recent decades. It has allowed for the continuing increase in production of fish for human consumption, whereas wild catches have been stabilizing (Figure 1). That's why today aquaculture supports about half of the fish production consumed worldwide, value that is predicted to reach 60% by 2030 (Calixto *et al.*, 2020; FAO, 2020).

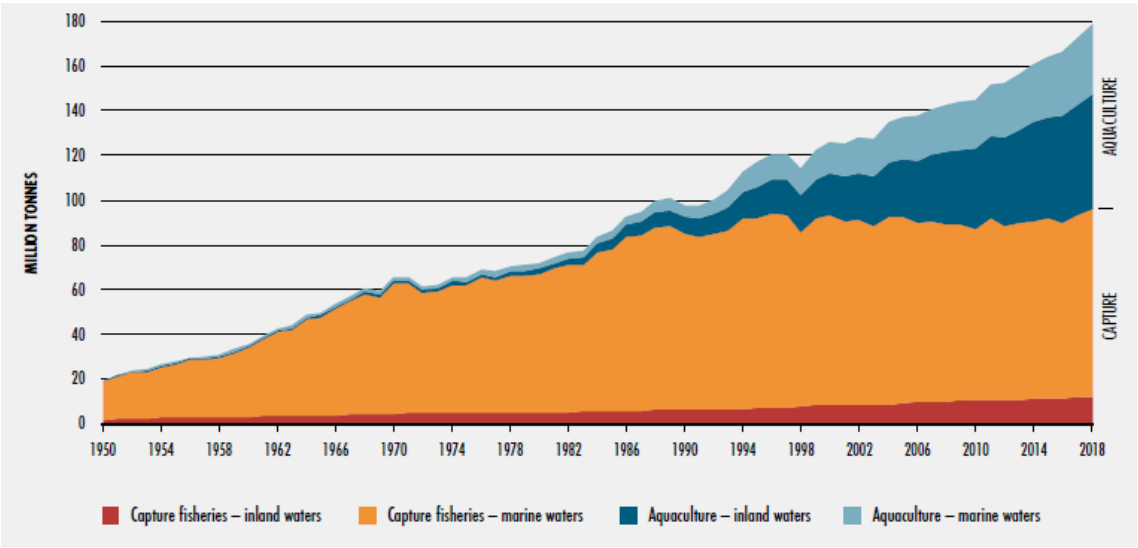

Figure 1: World capture fisheries and aquaculture production, between 1950 and 2018 (FAO, 2020).

Furthermore, the global food fish consumption, from 1961 to 2017, was higher than all other animal protein foods (meat, dairy, milk, etc.) and at the same time increased almost twice in comparison with the annual world population growth for the same period. Besides that, per capita food fish consumption grew from 9.0 kg (live weight) in 1961 to 20.5 kg in 2018, by about 1.5% per year (FAO, 2020). According with the latest data made available by FAO, in 2018, the total production in the aquaculture sector was of 114.5 million tonnes, including 82.1 million tonnes of aquatic animals and 32.4 million tonnes of aquatic algae (FAO, 2020).

However, the aquaculture sector is partially failing to deliver on its promises to improve global food security and relieve pressure on wild fish stocks. The negative impact of the toxic chemicals and antibiotics used on fish farms, the genetic contamination of wild stocks, and the reliance on wild caught fish to make fishmeal and fish oil for feed production are some of the problems that need to be resolved. Nevertheless, nowadays, with the support of new technologies and more innovative and sustainable practices, considerable improvements are being made to overcome the aforementioned aquaculture production pitfalls (Boyd *et al.*, 2020; Guillen *et al.*, 2019).

1.2. Aquaculture classification and IMTA

There are several ways to classify aquaculture facilities and production systems. Extensive, intensive and semi-intensive aquaculture are common ways of classifying aquaculture based on production per unit of volume (m^3) or unit of area (m^2) cultivated. Extensive aquaculture is the most primitive and takes full advantage of the natural conditions of the environment, with virtually no production control. It is characterized by low productivity, where cultivated species are kept in low density. The food used is that available in the natural environment and the level of investment and technology is low. In semi-intensive aquaculture, there is a low level of control over production, although the loading densities used are higher than in the previous case. The technology used is already more advanced (e.g. use of paddlewheel aerators) and, in addition to the naturally available food, feed is also provided. Lastly, intensive aquaculture is characterized by using high densities with high control over all production parameters (high technological level). In addition, all nutritional requirements of the fish are exclusively provided by commercial/artificial feeds (Lekang, 2007).

On the other hand, it is possible to divide aquaculture in three types: monoculture, polyculture and integrated aquaculture. In the first case, as the name implies, only one

specie is farmed. In contrast, two or more species can be farmed and share the same pond, tank, cage, pen, or nearby areas. Under these conditions, somehow, resources such as space, water, feed, or nutrients in general are shared among the different species. According with FAO, polyculture means the rearing of two or more non-competitive species in the same culture unit, whereas integrated multitrophic aquaculture (IMTA) (Figure 2) is defined as the culture of aquatic organisms based on the sharing of resources that originate from agricultural, agro-industrial, wastewater, power stations, and other activities (FAO, 2002).

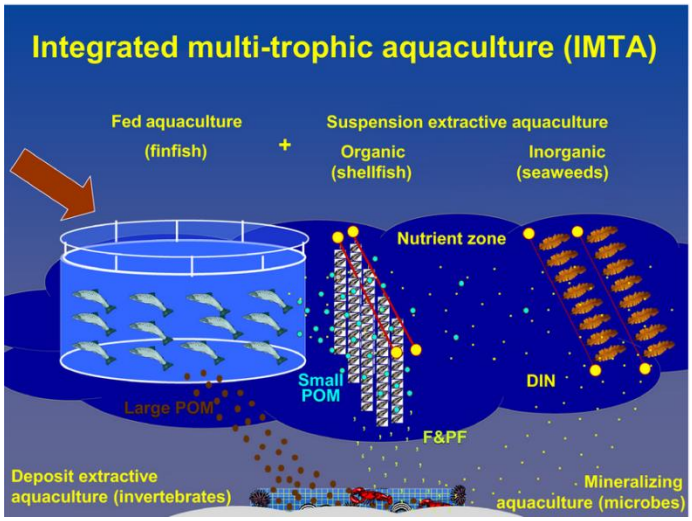

Figure 2: General design of an integrated multitrophic aquaculture system (Chopin, 2013).

Therefore, IMTA combines the cultivation of fed species (e.g. fish/shrimp) with organic extractive species (e.g. shellfish/herbivorous fish) and inorganic extractive species (e.g. seaweed) in the appropriate proportions to create balanced systems for environmental and economic sustainability, thus increasing social acceptability. Besides that, IMTA concept is very flexible, so it can include open-water, land-based, and aquaponics systems (Chopin, 2013; Ridler *et al.*, 2007). Research that investigates the potential of IMTA began during the early 1970s and since then this type of cultivation system has been gaining more and more prominence. IMTA systems allow greater reuse of water and reduction of waste that is released into the environment, while improving production conditions, generating better quality products and mitigating pathologies, which allows the reduction of the use of antibiotics (Biswas *et al.*, 2020; Rosa *et al.*, 2020). The increase in economic profitability in IMTA systems is generated not only by the production of different species with economic value, but also by the reduction of the economic risk of that exploitation. The use of more sustainable production practices also contributes to increase the social acceptance of products (Biswas *et al.*, 2020).

1.3. Assessing IMTA systems sustainability: stable isotopes and C, N and P elements

To get high production in a short period of time, high quantity of inputs, namely fertilizers and feeds (50–60% of operational cost) are used, as happen in most of intensive aquaculture farms. Many experiments indicated that about 85% of phosphorus (P), 80–88% of carbon (C) and 52–95% of nitrogen (N) originated from feed inputs are lost to aquaculture environment through uneaten feed, fish excretion and respiration. At the same time, only 13% C, 29% N and 16% P from the inputs used in aquaculture are retained by cultured animals and the remaining are lost to the water and sediment (Wu, 1995).

The analysis of stable C and N isotopes has been widely used to assess the nutritional sources of several species, to study trophic interactions in communities, and to analyse the organic matter derived from aquaculture systems. Considering the $^{13}C/^{12}C$ ($\delta^{13}C$) and $^{15}N/^{14}N$ ($\delta^{15}N$) ratios, it is possible to differentiate autochthonous organic matter from allochthonous, hence this is a versatile approach in environmental impact assessment studies (Mahmood *et al.*, 2016; Vizzini *et al.*, 2005). As the isotopic ratios of C in the tissues of organisms are generally close to those of their diets (by about 1 ‰), it is possible to get an idea of the origin of the organic matter. On the other hand, N isotopic ratios are enriched by 2 to 4 ‰ per trophic level and, therefore, are normally used to estimate the trophic position of the organism (Park *et al.*, 2015). The isotopic signatures of residues derived from aquaculture (usually enriched in ^{15}N and depleted in ^{13}C) tend to be distinct from those of natural origin, providing information on the flow of these residues that are incorporated into the adjacent food chain. This makes it possible to evaluate the efficiency, in terms of sustainability, of the IMTA systems, however, this efficiency is very dependent on the spatial, temporal contexts and the species used (Gondwe *et al.*, 2012; Park *et al.*, 2015; Yokoyama *et al.*, 2015).

1.4. Pathologies and stress in aquaculture

From an economic point of view, feed and infectious diseases are the two main expenses in aquaculture. In this context, the search for new sustainable ingredients and more effective “natural” ways to prevent diseases, as the use of immunostimulants, has gained increasing prominence (Bagni *et al.*, 2008; Carbone & Faggio, 2016).

Pathologies caused by bacteria, viruses, ciliates, dinoflagellates, myxozoans, nematodes, trematodes and crustaceans have been generating huge losses in many

aquaculture companies. Thus, one of the major challenges in the production of European seabass (*Dicentrarchus labrax*) is precisely the occurrence of bacterial outbreaks during the cultivation process, especially in intensive systems (offshore floating cages: where most of the production comes from) (FAO, 2005b).

Farmed fish are frequently exposed to different stress conditions which in some cases can have significant negative effects on their health status, often increasing susceptibility to pathogens. Stress has been described as a cascade of physiological events that occurs after perception of a threat, during which an organism attempts to restore homeostatic norms (Ramsay *et al.*, 2009). Fish own a neuroendocrine stress response that has a clear short-term adaptive value, however, when activated for prolonged periods, this response can be harmful, with implications for fish welfare. The way fish respond to a stress factor is quite variable, the physiological changes associated can be rapid or delayed, short-term or long-term, depending on factors such as species and life stage of the fish, nature of the stressor, and other environmental factors. In general, the exposition to a stressor develops through different stages, involving, in a first step, a response characterized by neuroendocrine modification (catecholamine release and cortisol production). The second stage is related to corticotropin-inter-renal axis activation, bringing numerous modifications in terms of haematological parameters. Lastly, if the homeostasis is not restored, a tertiary response acts on the entire organism, affecting growth, fecundity, disease resistance, and even inducing death (Roque *et al.*, 2010; Santulli *et al.*, 1999).

The hormone cortisol is the most informative and accessible marker of stress in fish, which typically increases during exposure to acute or chronic stressors. Elevated cortisol levels are known for generate changes in blood cells and plasma glucose and lactate, hence, these variables are also considered representative of the stress status of fish. Short-term increases in cortisol are correlated to adaptive responses, whereas repeated or chronic increases in cortisol are associated to maladaptive responses, resulting in decreased growth, impaired reproduction, and increased susceptibility to infectious diseases (Fernández-Alacid *et al.*, 2019; Ramsay *et al.*, 2009).

Seasonal changes, cultured environments, high stock densities and catch/harvest are some usual stressors in fish (Yılmaz *et al.*, 2016). Several studies have analysed the effect of increasing density on biological performance in fish since it represents a potential source of chronic stress, which may affect physiology and behaviour of farmed fish. Generally, high density is considered as a potential source of stress, with a negative effect on fish growth rate, survival and feeding rates. Water quality

deterioration or/and increase of aggressive behaviour are the main negative effects which arise from the high stock densities. In European seabass, no differences in survival, growth, plasma cortisol glucose, total proteins, triglycerides and cholesterol were observed in fish reared in a recirculation system arranged in tanks at different densities up to 45 kg/m^3 (Sammouth *et al.*, 2009). Furthermore, it was demonstrated that this species shows a typical stress response when exposed to acute stressors (e.g., chasing for 5 min and air exposure for 1–1.5 min), with peak plasma cortisol levels at 1h and plasma glucose and lactate concentrations at 2h post-stress (Fatira *et al.*, 2014; Millot *et al.*, 2014).

As already mentioned above, the stress tolerance of fish can be analysed by studying some of the blood parameters, giving us an idea about the welfare status of fish. Besides plasma cortisol, other blood constituents such as glucose, lactate dehydrogenase (LDH), triglycerides, aspartate aminotransferase (AST-GOT) and alanine aminotransferase (ALT-GPT), can be used for diagnosis of the physiological and health status of fish (Fernández-Alacid *et al.*, 2019; Sammouth *et al.*, 2009).

1.5. Marine algae as functional additives in aquafeeds

In order to curb the problems associated with pathogens, strategies like those used in other sectors of animal production (vaccination and chemical therapies) have been used in aquaculture. Antibiotics have been routinely administered, but sometimes with negative impacts on the environment and human health (especially in developing countries), namely the proliferation of bacterial strains resistant to antibiotics and the accumulation of chemical residues in the tissues of the organisms (Romero *et al.*, 2012).

Through the manipulation of diets, alternative preventive techniques such as strengthening the immunity of fish through the prophylactic administration of prebiotics/probiotics with immunomodulatory effects, and antioxidant supplements have been showing very satisfactory and promising results. These cost effective and sustainable methods may even be an alternative to vaccines, maximizing the use of natural components in the formulation of diets and reduce interference in the state of homeostasis of fish and in the environment (Akhter *et al.*, 2015; Peixoto *et al.*, 2019).

In this context, marine algae, that contain bioactive molecules with immunostimulant and antioxidant properties, deserves mention. Algae polysaccharides have been shown to stimulate non-specific host immunity and positively modulate intestinal health, enhancing the digestive abilities of fish. In addition, β-glucans that can

be found in algae, stimulate the immune system through the rapid release of reactive oxygen species (ROS). This process, called respiratory burst activity (RBA), is associated with the production of ROS by macrophages and neutrophils after contact with foreign particles (Peixoto *et al.*, 2019; Pilarski *et al.*, 2017).

Traditionally, forage fish have been the basic ingredient in the production of aquafeeds, because it is a very rich source of high-quality protein, micronutrients and lipids (especially LC-PUFA, such as eicosapentaenoic acid C20: 5 n-3 (EPA) and docosahexaenoic acid C22: 6 n-3 (DHA)). Fishmeal and fish oil are considered one of the main obstacles for the aquaculture feed industry so, finding alternative sources to minimize the pressure on forage fish will be essential, if not the growth of aquaculture production will remain costly and unsustainable (Hua *et al.*, 2019; Kok *et al.*, 2020; Kolanowski, 2010). The fact that ingredients extracted from algae can be included in the diets, contributes to a more sustainable aquaculture, since the levels of fishmeal and fish oil are reduced in part. Although prolonged feeding with this type of ingredients is controversial due to antinutritional factors, palatability and amino acids profile, specific transformation processes, capable of dealing with these problems, are increasingly being developed (Wan *et al.*, 2019).

Many compounds can be obtained from algae: proteins, carbohydrates, fatty acids, phycobiliproteins, carotenoids, etc. and applied not only in feed production but also in other types of industry. These target compounds can be highly variable (along with algae growth rate and sometimes even the morphology), depending on the species and environmental or cultivation conditions. Theoretically this can be advantageous, since, by controlling the conditions, the growth of algae is directed towards maximizing the production of a particular compound. However, it is not always possible to recreate the ideal conditions for this purpose and several other factors can have influence in the composition of algae, such as the age of culture or fronds and the life stage (macroalgae in particular). Besides that, another important issue is related to timescale, the compounds behave differently from each other, some can vary within seconds to minutes (e.g. enzyme activation/deactivation; antioxidant responses), others within a scale of hours to days (e.g. large polysaccharides; de novo protein synthesis). Likewise, this timescale is influenced by environmental parameters, such as light, temperature, salinity, pH/CO_2 and nutrients availability. On the one hand, changes in light intensity can trigger almost instantaneous responses (e.g. changes in short-chain carbon components); on the other hand, the effects associated to temperature changes usually takes longer (e.g. formation of membrane lipids) (Malta, 2017).

As already seen, the dynamic of compounds from algae can be highly variable, therefore it is very important to continue to deepen knowledge about the development and composition of different species of algae and how external factors can influence them. This will allow to improve the cultivation in order to increase the efficiency in terms of production of the selected and desired compounds. In the macroalgae group, two genera have been widely studied, mainly for their properties and potential in numerous sectors: *Ulva* and *Gracilaria*. In these two genera of algae for instance are present compounds capable of increasing lipase activity, improving antioxidant responses and promoting complement system and lysozyme activity, in European seabass (Subramaniam et al., 2019).

