Enhancement of feed management in an intensive aquaculture setting for *Solea senegalensis* juveniles

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To everyone, from the bottom of my heart,

Thank you.

Resumo

Sistemas de Recirculação de Aquacultura (RAS) têm vindo a expandir-se e estão atualmente a emergir como uma estratégia fundamental para fazer face à crescente procura global de alimento. O linguado senegalês, ou linguado-branco, (*Solea senegalensis*) é uma das espécies mais interessantes para ser explorada em RAS devido às suas caraterísticas biológicas ótimas para produção intensiva e à elevada procura por parte dos consumidores, associada a elevados preços no mercado. Nas últimas duas décadas, surgiu um conjunto substancial de literatura que demonstra que o linguado do Senegal (SnS) pode ser produzido de forma intensiva neste tipo de sistema. No entanto, ainda não atingiu o nível de industrialização observado noutras espécies. Sendo então o avanço de regimes alimentares mais otimizados em aquacultura um dos pontos cruciais para aumentar a eficiência, a rentabilidade e a capacidade de produção.

O Grupo Sea Eight é um conjunto de empresas especializadas na produção de SnS em RAS. Esta tese foi realizada na maternidade do Grupo, a Safistela, em colaboração com o laboratório Nutrimu, na FCUP.

Esta tese é composta por um segmento prático no departamento de produção e, posteriormente, por um segmento onde foi desenhado e estabelecido um ensaio nutricional, no departamento de I&D.

No primeiro, tive a oportunidade de efetuar as rotinas diárias de todas as salas da Safiestela, desde a criação dos reprodutores até à pré-engorda, a última fase antes do transporte dos peixes para as outras instalações do Grupo onde se realiza a engorda final. Esta oportunidade proporcionou-me o conhecimento e a prática para compreender melhor o comportamento e as necessidades deste peixe plano ao longo de todo o seu ciclo de vida.

Na segunda, uma comparação de duas dietas comerciais isolipídicas, a Dieta A e a Dieta B, revelou diferenças consideráveis no crescimento e no desempenho económico. A composição, o preço total e outros pormenores relativos a estas dietas não foram incluídos nesta tese devido a acordos de confidencialidade com os fornecedores de rações.

Fatores como o Peso Corporal Inicial (Dieta A = 5,78 ± 0,17 g; Dieta B= 5,92 ± 0,20 g) vs Peso Corporal Final (Dieta A= 21 ± 1,07 g; Dieta B= 16,7 ± 0,86 g), Taxa de Conversão Alimentar (FCR) (Dieta A = 2,06; Dieta B = 2,92), Índice de Crescimento Diário (DGI) (Dieta A = 1,08; Dieta B = 0. 84), e Rácio de Coeficiente Económico (ECR) (Dieta A = 1,79; Dieta B = 2,92) mostram a vantagem significativa que resultaria da substituição da atual dieta utilizada na fase de Nursery na Safiestela, Dieta B, pela Dieta A.

Além disso, verificou-se uma correlação significativa entre o desempenho do crescimento e a turbidez. De acordo com o teste de correlação de Pearson, uma melhor qualidade da água está significativamente correlacionada com melhores desempenhos de crescimento. Assim, é discutida uma proposta de incorporação da medição de turbidez nas rotinas diárias da linha de produção. As medições diárias da turvação podem ser uma ferramenta adicional para avaliar a eficiência da filtração do sistema e o desempenho da alimentação, que são conhecidos por estarem correlacionados com o desempenho do crescimento.

Os resultados desta tese permitiram propor recomendações para mudanças no que diz respeito ao controlo da alimentação, que permitirá à Safiestela melhorar o rendimento da produção da área da Nursery.

Palavras-chave: Linguado Senegalês, *Solea senegalensis,* Aquacultura, Nutrição Aquática, Ensaio de Nutrição, Performance Económica.

Abstract

Recirculating Aquaculture Systems (RAS) have been expanding and are currently emerging as a key strategy for addressing the rising global demand for food. *Solea senegalensis* is one of the most interesting species to be exploited in recirculation aquaculture systems due to its optimal biological characteristics for intensive production and high consumer demand, coupled with high market prices. A substantial body of literature has emerged over the past two decades, demonstrating that Senegalese sole (SnS) can be produced intensively in this type of system. Nevertheless, it has yet to achieve the level of industrialization observed in other species. Thus the advancement of more optimized dietary regimens in aquaculture is a crucial point for enhancing efficiency, profitability, and production capacity, which is still underdeveloped

Sea Eight Aquaculture Group is a set of companies that specialize in the production of SnS in RAS. The Maternity of the group, Safiestela, is where this thesis was elaborated, in collaboration with the Nutrimu lab, in FCUP.

This thesis is composed of a practical segment in the production department, and after, a segment where a Nutritional trial was designed and established, in the R&D department.

In the first, I got to perform the daily routines of all the rooms within Safiestela, from the broodstock to the pre-ongrowing, the last stage before the transport of the fish to the grow-out facilities. This opportunity provided me with the knowledge and practice to better understand this flatfish's behavior and needs throughout the entirety of its life cycle.In the second, a comparison of two commercial isolipidic diets, Diet A and B, revealed considerable differences in growth and economic performance. The composition, full price and other details regarding these diets were not inserted in this thesis due to confidentiality agreements with the feed suppliers. Factors such as Initial Body Weight (Diet A = 5.78 ± 0.17 g; Diet B= 5.92 ± 0.20 g) and Final Body Weight (Diet A= 21 ± 1,07 g; Diet B= 16,7 ± 0,86 g), Feed Conversion Ratio (Diet A = 2.06; Diet B = 2.92), Daily Growth Index (Diet A = 1.08; Diet B = 0.84), and Economic Coefficient Ratio (Diet A = 1.79; Diet B = 2.92) clearly show the advantage that would result from the current diet used in the Nursery phase at Safiestela, Diet B, being replaced with Diet A.

Also, there was an observation of a significant correlation between growth performance and turbidity. According to the Pearson correlation test, better water quality correlated

with higher growth performances. Hence, a proposed incorporation of turbidity measurement in the daily routines of the production line is discussed. Daily turbidity measurements could be an additional tool to assess system filtration efficiency, feed performance, which are known to be correlated with growth performance.

The findings of this thesis have enabled the proposition of recommendations for changes in the management of feed that will allow Safiestela to improve the production yield of the nursery area.

Keywords: Senegalese Sole, *Solea senegalensis*, Aquaculture, Aquatic Nutrition, Nutrition Trial, Economic Performance.

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List of abbreviations

RAS Recirculating Aquaculture System

EPA Eicosapentaenoic Acid

ABS Adsorptive Bubble Separation

AOB Ammonia Oxidizing Bacteria

NOB Nitrite Oxidizing Bacteria

BioC Biofilter carriers (also known as bioballs)

ppt Parts per thousand or g L^{-1}

DAH Days After Hatchery

B.C. Before Christ

SnS Senegalese sole

ALT Alanine Aminotransferase

AST Aspartate Aminotransferase

TAG Triglyceride (also known as Triacylglycerol or Triacylglyceride)

FA Fatty Acids

VLDL Very Low Density Lipoprotein

Preface

In a company specialized in the aquaculture industry, with a unique focus on the *Solea senegalensis* species, the essence of the work carried out is to improve industrial processes to achieve the company's objectives. To equip myself with the skills, routines, and knowledge to best benefit this improvement, I began by working in the production department of Safiestela, the Sea Eight Aquaculture Group's maternity. In the first few months, I carried out day-to-day work alongside the other production staff at the different life cycle stages in which this facility operates, participating in every stage of production.

Being exposed to different tanks, techniques, and practices, which vary according to the phase of the fish's development, allowed me to learn from a wide range of experiences. This versatility has given me a more in-depth knowledge of the procedures used in this industry, something I didn't have until I was elaborating this dissertation.

In the 1st Chapter, I'll begin by contextualizing the Sea Eight Aquaculture Group, the Safiestela company, and my work while on the production team.

In the 2nd Chapter and subsequent subsections, the main focus of this body of work will be explored in depth. This thesis's primary objective is to identify and implement more effective feeding management practices, with the aim of enhancing production efficiency while reducing costs and maintaining suitable welfare conditions in intensive aquaculture production. Additionally, this work will contribute to the acquisition of specialized knowledge in this intensive RAS production and on the *Solea senegalensis* species.

1st Chapter

SEA EIGHT AQUACULTURE GROUP

Safiestela Sustainable Aqua Farming Investments, S.A.

1st phase of the thesis – internship in the production line

1. Sea Eight Aquaculture Group

Sea Eight was founded in 2012, originally Sea8, with the idea of producing high-quality, sustainable Senegalese sole (*Solea senegalensis*), using recirculating aquaculture systems, to the highest sustainability standards.

This company has well-defined targets in its ambition to develop, on land, a protein of the utmost quality in the present and for the future. Following imperative values of respect for the environment, sustainable development, and the best animal welfare management practices, the group's mission is to continually develop industrial processes to ensure maximum food safety, quality, and efficiency, achieving a final product at the lowest possible cost using the least possible amount of natural resources. The ultimate goal is to be the world leader in sole production by following these cornerstone principles.

With 3 companies under its umbrella, 2 grow-out farms, and 1 hatchery, the Sea Eight group covers all stages of the production of this animal. The on-growing plants Aquacria Arousa and Aquacria Píscicolas are located in Galicia and Aveiro, respectively, and the hatchery, Safiestela, is situated in Estela, Póvoa de Varzim, where this thesis was carried out.

Due to the Group's rapid growth, it is currently expanding, with a new facility being built in Gijón, Asturias, and the amplification of the Safiestela facilities, where the current production capacity is intended to double.

2. Safiestela S.A.

Safiestela's premises date back to the early 1990s. The company A. Coelho e Castro built these facilities as part of its commitment to producing turbot (*Psetta maxima*) and used them for this purpose for almost two decades. Despite this, at the end of the 2010s, the high level of competition in the market for turbot became unappealing, so the company shifted towards *Solea senegalensis* and adapted the production facilities accordingly.

However, these facilities, Figure 1, were acquired by the Sea Eight group in 2012 and this is where Safiestela began as we know it today, a hatchery that houses various stages of Senegalese sole production, from broodstock to pre-ongrowing. The juveniles produced here are then transported to the grow-out facilities where all the necessary procedures are carried out until they are sold to the end consumer. The stages of

production of juveniles in Safiestela emulate the natural progression of the life cycle of the Senegalese sole, being each stage dedicated to fulfilling the needs that the fish demand at each point of its development:

Broodstock → Incubation → Larvarium → Weaning 1 → Weaning 2

↓

Transport ← Pre-Ongrowing 2 ← Pre-Ongrowing 1 ← Nursery

Figure 1 - Safiestela - Sustainable Aqua Farming Investments, S.A. aerial view on the left and the facade of the Maternity on the right. Retrieved from Google Maps, 2024.

2.1. Stages in Safiestela's production

From October 3rd to December 18th, 2023, I was integrated into the different stages of production to acquire the necessary skills to carry out sole production routines independently.

The process of developing and enhancing the production implies an understanding of how each step of the system, of the routines, is guided daily, and, with these aspects as a basis, adopt measures that help to improve production, its efficiency, its sustainability and, as a result, enhance its economic returns.

2.1.1. Broodstock

This species is dioic and has no visual sexual characteristics that allow for its sexual dimorphism to be distinguished (Dinis et al., 1999; Martín et al., 2019). It achieves sexual maturity when it reaches 30 cm, around the age of three years *(Solea Senegalensis*, Senegalese Sole: FishBase, n.d.).

Broodstock can come from aquacultures in earthen ponds, wild captures, or generations of previously reared juveniles, called F1s.

To achieve a constant source of eggs throughout the year, around 13 batches are made each year, depending on the reproductive performance of the fish (Dinis et al., 1999; Imsland et al., 2004). This facility manipulates the temperature and photoperiod conditions in the tanks. The Breeding area is divided into 4 rooms, Figure 2, each with manipulated settings to emulate a specific season: summer, fall, winter, and spring. These conditions cyclically replicate the natural environment, so, at any time of the year, we see a room at its peak of sexual maturation ready to spawn (Dinis et al., 1999; Imsland et al., 2004; Villanueva & Alonso, 2014).

Figure 2 - Broodstock rooms in Safiestela. Photos taken by Alexandre Veloso.

The fish are kept in large, round polyethylene tanks with a water column 0.85 meters high. In each tank's upper area, an outlet is installed to prevent overflow by connecting it to an egg collector, a cylindrical-conical receiver with a mesh that prevents the eggs received from falling into the water outlet. This receiver is constantly oxygenated with a diffuser stone, as shown in Figure 3.

Figure 3 - Egg collector connected to each tank in the Broodstock rooms. Photo taken by Alexandre Veloso.

The broodstock are always fed *ad libitum*, but depending on the stage of the year the fish is in (spawning or resting), there are tailor made dry feeds suited to the nutritional requirements of the respective stage. In the spawning and pre-spawning phase, the fish are fed fresh food, for example, polychaetes and mussels, in order to provide extra stimulation (Imsland et al., 2004; Villanueva & Alonso, 2014).

Each fish is tagged with a microchip to achieve tracking and quality control of the stocks and, depending on the brooders used, to monitor their reproductive life. The sex of the fish is determined by palpation. It is done only by experienced staff with extensive empiric knowledge. This screening is important to distribute the males and females in a 1:1 ratio in each tank (Villanueva & Alonso, 2014).

Resuming, Safiestela attains sexual stimulation of the broodstock by way of manipulating the photoperiod, temperature of the water, male to female ratio, administering live feed, hormone injection

In SnS, only wild fish and F1 females are capable of reproducing naturally. F1 males do not have this innate ability, and even artificial insemination is not a viable option in these cases, as their sperm production is low and of poor quality (Carazo et al., 2011; Riesco et al., 2019).

Manipulating photoperiod and temperature conditions allows for frequent and continuous spawning. In addition, GnRHa injections are used both to facilitate the maturation of the fish's gonads and to achieve more efficient egg production (Howell et al., 2009; Villanueva & Alonso, 2014).

Safiestela uses both active and passive fertilization methods according to the maternity's needs. In the active method, gametes are manually removed from males and females, and in the passive method, the fish naturally carry out the normal mating rituals.

Artificial fertilization takes place moments after the gametes are removed. Each type of gamete has a useful life span after being activated, by making contact with salt water, at around 30 seconds. The extraction process must be carried out quickly and in a dry environment. After extracting the sperm, an analysis of quality and quantity is performed so that it is possible to determine the approximate amount of eggs necessary to extract from the females. Saltwater is added and mixed lightly with both gametes so that these can be activated and evenly distributed (Villanueva & Alonso, 2014).

The natural fertilization process does not require human intervention, and the fish, mainly during the night, perform the mating ritual, also known as "Follow", as demonstrated in Figure 4, and, routinely, in the morning, the fertilized eggs located in the collectors are harvested (Carazo et al., 2011, 2016; Dinis et al., 1999; Imsland et al., 2004; Villanueva & Alonso, 2014).

After the collection of the eggs, they are carefully washed and disinfected before being headed to Incubation.

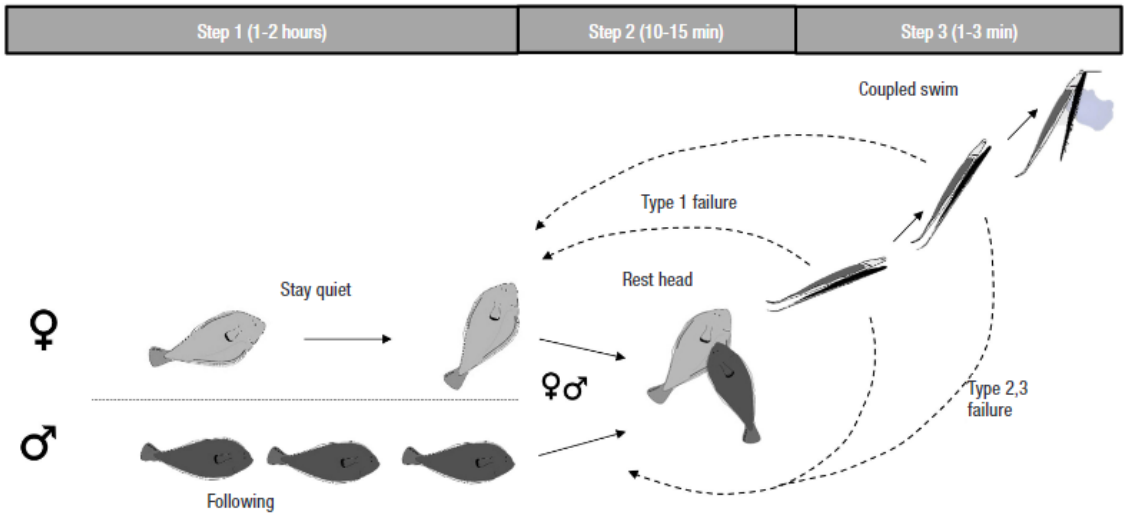

Figure 4 - Schematic of the behavior observed in mature adults in the reproduction. From Carazo et al. 2016.

2.1.2. Incubation

Once the fertilized eggs have been collected, either naturally or artificially, they are sent to a room with incubators, Figure 5.

Before the eggs are transferred to the incubators, they are acclimatized to the water's temperature in the incubation room, to avoid thermal shock.

The incubation room consists of several cylindrical-conical incubators with a capacity of 200 L each. The eggs are kept at a density of 500 to 1000 eggs L^{-1}, in tanks with a constant temperature of 19 ºC (around 800 degree-hours), with aeration and an upward flow of 0.5 L/min and a 400 µm filter at the water outlet, just as described in Villanueva & Alonso (2014). The incubation room uses an open system, as this life stage is very sensitive to any variation in water quality and this method is essential for the survival of the larvae.

The diameter of the eggs varies from 0.99 to 1.03 mm, with a slight decrease in size throughout the spawning season (Dinis et al., 1999; Imsland et al., 2004). The salinity of the water in the tanks is kept between 30 and 35 ppt, which allows the eggs to float. There is no illumination while the eggs are in the incubators.

