GROWTH TRIAL IN ATLANTIC SALMON (*SALMO SALAR*) TESTING SIX FISHMEALS IN RAS

Amalia Sanz Llorente
Department of Mariculture, Cuban Fisheries Research Center (CIP), Havana, Cuba.
sanzllorenteamalia@gmail.com

Supervisor:
Georges Lamborelle: georges@matis.is

ABSTRACT

Cuba, the largest island in the Caribbean, relied on capture fisheries for marine products until the 1980s, since then they have declined owing to various causes, including natural disasters such as hurricanes. The lack of seafood in the Cuban diet is the main reason why one of the government's current goals is to develop mariculture. To achieve this goal, Cuba has received funding and support from foreign institutions and international collaborations. One collaboration is between the Cuban Fisheries Research Center (CIP) and the Institute of Marine Research (IMR) from Norway, which has been sustained for over 20 years. The most recent stage of this collaboration resulted in the installation of a Recirculation Aquaculture System (RAS) at the Mariculture Experimental Station of Mariel, owned by CIP. The aim of this facility is to create broodstock and produce marine fingerlings in Cuba. Given that the management and initiation of RAS require qualified personnel, the outcome of the present study is to become a training tool for Cuban aquaculture workers. The present study was conducted within a RAS at the Matís Aquaculture Research Station (MARS) in Iceland, and its main objective was to compare performance parameters between six fishmeal treatments for Atlantic salmon. The experiment lasted 56 days, during which water quality parameters were measured daily, and the fish were weighed at the beginning and end of the experiment, which was used to calculate the Specific Growth Rate (SGR). Uneaten feed was collected daily to calculate the feed intake and feed conversion ratio (FCR). In addition, water samples were collected from the biofilter at three time points during the experiment to evaluate the variation in the biofilter community. FCR (0.83-0.87) and SGR (0.96-1.06) showed no significant difference between the six tested feeds during the trial. Statistical analyses based on the diversity of the sequenced DNA extracted from the samples did not reveal significant differences in community diversity. Nitrosomonas Genus, Alphaproteobacteria, and Gammaproteobacteria Class were present in all the water samples. In the end, all feed behaved similarly, and the biofilter function was not affected by the overfeeding process, guaranteeing water quality during the trial.

Keywords: RAS, Atlantic salmon, Biofilter, SGR, FCR.

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1 INTRODUCTION

1.1 Mariculture in Cuba

Cuba is the largest island of an archipelago in the Caribbean Sea. This archipelago includes a smaller island named Isle of Youth and 4195 cays and islets. Because of its geographical position, it has been known as "the key to the Gulf" since colonial times. Its closest countries are Haiti, Bahamas, the United States, Jamaica, and Mexico (FAO, 2023).

Given that Cuba is an island, its main source of marine products has been capture fisheries since the 1950s. However, the decrease in the productivity of this resource since the 1980s is evident mainly due to mismanagement and natural disasters such as hurricanes. Consequently, the majority of present-day capture fisheries in Cuba are overexploited, or even depleted (Baisre, 2017). Due to the lack of seafood in the Cuban diet, aquaculture of marine organisms is currently an important part of national food security. However, mariculture is not well developed in Cuba.

Since 2016, the country has followed a national strategy for mariculture development promoted by the Ministry of the Food Industry (MINAL) and the Cuban Fisheries Research Center (CIP). The cultivation of species such as oysters, sponges, and sea cucumbers has been conducted on a pilot scale. The main challenge is the production and acquisition of feed. In particular, there is a lack of raw materials for feed preparation, and the absence of national companies that process raw materials for feed preparation. The raw material for feed production is imported, and with barriers to foreign currency access, it is difficult to obtain funds to conduct such imports (Ministry of Food Industry and Fisheries Research Center, 2016).

The Cuban scientific sector, dedicated to aquaculture, has received support from international collaborations. Monetary and technological contributions have positively influenced the training and preparation of qualified personnel for aquaculture management. Collaboration between the Netherlands and the Aquaculture Technology & Development Company (EDTA) resulted in the installation of a freshwater Recirculation Aquaculture System (RAS) (Carlés, 2016). In addition, a RAS facility for mariculture was installed at the Mariculture Experimental Station of Mariel in relation to an international project called “Production of marine fingerlings in Cuba” between CIP and the Institute of Marine Research (IMR) from Norway.

International collaboration between CIP and the IMR for over 20 years has the main objective of reducing the need for imports in the marine aquaculture sector. Because of this collaboration, the installation of marine cages for cobia cultivation in the Bay of Pigs began in 2013 (Flores et al., 2016). The cobia fry were imported from Ecuador because Cuba did not have broodstock to obtain them. The culture was successful, but because of the lack of a fully equipped hatchery in Cuba, the specimens could not be preserved to start a broodstock (Isla et al., 2019). For this reason, it was decided that the next stage of this collaboration, with the prospect of starting in 2021, would focus on developing a hatchery equipped to create broodstock and begin the

production of marine fingerlings in Cuba. This hatchery also aims to support and conduct experiments to investigate and grow species with potential for Cuban aquaculture.

In preparation for this new stage, a RAS that included broodstock culture, pre-breeding, and breeding areas was installed at the Experimental Station of Mariel. RAS have been developed for more than 30 years, mainly in developed countries, with the aim of reducing the use of water in aquaculture production units and improving growth, disease control, and farming efficiency. This technology reuses the major possible amount of water for the cultivation of aquatic organisms by ensuring water quality through the constant utilization of several filtration mechanisms. These mechanisms include biological, mechanical, and physical filtration mechanisms (Isla M., 2008).

Qualified personnel are needed to operate RAS, and in Cuba, there is currently little practical knowledge about RAS technology. This study aims to compare performance parameters between treatments from a growth trial in a RAS. The above aims to produce a training tool that addresses the correct practices in the manual handling of RAS technology, as well as the conditions and parameters that are needed to maintain water quality and a functional biofilter within this system.

2 OBJECTIVES

A detailed evaluation of the function of RAS and the experimental conditions within this system would be a valuable tool to increase the qualifications of aquaculture professionals in Cuba. To achieve this, the study objectives of this study are as follows:

2.1 General objective

- Comparison of performance parameters between six fishmeal treatments in Atlantic salmon using RAS technology.

2.2 Specific objectives

- Calculation of Feed Conversion Ratio.
- Calculation of Specific Growth Rate.
- Evaluation of the biofilter community during the trial period for improved water quality in RAS.