Other species of algae with high potential as supplementary ingredients on fish diets include the sugar kelp, *Saccharina latissima* and the blue-green algae *Arthrospira platensis*. Sugar kelp (*S. latissima*) belongs to the class Phaeophyceae, Laminariaceae family and together with other Laminariaceae species, are commercially known as kombu. It is known for its richness in fibers, vitamins, minerals and antioxidants. Although biomass obtained from *S. latissima* has been used mainly for human consumption and as raw material for the alginate industry, in recent years, many other applications, such as animal feed, have emerged successfully with promising advantages. For instance, alginate and laminarin extracted from this alga are proven to fortify the immune system of fish (Ferreira *et al.*, 2020; Malta, 2017; Peteiro & Freire, 2013). On the other hand, the microalgae *Arthrospira platensis* (class Cyanophyceae, family Microcoleaceae) is widely cultivated to produce biologically active food additives and to treat several diseases. Nowadays, the main biomass is used in dried form for food supplements and is well known as an important ingredient in the world of aquaculture feed industry (presence of high value protein contents) (Dolganyuk *et al.*, 2020; El-Sheekh *et al.*, 2014; Malta, 2017).

Therefore, the use of algae in feed formulation, brings many benefits and to assess how advantageous and impactful diets containing algae compounds are for the full and healthy development of fish, methods such as immunoassays and histological analysis have been used, constituting themselves as biomarkers of the functional and nutritional status of the organism (Fernández-Alacid *et al.*, 2019; Peres *et al.*, 2014). Immunoassays are bioanalytical methods in which the detection and quantitation of the analyte in samples, depends on the reaction of an antigen (analyte) and an antibody (Ju *et al.*, 2017). Through them is possible to evaluate biochemically lysozyme (antibacterial enzyme present in the serum and mucus of fish that is particularly associated with leucocytes and leucocyte-rich tissues), antiprotease activity (serum substances that

inhibits the activity of proteases), and SOD (group of enzymes that catalyse the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide, being responsible for free radical generation and consumption) present in the serum (Duthie & Lorenz, 1949; Gui *et al.*, 2010; Hikima *et al.*, 2003; Luostarinen *et al.*, 1997). Histological analysis of the digestive system, particularly of the intestine and liver, is also considered a good indicator of the nutritional status of fish. For this, various methods of histological analysis are used, most often semiquantitative scoring system, histochemical and immunohistochemical methods (Raskovic *et al.*, 2011).

1.6. Ash content in marine algae

The ash content is an important quality parameter and can be defined as the amount of total minerals present in a biomass, in other words, the inorganic residue remaining after ignition or complete oxidation of organic matter in a biological material. Therefore, ash measurement is a common procedure whilst analysing biomass for nutritional or compositional evaluation. Ashes/minerals promote health and act on metabolism, thus are nutritious substances indispensable to the organisms (Aston *et al.*, 2018; Liu, 2019; Traugott *et al.*, 2020).

Algae are well known for having high levels and high variation in total ash. This high ash content constitutes, on the one hand, a hindrance to overcome since, generally, diminishes algae inclusion levels for food and feed, and leads to operational problems in biomass combustion systems for energy conversion. That's why methods to manipulate the C contents (carbohydrates, protein and lipids), while reducing ash would be beneficial in order to develop and maximize the use of algae, namely seaweeds as *Ulva* spp. (Malta & de Nys, 2016). An experiment with *Ulva ohnoi* cultivated under controlled land-based system over 6 months, where yields of biomass and bioproducts (fatty acids; soluble fibres; amino acids) were assessed, proved that ash was the second largest component of the biomass after carbohydrates. Furthermore, the peaks of ash content were coincident with peaks in biomass productivity and the concentration of soluble fibres was not affected by the drastic changes in the ash content over the study period (Mata *et al.*, 2016).

Due to its importance as an indicator of quality of a biomass, either relative to food, feed or industrial material and renewable fuel feedstock, ash content is generally measured gravimetrically by burning samples in a muffle furnace at a high temperature for a specified duration (process known as dry oxidation or dry ashing). The process of

dry ashing has been the primary method to measure ash content in biomass, because is a safe and convenient method, and requires no added reagents, no blank for subtraction and many samples can be analysed simultaneously.

1.7. Microbiomes

Currently, it is known that most existing eukaryotic organisms establish interactions with specific microorganisms, which contribute to their success (such as: human intestine, rhizosphere and sponge microbiome). The development of new technologies, such as metagenomics and metatranscriptomics, has allowed the discovery of relevant information for the understanding of interactions/symbiosis between microorganisms and their hosts (holobionts) (Graham *et al.*, 2015). It has been shown that the growth and development of macroalgae depend on the associated microorganisms, particularly the bacterial communities. Although several interesting studies have already been published on the interaction of macroalgae with bacterial communities, the part regarding the functional diversity and the connectivity of these communities with the host, is not yet properly explored. It is known that bacterial cells communicate chemically with those of the host, assisting the growth, morphogenesis and reproduction processes. For instance, the epiphytic bacterial communities contribute to the protection of the surface of macroalgae against fouling organisms through the production of biologically active metabolites.

A study by Provasoli (1958) reported that an axenic culture of *Ulva* developed abnormal thalli with a polymorphic morphology. Subsequently, it came to be observed that *Ulva* maintained the normal thalli only when its cultures were inoculated with specific bacterial communities (Singh & Reddy, 2016). Likewise, it appears that these microorganisms are an essential and integral part of fish such as European seabass, playing a fundamental role in the health and development of the animal, having already been proven that the composition of the microbiome is directly related to the resistance to pathogenic agents. Therefore, one way to promote species health and productivity is through the regulation of the functionally active microbiome (Rosado *et al.*, 2019).

1.8. Aquaculture species

1.8.1. Japanese oyster (*Crassostrea gigas*)

Currently, about 580 aquatic species are cultivated worldwide (Watts *et al.*, 2017). One of the main aquaculture species is the pacific cupped oyster or Japanese oyster (*Crassostrea gigas*) (Figure 3). Belongs to phylum Mollusca, Bivalvia class and it's native to the south east Asia, where habits the estuary and coastal areas of South Korea, North Korea, China and Japan. However, it has been introduced for aquaculture purposes in many different parts of the world due to its wide adaptation range at different environmental conditions (Ezgeta-Balić *et al.*, 2020; Nehring, 2011). The Japanese oyster has an elongated limestone shell, usually whitish, composed of two valves, a slightly convex or flat right valve, and a quite deep and moderately cup shaped left valve. These, in turn, are joined by adductor muscles and a ligament that control their opening. Oysters feed by filtration process, through gills cilia, which retain particles suspended in water. Thus, these non-selective filtering organisms have a very varied diet, mainly consisting of phytoplankton, bacteria and protozoa. *C. gigas* is a protandrous hermaphrodite species (most commonly, initially male and then becomes female throughout the life cycle) and has external fertilization, with each spawning female releasing about 50 to 100 million eggs (FAO, 2005a).

The success of the production of this bivalve is due to its biological characteristics, which allow it to tolerate several environmental conditions, such as great amplitudes of salinity and temperature, including the fact that they have a rapid growth, with the average length of an adult oyster being about 80-150 mm. The market size is reached in a period of 18 to 30 months (Ezgeta-Balić *et al.*, 2020; Nehring, 2011).

It is an interesting species for cultivation in IMTA systems and nowadays three main oyster farming methods are used depending on environmental conditions such as tidal range, water exchange rate, water depth and type of bottom substrates: off bottom culture, on bottom culture and suspended culture (FAO, 2005a).

Many studies have been conducted with the aim of improving the management of *C. gigas* aquaculture. In this sense, ecophysiological and commercial quality indicators (condition index; content of meat; shell), levels of organic pollutants (polychlorinated biphenyls; organochlorine pesticides) and nutritional quality parameters (proximate and mineral composition; glycogen content; fatty acid profile; cholesterol) are some of the parameters that have been used to evaluate variables such as growth, safety and

nutritional quality (Grangeré *et al.*, 2009; Li *et al.*, 2009; Orban *et al.*, 2004; Pouvreau *et al.,* 2000).

In 2018, the world production of the pacific oyster was 643.5 tonnes and most of it was in China (FAO, 2005a).

Figure 3: Japanese oyster (*Crassostrea gigas*) (FAO, 2005a).

1.8.2. European seabass (*Dicentrarchus labrax*)

One of the fish species that is much sought after and appreciated is the European seabass (*Dicentrarchus labrax*) (Figure 4). This was the first non-salmonid marine species to be grown commercially in Europe and is currently the most important and widely grown commercial fish in Mediterranean areas such as Greece, Turkey, Italy, Spain, Croatia and Egypt. It is a teleost fish, of the Moronidae family, which is distinguished by having an elongated body, showing a silvery-grey and bluish colour on the posterior part of the body, while the flanks have a silver coloration. It presents 8 to 10 dorsal spines, 12 to 13 dorsal rays, 3 anal spines and 10 to 12 anal rays. The posterior border of the operculum is serrated, and the lower part has strong denticles directed forward. It has 2 flat opercular spines and the mouth is moderately protractile. This species can reach up to 1 meter in length and weigh up to 12 kg. It is distributed throughout the Northeast Atlantic, from Norway to Senegal, as well as in the Mediterranean and the Black Sea. Sexual maturity is reached earlier in males (2 - 4 years) than in females (3 - 6 years). There is only one breeding season per year that occurs in winter in the Mediterranean population (December to March) and until June in the Atlantic populations. Because it is an euryhaline and eurythermic species (ability to adapt to different salinities and temperatures, respectively), it can easily be found in estuaries, lagoons, rivers and in coastal waters.

It’s a carnivorous fish that feeds essentially on small fish, molluscs and crustaceans, therefore, in a context of cultivation, requires a diet rich in protein (FAO,

2005b; Haffray *et al.*, 2000). The estimated protein content for maximum growth is around 50% of diet (Peres & Oliva-Teles, 1999). As for temperature, optimal growth was estimated at around 24ºC (Besson *et al.*, 2016).

Most of the production comes from sea cage farming, although cultivation in seawater ponds and lagoons is also common. In 2016, the world production of this species was 191 003 tonnes (FAO, 2005b).

Figure 4: European seabass (*Dicentrarchus labrax*) (FAO, 2005b).

1.8.3. Sea lettuce (*Ulva*)

The genus *Ulva* (Figure 5) is classified under phylum Chlorophyta, order Ulvales, family Ulvaceae and comprises approximately 120 species currently accepted taxonomically that generally stand out as locally dominant individuals in rocky coastal areas, oceanic intertidal areas and estuaries worldwide. Its macroscopic shape is membranous, growing in the form of typical tubular or flat/blade-like thalli, branched and connected to the substrate by rhizoid, or else it can appear as free life. Both the haploid form and the diploid form are morphologically similar, so the life cycle is isomorphic (An & Nam, 2017; Coste, 2018; Favot, 2017; Vázquez-Rodríguez & Amaya-Guerra, 2016). Due to the ability to thrive in environments rich in N and with lots of light plus the ability to live free and fragmented, species are often linked to “green tides” and other algal blooms (Hiraoka *et al.*, 2004). The identification of species of this genus is quite complex since conventional methods, such as morphological and anatomical characters, are insufficient, making it necessary to resort to molecular techniques. It is a genus that has aroused interest in recent decades as a useful organism in environmental bioremediation processes and for the value of the biomass obtained, with several studies showing its great potential in IMTA systems (Favot, 2017). The use of its biomass in animal and human nutrition is especially important due to nutraceutical qualities (highlighting the presence of the characteristic sulfated polysaccharide: ulvan). The potential use as an

energy resource and feedstock for biomaterials is also very promising (Favot, 2017; Vázquez-Rodríguez & Amaya-Guerra, 2016).

Figure 5: Sea lettuce (*Ulva* sp.) (FAO, 2019).

Regarding to growth dependence to environmental factors, it is noteworthy to remark that growth rate responses of algae to temperature typically follow a bell-shaped curve, leaving a temperature window in which a peak is reached (maximum growth). For *U. pertusa* and *U. rigida* was reported a significant impact on growth rates, after exposition to only one or two degrees decrease at night. It's quite difficult to find out specific temperature-growth responses because specimens within species may display different growth optima. This is well applicable for *Ulva*, and we only should indicate best growth temperatures in a general way. For example, in *U. intestinalis* maximum growth occurs at water temperatures below 20ºC, whereas *U. ohnoi*, seems to growth fast at a high temperature range (25 – 35°C) (Malta, 2017).

Light is an energy source that allows capture and conversion of CO2 into sugars, thus influencing algal growth through its effect on photosynthesis. In what concern to algae and other photosynthetic organisms, light refers to the light spectre of 400 to 700 nm that they can use for photosynthesis (PAR: photosynthetically active radiation, expressed as µmol photons m^{-2} s^{-1}). So, basically, there is a maximum light intensity (assuming optimum growth conditions) at which photosynthetic activity no longer increases (saturation) and beyond which it decreases again. For instance, in *U. intestinalis* was reported a value of saturation varying between 174 and 245 µmol photons m^{-2} s^{-1} and a value between 245 and 465 µmol photons m^{-2} s^{-1} for the other temperate water species of *Ulva*. Nevertheless, algae have enormous plasticity, being able to adapt to different light conditions, by modulating the content of chlorophyll a and accessory pigments (Malta, 2017).

In general, maximum growth of algae occurs around neutral pH, at high pH growth slows down as it reduces availability of CO2 and bicarbonate. However, due to

great plasticity, some species can keep maintaining high growth rates at pH values away from neutral, such as *Ulva* species which are able to thrive at pH above 9 (Malta, 2017).

It is also reported that most of *Ulva* species have a better growth performance with a salinity varying between 15 and 35‰. *U. lactuca*, *U. rigida*, *U. scandinavia* and *U. curvata* growth are known to be inhibited at a salinity higher than 30‰. Nevertheless, each species has their own flexibility, some may even thrive in fresh water and salt water (Malta, 2017).

N and P are two crucial nutrients and along with C, represent the macronutrients. The micronutrients are represented mainly by trace metals, as iron (Fe). N is key in the formation and composition of proteins and nucleic acids. Its content range in seaweeds between 0.5 – 9% dry weight (DW). On the other hand, P is part of essential molecules as ATP, the energy carrier of cells, and of DNA and RNA, besides being also important in the constitution of phospholipids. Its content range in seaweeds between 0.1 – 1% DW. These two nutrients are the main responsible for promoting growth of algae, so in situations of starvation or limitation a decrease in growth will practically always be expected (Malta, 2017).

Due to the highly nitrophilic nature, protein content in *Ulva* is one of the highest among seaweeds (although this value can vary a lot, between 0.4 and 44.3 % DW, as seen before several aspects enter the equation). Besides that, according to many authors, high acceptance and digestibility are two aspects that characterize the protein obtained from *Ulva*. Regarding the amino acid profile, *Ulva* is particularly rich in leucine, lysine, valine and threonine (essential amino acids); glycine (semi-essential amino acids); glutamate, aspartate and alanine (non-essentials). Although little studied, impact of different growth temperatures in protein content of seaweeds is a factor that should be consider. For example, a study carried out with *Ulva pertusa* shows a decrease in protein synthesis at temperatures beyond the optimum for growth, and increases at reduced temperature, or during the night in a day/night cycle with temperature decreases at night (Malta, 2017; Shuuluka *et al.*, 2013). Furthermore, it is known that trend goes towards decrease of protein content when algae are exposed to higher light intensities (up to saturation), hence the important influence of light on protein content (Malta, 2017). An experiment with *U. ohnoi*, cultivated in a salinity range of 10-60‰, reported a total protein content more or less constant. However, amino acid composition suffered a deep change between salinities (Malta, 2017). Other study, in this case with *U. prolifera*, shows a different panorama, where an increase protein content was registered with salinity (Malta, 2017). In general, when we have situations of N limitation or starvation, protein

content tends to decrease. Same happens to P limitation, lower protein contents are associated with a lack of P (Malta, 2017).

In terms of carbohydrates, glucose, rhamnose and xylose are the main sugars found. Moreover, *Ulva* and all other seaweeds are a rich source of polysaccharides that are present mainly in the cell walls. Beyond the polysaccharides shared with other macroalgae, order Ulvales, where *Ulva* is included, own a characteristic exclusive component named ulvan. This sulphated polysaccharide has been arousing a lot of interest, because its high versatility (antiviral, anticoagulating, antioxidant, immunomodulating, etc.). The content of carbohydrates may change when exposed to different temperature conditions. However, these changes can often be dependent not only on temperature, but also on other factors (namely dissolved CO_2), making it difficult to determine the exclusive effect of temperature (Malta, 2017). For instance, an experiment carried out by Wang *et al.* (2007) in *U. pertusa* shows a slightly higher carbohydrate content after 20 ± 2ºC and 20 ± 4ºC circadian rhythms of fluctuating temperature conditions, in comparison with those maintaining at constant temperature of 20ºC. Influence of light manifests itself with an increasing of carbohydrates content when algae are exposed to higher light intensities (up to saturation). Furthermore, the parameter salinity, in *U. fasciata*, was reported to have influence in carbohydrates content, where an increase in salinity (only until the optimum for growth, 35‰ in this case) leaded to an increment in sugar content. Higher accumulations of carbohydrates in *Ulva* were also reported in situations of N limitation or starvation and in P limitation conditions (Malta, 2017).