The eggs are kept in the incubators for 5 days, with the age countdown starting on the day -2. Hatching occurs on day 0; from then on, age is counted with reference to this

day, the day of hatching. On the 2nd DAH (days after hatchery) the hatched larvae are removed from the incubation area and taken to the Larvarium.

During spawning, the incubation tanks are replenished daily with eggs originating from the Broodstock fertilization. The number of larvae produced is monitored and each tank is sanitized after each transfer to the Larvarium, to make them available to receive the next batch of fertilized eggs. The water flow, aeration, and UV lamps are monitored regularly, and the sand filter is backwashed 3 times a day.

Small samples are also taken from the tank on a daily basis to monitor the progress of the eggs' maturation under a microscope. This makes it possible to know the quality, density, and general condition of the batch of fertilized eggs, and thus to establish the distribution of the fertilized larvae when they are transferred to the tanks in the Larvarium.

After hatching, the larvae have an average length of 2.4 ± 0.1 mm, weighing around 0.35 mg (Dinis et al., 1999). There is variation within the population in terms of the weight and length of the larvae, this variation will depend on the stock and size of the eggs. Post hatch, they have reduced motility, a closed mouth and anus, and a symmetrical body.

Figure 5 - Incubation room. Photo taken by Alexandre Veloso.

2.1.3 Larvarium

At the Larvarium, Figure 6, the tanks that receive the larvae at the 2nd DAH have a volume of 2700 L, where they are kept at a density of 15 larvae L^{-1} at an O_2 saturation of around 130 %. The tanks have a cylindrical-conical shape and a self-cleaning structure with a slope at the base, with a central filter to prevent the larvae from falling into the sewer. The photoperiod is 16 hours of light, and 8 hours of darkness, provided by lamps suspended over each tank.

The larvae are born pelagic, where they prey on their live food along the water column, and the extrinsic feeding only begins at 3 DAH onward. Until this time, the larvae use their yolk sac as a food source. From 3 DAH until 5 - 7 DAH, the larvae are fed exclusively on rotifers (*Brachionus plicatilis*).

Figure 6- Larvarium room on the left and tank closeup on the right. Photo taken by Alexandre Veloso.

During these first few days, microalgae enrichment is started, simultaneously, to maintain the nutritional value of the rotifers present in the water column. The microalgae, not only maintains the nutritional value of the rotifers but also benefits the quality of the water in the tanks. These algae consume the nitrogen compounds that are toxic to the larvae and thus also promote optimal maintenance of the quality of the water column, as well as improving oxygen production (Abowei & Ekubo, 2011). In addition, the green hue of the water helps underdeveloped larvae, with low predatory efficiency and poor sight, to see their prey better. The low predatory efficiency is also exacerbated by their reduced mouth size and reduced mobility. For these reasons, they are exposed to a higher light intensity than that applied at later stages, providing the rotifers a higher contrast between the prey and the surrounding environment.

Between 5 - 7 DAH, the diet undergoes a transition from rotifers to brine shrimp (*Artemia salina*), more specifically *Artemia metanauplia*. The *Artemia* fed at this stage,

due to their nutritional composition, which is previously enriched with *Isochrysis galbana*, helps rapid larval development. In fact, Senegalese sole larvae can be fed on *Artemia* from the start of the exogenous feeding phase. However, although this feed is very effective at boosting rapid larval development (Madkour et al., 2023; Rønnestad & Conceição, 2012), rotifers must first be administered as early as 3 DAH. Rotifers, which are enriched with microalgae, have a composition rich in oligo-nutrients that improve the physiological development of the larvae (Villanueva & Alonso, 2014).

Between 10-15 DAH, the larvae undergo a process of transition and physiological change, called metamorphosis, which lasts until 20 DAH. Before this stage, they have pelagic behavior and body symmetry. During metamorphosis, the larvae's body undergoes changes: migration of the left eye to the right side; and torsion of the internal organs, among others. The larvae begin this process at around 4.1 mm in standard length and end up at ± 8 mm, with benthic behavior (Dinis et al., 1999).

The larvae remain in this room until metamorphosis is complete, after which they are transferred to the Weaning Area, where the gradual transition from live feed to inert food takes place.

2.1.4. Live feed

In aquaculture, live feed is generally used during the larval stage. There is still a large deficit when it comes to simulating the benefits of this type of food using inert food (Wikfors, 2004). The interest in being able to replicate this type of food is due to the potential scarcity of *Artemia* and the difficulty and instability in producing microalgae and rotifers, as well as the high cost involved in these maintenance and cultivation operations (Villanueva & Alonso, 2014).

However, the use of these feeds is still the only viable industrial option for producing high-quality juveniles of SnS.

Senegalese sole larvae are altricial, which means that at the beginning of exogenous feeding, their digestive system is still at a precarious stage of development, and they are unable to digest inert food. In addition to this slow maturation of the digestive system, these larvae have reduced mobility. For these reasons, it is necessary to use live food. Formulated inert feed tends to sink in the water column and is less available than live feed, which, by moving continuously, not only provides better distribution but also

stimulates the larvae. Palatability is also an important factor to mention, as inert feed is usually drier in texture and more difficult to accept than Rotifer/*Artemia*, which is, for the most part, very similar to the food that the larvae are adapted to hunt in the wild (Wikfors, 2004).

2.1.4.1. Rotifers

Rotifer, *Brachionus plicatilis*, are small metazoan organisms. This species was first described as a pest in eel culture in the 1950s. In Japan, these organisms were exploited for the first time as food for the early stages of marine fish development, where they began to show how good a resource, they are due to their biological factors, from the perspective of industrial production. Rotifers demonstrate high resistance to a wide range of environments and diseases and can replicate with a high reproduction rate and in high densities. These capabilities make rotifers very attractive to produce on a large scale.

Rotifer reproduction is a choice between sexual and asexual reproduction, as shown in Figure 7, depending on the environmental conditions. They are euryhaline and can withstand large variations in salinity, which is why, in the wild, they are found in marine, brackish, and freshwater ecosystems.

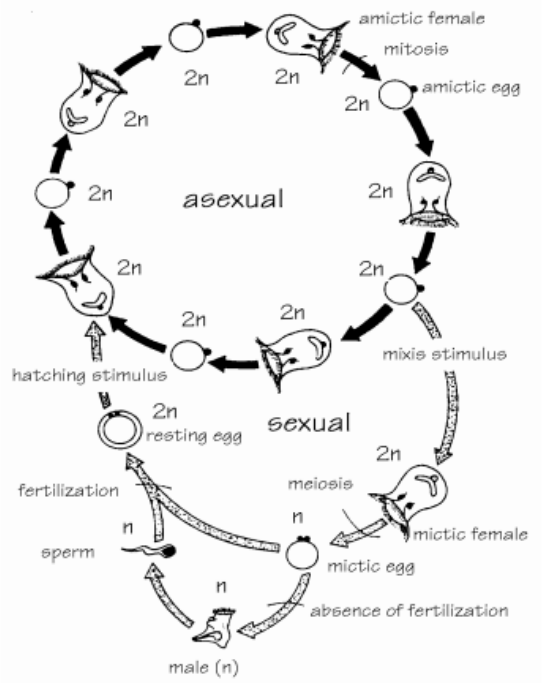

Figure 7 - FAO – Manual on the production and use of live food for aquaculture, modified from Hoff and Snell, 1987.

However, they do present challenges, as rotifers have a very short life cycle, 3.4 to 4.4 days, in the event of any spread of disease or problems supplying food to batches of rotifers (Hagiwara, 2017; Hagiwara & Yoshinaga, 2017), we could be faced with high mortality rates in a short period of time and jeopardize the stable supply of food to the larvae at one of the most crucial stages of larval development.

Rotifers are commonly described as living capsules that act as a nutrient transporter for the larvae. They are fed very dense pastes of microalgae (*Chlorella sp*. and *Nannochloropsis oculata*) (Hagiwara & Yoshinaga, 2017; Lubzens et al., 1995; Wikfors, 2004). This resource provides essential nutritional enrichment for the larvae, supplied by the rotifers. Chlorella is naturally enriched with vitamins and n3-HUFA (Highly Unsaturated Fatty Acids) and *Nannochloropsis oculata* with Eicosapentaenoic acid (EPA), essential elements in the physiological development of marine fish larvae (Villanueva & Alonso, 2014). These pastes are also very advantageous as they can be stored frozen for several months without losing their nutritional benefits.

The Rotifer room, Figure 8, is made up of 4 cylindrical-conical tanks, each with a capacity of 600 L, and another used solely for enrichment. Being kept at O2 saturation levels between 80-100%.

Figure 8 - Rotifer room in Safiestela. Photo taken by Alexandre Veloso.

At Safiestela, the procedure used for growing rotifers is the batch method, which is most commonly utilized in marine fish hatcheries, as described in Hagiwara & Yoshinaga, 2017. Cultivated in a constant volume while the rotifer density grows, as shown in Figure 9. Samples are taken daily to monitor the density of rotifers in each tank. When the ideal density is reached, most of the crop in the tank is harvested and washed. Some of these rotifers will be used for larval feeding and are placed in other tanks so that the larvae can be fed 5 times a day. What is not used for feeding is used as inoculation to start new cultures. The quantities for each purpose are stipulated in the “Rotifer Room Cultivation and Enrichment Protocol”. Each production cycle lasts 3 days, and a stable culture of these rotifers is maintained to have a constant source of food for the larvae between 2-5 DAH.

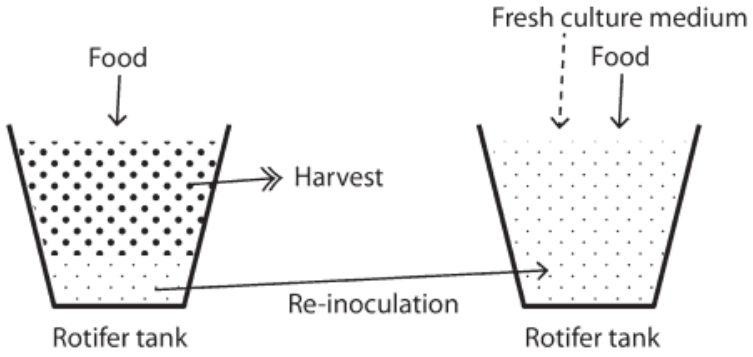

Figure 9 - "Batch method" schematic of the protocol used in Safiestela Rotifer cultivation. From Hagiwara, A. & Yoshinaga. 2017.

2.1.4.2. *Artemia*

Well-known for their frequent use as live food in aquaculture, more specifically for the larval stages of marine fish and crustacean species. *Artemia* has several characteristics that make it very attractive to this industry, from dormant embryos to its excellent nutritional composition (Lucas & Southgate, 2013; Wikfors, 2004).

The use of *Artemia* as a primary food in the production of marine fish larvae has undergone adaptations to make feeding using living organisms more efficient and as short-lived as possible, while still providing the nutrients that remain essential, where *Artemia* is still the only way to supply these nutrients (Wikfors, 2004).

However, it is clear that the use of *Artemia* in the long term will not be a sustainable choice when the industry demands quantities above the natural capacity of salt lakes

around the world. In addition to the finite capacity of the lakes, the fluctuation of the nutritional composition depending on climatic conditions makes the use of this organism an unsafe and unstable option as a food of one of the most fragile stages in aquaculture (Conceição et al., 2010; Wikfors, 2004). As a consequence of this shortcoming, there has been a continuous investment in improving inert feeds to eventually replace live food.

In the wild, when subjected to harsh environmental conditions, these crustaceans produce cysts that float. These cysts are caught and, when kept dry, can be preserved for long periods. It is in this condition that they are marketed all over the world (Lucas & Southgate, 2013; Madkour et al., 2023; Wikfors, 2004).

Unlike the Rotifers that are produced in-house, *Artemia* cysts are sourced from a commercial supplier.

In the *Artemia* room, Figure 10 on the left, to activate the cyst's metabolism and restart their development, they're immersed in salt water, which is subjected to intense aeration, slight oxygenation, salinity around 35 ppt, and a temperature of 25 ºC, as represented on the right in Figure 10. At this stage their shape changes from biconcave to spherical. Around 24 hours after contact with water, the outermost layer is broken off and the embryo still appears surrounded by the cyst wall. Meanwhile, the development of the embryo continues and when it is about to reach the INSTAR I stage, the shell is discarded so that it can move freely (Madkour et al., 2023; Villanueva & Alonso, 2014; Wikfors, 2004).

In Safiestela, the EG strain is used in this process; this strain was previously treated with iron. This treatment introduces iron into the chorion of the cyst shell, which means that at the stage where the shell is discarded, this process can be carried out with the aid of a magnetic separator and thus remove all the shells from the *Artemia nauplii* (Villanueva & Alonso, 2014).

After the hatching and enrichment phase, the *nauplii* are transferred to cylindrical-conical tanks where, under strong aeration and oxygenation, they will be filtered, washed, and placed in a container with a predefined volume. From this predefined volume, the necessary dose to be extracted to feed the larvae tanks will be measured and calculated.

Figure 10 - *Artemia* room in Safiestela (on the left) and cyst activation (on the right). Photo taken by Alexandre Veloso.

2.1.5. Weaning

The larvae from the Larvarium, after finishing metamorphosis, goes to the tanks in the Weaning area, more specifically in Weaning 1.

There are two different weaning zones, Weaning 1 and Weaning 2. The difference between the two is the life stage they contain and the type of water system utilized, which is different. In Weaning 1 the system is open. In Weaning 2 is a closed RAS.

Weaning 1 receives the larvae at 13 DAH and keeps them until 45 DAH. It has an open system, as described later in "Safiestela's open systems". The larvae that enter Weaning 1 will be introduced into squared polyethylene tanks with rounded corners, the same as in Weaning 2, with the distinction being in the smaller grade mesh in the center of the water outlet to prevent the larvae from being lost. These tanks have a water column height of approximately 12 -15 cm. After a few days, the larvae are relocated to the next stage in this area.

Weaning 2 receives the larvae already sorted by size from Weaning 1 and they remain here until they are transferred to the next stage, the Nursery, at 75 DAH. In Weaning 2, the squared-shaped tanks possess the same water column height, with a larger mesh filter in the center.

In Weaning, Figure 11, the purpose of this section is to transition the larvae gradually from live food, *Artemia*, to inert feed (Villanueva & Alonso, 2014).

The feeding regime at Weaning starts the same as seen when they left the Larvarium, with enriched EG *Artemia*, with 12 meals a day for 23 days, until 40 DAH. The introduction of inert feed begins at 31 DAH. The amount of *Artemia* fed is reduced in percentage until it reaches zero at 40 DAH, and the opposite happens with the inert feed. From 0 % on the 30 DAH to 100 % on the 41 DAH. This is how weaning from live feed takes place.

A concern that also begins to emerge at this stage is the difference in the growth rate in each tank's population. Naturally, different fish have more capacity to fight for food and so, over time, there will be a greater variance between the fish that grow the fastest and those that grow the slowest. Because of this, manual size grading is carried out as soon as the fish starts to transition between the different stages of Weaning. In these screenings, the fish populations are separated according to size, so that the population in each tank is uniform, and consequently their development will also be more homogeneous. It is also at this time when fish that are below the expected growth curve are discarded (Villanueva & Alonso, 2014).

The daily routines in the Weaning sector include counting fish and calculating the average weight of each tank, calculating the feed for the tanks according to average weight and the respective life cycle stage, grading (when necessary), cleaning and disinfection of the tanks, floors, and equipment, receiving and transferring fish (when planed).

The calculated inert feed is supplied to each tank using a system of automatic feeders connected to a silo that is periodically replenished with feed, which is monitored by a central computer system that programs the frequency and quantity of feed to be

Figure 11 - Weaning room in Safiestela. On the left is the tank where the manual grading is done. On the right, the tanks from Weaning area. Photos taken by Alexandre Veloso.

distributed to each tank daily. The *Artemia*, also under similar calculations with the same considerations, is administrated manually.

2.1.6. Nursery

In the broad sense, the Nursery involves receiving the larvae from Weaning, already sorted and weighing around 1 g, and maturing them to the juvenile stage, when they reach a weight of around 7 - 10 g at 140 DAH. When they reach this weight, they are transferred to the Pre-Ongrowing Area, the last phase present in the Safiestela hatchery.

The Nursery, Figure 12, consists of raceway-shaped polyethylene tanks with a water column height of around 12 centimeters. These tanks are arranged vertically. All the tanks in this section share the same recirculation system (RAS).

In this room, the water temperature is maintained at 19 ºC, at a salinity of 35 ppt, and with the oxygenation saturation always above 100%, at which point the water is considered supersaturated with O_2 (Lawson, 1995; Lekang, 2007).

As for the feeding, in the Nursery area, already exclusively feeding inert diets, each tank is supplied using a system of automatic feeders connected to a silo that is periodically restocked with feed, which is monitored by a central computer system. The frequency and quantity of feed supplied are calculated daily using the average weight in each tank; 200 fish are counted and weighted for this calculation. Following this calculation, a feeding rate is applied that is within the protocols defined by the company, which have been calculated and optimized over the years.

During the daily cleaning routines, the lighting in the Nursery room is dimmed to reproduce as accurately as possible the light conditions of a benthic environment.

The daily routines in this room follow the same parameters as in the Weaning section, but the techniques used are different due to the fish's maturity and the tanks' shape. The tanks are cleaned using brushes while, at the same time, the condition of the fish in the tanks is visually checked. Dead fish or other unusual symptoms are discarded and then registered. Every day, the materials used to clean the tanks, and the Nursery room are sanitized and disinfected, following Safiestela's standard disinfection protocols.

On the fish's journey in the Nursery stage, the fish will be submitted to a selection process (only once in the Nursery), the purpose of which is to check for possible diseases, discoloration, vertebral fusions, or any other type of anomaly that would make the fish unviable for human consumption or unfit. This inspection is carried out manually before

the fish enter the machine, which sorts them according to their size, and is carried out by the grader, here the fish are separated according to their thickness.