3 LITERATURE REVIEW

3.1 Growth Trial and Experimental Design

A growth trial is an experiment in which the main objective is to compare the performance parameters of fish to detect significant differences. To achieve this, it is important to establish the feed as the only variable factor during the experiment. The remaining parameters should be equal. The expected results in these assays must then be due to an experimental feed or some

specific ingredient within the feed. To ensure accurate results, the experimental design should include replication of treatments (Thorarensen, et al., 2015; Hardy & Kaushik, 2022).

3.1.1 Feed Management and Duration of Experiment

The most utilized method to perceive significant differences within the growth of individuals in a growth trial is by the weighing of an individual at the beginning and end of the experiment to calculate the Specific Growth Rate (SGR). In addition, calculation of feed intake is necessary to determine the Feed Conversion Ratio (FCR). Both values provided an approximation of the efficiency of the feeding process and nutrient utilization. To achieve maximum growth, the animal must be overfed during the trial. Uneaten feed should be recovered daily, to determine how much the fish consume. Overfeeding ensures that there is no energy limitation affecting the fish and guarantees that the feed reaches every animal within the rearing unit (Jobling, 2011).

The duration of a growth trial depends on several factors, such as the species used for the experiments and response to the experimental diets. According to Cowey (1992), a reasonable time for these assays would be 8 -12 weeks, but this will be dependent on the factors described above. It is risky to end an experiment before a time that allows a significant response to the tested ingredient or diet (Jobling, 2011).

3.1.2 Diets

For growth trials, it is common to utilize diets that contain at least one ingredient capable of introducing a variation in animal response to the feed. To observe this response, it is necessary to compare this diet with another diet in which the ingredients are known. This last diet must also have a previously known response in the animals, to function as a reference point. This reference feed functioned as a control during the experiment (Jobling, 2011; Hardy & Kaushik, 2022).

3.1.3 Organisms

Information regarding the experimental organisms is extremely important in every trial. Such knowledge is essential for explaining the response to treatment during assays. Ideally, every organism should come from the same batch to minimize responses due to genetic factors. This will also reduce the influence of other factors such as age and weight (Jobling, 2011; Dong, et al., 2023).

3.1.3.1 Atlantic salmon

Atlantic salmon (Salmo salar) is a carnivorous fish species with a complex life cycle that includes both freshwater and saltwater stages. Salmon´s diet requires an elevated level of protein, and as a consequence, the fish produces a lot of waste. The sensitivity to ammonia compounds (resulting from fish waste) is high in this species. This shows the need to keep salmon in a space where the amount of nitrogen compounds within the water can be regulated. In addition, salmon culture stages include temperature and salinity variations. Therefore, salmon farming should be conducted within a culture system that allows the regulation of factors such as nitrogen compound levels, temperature, and salinity to guarantee a favorable growth rate. RAS systems are the best choice to accomplish these requirements (Golfand, 2023).

3.2 Recirculation Aquaculture Systems (RAS)

RAS is a technology based on the reutilization of the largest possible percentage of water and its minimal exchange with the environment. To achieve this, the system includes filtration methods that guarantee the quality of the water. Typically, RAS systems contain production units (culture tanks) equipped with feeders and control sensors for water chemical parameters, such as pH and Oxygen. To maintain the quality of the environment inside the tanks, the RAS includes a water treatment system. The treatments classically include a solid removal system, aerator/oxygenator system, biofilter, and UV lamp disinfection (Figure 1). Although the initial costs of installation can be remarkably high, the system is very cost-effective when functional. High productivity can be achieved by controlling performance parameters such as temperature to improve and accelerate growth. RAS provides a controlled environment for the culture and is an alternative to traditional outdoor fish farming (Mongirdas, et al., 2017; Pulkkinen, 2020; Balami, 2021).

However, RAS management must be conducted by qualified personnel, and daily measurements of parameters such as dissolved oxygen, temperature, salinity, organic compound levels, Total Ammonia Nitrogen (TAN) concentration, CO_2, and pH must be performed. Given that the key to successful production in RAS is water quality, the above parameters that directly affect the filtration methods, mainly the biofilter, should be kept at suitable concentrations (Isla, 2008).

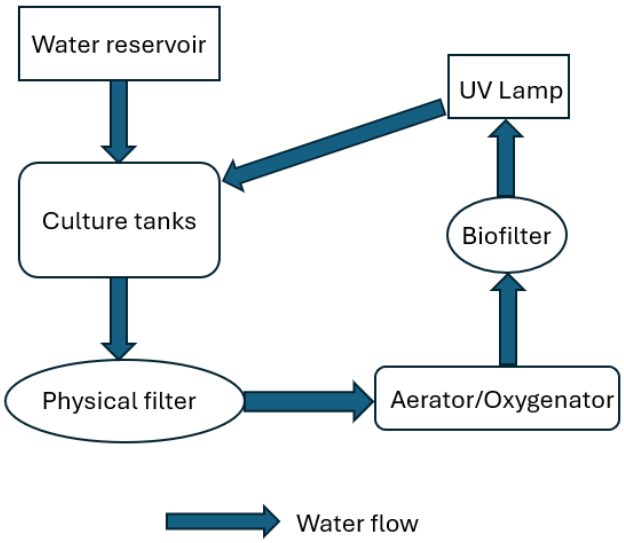


Figure 1: General diagram of a RAS.

3.3 RAS components

3.3.1 Solid Removal System

The accumulation of solid compounds can be harmful to cultured organisms in RAS and can significantly decrease the water quality of the system. Suspended solids can increase biochemical oxygen demand, cause damage to fish gills, reduce biofilter nitrification, and increase ammonia in the system (Khater, et al., 2011; Balami, 2021).

Sensitivity to solid compounds differs between species. Whiteleg shrimps (*Litopenaeus vannamei*) do not present high sensitivity to solids, but some species, such as Atlantic salmon (*Salmo salar*), are sensitive. Therefore, these solids must be removed as quickly as possible, particularly settable solids. The most common methods for removing settable solids are sedimentation tanks (clarifiers), mechanical filters (granular or screen), and swirl separators. All these filters are commonly called physical filters and are effective in filtering out relatively large particles, such as feces, mucus, and uneaten feed (Balami, 2021).