Like happen with most seaweeds, *Ulva* species tend to have a total lipid content considerably lower than microalgae and, generally, about 50-60% is present in the form of fatty acids. However, due to this great percentage of fatty acids and high growth rate that this alga can achieve, *Ulva* can be considered a potentially rich source of harvestable fatty acids, namely mono- and polyunsaturated FAs (MUFAs and PUFAs) (Malta, 2017). Profile and total content of FAs is quite variable and according to McCauley *et al.* (2016) the profile on average consists of 35.3 ± 13.7% saturated fatty acids (SFAs), 15.9 ± 6.6% MUFA and 41.8 ± 15.6% PUFA. One of the most commonly visible effects of temperature variation on lipids is related to alterations in the levels of unsaturation of membrane fatty acids. Saturated fatty acids levels tend to increase with temperature, whereas at lower (suboptimal) temperatures PUFA levels have been reported to be higher (Malta, 2017). In *U. pertusa* total fatty acids levels increase when exposed to lower temperatures (Malta, 2017). Same study also showed that lower temperatures led to increase levels of oleic and linolenic fatty acids (unsaturated FAs).

Along with an increasing on light intensity, lipid content tends to increase, but at same time, percentage of unsaturated fatty acids (mainly PUFAs) tends to decrease (associated with damage and oxidation processes) (Malta, 2017). Although this is far from be linear, as reported by Floreto & Teshima (1998), where in *U. pertusa* a higher proportion of PUFAs were found at high light, without changes in total lipids. The pH of water is closely related with CO2, determining the availability of this compound for the algae. For instance, in *U. rigida* was found that an acidic pH (CO2 addition) essentially mediates the effect of N on lipids. Moreover, effects of CO2 were found on lipid class composition but not total lipids. Regarding salinity, many studies were made with *Ulva* species and, for example, in *U. fasciata and U. pertusa* total fatty acids have been found to increase with salinity (Malta, 2017). Generally, lack of N in the system leads to an increase in percentage of lipids, but omega 3 fatty acids and other PUFAs tend to decrease their percentual levels. Effects of P limitation follow the same trend, so an accumulation of lipids is expected, but with a decrease in PUFAs and a change in lipid composition (decreased in phospholipids, etc.) (Malta, 2017).

Moreover, sea lettuce is also known for be rich in several minerals and vitamins, such as C, B3 and B12 (Malta, 2017; Taboada *et al.*, 2009).

U. ohnoi is one of the most promising species of this genus for cultivation. A better performance in terms of growth, protein content and balance between macro minerals and trace elements is proven, compared to species such as *U. fasciata*, *U. rigida* and *U. australis* (Caamaño, 2016; Rodríguez, 2016).

1.8.4. Sea moss (*Gracilaria*)

Another genus that has a relevant global importance is the genus *Gracilaria*. It belongs to the group of red algae (Rhodophyta), and is represented by more than 150 species. It is one of the most cultivated algae in the world (China and Indonesia dominate the production), and a large part of its biomass is directed to the phycocolloid industry, constituting the main source of agar-agar (66% of the total produced). Typical habitats for these species are protected environments, such as bays, estuaries or river mouths. It is cosmopolitan and grows in areas of intertidal and subtidal rocky, sandy and muddy, up to 20 m deep. Thalli are erect and arise from a small discoid structure. They are generally cylindrical, depressed or blade-shaped, with lateral, alternating or subdichotomous branches. Its life cycle is triphasic and isomorphic (FAO, 2014; Santelices & Doty, 1989). As with many algae, the life cycle, growth and phycocolloids

content in *Gracilaria* species depend on different sea water parameters, such as temperature and salinity, dissolved nutrient salts, irradiation, presence of other algae species and epiphytes (Gioele *et al.*, 2017).

Gracilaria gracilis is one of the best-known species and whose biomass can be used, either in the production of agar, or in the manufacture of bio-oils and mesoporous material. In addition, it is a promising source of r-phycoerythrin, arachidonic acid (ARA: PUFA ω-6), proteins and carbohydrates. Several studies point to high antioxidant and radical scavenging activity, so the biotechnological, nutraceutical and pharmaceutical applications of this alga are very interesting (Francavilla *et al.*, 2013). *G. gracilis* is a source of a panoply of organic compounds, depending on the harvesting season (considering a temperate region). Therefore, it was found that r-phytoerythrin, ARA, proteins and carbohydrates contents were higher in winter, while in summer stood out the concentration of total phenols, greater antioxidant properties and radical scavenging activity (Gioele *et al.*, 2017). Its fast growth rate, ease of vegetative reproduction and other characteristics, have promoted the start of this algal culture and, in general, the optimal temperature for growth in this species falls in the range 19°-23°C (alga appears to start suffering at temperatures ≥ 28°C) (Gioele *et al.*, 2017; Raikar *et al.*, 2001).

1.9. Objectives

This work was essentially practical and developed in the scope of the projects INTEGRATE, BIOSEA and SIMBA. The main objectives of this report were: 1) to monitor an IMTA system at pilot scale through the collection and analysis of samples and calculation of variables such as the condition index; 2) evaluate the growth of *Ulva ohnoi* in the different cultivation systems and established conditions; 3) investigate the influence of different temperature conditions on *Ulva ohnoi*, taking into account its growth and ash content; 4) analyse data obtained from previous experiments using *Ulva ohnoi* to evaluate the influence of salinity, N source and P concentration on ash and macronutrient content; 5) evaluate the inclusion of algae in European seabass diets, taking into account parameters related to growth, health, immunity and histology; 6) between fish fed different diets, investigate the presence of differences in blood serum after stress exposure.

2. Internship description

This internship was held at the facilities of Andalusian Aquaculture Technology Center (CTAQUA), located in the province of Cádiz (Spain), in the region of El Puerto de Santa María.

For about five and a half months (supposed to be 9 months, but due to COVID-19 pandemic restrictions my stay period was interrupted) I had the opportunity to work with qualified personnel and to participate in various experiences, within the scope of three European projects: INTEGRATE ("Integrate Aquaculture: an eco-innovative solution to foster sustainability in the Atlantic Area"), BIOSEA ("Innovative cost-effective technology for maximizing aquatic biomass-based molecules for food, feed and cosmetic applications") and SIMBA ("Sustainable Innovation of Microbiome Applications in Food System").

2.1. About CTAQUA

CTAQUA is a non-profit research and consultancy institution in the aquaculture and sea food sector, financed by the research and consultancy services it provides (namely to private companies), and by European subsidies. It works together with partners on a regional, national and European scale, with the mission of encourage competitive innovation in companies, in response to the business needs of the aquaculture sector, through the development of applied research to different production processes. CTAQUA operates, basically, at these following six levels: food and nutrition; new species; animal health and welfare; environment; commercialization; applied engineering. Parallel to the support of consultancy to several companies, namely DIBAQ, BIOMAR, CULMAREX and NUTRIAD, CTAQUA has been establishing itself and participating in several projects, both on a regional, national and even international level (e.g. SMARTFEED, PISTRESA +, VALORA, ACCESS2SEA and NOVELFISH projects).

CTAQUA facilities are adapted to carry out multifaceted research and investigation activities. Thus, essentially, there are 6 test rooms adapted to each sector of activity: nutrition (diet tests); diversification (experimentation with new species); molluscs and crustaceans (specific for these groups of organisms); phytoplankton and zooplankton (tests with production of these organisms); microbiology and pathology (resistance tests); transformation (processing tests and treatment of raw marine products into processed products). Moreover, the recirculating aquaculture systems are the

predominant culture systems in the facilities. In addition, there are also 3 laboratories organized according to the purpose for which they were designed: materials (where the large part of the material is accommodated and raw samples from fieldwork are normally processed); physical-chemical tests (analysis of water quality, residues and substrates); food technology (analysis of feed and marine products, both processed and fresh). In addition, the center has several rooms where meetings are held and where data analysis/production of reports are made.

2.2. Routine and tasks

I started my work with the INTEGRATE project (see chapter 3), providing support in the monitorization of a semi-extensive IMTA system. The routine consisted at following the development of algae (*U. ohnoi* and *G. gracilis*), through harvesting, weighing and maintenance of the cage culture structures. Samples of algae, fish (*Sparus aurata*), oysters (*C. gigas*), and abiotic components, such as water and sediments, were also obtained to be further processing and analysed, namely for determination of isotopic composition. Besides that, some of the fish were weighed, oysters were separated by size, and then their weights were also obtained (to calculate the condition index).

Regarding to BIOSEA project (see chapter 4), I was responsible for monitoring several macroalgae cultivation systems, both indoor and outdoor. In this way, I kept, in a small scale, *Ulva ohnoi* and *G. gracilis* stocks, under constant conditions of temperature and light. For the case of *Ulva*, a follow-up of its growth patterns on a larger scale, in tanks on the outside and photobioreactors on the inside, was made. Moreover, for the photobioreactors, I developed an experiment related to temperatures, to evaluate its influence on elements like growth and ash content. In addition, samples of *Ulva* (both dry and fresh) from the tanks and photobioreactors were regularly obtained for subsequent analysis (including determination of bioactive compounds). Within this project I was also assigned the task of analysing some results obtained from three different experiments with *U. ohnoi* carried out on photobioreactors (salinity trial; N-source trial; P-concentration trial), aiming to compare ash, lipids, protein and carbohydrates contents, between treatments. Overall, the monitoring of these cultivation systems, required, weekly, the weighing of biomass, cleaning the containers, renewing of water and adding the respective nutrient medium.

Still concerning the BIOSEA project, in a trial with European seabass, I assisted, three days per week, in the administration of feeds, cleaning of RAS components and

parameter measurements. The objective was to compare the diets (one control diet and three experimental diets containing algae extracts), bearing in mind the growth and health conditions of the fish. Immunoassays analysis were carried out at the end of this experiment to evaluate the immune status of the different fish groups (three blood samples from each group). Additionally, the histology of the intestine, in four fish of each diet, was also done (to evaluate the "intestinal score"). After, a physiological challenge was carried out, where the fish were submitted to a stress factor (confinement + netting). The goal was to analyse some metabolites (lactate dehydrogenase, triglycerides, transaminases and glucose) in the blood serum as stress indicators.

In the scope of the project SIMBA (see chapter 4), three samples of water from an outdoor pond and five samples of *U. ohnoi* from an outdoor tank were obtained to study seaweed-associate microbiome. For that, I followed a bacterial DNA isolation protocol.

During my internship period, I also helped an Erasmus colleague who was, like me, an internship at CTAQUA. He mainly carried out an experiment with *U. ohnoi* cultivated in photobioreactors, where he sought to evaluate the influence of different sources of N (nitrate and ammonium), and the nitrate uptake capacity after exposure to ammonium, bearing in mind the growth and composition of the alga. Therefore, I helped him with the determination of parameters like chlorophyll and total P, for each of the established treatments.

3. INTEGRATE project

3.1. Overview

The INTEGRATE project involved the participation of institutions from 5 European countries (Portugal, Spain, France, Ireland and United Kingdom) and was funded by the ERDF, through the INTERREG Atlantic Area 2014-2020 program. Essentially, the main focus of this project was on consolidating the work and the market associated with eco-efficient aquaculture techniques, communicating the principles and benefits of IMTA systems, based on a holistic approach, and encouraging the "blue and green" growth of aquaculture in the Atlantic Area and European Union. Thus, an articulated set of 6 work packages (WP) were defined *a priori*, where the plans and guidelines are listed. My job under this project was mostly practical, in the field, and focused, more precisely, on WP 4. The aim of this WP was to assess the state of IMTA in the Atlantic region to prepare the way for the development of IMTA on a commercial scale. Therefore, one of the measures consisted in implementing a pilot action with the objective of develop and consolidate an IMTA eco-friendly standard model for land-based semi-extensive aquaculture industry. In this sense, the cultivated species (algae, fish and oysters) and the abiotic factors (water and sediments) of the system were monitored, namely by taking samples for subsequent analysis.

3.2. Material and methods

As leader of the INTEGRATE project, CTAQUA promoted the implementation of a pilot experiment in one of the old salt pans included in the Natural Park of the Bay of Cádiz (Figure 6), in the locality of Puerto Real. Thus, in this salt pan, denominated "Salina de Belén de Levante y Poniente", managed by the company Estero Natural S.L., was developed a semi-extensive IMTA system, compartmentalized in two different sectors. In one of the compartments, were introduced about 1500 gilthead seabream and approximately 1900 oysters (*C. gigas*), kept in hanging mesh baskets. In the other section, algae (*U. ohnoi* and *G. gracilis*) were introduced, in a first stage arranged in long line, but due to the inefficiency of this method (including a problem associated to epiphytic bryozoan *Amathia verticillata* on the algae), plastic mesh cages started to be used. The system was controlled by tidal flows and a series of floodgates attached. So, basically, the water present in the sector of gilthead seabream + oysters flowed towards the algae compartment, nourishing them.

Figure 6: Aerial view of the intervention site (obtained from Google Earth).

When I arrived, the work was already going on and, therefore, the work I developed, corresponds to a final phase of this part of the project. In this way, I was involved in the weekly monitorization of the system, carrying out the harvest (with the help of nets and baskets), weighing (after centrifugation, using a professional salad spinner) and sampling of the algae, plus cleaning the respective cages with a brush. Then, the seaweed samples were dried (using a homemade dryer) and stored in zip lock bags. In the lab, these samples were later grinded (with a mortar and pestle) and placed in Eppendorf tubes for further analysis. Water and sediment samples were also collected and processed. So, I utilized vacuum filtration for water samples, using glass microfiber filters (Whatman 1822-090, Grade GF/C Glass Microfiber Filter), each of them previously weighed in an ADAM PW 254 precision balance. Afterwards, the discs with the retained particles were dried in an INDELAB digital drying oven (at around 100ºC), then weighed again, wrapped in aluminium foil and placed in zip lock bags. In the case of sediments (Figure 7), firstly, they were dried in the oven (at about 60ºC), then sieved and, finally, using a mortar and a pestle, milled to be stored in Eppendorf tubes.

Figure 7: Sediment samples preparation.

At the end of the pilot experiment, I was also involved in the final sampling of fish and oysters. In the case of fish, the process was carried out with the help of a trawl and with the support of personnel linked to the company Estero Natural S.L. Some of the fish were sacrificed to obtain samples, and others were weighed and measured, for later comparison with the records made at the beginning of the experiment. During this process, fish were properly maintained (with the necessary aeration in the containers to which they were transferred) and anesthetized using clove oil (Figure 8).

Figure 8: Sampled fish (*Sparus aurata*).

Oysters were all separated by sizes (small, medium or large) and then weighed. Moreover, some of them were selected (5 specimens for each size), measured with the aid of a calliper and weighed (specifically: dry meat weight; dry shell weight; total fresh weight; fresh meat weight), in order to calculate the condition index. Fresh weights were determined directly using a scale and dry weights estimated after drying to constant weight at about 100ºC in an oven. A few other oysters were also selected to be stored in zip bags, for later analysis (namely stable isotopes determination).

All samples collected, at a later stage, were sent to a specialized laboratory located in Seville, where the analysis, namely of stable nitrogen isotopes, were carried out.

The following formulas were used to calculate the condition index, through three different approaches (CI1; CI2; CI3) (adaptated from Crosby & Gale, 1990; Mann, 1992; Phernambueq & Vroonland, 1983; Rupendra, 2013; Yildiz *et al.*, 2011):

$\mathbf{CI1} = \frac{\boldsymbol{PSV}}{\boldsymbol{PSC}} \boldsymbol{x100}\%$; where PSV is dry meat weight (g) and PSC is dry shell weight (g);

$\mathbf{CI2} = \frac{\boldsymbol{PSV}}{\boldsymbol{PV-PSC}} \boldsymbol{x100}\%$; where PSV is dry meat weight (g), PV is total fresh weight (g) and PSC is dry shell weight (g);

$\mathbf{CI3} = \frac{\boldsymbol{PFV}}{\boldsymbol{PV}} \boldsymbol{x100}\%$; where PFV is fresh meat weight (g) and PV is total fresh weight (g).

Unfortunately, due to the unreliability of the data obtained for algae (problems related to pests and accumulated dirt), only data obtained for oysters were included in this report.

3.3. Results and discussion

The CI represents an ecophysiological approach to estimate meat quality and yield in cultured bivalve molluscs, namely oysters. As an ecophysiological index, the CI can be affected by multiple biotic factors (e.g. food availability), abiotic factors (e.g. temperature) and physiological activities (e.g. spawning) (Lawrence & Scott, 1982; Rebelo *et al.*, 2005).

Table 1 shows the mean CI values obtained for the 3 final sampling stages of the experiment (knowing that seed oysters were planted on 21/01/19).

Table 1: Mean oyster condition indices (CI1; CI2; CI3) ± SD recorded throughout the experiment (planted on 21/01/2019).

Date	CI1	CI2	CI3
28/05/2019	39.7 ± 38.7	31.1 ± 32.1	22.5 ± 16.1
30/08/2019	11.2 ± 7.0	14.3 ± 14.3	18.2 ± 18.2
05/11/2019	6.1 ± 2.1	7.0 ± 5.3	14.2 ± 6.1

As mentioned by Castillo-Durán *et al.* (2010), the value of the condition indices for this species of oyster (considering a temperate climate) tend to be higher in winter and lower in summer, because the high temperatures assume themselves as a physiological stress factor. Moreover, autumn is the most favourable period to start cultivating *C. gigas*.