Fish are graded into three categories: small, medium, and large. Small fish, due to their low growth capacity, are discarded. This is done to maintain maximum efficiency in the use of system resources such as space, food, and oxygen (among others) (Villanueva & Alonso, 2014). Medium and large fish are then split up and assigned to separate tanks. This is done to homogenize the populations within each tank so that there is no major discrepancy in the growth of the fish. After sorting, when the large fish reach 10 g and the medium fish reach 7 g, they are transferred to the next phase in the hatchery, the Pre-Ongrowing.

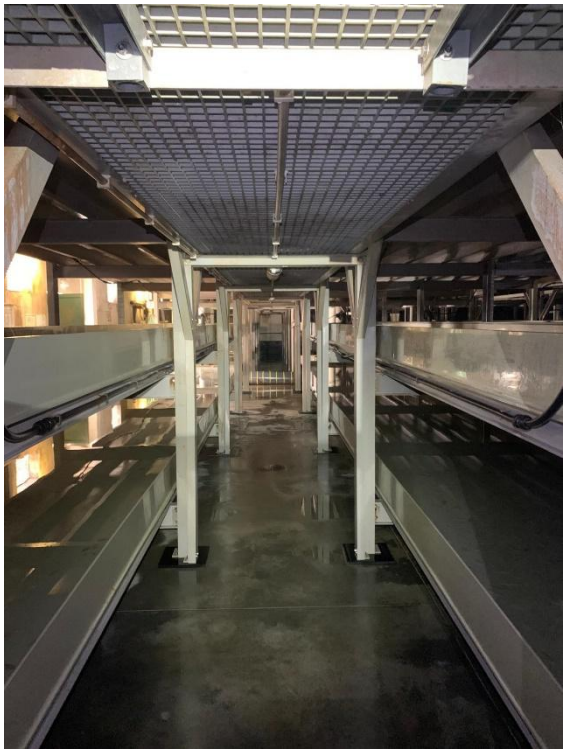

Figure 12 - Nursery room at Safiestela. Photo taken by Alexandre Veloso.

2.1.7. Pre-Ongrowing

As previously described, the juveniles that arrive at Pre-Ongrowing, 170 DAH, come in two grades, the large ones usually weighing at least 10 grams and the medium ones weighing between 4 and 7 grams.

Pre-Ongrowing, Figure 13, is the last stage of development at Safiestela before the fish are shipped to the SEA EIGHT group's Grow-out plants. This area is composed of tanks, stacked in vertical columns, and organized into Pre-Ongrowing 1, where the fish arrive from the nursery, and Pre-Ongrowing 2, from where the fish are removed for transport. Pre-Ongrowing 1 refers to the tanks on the lower level, the 2 tanks closest to the ground in the 10 columns, and Pre-Ongrowing 2 encompasses the tanks on the upper levels. The tanks in the Pre-Ongrowing stage are raceway-shaped, with the water inlet on the north side and the outlet on the opposite side, to create a watercourse with unidirectional flow.

Figure 13 - Pre-Ongrowing room at Safiestela. Photo taken by Alexandre Veloso.

As mentioned above, each of the Pre-Ongrowing sections is integrated into its own individual RAS, both are similar to each other, with the difference only being in the elevation (difference of 2 m) on which they are built, as demonstrated in Figure 14.

Figure 14 - Pre-Ongrowing's RASs in Safiestela. On the left the RAS from Pre-Ongrowing 1, and on the right the RAS from Pre-Ongrowing 2. Photo taken by Alexandre Veloso.

The daily routines are very similar to those in the nursery stage, both in terms of the maintenance and grading of fish. The differentiating factor between both stages is in the feed size, which is larger for this later stage. Additionally, the grading machine is also slightly distinct. Unlike in the nursery, where the fish are sorted due to their thickness, in Pre-Ongrowing, the triage is done concerning their weight, as shown in Figure 15. This can separate the fish by their most important factor to monitor in production, the weight. This allows the fish to be sent to the growing facilities with the most accurate homogenization.

When the tank's medium weight reaches 10 to 20 g, and in coordination with the Growing plants, it is scheduled for transport. Then, the fish will go on to the grow-out stage until it reaches the commercial weight.

In this and the other areas with a closed system at Safiestela, the water parameters are held stable and under control, the temperature kept around 20 ºC, oxygen around 140 %, pH at 7.5, Redox potential from 150 to 250 mV, and salinity at 35 ppt. Any significant change in the values of the mentioned parameters will result in fast and effective action from the production staff to set the conditions back to standard quickly and avoid any prolonged stress exposure to fish.

2.2. Safiestela's Recirculating Aquaculture System (RAS)

One of the most promising intensive aquaculture production systems is RAS. Recirculating water systems are utilized to achieve the goals of high-quality, and sustainable production in a controlled marine environment with minimal to no water needed per kg of fish. Only the Broodstock, Weaning 2, Nursery, and Pre-Ongrowing (1 and 2) stages have, each, their recirculation and water treatment system. The RASs are made up of mechanical filters, biological filters, disinfection systems (ozone and ultraviolet), skimmers, and a degassing tower. To prevent interruptions, the systems are built with redundancies, such as alternative pumps, bypasses in every stage of the RAS, and backup generators in case of a power outage (like the one in Figure 16), so that the tanks' water supply is not disrupted.

Figure 15 - Backup generator operated in Safiestela. Photo taken by Alexandre Veloso

2.2.1. Mechanic Filters

The function of mechanical filters is the removal of suspended solids, particulate matter, and dissolved solids present in the water.

2.2.1.1. Rotofilter

This type of filter specializes in removing the largest solids, and particulates, larger than 60 µm from the water column (Xiao et al., 2019). Said removal is performed by a drum with a rotating belt that constantly filters the water with suspended solids during constant mechanical agitation, as shown in Figure 17. This filtration produces a sludge (cake) that is subsequently discharged to the sewer. The rotofilter undergoes frequent high-pressure spray washing to avoid clogging and to keep the process efficient.

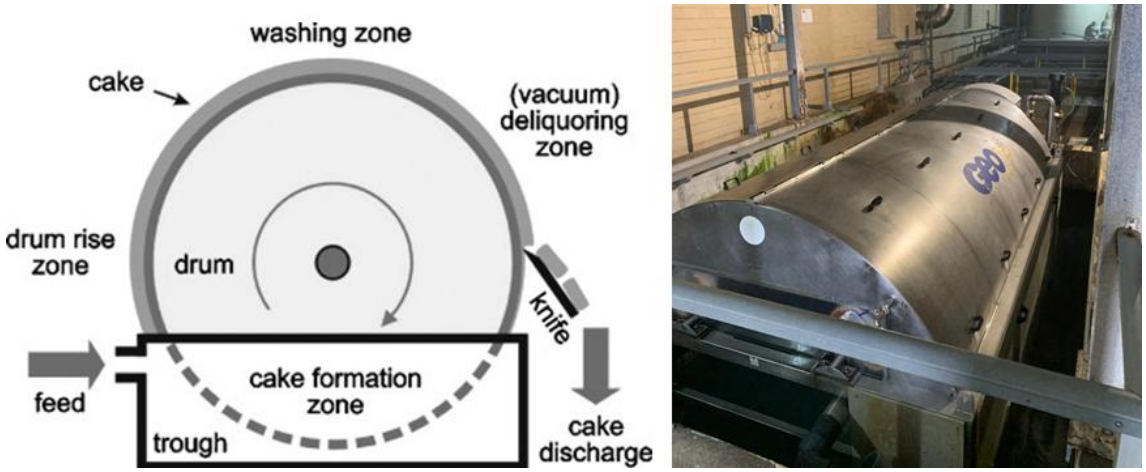

Figure 16 - Rotofilter schematic from Tarleton, S., & Wakeman, R. (2006) on the left and Rotofilter utilized in Safiestela in the RAS of the nursery. Photo taken by Alexandre Veloso.

2.2.1.2. Sand Filter

Pressurized sand filters are used to remove the size range of smaller particulate solids and larger dissolved solids, from 30 to 75 µm (Xiao et al., 2019). These filters (Figure 18) contain graded quartz sand which is laid down on pebbles and gravel. The water enters at the top and is introduced into the sands evenly to allow for homogenized filtration, preventing the creation of easier streams of water and thus not filtering as it should. The outlet for the filtered water is at the base of the filter, where it then goes on to the next filtration system. The filter has around 30% free space, so it is possible to backwash frequently, a similar process as with the rotofilter, to keep the sand filter's efficiency at its optimum (Basu & Debnath, 2015).

Figure 17 - Sand filters used in Safiestela. Photos taken by Alexandre Veloso.

2.2.1.3. Protein Skimmer

The process of foam fractioning of the water column is used to remove colloids and particles that are still dissolved. ABS or "Adsorptive Bubble Separation" explains the physical and chemical interactions that occur on the surface area of the bubbles formed in the foam, which trap the impurities dissolved in the water and remove them (Lekang, 2007). In the case of Safiestela, due to seawater abstraction, this process is all the more efficient because the bubbles in saltwater have greater surface tension, which is essential for bubbles to form. In these cases, the salinity stabilizes the foam, making it more difficult for the bubbles to burst. In fresh water, the bubbles are larger and burst more easily, so the ability to transport impurities is diminished in these circumstances. The following diagram (Figure 19, on the left) shows how the process of foaming and filtering the water from the tanks is carried out, like the one used in the recirculation systems at Safiestela (Figure 19, on the right).

This device is also used to inject ozone. When this gas is inserted into the skimmer, it causes oxidation reactions and breaks down biomolecules. Ozone will also form small bubbles, so, as well as being a disinfectant, ozone also enhances the efficiency of the skimmer.

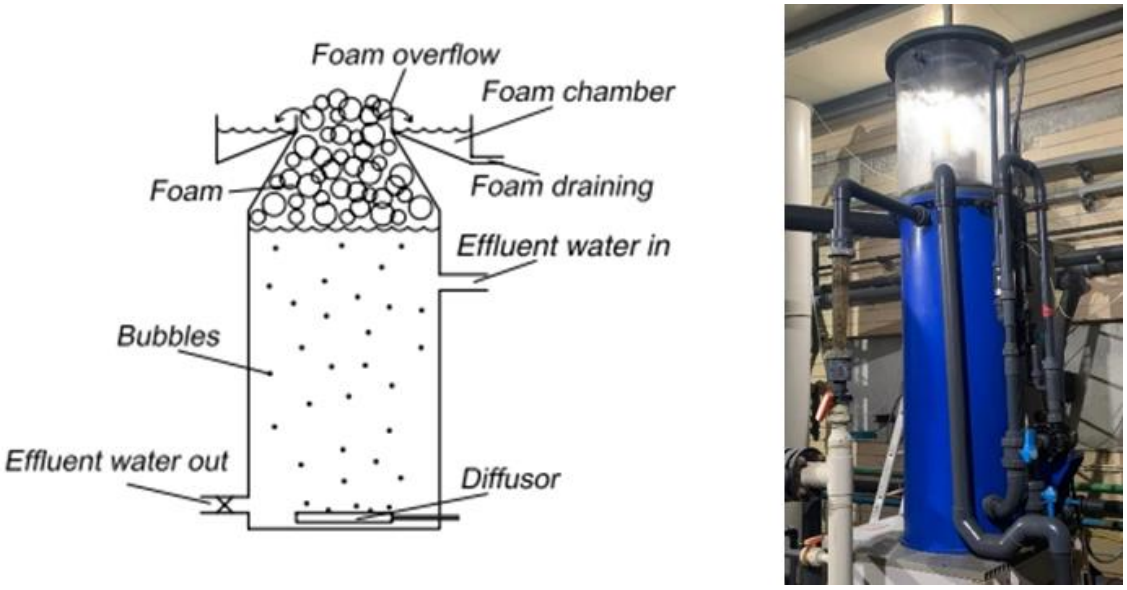

Figure 18 - On the left, protein skimmer schematic from Odd-Ivar Lekang (2013). On the right, protein skimmer used in Safiestela. Photo taken by Alexandre Veloso.

2.2.2. Biological filters

As a by-product of feeding, fish produce ammonia compounds. It is extremely important that these substances, which are toxic to fish, are extracted from the system to non-toxic levels, especially when you take into account that the system reuses 95% of the water it uses daily.

Using a biological filter relies on having a biofilm of bacteria that oxidize ammonia to nitrite, nitrite to nitrate, and, depending on the bacteria used in the biofilter, nitrate to molecular nitrogen. Turning ammonia into nitrites and nitrites into nitrates is called nitrification and is an aerobic process, so adding air to the biofilm is necessary to enable these reactions. Turning nitrates into molecular nitrogen is called denitrification and is an anaerobic process, in which case the medium in which this reaction takes place must be completely airtight. It is important to note that fish are more tolerant of the presence of nitrates in water when compared with other ammonia compounds. In other words, nitrification is seen as a priority in most aquaculture cases. Although denitrification is indeed a way of making the system have low total ammonia levels, it is not as relevant to fish as nitrification. Denitrification only becomes relevant when systems with very high-density levels are used and nitrate levels become unsustainable (Datta, 2012; Lucas & Southgate, 2013; Preena et al., 2021; Ruiz et al., 2020; Stien et al., 2020; Zhang et al., 2011).

Nitrification relies on bacteria that use atmospheric oxygen to oxidize ammonia and use carbon dioxide as a source for their growth. Ammonia Oxidizing Bacteria (AOB) transform the ammonium ion into nitrite and Nitrite Oxidizing Bacteria (NOB), nitrite into nitrate. The development of these bacteria in the biofilter is formed through a biofilm that extends over the surface area of the biofilter carriers (BioC). (Alexander & Clark, 2016; Preena et al., 2021; Spieck & Bock, 2015)

The efficiency and growth of the biofilm have requirements regarding the physico-chemical conditions of the water. Factors such as ammonia concentration, temperature, alkalinity, oxygen concentration, pH, salinity, organic substances, and toxic substances must be monitored and controlled within levels that the nitrifying bacteria can tolerate so that the efficiency of the biofilter is not jeopardized (Jafari et al., 2024; Lucas & Southgate, 2013).

The biofilter system used in the recirculation systems at Safiestela makes use of fluid bed bioreactors. In these systems, the bacteria are attached to BioC and are floating in

an upward current of water and air supplied by diffusers at the bottom of the tank, as shown in Figure 20. The BioC have a density just below the density of the water, which prevents them from clogging the outlet for the water and maintains a constant state of "fluidity" in the tank (Lekang, 2007).

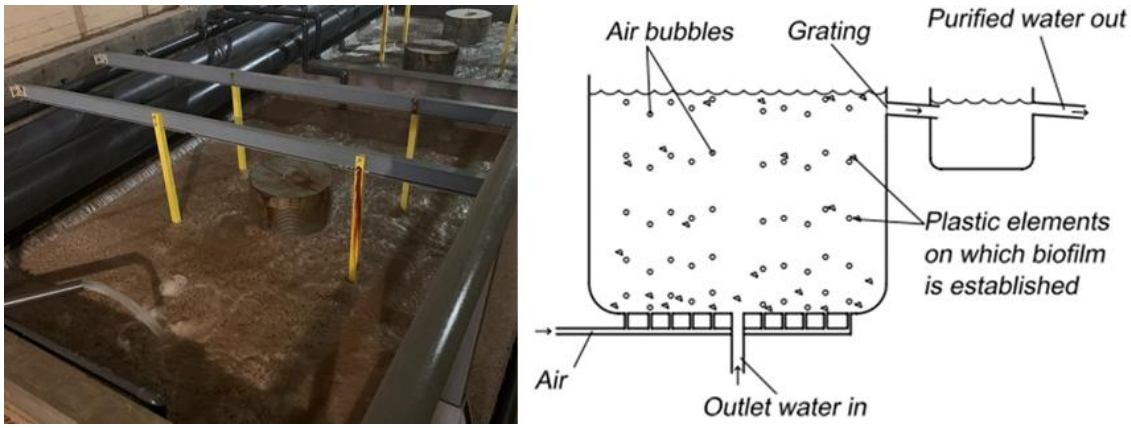

Figure 19 - On the left, moving bed biofilter in Safiestela. Photo by Alexandre Veloso. On the right, Moving bed biofilter schematic from Odd-Ivar Lekang (2013).

2.2.3. Disinfection systems

2.2.3.1. Ozone

The use of ozone in aquaculture environments, especially in hatchery RAS, has proven to be a tool for improving the efficiency of water quality maintenance in aquaculture systems by way of clarification of the water, reducing odor and off-flavor compounds, microflocculation of suspended and dissolved particulates, and oxidization of nutrients, cells, and other compounds, being especially effective oxidizing bacteria and viruses (Rueter & Johnson, 1995; Summerfelt et al., 2001; Timmons & Ebeling, 2010).

The ozone molecule has chemical properties that are highly harmful to all life forms due to its high oxidizing power. Damaging cell membranes and nucleic acids characterize it for the role it plays in breaking down long-chain molecules into simpler elements (Gonçalves & Gagnon, 2011; Lekang, 2007; Van Rijn, 2013).

In all the RAS implemented at this hatchery, ozone is only inserted in the skimmer area, the retention time used is as recommended by the skimmer manufacturer, which is calculated to achieve adequate disinfection. This is carried out with the intention that ozone molecules decay long before they reach dangerous concentrations in fish tanks. The miscalculation of this parameter could bring upon the fish a plethora of damage to

the tissues of the fish provoking greater susceptibility to infections or even death (Bullock et al., 1997; Summerfelt et al., 2001).

Its implementation in the system is done in programmed quantities and frequencies according to the feeding plan (Gonçalves & Gagnon, 2011).

Ozone utilized in this facility is produced in-house. The utilization of an Ozone Generator, as shown in Figure 21, generates this highly unstable molecule to be administrated in the system.