However, some suspended solids cannot settle at the bottom. These solids float in the water column and can potentially obstruct the gill function of fish by causing irritation and, consequently increasing the oxygen demand in the system. This type of solid can be removed by a protein skimmer, also known as foam fractionator, which contributes to controlling the foaming agents. Nonetheless, these floating solids can be removed using a drum filter (Balami, 2021).

3.3.2 Aerator/Oxygenator System

Maintaining an appropriate oxygen concentration within RAS is important. Hence, the required concentrations of this gas can be supplied to tanks through continuous aeration. Aeration can be performed by an air diffuser system and aerators using either pure gaseous oxygen or atmospheric oxygen (air) (Balami, 2021).

3.3.3 Biofilters

Biofiltration in RAS consists of the removal of total ammonia nitrogen by a community of microorganisms that colonize the biofilter's surface. The surface of the biofilter consists mainly of polyethylene particles, which are then coated with bioactive media (Isla, 2008).

TAN includes the two forms in which the ammonia is present in aqueous solution, (NH_3) gaseous and (NH_4^+) cationic. The variation between both forms depends on environmental factors such as temperature and chemical factors like pH and salinity. The presence of TAN can be very toxic for the fish and needs to be removed from RAS (Pulkkinen, 2020).

TAN are removed in a nitrification process that is performed in two steps, traditionally conducted by two types of nitrifying bacteria. The first step of the nitrification reaction consists of the ammonium (NH_3/ NH_4^+) oxidation to nitrite (NO_2) and the second step is the nitrite oxidation to nitrate (NO_3). But organisms that perform both steps were recently discovered (van Kessel, et al., 2015), (Pulkkinen, 2020; Burut-Archanai, et al., 2021). Studies based on the application of molecular biological techniques such as Polymerase Chain Reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and DNA sequencing have been used to identify the bacterial community that is present on biofilters in RAS. The results from these studies revealed the presence of *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus* performing the first step of the nitrification process. In addition, *Nitrospira* and *Nitrobacter* were found performing the second step of the nitrification process (Burut-Archanai, et al., 2021).

There are a variety of microorganisms living within the biofilter other than autotrophic nitrifying bacteria. The most studied biological interaction inside biofilters has been between heterotrophic and autotrophic bacteria. Heterotrophic microorganisms use organic compounds as carbon sources and present faster growth compared to autotrophic bacteria. In conditions where organic carbon is in higher concentrations, for example with considerable amounts of uneaten feed or feces, the growth of heterotrophic organisms may increase and displace nitrifying bacteria (Pulkkinen, 2020).

Organic compound concentration is not the only factor that influences the biofilter´s community in a RAS. pH levels and dissolved oxygen concentrations are also critical parameters that can directly affect the efficiency of biofiltration processes (Balami, 2021).

3.3.3.1 pH levels

Nitrification is a process that controls the concentration of un-ionized ammonia-nitrogen in RAS. The efficiency of this process is very related to pH levels. Lower pH levels convert ammonia into ammonium or ionized ammonia (NH_4^+). Higher pH levels convert ammonium into unionized ammonia (NH_3). Unionized ammonia is extremely toxic for fish. In general, it is recommended to maintain the pH levels in the range between 7.0 - 8.0. (Balami, 2021).

3.3.3.2 Dissolved Oxygen concentrations

Dissolved Oxygen (DO) levels below 5 ppm will result in poor health and performance from the fish. However, levels below 2 ppm can lead to biofilter malfunction because the rate of O_2 diffusion into bacterial film for *Nitrobacter* and *Nitrosomonas* begins to limit the nitrification process. As a result, free CO_2 accumulates and lowers the pH. The accumulation of CO_2 can be a problem for fish health and is dangerous when the RAS has high stocking densities (Balami, 2021).

3.3.3.3 Organic compounds levels

Fish feces, uneaten feed, algae, and sloughed micro-biological cell mass are all organic compounds and sources of solid production within RAS. The accumulation of these compounds decreases the water quality in the system and attracts opportunistic pathogens. These compounds are also a source of organic carbon that can speed up the growth of heterotrophic bacteria and affect the nitrifying bacteria community within the biofilter (Isla, 2008).

3.4 Advantages

RAS system offers potential benefits in comparison to a pond or cage culture such as less water usage, flexible site selection, and better environment management because of the minimal volume of effluent. Also, it allows better control over the culture performance parameters and provides a higher intensity of production (Balami, 2021).

With the RAS technology, it is possible to regulate the temperature. This speeds up the development of various reared fish and avoids the seasonal prevalence of fish. Regardless of the maximum reutilization of the water, there is less disease occurrence in this system and a

shorter production cycle due to a controlled environment (Mongirdas, et al., 2017; Balami, 2021).

3.5 Disadvantages

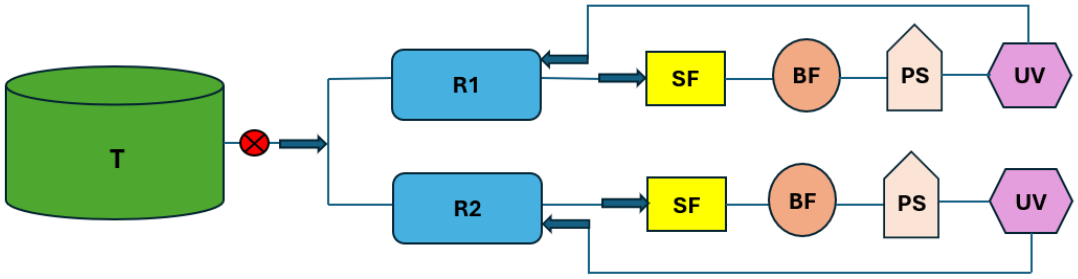
Installation of RAS facilities is expensive. All equipment, infrastructure, treatment systems, construction, and management projects require significant funding. In addition, maximum recirculation of water can be challenging for the prevention and treatment of diseases. The presence of pathogens in a RAS compromises the entire system, and the use of antibiotics for treatment could disrupt the microbiome of the biofilter. Breakdown of the biofilter can cause varying levels of nitrite or ammonia, which are highly toxic chemical compounds for the fish. A less studied disadvantage is the accumulation of microplastic particles in RAS, which may serve as ideal floating carriers for heavy metals, antibiotics, and antibiotic resistance genes (Almeida, et al., 2019; Balami, 2021; Wei, et al., 2024).