For all these indices there have been a decrease over the period considered. Taking into account the parameters used to calculate the indices and the information described above, in general, these results seem to be in line with expectations. Based on temperature data from the Cádiz agroclimatic stations, it was also observed that the

values are higher for 28/05/19 (lower temperatures) compared to 30/08/19 (higher temperatures). Although the minimum values were recorded for 05/11/19, it is expected that they would increase from then on (throughout the winter). In this experiment it is assumed that food availability is not the limiting factor since this is an IMTA and organic matter input was supposed to be almost constant (without much variation). Thus, the season/temperature of the water should have been one of the main factors influencing the CI values obtained.

As already showed in several studies, in IMTA systems, *C. gigas* tends to have higher condition indices and therefore, better quality oysters compared to traditional culture systems (Castillo-Durán *et al.*, 2010; Schupp, 2015). Hence the need to promote this type of production system for oyster farming.

4. BIOSEA+SIMBA projects

4.1. Overview

These two projects were both developed under the European Program Horizon 2020. Starting with the BIOSEA project, its main focus was, essentially, promote the development and validation of innovative, competitive and economical upstream (cultivation + biomass stabilization), and downstream (cell disruption + extraction and purification) processes for the cultivation of 2 microalgae (*Spirulina platensis* and *Isochrysis galbana*) and 2 macroalgae (*Ulva ohnoi* and *Saccharina latissima*), in order to produce and extract high-value active principles at low cost (up to 55% less compared to current processes) for use in food, feed and cosmetics/personal care as products with high added value. Various institutions from Spain, Belgium, Turkey, Germany and Netherlands, participated in this project. Once again, the structural organization of this project was based on 7 work packages and my work was focused on WP 1 (with the aim of cultivating micro and macro algae and optimization of their growth), WP 2 (with the purpose of extraction and purification of active principles, using innovative approaches to maximise the yield, optimize the cost and the valorisation of the biomass components), WP 3 (where the active principles and different formulations for each industrial sector were evaluated and developed, respectively) and WP 4 (validation of final application products).

On the other hand, SIMBA project involves the participation of 11 different countries, in a total of 23 European partners. Its fundamental objective is to harness complex soil and marine microbial communities for the sustainable production of food in the agricultural and aquaculture sector. This project is divided into 10 work packages and I was involved on WP 3 whose overall objective was to optimize the application of marine microorganisms in the production of feed for fish, algae and plants.

4.2. Material and methods

- Stock cultures

Stock cultures of *U. ohnoi* and *G. gracilis* (Figure 9) were established in a growth chamber (Radiber GERHR-360), under controlled conditions, at a constant temperature of 20ºC, with light being provided by 6 tube lamps 18W T8 LED 5000K. The development of these cultures in glass flasks in triplicate for each species (500 mL, 1000 mL or 2000

mL, depending on the biomass sown; with densities being variable over the weeks) was followed weekly. The biomass (which would later be sown) was weighed after centrifugation (utilizing a salad spinner), and flasks were cleaned with a bottle cleaning brush (if too dirty, chlorine bleach was used). A total renewal of the water (previously mechanical filtered, through porous filters: 40 µm, 20 µm and 1 µm) was made and the respective nutrients, whose volume was already established for each species, were added. So, in the case of *Ulva*, the modified F/2 medium (Fitoplancton Marino: easy algae®), commercially available, was used, in the proportion of 1 mL/L. In *Gracilaria*, the nutrient medium to be used was prepared according to the protocol proposed by Von Stosch (Yarish *et al.*, 2012), which also followed the proportion of 1 mL/L. The aeration in the flasks was provided by a pressurized tube connected to plastic serological pipettes by aquarium airlines fitted with control valves and connectors. The rate of aeration was adjusted visually, avoiding the fragmentation and trying to maintain the same airflow in all the flasks. Based on the weight data that was collected, Specific Growth Rate (SGR) values were calculated.

Figure 9: Stock cultures of *Ulva ohnoi* and *Gracilaria gracilis.*

- Outdoor tanks and indoor photobioreactors

U. ohnoi was also cultivated in two outside tanks of 500 L (Figure 10) each and in six inside photobioreactors of 75 L, under exposure to artificial light, provided by a LED tube T8 18W 6000K (16:8 Light:Dark cycle) per photobioreactor.

Regarding the 500 L tanks, that were exposed to variable weather conditions, monitoring was done once a week, and the procedure went through the following steps, for each tank:

- Firstly, to determine the amount of nutrients to be added further ahead, it was necessary to collect a water sample, before starting with the harvest and cleaning process. So, the sample was collected to a 5 mL Eppendorf tube with the aid of a 10 mL syringe with a 0. 45 µm filter on top. Besides that, salinity was also measured with a refractometer.
- Hereupon, the air pump was turned off and then I proceeded to the harvest the algae using a net, placing them in a plastic box. After they are all collected, were transferred to a professional salad spinner for centrifuge. Finally, they were weighed using a digital kitchen scale (Lacor 61735) and put back in the plastic box.
- After this, the tap was opened, letting the water go out and, meanwhile, the tank, the temperature sensor (iButton thermochron) and the aeration tube, were cleaned using a broom or a brush. Afterwards, the tank was rinsed and filled with saltwater (with a filter bag at hose tip) and the air pump was switched on. When the tank was too dirty and/or when the water was murky which could compromise the growth, a total change of water was made. On the other hand, if the water was neither dirty nor murky, the change was partial (10% of water change).
- After the harvest and cleaning, salinity and water sample collection was repeated as initially.
- When replanting, the portion of algae to be used was taken from the plastic box, weighed and placed back into the tank. The leftover biomass was rinsed with freshwater, spun dry and dried in a homemade dryer (constituting the samples that would later be sent to VITO, a Belgium partner of the project BIOSEA, in order to analyse the presence of bioactive compounds).
- Finally, the water samples collected earlier were analysed to determine the amount of nutrients present in the water and calculate the amount required to add to the tank in order to avoid a nutritional deficit capable of compromising growth. The absorbance's at 220 and 275 nm were measured, using a Zuzi model 4251/50 spectrophotometer. For the "white" demineralized water was used. The absorbance values were then computed in an excel database with pre-established formulas (Gross *et al.*, 1999). The nutrients mix utilized was the Cell-Hi F2P (Varicon Aqua), a powder mixture based on the Guillard F/2 medium that has the same N, P, trace element and vitamin content. Besides that, it's probably the only medium for culture of micro- and macroalgae available commercially in powder form. So, after weighing using an ADAM PW 254 precision balance, the medium was added to the tank.

Based on the SGR, water temperature (directly through the OneWireViewer) and light (taken from the Cádiz agroclimatic stations ID station 10, ID station 5 and ID station 11; basically downloading the data from ID station 10 or, if there is no data at this station for some period of interest, by averaging ID station 5 and ID station 11) data obtained, further ahead is presented a graphical approach that relates these variables.

Figure 10: *Ulva ohnoi* cultivated in tanks.

In the photobioreactors (Figure 11), the methodology used was similar to that of tanks and monitoring was also weekly. Thus, basically, for each photobioreactor, the following tasks were performed:

- First, algae were collected using a net and put in a mesh bag. Then, aeration provided by a pressurized tube connected to the bottom of the cylinder by aquarium airlines fitted with a valve, was turned off. The tap was opened, letting all the water goes out. Meanwhile, this mesh bag containing the algae was placed on the professional salad spinner for centrifuge.
- After this, the photobioreactor (and the temperature sensor, if present) was cleaned with freshwater and scrubbed with a cleaning brush. If too dirty, chlorine bleach was used.
- Cylinder was rinsed and filled with saltwater, previously filtered by a sequence of mechanical filters (40 µm, 20 µm and 1µm) to avoid fouling by particles and organisms. Aeration was turn on and visually adjusted.
- Then, already spun dry, the mesh bag containing the algae, was weighed (weight includes algae + mesh bag that had 31 g after spun dry) utilizing a digital kitchen scale (Lacor 61735). Part of the biomass was used for restocking, the remaining biomass was rinsed with freshwater, spun dry and dried in a homemade dryer or

frozen in a Liebherr freezer at -21ºC until future analysis (namely by VITO, for analysis of bioactive compounds).

- Finally, salinity was measured, and the nutritional medium was added. Here the medium used was the modified F/2 (Fitoplancton Marino: easy algae®) and the volume applied varied throughout the cultivation (base value of 30 mL per photobioreactor).

Biomass weight and water temperature (through the OneWireViewer) data from photobioreactors, were, posteriorly, used to calculate and graphically represent the SGR vs. temperature.

Figure 11: *Ulva ohnoi* cultivated in photobioreactors.

- Photobioreactors – temperature experiment

At a later stage, I started a new experience that lasted 3 weeks, aiming to observe the influence of different temperatures on the SGR and ash content in *U. ohnoi*. Basically, 3 heaters EHEIM JAGER 150W were installed on 3 of the 6 photobioreactors and were set for a temperature of about 25ºC. The other photobioreactors remained at room temperature (water temperature around 18-19ºC average) which, in this case, corresponded to lower values than the heated ones. Additionally, 3 more temperature sensors (iButton thermochron) were placed in each heated photobioreactor, so 4 sensors in total were installed (1 in one of the photobioreactors at room temperature, the remaining 3 in each of the heated photobioreactors), allowing a more reliable temperature follow-up. During these weeks, the before mentioned methodology was followed, but with some remarks that should be pointed:

- First and second weeks were like a period of acclimation of *Ulva* to the stipulated temperatures.

- Previously to the process of restocking, extreme careful was taken not to mix the "heated" and "unheated" algae, so the algae harvested in a photobioreactor, were always replaced in that same photobioreactor. Furthermore, in the case of heated photobioreactors, algae were added only when the temperature reached the desired level, to avoid thermal shock.
- Algae were weekly replanted with 80 g of biomass per photobioreactor and 40 mL of F/2 medium.
- At the end of the experiment (third week), 50 g of algae from each photobioreactor were harvested, stored in plastic bags and frozen at -21ºC, for further analysis. At same time, two small portions of algae from each photobioreactor were collected to an aluminium foil container for ash content determination.

In this experiment, ash percentage was measured through the process of dry ashing (Figure 12). Algae were previously weighed, dried at 60°C (> 24 h), then incinerated at 550ºC during around 4 h and finally weighed again (Liu, 2019).

Results were analysed statistically using the statistical analysis software IBM SPSS statistics 26. The means were compared through the two-way blocked ANOVA (for SGR: temperature as experimental variable and date as blocking factor) and the independent t-test (for ash content) to assess significant differences between distributions (95% confidence interval). When a significant interaction between treatment and date was found the simple main effects were analysed thourgh a simple ANOVA. Prior to the analysis, normality and homoscedasticity of distributions were verified (Shapiro Wilk and Levene's test, respectively). Data is presented as means ± standard deviation.

Figure 12: *Ulva ohnoi* samples preparation for dry ashing.

- Photobioreactors – VITO data analysis

Additionally, I also carried out the analysis of results obtained in the scope of 3 different experiments, also performed in the same before mentioned 6 photobioreactors:

- A salinity trial where *U. ohnoi* was exposed to different salinities (low salinity: 25 ‰; control: 37 ‰; high salinity: 50 ‰), in duplicates.
- A N-source trial where *U. ohnoi* was exposed to either nitrate (NO_3) or ammonium (NH_4^+), in triplicates.
- A P-concentration trial where *U. ohnoi* was exposed to low phosphate (N:P, 48:1), normal phosphate (N:P, 24:1) and high phosphate (N:P, 12:1) media, in duplicates.

The results determined and sent by VITO partner were analysed statistically using the statistical analysis software IBM SPSS statistics 26. The means were compared through several two-way blocked ANOVA's in each of the experiments (treatment: experimental variable; date: block; protein, sugars, lipids and ash contents: dependent variables) to assess significant differences between distributions (95% confidence interval). When a significant interaction between treatment and date was found the simple main effects were analysed thourgh a simple ANOVA. Prior to the analysis, normality and homoscedasticity of distributions were verified (Shapiro Wilk and Levene's test, respectively). Data is presented as means ± standard deviation.

- Trials with European seabass (nutritional and physiological tests)

Regarding the European seabass nutritional test, three experimental diets were evaluated against a control diet (four replicates for each diet) (Figure 13). Based on previous observations (such as good indicator of immune system, great antioxidant power, etc.), the different extracts to be included in the diets were selected: the *A. platensis* protein extract, the *U. ohnoi* polysaccharide extract and the *S. latissima* alginate/laminarin extract. These diets were formulated by DIBAQ GROUP and elaborated following the corresponding feed formulas by SPAROS R&D. So, diet formulations were developed according with the different percentage of extract inclusion shown in the Table 2.

DIETA CONTROL MATERIA PRIMA	%
HARINA DE PESCADO (70% PB)	23,86
TRIGO	12,00
HARINA DE SOJA (47% PB)	9,84
GLUTEN DE MAIZ	9,62
CONCENTRADO DE SOJA (60%PB)	9,07
ACEITE DE PESCADO	7,00
ACEITE VEGETAL	6,44
HARINA DE GUAR	6,00
PROTEINA DE GUISANTE (72% PB)	5,00
LEVADURA	5,00
ALMIDON DE GUISANTE	2,65
FOSFATO MONOCALCICO	1,00
HARINA GIRASOL (34% PB)	0,98
LISINA	0,50
ANTIOXIDANTE Y ANTIFUNGICO	0,30
COLINA	0,20
TAURINA	0,15
PREMIX VITAMINICO	0,10
PREMIX MINERAL	0,10
VITAMINA C	0,10
BETAINA ANHIDRA	0,10

DIETA A MATERIA PRIMA	%
HARINA DE PESCADO (70% PB)	15
CONC. PROT. SPIRULINA	15
CONCENTRADO DE SOJA (60%PB)	15
TRIGO	9
PROTEINA DE GUISANTE (72% PB)	9,8
GLUTEN DE MAIZ	8,9
ACEITE VEGETAL	7,35
ACEITE DE PESCADO	7
LEVADURA	5
ULVA	3
ALMIDON DE GUISANTE	1,9
FOSFATO MONOCALCICO	1
LISINA	0,5
HARINA DE GUAR	0,5
ANTIOXIDANTE Y ANTIFUNGICO	0,3
COLINA	0,2
TAURINA	0,15
PREMIX VITAMINICO	0,1
PREMIX MINERAL	0,1
VITAMINA C	0,1
BETAINA ANHIDRA	0,1

DIETA B MATERIA PRIMA	%
HARINA DE PESCADO (70% PB)	15
CONC. PROT. SPIRULINA	15
CONCENTRADO DE SOJA (60%PB)	15
TRIGO	10
PROTEINA DE GUISANTE (72% PB)	9,8
GLUTEN DE MAIZ	8,9
ACEITE VEGETAL	7,35
ACEITE DE PESCADO	7
LEVADURA	5
SACCHARINA	2
ALMIDON DE GUISANTE	1,9
FOSFATO MONOCALCICO	1
LISINA	0,5
HARINA DE GUAR	0,5
ANTIOXIDANTE Y ANTIFUNGICO	0,3
COLINA	0,2
TAURINA	0,15
PREMIX VITAMINICO	0,1
PREMIX MINERAL	0,1
VITAMINA C	0,1
BETAINA ANHIDRA	0,1

DIETA C MATERIA PRIMA	%
HARINA DE PESCADO (70% PB)	15
CONC. PROT. SPIRULINA	15
CONCENTRADO DE SOJA (60%PB)	15
TRIGO	9,5
PROTEINA DE GUISANTE (72% PB)	9,8
GLUTEN DE MAIZ	8,9
ACEITE VEGETAL	7,35
ACEITE DE PESCADO	7
LEVADURA	5
ALMIDON DE GUISANTE	1,9
ULVA	1,5
SACCHARINA	1
FOSFATO MONOCALCICO	1
LISINA	0,5
HARINA DE GUAR	0,5
ANTIOXIDANTE Y ANTIFUNGICO	0,3
COLINA	0,2
TAURINA	0,15
PREMIX VITAMINICO	0,1
PREMIX MINERAL	0,1
VITAMINA C	0,1
BETAINA ANHIDRA	0,1

Figure 13: Composition of experimental diets (control, A, B and C).

Table 2: Experimental diets according with % algae extracts included.