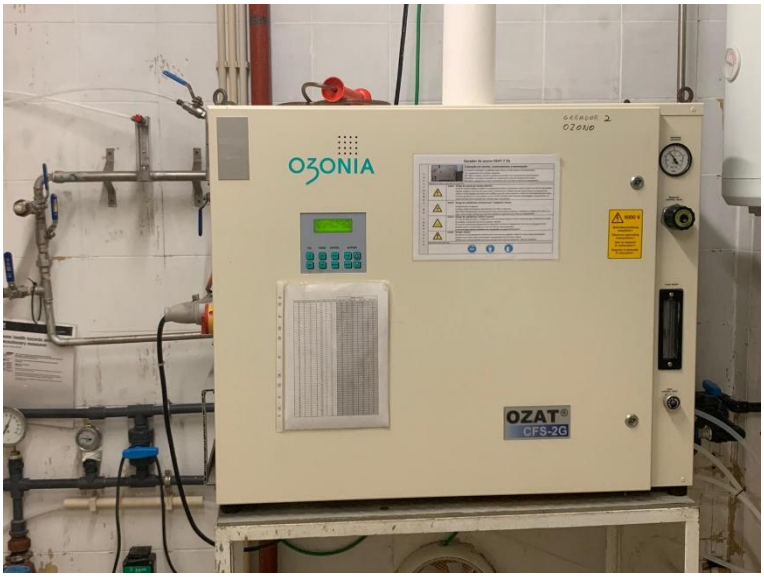

Figure 20 - Ozone machine utilized in Safiestela. Photo taken by Alexandre Veloso.

2.2.3.2. Ultraviolet

Ozone can be very effective at eliminating uni- and multicellular organisms; however, it has the problem of also being extremely toxic to fish and nitrifying bacteria, the high concentration that could make this a highly effective disinfection system is what also makes it limited (Kasai et al., 2002; Lucas & Southgate, 2013).

The UV mechanism, such as the one shown in Figure 22, when used exclusively, is very efficient at disinfecting water but has very specific requirements and limitations. This efficiency is only observed when there are little to no particles in the water, or other suspended solids, which "shade" the pathogens in the water that the UV light is supposed to destroy (Lekang, 2007; Zheng et al., 2011).

For these reasons, the use of an element with high organic matter oxidizing power followed by a nucleic acid destruction system is the most destructive way of achieving

maximum water disinfection, taking into account the health of the fish and the integrity of the biofilm in the biofilter (Kasai et al., 2002; Lekang, 2007; Lucas & Southgate, 2013; Summerfelt, 2003).

The recirculation systems at Safiestela use UV lamps that irradiate the water, which has already been previously filtered by mechanical and biological mechanisms after the first ozone disinfection has been carried out. The placement of UV after Ozonation in a RAS happens because of the UV's ability to, besides being a powerful tool in removing pathogens, particulate matter, and other foreign bodies, increase the dissociation speed of Ozone (Summerfelt et al., 2004).

This technology protects fish, both from potential pathogens and Ozone residues, while also improving water purity.

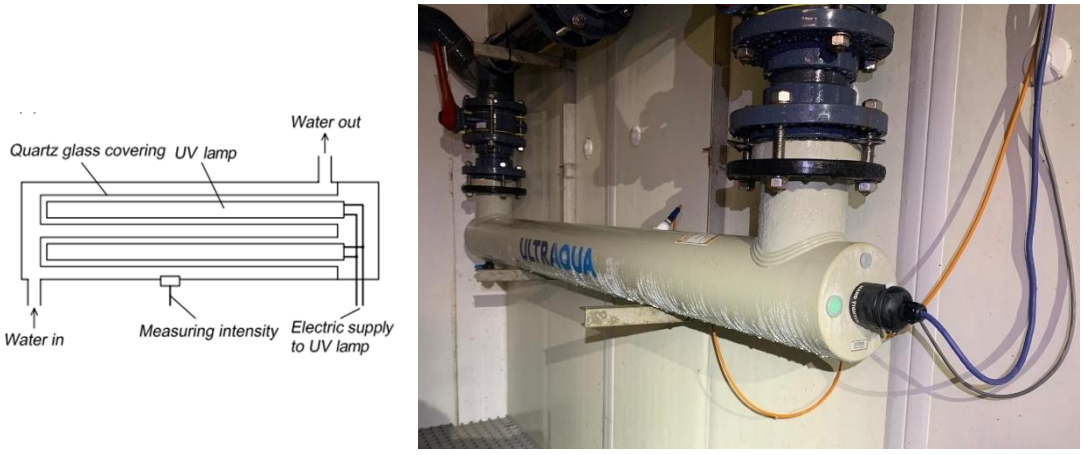

Figure 21 - Ultraviolet lamp schematic from Odd-Ivar Lekang (2013) on the left and UV lamps utilized in Safiestela on the right. Photo taken by Alexandre Veloso.

2.2.4. Aeration

Systems that recycle the water used, with levels of recirculated water around 95%, have a high need for disposal of Carbon Dioxide, a product of fish and bacteria respiration. The high presence of gases in the water such as nitrogen or carbon dioxide is toxic to fish, AOB, and NOB present in the biofilter. These by-products can cause several acute, or chronic, health problems or even death (Bregnballe, 2022; Lawson, 1995; Lekang, 2007; Timmons & Ebeling, 2010).

Aeration of the water column allows these toxic compounds to be removed.

In the degassing tower, Figure 23, as the one presented on the left in Figure 9, the inlet of water is above a column composed of elements with a high surface area, and with air pumped from the bottom, this creates a downward water flow high contact surface between air and water. As a form of redundancy, the use of pure oxygen is also available to augment water oxygenation.

Being the water over-saturated with gases, such as Carbon Dioxide, Nitrogen, and other noxious gases, and under-saturated with oxygen, the contact between air and water will provide an efficient, low-energy method of removing toxic gases and inserting oxygen. This happens due to the delta in saturation of the different gases in water and in the air. High-saturated dissolved gases will transfer to where the same gas has lower saturated levels. So, high CO_2 and N in the water will transfer to the air, and low O_2 in the water will increase its level with oxygen received from the air until a stable and equal level is reached in the concentrations of all gases (Timmons & Ebeling, 2010).

Figure 22 - Degassing tower used in Safietela.
Photo taken by Alexandre Veloso.

2.3. Safiestela's open systems

In the systems: Incubation, Larvarium, Live food (Rotifers and *Artemia*), and Weaning 1; an open system design is adopted. The saltwater needed for renewal comes from the sea catchment located in the coastal area of Rio Alto (Póvoa de Varzim).

After the water is collected from the ocean, it goes through filtration and disinfection systems to have the necessary biological, physical, and chemical conditions. These systems are considerably simpler than recirculation systems. Filtration begins with sand filters, then the water passes through cartridge filters, Figure 24, which deal with the smallest particles.

Figure 23 - Cartridge filters utilized in Safiestela to remove impurities from the sea sourced water. Photo taken by Alexandre Veloso.

UV lamps are used for disinfection and at the end of these processes the water enters the heat exchangers which ensure that the water goes into the tanks at a stable temperature of 19ºC (except for the Broodstock). In these systems water quality is of great importance and must be maintained at the optimum and constant levels. Being high-sensibility zones for the entire production, there cannot be a system failure that jeopardizes the survival, or well-being, of the initial stages in the life cycle and, consequently, the failure (or underperformance) of these batches in the following production stages.

2nd Chapter

NUTRITIONAL TRIAL

A study of two diets from different suppliers on growth, feed use, and costs.

2nd phase of the thesis – Nutritional trial in R&D and Nutrimu

1. Introduction

1.1. Historical contextualization of Aquaculture

The production of aquatic animals for human consumption is an enduring aspect of our nutritional heritage. The culture of aquatic organisms is one of the many examples that illustrate the remarkable resourcefulness of the human species in sourcing animal protein.

Aquaculture, or the mention of this praxis, can be dated back to 3 500 to 4 000 years Before Christ (B.C.). In East Asia, mainland China, the beginning of this “Art” was mainly restricted to capturing juveniles of common carp, followed by enclosing these organisms in ponds and growing out until ripe enough for consumption. These basic, rudimentary, and simple practices were the norm for aquaculture worldwide and kept its progression ceiling limited for thousands of years (Ling, 1977; Stickney & Treece, 2012). This knowledge was expanded by Chinese emigrants who continued the carp culture in different environmental and geographic contexts, first in South-East Asia and then the world. The practices introduced to local indigenous populations were then improved and adapted to local conditions and species based on simple trial and error (Ling, 1977; Nash, 2011; Stickney & Treece, 2012). The introduction of aquaculture in Europe can be traced back to the peninsula of Anatolia, where it was first developed. It subsequently spread to Greece and Italy, facilitated by the Roman Empire (Nash, 2011; Stickney & Treece, 2012).

Prior to the establishment of hatcheries in the nineteenth century, the prevailing techniques for the capture, containment, and grow-out of fish were relatively unsophisticated. While the advent of hatcheries led to an increase in the stocking of larvae and post-larvae in the early 1900s, there remained a gap in the knowledge regarding the feeding of younglings. Until this bottleneck was surpassed, only species with extensive yolk sack feeding phases, diadromous fish species such as salmon and trout, could be increased in production because of the increased ability to artificially engineer these organisms’ life cycles (Shelbourne, 1964).

At the beginning of the 20th century, it began to be apparent an increase in North America's interest in improving natural fish stocks due to the high dependence of populations on food sourced from fisheries. Thus, a government-backed plan was developed to have facilities able to produce larvae to replenish natural populations with artificial fingerlings, an approach later proved to be inefficient (Stickney & Treece, 2012;

D. B. White & Stickney, 1973). However, the research and development behind this project led to a significant increase in knowledge of controlling the first phases of marine fish culture, with the adoption of several new techniques (up until that point in time).

But it wasn't until the 1970s that great monetary and personnel investments were allocated to intensive aquaculture-enhancing research in all its avenues of study. Aquatic farmers started to raise knowledge of controlling the life cycle of a broader range of selected species (Asche et al., 2008).

The development of basal technologies has helped to cheapen the production costs of aquatic products and thus increase the competitiveness of aquaculture against fisheries. This brought interest, first from scientists and governmental research institutions and then from large international corporations intending to intensify and modernize aquaculture production (Nash, 2011).

The rise in the ability to control the feeding, breeding, hatching, etc, allowed an increased level of value and quantity of reared produce. Species diversification became inevitable due to market demands and the depletion of the natural fishing stock. So, through research and development of both public and private entities, the species range with rearing knowledge also improved immensely (Nash, 2011).

Improving expert knowledge in the intensive aquaculture of commercial fish provided conditions for the production yields to extensive growth throughout the following decades (Figure 25).

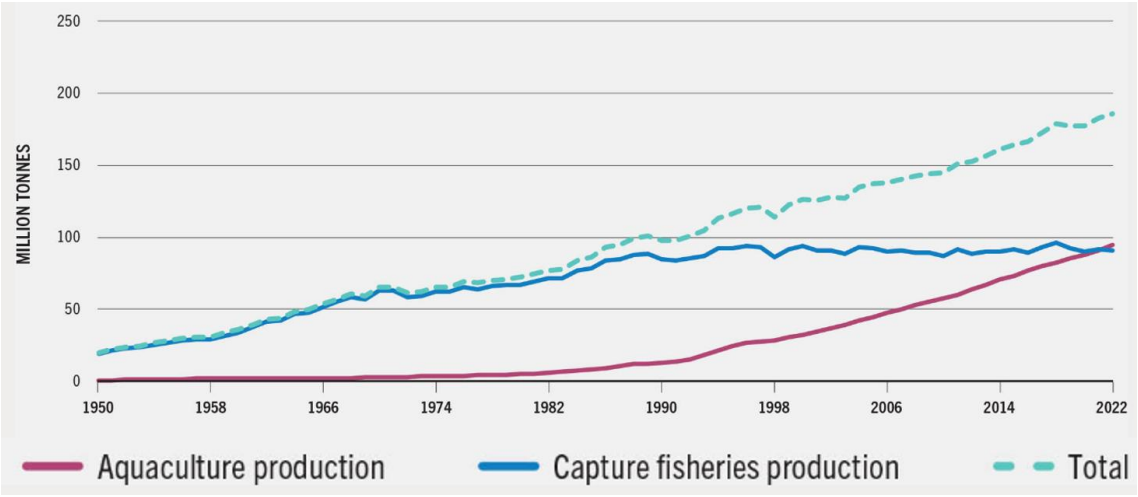

Figure 24 - World Fisheries and Aquaculture productions, excluding algae. Units represented in live weight of the aquatic animals. Retrieved from 'The State of World Fisheries and Aquaculture', FAO 2024

The diversification of cultivated species was, and will continue to be, a crucial step in expanding the industry's geographical reach and enhancing its resilience to market price fluctuations, disease proliferation, climate change, and other factors that could potentially limit the sustainability and stability of food sources. By diversifying the species cultivated, the industry can overcome the limitations of seasonal harvests and other factors that could otherwise hinder its growth and profitability. The prospect of diversification also naturally develops as a result of the emergence of new economic opportunities due to the market over-saturation of selected species (Boyd et al., 2020; Cai et al., 2023; Harvey et al., 2017; Le François et al., 2010; Schmidt et al., 2011). Farmed species diversification plays a special role even when considering the limitations of fisheries. The wild-sourced aquatic products captured levels have stabilized since the 1990s, but the number of inhabitants and seafood consumption have risen significantly (FAO, 2024). Subsequently, to supply proper food for such demand, aquaculture must expand, and so it has.

Since tracking Aquaculture’s production output began, this industry has maintained an average annual growth of 7.0 % (nominal terms). In comparison, fisheries remained stable between 86 – 94 million tonnes per year since the 1980s.

In 2022, Aquaculture reached never-previously seen levels of aquatic animals produced for human consumption, a record high 51 % (corresponding to 94 million tonnes) of the total weight of animals produced. For the first time, this represents higher production than fisheries (91 million tonnes, or 49 % of the total aquatic animals' production for human consumption) (FAO, 2024).

1.2. Recirculation Aquaculture Systems

Most fish farms use low-cost, minimal-intervention methods that face many challenges. These issues include extracting resources from the natural environment, lack of biosecurity control, inability to predict production levels due to unexpected weather, and many others. However, more predictability, control, and capacity to scale production is needed to meet the growing demand for seafood. It is unreasonable to expect these systems to fulfill these needs (Lucas & Southgate, 2013; Martins et al., 2010; Timmons & Ebeling, 2010). Juxtaposing, RAS systems have demonstrated their capacity to provide specialists with the most sustainable and efficient methods for controlling all aspects of production with minimal environmental impact. To maintain the profitability

and continuous operation of these types of facilities, high initial and maintenance costs, as well as highly knowledgeable personnel, are required, which implies an economic barrier to deploying such systems (Ahmed & Turchini, 2021; Boyd & McNevin, 2015; Lucas & Southgate, 2013; Tidwell, 2012; Timmons & Ebeling, 2010). Despite the current scarce global deployment of this technology, experts foresee that the expansion of aquaculture production through the utilization of aquaculture systems with reduced or no environmental impact, such as RAS, will seek to satisfy the rising global demand for sustainable and cost-effective seafood, while also addressing concerns surrounding food security (Ahmed & Turchini, 2021; Aich et al., 2020; Boyd et al., 2020; Campanati et al., 2022).

1.3. Species explored in RAS

Asche et al., in 2008, put forward that although RAS provides several benefits due to its ability to deliver “complete environmental control”, it does not show proficient economic results when applied to grow-out stages of certain well-known marine finfish species like salmon, sea bass, and sea bream. So, in most species, RAS is used for hatching and initial larval development only. But, even with that in mind, Asche recommends that Pleuronectiformes species, also known as flatfish, be produced in RAS for the entire life cycle.

This is consistent with the evidence presented in other literature, suggesting that this particular order may possess certain characteristics contributing to its perceived competitive superiority relative to other commonly commercialized fish. Flatfish species are benthonic, which implies that in their rearing (after metamorphization), a high column of water is not a necessity to maintain them, bringing a direct beneficial impact on the tank choice, space management, lower maintenance, and overall reduced production costs that make it easier for aquaculture professionals to retain a higher yield per hectare of land used in their fish production (Brown, 2002; Kamstra et al., 2001; Labatut & Olivares, 2004; Øiestad, 1999). Furthermore, flatfish demonstrate elevated market prices, Figure 25, and consumer acceptance, particularly in European markets (but also in other regions of the world), and are increasingly sought after for their white, low-fat, high-protein fillet (or whole fish) (Bjørndal et al., 2016; Dinis et al., 1999; Imsland et al., 2004; Morais et al., 2016).

Although most flatfish species share these same biological benefits and market preferences, some species are more attractive to intensive exploitation than others. Sole has been, for more than forty years, identified as a high-potential alternative for marine aquaculture (Howell, 1997; Shelbourne, 1975).

1.4. Farmed Sole species, the advantage in *Solea senegalensis*

Sole has been a focus of interest for aquaculture researchers and practitioners due to its potential for highly lucrative intensive aquaculture. The two species included in this classification (*Solea solea*, Linnaeus, 1758, and *Solea senegalensis*, Kaup, 1858) have ranked among the priciest in the consumer market for more than 10 years (Imsland et al., 2004), as Figure 25 demonstrates. Both are jointly represented in numerous institutional reports on (both fisheries and aquaculture) market performance and production and also mistakenly identified as the same species by the end consumer (Bjørndal et al., 2016; Fao, 2015).

Common sole and Senegalese sole (SnS) are similar. Both larvae are hard to distinguish from one another. Both have similar life cycles and are gonochoric (Lagardere et al., 1979; Russell, 1976). SnS has a shorter time to reach maturation, age 3 or more, whereas Common sole females take 4-plus years. When females reach maturation, they show a total length of 32 cm in SnS and 27 (in the Mediterranean Sea) to 30 cm (in the Atlantic Ocean) in Common sole (Ramos, 1982). SnS shows a higher overall growth rate than Common sole. Being a more southern inhabitant, SnS has a better tolerance for higher rearing temperatures when comparing natural habitats, which benefits its culture in south European countries (Dinis et al., 1999; Fao, 2015).

Both are teleost finfishes from the Flatfish order (Pleuronectiformes) and the Solidae family (Ben-Tuvia, 1990; Costello, 2001). SnS is an oval, asymmetrical fish with eyes on the right side. It has a pectoral fin on the eye side and a dark interradial membrane with yellowish-grey rays. The color of the eye side varies from greyish to reddish brown, this coloration is characterized by a series of large, diffuse, dark spots. the blind side is white; the pectoral fin on the eye side is marked by an elongated, black spot at the distal end; the posterior part of the tail displays a darker pigmentation than the rest of the fin. The average adult size is 30 to 40 cm but can reach up to 70 cm. This species has a life span of up to 11 years (Ben-Tuvia, 1990; Imsland et al., 2004; R. Martins & Carneiro, 2018).