4 MATERIALS AND METHODS

4.1 Growth trial

The experiment was conducted for 56 days from the 13th of February 2024 until the 8th of April 2024 at the Matís Aquaculture Research Station (MARS) in a RAS with 36, 200-liter tanks (Kunststoff Spranger, Plauen, Germany) (Figure 2). The system includes a mechanical (sponge filter), biological (moving bed), physical (protein skimmer and UV), and chemical (ozone) water treatment (Figure 3).

Figure 2: RAS facility at MARS.



T: Reservoir tanks.
R1: Row I (12 culture tanks).
R2: Row II (12 culture tanks).
SF: Sponge filter.
PS: Protein skimmer.
BF: Biofilter.
UV: UV Lamp.
: Valve.
: Water flow.

Figure 3: General diagram of the RAS system used for the experiment.

Six experimental diets were evaluated in the trial. They were all equal in energy and Crude Protein content (Table 1) to analyze only the influence of the different fishmeal contents of these diets on fish growth. Given that the rest of the feed ingredients are known and uniformly distributed in all the diets, it is correct to assume that any variation obtained in the experiment results will be due to the differences between the fishmeals (Table 2). All feeds have been extruded and were labeled with A, B, C, D, E, and F.

Table 1: Approximate composition of the experimental diets (% based on DM).

Experimental feed	Crude Protein*	Crude Fat**	Dry Matter
A. Fish Feed	46.1%	16.7%	96.8%
B. Fish Feed	44.4%	16.1%	95.7%
C. Fish Feed	43.5%	15.5%	95.6%
D. Fish Feed	44.4%	15.3%	95.6%
E. Fish Feed	43.4%	17.0%	95.7%
F. Fish Feed	43.3%	16.3%	95.8%

*Protein Method (ISO 16634-1:2008 (E)) ** Soxhlet Method (AOCS Ba 3-38 (2017))

Table 2: Feed formulation (Raw materials percentages).

Raw materials	A	B	C	D	E	F
Corn gluten meal	7.62	6.00	6.00	6.00	6.27	6.27
Soy-protein concentrate	21.00	20.17	20.05	20.10	21.00	21.00
Sunflower meal	5.00	5.00	5.00	5.00	5.00	5.00
Wheat gluten meal	13.00	13.00	13.00	13.00	13.00	13.00
Wheat grain	12.38	13.31	13.42	13.50	13.00	12.81
Fish oil	10.55	10.90	10.89	10.86	10.64	10.39
Rapeseed oil	9.71	11.31	11.33	11.02	10.67	11.23
L-Lysine HCL	0.18	0.06	0.04	0.01	0.08	0.05
DL-Methionine		0.06			0.02	0.02
Mono ammonium phosphate	1.15	1.17	1.17	1.17	1.16	1.16
Minerals	0.20	0.20	0.20	0.20	0.20	0.20
Vitamins	0.69	0.69	0.69	0.69	0.69	0.69
Water	-1.47	-1.86	-1.78	-1.55	-1.72	-1.82
Fishmeal 1	20					
Fishmeal 2		20				
Fishmeal 3			20			
Fishmeal 4				20		
Fishmeal 6					20	
Fishmeal 7						20

Eighteen tanks were included in the trial. The fish used for the trial were Atlantic salmon from Benchmark Genetics in the post-smolt stage. Each tank contained 16 fish that were fed 21 times per day, once an hour (from 00:00 to 23:00, excluding the time between 08:00-11:00), 30 minutes each time. All diets were evaluated in triplicate (Figure 4). Feeding was conducted with an automatic feeding system. During the trial, an overfeeding of 15 % was ensured. The seawater (33 ppt) used for the experiment was made with artificial sea salt (Aquamedic, Bissendorf, Germany).

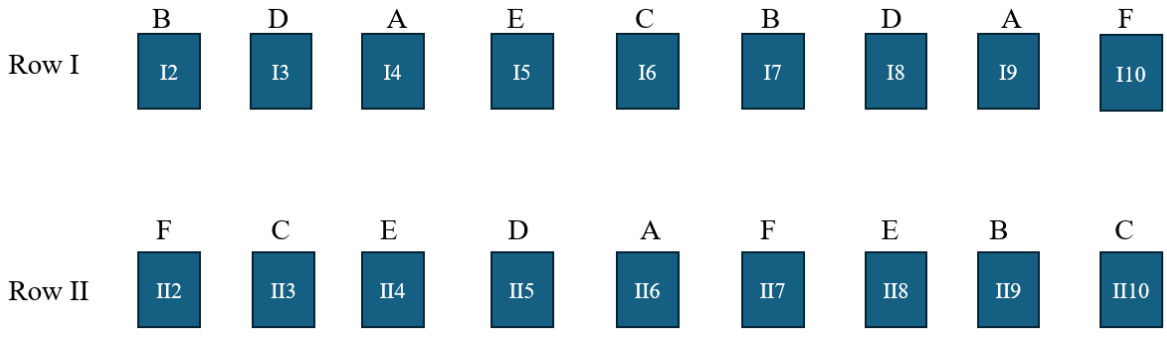


Figure 4: Randomization of the feeds over the tanks.

The individual weight was measured at the beginning and the end of the experiment to calculate the Specific Growth Rate (SGR) as a measure of growth. During the trial, the uneaten feed was collected daily to calculate weekly feed intake and Feed Conversion Ratio (FCR). All the water quality parameters measured during the experiment were within the recommended range (Table 3).

Table 3: Measurement of water quality parameters during the experiment.

	Measuring frequency	**Target Values**	**Daily Average**	
Temperature	Every hour	12	12.10 ± 0.73	°C
Oxygen	Every hour	> 7	10.16 ± 0.51	mg l^{-1}
Salinity	Once a day	33	32.38 ± 0.87	ppt
pH	Once a day	> 7	7.11 ± 0.15	mg l^{-1}
Ammonia	Once a day	< 1	0.70 ± 0.10	mg l^{-1}
Nitrite	Once a day	< 0.2	0.18 ± 0.03	mg l^{-1}
Nitrate	Once a day	< 300	97.31 ± 25.50	mg l^{-1}

4.2 Biofilter community analyses.

4.2.1 Sample processing

The water samples were taken at three time points during the experiments.
T1: 29th February
T2: 14th March
T3: 4th April
All samples were taken in triplicate (a, b, c).
One additional feces sample (triplicate) was taken from one of the tanks.
The water samples from the biofilter were filtered using a sterile membrane filter of 0.2 µm pore size and a diameter of 47 mm.
The DNA was extracted from the membrane filter using the QIAamp PowerFecal Pro DNA Kit (Qiagen). The extraction protocol was modified as follows:
Step 1: 800 µl of salt water was added followed by 800 µl of C1 solution. Saltwater was added because of the absorption of the liquid by the filters.
Step 2: Samples were vortexed at maximum speed for 20 minutes instead of 10 minutes.
Step 15: Centrifugation was performed 2 times (16000 x g for 2 minutes) before placing the MB Spin Column into the Elution Tube.
Step 16: 25 µl of Solution C6 was added instead of 50-100 µl.