DIET	INGREDIENT	SOURCE	% INCLUSION
A	Protein concentrate	*Arthrospira platensis*	15.0
	Polysaccharides rich fraction	*Ulva ohnoi*	3.0
B	Protein concentrate	*Arthrospira platensis*	15.0
	Alginates/laminarin rich fraction	*Saccharina latissima*	2.0
C	Protein concentrate	*Arthrospira platensis*	15.0
	Polysaccharides rich fraction	*Ulva ohnoi*	1.5
	Alginates/laminarin rich fraction	*Saccharina latissima*	1.0
CONTROL	N / A	N / A	N / A

The experiment period was 10 weeks (including 1-week acclimatization) and the performance of the diets was evaluated by comparing fish growth and health indices. In terms of cultivation system, fish, obtained from a commercial fish farm (Andromeda Group, Spain), were maintained in a recirculating aquaculture system (RAS) (Figure 13). Specifications of RAS selected for this trial are summarized below:

- 16 tanks of 300 L each
- 5-10% daily water renewal
- Air stone
- Controlled water flux according to fish size
- Temperature control (21 ± 1°C)

- Photoperiod control (12light:12 dark)
- Mechanical filtration (filter wool)
- Biological filtration (bio balls)
- Protein skimmer

A preliminary survey of the population was carried out to select fish with similar weight in all groups. A coefficient of variation of 15% was applied. Selected fish had an average weight of 1.7 ± 0.1 g fresh weight and 60 were randomly allocated to each tank (same initial biomass for each tank) in the beginning of the experiment. Fish and tanks were tended and monitored on a daily basis: cleaning and maintaining the different components of the RAS; verifying the fish status and behavior; registering and controlling water parameters (dissolved O_2 and temperature with an OxyGuard Handy Polaris 2; NO_2^- and NH^{4+} with Merck KGaA MColortest™ kits). Fish were fed by hand *ad libitum* 3 times a day for 6 days a week. I was also involved in the process of sampling that took place every 14 days. During this experiment, a total of 5 samplings were made, consisting on weighing all the fish in groups of 5 individuals for all the tanks. However, in the final sampling the fish were weighed individually. Before each sampling, study fish were kept off feed for 24 h and clove oil (0.02 mL/L) was used as an anesthetic for handling and weighing them.

Overall, the parameters determined were the following: SGR; weight gain (ΔW); survival (SV); feed conversion ratio (FCR); feed intake; Fulton's condition factor (K); hepatosomatic index (HSI); viscerosomatic index (VSI).

Figure 14: General view of the recirculating aquaculture system (C-300).

The immunoassays analysis was carried out at the end of the trial. Three blood samples (serum) from three randomly selected fish per tank were collected to evaluate the immune status of fish from each group by colorimetric assay kits. Parameters analyzed were lysozyme, antiprotease and superoxide dismutase activity, functioning as biomarkers of immunostimulant and antioxidant activity.

Apart to the immunoassays, histology of the middle intestine (one fish per tank, four replicates per diet) was also done. Samples of midgut from the 16 fish in total (4 fish for each diet) were fixed in 10% buffered formalin and sent to a specialized lab (Ictiovet S.L.) for the histopathological analysis. An intestinal score was established for each sample as an indicator of global intestinal status and incidence of intestinal changes (range 0-24, from less to highest incidence, respectively). The determination of the intestinal score took into account the following parameters: infiltrate of the mucosa; infiltrate in the lamina propria; apoptotic forms; increment of mucous cells; infiltrate in the submucosa; increment of the thickness of the lamina propria; increment of the thickness of the submucosa; congestion of the submucosa.

Soon after the end of the trial, experimental fish were also subjected to a physiological test to determine if differences of stress in fish blood serum exist between groups after stress exposure. The challenge was performed at same holding RAS and ambient conditions and consisted on the determination of the fish stress status of all groups (control diet; diet A; diet B; diet C) at different times:

- T0: Basal fish stress status right before stress exposure
- T1: Fish stress status 1 h after stress exposure
- T24: Fish stress status 24 h after stress exposure

Firstly, prior to stress (T0), 4 fish from 2 replicates of each group/treatment were sampled for blood parameters. The stress exposure was conducted by reducing the water volume in the tanks, obtaining a water volume around 30 L/tank and therefore increasing the stocking density in the tank (confinement). The water volume reduction was followed by disturbing fish with a net (chasing them, simulating fish catching) during 1 h in intervals of 5 min (netting). After that, again 4 fish from 2 tanks of each treatment were sampled 1 h after the stress. For the other 2 tanks of each treatment, blood samples from 4 fish were also collected 24 h post-stress. In the final, 32 samples in basal conditions, 32 samples 1 h post-stress and 32 samples 24 h post-stress (total of 96 blood samples) were obtained and the following parameters were analyzed as stress indicators: transaminases (AST-GOT and ALT-GPT), glucose, triglycerides and lactate dehydrogenase (LDH) (Figure 14) (Fernández-Alacid *et al.*, 2019).

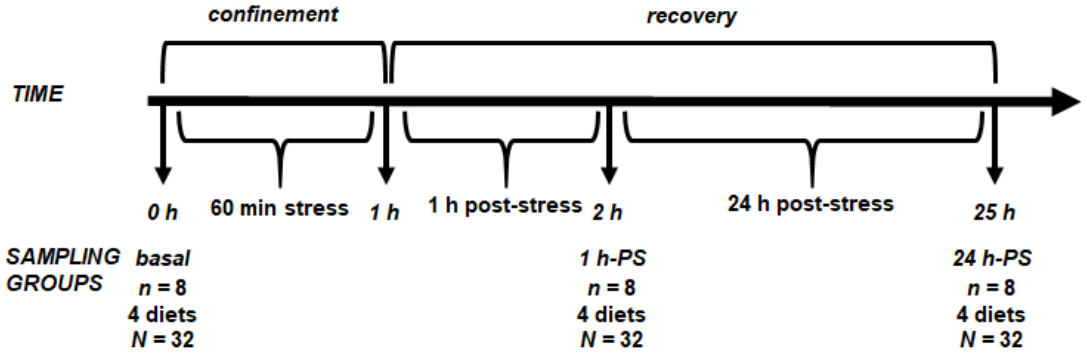

Figure 15: Schematic representation of the physiological stress test.

Globally, results were analysed statistically using the statistical analysis software IBM SPSS statistics 26. The means of all experimental groups were compared through the one-way ANOVA to assess significant differences between distributions (95% confidence interval), followed by a post-hoc analysis (Tukey test) to compare all possible pairs of means. Prior to the analysis, normality and homoscedasticity of distributions were verified (Shapiro Wilk and Levene's test, respectively). Data is presented as means ± standard deviation.

- Seaweed-associate microbiome

Regarding the study of microbiomes, I only participated in an initial stage of the experiment related to the evaluation of *U. ohnoi* associate microbiome, where we tried to ascertain the functionality of the established and proposed protocol by NIOZ (one of the partners of SIMBA project). Therefore, the procedure was developed in 2 phases, one relative to *U. ohnoi* sample collection, other to water sample collection.

Starting with the methodology for *U. ohnoi* sample collection (Figure 15), firstly algae were collected (approximately, 5 thalli, the sufficient to cover 5 petri dishes of 100 mm diameter), in this case, from one of the two outdoor tanks. Then, excess of water was removed and algae were placed in a clean beaker glass. In a laminar flow cabinet, the thalli were rinsed (~5 times) with excess sterile 3% NaCl, solution previously prepared and autoclaved. After, each thallus was spread in 5 separate sterile Petri dishes. Avoiding damage of the samples as much as possible (to prevent the release of *Ulva* cells), a sterile cotton swab was used for each sample, swiping the thallus on one side and applying a slightly pressure (avoiding the cotton to become green) to ensure obtention of associated bacteria. Each cotton swab corresponding to each sample, was

placed in a tube and frozen immediately at -80ºC in a Froilabo BIO Memory 690 L, until shipping.

On the other hand, water sample collection is essential to discriminate between the “true associated microbiome” and non-associated (free-living) microbial species. Thus, using a clean bottle, 3 L of water from an outdoor pond were collected. Resorting to vacuum filtration, every 1 L of water (corresponding to 1 sample), was filtered using sequentially 5 µm and 0.45 µm Sterivex filters. Then, water coming from 0.45 µm was filtered using a syringe of 60 mL fitted with a 0.22 µm Sterivex cartridge (must be completely clogged, if not, means that we should filter more water for each sample). After this, the cartridge was sealed using a sticky tack and stored at -80ºC in a Froilabo BIO Memory 690 L, until shipping. The time since the sample collection until the final 0.22 µm filtration should not exceed 30 min, since it can compromise data reliability.

Later, the 5 *Ulva* and 3 water samples were sent to the partner NIOZ, to carry out the DNA analysis and determine the variety of prokaryotes existent.

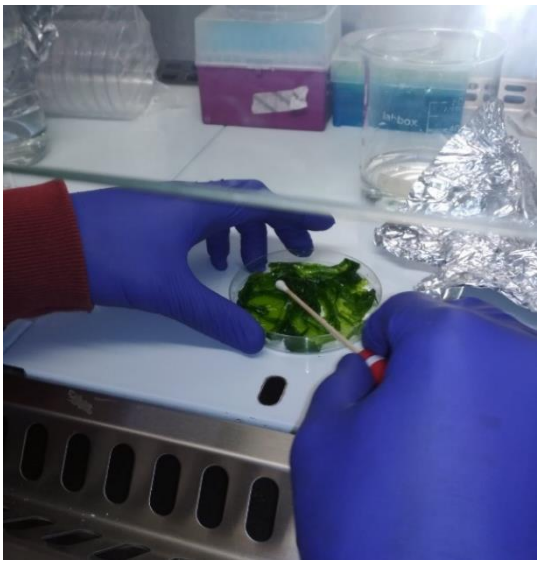

Figure 16: *Ulva ohnoi* sample collection.

Hereafter are presented the formulas used to calculate all the different, before mentioned, parameters:

$\mathbf{SGR} = \frac{(\ln Wf - \ln Wi)}{t} \boldsymbol{x100}\%$, where Wf and Wi are final and initial fresh weight (g) respectively and t is time in days;

$\mathbf{Ash\% \ of \ DW} = \frac{(\boldsymbol{Ash\ weight\ (g)})}{(\boldsymbol{Dry\ weight\ (g)})} \boldsymbol{x100\%}$;

$\mathbf{\Delta W} = \frac{\boldsymbol{Wf-Wi}}{\boldsymbol{Wi}} \boldsymbol{x100\%}$, where Wf and Wi are final and initial fresh weight (g) respectively;

$\mathbf{SV} = \frac{\boldsymbol{Nf\ individuals}}{\boldsymbol{Ni\ individuals}} \boldsymbol{x100\%}$, where Nf and Ni individuals are the number of fish at the end and the beginning of the study respectively;

$\mathbf{FCR} = \frac{\boldsymbol{Total\ feed\ supplied\ (g)}}{\boldsymbol{Total\ fresh\ weight\ increase\ (g)}}$;

$\mathbf{K} = \frac{\boldsymbol{Fresh\ weight\ (g)}}{\boldsymbol{Total\ length\text{^}3\ (cm)}} \boldsymbol{x100\%}$;

$\mathbf{HSI} = \frac{\boldsymbol{Wl}}{\boldsymbol{Wt}} \boldsymbol{x100\%}$, where Wl is liver weight (g) and Wt is total weight of the fish (g);

$\mathbf{VSI} = \frac{\boldsymbol{Wv}}{\boldsymbol{Wt}} \boldsymbol{x100\%}$, where Wv is viscera weight (g) and Wt is total weight of the fish (g).

4.3. Results

- Stock cultures

Since the cultivation conditions were always the same over a period of 14 continuous weeks of cultivation (in a growth chamber, with a constant luminosity and temperature, and equal concentration of nutrients), through the observation of Figure 17, it is clearly visible that the amount of algae that was sowed, each week, in each of the 3 glass flasks, had influence in the measured SGR values, insofar as lower sowed biomass is associated with higher SGR values, and higher sowed biomass is associated with lower SGR values.

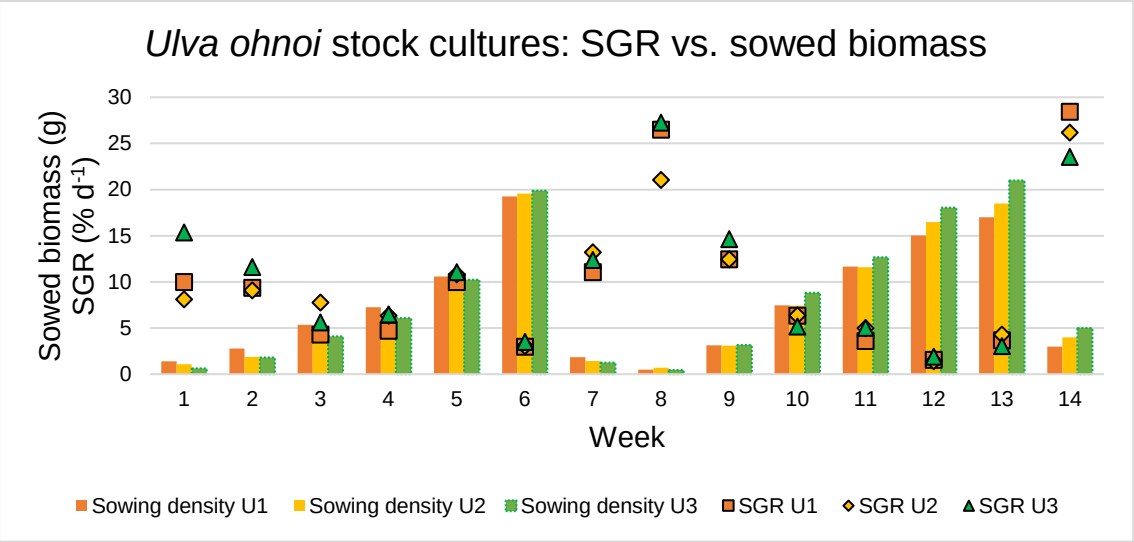

Figure 17: *Ulva ohnoi* specific growth rate (SGR) and sowed biomass for each replica (out of 3) throughout 14 weeks of cultivation in the growth chamber.

- Outdoor tanks

Based on Figure 18, it can be seen that the SGR values were quite variable throughout the cultivation in the two outdoor tanks, with an average maximum recorded of about 30% d^{-1} in the week 04/12/19-11/12/20 and minimum values registered in the period 03/03/20-18/03/20. Moreover, there does not seem to be an obvious correspondence between these values and the different values of temperature and solar radiation. Thus, from what is observed, it can be said that it is not possible to establish a pattern between the SGR and the temperature and solar radiation.

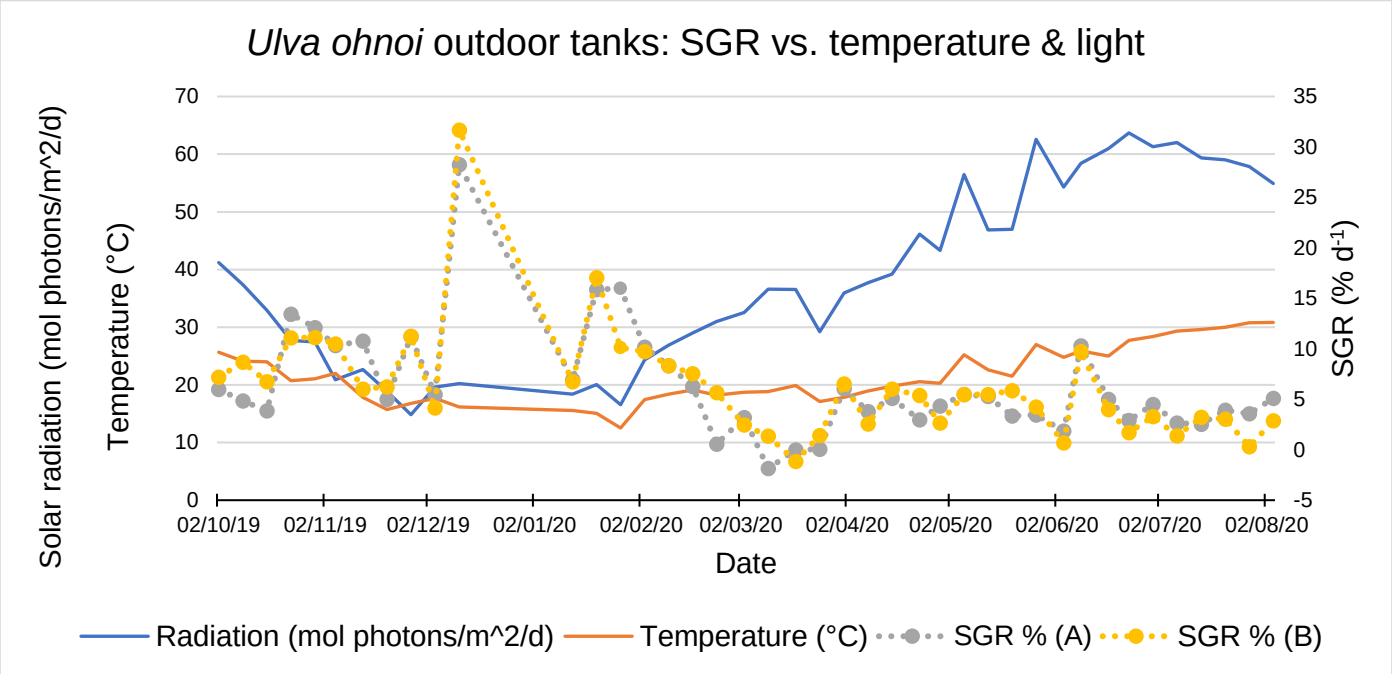

Figure 18: *Ulva ohnoi* specific growth rate (SGR), grown in two outdoor tanks (A and B), along with weekly means of water temperature and light/solar radiation, during the period 02/10/19-04/08/20.