This species is found in the eastern Atlantic Ocean, from the Bay of Biscay down to the south, and in the Mediterranean Sea, although it is rare. It is also present in the waters to the south of Senegal (Golani & Brand, 2016).

This benthic species inhabits sandy or muddy substrates in coastal areas, at depths of up to 1000 meters, and in salt lakes. A predator that feeds on benthic invertebrates, including polychaete annelids, bivalve mollusks, and small crustaceans (*Solea senegalensis*, Senegalese Solesole: FishBase, n.d.).

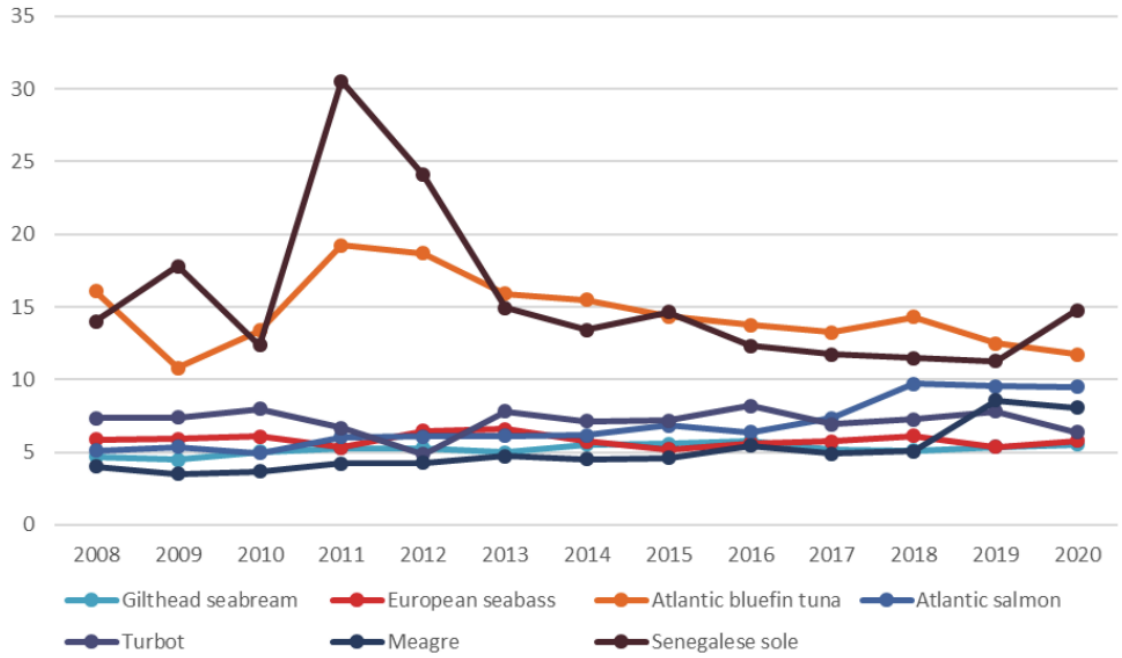

Figure 25 - Price evolution (€/kg) of the main species produced in EU marine aquaculture: 2008-2020. Source: Scientific, Technical and Economic Committee for Fisheries – Economic Report on the EU aquaculture (STECF-22-17) (Nielsen et al., 2023)

The depletion of natural stocks due to overfishing and anthropologic destruction of natural habitats, wild captures of Sole have declined since 1994 (Bjørndal et al., 2016; Pauly & Zeller, 2016). Consequently, a market hole has been created, where, through investigation and investment, both Common Sole (also known as Dover Sole), *Solea solea,* and Senegalese Sole, *Solea senegalensis,* surged as species of interest to explore intensive aquaculture systems, which has shown a significant yield growth since the beginning of the 2010s (Fao, 2015; Morais et al., 2016).

Intensive research and culture of this species since the 1980s (Arias et al., 1984; Dinis, 1986; Rodríguez & Arias, 1978) highlighted several challenges in SnS production. A lack

of control over broodstock, on-demand hatching, and knowledge of the specifics of larvae nutrition has severely hampered the quality and quantity of eggs and fry produced (Dinis et al., 1999; Rodríguez-Gómez et al., 1999).

The culmination of high investment in research and development during the early 2000s changed this paradigm at the beginning of the 2010s, where novel practices applied to the production of this species were then economically possible, and the potential for the coming future seems bright (Bjørndal et al., 2016; Bjørndal & Guillen, 2014; Fao, 2015).

RAS and low-height raceway systems have been the standard in high-intensive sole farming (Morais et al., 2016; Muñoz-Cueto et al., 2019). These systems have increased production and quality exponentially since the beginning of the last decade, as seen in Figure 26.

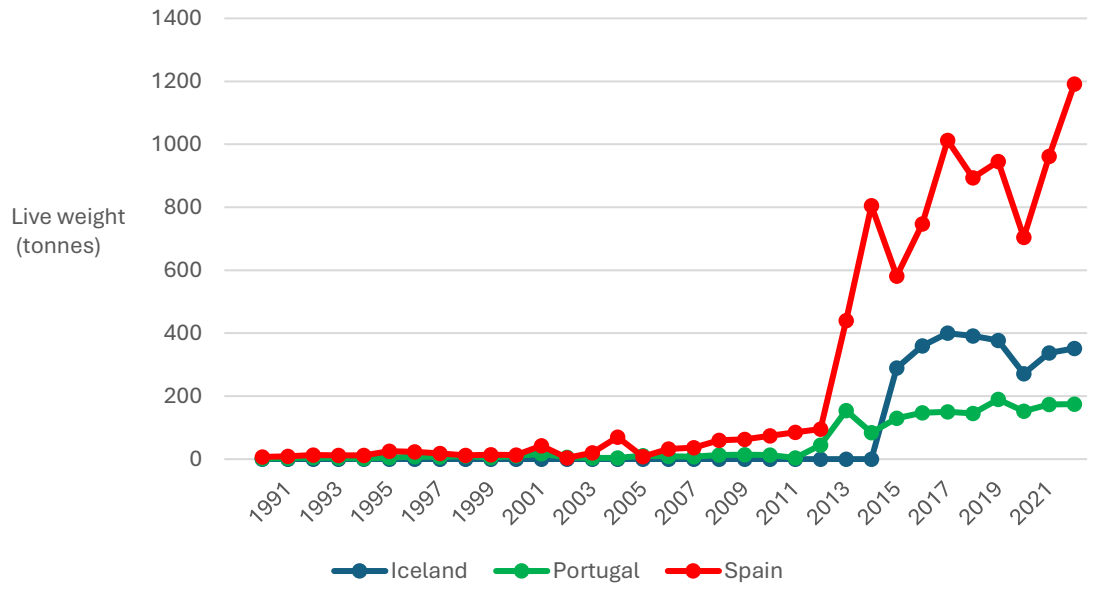

Figure 26 - Global aquaculture production (tonnes, live weight) of *Solea senegalensis* since 1990. Source: FishStat, FAO, 2024.

Increased know-how and control over the life cycle of the sole in artificial environments has been the main motivator for this accentuated growth. Although current production levels of Sole are considerably higher than those in 2010, there is still massive room for improvement compared to more industrialized species, such as optimizing feed management and composition.

1.5. Nutrition as a means of catapulting aquaculture production efficiency of SnS

Fed aquaculture (intensive and semi-intensive), the majority of global aquaculture (73%) (FAO, 2024), has feeding-related costs as one of the highest production expenditures, which presents a critical challenge when increasing production chain profitability. In Sole farms utilizing RAS systems, Bjørndal et al., (2016) reported these on 15.8% and Imsland (2004) on 17.9%. In earth ponds of intensive production, the percentage of feed in the production cost is much higher, García García & García García, (2006) reported – depending on cultured densities – values between 30 and 38%. This and other current challenges regarding SnS production (increase fish growth rates, welfare, stress tolerance, resistance to diseases, reduce risks in Sole production, etc) may be resolved – or significantly reduced – through the implementation of novel advanced aquafeed technology and innovative management practices (Oliva-Teles, 2012; Pohlenz & Gatlin, 2014), thereby creating additional lucrative expansion opportunities.

Over the past fifty years, since Howell in 1973, there has been a significant challenge in both academic and industrial research to attain the expertise required to fulfill nutritional requirements throughout Sole's life span.

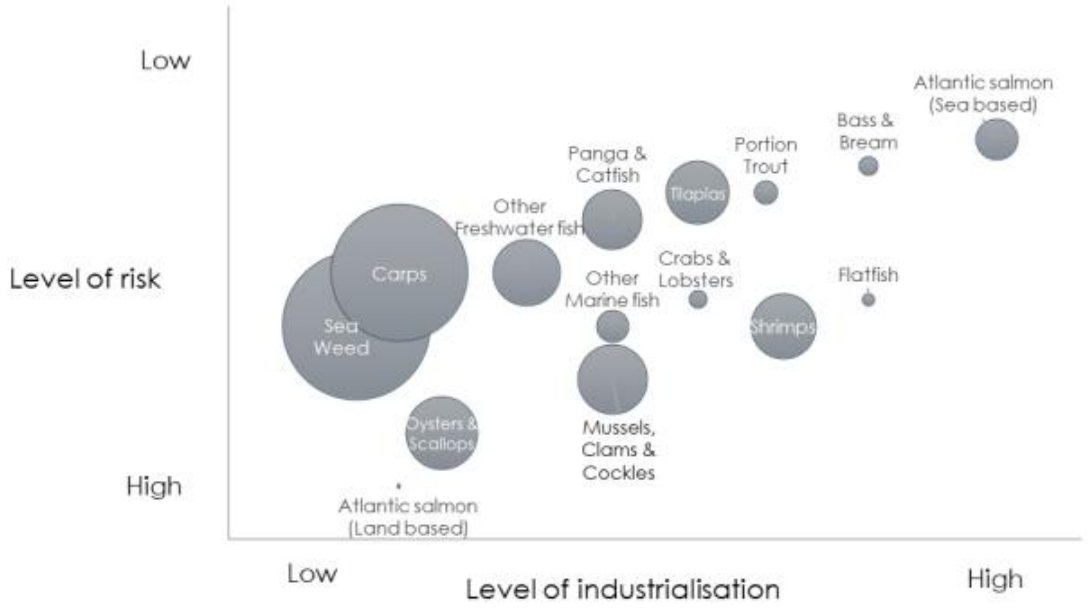

Figure 27 - Illustration relating "Level of Risk" and "Level of Industrialization" in a range of different farmed aquatic products from around the globe. The size of each circle represents the total volume of production. Source: Mowi (2024)

The extensive literature that now exists on the subject provides a valuable resource to culturists (Alves Martins et al., 2013; Aragão et al., 2004; Beirão et al., 2015; Darias et al., 2012; Hachero-Cruzado et al., 2014; Matias et al., 2024; Morais et al., 2006; Villalta et al., 2008a, 2008b; Vizcaíno et al., 2018), particularly over the past two decades, which have been pivotal in enhancing feeding efficiency, feed sustainability, and optimizing yield and survival rates. The knowledge in the fields of metabolism, nutrient requirements, and other nutrition-related disciplines served as the foundation for the production growth observed over the past decade, Figure 26.

Notwithstanding the aforementioned improvements, there is still considerable potential for further enhancement of the quality of feed related areas in SnS production.

Flatfish farming output levels continue to exhibit marginal levels when compared to other species that are subjected to more intensive cultivation procedures, such as salmon, sea bream, and sea bass – as seen in Figure 27. At this stage, Pleuronectiformes and SnS, in particular, are considered to pose a moderate risk in production, with a medium level of industrialization and low yield levels, but with high end-consumer acceptance and high market prices. This situation offers great potential for future valorization. Further research and development could potentially elevate SnS to a level of importance in the global food supply that is comparable to that of species that have already undergone market consolidation.

According to FAO (2024), the optimization of feed according to the species and life stage was one of the main proponents of the growth seen in aquaculture production in recent decades. As a consequence of the accumulation of new knowledge over the years, alternative feeds with superior performance at a reduced cost have been developed and introduced to the fish feed market by various commercial suppliers and subsequently employed by aquaculture. A competitive fish feed market pushes for further development to even better, more sustainable, and more cost-efficient aquafeeds, even though it is a process with associated challenges.

In the early 2000s, the future of aquaculture expansion was questioned, given the high reliance on fishmeal and fish oil sourced from wild fisheries. At the time, the high "fish in: fish out" ratio was one of the main arguments for the then-proposed limitation in the production of high-value, carnivorous fish species because of its dependence on such a unsustainable food source (Cressey, 2009; Hannesson, 2003; Naylor et al., 2000; Tacon et al., 2006; K. White et al., 2004). Hence, improvements in feed management will

continue to be key to the future development of sustainable, scalable, and resilient SnS aquaculture (Bjørndal & Guillen, 2014; Glencross et al., 2023).

Consequently, the main objective of the present trial developed in this thesis is to evaluate and optimize the choice of Nursery feed between two diets from different commercial suppliers in the current fish feed market, specifically within a RAS shallow raceway tank system. Selecting a superior aquafeed may improve growth rates, welfare, and health and reduce feed wastage. This, in turn, would enable Sea Eight to achieve higher levels of sustainable and economically efficient standards in the production of Senegalese sole.

2. Materials and Methods

2.1. Diets

A proximate analysis of two distinct commercial diets, designated here as "Diet A" and "Diet B" was conducted on both diets prior to the start of the trial; the results are detailed in Table 1. Under the terms of the confidentiality agreement between the parties involved in this trial, the names of the suppliers, the real price, and their respective ingredient composition will be excluded from this paper.

Table 1 - Proximate analysis of commercial Diets utilized in this trial.

	Diet A	**Diet B**
Dry matter (%)	8.1	9.2
Protein (% dry matter)	62.2	59.7
Lipids (% dry matter)	17.4	17.7
Ash (% dry matter)	12.2	12.7

2.2. Experiment and system design

In this trial, it was adopted a “Randomized Blocks” experimental design, first described by Fisher. The trial was conducted in three independent systems, with each diet tested in duplicate in each system. The distribution of the diets was randomized (Zar, 2014).

Albeit this system design offers a more robust analysis and an implied certainty to rule out environmental differences, the systems were still kept in stable biological, physical, and chemical conditions.

Three similar thermo-regulated RAS systems were utilized, emulating the larger RAS installed in the production facilities. Each of the three systems had four shallow raceway-type polyethylene tanks (40 cm x 80 cm x 20 cm) assembled in parallel, with a self-cleaning design (a slight tilt in the tank's floor towards the outlet). Every tank had a manually regulated water inlet at the top and, at the bottom, an outlet with a mesh to prevent fish escape. The water renovation rate in the systems was maintained at 5% of the system's total water volume. At certain times, due to the low volume of the system, this rate was manually overridden to maintain the water quality level. Once the optimal quality was achieved, the renovation rate was readjusted accordingly.

Each RAS is comprised of a sump with three interconnected divisions, including a mechanical and biological filter, in addition to a skimmer, as demonstrated in Figure 29.

The mechanical filter was set up as an aggregate of different mesh gradient sponges, wherein the mesh grade diminished following the progression of the water flow. The gradual reduction in mesh grade as the water flowed through the sponges effectively prevented clogging, ensuring a continuous and uninterrupted water flow. The sponges were situated in the immediate vicinity of the point of influx of the residual waters.

After the mechanical filters, a biofilter, with BioC sourced from production's RAS with an already matured biofilter, provides for the elimination of ammonia compounds. Furthermore, this division is equipped with a thermostat, which enables the regulation of the water temperature. In this area, it is also provided constant and intense aeration, to provide fluidity to the BioC.

Subsequently, the water is conveyed to the final division. The skimmer is set up in this division. As previously described, the protein skimmer facilitates the removal of colloids and other particles that remain dissolved in the water column. Subsequently, the water flows to the pump, where a diffuser stone is situated to facilitate oxygenation.

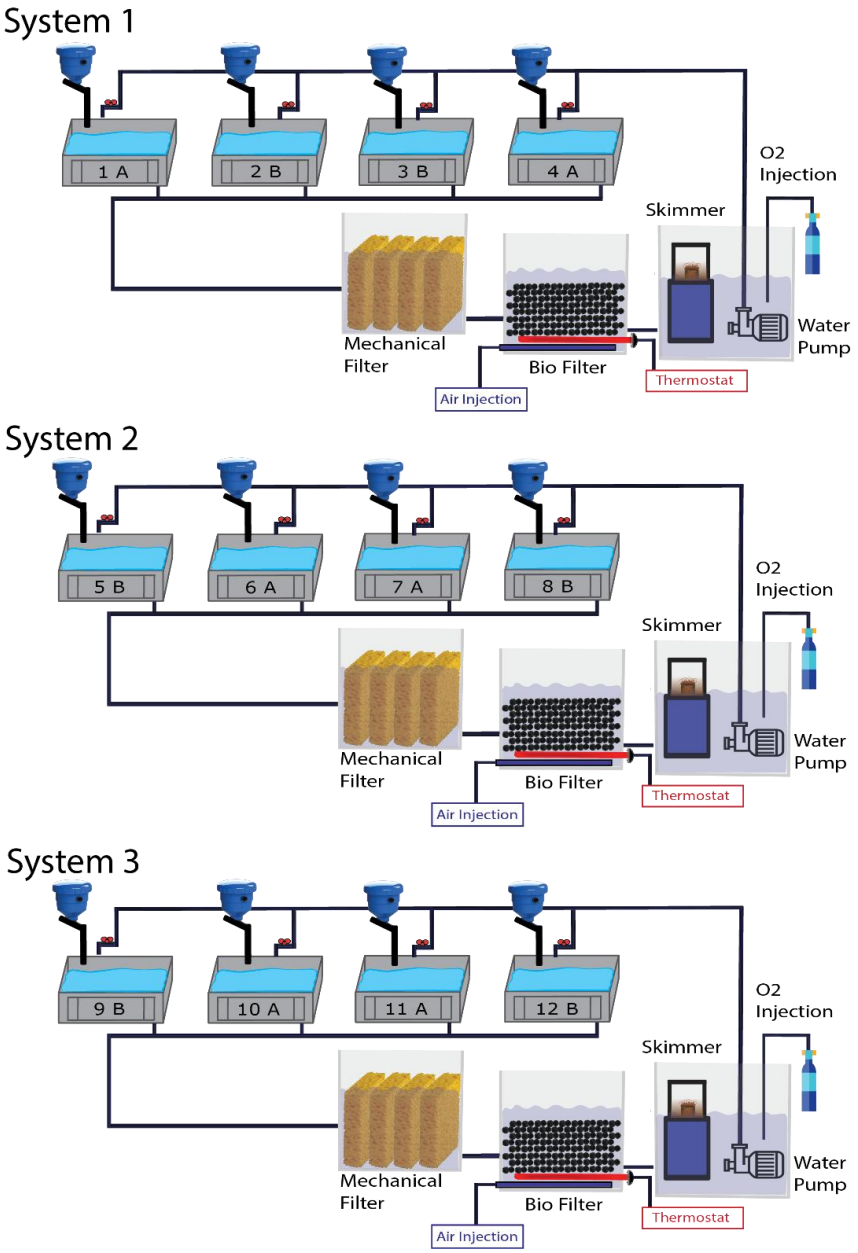

Figure 28 - Trial's RAS design with the 3 systems. Design by Alexandre Veloso.