4.2.2 Gene amplification

PCR was performed to amplify the 16S rRNA gene from the samples (Table 4). Primers targeting the V4 hypervariable region with Illumina overhang 520F-Ill (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-AYTGGGYDTAAAGNG) and 802R-Ill (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-TACNVGGGTATCTAATCC) were utilized (Table 5). Amplification was verified by electrophoresis on 2% agarose TAE gel, and the expected amplicon size was 253 bp. The resulting amplicons were purified using Aline PCRClean DX beads in pooled duplicate reactions.

In a second PCR reaction, the purified amplicons were amplified and individually barcoded using the Nextra XT v2 Index Kit (Illumina) and Q5 polymerase (NEB). Amplification was verified on gel as before and an expected indexed amplicon size of 439 bp.

Table 4: PCR programme.

	Reaction	
Initial Denaturation	98°C	60sec
30 cycles of	98°C	10sec
	50°C	30sec
	72°C	30sec
Final Extension	72°C	2min
Hold	10°C	Forever

Table 5: PCR reaction.

	Reaction	**Mastermix**
DNA Template		5uL
H2O	6.75	Mastermix = 20uL each sample
5X Buffer	5.00	
5X Enhancer	5.00	
dNTP 10mM	0.50	
520F-Ill	1.25	
802R-Ill	1.25	
Q5 Pol	0.25	
Total	25	25uL

4.2.3 Gene sequencing

To remove free index adaptors and normalize sample concentrations the SequalPrep™ Normalization Plate (96) Kit was used (Applied Biosystems™). To measure the concentration of the pool the Qubit High-Sensitivity dsDNA assay (Invitrogen) was conducted. The normalized amplicons were pooled and sequenced on the iSeq 100 sequencing platform with the v2 cartridge chemistry (Illumina).

4.3 Data analysis

4.3.1 Growth trial

FCR was calculated using the formula:

$$FCR = \frac{feed\ intake\ (g)}{growth\ (g)}$$

SGR calculations were made as follows:

$$SGR = \frac{(\ln(Wt) - \ln(W0))}{days} * 100$$

with:
$\ln(Wt)$ = natural logarithm of the final individual weight
$\ln(W0)$ = natural logarithm of the initial individual weight

The FCR and SGR values obtained for each feed were analyzed on RStudio using One Way ANOVA.

4.3.2 Biofilter´s community analyses.

Data analysis was done in RStudio using packages dada2 (v.1.28.0) and phyloseq (v.1.44.0) with the Silva database (v138), plotting was done using ggplot2 (v.3.5.0). Alpha Diversity was compared between time points using the Shannon, Simpson, and Fisher's Diversity Indexes. Statistical significance was assessed using the non-parametrical Kruskal Wallis test, given that the data did not follow normal distribution.

5 RESULTS

5.1 Growth trial

The fish fed with the experimental diets nearly doubled their body weight during the 56 days of the experiment (Table 6, Figure 5).

Table 6: Average weight of individuals at beginning and end of the trial.

Experimental Feed	Tanks	Initial individual average weight	Final individual average weight
A	I4	134.00	235.88
A	I9	132.63	238.69
A	II6	131.00	225.94
B	I2	132.00	220.69
B	I7	134.31	233.75
B	II9	136.19	234.69
C	I6	135.38	246.75
C	II3	137.00	247.27
C	II10	140.44	243.00
D	I3	128.81	235.56
D	I8	136.69	236.13
D	II5	134.88	224.44
E	I5	132.56	240.33
E	II4	132.50	242.56
E	II8	139.31	249.94
F	I10	140.06	249.25
F	II2	130.56	233.27
F	II7	129.75	234.88

Figure 5: Atlantic salmon during final individual weighing.

The SGR as a measure of the fish growth during the trial had values from 0.96-1.06. FCR for the experimental feed was from 0.83-0.87 (Table 7).

Table 7: FCR and SGR of tested diets.

Feed	FCR	SGR
A	0.83 ± 0.05	1.01 ± 0.04
B	0.87 ± 0.05	0.96 ± 0.04
C	0.85 ± 0.06	1.04 ± 0.05
D	0.85 ± 0.03	0.99 ± 0.08
E	0.84 ± 0.06	1.06 ± 0.02
F	0.85 ± 0.10	1.04 ± 0.02

5.2 Biofilter community analyses.

5.2.1 DNA Extraction

The DNA extraction results were checked on a Spectrophotometer to verify the Nucleotide Acids presence (Table 8).

Table 8: DNA Extraction results.

Sample ID	DNA concentration (ng/ul)	260/280	260/230	Volume (uL)
T1a	19.7	1.82	0.33	25
T1b	27.5	2.01	0.75	25
T1c	24.6	2.24	0.20	25
T2a	23.7	1.90	0.09	25
T2b	44.2	2.02	0.31	25
T2c	47.8	2.06	0.71	25
T3a	18.5	2.19	0.54	25
T3b	31.3	2.10	1.49	25
T3c	13.9	2.23	0.21	25
FaecesA	397.9	1.96	1.93	50
FaecesB	458.8	1.96	2.17	50
FaecesC	468.6	1.97	1.44	50

5.2.2 Gene amplification

The initial PCR showed the presence of amplicon size of 253 bp as expected on the electrophoresis gel verification (Figure 6).