On the other hand, Figure 19 shows an important aspect, already mentioned for stock cultures, and that must be taken into account: the amount of alga that was reseeded each week (sowing density). Thus, in general, once again, lower values of sowing density corresponded to higher values of SGR, and higher values of sowing density were associated with lower values of SGR. In fact, this is visible when comparing the two tanks whose sowing densities were variable throughout the weeks. Mostly, the bars corresponding to "other density" (tank with lower SGR) are above the crosses that signal the values of "related density" (tank with higher SGR), demonstrating that trend.

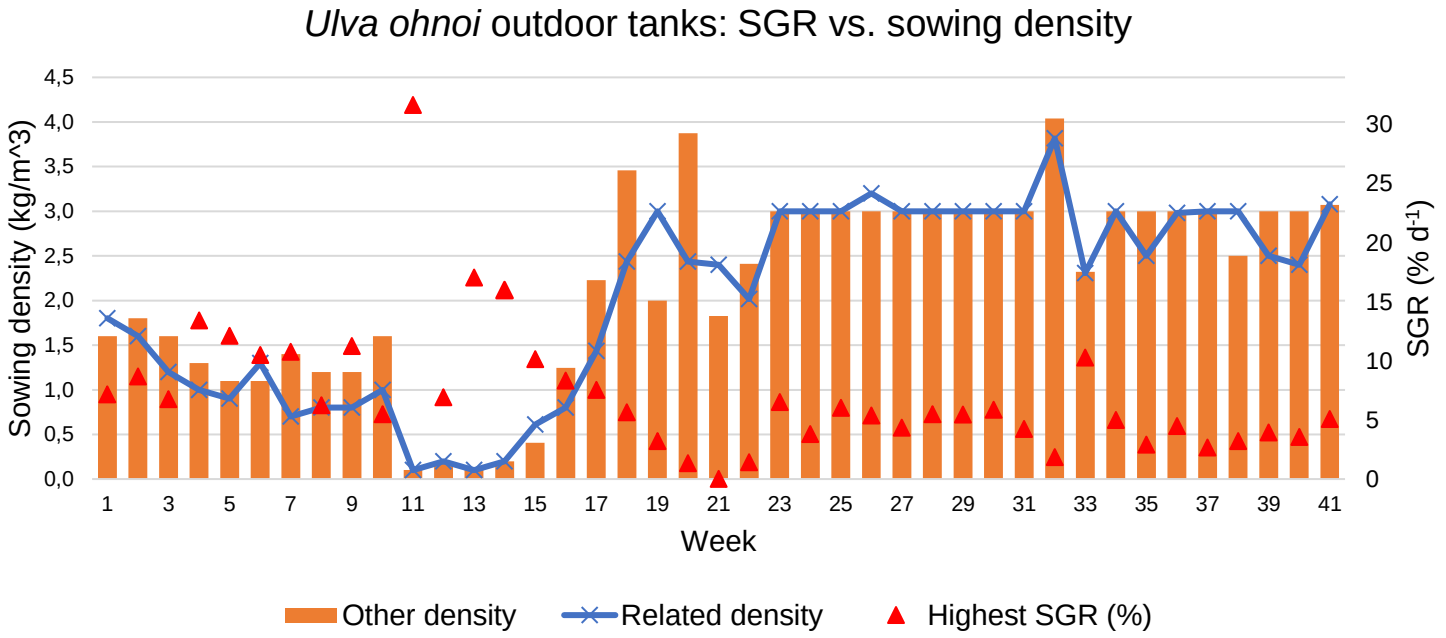

Figure 19: *Ulva ohnoi* outdoor tanks cultivation, during the period 02/10/19-04/08/20 (represented by week 1 to week 41), where weekly "Highest SGR" values correspond to the tank with the highest growth rate (out of 2). Sowing density is represented by "Related density", the density associated to the tank with highest SGR, and "Other density", the density used in the tank with lower SGR.

Some remarks considered while analysing these graphs (Figures 18 and 19) were the following:

- For the weeks 4, 11, 13, 23, 25, 29, 31, 33, 35, 37, 39 and 41 a total changed of water was effectuated, due to dirt, turbidity or microalgae growth. Hence, after these total exchanges, it is expected that SGR tends to increase suddenly in that particular week.

- Between the week 11 (ending at 11/12/19) and "week" 12 (starting at 11/12/19; ending at 13/01/20) no maintenance was carried out, nor any change or replacement of water, so, due to evaporation (possibility of the water temperature sensor have been in direct contact with the air), the temperature values, especially, maximum and minimum temperatures (25,375 and 5,125ºC of temperature, respectively), should be considered as doubtful values. Therefore, the SGR values calculated for "week" 12 should not be considered as reliable.

- Weeks 20, 21 and 22 registered the lowest values of SGR, possibly due to the phytoplankton bloom observed.

- Indoor photobioreactors

In Figure 20 it is difficult to observe an obvious pattern, but, once again, the highest values of SGR (weeks 2, 3 and 4) seem to coincide with the lowest values of sowing density (although temperatures do not seem to be influencing SGR, because they are different between weeks, makes this conclusion questionable).

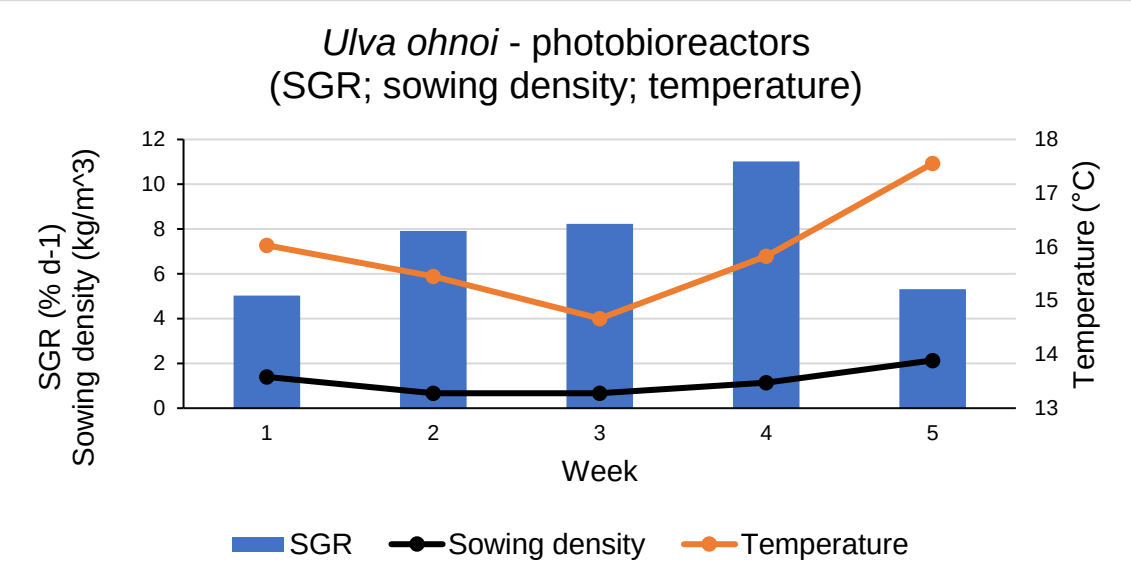

Figure 20: *Ulva ohnoi* specific growth rate (SGR), respective sowing density and weekly average water temperature recorded for the photobioreactor with highest growth rate (out of 6), during a period of 5 weeks.

- Photobioreactors – temperature experiment

Table 3 presents SGR data analysis from the temperature experiment. A two-way blocked ANOVA was carried out to assess whether there were significant differences in SGR, considering the treatment (fixed factor) and week (random factor/block). The data followed a normal distribution (p-value=0.156) and homogeneity of variance was confirmed (p-value=0.537). The interaction effect (Treatment*Week) shows a statistically significance value (p-value=0.000).

Table 3: Results of two-way blocked ANOVA showing the effect of the independent variables ("Treatment" and "Week") on the dependent variable "SGR".

Variable	Factors	*df*	*F* value	*P*-value	Levene's test	
SGR					*F* Value	0.856
	Treatment	1	0.211	0.691	P-value	0.537
	Week	1	0.129	0.886		
	Treat*Week	1	32.101	0.000	*df1*	5
	Error	12			*df2*	12

Therefore, simple main effects were executed and analysed. Results are shown in Figure 21 and as can be observed no significant differences were found between week 1 and week 2. On the other hand, within each of these weeks, the SGR value for the treatment "Heated" was significantly higher compared to the treatment "Unheated". Compared to weeks 1 and 2, week 3 had the highest significant SGR value for "Unheated" and the lowest significant SGR value for "Heated". Moreover, within this week, the SGR value for "Unheated" was significantly higher compared to the treatment "Heated" (9.3 and 6.6% d^{-1}, respectively).

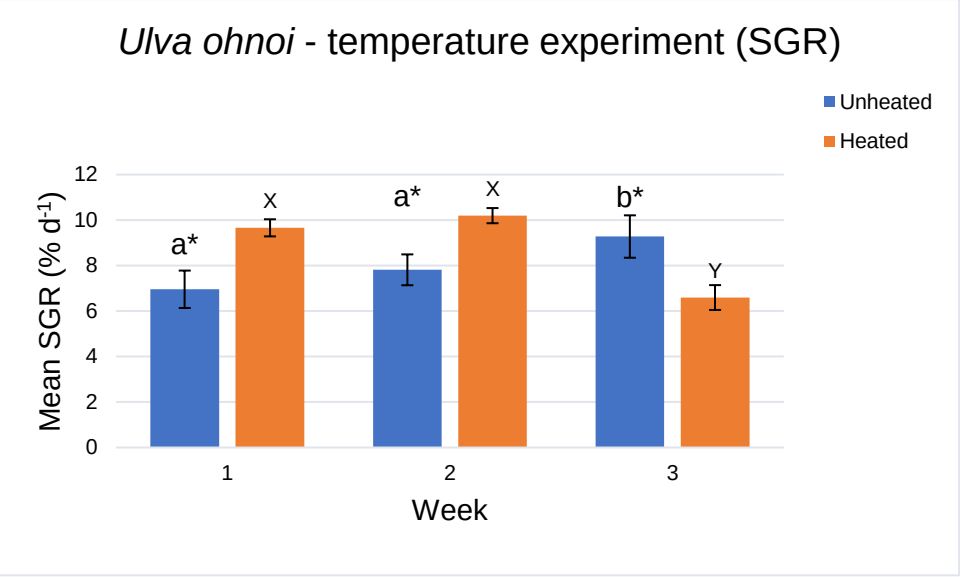

Figure 21: *Ulva ohnoi* average specific growth rate (SGR) ± SD during the 3 weeks of temperature experiment. Treatments (n=3) are represented by the blue ("unheated") and orange bars ("heated"). Different letters (compare weeks) and asterisk presence/absence (compare within the week) indicate significant differences between groups (p ≤0 .05).

An independent t-test was also performed to assess whether at the end of the third week of experiment the mean ash content in algae showed significant differences between the two treatments. Thus, given the p-value of 0.248 (Table 4), we can conclude that there are no significant differences between the "Unheated" and "Heated"

treatments and we can actually confirm this in Figure 22 in which the mean ash content is higher for the “Unheated” treatment, but very slightly, almost imperceptibly (29.7%/dry matter for “Unheated” vs. 29.1%/dry matter for “Heated”).

Table 4: Results of independent T-test testing the statistical significance of *Ulva ohnoi* ash content according to the treatments (“Unheated” and “Heated”).

Variable	Levene's test		t	df	*P*-value
Ash	*F* value	1.822	0,883	4	0.427
	P-value	0.248			

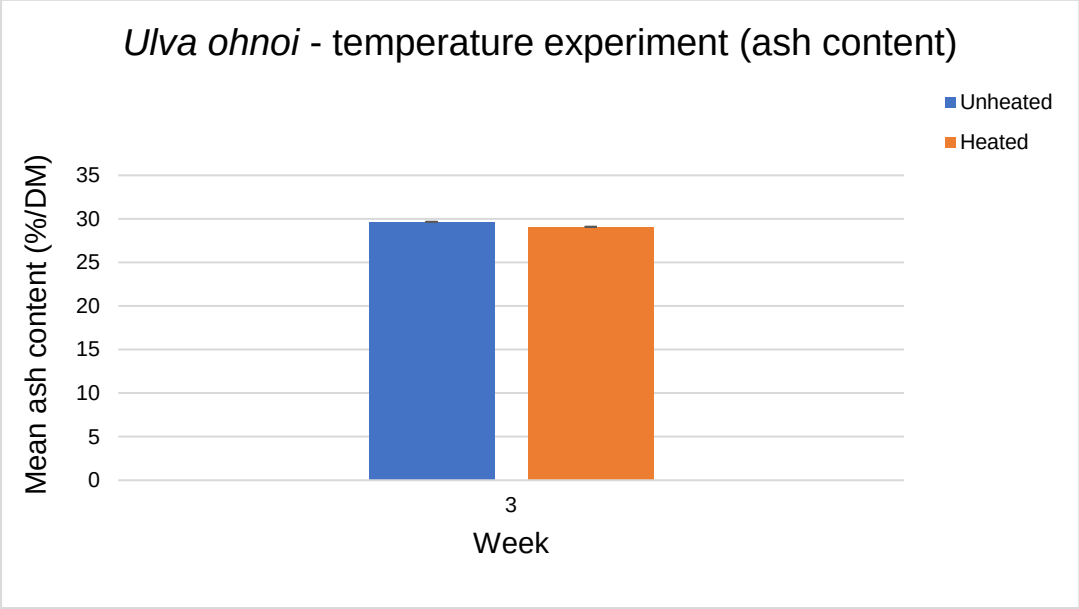

Figure 22: *Ulva ohnoi* average ash content ± SD registered in the third and last week of the temperature experiment. Treatments (n=3) are represented by the blue (“unheated”) and orange bars (“heated”).

- Photobioreactors – VITO data analysis

Regarding the salinity experiment (Tables 5 and 6) it can be concluded that in terms of ash content there were no significant differences, neither between treatments (p-value = 0.309), nor even between dates (p-value = 0.507). In addition, the interaction between treatment and date did not show to be statistically significant (p-value = 0.253).

As for the dependent variable lipids, the interaction effect (Treatment*Date) shows a statistically significant value (p-value = 0.012), so analysis of simple main effects was conducted. For the first week (date 19/07/2019) a significant higher value was recorded for high salinity (HS) compared to low salinity (LS), whereas in the second week (date 26/07/2019) all treatments were significantly different among them (with the highest

value for the high salinity treatment). In addition, comparing the weeks, a significant value was only recorded for LS, being higher for the first week.

In relation to protein content, the main effect of treatment was found to be statistically significant (p-value = 0.013), specifically between low salinity (LS) and control (C) plus low salinity (LS) and high salinity (HS), with an average of 23.0%/DM for low salinity, 19.3%/DM for control and 20.1%/DM for the high salinity treatment, over the two experimental weeks. For the date factor no significant differences were observed (p-value = 0.052).

Relatively to sugars content, the interaction effect was statistically significant (p-value = 0.020). Therefore, analysis of simple main effects was conducted and shown that in the second week (date 26/07/2019) a significant lower value was recorded for HS compared to LS and C. Moreover, between weeks, sugar content for HS showed to be significantly lower in week 2.

Table 5: Results of 4 two-way blocked ANOVA's for the salinity trial, showing the effect of the independent variables ("Treatment" and "Date") on the dependent variables ("Ash", "Lipids", "Protein" and "Sugars") (*P < 0.05; **P < 0.01; ns = not significant). LS = low salinity, C = control and HS = high salinity.

Two-way ANOVA	Date	Treatment	Date x Treatment	LS	C	HS
Ash	ns	ns	ns	-	-	-
Lipids	ns	ns	*	-	-	-
Protein (N-based)	ns	*	ns	a	b	a
Sugars	ns	ns	*	-	-	-

Table 6: Results of analysis made by VITO for the two weeks' salinity trial. Treatments are represented by the initials LS (low salinity), C (control) and HS (high salinity). All four dependent variables (ash; lipids; protein; sugars) are presented as mean %/DM ± SD (considering that for each treatment corresponds two photobioreactors, n = 2. The blue columns represent analysis of the simple main effects: different lower and upper case superscripts stand for statistical differences between treatments within weeks 19/7 and 26/7, respectively; * stands for statistical differences between weeks within the same treatment ($p \leq 0.05$).

Date	Treatment	Ash	Lipids	Protein (N-based)	Sugars
19/07/2019	LS	32.7 ± 1.8	$10.8 \pm 0.2^{b*}$	23.6 ± 1.9	20.1 ± 0.8
	C	34.6 ± 0.3	11.5 ± 0.7^{ab}	20.0 ± 0.7	20.6 ± 0.9
	HS	33.3 ± 1.2	12.4 ± 1.2^{a}	20.3 ± 0.5	$19.3 \pm 0.4^{*}$
26/07/2019	LS	32.3 ± 0.4	$7.0 \pm 0.2^{C*}$	22.3 ± 0.3	19.1 ± 4.9^{A}
	C	34.6 ± 1.5	10.3 ± 0.1^{B}	18.6 ± 1.6	23.5 ± 0.2^{A}
	HS	35.8 ± 1.4	11.8 ± 0.1^{A}	19.8 ± 0.1	$9.5 \pm 2.4^{B*}$

Regarding the N-source experiment (Tables 7 and 8), in terms of ash content, a significant difference was found for the interaction factor (p-value = 0.001). Therefore, analysis of simple main effects was performed and showed, in the second week (date 22/08/2019), a significant lower value for NH_4^+ compared to NO_3^-. Moreover, comparing weeks, a significant higher value for NO_3^- was observed for the second week.