2.3. Experimental fish

The fish in this trial were sourced from the Weaning stage of the production line. A total of 1 200 SnS juveniles (*Solea senegalensis)*, at 181 DAH, were kept for 90 days in the company's experimental unit.

Prior to the commencement of the trial, the fish were acclimated to the conditions within the experimental unit for a period of three days. During the acclimation period, the fish were kept fed with Diet B.

At the beginning of the trial, 12 homogenous groups of 100 SnS (initial body weight of 5.9 ± 0.2 g) were assigned to each tank of the 3 systems, corresponding to an average density of 1.83 ± 0.06 kg/m². Each experimental diet was randomly designated to each tank, so, each system had duplicates of both diets (two tanks with Diet A and the other two with Diet B), representing n = 6 for each Diet.

The sheltered experimental unit permitted stable environmental conditions in the employed recirculation aquaculture systems.

2.4. Experimental conditions

Throughout the trial, the experimental unit's facility, the tanks, the sumps, the skimmer, and the drainage infrastructure were maintained and cleaned daily.

Daily (temperature, oxygen, salinity, and turbidity) and biweekly (pH, Nitrates and Ammonia) measurements were kept throughout the trial to maintain the appropriate conditions for the fish's wellbeing: the temperature was kept at around 19ºC; oxygen was sustained in the range of 100 – 200 %; pH around 7; salinity at 35 ppt; ammonia maintained under 1 mg/L and the Nitrites under 0.8 mg/L. The systems had an applied photoperiod of 2 hours of light and 22 hours of darkness.

Ammonia, in the form of NH_3^- - N, was quantified utilizing a Hach ® Spectrophotometer DR3900, following the defined protocol and the designated kit "Nitrogen, Ammonia – Salicylate Method – Method 8155". Nitrates, in the form of NO_3 - N, were quantified with the same spectrophotometer, but in accordance with the protocol "Nitrate, MR – Cadmium Reduction Method – Method 8171". Both kits and protocols were obtained from Hach®.

Turbidity was assessed using a Hanna® Portable Turbidity Meter with Fast Tracker Technology HI-98713-02.

The measurements of turbidity, oxygen, and temperature of the tanks occurred every morning 2 hours after the last meal provided. Turbidity is attained by extraction of a water sample from each tank at the closest location to the outlet. Temperature was measured using a thermometer near the outlet also as it was the measurement of oxygen.

2.5. Feeding trial

After the acclimation period, the feeding trial was initiated. The feeding trial was conducted over a period of 90 days, at Safiestela's facilities in a recently constructed unit that is solely dedicated to the purpose of testing potential improvements to use in production line. The fish were fed daily, and the quantity administrated per day was calculated based on the expected total biomass of each tank. This calculation follows the company's internal protocol and growth references in this life stage, and as such, these values cannot be disclosed. The daily feed weight was updated weekly.

The feeding occurred 8 times a day, using automatic feeders. For this purpose, one "Fish Mate© – F14 – Aquarium fish feeder", as depicted in Figure 30, was set up on each tank.

Figure 29 - Automatic fish feeder "Fish Mate © – F14 – Aquarium fish feeder" utilized in this trial. Photo taken by Alexandre Veloso.

2.6. Sampling

Three weightings were performed in this trial: Initial, Intermediate, and Final. In each, the fish were stopped being fed 4 hours prior to sampling. In the Intermediate sampling, only

10 fish in each tank were individually weighed. Each fish was weighed at the beginning and the end of the trial.

At the Initial and Final sampling, three fish per tank were sacrificed (eighteen fish per dietary treatment, six per system) by an anesthesia overdose resourcing to fish immersion in 25 ml of Benzocaine (Aquacen Benzocaína 200 mg/mL, Cenavisa©), diluted in 10 L of freshwater.

Then, after the blood sample was extracted using heparinized syringes through the fish's caudal vessel, it was pooled with three fish per tank. Blood was then centrifuged for 5 min at 12 300 g. The blood's plasma samples were stored at -20 ºC until further analysis. The carcass was collected, 3 fish pooled by tank, and stored frozen at -20 ºC until later analysis.

This experiment was conducted in accordance with the guidelines set forth by the Federation of European Laboratory Animal Science Associations (FELASA) Category C recommendations and in compliance with the European Union Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals for scientific purposes.

2.7. Analytical methods

2.7.1. Proximate analysis

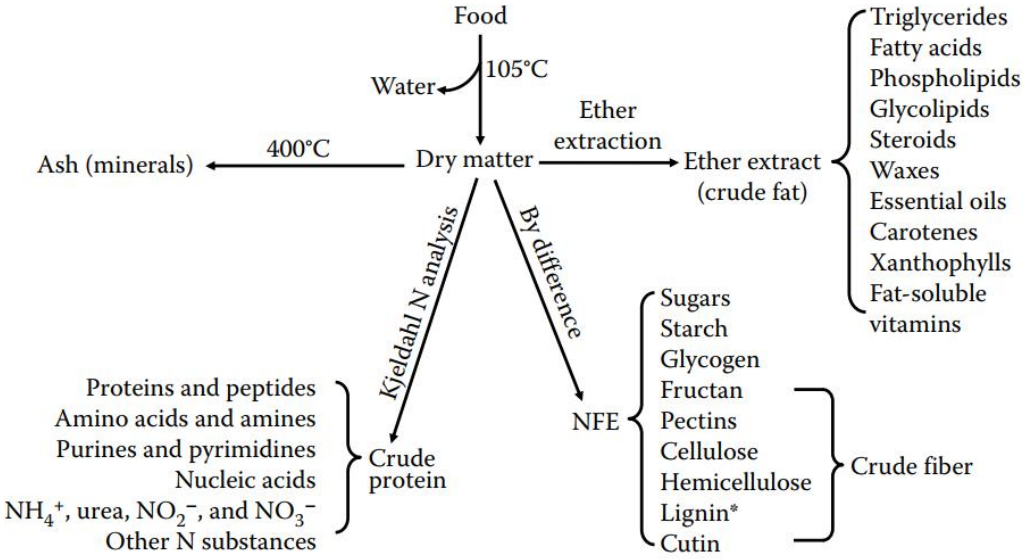

Figure 30 - Diagram of the results and methodologies utilized in a proximate analysis. Source: Wu, G. (2018). Principles of Animal Nutrition. CRC Press, Taylor & Francis Group.

The following parameters were measured: dry matter, crude protein, crude lipids, and ash. The methodology employed is outlined in the following sections:

2.7.1.1. Dry Matter

Was determined by drying them at 105°C until a constant weight was achieved. Dry matter can be defined as the aggregate of organic and inorganic substances. Organic matter, which comprises carbohydrates, lipids, proteins, peptides, free amino acids (AAs), nucleic acids, and other nitrogenous substances (e.g., urea, ammonia, nitrite, and nitrate), along with organic acids and vitamins, whereas inorganic matter is composed of minerals. The measurement of Dry Matter also provides a preliminary estimation of the water content, which can be further utilized to gain insight into the feed pellets' texture, durabili4ty, water stability, buoyancy, and space taken in storage (Wu, 2018).

2.7.1.2. Crude Protein

This value was calculated by multiplying the mean nitrogen content by a factor of 6,25. The protein nitrogen content was quantified through the Kjeldahl method following acid digestion with the Kjeltec 1016 Digestor and 1002 Distillation units. (Tecator, Hoganas, Sweden). Crude protein does not give full insight into the true protein content, due to two factors: firstly, some proteins contain varying amounts of nitrogen, dependent on their

amino acids (AA) composition; and secondly, different feedstuffs contain differing percentages of non-protein nitrogen.

2.7.1.3. Crude Lipids

The lipidic composition is aggregated and determined with the ether extract. The ether is obtained through the digestion of the samples in petroleum ether on a Soxtec System (Tecator, Hoganas, Sweden).

2.7.1.4. Ash

The ash was evaluated by incineration in a muffle furnace at 450 °C for 16 hours. Ashes give insight into the inorganic matter. As mentioned above, inorganic matter represents the minerals in the sample analyzed.

2.7.2. Plasma analyses

To obtain an understanding of the fish's health, metabolites (such as total cholesterol, glucose, and triglycerides) and enzymes (such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) in plasma, blood samples extracted from the fish were analyzed.

2.7.2.1. Metabolites

Total cholesterol: The SPINREACT® kit (Ref:1001090), SPINREACT, S.A. / S.A.U., Girona, Spain, following the “Cholesterol – CHOD-POD. Enzymatic colorimetric – Quantitative determination of cholesterol” protocol provided by SPINREACT® also, were utilized to measure the total cholesterol present on the plasma. The protocol utilized is designed for human blood analysis, as such, to adapt for the species in question, the reagents utilized were halved to refine the protocol for such animals.

Cholesterol esters undergo a process of hydrolase, whereby they are broken down into their constituent components: cholesterol and fatty acids. Subsequently, cholesterol undergoes oxidation, resulting in the formation of 4-cholestenone and hydrogen peroxide (H_2O_2). The aforementioned products, when exposed to phenol, undergo a reaction with peroxidase (POD) to produce quinonimine and water. Quinonimine is a colored compound whose color intensity proportionally correlates with cholesterol serum levels.

The samples were measured in duplicates. The Quinonimine color absorbance in the samples was read at 505 nm, and the cholesterol was then calculated with the following calculation stated in the protocol:

$$\frac{\text{(A) Sample - (A) Blank}}{\text{(A) Standard - (A) Blank}} \times 200\ (\text{Standardconc.}) = \text{mg/dL cholesterol in the sample}$$

Figure 31 - Formula to determine total cholesterol present in blood, with an absorbance (A) measured at 505nm. SPINREACT, SA

Triglycerides (TAG): To measure the levels of triglycerides the “Triglycerides-LQ – GPO-POD. Liquid – Quantitative determination of triglycerides” by SPINREACT protocol was followed using the SPEANREACT® Triglycerides kit (Ref: 1001312) the plasma levels of this fat were assessed. The protocol utilized is designed for human blood analysis, as such, to adapt for the species in question, the reagents utilized were halved to refine the protocol for such animals.

Incubation of triglycerides with the enzyme lipoprotein lipase (LPL) results in the liberation of glycerol and free fatty acids. Glycerol is subsequently converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase (GK) and adenosine triphosphate (ATP). Glycerol-3-phosphate (G3P) is then oxidized to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2) by glycerol phosphate oxidase (GPO). In the final reaction, hydrogen peroxide (H_2O_2) reacts with 4-aminophenazone (4-AP) and p-chlorophenol in the presence of peroxidase (POD) to yield a red-colored dye. The intensity of the color formed is proportional to the TAG concentration in the sample.

The samples were measured in duplicates. The color formed is measured with the absorbance in the samples, which was read at 505 nm, and the TAG was then calculated with the following calculation stated in the protocol:

$$\frac{\text{(A) Sample - (A) Blank}}{\text{(A) Standard - (A) Blank}} \times 200\ (\text{Standard conc.}) = \text{mg/dL triglycerides in the sample}$$

Figure 32 - Formula to determine triglycerides present in blood, with an absorbance (A) measured at 505nm. SPINREACT, SA.

Glucose: The assessment of Glucose levels in plasma was attained using the SPINREACT® kit (Ref: 100191) by following the protocol “Glucose-TR – Trinder test. GOD-POD – Quantitative determination of glucose” by SPINREACT, S.A.. The protocol utilized is designed for human blood analysis, as such, to adapt for the species in question, the reagents utilized were halved to refine the protocol for such animals.

In this method, Glucose oxidase (GOD) catalyzes the oxidation of glucose to gluconic acid. The formation of hydrogen peroxide (H_2O_2) is detected by a chromogenic oxygen acceptor, phenol-aminophenazone, in the presence of peroxidase (POD), the reaction results in a colored dye. The intensity of the color produced is proportional to the glucose concentration in the sample.

The samples were measured in duplicates. The color formed is measured with the absorbance in the samples, which was read at 505 nm, and the glucose was then calculated with the following calculation stated in the protocol:

$$\frac{\text{(A) Sample - (A) Blank}}{\text{(A) Standard - (A) Blank}} \times 100\ \text{(Standard conc.)} = \text{mg/dL glucose in the sample}$$

Figure 33 - Formula to determine glucose present in blood, with an absorbance (A) measured at 505nm. SPINREACT, SA.

2.7.2.2 Enzymes

AST and ALT are catalytical enzymes, so the enzymatic activity assessed in this trial was determined following the rate of the biological reactions described below. Enzyme activity will be expressed in U/L. One international unit is the amount of enzyme that transforms 1 μmol of substrate per minute, in standard conditions. The concentration is expressed in units L^{-1} of sample (U/L).

AST: Also known as ASAT, and previously called glutamic oxaloacetic transaminase (or GOT), was quantified in this trial using a commercial kit, (SPINREACT, Ref: 41273) following the protocol “GOT (AST)-LQ – NADH. Kinetic UV. IFCC rec. Liquid – Quantitative determination of aspartate aminotransferase GOT (AST)” by SPINREACT, S.A..

In this method, AST catalyzes the reversible transfer of an amino group from aspartate to α-ketoglutarate, forming glutamate and oxalacetate. The oxalacetate is then reduced

to malate by malate dehydrogenase (MDH) and NADH. The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of AST present in the sample.

To obtain the rate curve that most accurately reflects the gradual decrease in the rate of biological reactions, a series of sample concentrations were tested. The samples were prepared at ratios of 1:1 (1 part plasma and 1 part distilled water), 1:10 (1 part plasma and 10 parts distilled water), 1:100 (1 part plasma and 100 parts distilled water), and 1:300 (1 part plasma and 300 parts distilled water). The triplicate samples had a volume of 100 μL.

The calculation was performed for each concentration in accordance with the following formula:

$$\Delta A/min \times 1750 = U/L \text{ of AST}$$

Figure 34 - Formula to determine AST in blood, with an average absorbance per minute (Δ A/min) measured at 340 nm at room temperature (25 ºC). SPINREACT, SA

AST reference levels have been determined in the bibliography in Peres 2014, from 118 to 605 U L^{-1}.

ALT: Also known as ALAT, previously called glutamic pyruvic transaminase (or GPT), was quantified in this trial using a commercial kit, (SPINREACT, Ref: 41282) following the protocol “GPT (ALT)-LQ – NADH. Kinetic UV. IFCC rec. Liquid – Quantitative determination of alanine aminotransferase GPT (ALT)” by SPINREACT, S.A..

In this method, ALT catalyzes the reversible transfer of an amino group from alanine to α-ketoglutarate forming glutamate and pyruvate. The pyruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH. The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of ALT present in the sample.

To obtain the rate curve that most accurately reflects the gradual decrease in the rate of biological reactions, a series of sample concentrations were tested. The samples were prepared at ratios of 1:1 (1 part plasma and 1 part distilled water), 1:10 (1 part plasma and 10 parts distilled water), 1:100 (1 part plasma and 100 parts distilled water), and

1:300 (1 part plasma and 300 parts distilled water). The triplicate samples had a volume of 100 μL.

The calculation was performed for each concentration in accordance with the following formula:

$$\Delta A/min \times 1750 = U/L \text{ of ALT}$$

Figure 35 - Formula to determine ALT in blood, with an average absorbance per minute (Δ A/min) measured at 340 nm at room temperature (25 ºC). SPINREACT, SA

Unlike AST, Alanine aminotransferase is exclusively produced within the hepatic tissues and is tightly linked to the health of the liver. The concentration of the enzyme in plasma can serve as a marker for liver function, with higher levels of the enzyme being associated with more severe liver damage (De La Torre et al., 2000; Lemaire et al., 1991; Liu et al., 2014; Sherman, 1991). When compared with ASAT, ALT has a higher release after hepatocellular damage.