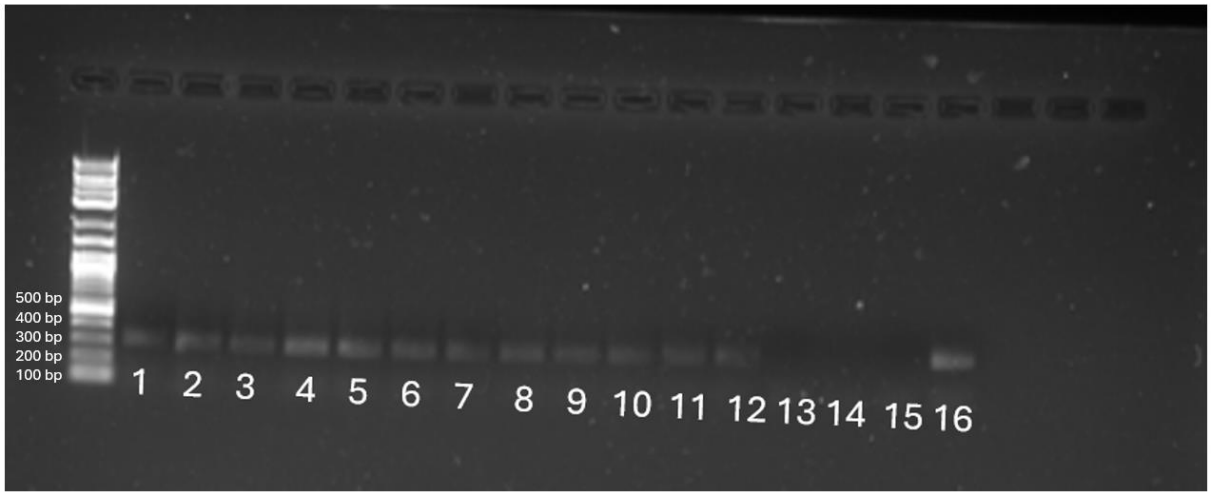


Figure 6: Electrophoresis gel verification of 253 bp presence on the PCR results (1:T1a; 2: T1b; 3: T1c; 4: T2a; 5: T2b; 6: T2c; 7: T3a; 8: T3b; 9: T3c; 10: Feces A; 11: Feces B; 12: Feces C; 13, 14, 15: Negative control, 16: Positive control).

The Index PCR showed the presence of amplicon size of 439 bp as expected on the electrophoresis gel verification (Figure 7).

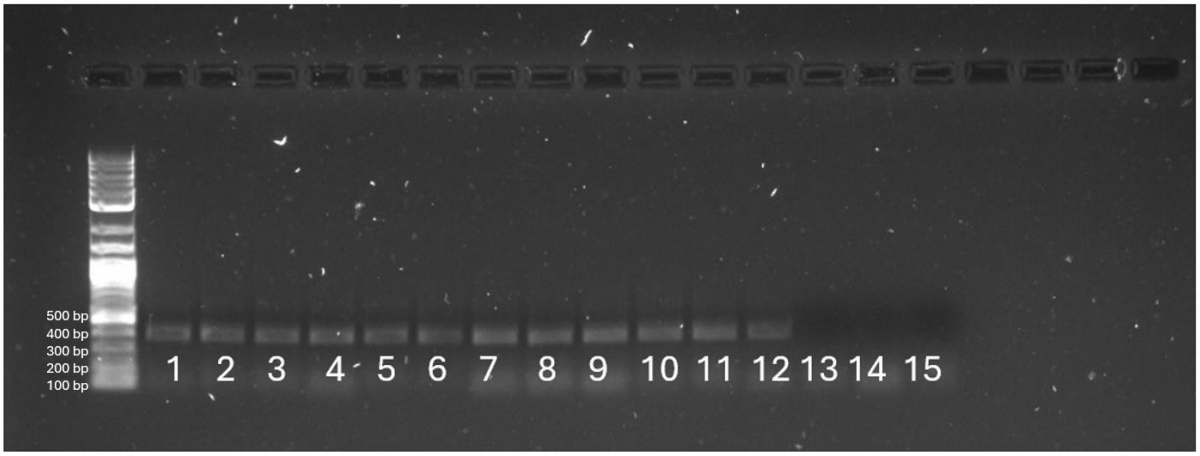


Figure 7: Electrophoresis gel verification of 439 bp presence on the Index PCR results. (1:T1a; 2: T1b; 3: T1c; 4: T2a; 5: T2b; 6: T2c; 7: T3a; 8: T3b; 9: T3c; 10: Feces A; 11: Feces B; 12: Feces C; 13, 14, 15: Negative control).

5.2.3 Gene sequencing

The sequencing results showed the presence of Alphaproteobacteria and Gammaproteobacteria Class (Figure 8) and *Nitrosomonas* Genus in all the samples (Figure 9).

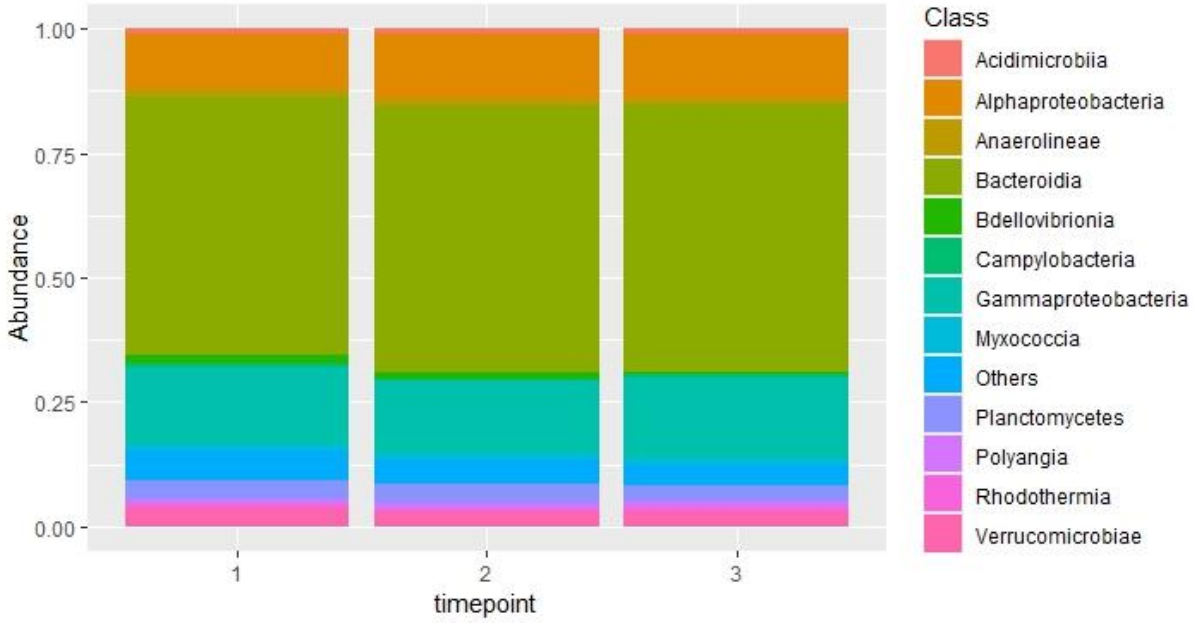


Figure 8: Abundance of Class above 0.5% in the samples.