In relation to lipids, significant main effects were found for the treatment factor (p-value = 0.036), where the average, over the experimental period, was 8.2%/DM and 9.3%/DM for NH_4^+ and NO_3^-, respectively. For the date factor (p-value = 0.068) no significant differences were found.

For the protein (N-based) content the interaction factor was significant (p-value = 0.006), thus analysis of simple main effects was carried out. Within each of the weeks (16/08/2019 and 22/08/2019) there were significant differences between the treatments, both with higher values for NH_4^+ compared to NO_3^-. In addition, comparing weeks, there was a significantly higher value in week 2 for NH_4^+.

For the variable sugars, significant main effects were found for the treatment factor (p-value = 0.017), with an average, over the experimental period, of 21.6%/DM and 24.2%/DM for the NH_4^+ and NO_3^-, respectively. Between weeks a significative difference was also found (p-value = 0.031), where an average of 23.6%/DM and 22.1%/DM was recorded for the first (16/08/2019) and second (22/08/2019) weeks, respectively.

Table 7: Results of 4 two-way blocked ANOVA's for the N-source trial, showing the effect of the independent variables ("Treatment" and "Date") on the dependent variables ("Ash", "Lipids", "Protein" and "Sugars") (*P < 0.05; **P < 0.01; ns = not significant).

Two-way ANOVA	Date	Treatment	Date x Treatment
Ash	ns	ns	**
Lipids	ns	*	ns
Protein (N-based)	ns	ns	**
Sugars	*	*	ns

Table 8: Results of analysis made by VITO for the N-source trial (19/08/19-22/08/19). Treatments are represented by the type of source of nitrogen, NH_4^+ (ammonium as source) and NO_3^- (nitrate as source). All four dependent variables (ash; lipids; protein; sugars) are presented as mean %/DM ± SD (considering that for each treatment corresponds three photobioreactors, n = 3). The green columns represent analysis of the main effects. The blue columns represent analysis of the simple main effects: # stands for differences between dates within the same treatment; * and * stand for differences between treatment within 16/08 and 22/08, respectively ($p \leq 0.05$).

Date	Treatment	Ash	Lipids	Protein (N-based)	Sugars
16/08/2019	NH_4^+	28.4 ± 0.7	7.9 ± 1.1	28.1 ± 0.6$^{\#*}$	22.4 ± 0.9
	NO_3^-	29.0 ± 0.8$^{\#}$	9.1 ± 1.1	20.4 ± 1.3*	24.8 ± 0.5
22/08/2019	NH_4^+	28.4 ± 1.0*	8.6 ± 0.7	31.3 ± 1.2$^{\#*}$	20.8 ± 0.6
	NO_3^-	34.0 ± 0.4$^{\#*}$	9.6 ± 0.7	18.8 ± 1.2*	23.5 ± 0.2

Relatively to the phosphate concentration experiment (Tables 9 and 10), the ash content did not show statistically significant differences for any of the considered factors: treatment, date and interaction (p-value = 0.930; p-value = 0.118; p-value = 0.103).

On the other hand, lipids showed significant differences between treatments (p-value = 0.026), with average values, over the experimental period, being significantly higher for the low phosphate (8.1%/DM) and normal phosphate (8.2%/DM) compared to the high phosphate content (7.8%/DM). Significant main effects were also found for the date factor (p-value = 0,001), with an average of 8.3%/DM and 7.8%/DM for the first two weeks (date 12/09/2019) and for the two weeks after (date 26/09/2019), respectively.

Regarding the protein (N-based) content, no significant differences were found between treatments (p-value = 0.083). However, significant main effects were found for the date factor (p-value = 0.004), where the average values were 20.0%/DM and 18.7%/DM for the first two weeks (date 12/09/2019) and for the two weeks after (date 26/09/2019), respectively.

In terms of sugars, no statistically significant differences were found for any of the considered factors: treatment, date and interaction (p-value = 0.604; p-value = 0.134; p-value = 0.420).

Table 9: Results of 4 two-way blocked ANOVA's for the P-concentration trial, showing the effect of the independent variables ("Treatment" and "Date") on the dependent variables ("Ash", "Lipids", "Protein" and "Sugars") (*P < 0.05; **P < 0.01; ns = not significant). LP = low phosphate, NP = normal phosphate and HP = high phosphate.

Two-way ANOVA	Date	Treatment	Date x treatment	LP	NP	HP
Ash	ns	ns	ns	-	-	-
Lipids	**	*	ns	a	a	b
Protein (N-based)	**	ns	ns	-	-	-
Sugars	ns	ns	ns	-	-	-

Table 10: Results of analysis made by VITO for the phosphate concentration trial (12/09/19-26/09/19). Treatments are represented by the initials LP (low phosphate), NP (normal phosphate) and HP (high phosphate). All four dependent variables (ash; lipids; protein; sugars) are presented as mean %/DM ± SD (considering that for each treatment corresponds two photobioreactors, n = 2).

Date	Treatment	Ash	Lipids	Protein (N-based)	Sugars
12/09/2019	LP	30.3 ± 0.5	8.4 ± 0.1	19.9 ± 0.2	24.6 ± 0.2
	NP	30.1 ± 0.5	8.4 ± 0.3	20.0 ± 0.2	24.6 ± 0.0
	HP	31.1 ± 0.2	8.1 ± 0.1	20.2 ± 0.8	24.4 ± 0.2
26/09/2019	LP	33.0 ± 0.3	7.8 ± 0.0	18.4 ± 0.3	22.0 ± 1.5
	NP	33.1 ± 1.4	8.0 ± 0.1	18.6 ± 0.5	23.3 ± 1.1
	HP	31.6 ± 0.7	7.6 ± 0.5	19.0 ± 0.6	23.8 ± 1.2

- Trials with European seabass (nutritional and physiological tests)

Regarding the nutritional test, initial weight of the fish was not significantly different between diets, whereas at the end of the study, final weights in fish fed with control diet were significantly higher than with all other diets, thus resulting in a significantly higher weight gain (ΔW) and SGR (Figure 23). No significant differences were found between diets A and B and A and C, whereas a small, but significant difference in SGR was found between diets B and C. The higher growth rate (SGR) for the control diet may be related to the higher feed intake; the control diet seemed slightly more attractive to the fish in comparison with the other diets (Table 11), however it must be noted that differences are very small. Feed conversion ratio (FCR) was significantly lower for the control diet and diet B compared to diet C, whereas diet A showed an intermediate value and did not differ significantly either from the control nor from the other diets (Figure 24).

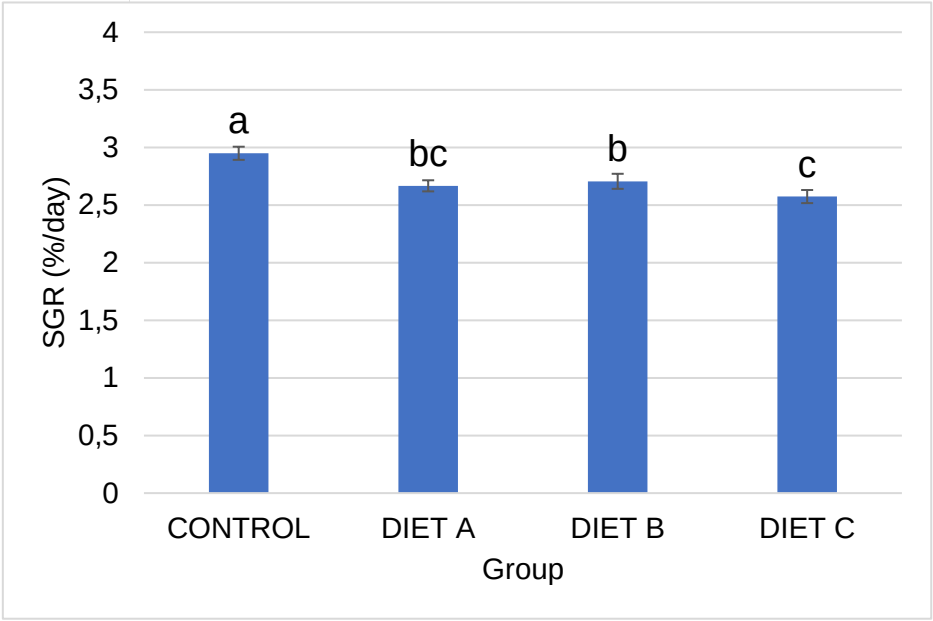

Figure 23: Specific growth rate (SGR, % d^{-1}) ± SD at termination of the study (day 70) of fish fed with different experimental diets versus the control diet of the fish nutritional test. Different letters indicate significant differences between groups ($p \leq 0.05$).

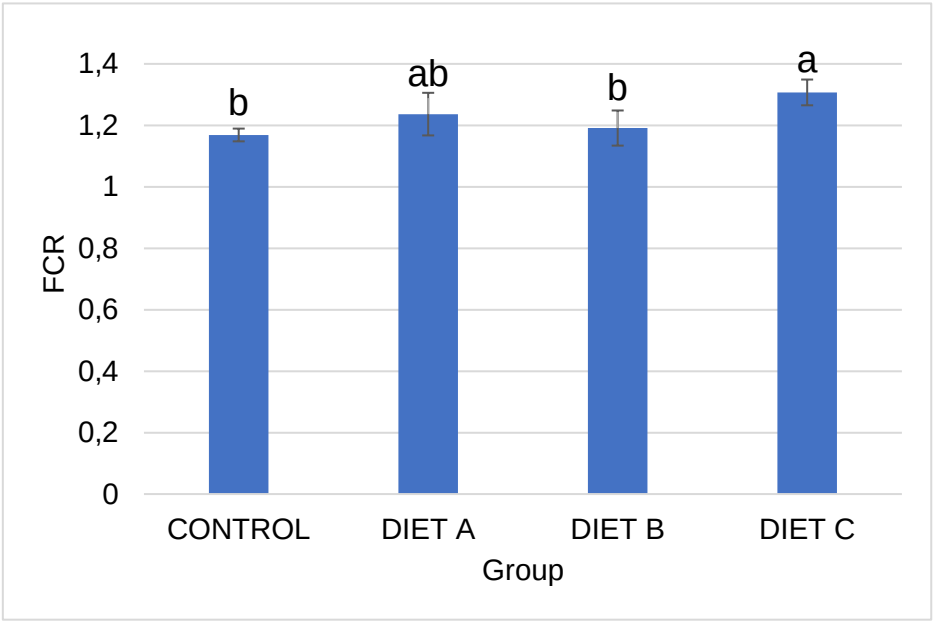

Figure 24: Feed conversion rate (FCR) ± SD at termination of the study (day 70) of fish fed with different experimental diets versus the control diet of the fish nutritional test. Different letters indicate significant differences between groups ($p \leq 0.05$).

The rest of parameters (SV, HSI and VSI) did not show any significant differences between experimental groups, except for K, where for control diet the value was significantly higher than diet C (Table 11).

Table 11: Results of growth and health parameters ± SD at termination of the study (day 70) of fish fed with different experimental diets versus the control diet of the fish nutritional test. Different letters indicate significant differences between groups ($p \leq 0.05$). Abw = average body weight, SGR = specific growth rate, FCR = feed conversion rate, SV = survival, K = Fulton's condition factor, HSI = hepatosomatic index and VSI = viscerosomatic index.

	CONTROL	GROUP A	GROUP B	GROUP C	ANOVA
Initial abw (g/fish)	1.73 ± 0.01	1.73 ± 0.01	1.73 ± 0.00	1.73 ± 0.03	F(3,12)=0.150 p=0.928
Final abw (g/fish)	13.7 ± 0.6^{a}	11.2 ± 0.4^{bc}	11.5 ± 0.5^{b}	10.5 ± 0.3^{c}	F(3,12)=33.219 p=0.000
Weight gain (g/fish)	11.9 ± 0.6^{a}	9.5 ± 0.4^{bc}	9.8 ± 0.5^{b}	8.8 ± 0.3^{c}	F(3,12)=33.335 p=0.000
SGR (%/day)	2.95 ± 0.06^{a}	2.67 ± 0.05^{bc}	2.71 ± 0.07^{b}	2.57 ± 0.06^{c}	F(3,12)=34.055 p=0.000
Feed intake (g/fish)	13.9 ± 0.9^{a}	11.7 ± 0.8^{b}	11.6 ± 0.3^{b}	11.4 ± 0.08^{b}	F(3,12)=14.911 p=0.000
FCR	1.17 ± 0.02^{b}	1.24 ± 0.07^{ab}	1.19 ± 0.06^{b}	1.31 ± 0.04^{a}	F(3,12)=5.832 p=0.011
SV (%)	98.3 ± 3.3	94.6 ± 4.8	96.7 ± 1.4	95.4 ± 1.6	F(3,12)=1.108 p=0.384
K	1.21 ± 0.02^{a}	1.19 ± 0.02^{ab}	1.18 ± 0.02^{ab}	1.17 ± 0.01^{b}	F(3,12)=3.871 p=0.038
HSI	1.99 ± 0.13	1.87 ± 0.28	1.83 ± 0.19	1.81 ± 0.62	F(3,12)0.782 p=0.526
VSI	10.9 ± 0.9	10.0 ± 1.1	9.8 ± 0.8	9.9 ± 0.6	F(3,12)=1.424 p=0.284

Biochemical analyses of serum samples are presented in Table 12. Lysozyme showed to be higher for diets A and C compared to the control, whereas diet B had an intermediate value, differing significantly from the other diets. Antiprotease activity was higher for diets A and B compared to the diet C and control, which did not differ significantly between them. As for superoxide dismutase, diets A and B registered higher values compared to the control, whereas diet C showed an intermediate value and did not differ significantly either from the control nor from the other diets.

Table 12: Results of biochemical analyses of serum samples ± SD of fish fed with different experimental diets versus the control diet at the beginning and end of the fish nutritional test. Different letters indicate significant differences between groups ($p \leq 0.05$).

Group	Lysozyme assay (µg/ml)	Antiprotease activity (%)	Superoxide dismutase assay (U/mg)
Initial pool	3.13 ± 0.08^{c}	70.01 ± 0.3^{b}	62.8 ± 0.2^{bc}
A	4.37 ± 0.10^{a}	71.2 ± 0.5^{a}	64.4 ± 0.8^{a}
B	4.10 ± 0.06^{b}	71.5 ± 0.5^{a}	64.3 ± 0.4^{ac}
C	4.38 ± 0.12^{a}	70.1 ± 0.3^{b}	64.0 ± 0.4^{ab}
CONTROL	3.21 ± 0.10^{c}	70.1 ± 0.3^{b}	63.0 ± 0.7^{b}

The intestinal score values obtained are shown in Figure 25. No statistically significant differences between diets were found ($F(3,12)=0.758$; $p=0.539$).

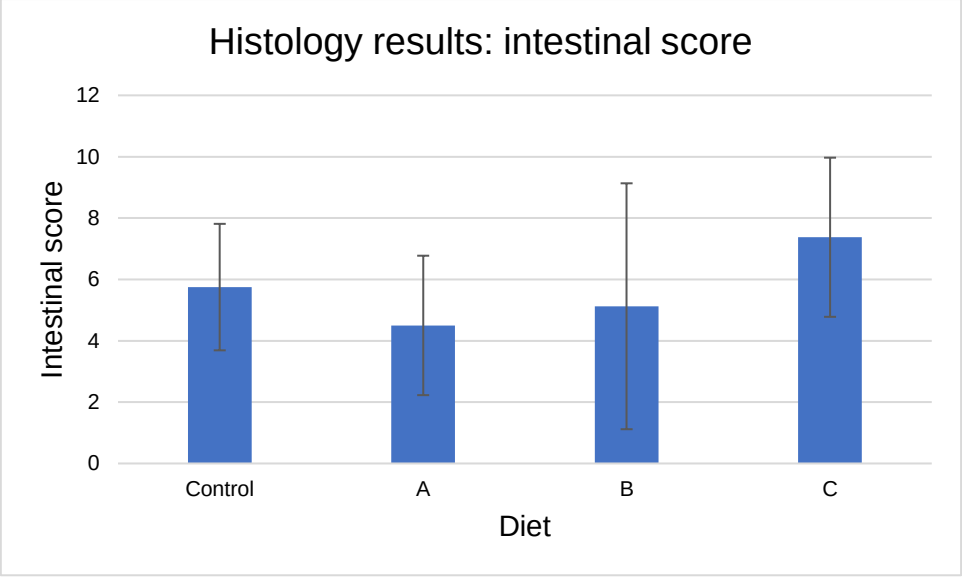

Figure 25: Mean intestinal score values ± SD recorded for each diet (control, diet A, diet B and diet C).

Values for transaminase (ALT-GPT) and triglycerides were below detection limit for most cases, hence these parameters will not be reviewed here.

Glucose levels (Figure 26) increased 1 h after stress for all diets (including the control diet). Significant differences were found between diets ($F(3,28)=6.846$; $p=0.001$), where a higher value for diets A and B (207.8 and 216.6 mg/dL, respectively) compared to the control (180.5 mg/dL) was recorded. For diet C intermediate values were found, without differing significantly from the control or the other diets.