2.8. Index calculations

Several performance indexes have been calculated following the formulas described below:

$$Weight\ gain\ (g) = final\ weight - initial\ weight$$

$$Weight\ gain\ (\%) = \left\{\frac{final\ weight - initial\ weight}{initial\ weight}\right\} * 100$$

$$Daily\ Growth\ Index\ (DGI) = \left\{\frac{final\ weight^{\frac{1}{3}} - initial\ weight^{\frac{1}{3}}}{time\ in\ days}\right\} * 100$$

$$Average\ Body\ Weight\ (ABW) = \frac{(initial\ weight + final\ weight)}{2}$$

$$Feed\ Conversion\ Ratio\ (FCR) = \frac{total\ feed\ intake\ (g)}{weight\ gain\ (g)}$$

$$Protein\ Efficiency\ Ratio\ (PER) = \frac{weight\ gain\ (g)}{crude\ protein\ intake\ (g)}$$

$$Feed\ Intake\ (FI)\ (g\ kg\ ABW^{-1}\ day^{-1})$$
$$= (feed\ consumption\ (g))/(Average\ Body\ Weight * time\ in\ days)$$

$$Survival\ Rate\ (\%) = \frac{final\ number\ of\ individuals}{initial\ number\ of\ individuals} * 100$$

$$Economic\ Profit\ Index\ (EPI)(€\ fish^{-1}) =$$
$$= (weight\ gain\ (kg) * selling\ price\ (€\ kg^{-1}))$$
$$- (weight\ gain\ (kg) * diet\ price\ (€\ kg\ of\ )$$

$$Economic\ Conversion\ Ratio\ (ECR)(€\ kg\ of\ fish^{-1}) =$$
$$= FCR\ (kg\ diet\ kg\ of\ fish^{-1}) * diet\ price\ (€\ kg\ of\ diet^{-1})$$

The formulas of Weight gain (g), Weight gain (%), Daily Growth Index (DGI) and Economic Conversion Ratio (ECR) were calculated as described in Moutinho et al. (2017). Feed Conversion Ratio (FCR) as in Boyd & McNevin (2022). Protein Efficiency Ratio (PER), Feed Intake (FI), and Economic Profit Index (EPI) as depicted in Martínez-Llorens et al. (2007).

In the context of the economic calculations, the euro (€) was employed as the reference currency.

Additionally, due to the confidentiality agreements in place, the full price of the diet per kilogram cannot be disclosed. However, for the purposes of this trial of comparison between the two diets, a proportion was used that is consistent with the diet's real market price. This proportion is calculated as follows: Diet A = 0.867€; and Diet B = 1€.

The selling price is calculated as an approximate reference , relatively close to real juvenile production costs to Safiestela, in order to get a more real representation of the costs of production in this facility. The selling price (€ kg^{-1}) was set at 17.5€, being the mean weight of each fish in this phase around the 20 g mark, variating according to the batch performance.

2.9. Statistical analysis

To facilitate a comprehensive examination of the statistical data, the IBM SPSS software version 29 (IBM® SPSS® Statistics, New York, USA) was utilized.

As it is outlined by Zarr (2014), the two-factor (diets and systems) analysis of variance with randomized blocks was analyzed using the resources of an ANOVA, duly tested for normality and homogeneity. In instances where the data did not meet the criteria for normality, the data were transformed and a Levene's test was conducted once more to guarantee the accuracy of the ANOVA analysis.

Pearson correlation test was also performed between the daily measured water parameters (Turbidity, Temperature, and Oxygen) in each tank and growth performance.

3. Results

3.1. Physical and chemical parameters in the water

Table 2 - Effects of the diets and systems (blocks) on the physical and chemical parameters in water.

	Diet A		**Diet B**		*p*-Value *diets*	*p*-Value *blocks*
	Mean	SD	Mean	SD		
Temperature (ºC)	19.8	0.21	19.8	0.23	***0.31***	***<.001***
Oxygen (%)	106	9.36	109	8.75	***0.02***	***<.001***
Turbidity (NTUs)	1.53	0.16	1.94	0.13	***<.001***	***0.12***

Mean (n=6) calculated alongside standard deviation “SD”.
Turbidity measured in NTU (Nephelometric Turbidity Unit). 1NTU = 1 mg L^{-1}.

The effects of the diets and the block on the variation of the physical parameters are presented in Table 2.

Temperature had no variation explained by the diet type, although Oxygen (%) and Turbidity (NTU's) did. Both Oxygen (%) and Turbidity (NTUs) were lower in the tanks fed with diet A. Representing the diet effect of p-value = 0.024 in the Oxygen variation and p-value = <0.001 in the turbidity variation. The blocks showed significant effect on both temperature and oxygen.

Table 3 - Table of correlations between growth and water parameters.

	Turbidity (NTU's)		***Oxygen (%)***		***Temperature (ºC)***	
	R	P	R	P	R	P
Weight Gain (g)	-0.85	***0.00***	0.57	***0.05***	-0.57	0.06
Weight Gain (%)	-0.87	***0.00***	0.56	0.06	-0.53	0.08
Daily Growth Index	-0.87	***0.00***	0.57	***0.05***	-0.54	0.07
Average Body Weight (g)	-0.82	***0.00***	0.56	0.06	-0.60	***0.04***
Feed Conversion Ratio	0.85	***0.00***	-0.57	***0,05***	0.55	0.07
Protein Efficiency Ratio	-0.,85	***0.00***	0.55	0.06	-0.55	0.07
Specific Growth Rate	-0.87	***0.00***	0.57	***0.05***	-0.52	0.08
Feed Intake (g kg /ABW /day)	0.82	***0.00***	-0.57	***0.05***	0.60	***0.04***
Economic Profit Index (€/fish)	-0.06	0.86	-0.41	0.19	0,18	0.59
Economic Conversion Ratio (€ kg of fish^-1)	0.87	***0.00***	-0.63	***0.03***	0.61	***0.04***
Turbidity (NTU's)	1.00		-0.47	0.13	0,51	0.09
Oxygen (%)	-0.47	0.13	1.00		-0,83	***0.00***
Temperature(ºC)	0.51	***0.09***	-0.83	***0.00***	1.00	

Correlation analysis was performed on the water parameters measured daily on each tank with the indexes resulting from growth performance calculations, Table 3. Data gives an overall high degree correlation on growth parameters with the turbidity variation. For the exception of EPI, every index showed a high correlation and significant levels (p-value < 0.05). The only correlation seen in Oxygen and Temperature was between each other (R = -0.83) and in a significant degree (p-value = 0.00).

3.2. Growth and Economic performance

Throughout the duration of the trial, no pathological alterations were observed, and the survival rate remained consistent across all tanks and systems, exhibiting no dietary-related effects (p-value = 0.9).

Growth and feed utilization data are presented in the table (Table 4), the initial body weights were found to be similar (p-Value = 0.17). There is a notable discrepancy in

growth performance between the two diets with elevated levels of significance from dietary influence.

The quantity of feed consumed daily by kilogram of fish, in comparison, on Diet A demonstrated a lower quantity of feed consumed by the fish relative to its body weight over the course of the trial. Furthermore, the type of diet employed is a significant factor influencing the variation of this index (p-value = <.001).

EPI, or more easily interpreted as the valued price of each fish produced, although higher in diet A. The difference between the diets cannot be significantly explained due to the influence of the type of diet utilized (p-value =0.853).

ECR, the cost of feed-related expenses to produce 1 kg of fish, is higher in Diet B. It is significantly more expensive, and it is explained by the effect of the dietary treatments executed (p-value = <.001).

The block had no significant effect on the growth of fish.

Table 4 - Growth and Economic performance of fish fed with Diet A and Diet B.

	Diet A		**Diet B**		*p*-Value *diets*	*p*-Value *blocks*
	Mean	SD	Mean	SD		
Initial Body Weight (g)	5.78	0.17	5.92	0.20	***0.17***	***.250***
Final Body Weight (g)	21.0	1.07	16.7	0.86	***<.001***	***.565***
Weight Gain (g) [a]	15.2	1.06	10.8	0.89	***<.001***	***.521***
Weight Gain (%) [b]	263	20.4	182	18.0	***<.001***	***.456***
DGI [c]	1.08	0.06	0.84	0.06	***<.001***	***.494***
Average Body Weight (g) [d]	13.4	0.55	11.3	0.43	***<.001***	***.581***
FI (g kg /ABW /day) [e]	26.3	1.13	31.1	1.26	***<.001***	***.699***
FCR [f]	2.06	0.16	2.92	0.25	***<.001***	***.521***
PER [g]	0.86	0.06	0.63	0.05	***<.001***	***.757***
Survival Rate (%) [h]	91.5	2.07	91.8	6.31	***0.90***	***.452***
EPI (€ fish^{-1}) [i]	0.25	0.02	0.18	0.01	***0.85***	***.684***
ECR (€ kg of fish^{-1}) [j]	1.79	0.14	2.92	0.25	***<.001***	***.968***

[a] Weight Gain (g)(WG) =[Final Body Weight(FBW)] - [Initial Body Weight (IBW)]
[b] Weight Gain (%) = [(FBW – IBW)/ IBW]*100
[c] Daily Growth Index = [(FBW$^{1/3}$ – IBW$^{1/3}$) / days]*100
[d] Average Body Weight (ABW)(g) = (IBW + FBW) / 2
[e] Feed Intake (FI) (g kg ABW-1 day-1) = ((total feed intake (g))/(Average Body Weight (kg)))/(time in days)
[f] Feed Conversion Ratio (FCR) = Dry Feed Intake / Wet Weight Gain
[g] Protein Efficiency Ratio (PER) = WG / Crude Protein Intake
[h] Survival Rate (%) = (Number of final fish / Number of initial fish)*100
[i] Economic Profit Index (EPI) (€ fish-1) = [WG (kg) * selling price (17.5 € kg-1)] – [WG (kg) * diet price (€ kg of diet-1)]
[j] Economic coefficient ratio (ECR) (€ kg of fish^{-1}) = FCR (kg diet kg of fish^{-1}) * diet price (€ kg of diet^{-1})

Mean (n=6) and with standard deviation represented in "SD".
Solea senegalensis juveniles' prices were defined to represent a close representation of the production cost per fish for Safiestela. Set price = 17.5€ at a mean weight of 20g per fish.

The selling price of feed represents the proportion of true cost: Diet A = 0.867€; Diet B = 1€. True cost is not disclosed due to confidentiality agreements with the commercial partners.

3.3. Proximal analysis

At the end and the beginning of the trial, whole-body composition was measured, Table 2. Diet A had more protein content, 47.6 % against 46.9% in B, and less lipids (A with 12.9 %, B with 13.3), and ash showed a higher percentage in the carcass composition in fish fed with the Diet A (9.46 % in A and 8.43 %). While differences were observed in all analyzed aspects, only the ash content exhibited a statistically significant variation as a result of the dietary treatments (p-value = 0.01), and also due to the block effect (p-Value = 0.02).

Table 5 - Proximal analysis of the carcasses at the beginning and at the end of the trial.

	Initial	**Diet A**		**Diet B**		*p*-Value *diet*	*p*-Value *block*
		Mean	SD	Mean	SD		
Dry matter (%)	71.0	71.9	1.09	72.2	1.30	***0.36***	***0.43***
Crude Protein (% dry matter)	44.7	47.6	1.75	46.9	2.11	***0.36***	***0.86***
Crude Lipids (% dry matter)	11.2	12.9	0.59	13.3	0.43	***0.63***	***0.40***
Ash (% dry matter)	9.21	9.46	0.61	8.43	0.81	***0.01***	***0.02***

Mean (n=6) calculated alongside standard deviation “SD”. Initial is composed of a pool of five fish within the population from which the trial was initiated.

3.4. Plasma Analyses

3.4.1. Metabolites

Table 6 - Serum analysis of the metabolites.

	Diet A		**Diet B**		**Initial**	
	Mean	SD	Mean	SD	Mean	SD
CHO (mg/dL)	216,3	69,3	204,2	54,2	212,9	83,9
TAG (mg/dL)	737,7	174,2	637,4	243,4	364,1	288,5
GLU (mg/dL)	326,1	143,6	314,5	165,8	361,6	219,3

Mean (n=6) calculated alongside standard deviation “SD”.
Each tank had duplicate measurements, the value of each n is the average of the duplicates.

Upon the examination of serum metabolites in the fish, statistical analyses were conducted to evaluate the impact of the diet on these levels in the blood. Following the examination of homogeneity in these parameters, it was determined that no metabolite exhibited the requisite homogeneity, thereby precluding the possibility of undertaking an ANOVA analysis. The transformation of the data was then attempted, yet this proved unsuccessful, as the Levene test continued to yield p-values less than 0,05. The values obtained from the analysis of blood metabolites are presented in Table 6.

3.4.2. Enzymes

The analysis of enzymes yielded no results. The photometric measurement of all attempted concentrations of the sample produced inconclusive results with regard to the quantity of enzymes in the plasma, as evidenced by the failure to obtain a possible outcome in calculations for both ALT and AST.

As such, no relation between the diets and enzyme quantities in blood was possible to test in this thesis.

4. Discussion

4.1. Implementation of Improved Feed Selection Practices in Aquaculture

The implementation of improved feed selection practices represents a pivotal element within intensive aquaculture systems. These modifications have the potential to increase production rates, reduce costs, and enhance the quality of the final product, rendering it more competitive in terms of pricing.

A comparative analysis of two commercial diets will enable Safiestela to make informed decisions regarding adjustments to the feeding regimen during the Nursery phase, grounded in scientific evidence. To facilitate this, three RAS systems were constructed to replicate, on a smaller scale, the current RAS system employed by Safiestela. This experimental approach, with consideration of the scalability of the systems, ensures that the results can provide predictive insights into the impact of dietary changes on the production line.

4.2. Dietary Effects on Growth Performance

The comparative analysis of Diet A and Diet B revealed significant differences in the growth performance of the fish. Data including Feed Conversion Ratio (FCR), Daily Growth Index (DGI), Feed Intake (FI), and Weight Gain percentage, demonstrate that fish fed with Diet A exhibited superior growth performance, requiring less feed. Furthermore, turbidity measurements demonstrated the differing effects of the diets on water quality. Based on the data and its correlation with the growth of juvenile sole, it can be concluded that better water quality provides optimal growth conditions for the fish, as expected (Grobbelaar, 2009; Schroeder, 2003).

The growth rate in Diet A was markedly superior compared to Diet B. The significant differences in FCR, FI, and ECR raise questions about whether daily feed administration may have been overestimated in tanks fed Diet B. This suggests lower feeding activity in these fish, and an adjustment of the feeding parameter could have prevented feed waste (Cho et al., 1994; Sun et al., 2016).

The two diets have very different effects on growth. The DGI levels are similar to those seen in other studies (Table 7). Lower dietary lipids that provided a better protein/lipid ratio, along with higher rearing temperatures, boosted growth in the trials with similar growth levels. This is intriguing, as this is not observed between diets A and B. In the case of the diets of this trial, proximate analysis does not give clues to the “why” of the enhanced growth observed in Diet A. So, other parameters in the feed must be the reason for the better growth performance, factors such as source and origin of the dietary ingredients, feed formulation, and feed manufacturing technologies (Gatlin III, 2010; B. D. Glencross et al., 2007) could probably explain what the proximate analysis of the diets does not.

Additionally, the direct comparison of the growth indexes demonstrates that, for an identical weight of feed, Diet A produced considerably more favorable results. This advantage, alongside a randomized block design with n=6, highlights the benefits of adopting Diet A in the Nursery dietary regimen.

4.3. Economic Performance

From an economic perspective, the Economic Conversion Ratio further supports the use of Diet A. Despite Using the same amount of feed throughout the trial, the cost of producing 1 kg of fish was 1.6 times more expensive with Diet B than with Diet A. To give an idea of the magnitude of the cost reduction from Diet A to B, it costs 2 920 euros in feed to grow 1 ton of fish on Diet B, but it only costs 1 790 euros to raise them with Diet A. Showing that utilizing Diet A would significantly reduce production costs at an intensive aquaculture facility like Safiestela.

4.4. Carcass Proximal Analysis

In the values observed in the proximal analysis of the farmed fish carcasses, there was no significant difference between the diets studied. The body composition of the fish did not exhibit a notable change in response to the dietary influence, a finding that is

consistent with the observations documented in Table 7, like the 2015 study by Bonacic et al.

4.5. Water Quality Parameters

Oxygen and turbidity showed variations correlated with the diets. Oxygen levels (p-value = 0.024) were lower in tanks fed Diet A. This, combined with lower FI and higher weight gain, suggests that lower oxygen levels could be linked to a higher metabolic rate due to more active feeding behavior (Jobling, 1981; Li et al., 2020). While oxygen levels were lower, they remained within supersaturation limits.

Water turbidity also varied significantly between diets (p-value < 0.001). The type and amount of feed influenced the accumulation of matter in the water column, with lower turbidity observed in tanks fed Diet A (Davidson et al., 2013; Ghosh et al., 1984). Turbidity was strongly correlated with growth performance, as previously mentioned by Bilotta & Brazier (2008) and Grobbelaar (2009). Measuring turbidity could be a valuable tool for daily monitoring of feed leftovers.

4.6. Plasma Analysis

In aquaculture, diagnosing malnutrition due to inadequate feed and feeding practices, stressors or disease is of great significance. The analysis of blood has been theorized as a potential method for evaluating such situations as a few selected blood parameters can provide an in-depth understanding of the physiological processes and the animal's health status (Hrubec et al., 1996; Lemaire et al., 1991). Changes in feed suitability, feeding practices, water quality, stress, disease progression, and therapeutic efficacy are often reflected in the biochemical profile of plasma, as noted in SnS (Nakagawa et al., 2007; Peres et al., 2014).

4.6.1. Metabolites

4.6.1.1. Cholesterol

Plasma lipids play a fundamental role in fish biology, with cholesterol being an essential component in the composition of all animals. In its various forms and consequently various functions, cholesterol plays an essential role in the structure of cells and as a precursor of steroid hormones. Its functions and applications in animal biology are therefore extensive. It is thus imperative that fish maintain appropriate cholesterol levels

to ensure the optimal functioning of biochemical processes in which cholesterol plays a role (Arnold & Kwiterovich, 2003; Luo et al., 2019; Wang et al., 2010).

The cholesterol levels in the blood are proportionally related to dietary cholesterol levels (Luo et al., 2019). An increase in lipid intake will result in an elevated cholesterol level in the blood, and a decrease in lipid intake will have the opposite effect (Valente et al., 2011). Therefore, levels of total serum cholesterol that diverge from the normal reference in SnS (0,1 to 0,9 g /dL) (Peres, Costas, et al., 2014) can give insight into how well the dietary source is performing in the cholesterol homeostasis of the body. Anormal levels of cholesterol can result in several problems, which could lead to increased susceptibility to diseases like hepatic steatosis, fat liver disease, etc, which undermines the healthy growth of fish (Jin et al., 2023; Lemaire et al., 1991). Both in yellow tale fish artificially infected with *L. garvieae*, and rainbow trout infected with *Vibrio anguillarum* it was established a correlation between low cholesterol levels and mortality in animals (Maita et al., 1998).