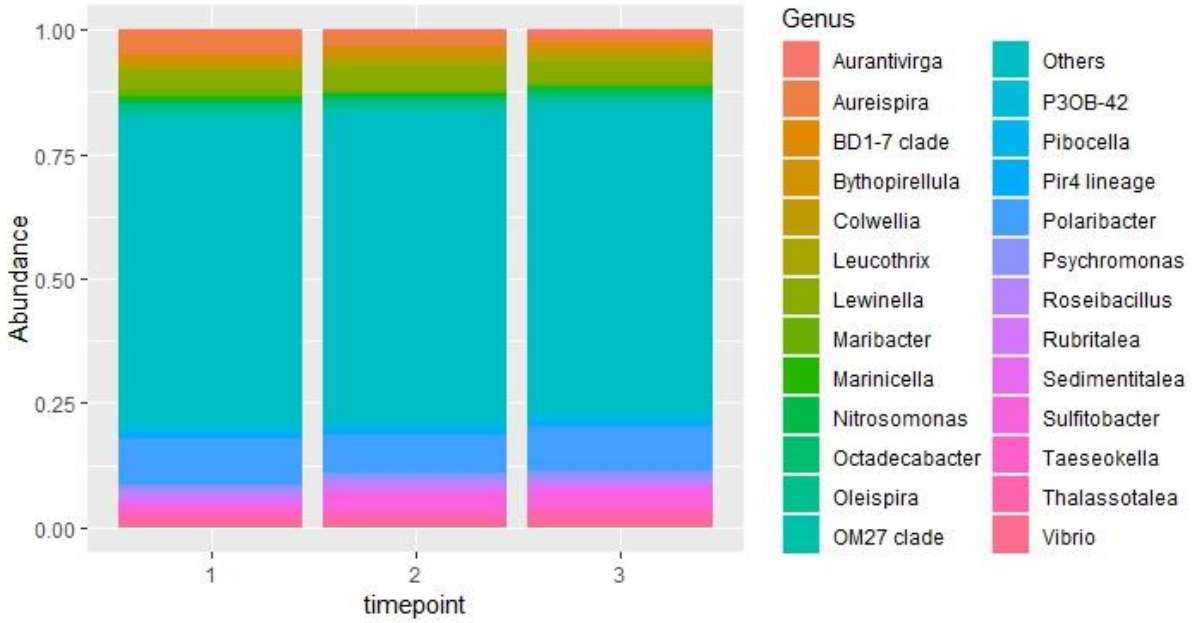


Figure 9: Abundance of Genus above 0.5% in the samples.

The results from sequencing were analyzed considering the Alpha Diversity present in the samples taken from the RAS biofilter at three points during the trial (Figure 10).

Alpha Diversity expressed a summary of the richness and evenness of the biological community present in the sample. Richness represents the number of taxa groups and evenness refers to the distribution of abundance of the groups in the samples. Diversity Indexes measure the Alpha Diversity of a community considering either richness or evenness (Fisher´s Index), or both (Shannon Diversity Index, Simpson´s Index) (Willis, 2019) (Wilson & Gownaris, 2024).

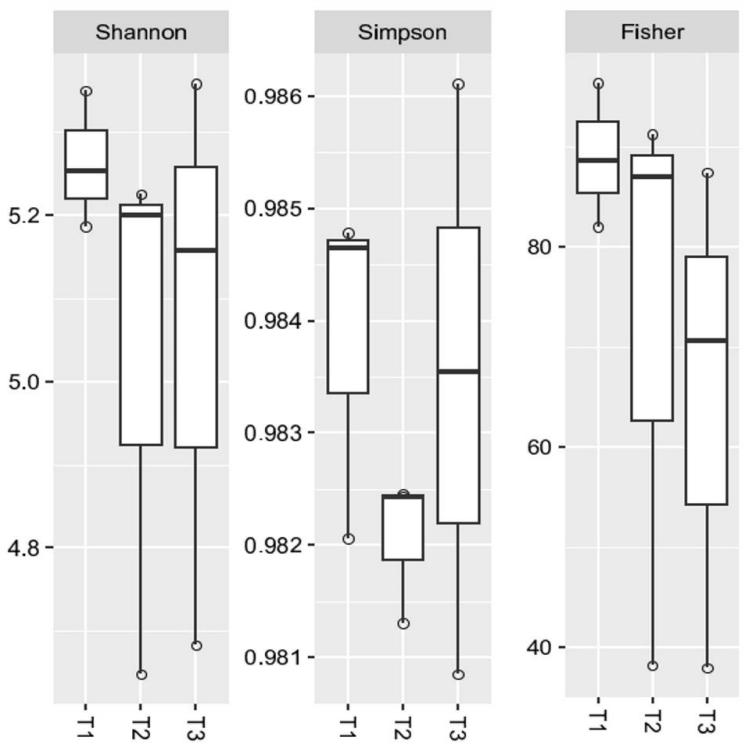


Figure 10: Alpha Diversity Measure as (from left to right): Shannon Diversity Index, Simpson´s Index, and Fisher´s Index.

The statistical analyses of the considered Diversity Index showed no significant differences in the biodiversity of the biofilter community between the three points sampled in the experiment (Table 9).

Table 9: Statistical Analysis of Diversity Index of the samples.

Diversity Index	Kruskal-Wallis (p value*)
Shannon	0.5611
Simpson	0.5611
Fisher	0.3292

* $p < 0,05$

6 DISCUSSION

6.1 Growth trial

The statistical analyses showed no significant difference between the feeds utilized in the experiment according to FCR (Figure 11) and SGR of the fish (Figure 12).