No significant differences were found before the stress or 24 h after the stress test when values had decreased again, however it must be noted that T0 values were considerably higher than T24 values (Figure 26).

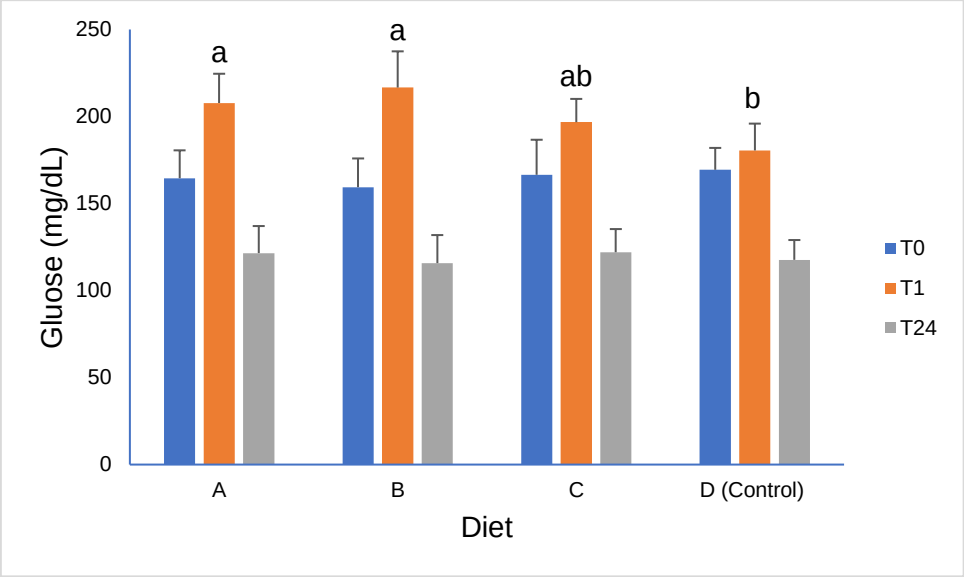

Figure 26: Glucose levels (mg/dL) ± SD in blood plasma from fish fed different diets before (T0), 1 h after (T1) and 24h after a physiological stress challenge. Different letters (T1 only) indicate significant differences between treatments.

AST values at T0 and T1 were higher for diets A and B compared to diet C and the control, however, variability was also high and it was only significant at T1 between diets A and B (127 and 134 units per litre, respectively) and diet C (84 units per litre). As also was the case for glucose, T24 levels were generally lower than T0 values, especially for diets A and B (Figure 27).

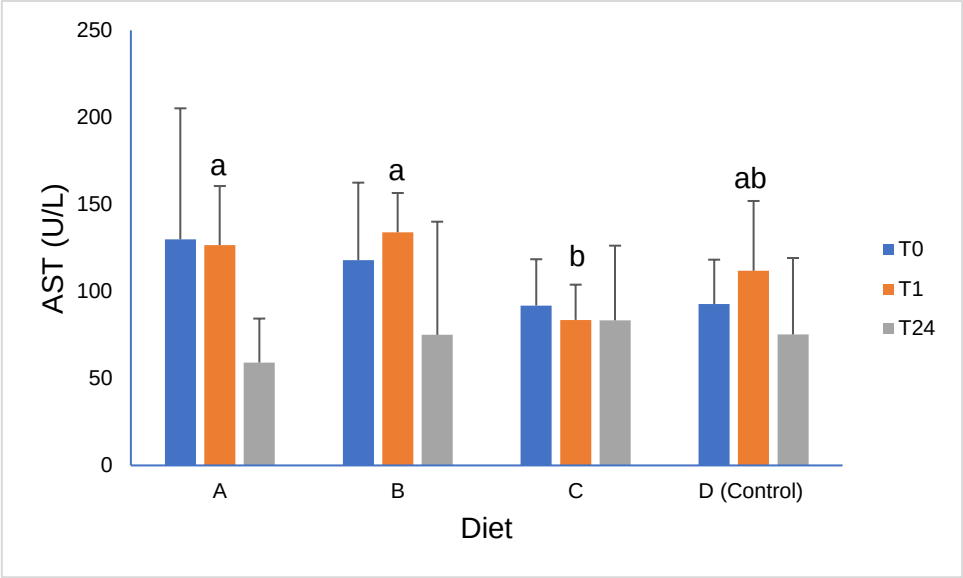

Figure 27: Transaminase (AST-GOT, U/L) ± SD in blood plasma from fish fed different diets before (T0), 1 hour after (T1) and 24h after a physiological stress challenge. Different letters (T1 only) indicate significant differences between treatments.

As for lactate dehydrogenase levels (LDH), variability was also quite high and at T1 only was found a significant difference between control diet (110 units per litre) and diet C (57 units per litre). Moreover, T24 levels appeared to decrease to the lower values in diets A and B compared to T0 and T1 levels (Figure 28).

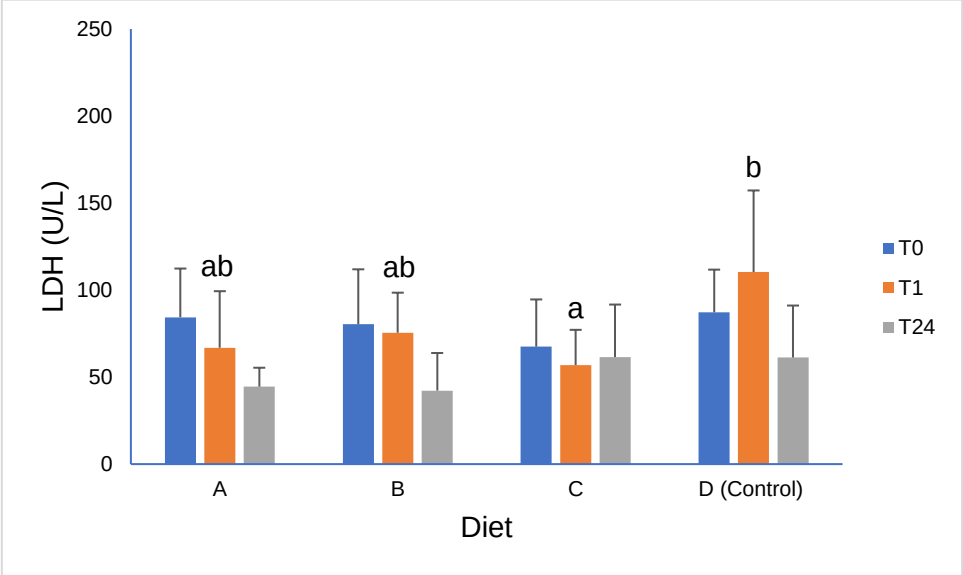

Figure 28: Lactate dehydrogenase (LDH, U/L) ± SD in blood plasma from fish fed different diets before (T0), 1 h after (T1) and 24h after a physiological stress challenge. Different letters (T1 only) indicate significant differences between treatments.

4.4. Discussion

- Stock cultures – Outdoor tanks – Indoor photobioreactors

The results presented for "Stock cultures", "Outdoor tanks" and "Indoor photobioreactors" showed that initial density was the factor that most likely influenced the SGR values recorded for *U. ohnoi* (higher density led to a lower SGR; lower density led to a higher SGR). Therefore, this demonstrates that initial density is an important factor when dealing with variables related to growth and gives us a general idea of the carrying capacity of the system (when stocking density starts to lead to limitations in growth rate). These are important points that must be taken into account when assessing the productivity of cultivation systems, particularly when moving to a commercial scale (Carl *et al.*, 2014). According to Mata *et al.* (2010) the optimum stocking density to obtain a maximum harvest is between 2 and 4 kg/m^3, which is in line with what was verified during the cultivation process in CTAQUA (it has been observed that with 2 kg/m^3 the optimum biomass cannot yet be achieved to obtain a maximum harvest). For scientific research it might be useful to maintain lower stocking densities (where we may have higher SGR values). However, on an algae production farm it is all about absolute growth, so higher stocking ratios should be used (where we may have lower SGR values in comparison).

Temperature and solar radiation are factors that directly influence the growth of algae, however the results obtained here cannot unanimously prove this relationship. In this sense, it would be expected that for water temperatures between 25 and 30ºC the highest SGR values would be recorded (Malta, 2017). However, according to Lawton *et al.* (2013) there are also strains of *U. ohnoi* from temperate climate that thrive better at lower temperatures, which probably is the case of the strain cultivated at CTAQUA. In addition, it should be noted that the amount of nutrients added to all the cultivation systems has been determined so that this does not become a limiting factor.

- Photobioreactors – temperature experiment

At first sight, the recorded SGR values seem to be somewhat unexpected, particularly in week 3. Given that the conditions established for each treatment were practically the same over the 3 weeks (namely a specific temperature established for each treatment; same stocking density in each week; same amount of nutrients added per week in each photobioreactor), would be expected no significant differences between the different weeks, only possibly between treatments could be expected. However, in

week 3 there were significant differences in relation to weeks 1 and 2. In addition, the "Unheated" treatment had a significantly higher SGR value than the one registered for the "Heated" treatment which had a lower value (this did not happen for any of the other weeks). On the one hand, the acclimation factor could explain the variation in the week 3, especially in the case of the "Heated" treatment (significant decrease in SGR), since algae, and this species in particular, has a great adaptive capacity, but that may require some time and therefore this could be a hypothesis to explain the results obtained (Gao *et al.*, 2016). However, this would not explain why there was a significant increase of SGR in the "Unheated" treatment, since, in this case, there was not exactly a period of adaptation to temperature (before the experiment started, the algae that would be used were already being cultivated at room temperature, so with an average water temperature around 18-19ºC). On the other hand, the fact that there is no temperature control in the photobioreactors room, means that there is another factor to take into account: the daily thermal amplitude (although, at the time of the experiment, not very pronounced) (Setchell, 1915). In this way, the thermal amplitudes recorded along week 3 may have presented a different pattern and, therefore, triggered the increase of the SGR in the "Unheated" treatment. However, this does not explain why there was a significant decrease of the SGR in the "Heated" treatment since thermal amplitude recorded was practically zero in this case. In conclusion, the combination of these two factors would be one of the possibilities that could explain the results obtained.

Regarding the ash content measured at the end of this experiment (week 3), taking into account the treatments and the time period considered, the conclusion is that there is no influence of temperature on the ash content in *U. ohnoi*. Although this has occurred for these experimental conditions, several studies show temperature as one of the factors that significantly influences the chemical composition of macroalgae, namely the ash content. This is an important parameter in the composition and biochemical functions of algae, but from a human point of view ashes have low commercial value and are major hurdles for biomass processing. Hence, for instance, it is interesting and useful to understand under which cultivation conditions lower levels can be obtained (D'armas *et al.*, 2019; Khairy & El-Shafay, 2013; Robin *et al.*, 2018).

- Photobioreactors – VITO data analysis

- Salinity experiment

Salinity experiment showed that ash content didn't differ significantly between treatments. On the other hand, for lipids, protein content and sugars statistical

differences were found. In general, lipid content seemed to show a positive correlation with salinity (up to a certain limit) (Rozentsvet & Nesterov, 2012). For the protein content, the results obtained are not in line with those obtained in a study conducted with *U. prolifera* (positive correlation between salinity and protein content), however are in agreement with another who has used *U. ohnoi* (more or less the same protein content in a salinity range of 10-60‰) (Malta, 2017). In turn, sugar content results showed to be not in line with an experiment conducted with *U. fasciata* (positive correlation between salinity and sugars content) (Malta, 2017).

- N-source experiment

Regarding the N-source experiment, in terms of ash content, it seems that ammonium as a source of nitrogen for *U. ohnoi* tends to make the ash levels lower compared to nitrate as a source of N. For the lipid content, based on the results, it is speculated that nitrate, as a source of N, may lead to higher lipids content in *U. ohnoi* tissues compared to ammonium as a source of N. As for the protein content, results show that levels appear to be higher when using ammonium as a N source and lower when using nitrate. This observation is quite interesting, since Shahar *et al.* (2020) recorded similar results with *U. fasciata*, not using ammonium, but ammonia. It was seen that its use favoured the production of biomass richer in protein than using nitrate. For the sugar content, results have shown that levels tend to be higher when nitrate, instead of ammonium, is used as a source of nitrogen. Shahar *et al.* (2020) also identified this pattern, although the biomass produced was lower, the sugar levels were higher for nitrate. Even though ammonium has been identified in several reports as a better N source compared to nitrate (promotes better growth) in *Ulva* cultivation, depending on the final desired product, it may be more advantageous to choose nitrate or even another source of N (Ganesan *et al.*, 1999).

- P-concentration experiment

Relatively to the phosphate concentration experiment, the ash content did not differ significantly between any of the treatments. Overall, lipid content results showed that cultivation of *U. ohnoi* in environments with high phosphate concentration (N:P ratio of 12:1) may be less profitable if the aim is to obtain lipid-rich biomass. It is well known that P is an important element in the constitution of lipids, namely phospholipids. Thus, an increase in P availability would be advantageous for lipid synthesis, however the results observed do not seem to indicate exactly that (Kumari *et al.*, 2014). Regarding the protein content, only a significant decreased was recorded for the date factor. As for sugar content, no statistically significant differences were found.

- Trials with European seabass

- Nutritional test

Nutritional test results evidenced that control diet had the highest significant SGR compared to the other diets containing seaweed extracts. This was most likely due to the slightly, but significant higher feed intake recorded, so algae extracts may have negatively affected the palatability of the other diets (Morais *et al.*, 2020). Overall, results obtained for FCR, Fulton's Condition Index, survival and viscerosomatic and hepatosomatic indices, show that diets A (Spirulina + *U. ohnoi*) and B (Spirulina + *S. latissima*) seem to be more advantageous than diet C (Spirulina + *U. ohnoi* + *S. latissima*). Similar previous studies with European seabass indicate that dietary supplementation of *Ulva* up to 10% inclusion level is an adequate source of protein and does not affect SGR and FCR (Peixoto *et al.*, 2016; Valente *et al.*, 2006). Another study conducted by Ferreira *et al.* (2020) with rainbow trout (*Oncorhynchus mykiss*), a carnivorous fish like European seabass, showed that supplementation of diets with up to 2% *S. latissima* does not affect growth. Therefore, possibly the additional 15% of *Spirulina* in diets A, B and C may have contributed to the SGR being significantly lower compared to the control.

On the other hand, relatively to the immune responsiveness of the fish, diets supplemented with *Ulva* or *Saccharina* (diets A and B, respectively) stood out from the control diet in the sense that the activity levels were always significantly higher for all the indicators analysed (lysozyme; antiproteases; superoxide dismutase). These indicators are key components of fish immunity and, generally, are positively correlated with disease resistance. Besides the fish species under study, the type of algae and the percentage of inclusion in the diet are factors that influence the level of activity of these indicators (Ellis, 1999; Lobo *et al.*, 2018; Peixoto *et al.*, 2016).

In all diets the values for the intestinal score were quite variable among the replicates (visible in the high standard deviation values recorded), for example, values in diet B varied between 2 and 11. No statistically significant differences were found between group means, which indicate that the use of this diets containing seaweeds, in terms of consequences on global intestinal status, is not disadvantageous compared to control diet. In fact, many studies have been used histological analysis as one of the reliable ways of assessing the effect of diets on fish health. Magalhães *et al.* (2020) resorted to histology as one of the indicators to assess the effects of dietary ratios of essential fatty acids in gilthead seabream, and Heikkinen *et al.* (2006) also did it in their study with rainbow trout to assess the effects of soybean meal based diet. Even with European

seabass, there are already some studies, such as the one carried out by Messina *et al*. (2019) who investigated, namely through histological analysis, the effects of replacing graded levels of dietary fish meal by a blend of two marine microalgae.

- Physiological test

For the stress test it was found, as could be expected, an increase on glucose levels at T1 and compared to control diet, the values were significantly higher for diets A and B. Similar results in European seabass were reported by Fanouraki *et al.* (2008) with plasma glucose levels peaking at 2 h post-stress. So, if the 2 h post-stress period had been assessed, levels would most likely be even higher than those registered for T1. Results presented for the enzymes AST and LDH in serum were quite variable, making it difficult to establish a clear pattern, although it would be expected that their pattern would follow the same pattern as glucose (Dar *et al.*, 2020; Pakhira *et al.*, 2015). Even though the use of these blood constituents as stress indicators is an issue that depends on many factors, several studies show that there is a negative correlation between the growth and development of fish and the levels of cortisol and, consequently, of glucose (and even other substances) (Jentoft *et al.*, 2005; Kubilay & Uluköy, 2002; Malini *et al.*, 2018; Martínez-Porchas *et al.*, 2009; Odhiambo *et al.*, 2020; Yavuzcan *et al.*, 2011). Thus, focusing mainly on the results obtained for glucose, for diets control and C at T1 the lowest values were recorded, so it could be argued that these diets may be the most advantageous in the sense that they may better cushion the impact of the stress factors implemented in this experiment. However, as already mentioned, this is a complex issue (among others, factors such as adaptation can enter the equation), so it is quite complicated to draw generalized and long-term conclusions.

5. References

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