The influence of dietary cholesterol levels is proportionally related to cholesterol levels in the blood. An increase in lipid intake will result in an elevated cholesterol level in the blood, and a decrease in lipid intake will have the opposite effect (Valente et al., 2011). Therefore, the level of cholesterol in the blood can provide information on the suitability of the lipid composition of the feed, while also giving a clue of how well the fish's lipid metabolism is performing.

In this trial, both diets showed serum cholesterol within the reference levels for this species, Diet A = 216.3 ± 69.3 mg/dL and Diet B = 204.2 ± 83.9. So, considering that between both diets there was no significant difference, and both are within healthy values, the dietary cholesterol levels on both diets are appropriate for this species. Compared to other carnivorous fish species, the serum cholesterol of Diets A and B are also similar.

4.6.1.2. Triglycerides

A Triglyceride (TAG), also known as Triacylglycerol or Triacylglyceride, is a simple lipid molecule comprising one glycerol backbone esterified with three fatty acid molecules. Triglycerides represent the primary storage form of lipids, serving as a source of energy. TAG is supplied through the diet, and following the ingestion of food, dietary lipids are hydrolyzed within the intestinal lumen. Upon intestinal uptake, fatty acids (FA) are

reesterified to form TAG molecules, which are packaged into chylomicrons and delivered primarily to muscle and adipose tissue. The remaining TAG present in chylomicron is then transported to the liver and processed intracellularly, leading to FA release within hepatocytes. The FA is then used to produce TAG again, which could then be stored as lipid droplets in the liver or bundled with VLDL and sent again back to muscles or stored in adipose tissue (Jain et al., 2021; Nguyen et al., 2008; Wilson, 2012). When fish are subjected to stressful conditions, such as malnutrition or insufficient feed, there is a depletion of those stored lipid reserves, which results from the fish resorting to consuming their TAG reserves, either the lipid droplets or the adipose tissue reserves (Alves-Bezerra & Cohen, 2018), to maintain the necessary energy levels. However, the variation in triglyceride values observed after stress differs depending on the species under study. In SnS, low TAG values have been linked to short-term stress related to food deprivation, which has been theorized to happen due to the slow rates of lipid absorption and metabolization (Valente et al., 2011).

An excessive consumption of dietary lipids could lead to high TAG levels in relation to the reference value for this species, in the case of SnS between 0.3 and 1.8 g/dL (Peres, Costas, et al., 2014). High TAG has been observed to indicate potential liver damage (Lemaire et al., 1991). However, both collected samples from this experiment showed similar values between both diets and within healthy values (Diet A = 737.7 ± 174.2 mg/dL; Diet B = 637.4 ± 243.4 mg/dL). Then, dietary TAG levels are at appropriate levels for SnS.

4.6.1.3. Glucose

Solea senegalensis's regular levels of glucose lie lower than most teleost fish, between 19 to 86 mg dL^{-1} (Peres et al., 2015). Interspecies variation of glucose levels has been associated with the level of activity, fish that exhibit reduced levels of locomotion tend to have lower serum glucose. On the other hand, there has been reported significant intraspecies glucose variation as well. Intraspecies variation can occur due to individual differences in feeding behavior, size, age, and reproductive status (Mc Donald & Milligan, 1992). Being the dietary conditions the main influencers on glucose variation (Conde-Sieira et al., 2015) .

It has been demonstrated the existence of spikes in glucose levels in teleost fish when they are subjected to stressful conditions. This physiological response has been linked

to various stressors, including environmental, hypoxia, disease, nutrition, handling, and stocking density-related factors. However, glucose-related mechanisms in SnS have been observed to exhibit greater resilience to these stressors. In long starvation periods, after 21 days, SnS is able to maintain glucose homeostasis (Costas et al., 2011). And also maintains stable glucose levels in several stocking densities, from 17 up to 40 kg/m^2 (Andrade et al., 2015; Azeredo et al., 2019). This stress resilience in SnS could be explained by a good metabolic capacity to deal with different glucose levels (Conde-Sieira et al., 2015), which are essential to survival (Navarro & Gutiérrez, 1995). This reveals this species' capacity to deal with different stressors in intensive aquaculture production.

However, in this trial Glucose showed quite high values when compared to the reference levels in SnS set by Peres et al (2015), tanks fed with diet A =326.1 ± 143.6 mg/dL; tanks fed Diet B = 314.5 ± 219.3 mg/dL, which represents almost 4 times as much as it should have in healthy conditions. The duration of the starvation period before sampling could be the determinant factor in this case. SnS has been found to maintain stable glucose levels in long-term starvation periods to maintain glucose homeostasis. Nonetheless, short-term starvation, 4 hours, as it was applied before sampling, may not show similar regulation capacity. The ability to respond to such stressor after 4 hours is diminutive in carnivorous fish species (Garcia-Riera & Hemre, 1996; HEMRE et al., 1995), and thus such spikes were observed in both sampling occasions, at the beginning and at the end of the trial.

The trial's glucose levels were also quite elevated when compared to other carnivorous fish species, except for farmed and wild seabass, when comparing the TAG levels on seabass, juvenile SnS from this trial performed similarly (Peres et al., 2013).

Then, interpreting the measure of glucose as a way to access the best feed choice for these juveniles may be altered, as the elevated glucose levels are not explained by the diet choices but due to short-time starvation. Conde-Sieira et al. (2015) demonstrated that SnS needs a minimum of 10 hours to re-establish basal levels of blood glucose. If the samplings were performed later (Costas et al., 2011; Peres et al., 2015) (made the blood sampling 24h after initiating starvation), the glucose might have shown a difference in the basal levels which could have provided a way to assess the impact of each diet on the fish's glucose metabolism. This, however, is still a conjecture since the cholesterol and triglycerides showed similar levels in both diets and glucose could have still the same behavior.

4.6.2. Enzymes

4.6.2.1. AST

Aspartate aminotransferase has been established as a biomarker for overall cellular health in humans, fish, and other animals. As it is a compound characterized for being present on the cell's cytosol, a high activity of this enzyme in serum is indicative of cellular degeneration. Although it is commonly used to assess liver damage, AST cannot be the sole factor in determining if there are injuries in the liver. This compound is also present in many other tissues, and a diagnosis on anything specific can't be clarified only with the study of the AST level variation (Harris, 2009) .

4.6.2.2. ALT

ALT is predominantly present in the cytosol of hepatocytes, exhibiting substantially elevated ALT activity relative to that observed in plasma. In cases of hepatocyte damage, ALT is secreted by the affected cells into the extracellular space and eventually the plasma. Consequently, ALT levels and activity are elevated in animal blood exhibiting damaged hepatocytes relative to those with intact hepatocytes. Additionally, ALT is present in other tissues, albeit at considerably lower concentrations (Aulbach & Amuzie, 2017; Huang et al., 2006).

Reference levels of the serum ALT have not been set yet in the literature for SnS. However, in environmental stress-inducing situations, in SnS, ALT showed sensitivity to low salinity in the water, where glucose production pathways were activated in response to such stress. Additionally, Borges, Medale, Dias, et al. in 2013 did not saw activation of ALT with variations of both dietary lipid and protein levels. In other species, nonetheless, ALT showed sensitivity to high protein levels in the diets (Fynn-Aikins et al., 1995; Lupiánez et al., 1989; Metón et al., 1999).

Although the presence of aminotransferase in plasma indicates damage in hepatic cells, the elevation of these enzymes does not translate into higher liver dysfunction (Telega, 2018; WROBLEWSKI & LADUE, 1955).

Blood analysis in this trial had underwhelming results. The serum metabolites showed no difference between the diets and the analysis of the enzymes ALT and AST were not possible to determine. The lack of catalytic enzyme activity on the substrate of the

Author	Diet's name	Proximal Analysis of the Diets				Proximal Analysis of the carcasses				FCR		DGI		Temperature (ºC) on the trial		Objective in study	Effects of the compound
		Dry matter	Crude protein (%DW)	Crude lipids (%DW)	Ash (%DW)	Dry matter	Crude protein (%DW)	Crude lipids (%DW)	Ash (%DW)	Mean	SD	Mean	SD	Mean	SD		
Borges et al. (2014)	CTR	91.1	56.8	8.67	13.5	23.4	73.5	20.3	7.52	1.13	0.04	1.24	0.09	20.0	1.00	Replace the fish oil supplementation with vegetable oil-based diets. Experiment with different levels of VO against a 100% fish oil diet.	Full replacement is possible without undermining fish growth and feed utilization.
	VO50A	90.9	57.1	8.70	13.4	23.7	72.7	20.1	7.44	1.22	0.05	1.25	0.14				
	VO50B	91.1	57.4	8.70	13.6	24.1	71.5	19.8	7.31	1.12	0.04	1.25	0.09				
	VO100A	92.8	56.8	9.39	13.5	23.9	72.1	19.9	7.37	1.08	0.15	1.31	0.13				
	VO100B	92.8	57.0	8.57	13.2	24.1	71.5	19.8	7.32	1.19	0.08	1.24	0.07				
	VO50PP	91.1	56.7	10.5	10.6	23.4	73.5	20.3	7.51	1.07	0.06	1.14	0.11				
Borges, Medale, Veron, et al. (2013)	L4	91.4	54.4	4.74	7.64	26.1	73.6	18.4	9.20	1.33	0.10	0.92	0.05	20.0	1.00	Access the effect of high lipidic diets in lipid metabolism and digestion.	SnS can digest lipids well, growth impairment is not due to lack of lipid digestibility. Liver receptors for VLDL are enhanced by lipid rich diets, but the muscle does not.
	L17	92.7	53.9	17.3	7.37	28.1	64.8	25.6	8.90	1.45	0.10	0.84	0.06				
Valente et al. (2011)	S-15	95.6	59.5	15.9	7.01	28.8	62.6	28.2	8.15	2.03	0.59	0.59	0.12	19.0	1.00	Long-term effects of plant protein- against a fishmeal-based diet. Long-term effects of Low energy diet on SnS juveniles and respective growth and fillet quality.	Replacing fishmeal with plant protein doesn´t affect FI, growth or PE, but here is liver vacuolization and necrosis.
	S-15PP	94.8	59.1	15.3	6.03	29.7	63.9	20.3	8.96	2.11	0.14	0.50	0.07				
	S-8	95.7	58.6	8.19	7.35	28.0	65.4	24.7	8.62	2.35	0.39	0.52	0.07				

Table 7 - Review table of diets utilized in Senegalese sole nutrition trials showing the proximal analysis of the diets and carcasses and growth performance indexes (FCR and DGI).

Authors	Diet's name	Proximal Analysis of the Diets				Proximal Analysis of the carcasses				FCR		DGI		Temperature (ºC) on the trial		Objective in study	Effects of the compound
		Dry matter	Crude protein (%DW)	Crude lipids (%DW)	Ash (%DW)	Dry matter	Crude protein (%DW)	Crude lipids (%DW)	Ash (%DW)	Mean	SD	Mean	SD	Mean	SD		
Silva et al. (2009)	F45	93.1	57.2	6.60	9.00	25.3	66.5	21.1	2.06	0.85	0.03	1.54	0.05	20.0	1.00	Develop a plant-based diet with similar growth performance and protein deposition to fish meal-based diets. Assess the better aa balance (EAA vs Lysine).	Fishmeal can be completely replaced by plants as long as if the diet is supplemented with the essential amino acids for SnS.
	F15+IAA	94.9	56.7	5.80	5.90	24.9	68.0	18.2	2.72	0.85	0.04	1.65	0.01				
	F15+Lys	96.4	56.1	5.90	6.10	24.6	68.1	18.0	2.46	0.92	0.02	1.31	0.07				
	F5+IAA	96.0	55.8	5.50	6.20	24.3	69.6	16.9	2.56	0.94	0.09	1.50	0.09				
	F5+Lys	95.0	55.4	6.20	6.20	23.2	69.6	15.1	2.66	1.04	0.05	1.29	0.06				
Borges et al. (2009)	L4	91.3	57.0	4.07	8.48	23.6	75.1	15.8	7.59	1.04	0.05	1.22	0.16	20.0	1.00	Set the optimal dietary lipidic level that improves growth without compromising flesh quality.	The lipid tolerance of SnS is low, with the maximum percentage of lipid that can be tolerated without impairing growth being 8%.
	L8	91.7	56.1	8.40	8.44	24.9	73.2	20.1	6.94	1.15	0.03	1.20	0.03				
	L12	92.2	56.8	13.7	8.30	23.8	71.3	21.6	7.96	1.66	0.09	0.94	0.02				
	L16	92.4	57.5	17.4	7.85	23.4	68.2	24.3	6.95	1.68	0.06	0.93	0.07				
	L20	92.6	56.1	22,5	7.82	24.4	66.4	24.0	6.53	2.16	0.18	0.81	0.08				
Bonacic et al. (2016)	8FO	94.5	56.0	7.90	10.5	25.6	50.3	16.4	9.38	1.31	0.12	-	-	19.3	1.20	Test if the effects of alternative sources of dietary lipids are modulated by their levels of inclusion in the diet and if their long-term use has different effects pre- and postprandially.	SnS deals well with the inclusion of VO, up to 100%. Dietary lipid levels can be increased higher than 10 % without affecting growth, only with diets with FM and at least 25% FO. SnS is weak in regulating FA absorption when fed high lipidic diets.
	8VO	95.4	56.9	7.40	10.7	24.8	50.9	17.1	9.68	1.30	0.06	-	-				
	18FO	95.7	58.0	17.6	10.4	27.6	45.0	23.2	8.33	1.16	0.10	-	-				
	18VO	95.6	57.2	17.4	10.3	26.9	45.5	21.5	8.92	1.30	0.09	-	-				

Authors	Diet's name	Proximal Analysis of the Diets				Proximal Analysis of the carcasses				FCR		DGI		Temperature (ºC) on the trial		Objective in study	Effects of the compound
		Dry matter	Crude protein (%DW)	Crude lipids (%DW)	Ash (%DW)	Dry matter	Crude protein (%DW)	Crude lipids (%DW)	Ash (%DW)	Mean	SD	Mean	SD	Mean	SD		
Benítez-Dorta et al. (2013)	100FO	-	56.7	12.3	10.1	-	-	-	-	1.58	0.26	-	-	22.2	0.85	Access effects of total replacement of FO with VO on growth performance FA composition and welfare.	Replacement with VO altered FA profile on muscle, liver, and intestine. ARA/EPA ratio remains the same with different oils in the diet. VO affected GR expression and HSP levels.
	100LO	-	56.8	13.0	10.5	-	-	-	-	1.60	0.43	-	-				
	100SO	-	56.6	12.6	10.3	-	-	-	-	1.66	0.51	-	-				
Guerreiro et al. (2012)	55P16L	90.6	55.7	17.0	8.14	16.3 / 14.2	-	-	-	1.56 / 1.23	-	1.11 / 1.73	-	15.8 / 21.9	1.1 / 0.6	Analyze the effects of different rearing temperatures, 16 and 22 ºC, and dietary Protein/Lipid ratio on growth performance, feed utilization, and carcass proximal composition.	No matter the water temperature, 55 % protein, and 8% lipid dietary level promote optimal growth in SnS. Increasing temperature from 16 to 22 ºC enhances growth rate and FCR.
	55P8L	86.6	55.3	8.19	8.02	12.5 / 15.1	-	-	-	1.03 / 0.91	-	1.30 / 1.91	-				
	45P16L	93.3	45.9	17.1	8.46	16.0 / 18.5	-	-	-	1.27 / 1.16	-	1.37 / 1.84	-				
	45P8L	92.4	43.4	7.75	8.12	15.2 / 19.3	-	-	-	1.61 / 1.22	-	0.98 / 1.81	-				
Hachero-Cruzado et al. (2024)	CTRL	93.5	54.0	15.6	8.07	-	-	-	-	1.76	0.20	-	-	21.5	3.54	Study the effects of replacing marine and plant-origin ingredients with *Tenebrio molitor* (TM) on growth performance and FA profile of SnS.	Replacing up to 10% FM with TM has no negative effect on SnS rearing. Replacing up to 15% PM with TM increases the growth rate. n-3 PUFA and DHA remained stable and n-3: n-6 index was improved with inclusion of TM. TM is a viable ingredient to utilize in SnS rearing.
	FM5	93.8	53.8	15.5	7.68	-	-	-	-	1.58	0.14	-	-				
	FM10	93.8	53.8	16.5	7.37	-	-	-	-	1.61	0.02	-	-				
	PP10	92.7	53.0	16.2	8.03	-	-	-	-	1.71	0.17	-	-				
	PP15	93.5	52.7	17.1	8.17	-	-	-	-	1.42	0.23	-	-				

5. Conclusion

The results of this trial exhibit the benefits of utilizing Diet A for the juvenile Senegalese sole during the Nursery stage at Safiestela, both growth and economic performance are enhanced compared to Diet B.

Sourcing the dietary advantage that gives Diet A an advantage in this trial was not feasible given the framework in which this experiment was carried out. The confidentiality agreements with the commercial partners that provided the diets included the details of the composition and source, which are commonly the main factors in diet modeling to improve rearing performance, thus limiting certain conclusions in this regard.

It is also important to reiterate that the welfare assessment of the fish in this trial is incomplete. While triglycerides and cholesterol levels remained stable and in appropriate levels, which could indicate that the basic lipid metabolism is functioning within healthy values, other physiological mechanisms could not be assessed. In future trials, it would be beneficial to conduct additional analyses that provide more comprehensive insights into the fish's health.

The turbidity analyzed daily showed a close relationship with how the growth performance behaved, better water quality related closely with better growth performance. The incorporation of the Turbidity measurement protocol into the daily routine could potentially enhance the predictability of the quality of water renewal in the RAS and the growth performance of the fish.

The data gathered in this work may serve as an effective index for establishing reference values and relating them to production performance, if the proposed diet is implemented in the production line, and even in future nutrition related trials with this species.

The conclusion of this thesis was attained with the fulfillment of the main objectives that were previously proposed, although with some described caveats that could be implemented to enrich future diet improvement trials in Safiestela, Sea Eight and the Aquaculture Industry in a broader sense.

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