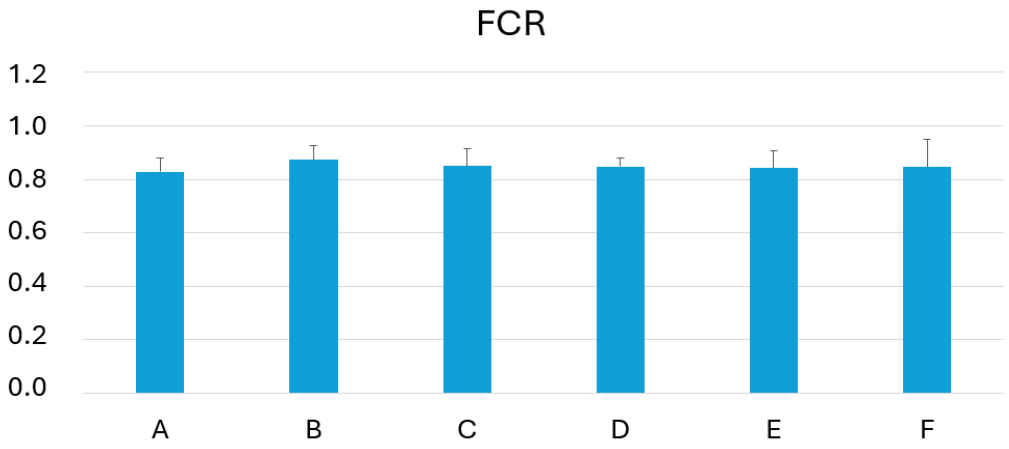


Figure 11: Results of the Tuckey test for the FCR values of diets A, B, C, D, E, and F.

FCR describes the ratio of the feed amount (kg) used to produce 1 kg of fish. The calculation of this value guarantees the optimal utilization of expensive resources like feed (Jobling, 2011).

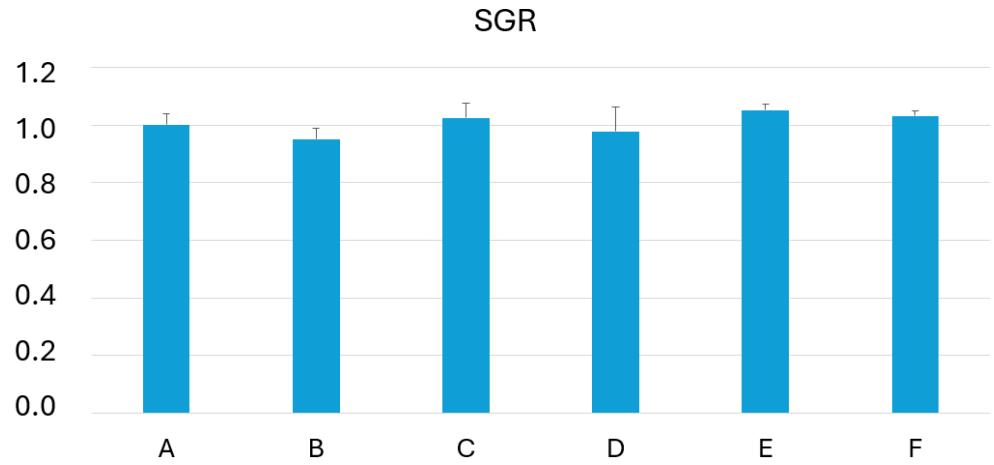


Figure 12: Results of the Tuckey test for the SGR values of diets A, B, C, D, E, and F.

SGR is a measurement that expresses the growth of an individual considering their weight at two specific points in time (Crane, et al., 2020).

The FCR and SGR values obtained in the present study were similar to those found by Storebakken, et al. (2000) for Atlantic salmon fed with fish meal and soy-protein concentrate as the main sources of protein (FCR for fishmeal = 0.81; FCR for soy-protein meal= 0.89; SGR for both feed = 0.88 - 0.89, water temperature 8-9°C). On the other hand, SGR obtained by Olsen, et al. (2004) with Atlantic salmon fed with *Calanus finmarchicus* oil as a substitute for fish oil had lower values (SGR= 0.75) and obtained higher FCR values (FCR= 1.02, water temperature 10°C).

Highly nutritious feeds are more likely to have lower FCR values given that lower amounts contain higher concentrations of compounds that satisfy the nutritional requirements of the organisms. In the case of salmon, this means a high content of protein (Golfand, 2023). The recommended dietary protein requirement for Atlantic salmon in a weight range of 20-200 g is 44% (Jobling, 2011). The average protein content in all the feed utilized in the present study represents 48.32 %.

6.2 Biofilter community analyses

Alphaproteobacteria and Gammaproteobacteria Class were present in the three sampling times. According to van der Meeren, et al. (2011) Gammaproteobacteria Class is commonly found in RAS systems. The presence of Alphaproteobacteria was also found in the previously mentioned study.

Pulkkinen (2020) obtained that the majority of bacterial communities in a freshwater RAS system belonged to Class Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, and Planctomycetes. On the other hand, the research showed only a 2% abundance for Class Bacteroidia. In the present study, performed in saltwater, the Class Bacteroidia represented the majority of the found taxa.

Class Planctomycetes was also found in our study. This taxa includes bacteria capable of performing the direct oxidation of ammonia and nitrite into nitrogen gas (anammox). Other taxa as Acidimicrobiia and Verrucomicrobiae have been also described for RAS (Pulkkinen, 2020).

Nitrosomonas Genus was also present in the three samples. The presence of this Genus was expected given its function in the nitrification process.

The presence of *Vibrio* Genus has been previously described in biofilters and it was also found in our study. These taxa are potentially pathogenic (Burut-Archanai, et al., 2021).

In general, the obtained taxa were expected and previously described for biofilters in RAS systems. The statistical analyses showed no significant differences between the three time points that were sampled. The daily TAN measurements showed the efficient performance of the nitrification process within the biofilter.

7 CONCLUSIONS

- There were no significant differences between the FCR of the six tested diets in the experiment. The average FCR obtained was 0.85.
- There were no significant differences between the SGR of the fish fed with the six tested diets in the experiment. The average SGR obtained was 1.02.
- The function of the biofilter was not affected during the experiment ensuring the water quality in the trial.
- There were no significant differences in alpha diversity and in comparable composition of most abundant taxa at both genus and class level of the biofilter during the experiment.

8 RECOMMENDATIONS

The experiment showed decent performances for SGR and FCR. This indicates the importance of good management of a scientific aquaculture trial. The different treatments did not show statistical differences, so no scientific recommendation can be given regarding the feeds and fishmeals. However, an experiment over a longer period could have shown significant changes in performance parameters.

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